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Emerging paradigm of intracellular targeting of GPCRs

Madhu Chaturvedi[1], **Justin Schilling**[2], **Alexandre Beautrait**[3], **Michel Bouvier**[3,4], **Jeffrey L. Benovic**[2], **Arun K. Shukla**[1,*]

[1]Department of Biological Sciences and Bioengineering, Indian Institute of Technology, Kanpur 208016, India

[2]Department of Biochemistry and Molecular Biology, Thomas Jefferson University, Philadelphia, PA 19107 USA

[3]Institute for Research in Immunology and Cancer (IRIC), Université de Montréal, Quebec, Canada H3T 1J4.

[4]Department of Biochemistry and Molecular Medicine, Université de Montréal, Montreal, Quebec, Canada H3T 1J4.

Abstract

G protein-coupled receptors recognize a diverse array of extracellular stimuli, and they mediate a broad repertoire of signaling events involved in human physiology. Although the major effort on targeting GPCRs has typically been focused on their extracellular surface, a series of recent developments now unfold the possibility of targeting them from the intracellular side as well. Allosteric modulators binding to the cytoplasmic surface of GPCRs have now been described, and their structural mechanisms are elucidated by high-resolution crystal structures. Furthermore, pepducins, aptamers and intrabodies targeting the intracellular face of GPCRs have also been successfully utilized to modulate receptor signaling. Moreover, small molecule compounds, aptamers and synthetic intrabodies targeting β-arrestins have also been discovered to modulate GPCR endocytosis and signaling. Here, we discuss the emerging paradigm of intracellular targeting of GPCRs, and outline the current challenges, potential opportunities and future outlook in this particular area of GPCR biology.

Keywords

GPCRs; allosteric modulators; pepducins; intrabodies; β-arrestins; barbadin

G protein-coupled receptors: beyond the extracellular surface

G protein-coupled receptor (GPCR) superfamily consists of approximately 800 different members, and they are at the center stage of many different signaling pathways involved in various aspects of human physiology [1]. They can recognize a very diverse range of stimuli including small molecules, peptides, lipids and proteins despite having a common seven trans-membrane (7TM) architecture [2]. This highlights an exceptional ability of 7TM

*Correspondence: arshukla@iitk.ac.in (A.K. Shukla).

scaffold to provide context dependent and remarkably adjustable binding pockets which can accommodate an incredibly large spectrum of chemical structures. Upon activation, GPCRs can couple to different transducers, of which the heterotrimeric G proteins and β-arrestins (βarrs) are most extensively characterized, and they both can mediate downstream signaling cascades leading to various cellular and physiological outcomes [3].

A key focus in GPCR research continues to be the identification and design of small molecule orthosteric and allosteric ligands with a long-term goal of developing novel therapeutics [4]. In fact, about one third of the currently prescribed drugs are targeted at GPCRs in the form of receptor antagonists, agonists, and allosteric modulators [5]. Interestingly, almost all of these ligands are primarily directed at the extracellular surface of GPCRs which is not only easily accessible in cellular context but it almost always harbors the binding site of natural ligands. This perhaps stems from the conceptual framework of ligand recognition exclusively at the extracellular surface, and cell based high-throughput screening strategies which are typically not designed to identify intracellular binders [6]. The intracellular surface of GPCRs has been studied primarily with respect to transducer coupling which results in the initiation of cellular signaling events, receptor trafficking and regulation [7].

In the last few years however, a series of interesting studies have emerged which underscore the potential of modulating GPCR signaling and functions from the intracellular side, either directly or by targeting their common signal-transducers. For example, crystal structures of three different GPCRs have been determined in complex with small molecule allosteric ligands bound to the cytoplasmic face of the receptors [8-10]. Moreover, cell permeable peptides derived from the intracellular loops of GPCRs are developed as pepducins and they have been found to modulate receptor signaling from the inside [11]. Importantly, availability of purified receptor proteins has provided the opportunity to design ligand-receptor interaction based screening strategies such as surface plasmon resonance (SPR) [12] or identification of antibody fragment based binders to the intracellular surface of GPCRs [13] (Box 1). Moreover, targeting the universal transducers downstream of GPCRs such as βarrs and their interaction partners by RNA aptamer [14], small molecule compounds [15] or intrabodies [16] has also emerged as an alternative approach to modulate GPCR functions from the inside.

In this review, we discuss the recent discovery of intracellular modulators of GPCR signaling with particular emphasis on small molecules, aptamers, pepducins and intrabodies. We discuss the mechanistic framework of how these modulators alter the functional outcomes downstream of GPCRs and underline the novel therapeutic opportunities that are starting to emerge from these recent discoveries.

Targeting GPCRs from the intracellular side

Several distinct approaches have emerged in the past few years to target the intracellular surface of GPCRs. First, small molecule antagonists of GPCRs that bind to the cytoplasmic side of the receptor, unlike the conventional extracellular ligands, have been identified [17]. Crystal structures of the CC chemokine receptors CCR2 and CCR9, and the β2 adrenergic

receptor (β2AR) have been determined in complex with intracellular ligands, and they reveal high-resolution details of the intracellular binding pockets of these ligands [8-10]. Second, lipidated peptides derived from the intracellular loops of GPCRs referred to as pepducins have emerged as a unique handle to modulate GPCR signaling from inside, and in some cases, they also bias GPCR signaling by preferentially activating or inhibiting one of the transducers over the others [11]. Third, RNA aptamers targeting the intracellular surface of the βAR have been generated which can distinguish between the inactive and active receptor conformations, and inhibit the functional coupling of the receptor to Gαs [18]. Finally, intrabodies derived from β2AR targeting nanobodies have been expressed in cells and several of them have turned out to modulate the coupling and activation of the two different transducers i.e. G proteins and βarrs [19, 20]. Specific details of the design, functional attributes and therapeutic potential are discussed in the subsequent sections.

Small molecule intracellular allosteric modulators of GPCRs

As mentioned earlier, GPCR targeted drug discovery has focused almost entirely on extracellular binding sites. However, there have been scattered biochemical and pharmacological evidence in the literature to suggest the possibility of ligand interaction on the intracellular side of the receptors as well, especially for some chemokine receptors [21-25]. Recent crystal structures of two different CC chemokine receptors namely, the CCR2 and CCR9, now directly establish binding of ligands to the cytoplasmic surface and also reveal the structural details of the binding pockets [8, 9]. CCR9 has now been crystallized in complex with vercirnon, an antagonist that has progressed up to phase III clinical trial for the treatment of Crohn's disease [26]. Surprisingly, the crystal structure revealed the binding of vercirnon to the cytoplasmic side of the receptor (Figure 1A-B). CCR2 has been crystallized in complex with two ligands, an orthosteric antagonist, BMS-681 and an allosteric antagonist, CCR2-RA-[R][7]. Of these, CCR2-RA-[R][7] binds to the intracellular face of the receptor while BMS-681 binds to the conventional orthosteric ligand binding site on the extracellular side. More recently, screening of DNA encoded small molecule library on purified β2AR has yielded several allosteric compounds, and the crystal structure of β2AR with one of these compounds, referred to as cmpd-15A, reveals its binding site on the intracellular surface of the receptor [10, 17] (Figure 1C). Intricate details of these crystal structures and ligand-receptor interactions have been discussed elsewhere recently [27] and therefore, we will only present an overview of novel insights derived from these structures.

Comparison of these three different crystal structures reveals that the overall contribution of receptor transmembrane helices that constitute the binding pockets of these ligands are very similar and they primarily involve TM 1,2,6,7 and helix 8 (Figure 1A-F). In fact, some of the residues interacting with these ligands such as $Y^{7.53}$ in the NPxxY motif and $F^{8.50}$ in helix 8 are conserved not only in these three receptors but in GPCRs overall [28]. Thus, it is tempting to speculate that intracellular binding sites may be a common feature of GPCRs, and they might involve a significantly overlapping interface. Moreover, the overall receptor conformation in these crystal structures are very similar to the corresponding inactive state structures of these receptors which is not surprising considering that each of these ligands behaves primarily as an antagonist in functional assays. In addition, binding mode of these

ligands appears to preclude the interaction of G proteins and βarrs which provides an additional mechanism for the antagonistic profile of these ligands through steric hindrance with transducer coupling [29, 30] (Figure 2 and Text Text Box 2). Finally, an interesting aspect of these intracellular ligand binding pockets is that they harbor a balanced combination of hydrophobic and polar residues which is a desirable and promising sign for potential druggability of these binding pockets.

It is important to mention that the crystal structures of several GPCRs have now revealed somewhat unexpected extra-helical allosteric ligand binding sites [31, 32]. However, the three structures discussed above reveal binding pockets in the transmembrane bundle at the cytoplasmic surface of the receptor which are more than 20-30Å away from the orthosteric pocket on the extracellular side. Availability of these crystal structures and the high resolution details of these novel binding pockets now provides a previously lacking platform for virtual ligand screening VLS) to identify additional intracellular ligands not only for these receptors but perhaps for other GPCRs as well. An interesting avenue that can be explored now is whether additional intracellular ligands can be designed guided by these crystal structures to selectively preclude one of the two key transducers and thereby, biasing the downstream GPCR signaling from inside.

Regulation of GPCR signaling by pepducins

Pepducins are short, lipidated, cell-penetrating peptides derived from intracellular loops (ICLs) of G protein-coupled receptors that rapidly traverse and remain tethered to the membrane inner leaflet [33-36]. Once inside the cell, pepducins can access allosteric binding sites within the key effector regions of cognate GPCRs where they can stabilize unique receptor conformations, which in turn can elicit pharmacological signaling profiles currently inaccessible via known orthosteric ligands. It is possible for a pepducin to specifically target its cognate receptor due to the relatively high sequence diversity within the ICLs [34]. As well, it has been shown that a pepducin can target and similarly affect signaling from closely related GPCRs due to relative similarity in ICL amino acid sequence [34, 35]. Such sequence-based specificity and promiscuity were apparent with the first description of pepducins [34, 35]. Derived from ICL3 of protease-activated receptor 1 and 4 (PAR1 and PAR4), respectively, P1pal-12 and P4pal-10 were shown to be antagonists of G protein signaling from their parental receptors. P1pal-12 was derived from the proximal region of the PAR1 ICL3, a region that shares little homology between PAR1 and PAR4. While P1pal-12 did not strongly antagonize PAR4, P4pal-10, on the other hand, was derived from the more highly conserved C-terminal region of the ICL3 and was also shown to block platelet aggregation downstream of PAR1 [35]. Additional studies found that P4pal-10 could inhibit an array of Gq-coupled receptors yet showed no effect on Gs-coupled (β2AR) or Gi-coupled (CXCR4) receptor signaling [37].

Pepducins can also function as GPCR agonists. Initial studies on PAR1 demonstrated that ICL3 pepducins could effectively stimulate a Ca^{2+} flux and platelet aggregation through PAR1 while also activating PAR2, dependent on the length of the pepducin [34]. ICL3 pepducins from PAR2 and melanocortin-4 receptor were also shown to activate their cognate receptors [34]. Indeed, pepducins that function as agonists or antagonists have now been

identified for a large number of GPCRs [11, 33]. Moreover, an ICL1 pepducin from the chemokine receptor CXCR4 (called ATI-2341) can function as a biased agonist, promoting effective interaction of CXCR4 with Gαi to activate a Ca^{++} flux while promoting minimal interaction with Gα13, GRKs and βarrs [38] (Figure 3).

The concept that pepducins can function as biased agonists prompted a broad screen of pepducins targeting the intracellular loops of the β2AR. The initial screen identified four classes of β2AR pepducins that could either mediate balanced signaling, Gαs-biased signaling, βarr-biased signaling, or direct activation of Gαs [39]. In this study, ICL3 pepducins from the proximal region of the β2AR were found to directly activate Gαs independent of the receptor (e.g. ICL3-8) while a pepducin from the central portion of ICL3 promoted selective interaction of the β2AR with Gαs (ICL3-9) without inducing GRK-mediated phosphorylation, βarr recruitment, or β2AR internalization [39]. ICL3-9 was also able to activate Gαs signaling downstream of the closely related β1AR, likely due to the relatively high sequence conservation across this ICL3 region between the β1AR and β2AR [39]. Additional studies revealed that a pepducin comprising the entire first intracellular loop of the β2AR (ICL1-9) could induce effective β2AR phosphorylation, βarr recruitment, receptor internalization and βarr-biased signaling without promoting any coupling to Gαs [40]. Interestingly, ICL1-9 also promoted mouse cardiomyocyte contractility in a β2AR- and βarr-dependent and cAMP- and Ca^{2+}-independent manner [40]. ICL1-9 is specific for the β2AR and can induce a significant conformational change in the receptor that is sensitive to inverse agonist treatment, although the nature of this interaction remains unclear.

Despite the emergence of pepducins as allosteric modulators of GPCR signaling that, in some instances, are able to induce unique pharmacological profiles, strikingly little is known about precisely how they do so. In fact, just a few studies have begun to shed light on this question. The photo-linkable CXCR4 pepducin ATI-2766, a pepducin analog similar to the aforementioned ATI-2341, was shown to cross-link specifically with CXCR4, although the site of cross-linking was not identified [41]. The environmentally sensitive fluorophore monobromobimane (MBB) has proven to be a useful tool for directly assaying the ability of pepducins to affect changes in receptor conformation [42]. The addition of the pepducin ICL1-9 to Cys265 MBB-labeled β2AR red shifted and decreased fluorescence, while a scrambled version of the pepducin did not [40]. The addition of wild type or a partially activated version of βarr1 resulted in increasingly pronounced shifts in MBB-labeled β2AR fluorescence indicating that ICL1-9 can interact directly with its cognate receptor to stabilize conformations that facilitate βarr binding [40]. By employing an array of methods including targeted mutagenesis, NMR, fluorescence resonance energy transfer (FRET), and MBB-labeled PAR1, Zhang et al were able to demonstrate that the PAR1 pepducin P1pal-19 requires the H8 helix and TM7 tyrosine propeller of PAR1 to stabilize a receptor conformation that favors G protein signaling [43].

Elucidating precisely how pepducins can induce biased GPCR signaling is an area that is ripe for discovery. Moving forward, techniques that may facilitate advances in our understanding of these mechanisms include the aforementioned use of MBB-labeled receptors, cross-linking, hydrogen deuterium exchange mass spectrometry, double electron-electron resonance (DEER) spectroscopy [44], ^{19}F-NMR [44], and X-ray crystallography of

pepducins with their cognate receptors. Given the complexity of GPCR signaling, integrative approaches that interrogate a pepducin-receptor system using multiple methods may prove to be particularly insightful. For example, such an approach combining *in- silico* evolutionary lineage analysis, mutagenesis and machine learning to cluster receptor signaling profiles yielded new insights into how crucial β2AR motifs can transduce ligand binding into distinct signaling events [45].

A significant limitation to the potential therapeutic use of pepducins appears to exist, as delivering pepducins to particular organs or tissues has thus far proven difficult [11]. The pepducin PZ-128 appears to be an exception to this hurdle and in clinical trials has been shown to reduce PAR1 platelet aggregation in humans with coronary artery disease [46]. PZ-235 is a PAR2 pepducin being developed to treat non-alcoholic fatty liver disease (NAFLD). When injected into a chemically inducible mouse model of NAFLD, PZ-235 was shown to block PAR2-driven fibrotic progression of liver stellate cells [47]. While more than 50% localized to the liver, on a per gram of tissue basis PZ-235 was fairly evenly distributed to most tissues, any consequences of which have yet to be determined [47]. In the near term, however addition to their exciting therapeutic potential, pepducins should continue to be useful tools to de-convolute the ever-increasing diversity and complexity of GPCR signaling.

Intrabodies targeting the β2 adrenergic receptors

Members of the camelid family express single chain antibodies unlike conventional immunoglobulin molecules that consist of the heavy and light chains [48]. The variable fragment of these single chain antibodies are referred to as nanobodies due to their relatively smaller size. In a quest to stabilize and crystallize the active conformation of the β2AR, a series of nanobodies were generated after immunization of llamas with a high affinity agonist bound purified β2AR [49]. A set of these nanobodies were subsequently expressed in the cytoplasm of cultured cell lines as intrabodies and their effects on receptor-transducer coupling and signaling was determined [20]. Interestingly, several of these intrabodies inhibited agonist-induced cAMP response, receptor phosphorylation and βarr recruitment to varying extent [20]. These observations not only confirm the binding of these intrabodies to the cytoplasmic surface of the receptor but also establish the feasibility of this approach to target GPCR signaling. Interestingly, these intrabodies maintained their preference for active or inactive β2AR conformations in cellular context, thus providing a unique handle to fine-tune ligand-induced GPCR signaling and trafficking [20].

The ability of these intrabodies to inhibit β2AR-Gαs and β2AR-βarr coupling may result either from the stabilization of inactive receptor conformations or a direct competition with Gαs, GRKs and βarr binding to the receptor due to overlapping interaction interfaces [29, 30, 50]. The latter scenario appears to be more likely because even those intrabodies which stabilize an active β2AR conformation (e.g. intrabody 80) appear to exert an inhibitory effect on β2AR-Gαs coupling [51]. Although the generality of this approach across a broad spectrum of GPCRs still remains to be tested, this study unfolds a novel framework to target GPCRs from inside. An interesting challenge however that remains to be surmounted with intrabody-based approach is the limitation associated with their functional delivery in live cells. Although a number of methods and tools, e.g. nanoparticle mediated delivery [52],

cationic liposome encapsulation [53] and self-internalizing peptides [54], are currently being developed, improving their targeted and efficient delivery still requires a significant amount of optimization. It is also important to note that not all the antibody fragments will exhibit functional expression as intrabody, and this is determined by their primary sequence, scaffold design and the dependence on disulphide bond for functional folding. Recent advances and knowledge dissemination in this area, especially the development of a completely *in-vitro* yeast display library of nanobodies [55], now makes this platform widely and readily accessible to many researchers and it is likely to catalyze broader utility and application of this approach in the near future.

RNA aptamers targeting intracellular surface of β2AR

Aptamers are small oligonucleotides (DNA or RNA) that can be tailored to recognize specific interfaces on proteins typically with high affinity and specificity [56]. Target recognition by aptamers is typically driven by their secondary and tertiary structures and hence, they offer the possibility of disrupting bimolecular interactions, *in-vitro*, and in cellular context [56]. Pegaptanib, a VEGF (vascular endothelial growth factor) binding aptamer, is currently in use as a drug for age related macular degeneration [57] indicating the therapeutic potential of aptamers. A recent study has utilized a state-of-the-art SELEX (Systematic Evolution of Ligands by Exponential enrichment) library screening and NGS (next generation sequencing) approach to identify several aptamers binding to cytoplasmic surface of the β2AR [18]. Several of these aptamers display selectivity for agonist-bound conformation of the β2AR over apo- (i.e. ligand free) or antagonist-bound conformations [18]. In fact, some of these aptamers not only prefer the agonist-bound β2AR but they also stabilize an active conformation of the receptor. Single particle negative staining has revealed their interaction with the intracellular receptor surface, and interestingly, some of these are able to inhibit agonist-induced cAMP accumulation suggesting their inhibitory effect on functional coupling between the β2AR and Gαs [18]. Although this study has not measured the effect of these aptamers on β2AR-βarr coupling, considering the overlap in Gαs and βarr binding sites on the cytoplasmic surface of the receptor, it is plausible that these aptamers may inhibit physical interaction with βarrs as well. It is also possible that these aptamers will specifically block the core interaction between the receptor and βarrs, and thereby stabilize the partially-engaged β2AR-βarr conformations associated solely though the phosphorylated carboxyl-terminus of the receptor [58, 59]. Again, similar to intrabodies, the generality of aptamer-based strategy to target the intracellular side of GPCRs remains to be evaluated further, and novel approaches to deliver them in live cells have to be designed for considering them as potential therapeutic agents.

Targeting βarrs: universal transducers of GPCRs

In addition to directly targeting GPCRs, another possibility that has emerged in the last couple of years is to modulate GPCR function by targeting their common effector, βarrs. The two isoforms of βarrs, βarr1 and Barr2, are involved in a range of functions downstream of GPCRs which include receptor desensitization through a steric hindrance mechanism, endocytosis by scaffolding clathrin and AP2, ubiquitination by serving as E3 ubiquitin ligase adaptors and signaling by nucleating the components of various signaling cascades [60-62].

As the functional multiplicity of βarrs arises from their scaffolding role and their direct interaction with numerous cellular partners, it is not feasible to selectively target specific βarr functions by global genetic manipulations (e.g. knock-down and knock-out approaches) which result in depletion or elimination of βarrs at the protein level. This is particularly important when considering that βarrs may have opposite effects on global responses such as desensitization vs. activation of G protein-independent signaling pathways. However, it is conceivable that selective disruption of βarr interactions can be utilized to modulate the corresponding functional responses in a spatio-temporal fashion. There have been two different strategies that are recently described to target βarrs and thereby modulate downstream signaling and agonist-induced GPCR endocytosis. These approaches involve an RNA aptamer targeting βarr2 [14] and a synthetic intrabody fragment recognizing βarr2 [16], and the details of these innovative tools are discussed in the following sections.

An RNA aptamer targeting βarr2

Similar to β2AR binding aptamers described above, SELEX based screening of a large RNA aptamer library on purified βarr2 has also identified a series of binders against βarrs [14]. Of these aptamers, several bind βarr2 with nanomolar affinity and they exhibit substantial selectivity for βarr2 over βarr1. Interestingly, some of the aptamers inhibited the interaction of βarr2 with ERK MAP kinase and PDE (phosphodiesterases) *in-vitro* and therefore, offered the possibility of altering βarr signaling in cellular context [14]. In fact, when delivered in live cultured cells, these βarr2 targeting aptamers maintained their functionality and displayed significant inhibition of several signaling readouts downstream of Frizzled and Smoothened receptors [14]. Furthermore, these aptamers were also able to inhibit the growth of a cultured human immortalized myelogenous leukemia cell line referred to as K562, and leukemic cells isolated from CML (chronic myelogenous leukemia) patients [14]. Although the effect of these aptamers is tested only on βarr-ERK and βarr-PDE interactions, considering the distinct binding interface of various interaction partners on βarrs, it is plausible that they might have influence other βarr interactions differently. Nonetheless, these findings establish the feasibility, and potential therapeutic implications of targeting βarrs with aptamers.

Synthetic intrabodies targeting β-arrestins

As mentioned earlier, not only the two isoforms of βarrs exhibit a significant functional divergence but their ability to interact with several different proteins lies at the heart of their multifunctional behavior [63]. In an attempt to target the individual isoforms and their distinct interfaces, a series of synthetic antibody fragments were recently generated using a phage display library of Fabs (antigen binding fragments) [16]. Interestingly, several Fabs not only distinguished between the two isoforms but they were also able to selectively recognize the activated conformation of βarrs. Importantly, several of these Fabs can modulate the interaction of βarrs with clathrin and ERK MAP kinase differently, and therefore, provide an interesting handle to selectively modulate βarr functions in cellular context [16]. One of these Fabs, referred to as Fab5, is able to disrupt βarr2-clathrin interaction but not βarr2-ERK2 interaction, *in-vitro*. When expressed in cultured cells as an intrabody, Fab5 is able to robustly inhibit agonist-induced internalization of several GPCRs

[16] (Figure 3C). As expected based on *in-vitro* interaction data, it did not affect agonist-induced ERK MAP kinase phosphorylation for the same set of GPCRs. This finding, taken together with other emerging studies in the literature, suggest that receptor endocytosis and ERK MAP kinase activation are not necessarily linked to each other as anticipated originally [16, 64].

This intrabody-based experimental framework therefore raises the possibility that it should be possible to further fine-tune βarr signaling, for example, by inhibiting the interaction with one of the signaling partners over the others. Similarly, it may also be possible to use intrabody-based approach to induce biased GPCR signaling from inside, for example by specifically interfering with receptor-βarr interaction. As mentioned earlier, the interaction of GPCRs with βarrs is a biphasic process and it involves both, the phosphorylated receptor tail (carboxyl-terminus) and the cytoplasmic surface of the transmembrane receptor core [30]. Several recent studies have documented the ability of partially engaged GPCR-βarr complexes to initiate receptor endocytosis and signaling, while the fully-engaged complexes appear to be critical for receptor desensitization [58, 59, 65]. Therefore, it is conceivable to selectively disengage one of these interactions by intrabodies to specifically tweak the spatio-temporal pattern of receptor signaling and trafficking.

Targeting the interaction partners of βarrs: an inhibitor of βarr-AP2 interaction

In addition to targeting βarrs directly, is it possible to modulate GPCR functions by targeting the interaction partners of βarrs? Such an approach, if feasible, should in fact facilitate the modulation of a specific functional outcomes imparted by this particular interaction partner. This has recently been accomplished by identifying a pharmacological inhibitor, referred to as barbadin, which selectively controls the interaction between βarrs and the β2-adaptin subunit of the clathrin adaptor protein, AP2 [15]. Barbadin was identified by combining *in-silico* screening and bioluminescence resonance energy transfer (BRET)-based cellular assays. Using the crystal structure of a complex between the β2-adaptin ear domain of AP2 and a carboxyl-terminal peptide of βarr1 [66] as the template, a virtual screen was designed to target this interaction interface, and it identified a number of potential hits. Among them, barbadin was the most potent βarr-β2-adaptin interaction inhibitor, and it was subsequently confirmed as a binder of β2-adaptin. Barbadin inhibits interaction between βarrs (i.e. both βarr 1 and 2) and AP2 in a concentration dependent fashion without interfering with the recruitment of βarrs to GPCRs or the binding of AP2 to clathrin or epsin [15]. As mentioned earlier, the interaction of βarrs with AP2 is a key driver for agonist-induced internalization of GPCRs, and therefore, the inhibition of the βarr-AP2 association by barbadin blocks the agonist-promoted βarr-dependent endocytosis of prototypical GPCRs such as the β2AR, V2R (vasopressin receptor sub-type 2), AT1aR (angiotensin II sub-type 1a receptor) [15]. Interestingly, barbadin does not affect βarr-independent and AP2-independent receptor internalization. The selective inhibition of βarr-AP2 interaction without any significant effect on βarr recruitment to GPCRs allows to selectively assessing the role of these two processes in βarr functions. In fact, barbadin blocks agonist-induced activation of ERK MAP kinase downstream of V2R. This observation suggests that the association of the

receptor-βarr complex with the endocytotic machinery and, most-likely, the ensuing endocytosis, is required for βarr-dependent ERK MAP kinase activation. Interestingly, barbadin also inhibits the sustained accumulation of cAMP promoted by the V2R and the β2AR stimulation consistent with the emerging notion that GPCR-stimulated cAMP production continues after endocytosis of Gαs-coupled receptors [67-72], and that the βarr-AP2 interaction is required for this phenomenon. Clearly, barbadin should prove to be very useful in specifically dissecting the roles of βarr-AP2 interaction under different cellular and experimental conditions for GPCRs. Ongoing structure-based optimization of barbadin analogues with improved biophysical and pharmacokinetic properties for *in-vivo* studies should provide tools to dissect βarr functions in animal models.

Concluding remarks and future outlook

The paradigm of modulating GPCR signaling through small molecule ligands, pepducins, aptamers and intrabodies has just started to emerge. The rapidly emerging structural data on GPCRs now provide a platform to engage virtual screening efforts to identify and characterize novel ligands binding at the intracellular surface of the receptors [73, 74]. Moreover, cryo-EM based high-resolution structures of GPCR-G protein complexes is also appearing at a staggering rate [75-77], and it opens the possibilities of designing selective inhibitors of their interaction interfaces as well. Therefore, the area of cytoplasmic targeting provides an entirely novel potential to modulate and bias GPCR signaling and opens novel therapeutic avenues by itself or possibly in combination with conventional GPCR ligands.

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Glossary

Orthosteric and allosteric ligands
the binding pocket of natural ligand is referred to as orthosteric site while any other binding site is referred to as allosteric.

Intracellular interface
refers to the cytoplasmic side of the receptors which is present towards the cell interior.

Signal transducers
refers to GPCR interacting proteins which initiate the onset of downstream signaling e.g. heterotrimeric G proteins, GRKs, βarrs.

Pepducins
lipidated, cell permeable peptides derived from the intracellular loops of the receptors.

Aptamer
oligonucleotides, DNA or RNA, that can recognize target proteins with high affinity and specificity.

Biased signaling
the phenomenal of preferential signaling through a specific signaling pathway over the others; also referred to as functional selectivity or ligand-directed signaling.

Antibody fragments
the antigen recognizing domain of antibodies which can be in the form of a Fab (antigen binding fragment) or ScFv (single chain variable fragment).

Nanobodies
the variable fragment of single chain antibodies produced by the members of the camelid family e.g. camels, llama etc.

Intrabodies
cytoplasmic expression of antibody fragments, typically achieved by plasmid based expression in cultured cell lines.

Endocytosis
trafficking of the receptors from the plasma membrane to the cell interior upon stimulation by agonist ligands.

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Text box 1.

A potentially generic strategy for screening intracellular GPCR binders

Unlike the conventional high-throughput screening using cell-based set-up, purified receptors clearly offer unique advantages for identifying intracellular binders and modulators. Considering that expression, purification and reconstitution (in liposomes or nanodiscs) of several GPCRs have now been streamlined, wider application of this strategy is clearly feasible and within reach of the broader GPCR community. Several studies have used surface plasmon resonance (SPR) based screening to identify small molecule GPCR ligands on either native or thermo-stabilized purified receptor preparations [12, 78]. However, validation of hits has focused primarily on radioligand displacement or cell-based functional assays which may not be suitable to report intracellular binders. Therefore, an extensive and appropriately designed characterization strategy should be used to evaluate the potential hits to identify non-conventional intracellular receptor binders. In addition, successful selection of aptamers and intrabodies targeting GPCRs has also utilized purified receptor preparations for screening, and the protocols described for these case-examples should be applicable to other receptors as well. Now, the availability of three different crystal structures in complex with intracellular ligands and a significant commonality in their binding pockets provides a promising starting point for virtual ligand screening to identify novel intracellular binders. It is also conceivable that assays measuring receptor-transducer interactions *in-vitro* or in cellular context can also be tailored to identify intracellular modulators of GPCRs. A recent study has used membrane preparation from cells overexpressing 5-HT and GLP-1 receptors to identify allosteric compounds to these receptors using a mass-spectrometry based approach, and this alternative strategy should also be generic and also amenable to intracellular ligand screening [79]. Finally, the successful identification of agonists by screening small chemical libraries on β2AR-nanobody fusion protein also highlights the possibility of tailoring *in-vitro* screening strategies to identify orthosteric and allosteric ligands of varying efficacies [13].

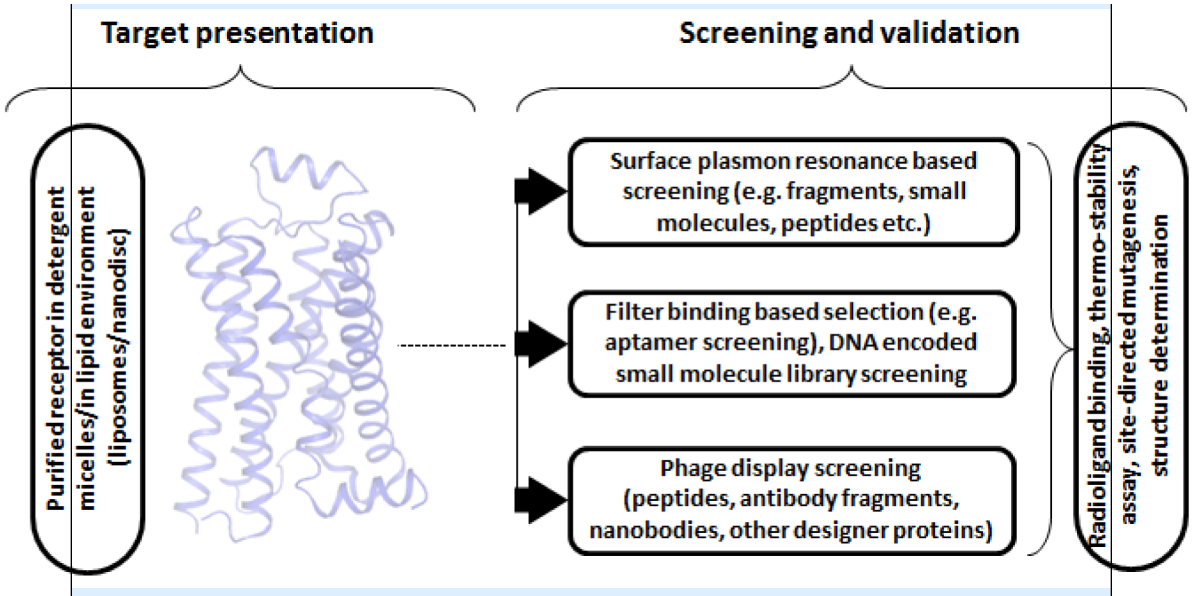

Figure for Text Box l.

A potentially generic pipelinefor identification and characterization of intracellular GPCR modulators.

Text box 2.

The mechanistic basis of intracellular modulation of GPCR functions

Recent discoveries suggest that there are several possibilities to fine-tune GPCR functions from the intracellular side for example, by targeting either the receptor directly or via common signal transducers such as βarrs. Understanding the fine details of the mechanistic framework for these intracellular modulators is a key to improve their efficiency, design even better derivatives and tweak the screening strategies going forward. Crystal structures of three different GPCRs reveal that intracellular antagonists stabilize an inactive conformation of the receptors as deciphered by the position of TM6 in these structures compared to fully-active conformation of GPCRs. In addition, binding sites of these intracellular ligands also overlaps to some extent with the interaction interface of Gαs and βarrs suggesting an additional contribution of steric hindrance based mechanism that precludes transducer coupling. Structural mechanism of intrabodies targeting β2AR also appears to be overall similar to that of intracellular ligands while direct high-resolution structural information is currently lacking to convincingly establish the mechanistic framework of pepducin and aptamer-based modulation of GPCRs. On the other hand, modulation of GPCR endocytosis and signaling by targeting βarrs appears to result primarily from direct inhibition of protein-protein interaction. For example, βarr2 targeting aptamer inhibits the interaction of ERK2 with βarr2 while barbadin inhibits the binding of AP2 with βarr2. Similarly, intrabody 5 selectively inhibits the interaction between βarr2 and clathrin and thereby suppresses agonist-induced GPCR endocytosis. However, it should be noted that βarrs also explore a broad repertoire of conformations linked with specific functional outcomes, and therefore, it should be possible to modulate functional outcomes downstream of GPCRs by allosterically regulating βarrs.

Highlights

- **i.** Targeting the intracellular surface of GPCRs or their effectors has emerged as a feasible approach for modulating GPCR functions and it has significant therapeutic potential
- **ii.** Small molecule allosteric ligands for CCR2, CCR9 and β2AR have been identified, and their binding mode is revealed by X-ray crystal structures
- **iii.** Pepducins, intrabodies and aptamers targeting the intracellular surface of GPCRs have been identified and they modulate receptor signaling in cellular context
- **iv.** RNA aptamer targeting βarr2 and a small molecule inhibitor of βarr2-AP2 interaction have been described and they can modulate GPCR signaling and endocytosis
- **v.** Synthetic antibody fragments targeting βarrs have been generated and when expressed as intrabody, they can selectively inhibit agonist-induced GPCR endocytosis

Outstanding Questions Box

i. How conserved is the binding pocket identified on the intracellular side of GPCRs? Is it possible to design intracellular ligands that can activate GPCRs? Are there natural examples of intracellular GPCR ligands?

ii. What is the exact mechanism of intracellular GPCR antagonists? Do they stabilize inactive receptors conformation; preclude transducer coupling by direct competition or a combination of both? Can their mechanism of binding be exploited to bias GPCR signaling from inside?

iii. How efficient and feasible will it be to develop intracellular ligands, pepducins, aptamers and intrabodies as novel therapeutics, either alone or in combination with some of the existing GPCR drugs?

iv. Whether additional small molecule compounds, similar to barbadin, can be identified and developed to selectively modulate βarr functions? Is it possible to design small molecule compounds that can activate βarrs in receptor-independent fashion?

v. Whether intrabodies targeting the receptor-βarr interaction interface can be identified and tailored to bias GPCR signaling towards the G protein signaling pathway, even in the context of receptor stimulation with physiological ligands?

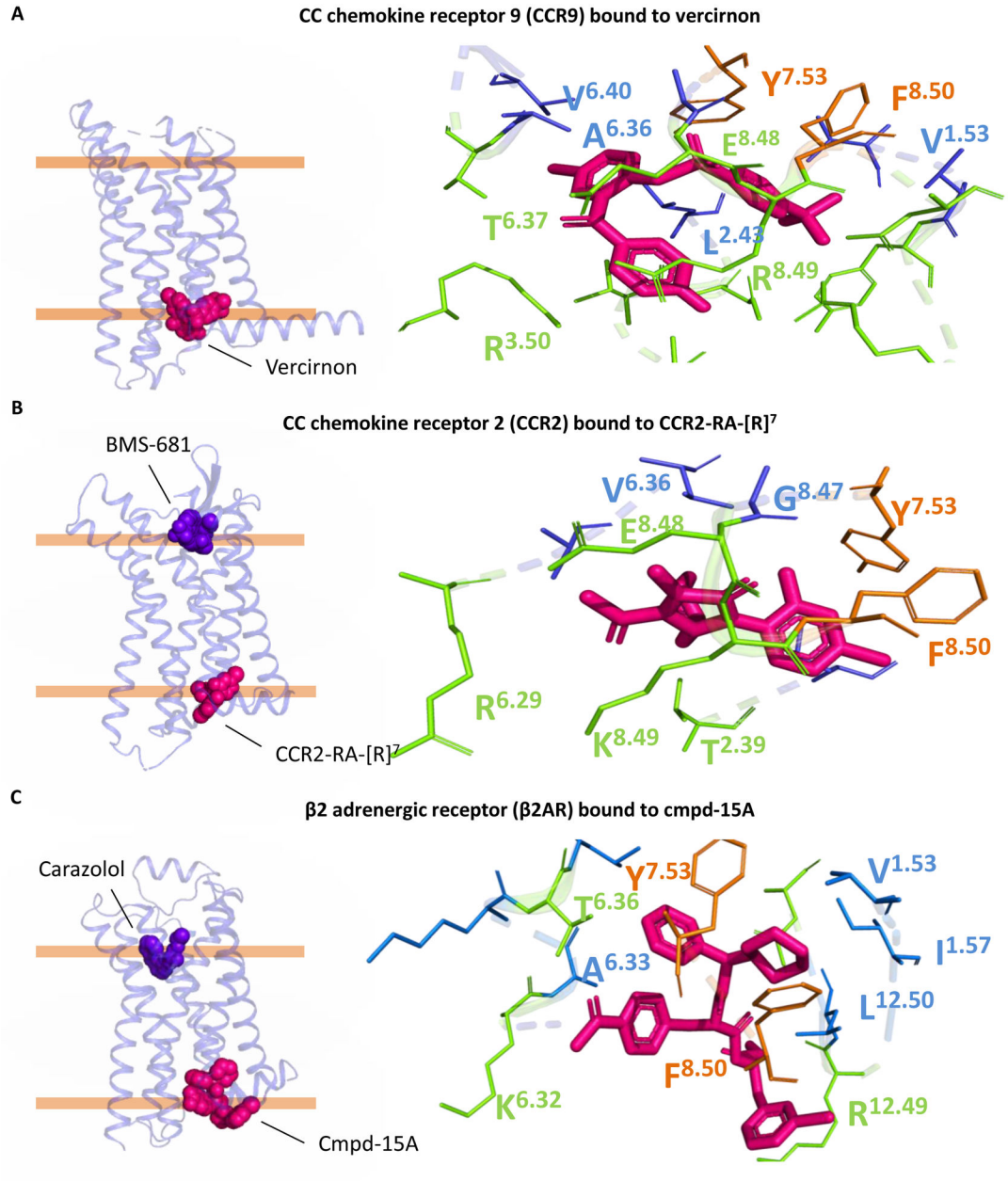

Figure 1. Binding of allosteric modulators to the intracellular side of GPCRs.

Crystal structures of CCR9 (A), CCR2 (B) and β2AR (C) in complex with vercirnon, CCR2-RA-[R]7 and cmpd-15A reveal the intracellular binding sites on these receptors. The left panels show an overview of the intracellular ligand binding while the right panels display a close-up of the binding pockets and ligand-interacting residues. In CCR2 and β2AR crystal structures, orthosteric antagonists (BMS-681 for CCR2; carazolol for β2AR) are also present. In the right panel, receptor residues within interacting distance are color coded (blue, hydrophobic; green, polar). Interestingly, the intracellular ligands in all three structures are anchored close to TMI, VI, VII and helix 8. Importantly, two residues (Y7.53 of the conserved NPxxY motif and F8.50 of helix VIII) involved in ligand binding are highly

conserved in the three receptors and in GPCRs in general. These images are created with PyMol using PDB entries 5LWE (CCR9), 5T1A (CCR2) and 5X7D (β2AR).

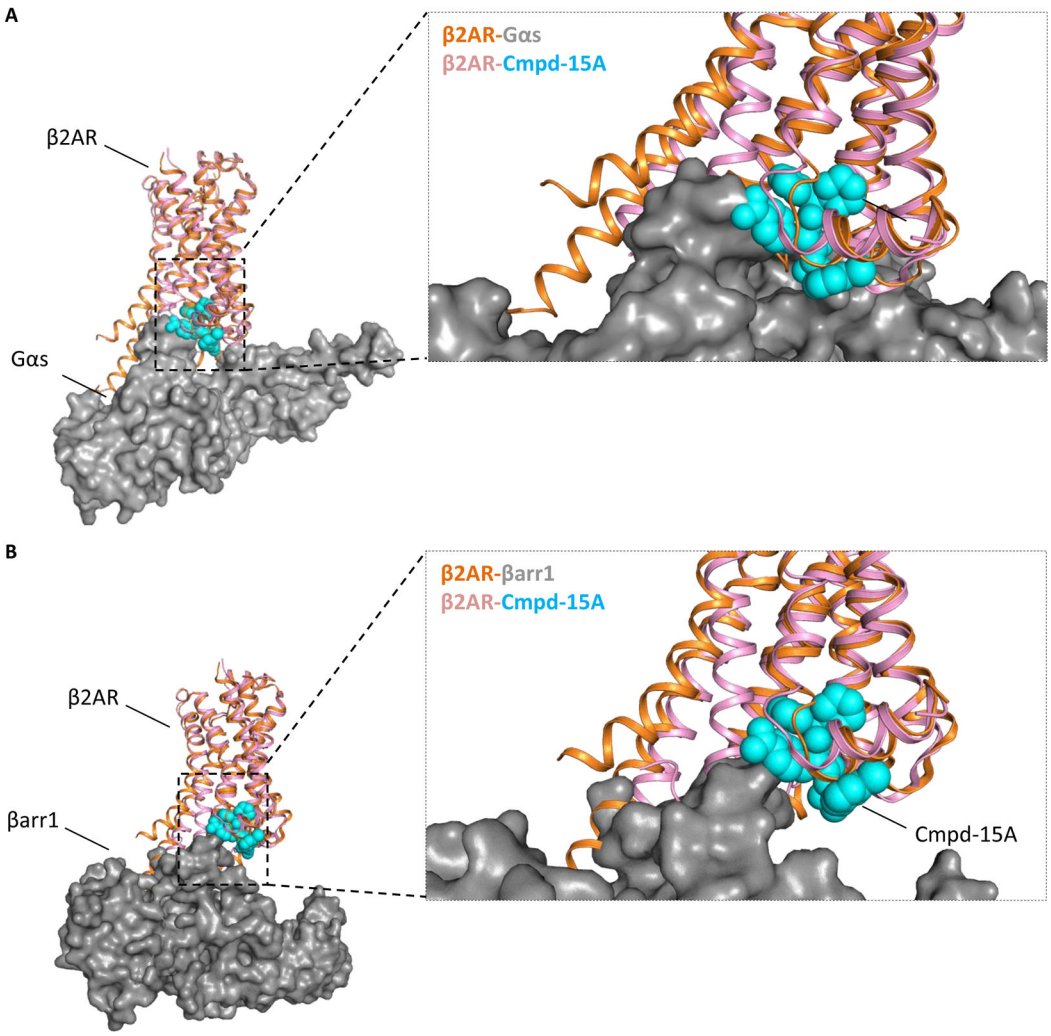

Figure 2. Overlapping interaction interface of intracellular β2AR antagonist with Gαs and βarr.

An overlay of the inactive β2AR (pink) crystal structure bound to Cmpd-15A (cyan) with an active β2AR structure (orange) bound to Gαs (grey) (A). The structural superimposition suggests a significant overlap between the binding site of cmpd-15A and the Gαs on cytoplasmic surface of the receptor. This overlap of the binding interface suggests that a direct competition between the cmpd-15A and Gαs may contribute to the antagonistic profile of the ligand. Similar to Gαs, the interface of the core interaction of βarrs on the β2AR also exhibits an overlap with the cmpd-15A binding site, albeit lesser than Gαs (B). Here, the crystal structure of β2AR (pink) in complex with cmpd-15A (cyan) is superimposed with a structural model of β2AR (orange)-βarr1 (grey) complex.

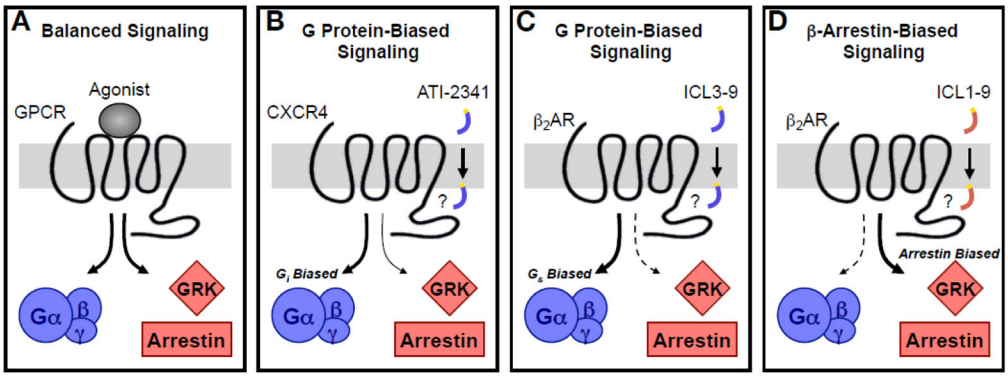

Figure 3. Pepducin-mediated biased GPCR signaling.

An unbiased agonist will induce balanced signaling by promoting coupling to heterotrimeric G-proteins, GRKs and βarrs (A). This will lead to G protein-dependent and β-arrestin-dependent signaling as well as GPCR desensitization, endocytosis and degradation. The CXCR4 pepducin ATI-2341 stabilizes a receptor conformation that selectively promotes Gαi-biased signaling with minimal coupling to Gα13, GRKs and βarrs (B). ICL3-9 is a Gαs-biased pepducin from the β2AR that stabilizes a receptor conformation that enhances Gαs signaling with minimal coupling to GRKs and βarrs and reduced desensitization and internalization of the β2AR (C). ICL1-9 is an arrestin-biased pepducin from the β2ARthat promotes βarr-dependent signaling with minimal G-protein signaling (D).

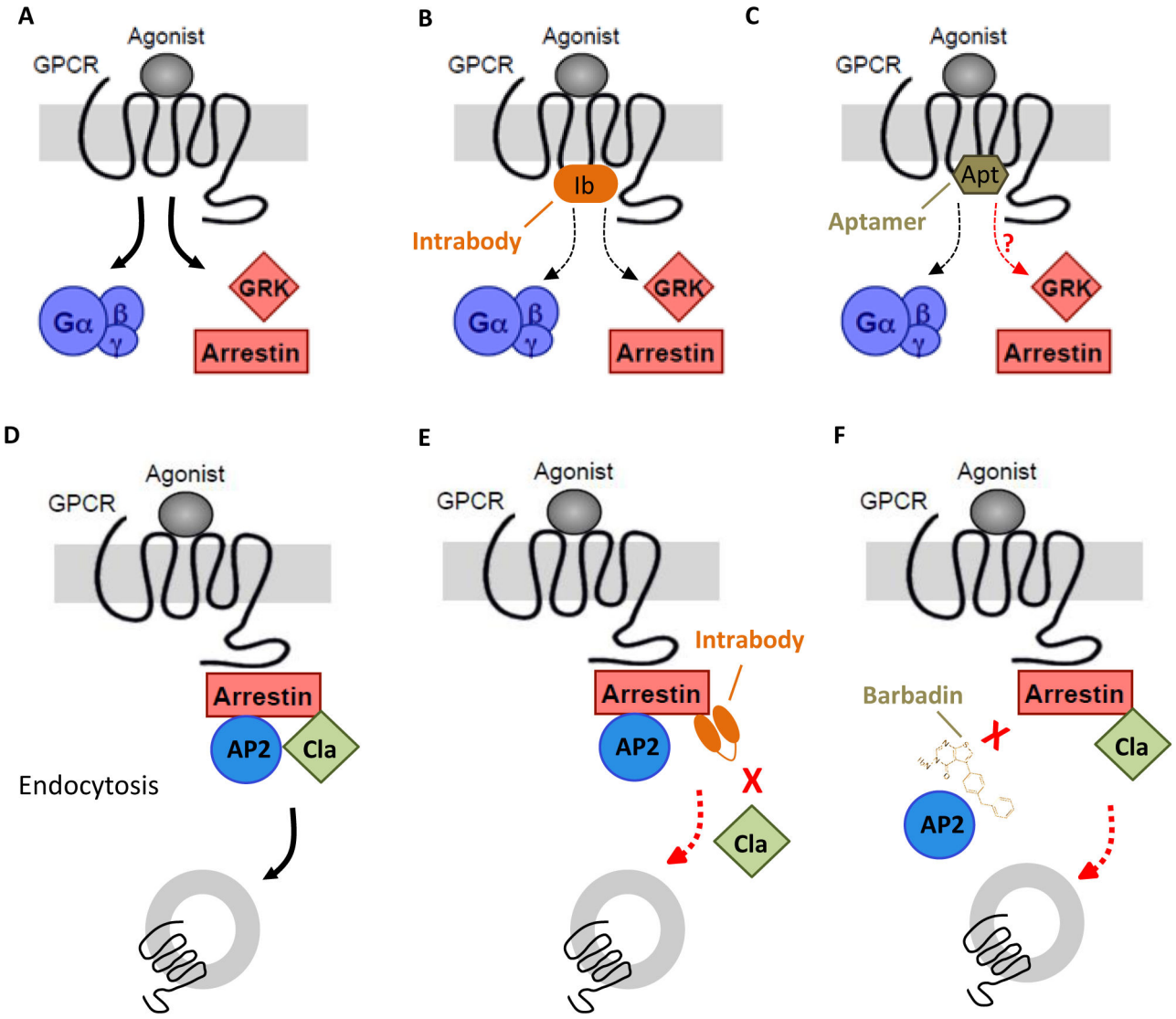

Figure 4. Emerging approaches to modulate GPCRs from the intracellular side.

A simplified schematic representation of bifurcated signal transduction pathways downstream of GPCRs (A). Upon agonist binding, GPCRs couple to heterotrimeric G proteins and βarrs. Nanobodies targeting β2AR when expressed as intrabodies (Ib) (cytoplasmic expression), can stabilize specific receptor conformation and/or interfere with transducer (G proteins and βarrs) coupling (B). These intrabodies recognize the intracellular surface of the receptor as they exhibit an effect on the functional response of β2AR in cellular context. RNA aptamers targeting the cytoplasmic surface of the β2AR have been developed that can exert an inhibitory effect on β2AR-Gαs coupling (C). A simplified schematic representation of GPCR endocytosis mediated by βarrs through their interaction with clathrin (Cla) and AP2 (D). Synthetic antibody fragments against βarr2 targeting the interface of βarr2-clathrin interaction have been developed (E). These antibody fragments when expressed as intrabodies can robustly inhibit agonist-induced GPCR endocytosis. A small molecule compound referred to as barbadin binds to the β2 adaptin subunit AP2 and

selectively inhibits its interaction with βarrs (F). As a result, barbadin inhibits agonist-induced GPCR internalization.