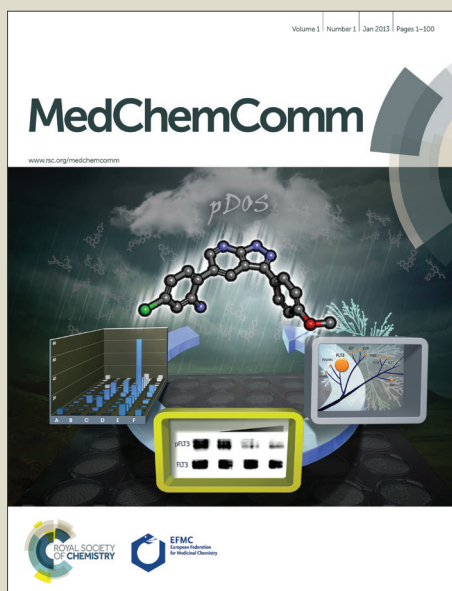


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## Review

# Insights into a defined secondary binding region on $\beta$ -adrenoceptors and putative roles in ligand binding and drug design

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**Abstract.** Increasing evidence points to the possibility of one or more secondary binding sites involved in the interaction of catecholamines on their receptors. However, the importance of these sites has not yet been clearly established. In this review, we examine the possibility that catecholamines reach a defined secondary binding region on beta adrenoceptors and attempt to analyze the approach of this ligand to this binding site. Inferences are made as to the possible effects on receptor activity when a compound interacts with the orthosteric binding site, the secondary binding region, or both in a concerted manner. Consideration is given to prolonged ligand interaction with orthosteric and allosteric binding sites, biased signaling, and feasible cellular responses, as well as to the importance of these effects in physiological processes when these receptors are targets for drug design.

## Introduction

The endogenous catecholamines —dopamine, noradrenaline and adrenaline— act on G protein-coupled receptors (GPCRs) as neurotransmitters or hormones in order to mediate biological responses. It is known that in humans their effects modulate functions in the cardiovascular, pulmonary and gastrointestinal systems, the peripheral and central nervous systems, and metabolic processes.<sup>1-3</sup>

Human catecholamine receptors are divided into five dopamine receptors (D1, D2, D3, D4 and D5) and nine adrenoceptors (AR) ( $\alpha_{1A}$ AR,  $\alpha_{1B}$ AR,  $\alpha_{1D}$ AR,  $\alpha_{2A}$ AR,  $\alpha_{2B}$ AR,  $\alpha_{2C}$ AR,  $\beta_1$ AR,  $\beta_2$ AR, and  $\beta_3$ AR), as well as some variants. These receptors were among the first classified according to the pharmacological profile of endogenous and exogenous ligands,<sup>4,5</sup> and the adrenoceptors were among the first cloned proteins.<sup>1</sup> It is thus not surprising that much effort has been exerted to obtain X-ray structural data about catecholamine receptors in the last few years.<sup>1,6,7</sup>

Computational procedures carried out with recent structural data, specifically from  $\beta_1$ ARs and  $\beta_2$ ARs,<sup>8-10</sup> have provided new information on ligand recognition and receptor activation, and are thus helping to predict key interactions in protein-ligand affinity. Through these theoretical studies a new concept is beginning to emerge: the importance of concerted binding by a ligand to two (or more) binding sites of a receptor, thus stabilizing a certain receptor conformation.<sup>8,11-13</sup>

Hence, the present study focuses on the phenomena that lead to the stabilization of certain receptor conformations induced by the binding of a ligand that follows its pathway to reach the orthosteric

binding site (the well-known site reached by endogenous ligands). Additionally, we discuss the phenomena involved in the ability of a ligand to reach this orthosteric binding site, including the relevance of a defined secondary binding site for physiological processes. Consideration is also given to the conceivable impact of this secondary site on the design of drugs that target these receptors.

For these purposes, we review state of the art theoretical simulations and structure-based drug design using X-ray data from crystal structures of ligand- $\beta$ -adrenoceptor complexes (only  $\beta_1$ ARs and  $\beta_2$ ARs have been crystallized), as well as some static and dynamic models of the three  $\beta$ ARs. Overall, the present analysis of interactions between adrenoceptors and ligands aims to identify well-defined orthosteric and possible allosteric binding sites (the latter with a focus on one defined binding region), the selectivity of ligands for binding on these sites, and the functional selectivity triggered by ligand-receptor contacts. Most importantly, implications for physiology, medicinal chemistry and therapeutics are explored.

## About the role of allostereism in $\beta$ -adrenoceptors

Nowadays it is well accepted that conformational changes occur in receptors in a ligand-free condition or during ligand-binding. It is also accepted that biological activity is a function of the population of receptors stabilized in a certain conformational state. Moreover, it has been recently proposed that we can design molecules with a fine-tuned control of cellular response by considering more than a simple integrated cellular response or a linear view of efficacy. That is, a ligand may cause a sophisticated conformational change in the receptor, leading it to express some, but not all, of its repertoire of activities in the cell.<sup>14</sup>

By limiting ourselves to the currently accepted assumptions (being aware that emerging topics could widen our analysis), we establish that ligand binding to a receptor induces a conformational state that results in activation, inactivation, or maintenance at basal activity.<sup>1,14</sup> In the case of an agonist, including partial or inverse agonists, the induced conformational state alters the basal kinetic activity of a receptor or a system coupled to this receptor. Contrarily, neutral antagonists (rare for adrenoceptors) block the binding of agonists but do not induce conformational changes relevant to cell kinetics.

A ligand can also stabilize a conformational state by binding to an allosteric binding region (a region different than that included in the cluster of residues forming the binding pocket for endogenous ligands). Such a ligand, known as an allosteric modulator,<sup>15</sup> scarcely affects kinetic processes but can greatly influence receptor activity in several ways, including the capacity of ligands to access the orthosteric binding site. Insights into this aspect of receptor activation should certainly afford even greater capacities in drug design<sup>16-20</sup> due to the promise of improved selectivity of ligands targeting these receptors.<sup>21-25</sup> This is particularly relevant for the design of new drugs aimed at the CNS.<sup>10,15,26</sup>

In light of the aforementioned aspects of ligand binding, a new nomenclature for ligands has come about that goes beyond the general terms of agonists, antagonists and allosteric modulators. New terms have been proposed and revised recently, describing molecules that activate or inactivate receptors, as well as those that just bind to allosteric binding sites (sometimes influencing the active/inactive state of receptors).<sup>15,26,27</sup> Recent advances in understanding the structural biology of GPCRs could greatly facilitate the study of allosteric modulation.<sup>15</sup> Hence, the concept of the stabilization of one or more conformational states through allosteric binding is expanding and increasingly attracting the attention of researchers in drug design.

Despite the growing evidence of allosteric modulation of GPCRs, this activity is still poorly utilized in drug design. Indeed, the only allosteric modulator of  $\beta$ ARs confirmed by experimental evidence is zinc (acting as a positive agonist modulator), and even for this ligand the location of the allosteric binding site is poorly defined.<sup>28</sup> The evidence in relation to other ligands is limited to a suggestion of allosteric modulation due to observed effects on the signaling of  $\beta$ ARs.<sup>29-32</sup>

It is worth noting that the probabilities of allosteric modulation increase when  $\beta$ ARs are found in oligomeric forms.<sup>33</sup> However, data on the putative potential of functional homo- and hetero-oligomers of  $\beta$ ARs are scarce, and to date have only described differences in ligand affinity to monomers or oligomers of  $\beta_2$ ARs.<sup>33-36</sup>

### The existence of a well-defined secondary binding region on $\beta$ -adrenoceptors

Considering  $\beta$ AR monomers, we can state that observation of ligand interactions on  $\beta$ ARs has provided experimental evidence of two different agonist affinity values in binding assays. This has led to the hypothesis of two binding sites and/or two or more conformational states involved in these interactions.<sup>10,37-42</sup> Evidence exists to support the idea that these binding sites or conformational states can influence not only the intrinsic activity of a ligand or selective signaling in the targeted cell, but also the time-dependent effect.<sup>10</sup>

Some experimental data support the hypothesis that ligand binding induces a certain conformational state of a receptor. Such evidence has been obtained through the use of radiolabeled ligands in binding assays,<sup>38,41-42</sup> as well as fluorescent or bioluminescent resonance energy transfer.<sup>10,43</sup> Moreover, some computational studies support

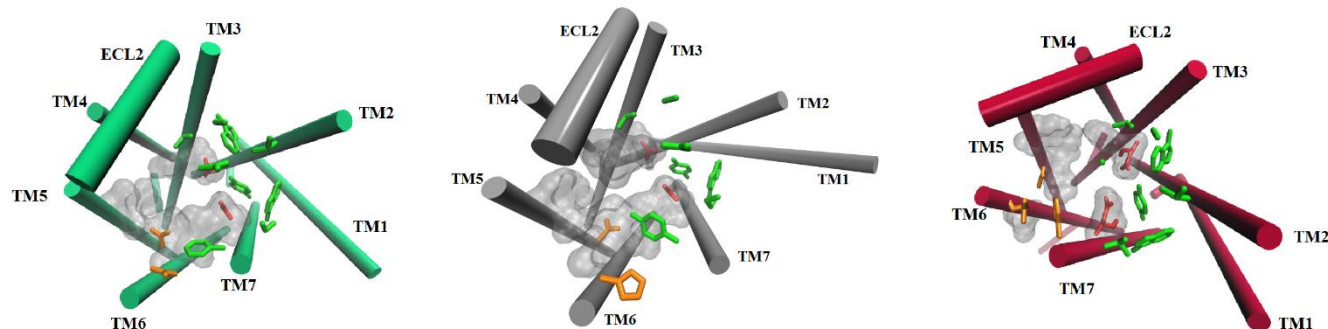
the existence of distinct GPCR conformational states, which may correspond to the variants in signaling found experimentally.<sup>44-49</sup> On the other hand, experimental ligand saturation analysis supports the existence of two distinct binding sites on  $\beta_1$ ARs (and probably on other  $\beta$ ARs)<sup>38,50,51</sup> for several ligands with agonist properties, including catecholamines.<sup>52-54</sup>

Each binding site must necessarily be associated with different binding residues within the receptor protein.<sup>15</sup> The recognition of some compounds by distinct amino acid residues has been evidenced by X-ray crystallographic studies as well as some docking simulations on  $\beta_2$ AR structures.<sup>10</sup> A similar condition has been found for cholinergic and dopamine receptors.<sup>21,55-56</sup> We can also consider that different ligands can interact at the same binding crevice as endogenous ligands, but with a greater contact surface.

Overall, this emerging evidence leads to the consideration of a more broadly defined binding site with different and well-defined regions. Accordingly, there is the region reached by endogenous catecholamines in the adrenoceptor pocket (the orthosteric binding site) as well as a second region in the same crevice (the allosteric binding site). We herein consider a secondary binding region that is near the orthosteric binding site, but located shallower in the crevice.

This suggestion is supported by theoretical and experimental studies with several compounds whose structure has two phenolic rings linked by a pair of ethylamine moieties sharing the amine moiety in the center, as is the case for fenoterol derivatives.<sup>57,58</sup> Some X-ray crystal structures of  $\beta_2$ AR-ligand complexes show that an aryethyl moiety is exposed to what we suggest to be the secondary binding region. In this respect, there are other crystallized structures including a non-adrenoceptor GPCR (e.g., ZM241385 on the  $A_2A$  adenosine receptor, JDTC on the  $\kappa$ -opioid receptor, and naltrindole on the  $\delta$ -opioid receptor) that also have an analogous moiety exposed to a similar binding region of their respective targeted receptor. This may mean that this secondary binding region in the crevice of  $\beta$ ARs is important for the modulation of selectivity by ligands acting on some other GPCRs.<sup>10</sup> Although in-depth discussion of this topic is beyond the scope of the present contribution, it should be mentioned that there is a wide variety of residue sequences in the receptors of these crystallized structures (considering class A GPCRs),<sup>59</sup> and that the receptors are related more by a similarity in the structure of the compounds targeting them.

In spite of the evidence that secondary binding regions may exist in some GPCRs, the precise coordinates of one or more secondary binding regions is an unresolved topic for  $\beta$ ARs. Site-directed mutagenesis studies coincide in suggesting some of the residues that may be involved in a secondary binding region, either inside or near the crevice of the orthosteric binding site. According to these studies, the residues involved are located in the TM2 to TM7 and ECL2 domains, as have been theoretically proposed and mapped on  $\beta$ AR structures (punctual examples for  $\beta_1$ AR and  $\beta_2$ AR have been described previously).<sup>10</sup> Although many differences in the sequence of extracellular and transmembrane regions near these binding sites have been described, some sub-regions among  $\beta$ ARs are conserved (Fig.1).<sup>60</sup> Interestingly, some theoretical simulations indicate that the secondary binding region is present in the conformational state that shows the highest ligand- $\beta_2$ AR affinity values. Taking into account all of this information, we propose that the secondary binding region consists of residues in the TM2, TM3, TM6 and TM7 domains, with a contribution from some residues in the second extracellular loop (Table 1).<sup>32</sup>



**Fig. 1.** The location of the secondary binding region in  $\beta$ ARs is marked with residues in green. The orthosteric binding site is depicted as a silver surface on each receptor, and some residues probably involved in ligand-induced actions in the two binding regions are represented in orange. See Table 1 for details of residues forming the second binding region shown as bonds in this figure.

**Table 1.** Putative amino acids included in the proposed secondary binding site for  $\beta$ ARs. They are colored in green if included in the putative secondary binding region, in red if shared with the orthosteric binding site, and in orange if in a region that seems to modulate the connection of these two parts of the binding site. Residues repeatedly mentioned in the bibliographic references are in bold.

Ballesteros-Weinstein position	With side chain contacts between ligands and		
	$\beta_1$ AR <sup>a</sup>	$\beta_2$ AR <sup>a</sup>	$\beta_3$ AR <sup>a</sup>
2.64	Leu101	His93	Leu97
3.28	Trp117	Trp109	Trp113
3.29	Thr118	Thr110	Thr114
3.32	Asp121	Asp113	Asp117
ECL2.50	Cys199	Cys191	Cys196
ECL2.51	Asp200	Asp192	Ala197
6.55	Asn310	Asn293	Asn312
6.58	Asn313	His296	Arg315
7.35	Phe325	Tyr308	Phe328
7.36	Val326	Ile309	Leu329
7.39	Asn329	Asn312	Asn332
7.40	Trp330	Trp313	Trp333
7.43	Tyr333	Tyr316	Tyr336

## Dualsteric-anchoring on $\beta$ ARs and its impact on cellular responses

Evidence exists that the interaction of compounds at the proposed secondary binding site (Fig. 2) could be responsible for modulating cellular responses. In the present contribution, we make inferences from studies employing  $\beta_2$ AR ligands with findings that seem to suggest a secondary binding site, evidenced by changes in the effects of signaling pathways, including in time-dependent  $\beta$ AR-ligand activity, or by phenomena described in functional assays on these receptors.

Recently, the interaction of ligands on GPCRs has been shown to modulate several pathways. Some of these ligands preferably induce the modulation of one of these pathways. This kind of ligand has been named a biased ligand and the phenomenon induced is denominated functional selectivity.<sup>61-67</sup>

Circumscribing the study to  $\beta$ ARs and their ligands, it should be borne in mind that these receptors trigger a biological response classically through  $G_{\alpha s}$ -protein coupling. However, it is known that these receptors can interact with other heterotrimeric G-proteins and other types of receptors, probably through mechanisms that regulate biological activity without involving protein interaction in the first transduction signaling step (e.g., calcium release and related phenomena).<sup>68</sup> In spite of this, current knowledge about the  $\beta$ AR-ligand interaction is centered on two pathways: G protein-dependent signaling and the  $\beta$ -arrestin pathway. The latter is closely related to ERK (Extracellular Signal-Regulated Kinases)-dependent signaling. Some ligands reportedly are able to induce biased signaling after interacting with these receptors.

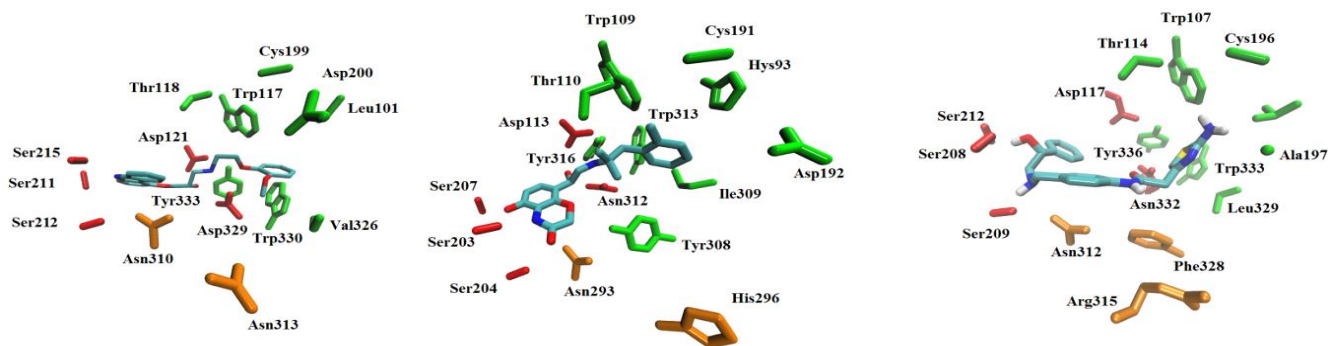
Regarding the biased action of ligands on GPCRs, evidence from theoretical and experimental studies corroborates the association of G-protein dependent pathways with the interaction of ligands on the orthosteric binding site involving the TM3, TM5, TM6 and TM7 domains, and the relation of the  $\beta$ -arrestin pathway to the contact of ligands with TM2 and TM7. Specific contacts have been suggested for the  $\beta$ -arrestin pathway, such as that between the motif NPXXY and switches involving the TM2, TM3, TM6 and TM7 domains.<sup>16</sup> Also, recent research on  $\beta$ -arrestin activity triggered by GPCRs supports the involvement of these transmembrane domains in this functionality, including studies that measure the conformational changes of the TM7 domain of  $\beta_2$ ARs by nuclear magnetic resonance.<sup>8,10,69,70</sup>

Some studies on crystal structures of  $\beta$ ARs or using theoretical simulations report compounds that fit into both the orthosteric binding site and the proposed secondary binding site in a concerted manner. Such studies could yield insights into biased or unbiased signaling by these ligands. In the case of unbiased signaling, ligands can modulate biological activity by activating both these pathways in a concerted manner that is almost simultaneous.<sup>69</sup> An example of this effect is provided by dobutamine, which through the  $G_{\alpha s}$  protein pathway induces 100% of the signaling found with isoproterenol, and via the  $\beta$ -arrestin pathway 70% of such signaling.

Most recent reports focus on biased signaling by ligands, with the greatest attention paid to the  $\beta$ -arrestin signaling pathway (e.g., the ERK pathway) mediated by binding in the aforementioned secondary region.



## Review



**Fig. 2.** Compounds on the binding site of the  $\beta_{1-3}$ ARs. Key residues in  $\beta_1$ AR,  $\beta_2$ AR and  $\beta_3$ AR (from left to right, respectively) for ligands contacting the putative binding site. Carvedilol on the  $\beta_1$ AR (from the structure with PDB code: 4AMJ; BI-167107) and on the  $\beta_2$ AR (from the structure with PDB code: 3POG). Miragebron on  $\beta_3$ AR from molecular modeling. Residues are colored in accordance with the related functional selectivity (see Table 1 and text for details).

These possibilities have been explored in recent years for  $\beta$ ARs as well as for other GPCRs. For example, carvedilol and bucindolol are antagonists on the adenylyl cyclase (AC) pathway but agonists on the  $\beta$ -arrestin pathway, and induce desensitization and internalization of receptors presumably by the latter mechanism.<sup>71,72</sup> Indeed, recent X-ray crystal structural data from complexes of  $\beta_1$ ARs suggest that these effects are caused by the binding of moieties of these ligands to a region shallower than the orthosteric binding site, leading to G protein-independent  $\beta$ -arrestin activation.<sup>10,73</sup>

In some crystallized complexes with a  $\beta_2$ AR, it can be observed that the ligand that reaches both the orthosteric and secondary binding sites.<sup>10,74,75</sup> This is the case with X-ray crystal structures of a  $\beta_2$ AR and BI-167107 or FAUC50. It is becoming apparent that these two compounds behave as long-term full agonists on the  $G_{\alpha s}$ -protein dependent pathway without any apparent activation of the  $\beta$ -arrestin pathway, which would of course imply that they are biased ligands.<sup>10</sup>

There are no crystal structures of  $\beta_3$ ARs, and few functional assays have approached the biased signaling of compounds on this receptor.<sup>76</sup> However, based on analysis of theoretical studies and experimental evidence measuring the biological activity of some proposed and synthesized compounds, it is possible to see that all known selective ligands for this  $\beta$ AR share a dual-core structure, elongated on one of the sides of the amine group (Fig. 3).

Furthermore, the binding of a ligand to this secondary binding site may not only control the intrinsic activity or signaling pathways, but also the time-dependent effect of ligand binding to  $\beta$ ARs. Hence, this secondary binding region could act as a modulator of time-dependent activation of  $\beta$ ARs by ligands, which would have several consequences in cellular responses.<sup>10</sup> For instance, when BI-167107 and FAUC50 reach the secondary binding site and orthosteric binding site in a concerted manner,<sup>77</sup> ligand binding could stabilize an active conformational state which favors prolonged activity. It is also possible that by means of this conformation of the receptor, which could be associated with the regulation of its expression on the cell membrane, the phosphorylation process is reduced or

prevented. A detailed structure-activity relationship about recently developed ultra-long acting  $\beta_2$ AR-ligands<sup>78-80</sup> can be found in previous studies.<sup>10</sup>

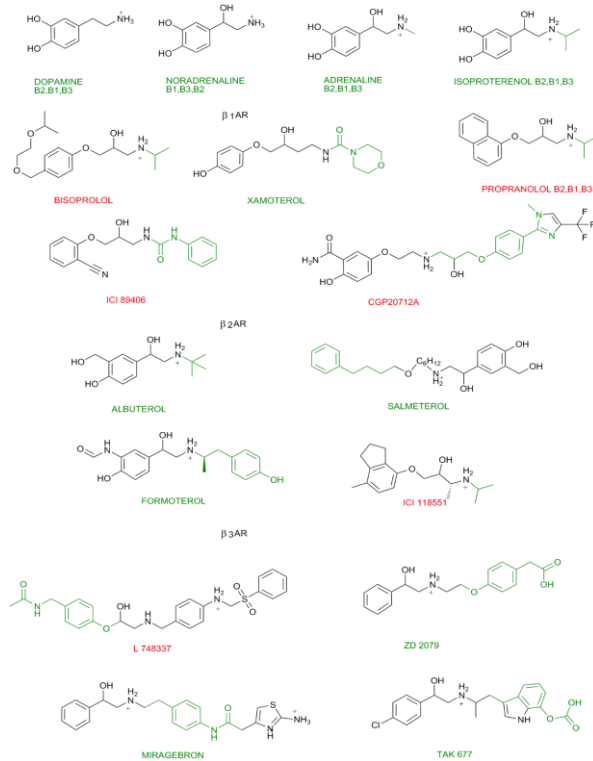
### The chemical features of ligands in the secondary binding site and functional implications

The detailed functionality of this secondary binding site is only beginning to be studied. In order to analyze the particular ligand moieties related to the activation or inactivation of the  $G_{\alpha s}$ -protein dependent and/or  $\beta$ -arrestin pathways, we should consider some structural features of ligands. With this aim, we turn to a 4-phenol-ethanolamine (catecholamine-like) moiety that is often considered the 'core' in  $\beta$ ARs-ligands (Fig. 3). This core, the shared structure of endogenous ligands having only a few variations, is frequently included in compounds proposed as innovative  $\beta$ AR-ligands. In the few cases in which a change in this core exists in the latter ligands, the compounds commonly behave as bioisosteres of the core structure.

It is interesting to compare adrenaline and noradrenaline. Whereas the former has a hydrogen atom attached to the amine group, the latter has a methyl group attached to the same core. Since the methyl group is somewhat bulky compared to the hydrogen atom, adrenaline can less easily fit into the binding pocket. Thus slightly bulkier ligands induce different conformational states and behave with a slightly biased effect.<sup>81,82</sup> In this sense, in experiments on  $\beta_2$ ARs, noradrenaline caused signals related to conformational changes in a proportion close to 50% of those induced by adrenaline. Furthermore, noradrenaline-induced changes were slower (almost by one third) than those induced by adrenaline. However, noradrenaline was almost as efficient as a full agonist in causing activation of the catalytic activity of  $G_{\alpha s}$  and ACs, while being inefficient for triggering  $\beta$ -arrestin2 recruitment to the cell surface, its interaction with  $\beta_2$ ARs, and the internalization of these receptors.<sup>54</sup>

It has also been observed that exogenous ligands, such as isoproterenol, bucindolol and propranolol, share a relatively bulky

isopropyl moiety linked to the amine group. All three of these ligands have high activity at the  $\beta$ -arrestin pathway, even though they are full, partial, and inverse agonists for the AC-pathway, respectively. Molecules with an even bulkier moiety attached to the amine group, including albuterol, clenbuterol and terbutaline (sharing a tertbutyl moiety), act as agonists for cAMP production, while displaying an even weaker effect on the  $\beta$ -arrestin-mediated phosphorylation pathway.<sup>82</sup> In these cases, it seems that steric hindrance near the amine group blocks activation of the  $\beta$ -arrestin pathway. This apparently does not occur if the ligand has a simpler moiety with carbons adjacent ( $\alpha$  or  $\beta$ ) to the amine group.



**Fig. 3.** Ligands with selectivity for the  $\beta$ ARs. The moieties proposed to contact the secondary binding region of  $\beta$ ARs are marked in green. The labels are in green letters for ligands considered as a full or partial agonist, and in red for ligands considered as an antagonist or inverse agonist.

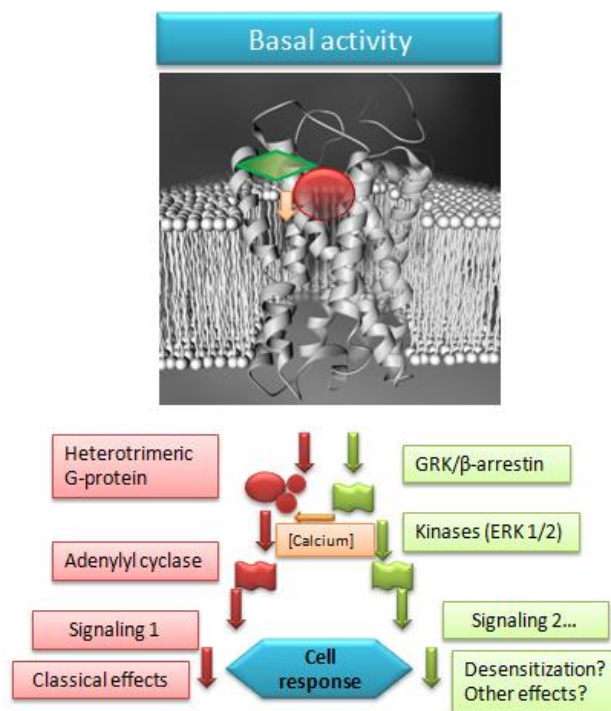
Additional studies should be carried out to identify moieties adjacent to the amine group in ligands, as well as to consider the whole binding site reached within the context of a dynamic perspective (due to observations that do not exactly correspond to our somewhat simplistic model). For example, other compounds such as procaterol and cimaterol (with only an isopropyl moiety linked to the amine group, and inflexible moieties in the catechol-related group) also show a weak effect on the  $\beta$ -arrestin pathway.<sup>82</sup> Recently developed compounds include a variety of other moieties linked to the nitrogen atom of the amine group (included in the core). These linked moieties fluctuate from isopropyl to 'long-tail' aryl groups.

In order to avoid a dispersion of attention among the variety of these linked moieties attached to the core, we focused the present analysis on some of the moieties often included in the structure of recently developed compounds. For instance, it has been suggested that large

non-polar groups are particularly effective in the activation of the  $\beta$ -arrestin pathway.<sup>73</sup> By trying to develop a more specific profile of the relationship between structural moieties in  $\beta_1$ AR or  $\beta_2$ AR ligands and activation of the signaling pathway of AC or  $\beta$ -arrestin (ERK1/2), and taking into account a previous bidimensional model, we proposed a Cartesian representation of the ligand profiles on  $\beta_1$ ARs and  $\beta_2$ ARs, based on the possible activation of one of these two signaling pathways.<sup>10</sup>

From this model, we observed that ligands require the core (4-phenol-ethanolamine) moiety for AC-activation (Figs. 3 and Suppl. Fig. 1). In order to trigger full signaling by this pathway, a compound must have an additional hydroxyl group (or a 'bioisosteric' nucleophilic moiety) in  $\beta$ -carbon to amine and in position 3 of the aromatic ring (Fig. 3). This moiety seems to establish more interactions with serines in the TM5 domain, particularly with Ser5.46, a residue involved in the contacts for full but not partial agonists.<sup>83</sup> On the other hand, it can be observed that the hydroxyl-propylamine moiety (Fig. 3 and Suppl. Fig. 1) confers ligands with the ability to act as antagonists or inverse agonists through the AC-pathway.

Judged from the compounds activating the  $\beta$ -arrestin pathway, it seems that this signaling mechanism is induced by a fragment group almost equivalent to one of the aryl-ethyl moieties activating the AC-pathway (Fig. 4). It is also notable that the moieties of the  $\alpha$ -carbon to amine<sup>84</sup> and/or that in the ortho or para position on the aromatic ring of the arylethylamine are relevant for activity induced through the  $\beta$ -arrestin pathway.<sup>10</sup>



**Fig. 4.** Plurimechanistic efficacy of  $\beta_{1-3}$ AR ligands affected by the interactions of compounds with the orthosteric binding site (in red) and the secondary binding region (in green), as well as additional interactions on the interphase and possible consequences.

Based on the aforementioned analysis as well as evidence and results from biophysics studies, one aryl-ethyl moiety appears to bind to the orthosteric binding site and the other to the nearby shallower region. Moreover, we proposed that the shallower secondary binding region can be anchored by ligands only when an adequate conformational

state allows it to be exposed in the  $\beta$ ARs, often induced by specific moieties in a ligand that can make the adequate arrangements between the TM3 and TM6 domains.<sup>10</sup>

Alternatively, there are molecules with a double-core moiety (e.g., dobutamine) that behave as an unbiased ligand,<sup>85</sup> and those with a long tail but without a bulky moiety attached contiguously to the amine group (e.g., salmeterol, formoterol and carmoterol, which share two core moieties in their structure) that can apparently fit into the orthosteric binding site and shallower secondary region in a concerted manner. Moreover, the synthetic agonist fenoterol and to a lesser extent terbutaline appear to display bias toward  $\beta$ -arrestin signaling.<sup>54</sup>

Taking all of this information into account, we propose that a concerted fit into both the orthosteric binding site and the secondary region seems to take place in one of two ways: by a long-tailed molecule (with a non-bulky moiety near the amine group) in a synchronized step, or by two small molecules in two consecutive steps. If the latter is the case, the binding of the small molecules (such as endogenous ligands) to each of the nearby surfaces on the receptor may happen almost simultaneously, which is apparently the case for  $\beta$ ARs.

### The putative roles of the secondary $\beta$ AR binding region in modulating the action of endogenous ligands

Most of the previously discussed data comes from studies on synthetic compounds acting as ligands. However, a critical issue is the relevance of the secondary binding region under physiological conditions, in which the endogenous ligands could fit into this binding site and either induce or not induce conformational changes related to the triggering of cellular signaling. The coupling of ligands in this region could be involved in many other processes, including recognition selectivity (e.g., the selectivity among endogenous ligands for reaching different receptors of a same group), biased signaling, and time-dependent effect. This idea is congruent with the fact that more diversity exists in the extracellular regions than in transmembrane domains among GPCRs.<sup>10,54</sup> Accordingly, we discuss potential physiological roles of these binding sites in  $\beta$ ARs based on data from X-ray crystal structures or theoretical simulations related to ligand- $\beta$ AR interactions.

Regarding the phylogenetic profile of this secondary binding site, among  $\beta$ ARs there are binding sites with similar dimensions and components in spite of the great diversity of these receptors (Suppl Fig. 2). Moreover, homology is high among the receptors incorporated in the aminergic cluster of the  $\alpha$ -group within the Rhodopsin family, including the crystallized Rhodopsin receptors, adrenoceptors, and dopamine and histamine receptors (Suppl. Fig. 3).<sup>86</sup> However, these binding sites are not clearly defined in other GPCRs families (data not shown). Specifically, among  $\beta$ ARs, the established phylogenetic proximity of the  $\beta_2$ AR and the  $\beta_3$ AR (judged from the analysis of the whole sequences) is lack if only the segments involved in the proposed secondary binding region are considered, because in these regions the highest sequence similarity is between  $\beta_1$ AR and  $\beta_3$ AR, which could be involved not only in the behavior of compounds with high/low affinity states reported from pharmacological assays (Fig. 5), but also in the physiological role of adrenoceptors and other GPCRs.<sup>42</sup> Actually, judging from data of other crystallized GPCRs, the analogous areas to this secondary binding site are conserved<sup>87</sup> and often are exposed to the extracellular face of the receptor (e.g., for opioids and chemokines) or unclearly delimited (e.g., for muscarinic, adenosine or sphingolipid receptors).<sup>10,86,88</sup>



**Fig. 5.** Phylogenetic tree of human  $\beta_{1-3}$ ARs by considering all the residues included in the transmembrane domains (on the left) or only those involved in the secondary binding region (on the right), as listed in Table 1. Plots done with GPCRDB software.<sup>110</sup>

In fact, considering that catecholamines reach the orthosteric binding site of  $\beta$ ARs, it cannot be discarded that endogenous (or other small) compounds can reach the secondary binding site at a different time (i.e., in a step previous or subsequent to the fit into the orthosteric binding site). It is noteworthy that several theoretical simulations have shown the possible pathways for reaching the orthosteric binding site as well as the proposed secondary binding region. For instance, docking and molecular dynamics simulations have been done with ligands that interact with this defined secondary region.<sup>10,19,89</sup> Moreover, some MD simulations have demonstrated that the ligand anchors itself to this alternative binding region.<sup>90</sup> Hence, the anchoring of a ligand to this secondary binding region is probably involved in determining which of two (or more) possible pathways a ligand follows to approach the orthosteric binding site, and possibly involved in whether or not there is functional selectivity (Fig. 4).<sup>91</sup> As aforementioned, different approaches applied to study the activity of endogenous ligands suggest that biased signaling also exists in their respective adrenoceptors, specifically in  $\beta_2$ ARs.<sup>54</sup>

To infer the possible mediated effects under physiological conditions for the binding of endogenous ligands to this secondary binding site, we assumed: (a) that ligands can reach this binding site, and (b) that this capacity is closely related to activity in the  $\beta$ -arrestin pathway (in agreement with the previous discussion) as well as to activity in other G-protein independent pathways.

We suggest a diversity of ways in which a ligand can anchor itself to this secondary binding site, based on the versatility of pathways by which a ligand reaches the orthosteric binding site of  $\beta$ ARs. It is also convenient to remember that  $\beta$ -arrestins, although originally discovered in relation to their ability to desensitize activated GPCRs, are now well established mediators of receptor endocytosis, ubiquitination, and G protein-independent signaling.<sup>72</sup> Among a multitude of possible effects, we emphasize that the reaching of this secondary binding site and the activation of arrestins is closely related to desensitization and modulation of cell signaling. And due to the aforementioned differences in the dynamics for reaching the orthosteric binding site, we infer that adrenaline has a greater effect than that induced by noradrenaline or dopamine. This down-regulation system can be related to a protective mechanism in the cases of abundantly expressed catecholamines and their prolonged availability to receptors and cells. The results of some studies agree with this viewpoint, and classify noradrenaline as a  $G\alpha$ -biased agonist, and adrenaline and bulkier agonists as compounds that have stronger action on  $\beta$ -arrestins and lead to desensitization.<sup>54,72</sup>



In order to illustrate these putative roles, we will use a specific example of each of the three known  $\beta$ ARs in humans. Although these receptors are ubiquitous, some tissues selectively express one subtype. Classically, the  $\beta_1$ AR is expressed in the heart, the  $\beta_2$ AR in smooth muscle, and the  $\beta_3$ AR in adipose cells and the genitourinary tract.

In the case of  $\beta_1$ ARs, several phenomena lend themselves to the inferences made herein. The contacts of ligands on this secondary binding site have implications for the down-regulation of  $\beta_1$ ARs and the response in the myocardium.<sup>92</sup> Indeed, studies with compounds that contact this binding site, specifically carvedilol and bucindolol (for which contacts have been demonstrated through X-ray crystal structures), have yielded insights into their probable role.<sup>93</sup> Initially, these compounds were only proposed as  $\beta$ -blockers. However, their use in this sense led to reports on atypical phenomena and additional protection for individuals who employed them, prompting researchers to analyze the particular pharmacological profile of these compounds.<sup>38,94</sup> Observations guided them to denominate these  $\beta$ -blockers as atypical, because they can block cAMP production while triggering signaling through the  $\beta$ -arrestin pathway. These phenomena have been recently studied, and results suggest that atypical behavior is related to our poor comprehension of ligand-receptor interactions, including those which occur outside of the orthosteric binding site.<sup>72,91,93,94</sup>

Additional elements may also be involved in the ability of compounds to reach this secondary binding site. Of particular interest is the phenomenon described as the dual behavior of  $\beta_1$ ARs due to the existence of two conformational states or binding sites with low or high affinity. Regarding the coordinates of this secondary binding site, some reports have suggested that residues in TM3 are involved, but additional features of this site are lacking. The importance of this secondary binding site has been related to alterations in the natural role of  $\beta_1$ ARs in myocardium and the physiopathology of some types of cardiac failure, as well as to the action of some drugs targeting these receptors (i.e., secondary effects during the treatment of orthostatic hypotension and neurocardiogenic syncope).<sup>38,93</sup>

Consequently, it can be suggested that when endogenous ligands reach this binding site, a G-protein independent signal is triggered, which would be related to the down-regulation of receptors and intimately linked to the clinical and experimental phenomena observed for atypical ligands on  $\beta_1$ AR. Also, taking into account the slight structural differences among endogenous ligands, we suggest that this binding site and the associated response are triggered in a more efficient form by adrenaline. This could be related to the fact that a catecholamine response exerted in the acute phase is mediated mainly by adrenaline. Given that this compound is designed for a short-term 'fight or flight' response, and that a sustained stimulation could generate dysfunction and greater damage, it should diminish the prolonged effect.<sup>95</sup> In other words, the proposed secondary binding site appears to be key to the molecular behavior of the receptors for preparing an acute modulation in the presence of some kinds of catecholamines (e.g., adrenaline), but to a lesser degree (or not at all) for other ligands even if they can activate a notable response in the target cell.

In the case of  $\beta_2$ ARs, atypical patterns have also been registered in relation to the pharmacological evaluation of some compounds. These atypical behaviors include high and low affinity states, ligand-dependent expression, and ineffective transduction related to collateral efficacy.<sup>96</sup> In this regard, differences among species have been clearly established, although the secondary state or binding region has been poorly mentioned for human  $\beta_2$ ARs (except for the

case of some human cells with receptor-overexpression).<sup>97</sup> Nevertheless, differences in cellular responses have been observed when using compounds that probably bind to the secondary region.

The same mechanism for regulating receptor expression allows the organism to regulate the action of noradrenaline or adrenaline on the expressed receptors in smooth muscle cells, thus modulating the cellular response. This effect is differently activated for endogenous and synthetic agonists, the latter of which often contain a bulkier than methyl group linked to amine (see discussion about  $\beta_2$ AR-ligands). Therefore, the phenomena related to an acute G-protein independent signal or to desensitization of receptors are poorly linked to the results found by testing endogenous ligands. Since these phenomena are linked to interactions in one or more secondary binding regions, their close examination is important for the development of new selective ligands on this receptor subtype.

Current experimental data for  $\beta_3$ ARs do not indicate any physiological relevance of a secondary binding site. Despite this experimental void, if we consider the possibility of high and low conformations or binding sites for endogenous ligands (as is the case for  $\beta_1$ ARs), as well as the suggestion that the secondary and the orthosteric binding sites overlap,<sup>98,99</sup> we can infer a key role for the secondary binding site in modulating acute or chronic exposure to a high concentration of endogenous catecholamines.

Interestingly, compounds have been developed with selectivity for this receptor over the other  $\beta$ ARs, and these compounds have a double catecholamine-like pharmacophore. Although this feature is similar to that of compounds that reach  $\beta_2$ ARs, there are some details that may be key in the high selectivity for this receptor, such as the inclusion of a carbonyl group in para-position of a ring that presumably fits in the secondary binding region (Fig. 4).<sup>100,101</sup> Indeed, the advances in this area could be key for developing compounds with high selectivity for either  $\beta_2$ ARs or  $\beta_3$ ARs.

### Consequences for drug design

Given that concerted multiple signaling pathways lead to collateral efficacy in a cell, acceptance and analysis of this secondary binding region near the classic orthosteric binding site in the  $\beta$ ARs may have important implications for drug discovery. If corroborated by future research, this perspective could be applied to other regions or fragments of GPCRs, or perhaps other receptors.

Hence, in the design of new compounds targeting  $\beta$ ARs, consideration should be given to reaching of this secondary binding region in order to improve the selectivity of ligands on a given subtype of receptors. This would selectively modulate the activity of cells expressing these receptors and the time-dependent effect. Additionally, by more broadly considering the pharmacodynamics and pharmacokinetic profile of new test ligands that reach the secondary binding region, more specific applications can be sought. In this sense, specific studies aimed at enriching the characteristics of moieties linked to the amine group in the core of ligands should improve selectivity on these receptors and may establish a quantitative structure-activity relationship.

Moreover, other studies should be designed to further elucidate the physiological and probably physiopathological importance of this binding region near the orthosteric binding site, paying attention to the analysis in humans of previously designed or well-known compounds (due to the difference between humans and other animal species<sup>98</sup>).

Furthermore, it should be mentioned that the importance of this secondary binding region in the modulation of signaling pathways has been suggested for non-adrenoceptor GPCRs, particularly for



those whose endogenous ligands are bioactive amines (catecholamine, acetylcholine, serotonin, etc.).<sup>102-107</sup> This secondary region may also be implicated with peptide-related ligands (e.g., JD1c on the  $\kappa$ -opioid receptor, naltrindole on the  $\delta$ -opioid receptor, and the neuropeptide enkephalines), which have an aryl-amine fragment in the first position that seems to reach a binding site in a region homologous to that discussed in this review. Moreover, it has been demonstrated that the modification of this fragment alters the potency and long-lasting action on opioid receptors.<sup>108,109</sup>

In addition, it is expected that each year many new compounds are developed and tested in its ability to act on  $\beta$ ARs, and increasing evidence is related to the use of different compounds for each case, triggering the personalization of prescription, yet if the available drugs share a mechanism of action like it is for  $\beta$ ARs-ligands.

Subsequently, as an additional and final commentary, we suggest an innovative way to present profiles for ligands targeting  $\beta$ ARs, which will likely be useful for ligands of other GPCRs. We suggest that when looking for a  $\beta$ AR-ligand, either in experimental or clinical use, a compound should be sought based on its ability to modulate the cellular activity of a selected group of cells expressing the targeted receptor. This activity can be changed by an agonist or maintained without change by an antagonist. In the former case, the ligand sought would have high affinity, high efficacy on a selected measurable pathway, and/or long-lasting action (probably the best combination of all these attributes). Hence, there are at least two reference values in order to check the possible effects on the system where the ligand is administered: the basal activity of targeted cell (if the endogenous ligand of a specific receptor is absent) and the cellular changes induced by the available concentration of endogenous ligands for which the receptors are expressed.

This perspective focusing on physiological conditions leads us to propose that the reference compound be an endogenous ligand (a chemical entity with an as yet conserved structure among species) with high efficacy and potency, and not a synthetic compound. The latter compounds would involve additional chemical moieties that imply conformational states different from those induced under physiological conditions. This is particularly relevant for  $\beta$ ARs, since isoproterenol (a synthetic non-selective  $\beta$ AR agonist) has frequently been accepted as a reference, which has been severely criticized from the incoming of pharmacological data from assays with high concentrations of old compounds and is especially disadvantageous for using in assays testing new compounds with higher efficacy, those denominated superagonists.<sup>27</sup>

Also, we recommend the consideration of a biological effect as the basal reference whenever this is possible. More specifically, the biological effect chosen as a reference should be that most relevant to the intended application of the test compound. For instance, for a potential bronchodilator, the biological effect to be measured should preferentially be its action on bronchial smooth muscle. We trust that by using such specific targets within a physiological context, the results can be presented in a standardized and thus comparable way for any tested compound. Furthermore, this would facilitate the identification of specific chemical moieties that induce a desired biological effect.

## Conclusions

Increasingly abundant evidence points to the location of at least one secondary binding region in  $\beta$ ARs that is shallower than the orthosteric binding site. Interactions of ligands with this secondary binding site (or sites) seem to be relevant for biased signaling and

the time-dependent effect of receptor activation, and possibly for other phenomena such as high- and low-affinity conformational states. We discuss the possible implications of one or more secondary binding sites in relation to the ability of endogenous ligands to reach their target receptors in human physiology. Based on the evidence herein cited, which is based on inferences from results of studies focusing on the binding process of ligands, we propose that contacts of ligands on this secondary binding site are related to a modulation of the cellular response through ligand binding to two or more sites in a concerted manner. One cellular response likely to be affected is the desensitization of activated catecholamine receptors with long-term exposure to their ligands. This down-regulation system can be related to a protective mechanism on the prolonged availability of catecholamines for receptors and cells where these receptors are abundantly expressed. We suggest that future research aimed at exploring the existence or absence of a secondary binding site focus on specific physiological models and use endogenous ligands as a reference. This should certainly facilitate the comparison of results from distinct studies and lead to new and useful knowledge for drug development targeting  $\beta$ ARs, whether or not the proposal of a secondary binding site is validated or proven to be irrelevant.

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## Conflict of interest

There are no conflicts of interest to declare.

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