Selective Signal Capture from Multidimensional GPCR Outputs with Biased Agonists: Progress Towards Novel Drug Development

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Abstract

G protein coupled receptors (GPCRs) are a superfamily of transmembrane-spanning receptors that are activated by multiple endogenous ligands and are the most common target for agonist or antagonist therapeutics across a broad spectrum of diseases. Initial characterization within the superfamily suggested that a receptor activated a single intracellular pathway, depending on the G protein to which it coupled. However, it has become apparent that a given receptor can activate multiple different pathways, some being therapeutically desirable, while others are neutral or promote deleterious signaling. The activation of pathways that limit effectiveness of a primary pathway or promote unwanted signals has led to abandonment of some GPCRs as drug targets. However, it is now recognized that the conformation of the receptor in its ligand-bound state can be altered by the structure of the agonist or antagonist to achieve pathway selectivity, a property termed biased signaling. Biased ligands could dramatically expand the number of novel drugs acting at GPCRs for new indications. However, the field struggles with the complexity and uncertainty of these structure-functions relationships. In this review we define the theoretical underpinnings of the biased effect, discuss the methods for measuring bias, and the pitfalls that can lead to incorrect assignments of bias. Using the recent elucidation of a β_2-adrenergic receptor agonist that is biased in favor of Gs coupling over β-arrestin binding, we provide an example of how large libraries of compounds that are impartial to preconceived notions of agonist binding can be utilized to discover pathway-specific agonists. In this case, an agonist that lacks tachyphylaxis for the treatment of obstructive lung diseases was uncovered, with a structure that was distinctly different from other agonists. We show how biased characteristics were ascertained analytically, and how molecular modeling and simulations provide a structural basis for a restricted signaling repertoire.

1 Introduction

1.1 Classic GPCR Signaling

G protein coupled receptors (GPCRs) are the largest protein superfamily in the body. They act as information portals from the extracellular environment to the cell interior, and are expressed on every cell type, responding to endogenous hormones, neurotransmitters, metabolites, chemokines, and many other molecules. As a superfamily, they are considered druggable targets for therapeutic agonists or antagonists, and in fact ~30%–50% of prescribed drugs act at GPCRs or their pathways [1]. G protein coupled receptors have a common structure, consisting of seven transmembrane (TM) domains,

three extracellular (ECL1-3) and three intracellular loops (ICL1-3), an extracellular amino-terminus and an intracellular carboxy-terminal tail (Fig. 1). Early conceptual models regarding activation of GPCRs might best be represented by a simple switch, where the receptor is either "on or off", fully activating a single intracellular pathway. Classically, this action was thought to occur due to an altered conformation of the agonist-bound receptor, which binds to the a subunit of a heterotrimeric G protein. The Gα then activates (or inhibits) an effector, which alters the generation of an intracellular molecule, which mediates a given function directly or after several additional steps. With receptors that couple to the G protein Gαs, the "first messenger" is the agonist (Fig. 1), and the Gαs subunit activates adenylyl cyclase (the "effector"), which catalyzes the conversion of ATP to cyclic AMP (cAMP, the "second messenger"). Through direct actions or through activating the cAMP dependent protein kinase A, the Gs coupled receptor via cAMP evokes

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an intracellular molecular or physiologic response, which may differ based on the cell type. For example, epinephrine acting on β_2-adrenergic receptors (β_2AR) on cardiac myocytes increases contractile force, while on smooth muscle cells causes relaxation. The dissociation of the G protein is followed by regeneration of the heterotrimer, and the cycle repeats if agonist is available to bind (Fig. 1). The essential elements of Fig. 1 remain correct and are useful for a general understanding of how extracellular agonists evoke intracellular events through GPCRs. The effectiveness of the system is readily apparent when one considers that these receptors (such as the adrenergic receptors) are typically expressed in tissues at femtomoles/mg protein, and endogenous agonists are present as low as the picomoles/mL range in the circulation. However, this binary nature of the actions from GPCRs is now recognized as being overly simplistic, which has led to more sophisticated models with the potential for development of highly targeted therapeutics, and for understanding maladaptive changes promoted by disease.

1.2 Multidimensional Signaling from GPCRs

The simple switch mechanism was considered inadequate by the 1990s to explain the plethora of signals, which were often cell type specific, that were being observed from activation of a given GPCR with receptor-specific agonists [2, 3]. Multiple mechanisms have since been uncovered which

reveal that GPCRs represent multifunctional signaling units. Figure 2 shows nine such mechanisms (see legend for further description). These include signaling via two different $G\alpha$ proteins, signaling by $G\beta\gamma$, and signaling via other proteins that are independent of a G protein. Receptor coupling to two G proteins was the first mechanism that clearly defined the multifunctional nature of a GPCR. The α_{2A}-adrenergic receptor was shown to couple to both Gi and Gs [2]. Subsequently, a small region of ICL3 near the membrane was shown to impact the Gs coupling, establishing a structural basis for the event, and showed that the conformational change evoked by agonist binding within the TM pocket is transmitted to the intracellular domains [4]. For this receptor, a study of both pathways with structurally diverse agonists showed that some agonists primarily (or exclusively) promote coupling to only one G protein [5]. At that time, two activated states were proposed for this receptor: R* which promoted coupling to both Gi and Gs proteins, and R^Δ, which preferentially promoted coupling to Gi. Studies from that same era indicated that merely overexpressing receptors, in the absence of agonist, could evoke signaling [6, 7], which indicated that the agonist-unoccupied receptor achieves an "active" conformation(s) spontaneously, albeit for very brief time periods, giving rise to basal levels of signaling or its product. This further cemented the idea that agonists do not "force" a receptor into a conformation, but rather stabilize and maintain one or more active conformations from a repertoire of many oscillating conformations when the receptor is in its free state. This concept is illustrated in Fig. 3 (insert), where multiple potentially activated receptor (R^{act}) conformations are represented by different colors. The equilibrium in this non-agonist bound state does not favor active conformations, as indicated by the different sized arrows between R and R^{act}.

2 Biasing GPCR Signaling Output

In the absence of agonist, basal levels of signals (such as cAMP) are measurable, and as indicated in Fig. 3 (where nine functions are illustrated), they may be at different levels in the free state. In this example, upon activation by agonist A, receptor conformations R^{*2} and R^{*5} are stabilized, significantly increasing signals directed by those conformations. When only considering these two signals, agonist A might be a "balanced" or "unbiased" agonist [8, 9]. In contrast, agonist B only promotes stabilization of the R^{*5} conformation, and thus this one signal is elicited when considering R^{*2} and R^{*5}. This agonist is termed a biased agonist, favoring signal 5 with no detectable signal 2 over basal levels. In this instance, there is no loss of signal 5 compared to the unbiased agonist. Agonist C does not promote R^{*2}, but the R^{*5} signal is somewhat impaired compared to the unbiased
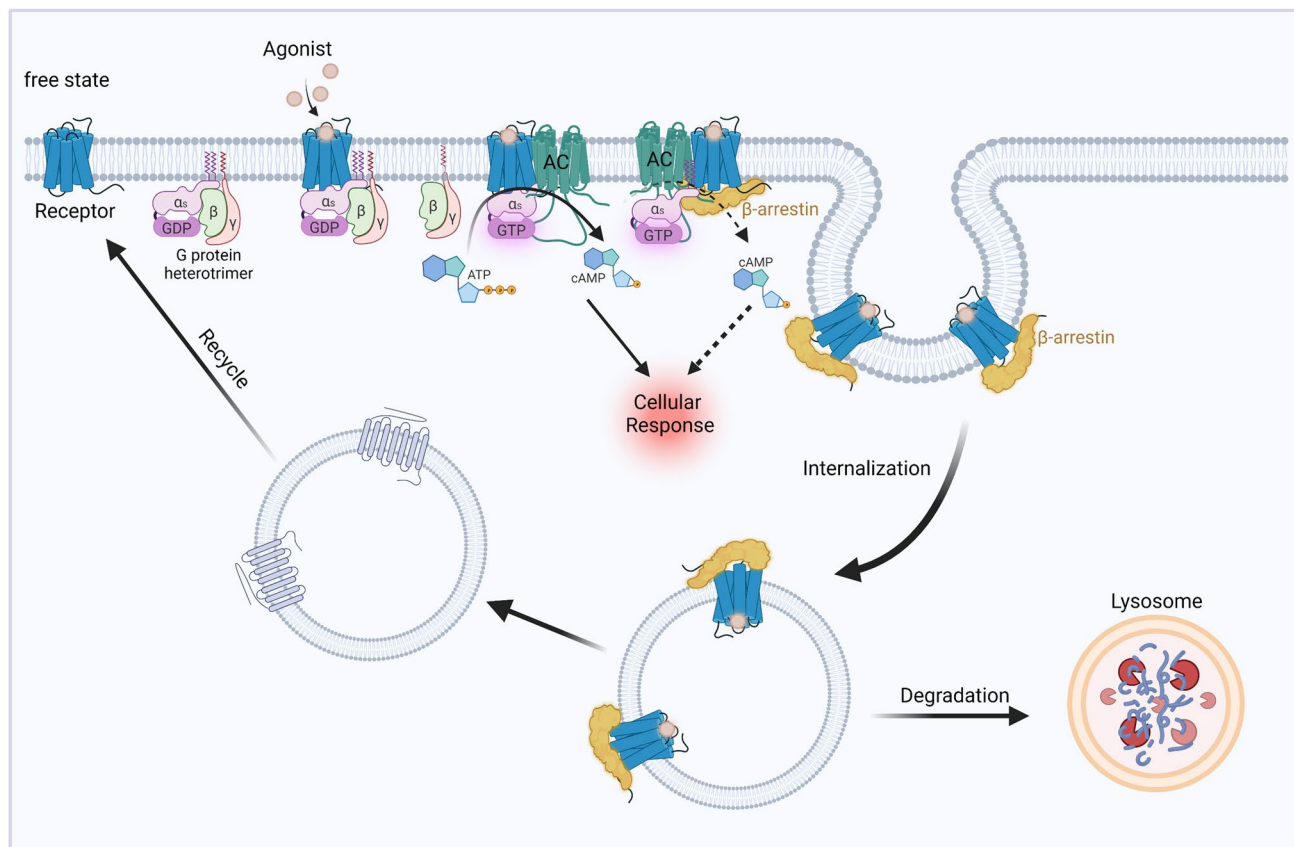
Fig. 1 One-dimensional signaling of a GPCR. The prototypic β_2AR with its 7TM spanning domains and the G protein Gs with its α and $\beta\gamma$ subunits, and the effector adenylyl cyclase, are depicted. With agonist binding, the heterotrimeric G protein binds and dissociates, with the α subunit binding to the receptor and ultimately activating adenylyl cyclase. Activated adenylyl cyclase catalyzes the conversion of ATP to cAMP. cAMP acts via activation of PKA and other mechanisms to evoke intracellular and physiologic events. The cycle repeats as long as agonist is available for receptor binding. For clarity, several intermediate steps between the agonist-unoccupied ("free") receptor and full G protein activation are not shown. β-arrestin can dampen receptor signaling to Gαs by competing with Gαs binding at the receptor, thereby depressing activation of adenylyl cyclase within the complex and subsequent cAMP production (dashed line). In addition, β-arrestin mediates internalization of receptors to intracellular vesicles. Depending on the receptor, the agonist and β-arrestin can be retained in the vesicle or not. Internalization leads either to recycling of receptors to the cell surface, or degradation of the receptors by lysosomes under conditions of long-term agonist exposure, a process termed downregulation. This view of function and regulation illustrates several key steps which is useful for orientation to the conventional signaling mechanisms of GPCRs, but is one dimensional (confined to a single signal, cAMP generation)

agonist A. Agonist C is also considered biased. If the R^{*5} signal is above the threshold necessary for the desired physiologic response, then agonist C might achieve therapeutic efficacy despite the modest impairment. Further complexity is evident when one notes that R^{*8} is activated by agonists A and B. If this is neutral to the pathophysiology or efficacy, then agonist B would be acceptable. Otherwise, agonist C, which does not activate R^{*8} might be a better choice. This scenario illustrates the importance of understanding the relevant signals to be measured based on the cell type and the disease phenotype. Note that agonist D promotes a gain of R^{*5} without affecting R^{*2}. Thus, agonist D is biased compared to agonist A. However, to date efforts to exploit biasing generally result in a loss in the magnitude of the undesirable event rather than a gain in the primary signal.

Recent studies with multiple GPCRs have begun to reveal technical and theoretical issues that need to be defined and addressed. Many of these revolve around what has been termed "system bias" [10, 11], which can lead to assignment of agonist biasing due to various artifacts. We propose to describe these issues with subsets of potential irregularities that may lead to false conclusions. The most straightforward type of bias that can lead to misleading results is "assay bias" (also termed "observational bias"). It is not uncommon for assays used to measure second messengers, direct receptor-G protein coupling, or other relevant agonist-promoted events to have different levels of sensitivity, maximal responsiveness, or signal-to-noise ratios. Thus, using an insensitive assay for one pathway and detecting little activation, while a sensitive and robust assay measures a significant signaling
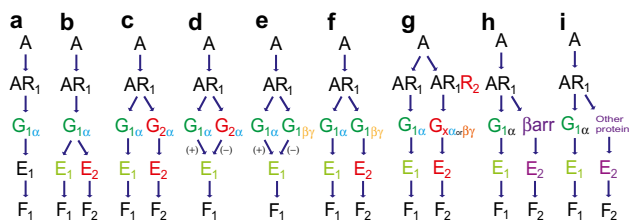
Fig. 2 Mechanisms of multidimensional GPCR signaling. **a** one-dimensional signaling; **b** signaling to two effectors from the same G protein; **c** signaling to two G protein α subunits by the same receptor to two different effectors; **d** the same as **c** except the two G proteins have opposing actions on one effector; **e, f** βγ signaling to one or two effectors; **g** agonist acts through a receptor monomer to activate an effector, and through a receptor heterodimer which has a different signaling mechanism through another G protein α or βγ subunit; **h** signaling occurs from the G protein and from β-arrestin; **i** same as **h** except the G protein-independent signaling is due to receptor interaction with another protein such as the Na^+/K^+-exchanger regulatory factor. *A* agonist, *R* receptor, *G* G protein, *E* effector, *F* a cellular function, *βarr* β-arrestin 1 or 2

event from the other pathway, could lead to the conclusion that the agonist is biased away from one pathway. One way to address this p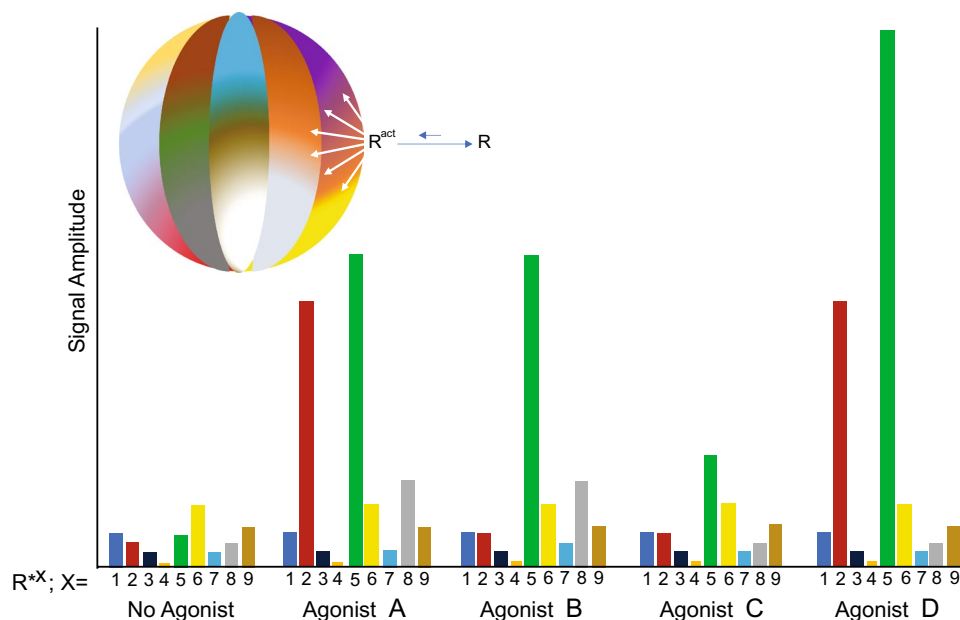otential problem is to perform assays with the proposed drug in tandem with a full, unbiased, agonist. Graphing data from the unbiased agonist from one assay against the other assay will reveal any incongruity between assays and potentially show regions within the plot where agonist biasing could be detected, despite assay bias [9, 10]. Another type of bias we term "intrinsic bias", which refers to the potential that of two agonist-promoted pathways being explored, one is in fact much more inefficient than the other, based on the intrinsic properties of the two pathways in the cell. In such a case, even with an unbiased agonist, the experimental data might appear to show ligand or assay bias, but in fact the results indicate the real-world of the cell. One can alter the efficiency of a pathway by overexpressing receptor, G-protein, GRKs, β-arrestin, etc., which could serve as a screening method. However, studies in the cell type of interest with endogenous expression of the signaling components remain necessary. The intrinsic bias phenomenon also brings out the issue of what defines the "reference". Ideally the compound would be "balanced", but such a term must be tied to the pathways of interest. Other pathways, whether or not their outcomes are measured, may well not exhibit balanced signaling. In some cases, such as the $β_2AR$, the endogenous agonist (epinephrine) or minor

Fig. 3 Biased signaling from different receptor conformations. The inset shows the free receptor oscillating to activated receptor that can take on multiple conformations, as indicated by the various colors. In the absence of agonist, the equilibrium favors the inactivated state. The bar graph illustrates the effect of agonist on nine receptor conformations (R^{*1} - R^{*9}). The y-axis represents the amplitude of the signal evoked by one of more of the agonist-stabilized active conformations. In the absence of agonist, basal levels of signaling are detectable due to the oscillating conformation of the receptor (or other non-receptor mechanisms). Upon agonist binding, certain conformations are stabi-lized, and the signal is increased over basal levels. Agonist A activates two signals (R^{*2}, R^{*5}, red and green bars, respectively), while agonist B activates only one signal (R^{*5}, green bar), consistent with biasing away from the R^{*2} state, while maintaining full R^{*5} activity. Agonist C is also biased but has compromised R^{*5} signaling. Another state (R^{*8}) is stabilized by agonists A and B, but not C. In comparison to these two agonists, agonist C is biased away from R^{*8}. Agonist D is potentially also biased, in that it has gained R^{*5} signaling without a change in R^{*2} signaling, compared to Agonist A

derivatives such as isoproterenol appear to activate several pathways with about the same potency and efficacy (taking into account assay bias). However, as more exotic receptors (or pathways) are studied, the endogenous agonist may not even be known, and there is no guarantee that it would necessarily be balanced. In such cases, the reference agonist should be the most potent and/or efficacious agonist that is recognized (for at least one pathway), while maintaining an open mind that novel compounds might be even more appropriate for this purpose as they are discovered. In such cases several benchmark agonists should be utilized to help form a more complete picture of signaling selectivity and to develop a rank-order. Another basis for erroneous interpretation is due to "kinetic bias". Agonist binding on-rates may differ due to structure, which may mean that the maximal signal for one pathway may be at a different time after agonist exposure compared to a second pathway, and ligand bias inferred because the two systems are not both at equilibrium. This concept can be extended to the kinetics of the downstream pathways, particularly deep pathways, where response times may differ between two or more measured events. Finally, we also consider "physiologic bias". To ascertain this type of bias, the relationships between the amplitudes of GPCR-promoted signals and the final functional outcomes of the pathways need to be considered. For example, the cell type of interest may generate a second messenger far in excess of what is needed to achieve maximal cellular response. If that response is considered deleterious, and the novel agonist is only partially biased away from the pathway, the deleterious events might still occur. Likewise, if "full" biasing by an agonist away from one pathway also significantly impairs signaling to the desired pathway, a therapeutic effect may not be realized.

3 β-Arrestin is a Nodal Point for Agonist Biasing of Three Therapeutic Outcomes: Efficacy, Desensitization, and Non-classical Effects

Figure 2h, i also indicates how multifunctional signaling from GPCRs can occur that is independent of G protein [12]. These events may involve direct interactions between receptor and another protein, but nevertheless are dependent on the conformation of the agonist-bound receptor. Of particular recent interest is the interaction between receptor and members of the arrestin family (β-arrestin 1 and 2) [13, 14]. Receptor desensitization (also termed tachyphylaxis and tolerance) is defined as a waning of receptor function over time during sustained agonist occupancy [15]. Studies using various techniques have clearly indicated that β-arrestin mediates short-term, agonist-promoted, homologous desensitization of most GPCRs [16]. β-arrestin interaction with

GPCRs, involves several phases, with two major phases shown in Fig. 4. Agonist binding promotes conformational changes in ICL3 or the C-terminal tail, which leads to receptor phosphorylation at Ser or Thr in these regions [17] by G protein-coupled receptor kinases (GRKs) (Fig. 4a). These phosphorylated regions become substrates for the binding of β-arrestin, which under confocal microscopy appears as a recruitment of the protein from the cytosol, where it is homogeneously expressed, to puncta at the cell membrane (Fig. 4b). This Phase 1 binding appears to be sufficient for the discreet packing of certain proteins within a signaling complex, due to the chaperone, adapter and scaffolding properties of β-arrestin. This assembly can act to evoke specific signals, such as activation of ERK1/2 (Fig. 4c). Note that β-arrestin signaling, as depicted here, is agonist dependent, but not affected by receptor-G protein interactions or downstream mediators. However, recent studies have indicated that there may be contributions by the G protein for some β-arrestin-promoted signaling [18]. Phase 2 β-arrestin binding involves additional binding to more proximal portions of the receptor including the G protein binding regions within the TM domains. This binding competes with receptor Gα binding [19, 20], thus attenuating G protein signaling, a process called uncoupling (Fig. 4c).

The Phase 1 β-arrestin binding is dependent upon the presence and location of the phospho-acceptors, the GRK isoforms expressed in the cell, and the agonist-promoted conformation of the loop or tail, transmitted from the binding pocket. Indeed, diverse ligand structure has been shown to differentially alter GRK activation and phosphorylation of specific residues, a phenomenon which has been termed "phospho-barcoding" [21–23]. Given these multiple factors, it is apparent that the "texture" of β-arrestin associated with a receptor-agonist pair in the cell can be variable, and could be influenced by agonist structure, leading to differential protein scaffolding and thus signaling by β-arrestin. Phase 2 binding and the subsequent desensitization can be influenced by agonist structure in several ways, including: by affecting Phase 1 binding (and thus the altering the conformation of β-arrestin prior to TM insertion) or by affecting the translocation of TM5 in the receptor core, which provides the space for β-arrestin interaction in that region (and thus altering the potential for competing with the G protein). In order to understand the mechanisms of β-arrestin bias, care must be taken to devise assays that can detect both phases, or alternatively, assays that detect the functional consequences of β-arrestin actions from each phase.

Finally, β-arrestin has been implicated in the assembling of components necessary for agonist-promoted internalization of GPCRs to the cell interior, usually in vesicles which can be routed to degradation with extended agonist exposure (Fig. 1). This latter process, termed downregulation, can lead to a significant loss of the cellular complement of receptors
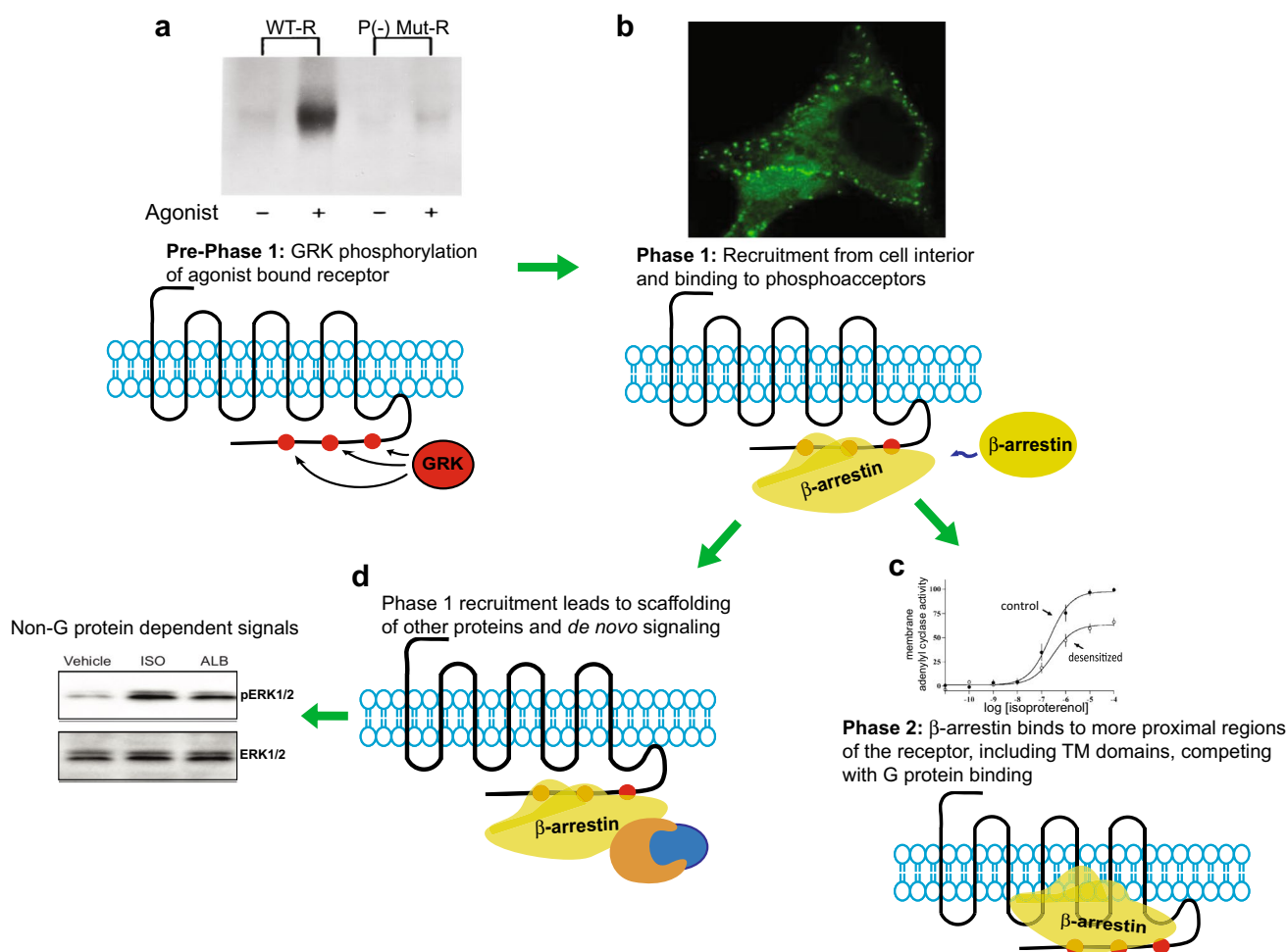
Fig. 4 β-arrestin interaction with GPCRs. **a** Prior to Phase 1 binding, the agonist-bound receptor is phosphorylated by GRKs in the indicated locations (red dots). **b** This event leads to Phase 1 binding which is a recruitment of β-arrestin from the cytosol to the phosphorylated receptor regions. **c** Phase 2 binding involves intercalation with the receptor core and competition with G protein α binding, resulting in desensitization due to this uncoupling. **d** The conformation of β-arrestin established by Phase 1 binding leads to scaffolding with other proteins and initiation of de novo signals, such as activation of ERK1/2. Multiple intermediate phases are not shown. The phosphorylation gel (**a**) is from reference [17], the confocal micrograph of β-arrestin recruitment (**b**) is from reference [24], the ERK1/2 phosphorylation gel (**d**) is from reference [37], and the desensitization data (**c**) are from reference [15] (all published by the authors, and used with permission)

resulting in marked desensitization. β-arrestin-mediated uncoupling occurs within seconds to minutes after agonist exposure, thus even with one dose, the therapeutic efficacy of GPCR agonists is almost immediately dampened [24, 25]. Thus, β-arrestin plays a role in establishing the therapeutic potency or efficacy of agonists signaling via G protein-coupled pathways. With the onset of internalization (the maximal extent is typically observed within 30 min of agonist exposure) the second wave of desensitization is initiated that can be reversed if the receptors have not been degraded. And then with hours of agonist exposure, the downregulation process leads to further desensitization [16]. This time-based continuum of events represents regulatory capacities of the cell to respond to its environment, but can also result

in tachyphylaxis, which limits the therapeutic effectiveness of administered agonists.

Note that β-arrestin action is at the center of agonist responsiveness, desensitization, and non-classical G protein independent signaling. Agonists that are biased away from β-arrestin while maintaining G protein signaling would therefore be expected to have improved efficacy, display less clinical tachyphylaxis, and less β-arrestin-mediated non-G protein signaling. This latter signaling may be deleterious (or not necessary for the therapeutic effect) in certain cell types under certain disease conditions, so the biasing may have additional therapeutic effects. As discussed below, there are also instances where β-arrestin signaling *promotes* the desired therapeutic endpoint and G protein signaling is

deleterious. So, an agonist that favors β-arrestin action, with minimal G protein coupling, would be the desired direction of the biasing under these circumstances.

4 The Diversity of Targets and Indications Under Development That Utilize GPCR Biasing

Of the known receptors that have been targeted by traditional agonists or antagonists [1], virtually all have been found to display multifunctional signaling by mechanisms similar to those depicted in Fig. 2 [26]. At least 30% of these receptors have been shown to be capable of biased signaling from structurally distinct ligands, amounting to > 250 compounds [26]. Generally, the goals of drug discovery efforts for biased agonists are to (a) selectively activate pathway(s) thought to impart the desired therapeutic response, and to not activate (or minimally activate) pathways associated with on-target but undesirable effects, (b) to increase signaling efficacy/ potency by minimizing rapid desensitization or (c) decrease tachyphylaxis by abrogating long term desensitization.

A few examples are provided here to illustrate how biased ligands have the potential to improve therapeutic responses. For the μ-opioid receptor, several agonists such as PTI-609 and TRV130, activate Gi coupling preferentially over β-arrestin signaling [27, 28]. This results in analgesia equal to or better than morphine, but with little desensitization (tolerance) to the drug, indicating that dose escalation would not be necessary and perhaps patients would be less prone to addiction. Interestingly, respiratory depression and constipation were less in patients treated with TRV-130, suggesting that these unwanted effects are also mediated through a β-arrestin pathway [27]. For the angiotensin II type 1 receptor, the agonist TRV120027 was found to be biased towards β-arrestin and away from G protein (Gq) activation [29]. In heart failure, antagonism of Gq has favorable effects on myocardial remodeling while β-arrestin activation appears to improve cardiac contractility and reduce afterload. While mouse models showed these salutary effects of TRV12007, the human clinical trial did not reach the defined clinical outcomes [30]. Agonists at the D2 dopamine receptor have been found to be positively and negatively biased to G proteins or β-arrestin. Studies to date suggest that certain agonists might have superior antipsychotic effects in schizophrenia with less extrapyramidal effects than unbiased agonists [31, 32]. The MC4 melanocortin receptor couples to Gi and Gs. MC4 agonists biased away from Gs, while maintaining Gi coupling, appear to significantly promote appetite [33] while those biased towards Gs initiate satiation signaling [34]. The GLP-1R receptor (glucagon-like peptide-1 receptor) regulates insulin secretion in a complex manner, and studies have indicated that an agonist biased away from β-arrestin

appears to be more effective than balanced agonists in lowering plasma glucose levels in diabetic mice [35]. Parathyroid hormone receptor agonists biased towards β-arrestin with attenuated G protein coupling promoted new bone formation (osteoblast formation) without bone reabsorption, in contrast to the dual effects observed with the endogenous hormone which is the current treatment for osteoporosis [36]. For the β2AR, the agonist C1-S (see below) is Gs biased with little to no β-arrestin engagement, which results in enhanced airway relaxation and the absence of tachyphylaxis to treat obstructive lung diseases [37]. These and other studies [26, 38] show the potential clinical utility of specific types of biasing, but also have revealed the complexity of measuring the relevant signals at the cellular level, calculating biased activity, and predicting clinical outcomes.

5 Screening for Unique β2AR Agonists for Treating Asthma

Efforts to discover biased ligands at GPCRs have often utilized known agonists and antagonists, or their derivatives, in screening studies for pathway selectivity. This low hanging fruit may be nearing exhaustion, and other ways of discovering ligands with unique properties such as biasing may be necessary. Here we demonstrate one such approach for the β2AR [37]. We note that structure-based drug design using in silico modeling techniques could be used for discovering biased ligands. However, little is known about how biasing is achieved at the structural level, there are relatively few x-ray crystal structures of GPCRs activated by biased ligands, and the mechanism(s) of achieving biasing appear to differ between various receptors. Thus, there is minimal information available to guide such modeling as a first-pass screening technique. Another approach is the individual screening of very large collections (tens of millions) of compounds for selected signals with model cells, which for all practical purposes requires automated/robotic infrastructures to assess so many separate compounds. Of particular importance for such screening at any level would be to use collections that are impartial to the apparent structural requirements for ligands to bind to a given receptor based on previous studies. This agnostic approach, with a collection that covers a broad chemical space, can also be accomplished with combinatorial mixture libraries, which use exponentially smaller samples to ultimately define single compounds with specific properties. These mixtures can be systematically arranged as scaffold ranking and positional scanning libraries [39–42], rapidly leading to structure-activity relationships. This has recently been accomplished for the β2AR, a target for β-agonists for the treatment of obstructive lung diseases such as asthma [37], and is discussed as an example

of how unique agonists can be found and testing for biasing in a specific manner can be investigated.

β2ARs expressed on human airway smooth muscle (HASM) cells couple to Gs, stimulate adenylyl cyclase and generate cAMP, resulting in HASM cell relaxation which dilates the constricted airways, improving airflow. Inhaled β-agonists such as albuterol and formoterol, are a mainstay in the acute treatment of asthmatic exacerbations as well as in long term maintenance treatment to prevent exacerbations and decrease chronic airflow restriction. However, multiple adverse effects have been associated with long-term β-agonist therapy [43–51], many of which appear to be related to a progressive loss of receptor function (tachyphylaxis) [43, 44, 46] and thus inability to maintain acceptable airflow. As described earlier, tachyphylaxis to prolonged agonist is initiated by β-arrestin, ultimately leading to receptor downregulation. In addition, the acute responsiveness to β-agonist of airway smooth muscle β2ARs is attenuated by β-arrestin [24] due to the β-arrestin uncoupling process. Thus, there is a clinical need for more efficacious β-agonists with minimal tachyphylaxis. Indeed, as many as 50% of asthmatics experience suboptimal disease control despite concomitant use of muscarinic receptor antagonists and anti-inflammatory agents [52].

A 40 million compound combinatorial scaffold ranking library consisting of 87 sample wells was utilized to test for potential β-agonists by measuring cAMP in cells recombinantly expressing human β2AR or non-transfected control cells. Figure 5a shows that several wells revealed positive signals (cAMP above vehicle, and no response in the control cells), particularly sample well 1319 which had the scaffold shown in Fig. 5b. All possible R-group substitutions were synthesized and arranged by position in mixtures (a positional scanning library), with the results of a portion of that cAMP screening shown in Fig. 5c. From the data, a deconvolution algorithm [40] was used to predict the most likely structures of the active β-agonists (denoted C1 through C12). The S-isomer structures of two of these candidates are shown on Fig. 5d. For reference, the structures of the endogenous β2AR agonist epinephrine, the synthetic full agonist isoproterenol, and the partial agonist albuterol (the most commonly prescribed β-agonist worldwide) are indicated in Fig. 5e.

6 Biasing Away From β-arrestin by a Structurally Novel β-agonist

6.1 Methods to Investigate Biasing

Additional studies confirmed activation of cAMP in a dose-dependent fashion for compounds such as C1-S in β2AR expressing cells, but not in non-transfected cells (Fig. 6a).

The R-stereoisomer of C1 was not active [37]. To examine β-arrestin interaction promoted by C1-S and other selected individual compounds from the screen, four assays were employed [37]: proximity ligation (PLA), enzyme complementation, confocal microscopy of β-arrestin-GFP, and ERK1/2 activation. Results from the PLA (Fig. 6b) showed no evidence for C1-S promoted association between receptor and β-arrestin, whereas the positive controls isoproterenol and albuterol readily promoted detectable association signals. This lack of agonist promoted β-arrestin binding was also observed with C1-S in the other three assays [37]. The closely related agonist C5-S (Fig. 5d) was noted to promote β-arrestin actions in a dose dependent manner and was balanced [37]. To ascertain the functional consequences of this β-arrestin phenotype, two types of desensitization experiments were performed. β2AR-expressing cells were treated with vehicle (control), albuterol, or C1-S for 10 min, washed, and then challenged with isoproterenol. The cAMP response (Fig. 6c) showed ~65% desensitization of the β2AR by albuterol, and no significant loss of function from C1-S pretreatment. Using magnetic twisting cytometry (MTC) [37, 53, 54] to measure the change in cell stiffness of HASM cells (relaxation), a similar protocol was employed to ascertain if functional desensitization was different between albuterol and C1-S. MTC represents an example of measuring a physiologic outcome, which as discussed earlier is important to ascertain if *relevant* biasing is present with an agonist, even when the more reductionist methods have been thoroughly employed. In the MTC experiments, Arg-Gly-Asp-coated ferrimagnetic microbeads are attached to integrin receptors on the HASM cell surface. Beads are magnetized horizontally to cell plating, and then twisted in a vertically aligned magnetic field. Small oscillating forced bead motions are optically detected with ~5 nm resolution (i.e. reflecting cellular stress that opposes forced bead motions), and changes in lateral bead displacements in response to the application of various β-agonists added to the media are measured in real time. A decrease in stiffness correlates with airway smooth muscle cell relaxation. Figure 6d shows that C1-S decreases the stiffness (i.e., relaxes) of HASM cells, consistent with the studies shown in Fig. 6a. To study desensitization of this response, HASM cells were treated for 30 min and for 4 h with vehicle, C1-S or albuterol. At the earlier time point, a ~36% desensitization with albuterol was observed, and a > 80% desensitization at the 4-h time point. In contrast, no statistically significant desensitization of the relaxation response was found with C1-S at either time point (Fig. 6e).

6.2 Potential Mechanisms of C1-S Biasing

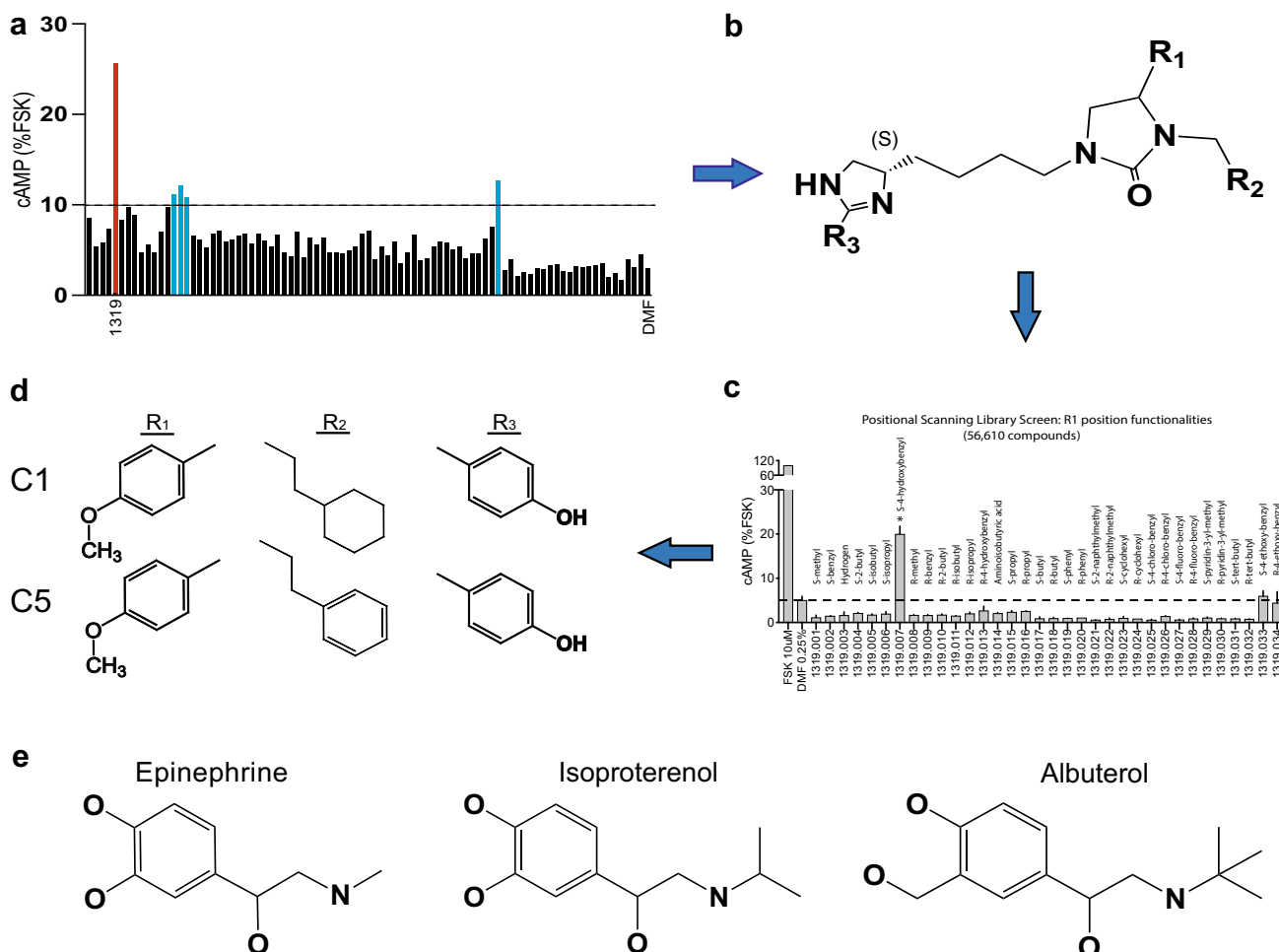Molecular modeling and simulations [37] were employed with the β2AR in explicit membrane and water with

Fig. 5 Elements of screening combinatorial scaffold ranking and positional scanning libraries for β₂AR agonists. **a** β₂AR transfected cells were treated with 87 samples, where each sample contained hundreds of thousands of compounds arranged by scaffold. Sample 1319 had a robust cAMP response over basal (DMF) levels. Line = value >10% forskolin. **b** A positional scanning library composed of the 1319 scaffold with all possible R-group substitutions

was screened by treating β₂AR expressing cells. Shown are results from 56,600 compounds as mixtures where R1 was fixed. Sample 1319.007 revealed a signal and the scaffold with the positions of the R groups is shown in **c**. **d** The structures of two compounds with the R group substitutions on the scaffold as indicated. **e** Structures of common βAR agonists

epinephrine, C1-S and C5-S. These were performed with the receptor alone (the inactive state) or with the receptor complexed with Gs (the active state). The full results are published elsewhere [37], and are summarized in Fig. 7, which shows a 3-D view of C1-S and its position in the TMs (a), and the pharmacophores for the three agonists (b–d). Epinephrine interactions included: hydrogen bonds (HB) to $Ser203^{5.42}$, $Ser207^{5.46}$, $Asn293^{6.55}$, $Asn312^{7.39}$ and $Asp113^{3.32}$; a salt bridge (SB) to $Asp113^{3.32}$; cation–π interaction with $Phe193^{ECL2}$; and a π-stacking interaction with $Phe290^{6.52}$ (Fig. 7b). For C1-S, several important deviations were noted, which results in a different conformation of the stabilized receptor that may contribute to the lack of C1-S-promoted interaction with β-arrestin (Fig. 7c). Both agonists form SBs with $Asp113^{3.32}$, but epinephrine also

forms a HB with $Asp113^{3.32}$. This SB if from the β-carbon hydroxyl of epinephrine, and there is no similar moiety in C1-S (Fig. 5d). Both compounds also have interactions with $Ser203^{5.42}$; however, epinephrine acts as a proton donor while C1-S is a proton acceptor. While epinephrine binds to residues in TM6 (Phe290 and Asn293) and TM7 (Asn312), the analogous interaction from C1-S is only at Asn312 (Fig. 7b,c). The $Phe193^{ECL2}$ interaction with C1-S is π-π stacking, while with epinephrine it is a much stronger cation-π interaction. Recently published data indicate that mutation of $Phe193^{ECL2}$ to Ala decreased β-arrestin binding upon isoproterenol activation [55], so the weaker interaction between C1-S and this residue may be a mechanism responsible for its biasing away from β-arrestin. The TM5 Ser207 interaction seen with epinephrine is not found with
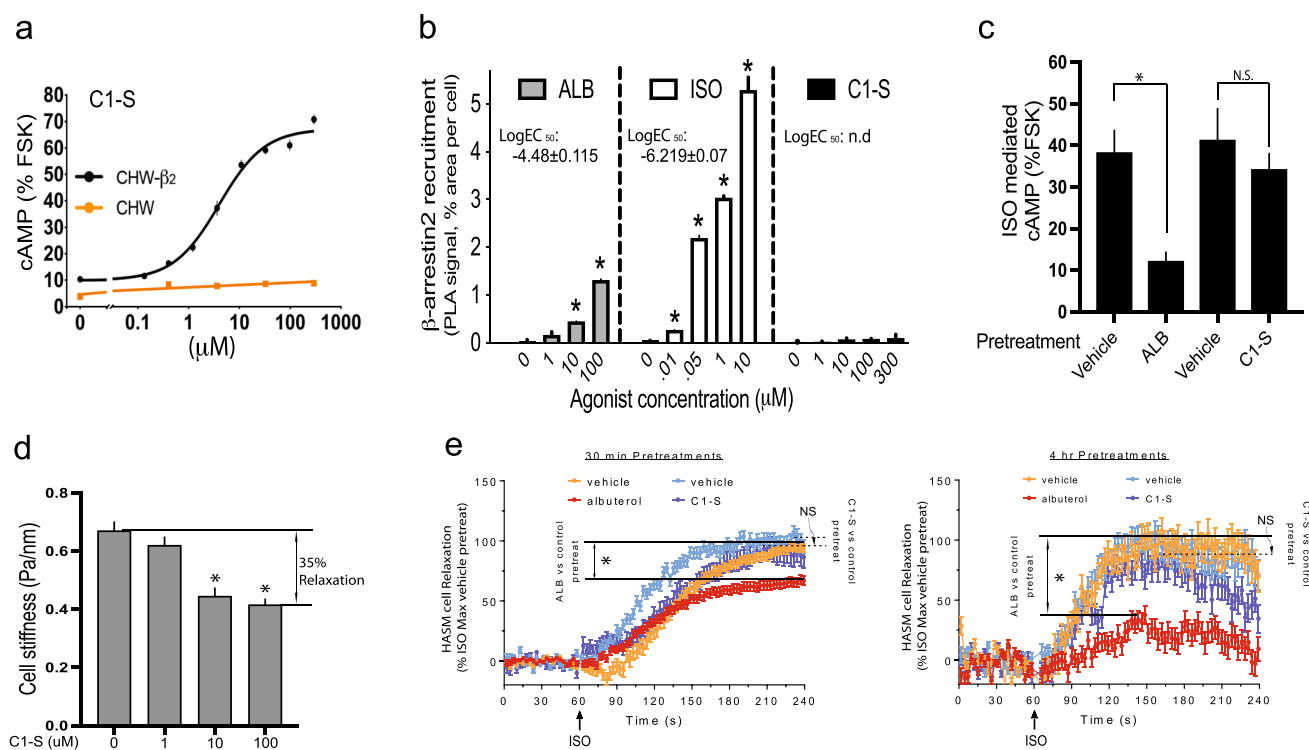
Fig. 6 The biasing of C1-S in pharmacologic and physiologic studies. **a** C1-S stimulated cAMP in a dose-dependent manner in cells transfected to stably express the human β_2AR (CHW-β_2), but not non-transfected parental cells (CHW). **b** β-arrestin binding to β_2AR by the agonists albuterol (ALB), isoproterenol (ISO) and C1-S as determined by the proximity ligation assay. While ALB and ISO promoted β-arrestin binding, C1-S did not, even at concentrations that caused maximal cAMP stimulation. **c** C1-S fails to promote β_2AR desensitization. Cells transfected to express human β_2AR were exposed to vehicle (control), 10 μM ALB or 150 μM C1-S for 10 min, washed, and then challenged with 10 μM ISO and the cAMP response measured. ALB pretreatment resulted in a loss of ISO stimulated cAMP, equivalent to ~68% desensitization. In contrast, C1-S evoked no significant desensitization. **d** C1-S relaxes human airway smooth muscle cells. Cells were harvested from a donor lung and passaged 3-5 times in cell culture as monolayers. Cell stiffness in response to the indicated concentrations of C1-S was measured by magnetic twisting cytometry. **e** C1-S fails to promote β_2AR-mediated relaxation of human airway smooth muscle cells. Cells were treated with vehicle (control), 1 μM ALB or 100 μM C1-S for 30 min or for 4 h, washed, and then challenged with 10 μM ISO and the decrease in cell stiffness (converted to relaxation) monitored in real time. ALB evoked ~ 35% and > 70% desensitization of β_2AR-mediated relaxation with the 30-min and 4-h pretreatments, respectively. C1-S evoked no desensitization. *$p < 0.01$ vs vehicle; *ND* not determined, *NS* not significant

C1-S (Fig 7c). These HBs along with Asn293[6.55] have been implicated in a polar network that promotes β-arrestin binding [56], and their absence in the interaction between C1-S and β_2AR may also contribute to the lack of C1-S-promoted β-arrestin binding. Ser207[5.46] is also implicated with the inward bulge of TM5 which is associated with the outward movement of TM6 upon catecholamine binding. The absence of this TM5 interaction with C1-S may also affect the cognate conformation for Gs coupling or β-arrestin binding. Interestingly, C5-S was found to recruit β-arrestin [37]. This agonist differs from C1-S only by having a terminal benzene in the R_2 position vs a cyclohexane in this position for C1-S (see Fig. 5d). When C5-S was modeled (Fig 7d), it lacked the Asn312[7.39] HB observed with C1-S, and gained the Asn293[6.55] HB, as is found with epinephrine

(Fig 7b). The reorientation of C5-S due to benzene being more exposed to solvent compared to cyclohexane appears to have promoted the TM6 binding, pulling TM3 and TM6 closer together near the top of the receptor. Mutagenesis of the receptor partially confirmed the binding interactions predicted for C1-S and C5-S. Two receptors were generated and expressed in HEK-293 cells: a Asn293[6.55] to Ala substitution, and a Asn312[7.39] to Ala substitution. It was reasoned that C1-S would be unaffected by the TM6 substitution, since there were no interactions found at that residue in the modeling. In contrast, C5-S coupling would be affected. The reverse was expected for the TM7 mutant. This TM7 mutation, however, appeared to markedly compromise the binding pocket, as antagonist radioligand binding was ablated and isoproterenol-stimulated cAMP levels were severely
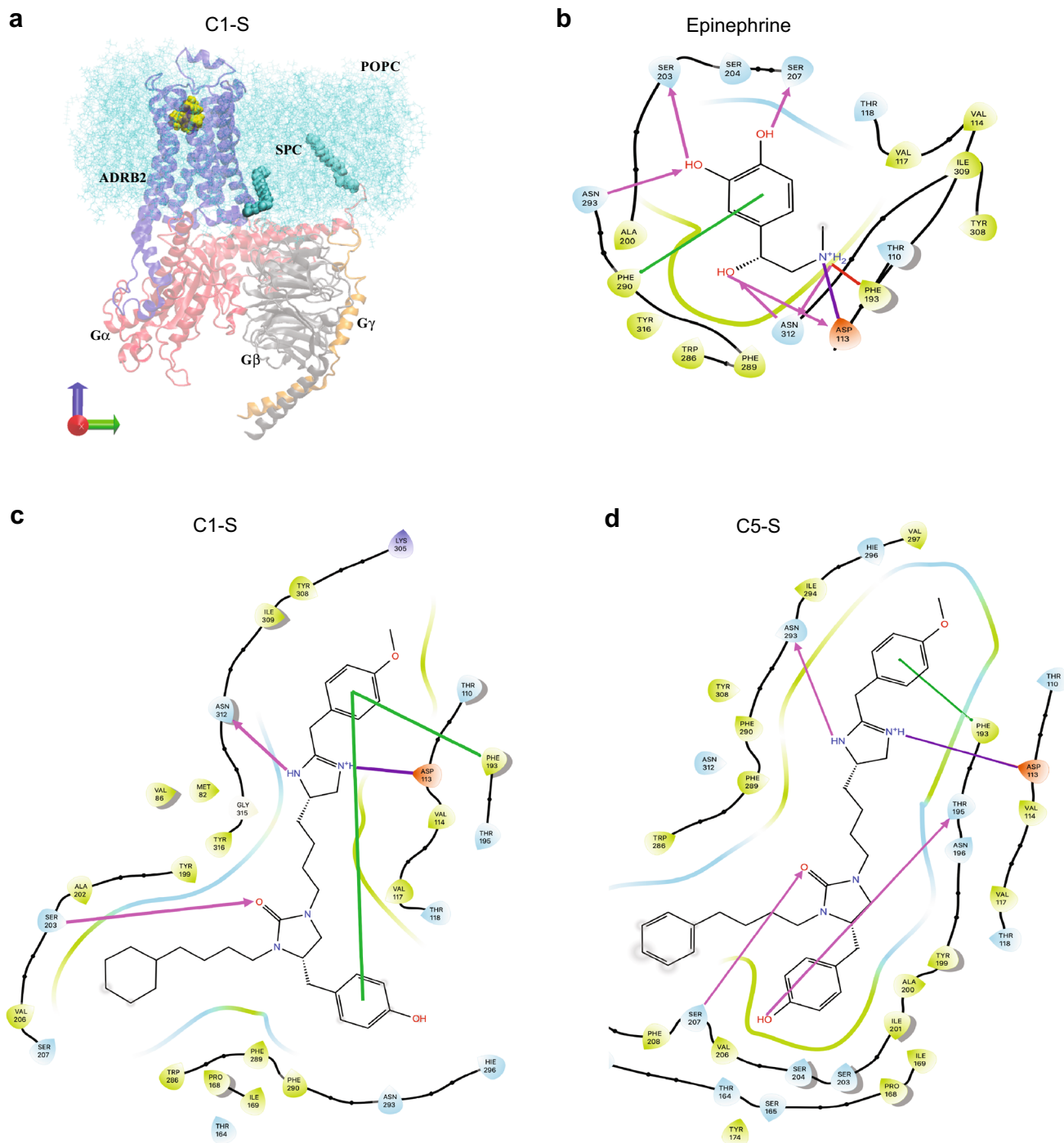
Fig. 7 Predicted binding sites for the indicated agonists at the β₂AR. **a** The activated β₂AR bound to C1-S in complex with Gs in explicit membrane and water as seen from the side. C1-S is indicated in yellow, the receptor is indigo, Gα is red, Gβ is grey, and Gγ is gold. **b–d** Binding interactions with the indicated agonists viewed as pharmacophores. See text for descriptions.

depressed [37]. The TM5 mutant receptor impaired C5-S signaling to cAMP (Fig. 8a), but C1-S signaling was unaffected (Fig. 8b). These results were consistent with the modeling, which showed differential binding of C1-S and C5-S at Asn293$^{6.55}$ (Fig. 7c vs d).

7 Concluding Remarks

Biasing the signaling outputs from multifunctional GPCRs by modification of agonist structure has now been demonstrated for many receptors. Multiple issues remain to be
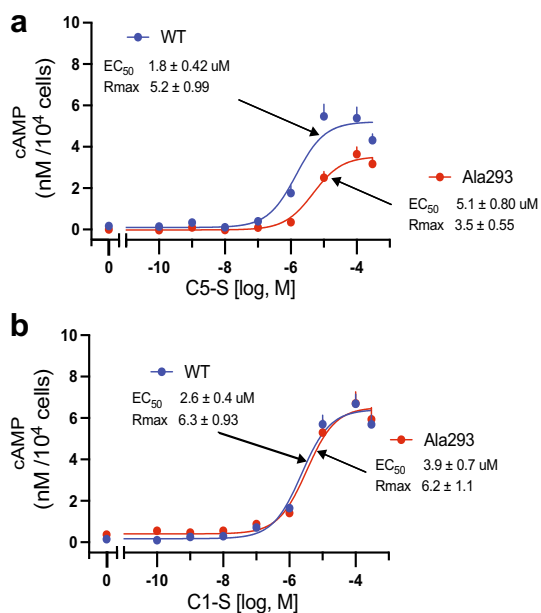
Fig. 8 Mutations of β_2AR confirm the modeling of C1-S and C5-S binding. **a** cDNA mutations were made to encode a mutant β_2AR where Asn293[6.55] was substituted with Ala. In transfected HEK-293 cells, C5-S stimulated cAMP studies revealed a decrease in the maximal response and a rightward shift in the dose-response curve, indicative of impaired receptor signaling with this agonist, and consistent with the modeling which showed a HB interaction between C5-S and Asn293[6.55]. **b** The Asn293[6.55] to Ala mutation had no effect on C1-S activation, consistent with the modelling which showed no interaction with Asn293[6.55] with this agonist, but instead an interaction with Asn312[7.39]. Mutation of the latter residue resulted in a severely distorted binding pocket for all ligands tested (see text) and was not further studied

clarified before these agonists are in routine use clinically. First, it is now of paramount importance to define which signals promote the desired pharmacologic response, which are deleterious, and which have no apparent effect. In addition, pre-clinical experiments with multiple model systems using various analytical methods will be necessary to identify true bias of a compound, as compared to apparent bias that is due to characteristics of the assays or other considerations. Studies using physiologic outcomes, as opposed to purely biochemical ones, are necessary to link a given bias to the relevant cellular event. Methods to identify these unique ligands in moderate to high-throughput ways, with large numbers of structurally diverse compounds, need to be employed. We showed how this was accomplished for the β_2AR, ultimately identifying the elusive biasing away from β-arrestin for agonists at this receptor. As more biased ligands are discovered and optimized, structural modeling at an atomistic level should be performed to aid drug discovery for specific types of biasing. In addition to computational modeling and mutagenesis as was used in the example described in this review, structural experiments using x-ray

crystallography and other methods can be utilized to define the conformational dynamics leading to bias. Whether the aforementioned approaches will show a mechanism that is common within the superfamily, or a subset of receptors, is yet to be realized and remains a challenge.

Declarations

Conflict of interest The authors declare no conflicts of interest.

Availability of data and material Not applicable.

Ethics approval Not applicable.

Consent Not applicable.

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