

Intracellular receptor modulation: Novel approach to target GPCRs

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Abstract

Recent crystal structures of multiple G protein-coupled receptors (GPCRs) have revealed a highly conserved intracellular pocket that can be used to modulate these receptors from the inside. This novel intracellular site partially overlaps with the G protein and β -arrestin binding site, providing a new manner of pharmacological intervention. Here we provide an update of the architecture and function of the intracellular region of GPCRs, until now portrayed as the signaling domain. We review the available evidence on the presence of intracellular binding sites among chemokine receptors and other class A GPCRs, as well as different strategies to target it, including small molecules, pepducins and nanobodies. Finally, the potential advantages of intracellular (allosteric) ligands over orthosteric ligands are also discussed.

Multiple binding sites to target a GPCR

G protein-coupled receptors (GPCRs, see Glossary) comprise one of the largest families of drug targets, with approximately 34% of the currently marketed drugs targeting this receptor class [1]. As lack of efficacy continues to be the main reason of failure in Phase II and Phase III clinical trials [2], novel approaches to successfully target these receptors are still necessary. As it is apparent from most GPCR crystal structures reported so far, small molecules often occupy a binding site exposed to the extracellular solvent—the so-called **orthosteric binding site** which is used by endogenous ligands [3] (Figure 1A). However, targeting GPCRs has proved to be quite challenging, especially when drugs need to compete with a high (local) concentration of the endogenous ligand, as is the case of targeting chemokine receptors during inflammatory conditions [4]. Hence, the development of **allosteric modulators** (Box 1) that bind to spatially distinct binding sites [5] has emerged as a promising approach to improve not only drug **efficacy**, but also selectivity and safety [6-8]. A variety of different **allosteric binding sites** have already been identified in GPCRs, most of them close to the orthosteric binding site; yet, unexpected ligand binding sites have recently been found in crystal structures of class A and class B GPCRs [5]. In this regard, the recent crystal structures of CC chemokine receptor 2 (CCR2) [9], CC chemokine receptor 9 (CCR9) [10], and β_2 -adrenergic receptor (β_2 AR) [11] have for the first time revealed a spatially conserved intracellular binding site for small molecules in class A GPCRs (Figure 1A), providing a new avenue to inhibit or modulate these receptors in different pathologies.

Intracellular region of GPCRs: Beyond signaling

In general, GPCRs share a similar structure consisting of three different domains (Figure 1A): the extracellular domain that includes three extracellular loops (ECLs) and the N terminus, which vary in length and structure depending on the GPCR subfamily [12]; the transmembrane (TM) domain that comprises seven TM helices; and the intracellular domain that includes three intracellular loops (ICLs), an amphipathic helix (H8) and the C terminus [3]. Traditionally, the upper TM section and the extracellular domain have been considered to encompass the ligand binding domain. In contrast, the lower TM section and the intracellular domain have been considered to be the signaling domain [3, 13]. Structurally, the intracellular domain is more highly conserved and flexible than the extracellular region containing the orthosteric binding site [3, 13], which is probably related to a common mechanism of receptor activation and G protein-coupling [14]. In this regard, analysis of several active- and inactive-state crystal structures has revealed a conserved rearrangement of residue contacts near the G protein-binding site, involving residues 3x46 in TM3, 6x37 in TM6, and 7x53 from the highly conserved NPxxY motif located in TM7 (residues according to **structure-based Ballesteros-Weinstein numbering** [15]) [14]. In addition, this region is also involved in the coupling and selective recognition of different G proteins [16, 17] and other signaling proteins such as β -arrestin [18], which can lead to a multitude of different signaling pathways upon activation of a GPCR. Recently, the traditional view of a separate ligand binding and signaling domain has been challenged as more evidence suggests that the intracellular domain of GPCRs can also be bound by ligands and thus be used for receptor modulation (Figure 1) [5, 9-11].

A common intracellular binding site in class A GPCRs

Among GPCRs there is now mutational, pharmacological and structural evidence of ligand binding sites located at their intracellular interface. This evidence is particularly extensive in the case of chemokine receptors (Box 2); thus, before extending to other class A GPCRs, we will first review the evidence available for chemokine receptors.

Intracellular binding site at Chemokine Receptors

The recent X-ray structure of CCR2 in complex with an orthosteric antagonist and the negative allosteric modulator (NAM, Box 1) CCR2-RA-[R] (PDB 5T1A) [9], and of CCR9 in complex with the NAM vercirmon (PDB 5LWE) [10] (Figure 2) have provided structural confirmation of such intracellular binding site in chemokine receptors. The two structures report an overlapping solvent-exposed binding site in the intracellular domain of these receptors, located more than 30 Å from the orthosteric binding site and enclosed by the intracellular ends of TM1 – TM3, TM6, TM7 and H8 (Figure 1A) [9, 10]. The NAMs bind this intracellular pocket where they interact with several conserved amino acid residues (Figure 1B).

Interestingly, before these crystal structures were solved, intracellular ligand binding sites had already been suggested for chemokine receptors. In 2008, a putative intracellular binding site for small-molecule compounds had been identified in CCR4, CCR5, CXCR1 and CXCR2 [19, 20]. Functional data from these studies suggested that a series of compounds required intracellular access in order to exert their activity. Specifically, for CCR4 it was shown that several compounds

similar to compound 1 (Figure 2) exhibited a lack of correlation in their potencies when measured in membrane or cellular assays. However, after permeabilization of the cells with saponin the potencies became comparable in both assays [19]. In CXCR2, the loss of cellular **potency** seemed to be dependent on the lipophilicity (logD) of the compounds. Lower lipophilicities resulted in a greater loss of potency, indicating that these compounds needed a certain level of lipophilicity to cross the cell membrane and reach the intracellular binding site [20]. A subsequent chimeric approach, with CCR4-CCR5 or CXCR1-CXCR2 chimeras, led to the suggestion that the C terminus was part of the binding site for these molecules [19, 20]. In CXCR2, this intracellular binding site was further mapped with help of homology modeling and mutational studies, which resulted in the identification of several C terminal residues as part of this allosteric binding site, including D84^{2x40}, T83^{2x39}, A249^{6x33}, Y314^{7x53}, and K320^{8x49} (Figure 1B) [20, 21]. Thus, these studies in CCR4 and CXCR2 provided the first biochemical evidence of the existence of such binding sites.

Using a similar approach, a homologous binding site was discovered in CCR2, where small molecules such as CCR2-RA-[R], JNJ-27141491 and SD-24 can bind (Figure 2) [22, 23]. Similar key residues were identified, including V244^{6x36}, K311^{8x49}, Y305^{7x53} and F312^{8x50} (Figure 1B) [22], which have now been confirmed by the X-ray structure [9]. A similar binding site has also been suggested in CX₃CR1 after pharmacological characterization of compound AZD8797 (Figure 2), a non-competitive inhibitor of CX₃CR1 with structural similarity to known CXCR2 intracellular ligands [24]. In addition, several **pepducins** derived from ICL1 of CXCR4 have been shown to interact selectively with CXCR4 in a non-competitive manner [25, 26]. Specifically, CXCR4 pepducin ATI-2341 has been predicted to interact with most of the residues located in ICL1 – ICL3

[27], indicating that this receptor can also be targeted from the intracellular side. Finally, the structure of the viral chemokine receptor US28 in complex with the chemokine ligand CX3CL1 and the **nanobody** Nb7 shows that Nb7 binds in a similar subpocket composed by the intracellular ends of TM3, TM5, TM6 and H8. Moreover, Nb7 interacts with several residues also involved in the binding of small-molecules or pepducins in human chemokine receptors, or in interactions with signaling proteins [28, 29].

Intracellular binding site at other class A GPCRs

This conserved intracellular binding site is not limited to chemokine receptors, as evidence for this site has been found in other class A GPCRs. In this regard, the crystal structure of β_2 AR (PDB 5X7D) has been solved with the small-molecule ligand 15PA (Figure 2), a polyethylene glycol-carboxylic acid derivative of compound 15 [30], co-crystallized at the intracellular interface [11]. Compound 15PA binds in a pocket formed by the intracellular ends of TM1, TM2, TM6, TM7, H8 and ICL1, where it interacts with key residues also identified in CCR2 and CCR9 [11] (Figure 3). Moreover, this binding pocket partially overlaps with the binding site of nanobodies Nb60 and Nb80 in β_2 AR [31, 32], Fab2838 in Adenosine A_{2A} receptor (A_{2A} AR) [33], Nb9-8 in M2R [34] and Nb39 in the μ -opioid receptor (μ OR) [35]. Previous to these crystal structures, different computational tools predicted intracellular binding pockets in rhodopsin and M2R [36, 37]. Molecular docking studies and virtual screening identified several rhodopsin inhibitors that bind at the interface between the GPCR and G protein [38, 39], in an intracellular pocket resembling that identified in chemokine receptors. More evidence for a generalized intracellular pocket comes from the proteinase activated receptor 1 (PAR1), where a series of small molecules such as compound

5-C (Figure 2) and ICL3-derived pepducins were shown to interact with residues located in TM7 and H8 [40, 41]. Similar ICL-derived pepducins have also been developed for PAR2 [42], PAR4 [43], sphingosine-1-phosphate receptor 3 (S1P₃) [44] and formylpeptide receptors 1 and 2 (FPR1 and FPR2) [45]. Taken together, there is mounting evidence for the presence of a spatially conserved intracellular pocket, not only in chemokine receptors but among several class A GPCRs.

Structural features of the intracellular binding site

The recent X-ray structures of CCR2 [9], CCR9 [10] and β_2 AR [11] are providing structural information on the features that determine binding and selectivity in this intracellular binding site (Figure 3, Key Figure). Moreover, these structures provide new opportunities for the application of structure-based drug design (SBDD) methods, such as virtual screening campaigns, which might allow the identification and/or optimization of novel intracellular ligands for these or other homologous receptors [5, 28]. Below, features of this site are discussed in terms of three component parts: a hydrophobic subpocket above H8, a central TM7-H8 binding region, and a region formed by TM3/6 and TM2/ICL1.

Hydrophobic subpocket

All ligands share a highly conserved hydrophobic subpocket above H8. Three highly conserved residues amongst class A GPCRs form the basis of this pocket: V^{1x53} (65% conserved), Y^{7x53} (89% conserved) and F^{8x50} (65% conserved) (Figure 3, upper panel). While there is only some evidence for the role of V^{1x53} in activation [46], numerous publications have shown the role of the latter two

residues in signaling and intracellular ligand binding at different GPCRs [14, 47, 48]. In terms of hydrophobicity, residues 1x56 and 1x57 are also highly conserved (Figure 3, upper panel). However, in CCR9 Y^{1x57} adopts an orientation that further opens up the pocket, allowing the large 4-tert-butyl substituent of the ligand to reach deeper into this pocket, indicating a role in conferring ligand selectivity.

Central TM7-H8 binding region

The central part of the pocket consists of the kink between H8 and TM7, formed by either P^{8x48} (β_2 AR) or G^{8x47} (chemokine receptors). This subpocket includes residues 8x47 to 8x49, which are conserved in terms of polarity, and residue 6x36 (Figure 3, central panel). For chemokine receptors this kink allows ligands to interact with the backbone of residues K/R^{8x49} and F^{8x50}. In CCR2, the specific conformation of this subpocket allows the ligand to bind closer to H8, where the negatively charged oxygen of the ligand is also able to interact with the backbone of E^{8x48}. In β_2 AR, P^{8x48} forces S^{8x47} inwards, allowing it to interact with the oxygen of the amide in the ligand, while a second interaction is formed between the nitrogen of another amide and D^{8x49}. Noteworthy is position 6x36 which is not strongly conserved (59% in terms of hydrophobicity) among GPCRs. This residue is key for ligand binding in both β_2 AR and CCR2: in β_2 AR, T^{6x36} forms a hydrogen bond with an amide of 15PA; in CCR2, V^{6x36} makes a hydrophobic interaction with the cyclohexyl substituent of the ligand. However, different effects have been reported upon mutation of this residue. While the mutation V^{6x36}A abolished ligand binding in CCR2 [22], it increased the stability of CCR9, facilitating its crystallization. In CXCR4, a T^{6x36}P mutation abolished signaling [49], whereas M^{6x36}T made the delta opioid receptor a **constitutively active mutant** (CAM) [50].

Finally, in the Adenosine A_{2B} receptor (A_{2B}AR) this residue acts as a determinant for G protein selectivity [51], indicating that this position might be crucial for target selectivity of intracellular ligands as well.

Region formed by TM3/6 and TM2/ICL1

The largest differences are observed in this region of the binding site; residues found in TM3 include R^{3x50} from the highly conserved DRY motif, and residue 6x40 conserved in terms of hydrophobicity (Figure 3, lower panel). Residue 6x37 seems to be important for selectivity, as exemplified by T^{6x37} in CCR9 that allows the chloro substituent of vercirnon to go deeper into this pocket. Interestingly, mutation of this residue has been implicated in altered signaling [51] and improved stability of A_{2A}AR to facilitate crystallization [52]. Polar residues found at the TM2/ICL1 interface interact with both the CCR9 and β₂AR ligand. For example, R^{12x49} (ICL1) forms a cation-π interaction with the β₂AR ligand while in CCR9 it interacts with both D^{2x40} and the nitro group of the ligand. However, these polar residues do not interact with the CCR2 ligand, indicating a different binding mode.

Strategies for intracellular modulation

In general, three main strategies have been used to target the intracellular side of GPCRs so far: small molecules, pepducins and nanobodies or “**intrabodies**”.

Small molecules

Small molecules currently account for the majority of drug types in clinical trials targeting GPCRs [1]. Although most of these small molecules are presumed to be orthosteric, the number of confirmed allosteric modulators targeting GPCRs is increasing in clinical trials[1]. In this regard, several intracellular small molecules have already been identified for a number of GPCRs, but few of these have progressed to clinical trials and none has made it to the market.

The largest number of small-molecule intracellular ligands reported so far target chemokine receptors, including CCR2 [9, 22], CCR4 [19, 53, 54], CCR9 [10], CXCR1 and CXCR2 [20, 21]. These intracellular ligands share similar chemical features such as the presence of acidic groups acting as hydrogen-bond acceptors when interacting with the target (Figure 2). A good balance of hydrophobic and polar residues make this binding site highly druggable, as described in a previous section [9]. However, intracellular ligands must cross the cellular membrane in order to exert their effect; therefore attention must be paid to the overall physicochemical properties of these intracellular small molecules, such as lipophilicity and molecular weight to ensure good permeability. Most of these intracellular ligands have been found using a traditional medicinal-chemistry approach. However, in the case of the β_2 AR, the co-crystallized compound 15PA (Figure 2) was derived from a novel β_2 AR NAM (compound 15) identified in a screening campaign using DNA-encoded small-molecule libraries, suggesting a novel approach to discover intracellular modulators in GPCRs [30].

One of the suggested intracellular ligands, the CCR2 antagonist CCX140-B from Chemocentryx (structure undisclosed) [55], has recently demonstrated positive results in a Phase II clinical trial

in patients with type 2 diabetes and diabetic nephropathy [56]. The CCR9 intracellular antagonist, vercirnon (Figure 2) [10], also showed promising results in Phase II clinical trials in patients with Crohn's disease [57]; however, it did not demonstrate clinical efficacy in the last Phase III study [58]. In case of CCR4, GlaxoSmithKline (GSK) has identified more than three different chemical scaffolds for intracellular antagonists—termed “site 2” antagonists by GSK [54]. Yet, only one of these ligands, GSK2239633 (Figure 2), progressed to Phase I clinical trials, before failing due to lack of efficacy [59]. Development of CXCR1-CXCR2 intracellular ligands such as SCH 527123 (Figure 2) has also resulted in several clinical trials for the treatment of chronic obstructive pulmonary disease (COPD) and asthma [60]. Although none of these ligands has been approved yet, this strategy has led to several clinical studies that might ultimately lead to a new marketed therapeutic agent.

Pepducins and Nanobodies

Another strategy for intracellular targeting of GPCRs is the use of pepducins, peptides derived from the ICLs of the target receptor, or nanobodies. As the use and pharmacology of several pepducins [61, 62] and nanobodies [63, 64] have been recently reviewed elsewhere, we will only briefly discuss them here. The pepducin approach has been explored with several GPCRs, including CXCR1, CXCR2 [65], CXCR4 [26, 66], PAR1 [41] and β_2 AR [67]. Although in many cases pepducins have been employed as pharmacological tools, several *in vitro* and *in vivo* preclinical studies support the role of pepducins as therapeutic agents [62]. In the case of PAR1, a recent clinical trial involving pepducin PZ-128 demonstrated positive results in patients with coronary artery disease [68]. Finally, the intracellular domain can also be targeted with intracellular

nanobodies or “**intrabodies**”, as exemplified by US28 [29], β_2 AR [16, 31, 32, 69], A_{2A} AR [33], M2R [34] and μ OR [35]. Although most of these intrabodies have been used to aid GPCR crystallization and understand receptor function, their therapeutic potential has also been highlighted [70].

Advantages and therapeutic implications of intracellular ligands

As a consequence of their ability to bind to distinct sites on a GPCR, intracellular allosteric modulators can have unique properties compared to compounds that target the (orthosteric) binding site of endogenous ligands [8]. Some of these key properties include the modulation of **affinity** and/or **efficacy** of orthosteric ligands, improved selectivity, **polypharmacology**, or biased signaling (Box 1, Figure 4).

Modulation of affinity and efficacy of orthosteric ligands

In β_2 AR, two allosteric intrabodies, a NAM and a PAM, were able to modulate the affinity of the orthosteric agonist isoprenaline by more than 15,000-fold, an unexpectedly large dynamic range (Figure 4A) [32]. Although both intrabodies insert into the pocket where the G protein binds, they modulate the functional state of the receptor differently by engaging with other residues within the binding pocket. The impact of the PAM and NAM intrabodies on a panel of orthosteric ligands of different efficacies was also shown to be consistent with the presence of multiple receptor states. The concept of more than two functional states—inactive and active—may allow for finer control of functional responses than previously thought. In addition, the demonstrated ability of allosteric ligands to differentially modulate the activity of distinct orthosteric ligands (referred to as probe

dependence) has important implications regarding the selectivity of drugs for receptors that are activated by multiple ligands, as is the case for chemokine receptors. Intracellular NAMs of CCR2 and CCR9 are thought to function by directly inhibiting the interaction with intracellular signaling proteins, while at the same time blocking the outward motion of TM6 and the upward motion of TM3, required for receptor activation [9, 10]. By preventing G protein coupling and stabilizing an inactive state, they also presumably reduce the affinity of the endogenous agonists. In addition, intracellular NAMs inhibit the receptor in an insurmountable manner (Figure 4B). As previously demonstrated in CCR2, CCR2-RA-[R] was able to decrease the maximum effect of the endogenous chemokine CCL2, even at the highest CCL2 concentration tested [23]. Another advantage of allosteric over orthosteric inhibitors is their saturability or the so-called "ceiling effect", which limits the allosteric activity to a certain level, despite further increments in the dose of the modulator [6-8]. Whether compounds targeting this site can be appropriately designed with the right level of saturability will become clear with more intracellular compounds in clinical studies.

Selectivity vs. Polypharmacology

As this intracellular binding site is likely present in most chemokine receptors, it may be a useful site for simultaneously blocking multiple chemokine receptors in disease contexts where polypharmacology has been deemed useful (Figure 4C). This may hold true in multiple sclerosis or rheumatoid arthritis where multiple chemokine receptors have been found to play a role [71]. As allosteric modulators, pepducins may prove useful for polypharmacology because they are derived from the intracellular loops of GPCRs, which often display a high degree of sequence similarity amongst related receptors [61]. For example, the pepducin P4pal-10 was shown to inhibit diverse G_q-coupled receptors without affecting β_2 AR (G_s) or CXCR4 (G_i) signaling [72]. Its broad

spectrum inhibition profile was exploited to investigate the effect of blocking G_q -mediated signaling from a number of receptors for the treatment of asthma, which involves multiple GPCRs. On the other hand, intracellular allosteric antagonists exhibiting >100-fold selectivity for CXCR2 over CXCR1 have been discovered indicating that selectivity can also be achieved in this binding site [20].

Biased signaling

Pepducins have also been shown to promote **biased signaling** of GPCRs. Biased signaling tends to involve preferential activation of G protein-dependent over G protein-independent signaling (e.g., via β -arrestin) or vice versa (Figure 4D). AT1-2341 is a pepducin derived from the ICL1 of CXCR4 that promotes specific G_i -mediated signaling without G_{13} -coupling or β -arrestin recruitment [66]. Similarly pepducin ICL3-9 derived from ICL3 of β_2 AR showed G_s -biased signaling [67], which may be advantageous for the treatment of asthma by limiting β -arrestin-mediated desensitization and potential tachyphylaxis from chronic use of β -agonists [73].

Concluding Remarks and Future Perspectives

There is now ample evidence from mutational, computational, and structural studies in class A GPCRs for novel allosteric binding pockets, located in close proximity to the G protein or β -arrestin binding site. This highly-conserved solvent-exposed intracellular pocket can be used to inhibit or modulate the receptor in an allosteric manner. Intracellular receptor modulation is not limited to small molecules, as intracellular pepducins and intrabodies have also been used to modulate

GPCRs from the inside. These intracellular agents bring new pharmacological opportunities, but also new challenges including optimization of their selectivity profile, and their permeability properties to allow access to the inside of the cell and ultimately to cross the intestinal wall. These and other key issues have been summarized in the *Outstanding Questions*. Although none of these intracellular agents is yet on the market, promising (pre)clinical results have been already reported, pointing to their clinical potential. Importantly, the recent crystal structures in complex with these ligands provide a detailed view of the intracellular pockets, allowing for a better understanding and a rational design of novel intracellular ligands to target these and other GPCRs in a wide variety of diseases.

Disclaimer Statement

The authors declare that they have no conflicts of interest.

Box 1. Allosteric modulation in GPCRs

G protein-coupled receptors (GPCRs) are considered natural allosteric proteins, as the site of interaction of the endogenous ligand—the orthosteric binding site—differs from the site of the signaling effectors, such as G proteins and β -arrestins [74]. In addition to the orthosteric site, GPCRs possess a variety of topologically distinct allosteric binding sites where ligands can bind [5]. When allosteric modulators bind, they modulate the activity of orthosteric ligands by inducing conformational changes in the receptor.

Orthosteric ligands are competitive and thus, they replace the endogenous ligand resulting in a single pharmacological state. In contrast, by modulating the activity of another ligand, allosteric ligands have the potential for fine-tuning a receptor response, maximizing the efficacy in some therapeutic contexts [6, 7], and/or minimizing the potential side effects and other liabilities [6, 8]. Depending on their effect, allosteric modulators can be divided in [6, 7, 75]:

- Positive allosteric modulators (PAMs): Enhance the affinity and/or efficacy of the endogenous or orthosteric ligand.
- Negative allosteric modulators (NAMs): Decrease the affinity and/or efficacy of the endogenous or orthosteric ligand.
- Ago-PAMs: PAMs with some inherent level of agonist activity on their own
- Silent allosteric modulators (SAMs): Have no effect on the affinity or efficacy of the endogenous or orthosteric ligand. Their presence may lead to for instance enhanced thermostability of the receptor and increased signaling lifetime.

Some key pharmacological properties of allosteric modulators are:

- **Insurmountability:** The ability of allosteric ligands to cause a decrease in the potency and/or efficacy of the endogenous agonist, even when the endogenous ligand is present at high concentrations.
- **Selectivity:** Generally, allosteric binding sites show less evolutionary pressure leading to a less-conserved amino acid sequence and thus, higher ligand selectivity than the orthosteric binding site. If an allosteric site is highly conserved, selectivity can be achieved via optimization of cooperativity with the orthosteric ligand or by targeting specific non-conserved amino acids.
- **Saturability or ceiling effect:** The limit of the pharmacological effect produced by the allosteric ligand due to saturation of the effect after full occupancy of the allosteric site.
- **Probe-dependence:** Both the magnitude and direction of the allosteric effect achieved by the allosteric modulator are dependent on the orthosteric ligand used as a “probe”.
- **Biased signaling:** The ability of a ligand to preferentially stabilize a conformation that leads to the selective activation of a signaling pathway.

Box 2. Chemokine Receptors

Chemokine receptors represent one of the largest subfamilies within class A G protein-coupled receptors (GPCRs). So far, 23 chemokine receptors have been identified that can be activated by more than 45 chemokine ligands (IUPHAR/BPS Guide to Pharmacology, <http://www.guidetopharmacology.org>, accessed on 04-12-2017). Chemokines and chemokine receptors are subdivided in four different families, according to the number and arrangement of conserved cysteine residues in the N-terminus of the chemokine ligands: C, with only one conserved cysteine present; CC, CXC and CX3C, with zero, one and three extra residues between two conserved cysteine residues. [76].

Both chemokines and chemokine receptors comprise the so-called chemokine system, which plays an important role in the migration and positioning of immune cells in homeostatic or pathological conditions [77]. According to their immune function, chemokine receptors can be classified as homeostatic, or dual inflammatory/homeostatic [78]. The chemokine system is a complex, seemingly redundant system in which one chemokine ligand is able to activate multiple chemokine receptors, and one chemokine receptor can be activated by multiple chemokine ligands. Yet, evidence suggests it is a highly fine-tuned system as it is tightly regulated by specific spatial and temporal control of chemokine expression [79, 80].

Dysregulation of this complex system has been implicated in a variety of inflammatory and immune diseases, including arthritis, diabetes, inflammatory bowel disease and cancer [81]. Three drugs targeting chemokine receptors have already gained market approval: maraviroc, a small-

molecule targeting CCR5; plerixafor, a small-molecule targeting CXCR4; and mogamulizumab, an anti-CCR4 antibody [76].

Glossary

Affinity: Parameter that describes how strong a ligand binds to its target.

Allosteric binding site: A binding site nonoverlapping and topographically distinct from the orthosteric binding site.

Allosteric modulator: Any ligand that binds to an allosteric binding site, from which they can modulate the activity of orthosteric ligands.

Constitutively active mutant (CAM): Mutation that leads to a permanent and agonist-independent active state of the GPCR, compared with the wild-type receptor.

Efficacy: Parameter that describes the degree of effect or response achieved by a specific ligand upon binding to its target.

G protein-coupled receptors (GPCRs): Family of transmembrane proteins that transduce a variety of extracellular signals into intracellular responses via G protein-dependent or -independent signaling pathways. In vertebrates, GPCRs are divided in four different classes or subfamilies: class A (rhodopsin-like), class B (secretin), class C (metabotropic glutamate), and class F (frizzled/smoothed).

Intrabodies: Intracellularly-expressed antibody fragments aimed at intracellular targets.

Nanobodies: Recombinant small size antibody (12 – 15 kDa), containing a variable-domain fragment derived from camelid heavy-chain antibodies.

Orthosteric binding site: The binding site recognized and used by the endogenous ligand for a corresponding receptor.

Orthosteric ligand: Any ligand that binds to the orthosteric binding site of the receptor. Orthosteric ligands include the endogenous ligands and non-endogenous agonists, antagonists, or inverse agonists.

Pepducins: Lipidated peptides derived from the intracellular loops or the C terminus of GPCRs, which specifically target their cognate receptor by acting as allosteric agonists or antagonists.

Polypharmacology: The ability of a ligand to (purposely and) effectively bind to several targets.

Potency: Parameter that describes the activity of a drug by defining how much of a ligand (concentration) is needed to produce a half-maximal effect.

Structure-based Ballesteros-Weinstein numbering: Numbering system for amino acid residues, which takes into account structural information to correct for bulges and constrictions. In this numbering scheme the first number denotes the transmembrane domain and the second number denotes the residue position in relation to the most conserved amino acid, the latter always in position 50. E.g. Y^{7x53} indicates that this tyrosine is located in TM7, in position 53. This numbering system is currently used by the GPCR database (GPCRdb, <http://www.gpcrdb.org>) [15].

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Figure Legends

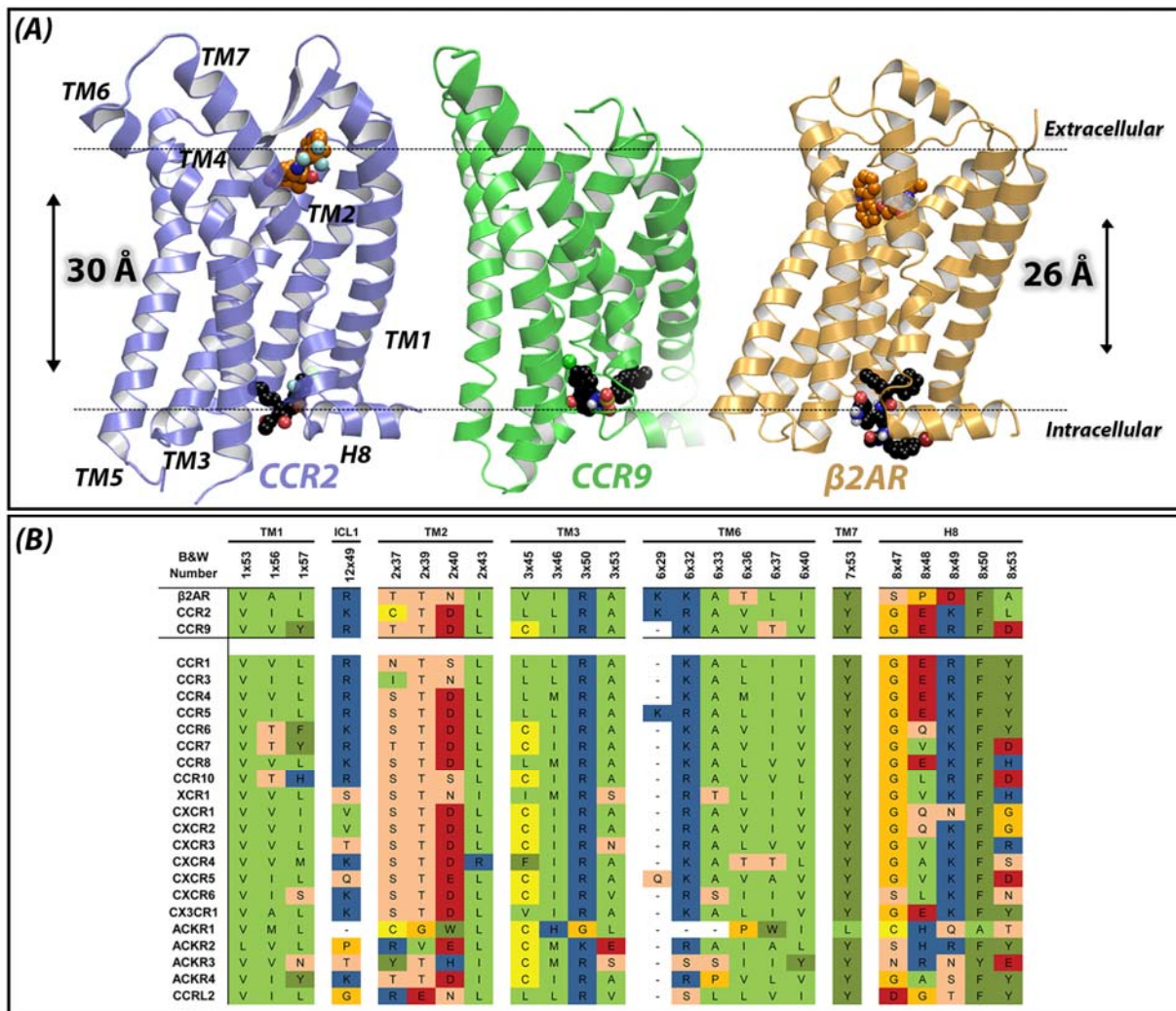


Figure 1. Novel allosteric binding site in class A GPCRs. (A) Endogenous ligands bind close to the extracellular region of GPCRs, in the so-called orthosteric binding site. Most of the co-crystallized small molecules also bind in this extracellular region, such as BMS-681 in CCR2 and carazolol in β₂AR. Recently, the crystal structures of CCR2 (purple, PDB 5T1A), CCR9 (green, PDB 5LWE) and β₂AR (yellow, PDB 4XT1) have revealed an allosteric solvent-exposed binding site, located in the intracellular region of GPCRs, around 30 Å away from the orthosteric binding site. This novel binding site challenges the traditional view of the upper 7TM region of GPCRs as

ligand binding domain and the intracellular region as signaling domain only. As shown in the structures, this intracellular binding site can also be targeted by small molecules such as CCR2-RA-[R] in CCR2, vercirnon in CCR9 and 15-PA in β_2 AR. Dotted lines represent the plane of the membrane. (B) Sequence conservation among chemokine receptors and β_2 AR, based on the GPCR database (GPCRdb, <http://www.gpcrdb.org>). Residues shown are residues involved in the intracellular binding site of CCR2, CCR9 and β_2 AR (upper three rows). Some of these residues have also been found to be important for ligand binding to other class A GPCRs, as well as for G protein and β -arrestin binding.

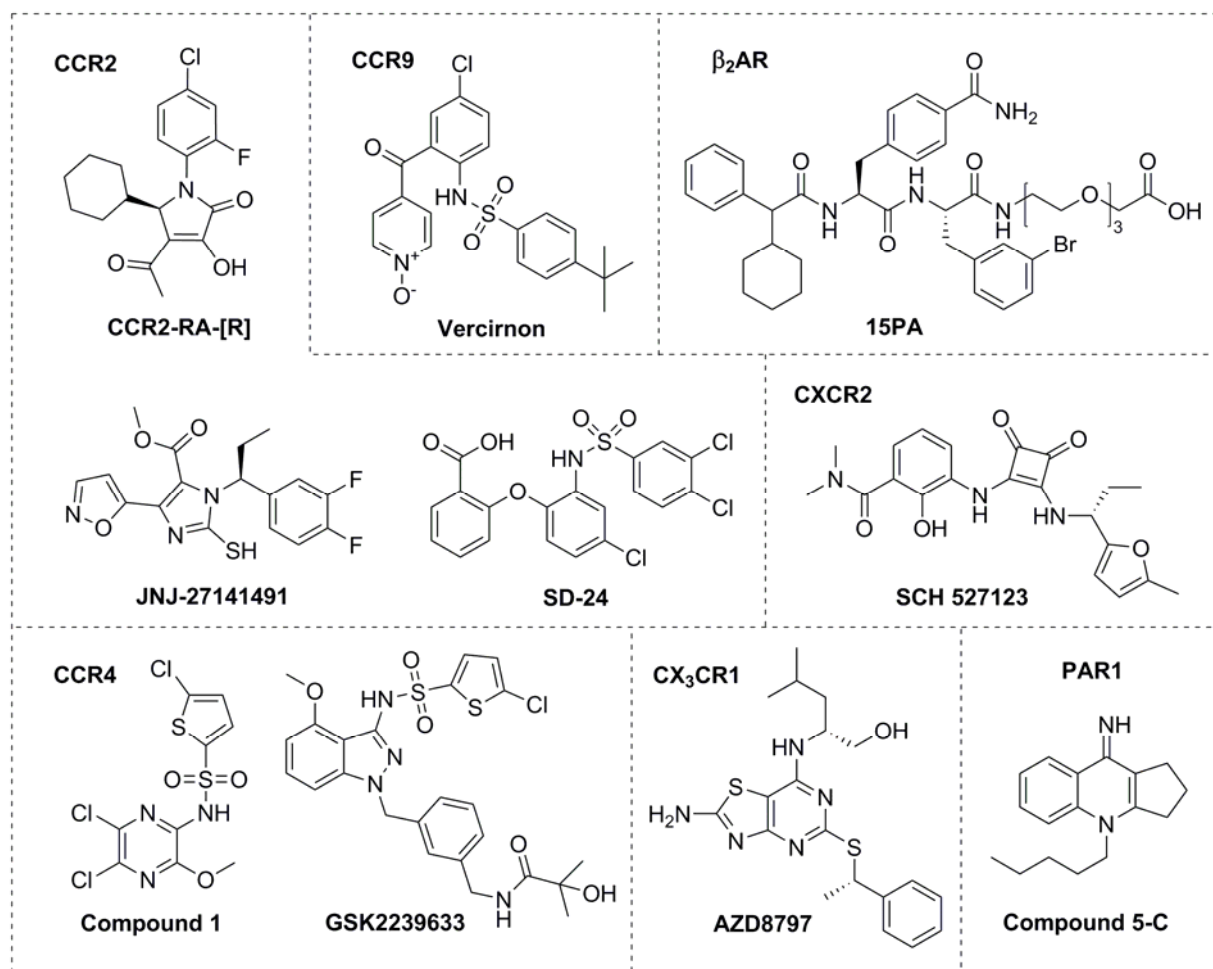


Figure 2. Chemical structures of selected intracellular small molecule ligands for different class A GPCRs. Upper row shows the chemical structures of cocrystallized intracellular ligands with their corresponding receptor: CCR2-RA-[R] with CCR2, Vercirnon with CCR9, and 15PA with β_2 AR. Vercirnon, SCH 527123 and GSK2239633 are examples of intracellular ligands that have progressed to clinical trials.

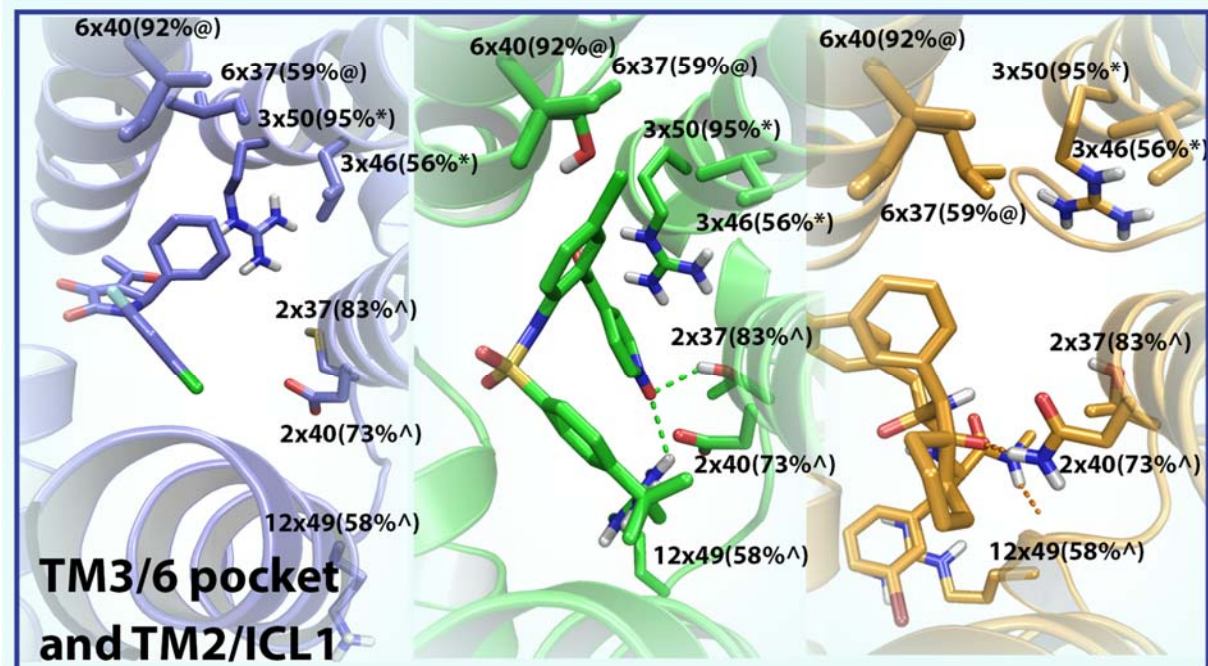
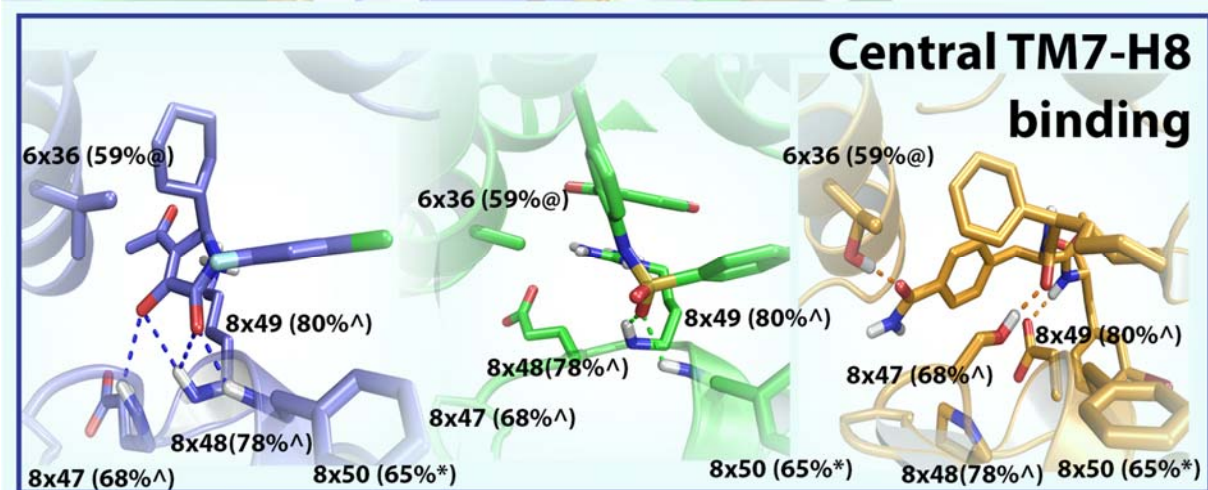
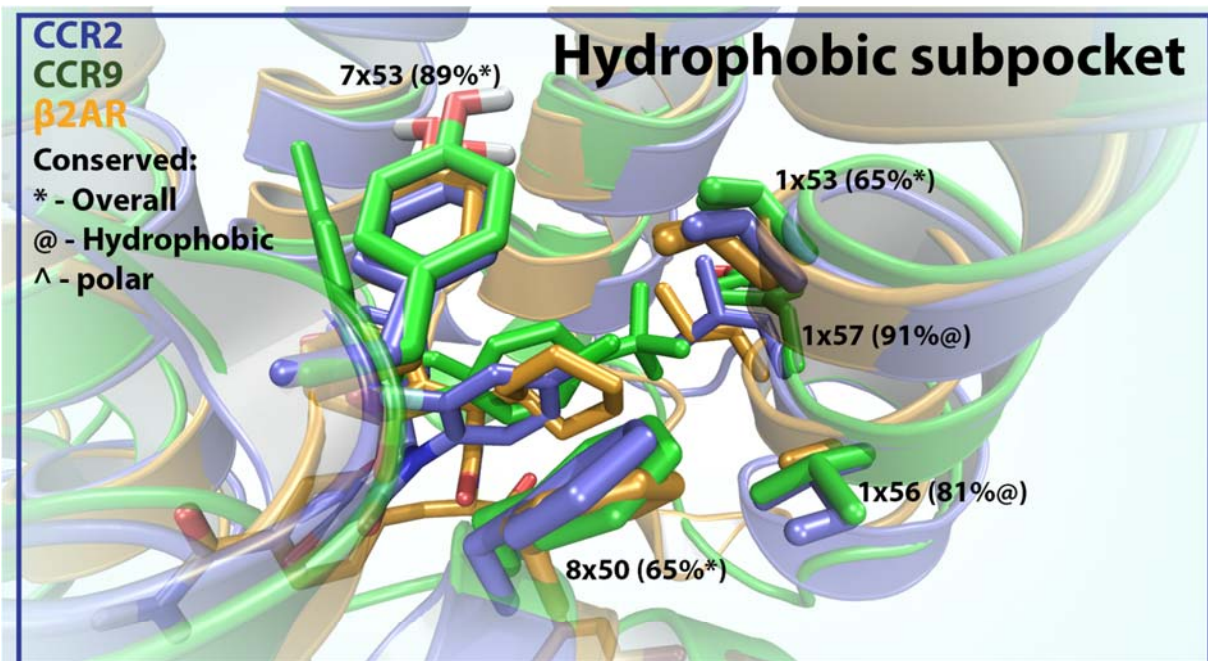


Figure 3. Overview of structural features of the intracellular binding site. Common features in intracellular ligand binding derived from the crystal structures of CCR2 (PDB 5T1A), CCR9 (PDB 5LWE) and β_2 AR (PDB 4XT1). Residues are numbered using structure-based Ballesteros-Weinstein numbers [15]. Residue conservation among all class A GPCRs is shown in the following way; residues that are overall conserved (identical) in class A (>50%) are shown first (*); for residues that are not conserved we show how conserved they are in terms of polarity (^) or hydrophobicity (@). The three different boxes represent three different sections of the intracellular binding sites, in the upper panel all receptors are superimposed while in the lower two boxes the receptors are shown separately. CCR2 is colored blue, CCR9 is colored green and β_2 AR is colored orange.

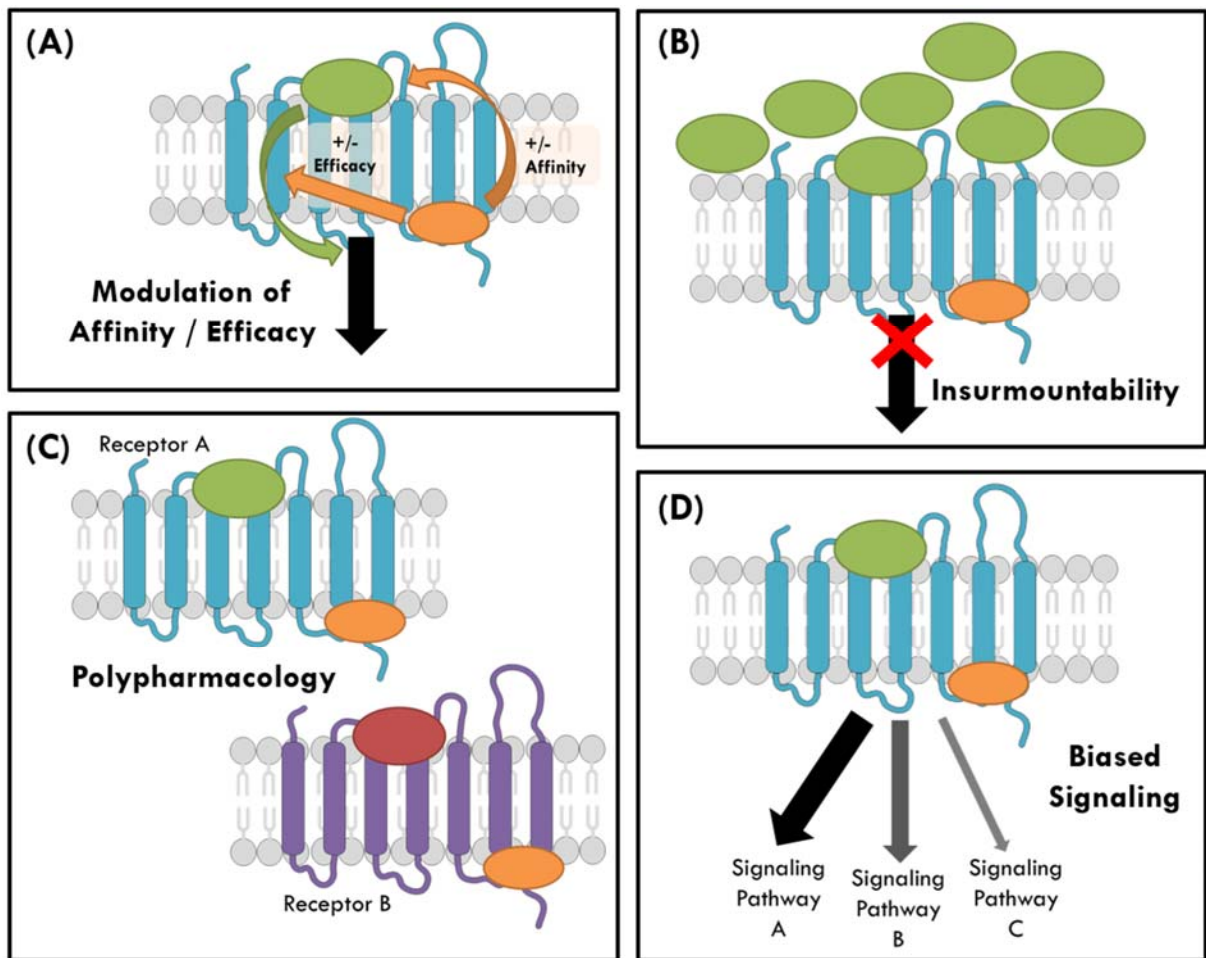


Figure 4. Potential advantages of intracellular allosteric modulators. (A) Intracellular allosteric modulators (small molecules, peptidics or intrabodies, shown in orange) have the potential to positively or negatively modulate the affinity and/or the efficacy of the endogenous ligand (shown in green or red) or any orthosteric ligand. The ultimate response depends on the level of positive or negative cooperativity between the two ligands. (B) Intracellular ligands can display insurmountability, as they can inhibit the receptor (shown in blue) even when high concentrations of endogenous ligand are present. (C) A highly-conserved intracellular binding site provides the possibility of designing intracellular ligands that bind and exert their effect in multiple receptors (receptor A in blue and receptor B in purple). These pharmacological ligands, as opposed

to selective ligands, might be advantageous in diseases where more than one receptor is involved.

(D) Intracellular ligands can also promote biased signaling, by preferentially modulating one signaling pathway over another upon activation by the endogenous ligand. For instance, they can stabilize G protein signaling over β -arrestin signaling. Source of cellular biology illustrations:

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