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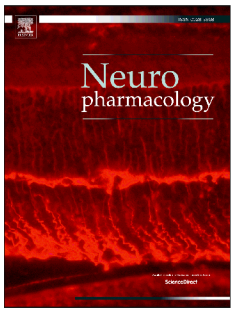

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ALLOSTERIC MODULATORS TARGETING CNS MUSCARINIC RECEPTORS

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ABSTRACT

Muscarinic acetylcholine receptors are G protein-coupled receptors (GPCRs) which are broadly expressed in the central nervous system (CNS) and other tissues in the periphery. They emerge as important drug targets for a number of diseases including Alzheimer's disease, Parkinson's disease, and schizophrenia. Muscarinic receptors are divided into five subtypes (M_1-M_5) of which M_1-M_4 have been crystalized. All subtypes possess at least one allosteric binding site which is located in the extracellular region of the receptor on top of the ACh (i.e. orthosteric) binding site. The former can be specifically targeted by chemical compounds (mostly small molecules) and binding of such allosteric modulators affects the affinity and/or efficacy of orthosteric ligands. This allows highly specific modulation of GPCR function and, from a drug discovery point of view, may be advantageous in terms of subtype selectivity and biased signaling. There is a plethora of allosteric modulators for all five muscarinic receptor subtypes. This review presents the basic principles of allosteric modulation of GPCRs on both the molecular and structural level focusing on allosteric modulators of the muscarinic receptor family. Further we discuss dualsteric (i.e. bitopic orthosteric/allosteric) ligands emphasizing their potential in modulating muscarinic receptor dynamics and signaling. The common mechanisms of muscarinic receptor allosteric modulation have been proven to be generalizable and are at play at many, if not all GPCRs. Given this paradigmatic role of muscarinic receptors we suggest that also new developments in muscarinic allosteric modulation may also be extended to other members of the GPCR superfamily.

Keywords: Muscarinic acetylcholine receptors, allosteric modulation, bitopic ligands, biased signaling, dualsteric ligands, subtype-selectivity

1. Introduction

Muscarinic acetylcholine receptors (muscarinic receptors) belong to the amine group of class A ("rhodopsin-like") G protein-coupled receptors (GPCRs) and comprise five distinct subtypes: M_1-M_5 (Fredriksson et al., 2003). The subtypes differ mainly in their expression pattern, G protein-specificity and in their molecular structure.

All subtypes are widely expressed in mammalian organisms and mediate a variety of physiological functions (Caulfield and Birdsall, 1998; Wess, 2004; Wess et al., 2007). For instance, muscarinic M_2Rs are the cardinal receptors mediating vagal modulation of heart tissue, whereas the muscarinic M_3Rs are located mainly on glandular and respiratory tissues, regulating glandular mucus secretion and bronchoconstriction, respectively (Alagha et al., 2014). Some of these functions are exploited therapeutically: muscarinic receptor antagonists are used in the treatment of chronic obstructive pulmonary disease, overactive bladder, Sjörengen's syndrome, and motion sickness (Novelli et al., 2012; Spinks and Wasiak, 2011; Wess et al., 2007). Alongside their peripheral effects, muscarinic receptors are abundant in the central nervous system where they play an important role in neuronal functions including the regulation of the dopaminergic system which is responsible for various cognitive and motor functions (Dencker et al., 2012). Imbalances in this system are implicated in various pathological conditions such as Alzheimer's disease, schizophrenia, Parkinson's disease and drug addiction, which enabled muscarinic receptors to emerge as potential drug targets for CNS disorders (Conn et al., 2009; Foster et al., 2014; Kruse et al., 2014a; Kruse et al., 2014b).

Activation of muscarinic receptors by agonists induces cellular signaling mainly by recruitment and activation of heterotrimeric G proteins. Three subtypes, M_1, M_3, and M_5, transmit their signals predominantly *via* the activation of $G_{q/11}$ proteins, which

stimulate phospholipase C that ultimately leads to an increase in intracellular Ca^{2+} concentrations, whereas M_2 and M_4 receptors favor activation of $G_{i/o}$ proteins, thereby inhibiting adenylyl cyclases and decreasing the intracellular levels of cAMP (Kruse et al., 2014b).

To date, four receptor subtypes (M_1-M_4) have been crystalized in an inactive conformation (Haga et al., 2012; Kruse et al., 2012; Thal et al., 2016). The overall structures are highly similar with the greatest structural homology within the ACh binding site (i.e. the orthosteric binding site). In line with countless mutagenesis studies, the receptor structures highlight why subtype-selective targeting of muscarinic receptors has not been achieved so far. However, there are also marked structural differences. Especially in the extracellular parts of the transmembrane domains and extracellular loops of the receptors there is low degree of sequence homology. The regions of low conservation comprise the 'common' allosteric binding site of muscarinic receptors (Ellis and Seidenberg, 1992). This gives the opportunity to exploit these allosteric binding sites as drug targets for subtype selective targeting of muscarinic receptors.

The concept of allosteric modulation at GPCRs was initially described at muscarinic receptors several decades ago (Lullmann et al., 1969; Mohr et al., 2013; Stockton et al., 1983). To date, they serve as the paradigm for allosteric modulation of GPCRs. A wealth of biochemical data and more recent structural data have revealed the basic molecular and structural mechanism of allosteric modulation at muscarinic receptors (Dror et al., 2013; Haga et al., 2012; Kruse et al., 2012; Kruse et al., 2014b; Kruse et al., 2013). In addition, numerous allosteric modulators are now available spanning an array of distinct pharmacological profiles. This makes targeting allosteric binding sites highly attractive for current drug development.

This review presents an overview of allosteric modulators of muscarinic receptors and their molecular and structural mechanisms in the light of potential advantages over classical orthosteric drugs. More recent developments, e.g. bitopic orthosteric/allosteric ligands, are described with regard to their molecular mechanisms and putative therapeutic advantages over purely allosteric modulators. The paradigmatic role of muscarinic receptors for allosteric modulation of GPCRs will be highlighted throughout this review.

2. Advantages of allosteric modulation of muscarinic receptors

Targeting allosteric binding sites of GPCRs appears to be a promising approach in particular for those receptors with structurally similar subtypes (i.e. muscarinic receptors) because high structural homology within the orthosteric binding site has severely hampered the identification of subtype selective ligands. In this light it is not surprising that although GPCRs are still one of the most important drug targets (Overington et al., 2006), and many still “un-drugged” GPCRs are associated with various diseases (Garland, 2013), novel drugs targeting GPCRs do not enter the market as frequent as one might expect. This may be because of unwanted off-target effects by engagement of different subtypes of the same receptor family (Allen and Roth, 2011). For instance, the M_1/M_4 preferring muscarinic partial agonist xanomeline was a promising compound for the treatment of Alzheimers disease. However, cholinomimetic adverse events such as gastrointestinal side effects have led to high dropout rates in clinical trials and discontinuation of the program (Bodick et al., 1997). In this regard, muscarinic receptors are a prominent example. They are implicated in the pathophysiology of Alzheimer’s disease, Parkinson’s disease, and schizophrenia. However, despite their prominent role in these CNS disorders and the great need for

improved therapeutic options in such due to frequent dose-limiting side effects, no modulators of CNS muscarinic receptors have entered the market until now (Foster et al., 2014).

In line with this, targeting allosteric binding sites can be highly advantageous as it offers specific modulation of GPCRs not achievable with orthosteric ligands. Most importantly, allosteric modulators exhibit a greater degree of subtype selectivity over classical orthosteric ligands. Mechanistically, this is mainly due to two mechanisms of selectivity. First, due to lower sequence conservation of allosteric binding sites, specific allosteric modulators can be identified with higher affinity for one subtype over the others. Second, subtype selectivity can also emerge from cooperativity rather than affinity, a phenomenon which has been termed 'absolute subtype selectivity' (Lazareno et al., 2004). The prime example is the allosteric modulator thiochrome (**Table 1**). Thiochrome has almost equal affinity for all muscarinic subtypes, however, it selectively enhances the binding of ACh at the M_4 subtype (Lazareno et al., 2004). Another advantage of allosteric modulators is that the allosteric effects only happen in the presence of an endogenous tone of the endogenous agonist (e.g. ACh in the case of muscarinic receptors). Given the fact that neuronal signal transduction is tightly controlled, allosteric modulation offers the opportunity to keep the spatial and temporal aspects of physiological signaling intact (Christopoulos, 2014; Christopoulos et al., 2014). In addition, allosteric effects are saturable, i.e. they exhibit a ceiling effect. This can be particularly advantageous in situations in which there is danger of overdosing. Beside these therapeutic advantages, two additional mechanistic considerations of allosteric modulators can be rated favorably. First, allosteric modulators mediate their effects in a probe-dependent manner. For instance, the allosteric modulator brucine (**Table 1**) enhances the affinity of ACh at M_1AChRs but shows neutral cooperativity with the

orthosteric antagonist *N*-methylscopolamine (NMS) (Lazareno et al., 1998). Another example is the alkaloid strychnine (**Table 1**) which is negatively cooperative with ACh at M_2Rs but is positively cooperative with NMS (Lazareno et al., 1998). In a physiological setting, probe dependence is especially important for receptors where multiple endogenous ligands are known, e.g. the chemokine receptor family. Second, allosteric ligands, in addition to their probe dependency, may preferably modulate a subset of all possible signaling pathways stimulated by the orthosteric agonist. This phenomenon termed 'biased allosteric modulation' adds another level of specificity to the pharmacological spectrum of allosteric modulators. For example, at M_1AChRs, the positive allosteric modulator VU029767 (**Table 1**) displays strong enhancement of ACh-stimulated intracellular Ca^{2+} release but does less so when ACh-stimulated phosphoinositide hydrolysis and phospholipase D activity are measured (Marlo et al., 2009). At M_4Rs, LY2033298 behaves as a positive allosteric modulator for ACh at multiple pathways. However, the degree of cooperativity between LY2033298 (**Table 1**) and ACh differs significantly between pathways (Leach et al., 2010). Hence, allosteric modulators may be able to fine-tune therapeutically beneficial signaling pathways.

3. Molecular principles of allosteric modulators

In the field of experimental pharmacology, the effects of allosteric modulators are commonly quantified by the allosteric ternary complex model (Christopoulos and Kenakin, 2002; Christopoulos and Mitchelson, 1997; Ehlert, 1988; Stockton et al., 1983). According to this model, binding of an allosteric ligand to an allosteric site modulates the affinity and/or efficacy of an orthosteric ligand and *vice versa* (Langmead and Christopoulos, 2014; Stockton et al., 1983). Conceptually, allosteric

modulators can be classified into three groups: positive allosteric modulators (PAMs), negative allosteric modulators (NAMs), and neutral allosteric ligands (NALs) (Christopoulos et al., 2014).

Positive allosteric modulators (PAMs) increase the affinity or efficacy or both of an orthosteric ligand or orthosteric agonist-receptor complex. *Negative allosteric modulators* (NAMs) decrease the affinity or efficacy of an orthosteric ligand or orthosteric agonist-receptor complex. *Neutral allosteric ligands* (NALs) bind to an allosteric site but have no effect on the affinity or efficacy of the orthosteric ligand (Christopoulos et al., 2014). Mechanistically, upon binding to an allosteric site, allosteric modulators alter the receptor structure in such a way that binding of an orthosteric ligand is enhanced (in the case of PAMs) or hampered (in the case of NAMs). NALs will also have an effect on receptor structure causing a conformational change, however, this does not affect the binding affinity of an orthosteric ligand. These allosteric mechanisms have first been identified at muscarinic receptors and are applicable to allosteric modulators of other GPCR families. Of note, allosteric modulators may have efficacy for receptor activation on their own (Christopoulos et al., 2014) which can therapeutically be beneficial, for instance, under particular pathological conditions in which the endogenous tone of neurotransmitter is reduced or even completely missing. Those allosteric modulators are termed 'allosteric agonists'.

The complex behavior of allosteric modulators is best illustrated by simulations of functional experiments using the operational model of agonism and allosterism (**Figure 1**). This model (Leach et al., 2007) is useful for analyzing experiments (e.g. measuring receptor activation or downstream signaling) when both an orthosteric and an allosteric ligand are present. This model allows quantifying the cooperativity between an orthosteric and an allosteric ligand with regard to modulation of binding

(i.e. affinity) and signaling (i.e. efficacy) of the orthosteric ligand using the parameters α and β, respectively (**Figure 1**). Dependent on the values of cooperativity, allosteric ligands can be classified with the aforementioned nomenclature (Christopoulos et al., 2014): $\alpha,\beta<1$; $\alpha,\beta=1$; $\alpha,\beta>1$ indicate negative, neutral and positive allosteric modulation of the orthosteric ligand's affinity (α) and efficacy (β), respectively.

In many cases, a PAM may display positive cooperativity with respect to both affinity and efficacy ($\alpha,\beta>1$). In functional experiments such allosteric behavior is detected as a left-ward shift of the concentration-effect curve of the orthosteric agonist upon addition of increasing concentrations of the allosteric ligand (**Figure 1 a,b**). This PAM activity can be accompanied with allosteric agonism itself (PAM-agonism) in which case an elevation of the lower plateau (i.e. in the absence of the orthosteric ligand) is also observed (parameterized by τ_A, **Figure 1a**). As an example, BQCA behaves as a PAM for ACh because it increases ACh binding and efficacy in Ca^{2+}-release and [^{35}S]GTPγS-binding assays (Ma et al., 2009). At high concentrations BQCA additionally behaves as a weak allosteric agonist (Ma et al., 2009). Another example is LY2119620 which shows similar PAM behavior for ACh and partial allosteric agonism at M_2 and M_4Rs in [^{35}S]GTPγS-binding assays (Croy et al., 2014; Schober et al., 2014). Interestingly, the effects of allosteric modulators on the affinity of the orthosteric ligand not always come along with their effects on the efficacy of orthosteric agonist-bound receptors (**Figure 1 c,d**). For example, LY2033298, a PAM with ACh at M_2Rs and M_4Rs, has also been shown to display allosteric agonism at M_2Rs (and less so at other subtypes) (Valant et al., 2012a). Moreover, LY2033298 also shows positive cooperativity in binding with the partial agonists pilocarpine and xanomeline, however, it displays negative cooperativity in signaling with both agonists when examined in ERK1/2 activation and [^{35}S]GTPγS-binding assays

(Valant et al., 2012a). This is particularly striking as LY2033298 itself is an allosteric agonist. Hence, albeit both the allosteric and orthosteric ligands alone produce active receptors, the ternary complex is inactive (at least with regard to the two signaling pathways). Such a behavior (simulated in **Figure 1 c,d**) is represented by opposite changes in the measured effect in the absence or presence of increasing concentrations of the allosteric ligand: in the absence of the orthosteric agonist increasing concentrations of the allosteric PAM-agonist lead to an increase of the lower plateau which is due to allosteric agonism. In contrast, at high concentrations of the orthosteric agonist increasing concentrations of the allosteric ligand lead to a decrease of the measured effect because the formed ternary complex produces less active receptors due to the negative cooperativity in signaling ($\beta<1$) of the allosteric ligand. The experimental signature of NAMs is represented by a right-ward shift of the concentration-effect curve of the orthosteric agonist (**Figure 1e,f**). For example, *N*-chloromethylbrucine is a NAM for ACh when measured in [^{35}S]GTPγS-binding assays at M_2Rs (Lazareno et al., 1998). Interestingly, the same allosteric modulator behaves as a PAM with ACh at M_3Rs – a clear example of allosteric subtype selectivity (Lazareno et al., 1998).

4. Structure and dynamics of allosteric modulation

The molecular details of allosteric modulation outlined above have been discovered using classical biochemical techniques such as radioligand binding experiments, mutagenesis and downstream signaling assays. Although they permit a mechanistic interpretation of allosteric modulation the structural insights obtained from these studies is limited. Technical breakthroughs in structural biology of GPCRs in the last decade have led to inactive-state structures of the M_1 (Thal et al., 2016), M_2 (Haga et al., 2012), M_3 (Kruse et al., 2012), and M_4R (Thal et al., 2016). Particularly interesting

from an allosteric point of view, the crystal structure of a ternary complex consisting of the active M_2R, the orthosteric agonist iperoxo and the PAM LY2119620 has been solved (Kruse et al., 2013). In addition, atomic-level molecular dynamics simulations of allosteric modulators for the M_2R have contributed to the understanding of the structural basis of allosteric modulation (Dror et al., 2013). These studies have shed light on the structural basis of allosteric ligand binding and have elucidated mechanisms of cooperativity between an allosteric and an orthosteric ligand.

The allosteric binding site of the M_2R is located in the extracellular vestibule of the receptor, 15 Å on top of the orthosteric binding site (Dror et al., 2013; Haga et al., 2012; Kruse et al., 2012; Kruse et al., 2013). Together, both binding sites form a large and coherent binding crevice in the inactive state of the M_2R (Haga et al., 2012). Upon activation of the receptor by the agonist iperoxo (Schrage et al., 2014; Schrage et al., 2013) the orthosteric site contracts and closes off towards the extracellular space. In the active state, both binding sites are virtually separated (Kruse et al., 2013). Noteworthy, also the allosteric binding site contracts upon receptor activation (Kruse et al., 2013). The allosteric binding site is mainly characterized by two aromatic centers. Center 1 is formed by $Y177^{ECL2}$ and $W422^{7.35}$ and center 2 is formed by $Y80^{2.61}$ and $Y83^{2.64}$ (Dror et al., 2013). These residues, among others, have been shown to be crucial for the affinity of classical bis-ammonium alkane allosteric modulators such as W84 (Huang et al., 2005; Lullmann et al., 1969; May et al., 2007; Prilla et al., 2006). The bis-ammonium alkane-type allosteric modulators (**Table 1**) form extensive cation-π interactions with both aromatic centers (Dror et al., 2013). The binding modes of other well-known allosteric modulators such as C_7/3-phth (Lanzafame et al., 1996), gallamine (Clark and Mitchelson, 1976), strychnine (Lazareno and Birdsall, 1995) and alcuronium (Jakubik et al., 1997) are highly similar and also involve cation-π interactions between their

positively-charged nitrogens and the two aromatic centers of the allosteric binding site (Dror et al., 2013). The PAM LY2119620, which is structurally different from the well-known allosteric modulators, has a different binding mode. However, LY2119620 also engages the residues of the aromatic center 1 albeit through π-π stacking rather than cation-π interactions (Kruse et al., 2013).

Besides the insights into allosteric ligand binding, the structural studies also suggest mechanisms for cooperativity (Dror et al., 2013; Kruse et al., 2013). This is best illustrated when comparing the active M_2R structure bound to iperoxo with the ternary complex with the PAM LY2119620 (Kruse et al., 2013). Both structures are remarkably similar which indicates that binding of iperoxo to the orthosteric binding site already shapes the conformation of the allosteric binding site. This mechanism defined as 'conformational coupling' between the orthosteric and allosteric binding sites can also explain the negative cooperativity observed between bis-ammonium alkane-type allosteric modulators and the orthosteric ligand NMS. The microsecond molecular dynamic simulations of M_2Rs show that the negative cooperativity between C_7/3-phth (and also gallamine) and the orthosteric ligand NMS are due to conformational coupling: NMS binding increases the volume of the allosteric binding site which makes C_7/3-phth (and also gallamine) binding less favorable as it prefers a smaller allosteric binding site (Dror et al., 2013). In addition to conformational coupling, electrostatic repulsion between positively-charged allosteric modulators (e.g. C_7/3-phth, W84, gallamine) and orthosteric ligands (e.g. NMS) has been suggested to be an underlying mechanism for negative cooperativity (Dror et al., 2013).

In contrast to the detailed structural insights into allosteric ligand affinity and allosteric mechanisms of cooperativity, little is known about the structural dynamics of allosteric modulation. Recent biophysical studies with the β_2-adrenergic receptor

(Manglik et al., 2015; Nygaard et al., 2013; Staus et al., 2016), the metabotropic glutamate receptor (mGluR) (Olofsson et al., 2014; Vafabakhsh et al., 2015) and the adenosine A_2A receptor (Ye et al., 2016) have provided evidence that GPCRs reside in a dynamic equilibrium of multiple inactive and active states. This equilibrium is modulated in a ligand- and G protein-dependent manner. In the light of GPCR dynamics, it would be interesting to understand how allosteric modulators influence the equilibrium of multiple receptor conformations in the absence and presence of different orthosteric ligands and intracellular signaling proteins. First evidence has been provided in a recent study where the influence of intracellular allosteric nanobodies has been studied on the equilibrium of the β_2 adrenoceptor upon stimulation with a library of different agonists (Staus et al., 2016). However, there are yet no data on how endogenous allosteric modulators - including the G protein itself - and other small-molecule allosteric modulators influence the conformational equilibrium of GPCRs. These future experiments will provide insight into allosteric mechanisms with a more dynamic focus.

5. Further developments of allosteric modulators: Bitopic orthosteric/allosteric ligands

The detailed molecular and structural knowledge of allosteric modulation of muscarinic receptors has enabled the design of bitopic orthosteric/allosteric (i.e. dualsteric) ligands (Bock and Mohr, 2013; Davie et al., 2013; Lane et al., 2013; Mohr et al., 2013; Mohr et al., 2010; Valant et al., 2012b; Valant et al., 2009). These ligands consist of an orthosteric moiety (either agonist or antagonist) which is connected by a chemical linker to an allosteric modulator (either PAM or NAM).

Bitopic orthosteric/allosteric ligands are a special case of bivalent ligands and target two distinct binding sites at the same receptor protomer.

Initially, the design of dualsteric ligands was based on the idea to combine the positive effects of orthosteric agonists with the higher subtype-selectivity of allosteric modulators (Disingrini et al., 2006). Such dualsteric agonists would retain the high binding affinity of the orthoster, be endowed with the subtype-selectivity of the alloster and, in contrast to allosteric modulators, would exert their effects also in the absence of an endogenous tone of ACh (Bock and Mohr, 2013; Lane et al., 2013). The first designed dualsteric ligands were 'hybrid 1' (i.e. iper-6-phth, **Table 2**) and 'hybrid 2' (i.e. iper-6-naph, **Table 2**) which were built from the orthosteric agonist iperoxo and parts of the bis-ammonium alkane NAMs (with ACh) W84 (iper-6-phth) and naphmethonium (iper-6-naph) connected *via* a flexible hexamethylene linker (Disingrini et al., 2006). Pharmacological studies have shown that the promise of muscarinic dualsteric agonists may hold true: they bind with high affinity to muscarinic receptors, exhibit a modest degree of selectivity for M_2Rs and robustly activate M_2Rs (Antony et al., 2009). Most interestingly, dualsteric activation of M_2Rs leads to preferential signaling through $G_{i/o}$ proteins. Activation of G_s proteins and β-arrestin recruitment are severely hampered, which classifies these dualsteric ligands as $G_{i/o}$-biased agonists (Bock et al., 2012). In addition, a number of dualsteric ligands for the M_2R and also M_1R have been described (**Table 2**). The spectrum of dualsteric ligands includes combinations of moieties with different functional properties, e.g. dualsteric antagonist-NAM (e.g. atr-6-naph)(Schmitz et al., 2014) or dualsteric agonist-PAM ligands (iperoxo-BQCAd)(Chen et al., 2015).

Dualsteric ligands have a complex chemistry, which results in an equally complex binding mode. Due to the two pharmacophores, which address distinct binding sites

of the receptor (i.e. the orthosteric and allosteric binding site), a dualsteric ligand can have multiple binding modes. This has been shown for iper-6-naph, a prototypical dualsteric ligand at the M_2R (Bock et al., 2016; Bock et al., 2014). Iper-6-naph (and also its congeners) can adopt at least two distinct binding modes (**Figure 2a**). One binding mode termed the 'dualsteric binding mode' is characterized by binding of iperoxo to the orthosteric site while the allosteric moiety 6-naph protrudes toward the extracellular part of the receptor and engages residues of the allosteric binding site (**Figure 2a**, left panel). The dualsteric binding mode produces active receptors and leads to activation of G proteins and cellular signaling albeit in a functionally selective manner (see below)(Bock et al., 2016; Bock et al., 2014; Bock et al., 2012). The second binding mode termed the 'purely allosteric binding mode' (**Figure 2a**, right panel) is characterized by the entire dualsteric ligand residing in the allosteric vestibule (both the orthosteric and the allosteric moieties), highly similar to the binding modes of typical allosteric modulators (e.g. W84, C_7/3-phth, gallamine) (Bock et al., 2016; Dror et al., 2013). The purely allosteric binding mode stabilizes inactive receptors and blocks receptor activation (Bock et al., 2016; Bock et al., 2014). Both binding modes occur in the same given ensemble of receptors and form a '*ligand binding ensemble*' (Bock et al., 2016) of active and inactive receptors bound to the same dualsteric ligand in two distinct binding modes. The extent of either binding mode is dependent on the affinities of either pharmacophore to its preferred binding site and the overall affinity of the dualsteric ligand in both binding modes (Bock et al., 2014). The formation of the ligand binding ensemble is theoretically possible for all dualsteric ligands but has so far only been shown for iperoxo-derived dualsteric ligands at the M_2R (Bock et al., 2016; Bock et al., 2014) and the M_1R (Chen et al., 2015). However, the existence of the dualsteric binding mode alone has been

demonstrated for a number of ligands, e.g. McN-A-343 (Valant et al., 2008) for M_2Rs, 77-LH-28-1 (Keov et al., 2014) and pirenzepin-BODIPY (Daval et al., 2013) for M_1Rs.

Dualsteric ligands display specific functional properties due to their complex binding mode(s). This has been studied most notably in terms of ligand efficacy and biased signaling (**Figure 2b**). Most of the dualsteric agonists, especially McN-A-343 and the iperoxo-derived dualsteric ligands for the M_2R are partial agonists. The molecular mechanisms underlying this special type of partial agonism at M_2Rs are highly complex and not yet fully understood. Multiple lines of evidence suggest that the partial agonist behavior of dualsteric ligands may come from both the dualsteric binding mode itself and from the equilibrium of active and inactive receptors, i.e. the ligand binding ensemble. With regard to receptor conformations, the dualsteric binding mode is likely to stabilize a conformation which will be different from the one stabilized by an orthosteric agonist alone. For example, in contrast to the parental orthosteric agonist iperoxo, the dualsteric ligands iper-6-phth and iper-6-naph stabilize a different M_2R conformation as measured by Fluorescence Resonance Energy Transfer (FRET) techniques using a M_2R FRET-sensor which reports on conformational changes between ICL3 and the C terminus of the receptor (Bock et al., 2012). In line with this, the activation of $G_{i/o}$ proteins by iper-6-phth and iper-6-naph is also reduced as demonstrated by Bioluminescence Resonance Energy Transfer (BRET) techniques using a Gi/o-BRET sensor which reports conformational changes of G protein-activation (Bock et al., 2012; Gales et al., 2005). Moreover, the dualsteric binding mode of McN-A-343 has also been suggested to be responsible for the resulting partial agonism because the allosteric moiety behaves as an antagonist and counteracts the agonism encoded in the orthosteric part of McN-A-343 (i.e. tetramethylammonium)(Valant et al., 2008). In addition to this mechanism the presence of dualsteric ligand-bound inactive receptors (the ligand is bound in the

purely allosteric binding mode, **Figure 2a** right panel) can reduce the overall efficacy of a dualsteric agonist (Bock et al., 2016; Bock et al., 2014; Chen et al., 2015). The greater the fraction of these inactive receptors, the less is the overall efficacy of the dualsteric ligand (**Figure 2b**).

Dualsteric ligands have been shown to exhibit functional selectivity, i.e. they preferentially activate one pathway over others (Kenakin, 2005). At M_2Rs, iper-6-phth and iper-6-naph have been classified as $G_{i/o}$-biased agonists (Bock et al., 2012) and McN-A-343 preferentially activates $G\alpha_{15}$ proteins (Griffin et al., 2007; Valant et al., 2008). At M_1Rs, the dualsteric ligands VU0357017 (=ML071) and VU0364572 are biased towards Ca^{2+} signaling and ERK1/2 activation and fail to recruit β-arrestin (Digby et al., 2012). The mechanism by which dualsteric ligand activation of muscarinic receptors leads to functional selectivity is not known. However, it is likely that dualsteric agonists stabilize a distinct subset of receptor conformations. In line with this, we have recently shown that the dualsteric binding mode (**Figure 2a**, left panel) interferes with the closure of the orthosteric binding site upon receptor activation. This may lead to different intracellular TM6 conformations which may result in altered signaling (Bermudez et al., 2017).

Lastly, alongside partial and biased agonism, dualsteric targeting of muscarinic receptors may offer a third, rather unexplored, signaling feature, i.e. protean agonism. Protean agonists behave as weak partial agonists when the receptor is inactive, however, when the receptor is spontaneously active, they show inverse agonism (Kenakin, 2007). In a recent study, we have demonstrated that dualsteric ligands with very weak efficacy (**Figure 2b**) can be protean agonists (De Min et al., 2017).

6. Summary and outlook

Allosteric modulation of GPCRs offers the possibility to fine-tune GPCR signaling in ways not achievable with classical orthosteric drugs. The importance of allosteric modulators is highlighted by drugs which have already entered the market: e.g. cinacalcet (Mimpara®), a PAM enhancing the effect of calcium ions at the calcium-sensing receptor used for the treatment of secondary hyperparathyroidism, maraviroc (Celsentri®), a NAM of the CC-motif chemokine receptor 5, blocking the HIV gp120 protein from binding to the receptor (Lagane et al., 2013), and plerixafor (Mozobil®), an allosteric antagonist at the CXC-motif chemokine receptor 4 that is used for stem cell mobilization in transplantations (Scholten et al., 2012).

Muscarinic receptors are the prime example of allosteric modulation of GPCRs and allosteric mechanisms identified at muscarinic receptors are paradigmatic for the entire GPCR superfamily. However, there are many aspects of allosteric modulation remaining to be discovered, two of them are highlighted here. First, although a plethora of allosteric ligands exist for all receptor subtypes, the chemical space of the modulators is far from being complete. With muscarinic receptor crystal structures at hand, many new scaffolds of allosteric modulators should be discovered by structure-based drug design and virtual screening. First evidence comes from a very recent study at the M_2R which used accelerated molecular dynamics simulations and has led to allosteric modulators with novel chemical scaffolds (Miao et al., 2016). A second aspect applies to the generalizability of the principles of dualsteric ligand binding and formation of ligand binding ensembles. One could argue that these mechanisms are somewhat specific for muscarinic receptors and will not be transferable to other receptors. However, dualsteric/bitopic ligands have been designed and discovered for other GPCR families, e.g. LUF6258 and VCP746 for the adenosine A1A receptor (Narlawar et al., 2010; Valant et al., 2014) and SB269652 for the dopamine D2 receptor (Lane et al., 2014). The dualsteric ligands for the A1A

receptor also display biased agonism which suggest that a dualsteric binding mode more generally induces an altered signaling profile of the receptor. Moreover, at serotonin 5-HT_{2B} receptors lysergic acid diethylamide and its precursor ergotamine are β-arrestin-biased agonists and the 5-HT_{2B} crystal structures reveal an 'extended' binding mode which is similar to the dualsteric binding mode discussed here at muscarinic receptors (Wacker et al., 2013; Wang et al., 2013). In addition, given the progress in understanding dualsteric ligands at muscarinic receptors, several ligands which were thought to be allosteric modulators were re-classified as dualsteric ligands (e.g. AC-42 at M_1Rs). Hence, one can be optimistic that more dualsteric ligands at different GPCRs will be discovered and, using structure-based drug discovery, even be designed.

Taken together, advancing our understanding of allosteric modulation at muscarinic receptors will not only be important to discover subtype-selective drugs for muscarinic receptors but, beyond, may lead to attractive allosteric strategies to improve drug targeting of other GPCR families.

Figure 1: Modulation of GPCR signaling by allosteric modulators: theory and experiments. Effects of allosteric modulators on orthosteric agonist-induced GPCR signaling can be described by an operational model of agonism and allosterism (see formula). E_{MAX} is the maximal effect of the system; [X] and [A] are the molar concentrations of the orthosteric and allosteric ligand, respectively; K_X and K_A are the equilibrium dissociation constants (reflecting affinity) of the orthosteric and allosteric ligand, respectively; τ_X and τ_A are operational measures of orthosteric and allosteric efficacy, respectively. Allosteric modulators have three key aspects which can be quantified with this model: cooperativity of binding (α), cooperativity of signaling (β) with the orthosteric agonist and allosteric agonism itself (τ_A).

(**a-f**) Simulation of experimental scenarios (ACh as the orthosteric agonist) which may be obtained with allosteric modulators of various properties. For simulations, the following parameters have been constrained: E_{MAX}=100, $\log K_X$=-6, $\log K_A$=-7, τ_X=10. The concentration of the allosteric modulator was increased from 0.3 nM to 3 µM (blue line to yellow line). The values for α and β are indicated in the graph. (**a,c,e**) allosteric agonism: τ_A=1. (**b,d,f**) no allosteric agonism: τ_A=0.01.

Figure 2: Ligand binding ensembles and functional implications. (**a**) Dualsteric/bitopic ligands can have at least two distinct binding modes: one dualsteric binding mode which produces active receptors (R*, left panel) and one purely allosteric binding mode which does not lead to receptor activation (R, right panel). Shown are snapshots from all-atom molecular dynamics simulations of active and inactive muscarinic M_2Rs bound to the dualsteric ligand iper-6-naph. (**b**) Depending on the nature of the ligand and its preference for either active or inactive receptors, the equilibrium of ligand-bound receptor ensembles determines the functional response detected in experiments. The efficacy spectrum can range from nearly full agonism (mostly biased, for details see text) to partial agonism or even inverse agonism (in spontaneously active systems).

Table 1

Receptor subtype	Modulator	Cooperativity with ACh		Reference
		Binding experiments	Functional experiments	
M1	brucine	+	+	(Birdsall et al., 1997)
	KT5720	+	n.d.	(Lazareno et al., 2000)
	BQCA	+	+	(Ma et al., 2009)
	VU0119498	n.d.	+	(Bridges et al., 2009)
	VU0027414	n.d.	+	(Marlo et al., 2009)
	VU0090157	+	+	(Marlo et al., 2009)
	VU0029767	+	+	(Marlo et al., 2009)
	ML137 (=VU0366369)	n.d.	+	(Bridges et al., 2010a; Bridges et al., 2010b)
	Lu AE51090	n.d.	+	(Sams et al., 2010)
	MK7622 (=PQCA)	n.d.	+	(Kuduk et al., 2011)
	ML169 (=VU0405652)	n.d.	+	(Reid et al., 2011)
	VU0456940	n.d.	+	(Tarr et al., 2012)
	VU0413162	n.d.	+	(Poslusney et al., 2013)
	VU0448350	n.d.	+	(Melancon et al., 2013)
	4-phenylpyridin-2-one derivatives	+	+	(Mistry et al., 2016)
	MT3	n.d.	-	(Jolkkonen et al., 1994; Olianas et al., 1999)
	MT7	-	-	(Olianas et al., 2000; Onali et al., 2005)
	staurosporine	-	-	(Lazareno et al., 2000)
	tacrine	-	n.d.	(Fang et al., 2010; Potter et al., 1989)
M2	(-)eburnamonine (=vinburnine)	+	n.d.	(Jakubik et al., 1997)
	LY2033298	+	+	(Valant et al., 2012a)
	LY2119620	+	+	(Croy et al., 2014; Kruse et al., 2013)
	W84	n.d.	-	(Lullmann et al., 1969)
	gallamine	n.d.	-	(Clark and Mitchelson, 1976)
	C7/3-phth	n.d.	-	(Lanzafame et al., 1996)
	alcuronium	-	n.d.	(Jakubik et al., 1997)
	strychnine	-	-	(Jakubik et al., 1997; Lazareno and Birdsall, 1995)
	brucine	-	n.d.	(Jakubik et al., 1997)
	WIN-51708	-	n.d.	(Lazareno et al., 2002)
	WIN-62577	-	n.d.	(Lazareno et al., 2002)
	dimethyl-W84	n.d.	-	(Maier-Peuschel et al., 2010)
M3	brucine	+	n.d.	(Jakubik et al., 1997; Lazareno et al., 1998)
	N-chloromethyl-brucine	+	n.d.	(Lazareno et al., 1998)
	N-Benzyl-brucine	+	n.d.	(Lazareno et al., 1998)
	brucine-N-oxide	+	n.d.	(Lazareno et al., 1998)
	WIN-62577	+	n.d.	(Lazareno et al., 2002)
	VU0119498	n.d.	+	(Bridges et al., 2009)
	alcuronium	-	n.d.	(Jakubik et al., 1997)
	brucine	-	n.d.	(Jakubik et al., 1997; Lazareno et al., 1998)
	WIN-51708	-	n.d.	(Lazareno et al., 2002)
M4	thiochrome	+	+	(Lazareno et al., 2004)
	LY2033298	+	+	(Chan et al., 2008)
	VU0010010	+	+	(Shirey et al., 2008)
	VU0152099	+	+	(Brady et al., 2008)
	VU0152100	+	+	(Brady et al., 2008)
	ML293	n.d.	+	(Salovich et al., 2012)
	ML253	n.d.	+	(Le et al., 2013)
	LY2119620	+	+	(Croy et al., 2014)
	VU0467154	+	+	(Bubser et al., 2014)
	MT3	n.d.	-	(Jolkkonen et al., 1994; Olianas et al., 1999)
	alcuronium	-	n.d.	(Jakubik et al., 1997)
M5	VU0119498	n.d.	+	(Bridges et al., 2009)
	VU0238429	+	+	(Bridges et al., 2009)
	VU0365114	n.d.	+	(Bridges et al., 2010a)
	VU0400265	n.d.	+	(Bridges et al., 2010a)
	ML326 (=VU0467903)	n.d.	+	(Gentry et al., 2013a)
	ML380	+	+	(Gentry et al., 2014)
	ML375 (=VU0483253)	n.d.	-	(Gentry et al., 2013b)
	VU6000181	n.d.	-	(Kurata et al., 2015)

Table 1: Allosteric modulators of muscarinic acetylcholine receptors. The most important allosteric modulators of muscarinic receptors are listed. The cooperativity with ACh is indicated ('+' = positive cooperativity, '-' = negative cooperativity, 'n.d.' not determined).

Table 2

Receptor subtype	Dualsteric/bitopic ligand	Functional properties	Reference
M1	VU0357017/ML071	agonist	(Digby et al., 2012)
	VU0364572		(Digby et al., 2012)
	TBPB		(Keov et al., 2014; Keov et al., 2013)
	77-LH-28-1		(Keov et al., 2014)
	iperoxo-BQCAd		(Chen et al., 2015)
	AC-42		(Avlani et al., 2010; Lebon et al., 2009)
	Lu AE51090		(Sams et al., 2010)
	Pirenzepin-BODIPY	antagonist	(Daval et al., 2013)
	para-LRB-AC42	n.d.	(Daval et al., 2012)
M2	McN-A343	agonist	(Valant et al., 2008)
	iper-6-phth (=hybrid 1)		(Antony et al., 2009; Bock et al., 2012)
	iper-6-naph (=hybrid 2)		(Antony et al., 2009; Bock et al., 2012)
	isox-6-phth		(Bock et al., 2014)
	isox-6-naph		(Bock et al., 2014)
	THRX-160209	antagonist	(Steinfeld et al., 2007)
	atr-6-phth		(Schmitz et al., 2014)
	atr-6-naph		(Schmitz et al., 2014)
	sco-6-phth		(Schmitz et al., 2014)
	sco-6-naph		(Schmitz et al., 2014)

Table 2: Dualsteric/bitopic ligands for muscarinic receptors. The most important dualsteric ligands for the M_1-and M_2Rs are listed. The functional properties (agonist/antagonism) are indicated. n.d.: not determined.

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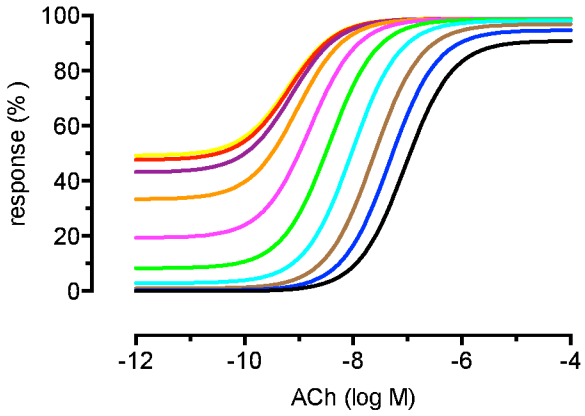
$$E = \frac{E_{MAX}}{1 + \frac{([X]K_A + K_X K_A + K_X[A] + \alpha[X][A])}{(\tau_X[X](K_A + \alpha\beta[A]) + \tau_A[A]K_X)}}$$

allosteric agonism

no allosteric agonism

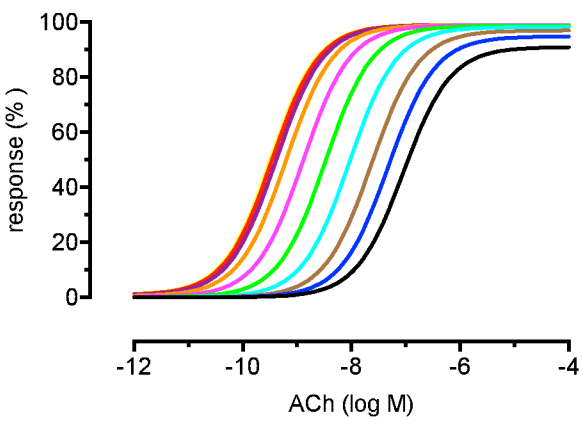
a

α=30; β=10



b

α=30; β=10



c

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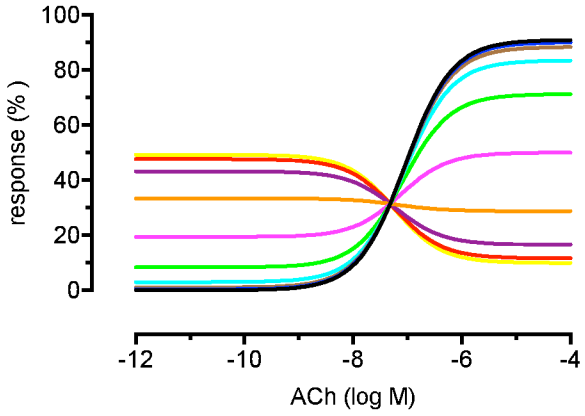

d

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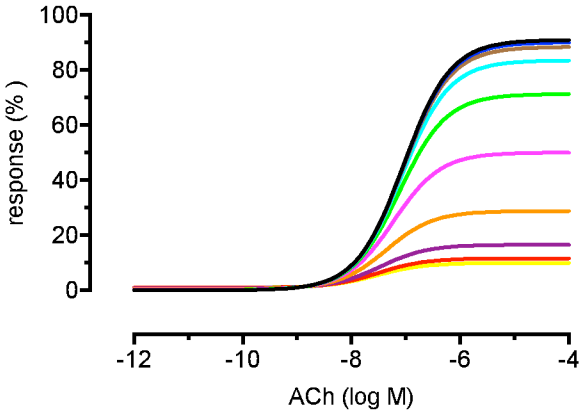

e

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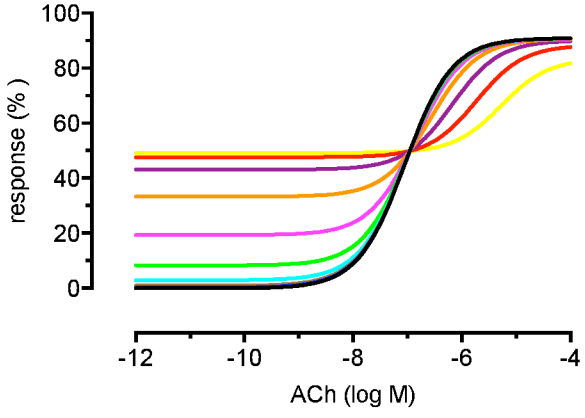
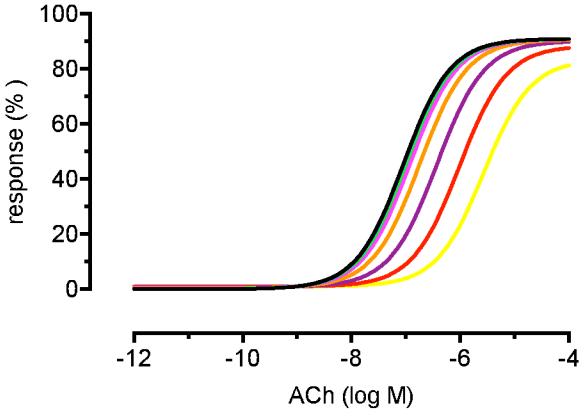
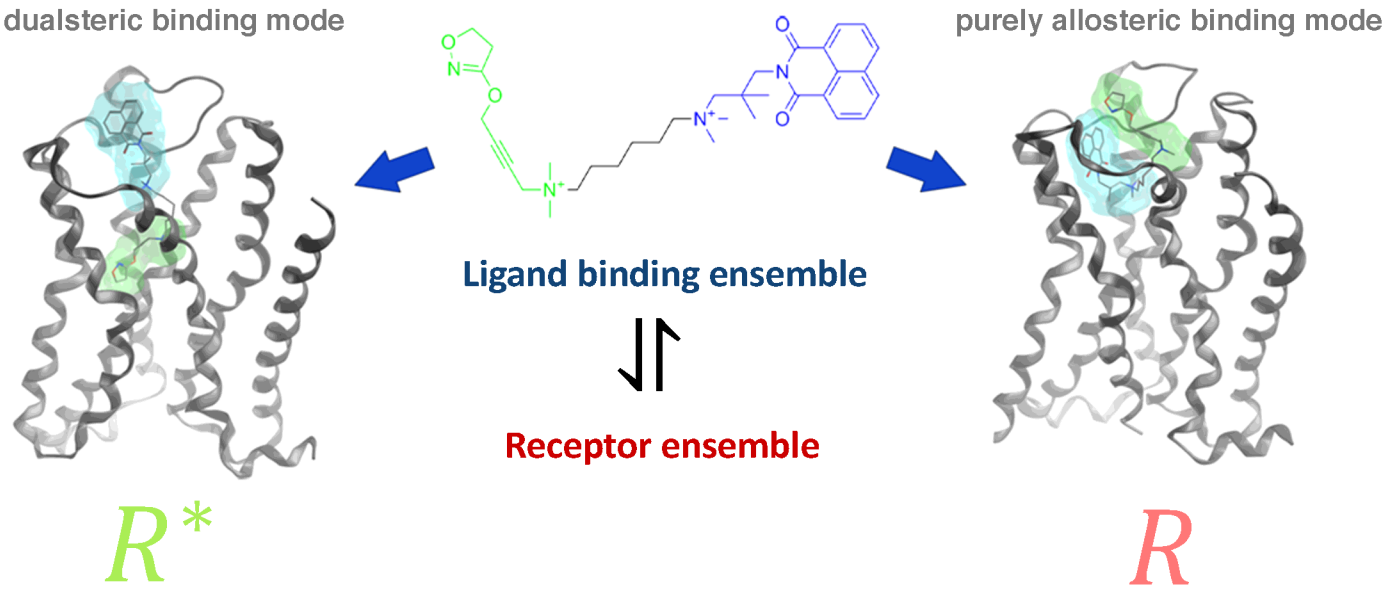


f

α=0.03; β=0.01



a



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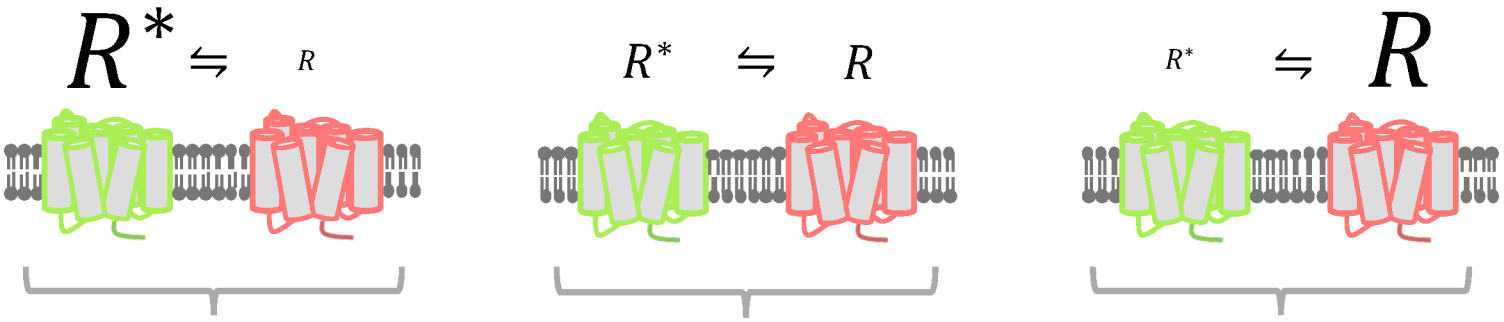

Partial to full agonism
(dependent on agonist)

Biased agonism

Partial agonism
(independent on agonist)

Biased agonism

Protean agonism to partial agonism
(dependent on agonist)

Highlights

In this review, we

- outline the importance of allosteric modulation for GPCR drug discovery,
- present the molecular and structural principles of allosteric modulation,
- provide tables of the most important allosteric modulators for all subtypes,
- and discuss more recent developments in the field of allosteric modulation of muscarinic receptors (e.g. bitopic ligands and biased signaling).