The Best of Both Worlds? Bitopic Orthosteric/Allosteric Ligands of G Protein–Coupled Receptors

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Abstract

It is now acknowledged that G protein–coupled receptors, the largest class of drug targets, adopt multiple active states that can be preferentially stabilized by orthosteric ligands or allosteric modulators, thus giving rise to the phenomenon of pathway-biased signaling. In the past few years, researchers have begun to explore the potential of linking orthosteric and allosteric pharmacophores to yield bitopic hybrid ligands. This approach is an extension of the more traditional bivalent ligand concept and shares some of the same challenges, including the choice and role of the linker between the two pharmacophores and the validation of mechanism of action. Nonetheless, the promise of bitopic ligands is the generation of novel chemical tools that have improved affinity and/or selectivity profiles. Previously identified functionally selective compounds (and medicines) also may act via a bitopic mechanism, suggesting that the phenomenon is more widespread than currently appreciated.

INTRODUCTION

GPCR: G protein–coupled receptor

TM: transmembrane-spanning

Bitopic ligand: a single chemical entity composed of an orthosteric pharmacophore covalently linked to an allosteric pharmacophore

Orthosteric site: the endogenous agonist-binding site on a receptor

Allosteric site: a binding site on the receptor that is topographically distinct from the endogenous agonist-binding (orthosteric) site

G protein–coupled receptors (GPCRs) are the largest family of cell surface receptors in living organisms, constituting ~2% of the human genome (1). Structurally, all GPCRs share a characteristic architecture that consists of an extracellular N-terminal domain, an intracellular C-terminal domain, and seven transmembrane-spanning (TM) domains (TM1–TM7) connected by three extracellular and three intracellular loops. Despite this common architecture, GPCRs can be activated by a diverse array of stimuli such as photons, ions, odorants, biogenic amines, lipids, and complex polypeptides to transduce extracellular signals into intracellular responses. Although the best-characterized intracellular GPCR coupling partners are the family of heterotrimeric G proteins, GPCRs also interact with other intracellular and membrane-associated signaling and scaffolding proteins. This remarkable flexibility allows GPCRs to modulate virtually any physiological process. Not surprisingly, GPCRs are highly tractable drug targets; approximately one-third of all medicines on the market target this protein family (2, 3). However, despite this proven success, only a small proportion of these receptors is currently targeted by therapeutics (3, 4), a fact that likely arises from a variety of reasons. For instance, not all GPCRs are necessarily of immediate relevance to the pharmaceutical industry; most olfactory GPCRs have not been traditionally targeted in this regard. Furthermore, some GPCR classes (e.g., biogenic amines) have been relatively easier to target than others (e.g., peptide receptors) and have simply benefited from more time spent on research into their properties. Orphan GPCRs present a challenge because of the lack of identified endogenous agonists. In all instances, however, additional issues are involved in the failure to appreciate novel paradigms of drug action at these receptors because this area is constantly in flux and rapidly expanding. Specific challenges in this regard include the fact that a single GPCR may couple promiscuously to more than one type of G protein in a cell- and tissue-dependent manner; signal though G protein–independent pathways; undergo complex regulatory processes; be allosterically modulated by small molecules and other proteins, including other GPCRs (5, 6); and adopt ligand-specific conformations that may be signal complex–biased or pathway-biased (5, 7, 8). Although these phenomena present novel avenues for achieving selectivity in drug action, the same functional versatility makes the identification and validation of effective therapeutics increasingly multidimensional. In recent years, this picture has become even more interesting owing to the discovery of bitopic ligands, i.e., molecules that can engender selectivity through concomitant engagement with orthosteric and allosteric sites on GPCRs. This review focuses on the key developments in the field that have led to the current interest in bitopic GPCR ligands.

GPCRs POSSESS MULTIPLE MODES FOR BINDING AND ACTIVATION

Although all mammalian members of the GPCR family possess a similar structural architecture, they are characterized by a relatively low sequence identity and can be distributed into families and subfamilies that have distinctive structural elements and themes for ligand binding, activation, and regulation (2, 9, 10). Family A (also termed Class I; 690 members) GPCRs constitute the rhodopsin-like group and include important drug targets such as the biogenic amine receptors and opioid receptors. Family B (Class II; 48 members) receptors constitute the secretin-like and adhesion GPCRs and include several current candidates for the development of potentially important drugs, such as the glucagon-like peptide 1 receptor. Family C GPCRs constitute the glutamate-like group (Class III; 22 members); they have a large amino-terminal domain that adopts a Venus flytrap–like structure containing the endogenous agonist-binding (orthosteric) site. The diverse

ligands for these receptors include very small molecules such as glutamate, γ-aminobutyric acid (GABA), or calcium. Although implicated in the action of many GPCRs, receptor dimerization is a particularly fundamental theme for Family C GPCRs (11).

At the turn of this millennium, structural studies of GPCRs utilized the high-resolution crystal structure of rhodopsin (12) as the prototypical template. Since 2007, more structures of Family A GPCRs have been determined, including the β_1 and β_2 adrenergic receptors, the adenosine A_{2A} receptor, the dopamine D_3 receptor, and the chemokine CXCR4 receptor (13–20). All these structures have highly similar helical bundles but reveal substantial divergence in the loop and tail regions (**Figure 1**). The structures not only highlight an intrahelical ligand-binding cavity but also indicate an important role of the extracellular loops for ligand binding and/or ligand entry. Until recently, most high-resolution GPCR structures were of antagonist- or inverse agonist–bound inactive states, but they nonetheless served to highlight the fact that different GPCR-targeting ligands can adopt strikingly different poses depending on the receptor type. The first minimally active conformation of a Family A GPCR was directly appreciated when the crystal structure was solved for opsin associated with a C-terminal peptide fragment of its G subunit, transducin (21). This structure suggested the presence of a substantial change in the arrangement of the helical bundle, with prominent movement of TM6. More recent studies have now provided insight into the mechanism of activation of other GPCRs, specifically the β adrenergic and adenosine A_{2A} receptors (22–25). Using a camellid antibody that stabilized the active state of the receptor (analogous to the action of a G_s protein), Rasmussen et al. (22) found that significant changes occur at the cytoplasmic face of the receptor, including the outward displacement of TM6 and TM5 and an inward movement of TM7 and TM3. In the recently published agonist-bound adenosine A_{2A} structure, significant movements of the cytoplasmic ends of TM3, TM6, and TM7 were observed, similar to those seen in both the opsin and the β_2 adrenergic receptor. In both cases, these changes are coupled to relatively subtle changes in the agonist-binding pocket when compared with the antagonist-binding pocket. This leads to the question: Which agonist-receptor interactions are essential to receptor activation? Although similar conformational changes occur at the intracellular sides of the agonist-bound β_2 adrenergic receptor and the agonist-bound A_{2A} adenosine receptor, these changes appear to be mediated by distinct agonist-binding interactions. In the β_1 adrenergic receptor, Warne et al. (24) observed that full agonists were able to form a hydrogen bond with a conserved serine from TM5. This interaction may reduce the energy barrier to allow the residue repacking and helix rotation that are observed in the β_2 adrenergic receptor–agonist-antibody complex. Conversely, binding of the A_{2A} adenosine receptor agonist does not directly affect TM5; instead, the most pronounced ligand-induced changes involve TM3, TM6, and TM7, as well as a significant movement of extracellular loop 3.

Collectively, the recently solved GPCR structures highlight how a common architecture can nonetheless accommodate a structurally diverse set of ligands to mediate conformational changes that lead to signal transduction. However, crystal structures only provide snapshots of discrete states and cannot directly interrogate the dynamic equilibria between conformational transitions. In this regard, alternative approaches are warranted to allow visualization of receptor structural changes not currently amenable to X-ray crystallography. For example, a recent study by Bokoch et al. (26) used nuclear magnetic resonance spectroscopy to investigate dynamic, ligand-specific changes around a salt bridge linking extracellular loops 2 and 3 of the β_2 adrenergic receptor. An important finding from this study was the identification of conformational coupling between the extracellular domain and the orthosteric binding site. This finding adds weight to the growing body of data indicating that the extracellular surface of a GPCR is remarkably diverse and therefore also represents another target region for the discovery of subtype-selective drugs.

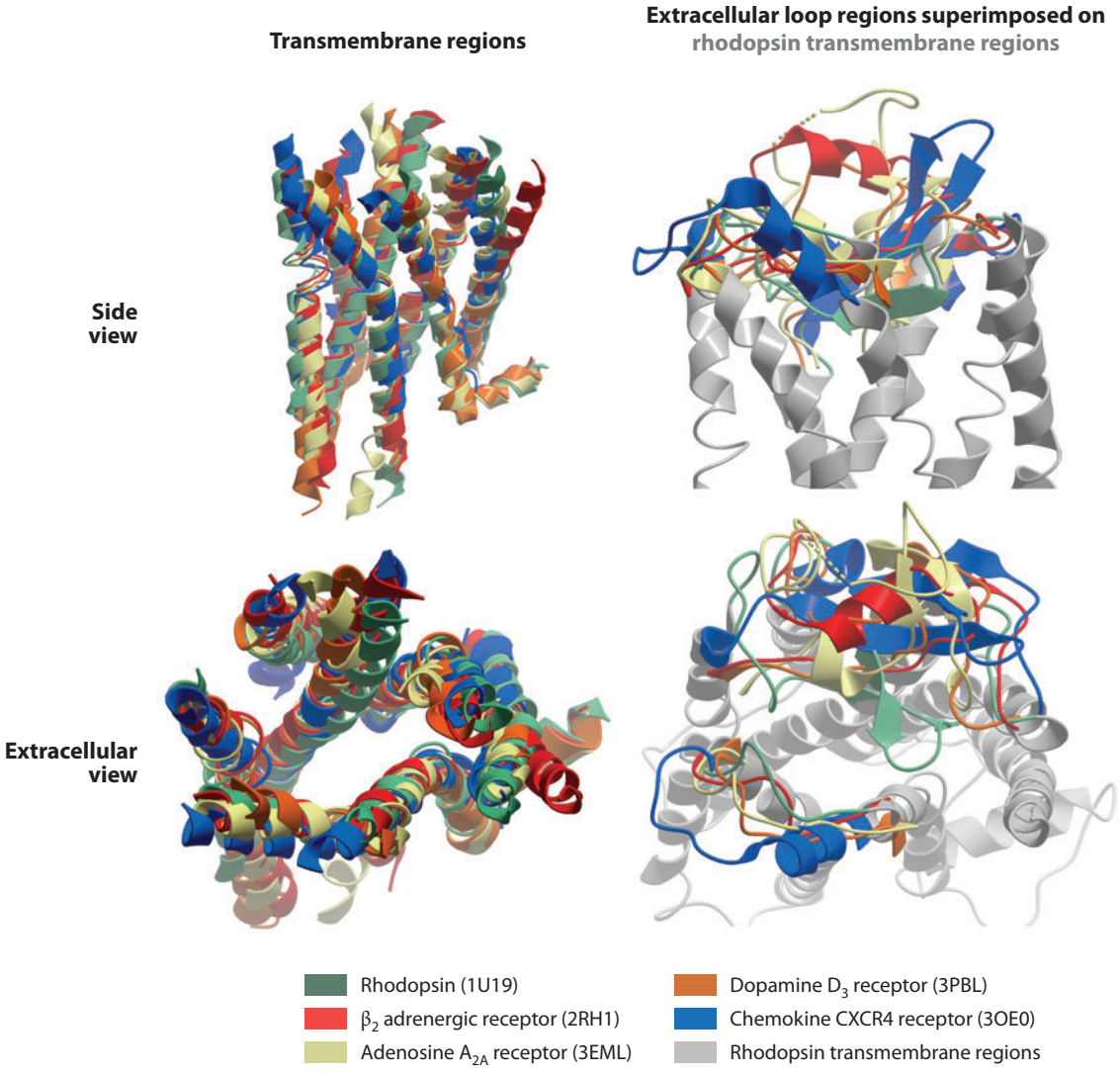

Figure 1

Overlay of high-resolution, inactive-state, crystal structures of G protein–coupled receptors reveals similar transmembrane helical arrangements but divergent extracellular loop regions. Left panels show alpha helical regions only from the side (*top panel*) or from an extracellular view (*bottom panel*). Right panels highlight equivalent views of the extracellular loops superimposed on the transmembrane regions of rhodopsin.

STIMULUS BIAS AND ALLOSTERIC MODULATION AS KEY PARADIGMS FOR GPCR SELECTIVITY

Given the increase in biochemical and biophysical data showing that GPCRs are highly dynamic proteins and that functionally distinct ligands can stabilize specific receptor conformations (5, 8, 26–35), it stands to reason that the vast array of behaviors that a GPCR can exhibit, e.g., from G protein activation to desensitization and internalization, need not be sequentially linked. Accordingly, different ligands acting at the same receptor and in the same cellular environment

cannot be assumed to promote similar repertoires of receptor behaviors. Numerous examples now exist to support the concept of a single ligand promoting distinct functional outcomes at a given receptor, depending on the pathway with which the receptor is engaged (5, 7, 8, 28, 30). A striking example of the lack of concordance between ligand efficacies has been observed with compounds traditionally classed as β-blockers at the β_2 adrenoceptor. Propranolol and ICI118551 are inverse agonists for G_s protein–mediated stimulation of adenylate cyclase but produce partial agonist responses for β arrestin–dependent activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) (28); i.e., they display opposite efficacies depending on the signal pathway being recruited. The ability of different ligands to engender discrete signaling activities at a given GPCR has been termed stimulus bias, stimulus trafficking, collateral efficacy, biased agonism, or functional selectivity (5, 7, 8). A common indicator of stimulus bias is the reversal of potency order or maximal effects for different agonists at a given receptor when they are examined across alternative signaling pathways, although such findings usually represent the extremes of the phenomenon; stimulus bias is also likely to be operative if the potency or efficacy preferences of a test agonist do not track with those of the endogenous agonist across different pathways (36–39).

The overall promise of stimulus bias is the ability to design ligands that selectively engage therapeutically relevant signaling pathways while sparing those that contribute to undesirable side effects; drug discovery emphasis is shifted from a traditional receptor subtype-centric view toward a receptor active-state-centric or pathway-selective view. The key challenges in this regard are twofold. First, it is usually not known which signaling pathway is most predictive of the desired therapeutic outcome. Second, it is necessary for functional screening of potential drug candidates to be performed across multiple assay formats. Nonetheless, recent provocative findings suggest that such functional selectivity may indeed be therapeutically advantageous. For instance, the GPR109A receptor agonist, nicotinic acid, is an effective antilipolytic, but its use is limited by serious cutaneous flushing (40). This flushing effect is mediated by a β arrestin–dependent pathway, whereas the beneficial lipolytic effect is believed to be mediated by a G_i protein–dependent reduction of cyclic adenosine monophosphate (41). Consequently, an agonist that selectively activates the G_i pathway but not the β arrestin pathway could prove to be a superior treatment for dyslipidemia. Interestingly, this pharmacological profile was recently identified in a novel series of pyrazole GPR109 receptor agonists (41, 42), providing proof of concept for such a pathway-targeted approach.

The phenomenon of stimulus bias effectively highlights the natural allosteric nature of GPCRs as signaling machines; the binding of an extracellular ligand promotes a conformational change that is transmitted to topographically distinct intracellular sites, which then selectively engage signaling proteins. There is no a priori reason why such allosteric effects cannot also be propagated between distinct binding domains on the extracellular surfaces of GPCRs. Indeed, the vectorial nature of information transfer across GPCRs (43) suggests that this should be the case (**Figure 2**). As discussed above, the diverse modes of ligand engagement within the various subfamilies of GPCRs indicate that a common structural topography is nonetheless flexible enough to provide different types of pockets for different types of ligands. Thus, the orthosteric-binding domain for one type of GPCR may represent an allosteric-binding motif in another type of GPCR that recognizes substantially different endogenous ligands. This clearly represents an exciting opportunity for novel drug discovery, especially with regard to synthetic small molecules, which remain a priority for the pharmaceutical industry.

The exploitation of allosteric sites on GPCRs provides a means by which numerous important issues associated with the difficulty in discovering small-molecule orthosteric ligands of certain GPCRs can be overcome, in particular for GPCRs in which the orthosteric site is highly conserved between subtypes (5, 6, 44, 45). Allosteric modulators are defined as ligands that (*a*) bind to GPCRs

ERK1/2: extracellular signal-regulated kinases 1 and 2

Stimulus bias (functional selectivity): a ligand-mediated stabilization of distinct receptor active states to the relative exclusion of others, leading to selective signaling via a subset of the signaling repertoire normally available to a receptor in a given cell type

Allosteric modulator: a ligand that binds to an allosteric site and modifies the binding and/or signaling of another cobound ligand

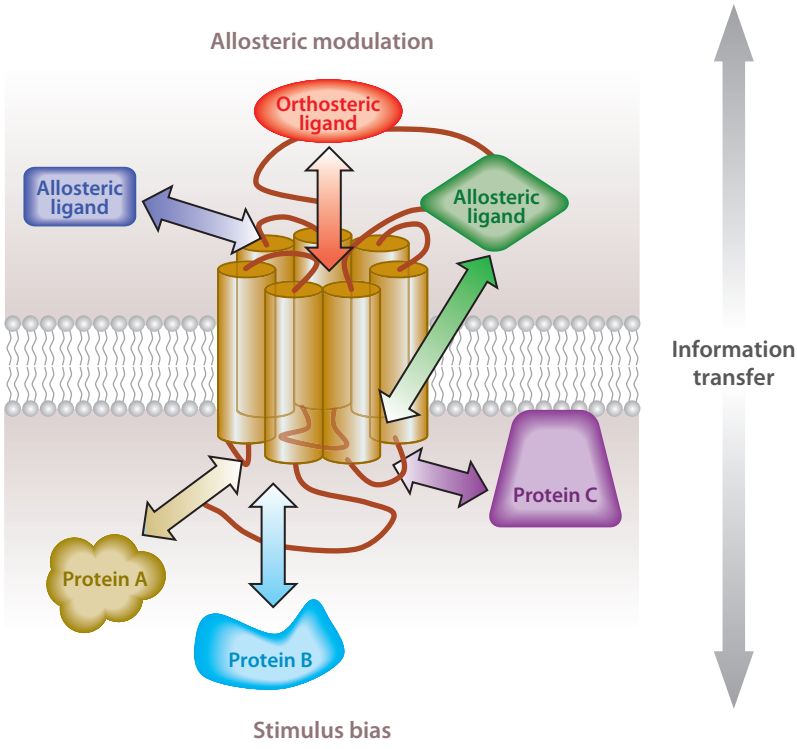

Figure 2

Bidirectional information transfer in G protein–coupled receptors (GPCRs) gives rise to the phenomena of allosteric modulation and stimulus bias. In both instances, the binding of a ligand to a GPCR promotes a conformational change that alters the interactive properties of other proteins or ligands at a topographically distinct region of the receptor.

at sites that are topographically distinct from the orthosteric site and (*b*) promote a conformational change that modulates orthosteric ligand affinity and/or efficacy. Some allosteric ligands can also act as (allosteric) agonists or inverse agonists in the absence of an orthosteric ligand (5, 6, 44, 45). In all instances, a receptor occupied by an allosteric ligand should be viewed as a novel receptor type with its own unique repertoire of behaviors.

Compounds that possess an allosteric mode of action can display numerous theoretical therapeutic advantages over orthosteric ligands. The most obvious advantage is the potential for greater selectivity across receptor subtypes because an allosteric site may have greater divergence in sequence between subtypes compared with the orthosteric domain. If the allosteric modulator displays minimal agonism in its own right, then it exerts its effect only in the presence of the orthosteric agonist, potentially maintaining both temporal and spatial aspects of endogenous physiological signaling. Because the magnitude of an allosteric effect is governed by the degree of cooperativity manifested between allosteric and orthosteric sites, the effects of allosteric modulators with limited positive or negative cooperativity have a ceiling level above which no further target-based modulation occurs, irrespective of modulator concentration. This means that large doses of allosteric modulators can be administered with a lower propensity toward target-based toxicity than that seen with orthosteric agonists or antagonists. Finally, given that allosteric modulators themselves promote a conformational change in GPCR structure, these compounds may

engender functional selectivity, either by themselves or by modulating the actions of the orthosteric ligand in a pathway-biased manner. Such pathway-selective modulation has already been observed in the actions of synthetic small molecules (46–49).

Interestingly, the recent focus on allosteric modulators of GPCRs has unmasked small molecules that can also bind to intracellular GPCR regions. For example, a combination of homology modeling, chimeric receptors, and mutagenesis studies implicated a potential intracellular binding pocket for a small-molecule inhibitor of the CXCR2 chemokine receptor (50). A similar intracellular site of interaction was suggested for small-molecule inhibitors of the chemokine CCR4 receptor (51). These studies suggest that such an intracellular site may exist across this receptor family and be exploited for drug discovery. This hypothesis is corroborated by the development of pepducins, which are created through the attachment of a lipidated group, such as an acyl chain, to a peptide that corresponds to a portion of one of the intracellular loops of a GPCR of interest (52). Mechanistic studies have revealed that the action of pepducins requires an intact receptor, and studies on a protease-activated receptor 1–directed pepducin highlighted the C-terminal tail of the receptor as a potential interaction site (53). Thus, pepducins can be regarded as novel allosteric modulators of GPCR function. Both agonistic and antagonistic pepducins have been developed against a variety of GPCRs, including protease-activated receptors, chemokine receptors, and the sphingosine-1-phosphate receptor 3 (52–63).

Irrespective of the location of the allosteric site, one of the key challenges associated with allosteric modulator drug discovery is the need to quantify allosteric effects in a manner that can, ideally, also be used to guide structure-activity and compound optimization studies. At the molecular level, allosteric modulation can be described thermodynamically in terms of the affinities that the orthosteric and allosteric ligands have for different receptor states in both the absence and the presence of the cobinding of one another as well as the cobinding of interacting cellular proteins that initiate signal transduction (5, 43). This is routinely presented in mass-action form as a variety of allosteric ternary complex models of different degrees of complexity, depending on the number of receptor states being represented (6, 64–67). A common feature of these models is the incorporation of one or more cooperativity factors that accounts for the effect(s) that each of the two ligands brings to bear upon the interaction of the other with the receptor. Unfortunately, these thermodynamic parameters are not easily attainable from routine experimental data except in the simplest cases of allosteric modulation. As a consequence, researchers have developed various operational models of allosterism that can be applied to experimental data in a manner that facilitates determination of the affinity of the modulator for the allosteric site on the receptor, as well as operational measures of the modulator's cooperativity and any signaling efficacy (68–71).

THE MESSAGE-ADDRESS CONCEPT IN THE DESIGN OF GPCR-TARGETING LIGANDS

Stimulus bias and allosteric modulation promise new modes of selectivity at GPCRs. A more historical approach to designing ligands is encompassed in the message-address concept, a term coined by Schwyzer (72) in the late 1970s and first applied to GPCRs by Portoghese and colleagues (73, 74). This concept posits that each ligand exhibits two components: the message, which is composed of the main receptor recognition motif and therefore promotes the transduction of the signal from the receptor to the effector, and the address, which is located near the message component and provides additional ligand-receptor interactions (**Figure 3**). This address component can be either on a region proximal to the message component (i.e., within the same binding site) or on a more distal region, such as a different binding site, within the same receptor or even on a different receptor. Incorporation of the address component proximal to the

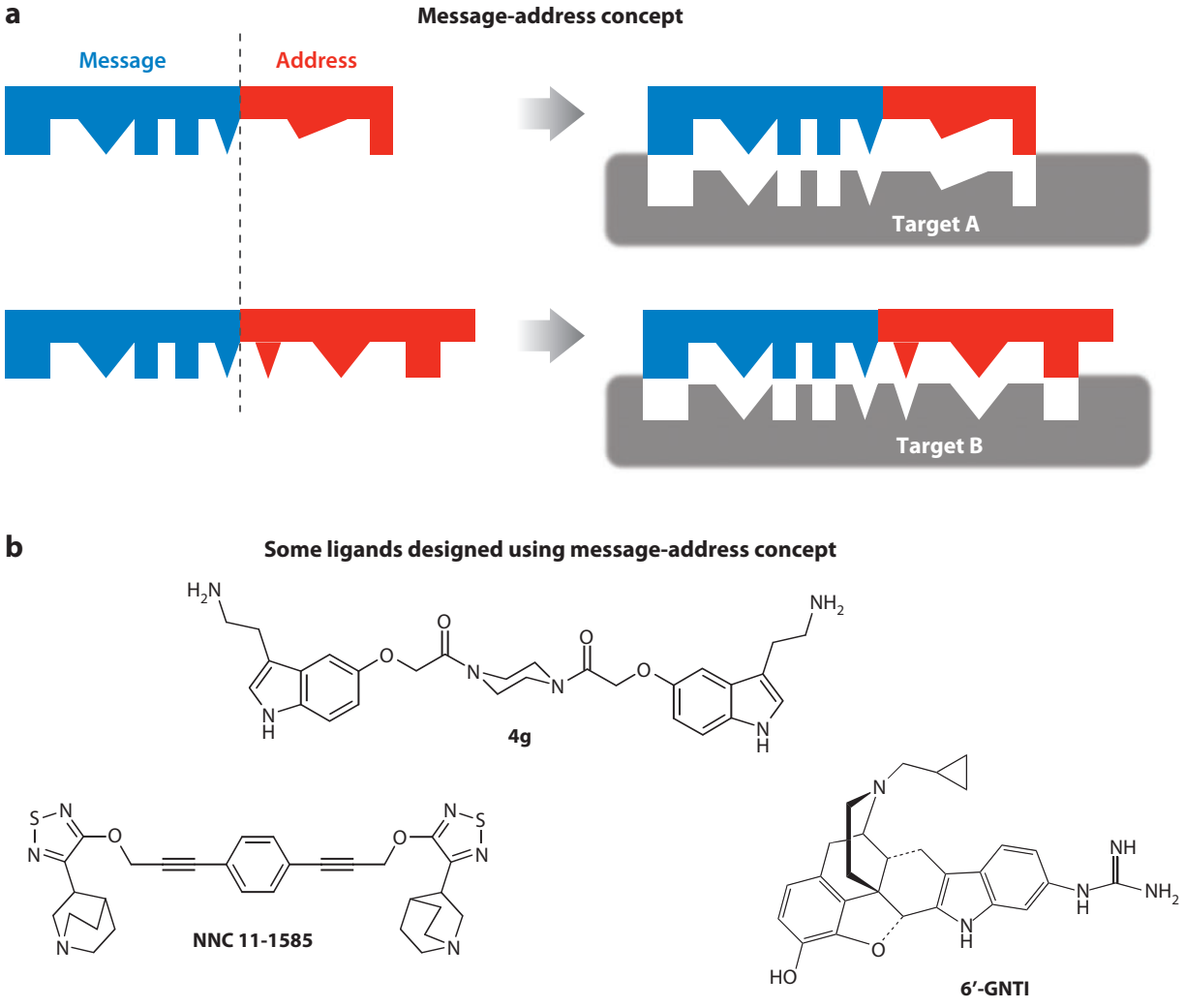

Figure 3

(*a*) The message-address concept defines selective drug design in terms of pharmacophore elements (message) that engage a conserved region of the target protein, responsible for signal transduction/termination, and additional moieties (address) that target less conserved regions associated with the target. (*b*) Some examples of ligands that have been designed using this principle are also illustrated: 4g ($5HT1_{B/D}$ receptors); NNC 11-1585 (mAChRs); and 6′-guanidinonaltrindole, also known as 6′-GNTI (δ-κ opioid dimers).

message component has been the most thoroughly utilized approach, as reflected by the plethora of endogenous orthosteric ligand analogs available for many GPCR families. Subsequent studies have focused on extending the distance between message and address components in ligands to exploit regions of the target receptor that are more distal to the main binding pocket. Furthermore, novel compounds have been synthesized by the linking of two distinct pharmacophores via an appropriately sized spacer to create bivalent ligands. The past three decades have witnessed the development of highly selective bivalent ligands for GPCRs (75), such as for the opioid receptors (76–78), muscarinic acetylcholine receptors (mAChRs) (79–81), serotonin receptors (82–84), cannabinoid receptors (85), and gonadotropin-releasing hormone receptors (86). Some examples

Bivalent ligand: a single chemical entity composed of two covalently linked pharmacophores

of such ligands are illustrated in **Figure 3**. This concept has even been extended to other proteins, such as acetylcholinesterases (87) and the tyrosine kinase receptors TrkA (88) and TrkC (85).

6′-GNTI: 6′-guanidinonaltrindole

HOMO- AND HETEROBIVALENT LIGANDS

A bivalent ligand is, by definition, a single chemical entity composed of two covalently linked pharmacophores. If the pharmacophores are identical, then the ligand is defined as a homobivalent ligand, whereas the linking of two different pharmacophores yields a heterobivalent ligand. Both homo- and heterobivalent ligands have been developed for GPCRs as pharmacological tools to achieve receptor subtype selectivity and/or, increasingly, as a means to study receptor dimerization. However, the earliest studies on GPCR bivalent ligands did not necessarily aim to target dimeric complexes, and many such ligands have relatively short linking groups between the pharmacophores (89). This suggests that these bivalent compounds may be interacting with neighboring binding sites on a single receptor rather than bridging two binding sites across a receptor dimer. One such example is the development of norbinaltorphimine, which is a homobivalent ligand selective for the κ opioid receptor (89). This compound consists of two naltrexone (opioid receptor antagonist) pharmacophores joined by a pyrrole spacer, but only part of the second pharmacophore was found to be necessary for κ opioid receptor selectivity (89). A similar finding relates to 6′-guanidinonaltrindole (6′-GNTI) (**Figure 3**), which was originally designed to be a κ opioid receptor agonist on the basis of the message-address concept but was subsequently revealed to be a more potent and selective agonist of the δ-κ opioid receptor heterodimer (90) (**Figure 4*a***). It is unlikely, however, that this heterodimer selectivity is achieved by simultaneous interaction of one molecule of 6′-GNTI with the two orthosteric sites from each of the δ and κ opioid receptors, given the molecule's size. However, other ligands selective for δ-κ opioid

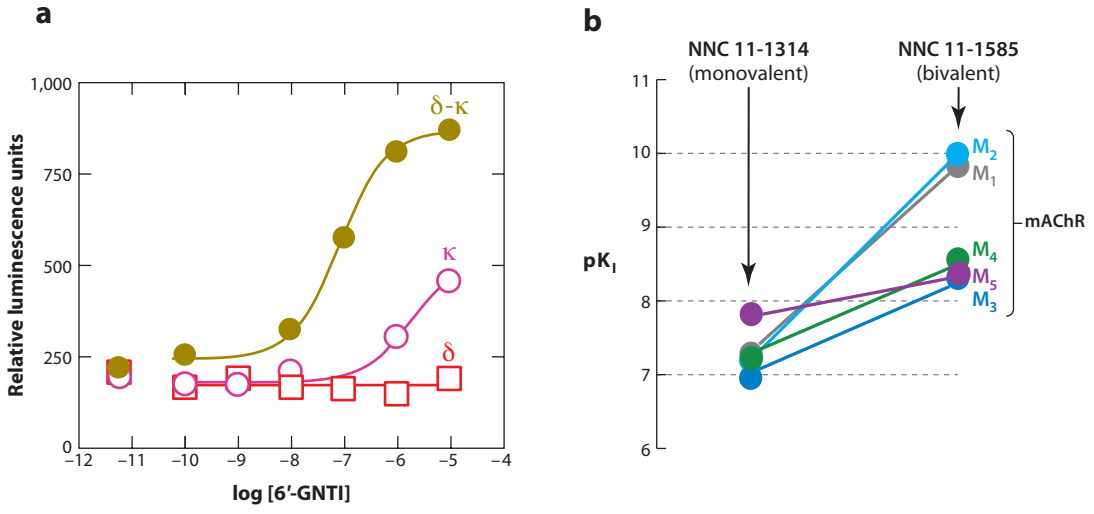

Figure 4

Bivalent ligands can lead to improvements in selectivity and/or affinity. (*a*) 6′-guanidinonaltrindole (6′-GNTI) has higher efficacy for promoting intracellular calcium mobilization in human embryonic kidney cells containing the δ-κ opioid heterodimer compared with cells individually expressing either δ or κ opioid receptors. Data replotted from Reference 90. (*b*) The homobivalent muscarinic acetylcholine receptor (mAChR) agonist NNC 11-1585 displays markedly higher affinity and selectivity for M_1 and M_2 mAChRs compared with its monovalent pharmacophore, NNC 11-1314. Data taken from Reference 79. The pK_I is the negative logarithm of the ligand equilibrium dissociation constant.

receptor heterodimers have been developed; these have substantially longer linker lengths that are more compatible with two distinct pharmacophores bridging a dimeric receptor complex. For example, the tethering of the κ-selective antagonist pharmacophore 5′-guanidinonaltrindole to the δ-selective antagonist pharmacophore naltrindole yielded optimal in vitro and in vivo potency when a linker region of 20–21 atoms was used (91, 92). Interestingly, all studies of μ-δ and μ-μ opioid receptor bivalent ligands have found similar optimal spacer lengths of 18–21 atoms (93, 94). Owing to the therapeutic potential of targeting GPCR homo- or heterodimers, numerous studies have utilized such a bivalent ligand approach. In other cases, heterobivalent ligands have been designed simply with the desire to combine pharmacophores that result in a common therapeutic endpoint. For instance, the conjugation of the β_2 adrenergic receptor agonist isoproterenol to the adenosine A_1 receptor agonist adenosine had been attempted in an effort to derive novel tools for modulating cardiac arrhythmias (95).

THE WORLD BETWEEN PHARMACOPHORES: THE CRITICAL ROLE OF THE LINKER

Because all bivalent ligands exhibit two pharmacophores joined by a linker, the nature, length, and flexibility of this latter structural feature itself can significantly influence the activity of the designed bivalent ligand. For instance, if the linker is too short, the ligand cannot bridge both binding sites simultaneously and shows little to no enhancement of activity compared with the monovalent ligand. Common types of linkers include polyethylene glycol, polyamide, or even polymethylene linkers, and each has its own chemical properties that also play a role in the final observed interactions. Another important consideration is the rigidity or flexibility of the structure of the linker because this contributes to the degrees of freedom available to the bivalent ligand. In the case of a rigid linker structure, if the orientation of the pharmacophores is not optimal, a reduction of activity may be anticipated. However, linker rigidity can also prove advantageous. For example, one way to elucidate the nature of GPCR homo- or heterodimers is to use bivalent ligands as pharmacological tools to facilitate the precise determination of the distance between each binding site from each monomer. However, simple linear linkers can be problematic because they can adopt variable secondary structures; at best, they can provide an estimate of the maximum distance between vicinal recognition sites. In contrast, a recent study has demonstrated that it is possible to target two different sites across GPCRs using a highly rigid bivalent ligand (96). Poly(L-proline) is a highly structured chain, which forms a helix that maintains a length of 0.9 nm per turn (**Figure 5*a***). Owing to the distinctive cyclic structure of proline's side chain, this amino acid provides exceptional conformational rigidity compared with that of others. Therefore, for a given number of prolines per linker, a precise linker length can be determined, rendering rigid bivalent ligands utilizing this linker extremely useful for providing topographical information. Within a range of linker lengths between 2 and 8 nm, followed by coupling with the appropriate pharmacophores, investigators found bivalent ligands of the CXCR4 receptor that exhibited the highest affinity when the linker length was approximately 6 nm (96). This result provides new insight for the design of bivalent ligands for this receptor as well as knowledge about the possible topography of the associated binding sites.

In the case of flexible linkers, one possible advantage is that they can allow the address component of the bivalent ligand to more freely move around and bind to its own binding site, which may result in a higher likelihood of increased affinity (but see the next section for caveats). Often, the introduction of a linker into a pharmacophore occurs on a phenyl ring, which raises the possibility of ortho-, meta- or parasubstitution; each of these influences the specific orientation of the linker relative to the pharmacophore. Although investigating all possibilities is certainly

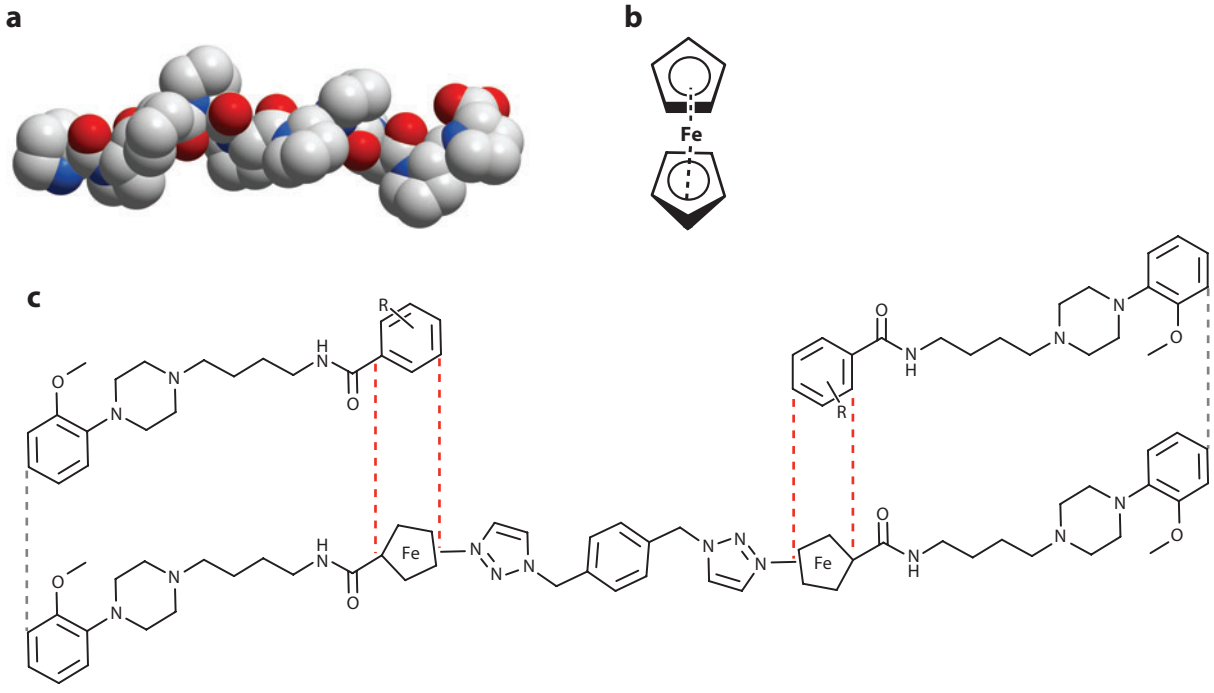

Figure 5

Rigid or flexible linkers can be used in bivalent ligand design. (*a*) The rigid helical structure adopted by a poly(L-proline) linker can be used as a "molecular tape measure." (*b*) The structure of ferrocene, which can be used in bioisosteric replacement of phenyl rings in bivalent compound linker regions. (*c*) An example of bioisosteric replacement of phenyl rings in a dopamine D_3 receptor–selective pharmacophore to yield a dopamine D_2–selective homobivalent ligand (see Reference 97).

possible, time and cost considerations have prompted the exploration of alternative approaches, such as the use of flexible analogs of the phenyl ring. One interesting study recently investigated the effect of highly flexible linkers by means of bioisosteric replacement using metallocene-based subunits (97). Specifically, owing to their similar physical and chemical properties, a phenyl may be interchangeable with a ferrocenyl that consists of two cyclopentadienyl rings bound on opposite sides of a central iron atom. A particular useful feature of the ferrocenyl is that the two cyclopentadienyl rings are able to rotate freely around each other; the absence of an individual bond between the rings and the iron metal causes this configuration to act like a hinge, which confers flexibility to the ferrocenyl (**Figure 5*b***). Following this principle, researchers designed a highly flexible homobivalent ligand that exhibited agonism at the dopamine D_2 receptor and antagonism at the dopamine D_3 and D_4 receptor subtypes (**Figure 5*c***). This type of profile may be associated with putative atypical antipsychotic activity (97).

IMPROVING AFFINITY AND SELECTIVITY WITH BIVALENT LIGANDS

Most bivalent ligands have been developed with a dual aim: (*a*) to improve affinity, by providing additional interactions, and (*b*) to improve selectivity, if these additional interactions involve less conserved regions across a family of receptors (**Figure 4**). Regardless of the pharmacophore (homobivalent or heterobivalent), there are generally two types of bivalent ligands: those with

relatively short linkers and those with long linkers. As indicated above, bivalent ligands with short linking groups may interact with a neighboring site on a single GPCR, via amino acid residues that are potentially less conserved, such as residues in the extracellular loops. Such additional interactions may be sufficient for an improvement of affinity, in addition to better selectivity; the previously mentioned example of the κ-selective opioid receptor antagonist, norbinaltorphimine, represents one such instance. In contrast, bivalent ligands with longer linking groups may utilize a neighboring site on a different receptor (or associated protein) to increase affinity and/or selectivity. The effect on affinity is predominantly due to a reduction of the overall energy required for the second pharmacophore to bind to its site. Simplistically, the binding of the first pharmacophore induces an increase in the proximity of the second pharmacophore to its binding site, therefore reducing the energy cost of the second pharmacophore to bind to its site compared with the energy required by two separate monovalent ligands. In theory, the affinity increase for appropriately designed bivalent ligands that meet this criterion should be equal to the product of the binding contributions of the individual pharmacophores (98), which can equate to a dramatic increase in ligand potency. Furthermore, if the two pharmacophores are identical, the "effective" concentration of the active pharmacophore within the immediate region of the protein binding site is elevated, which increases the probability of binding. This alone can also lead to an increase in the observed potency of the compound.

In addition to improving affinity, the linking of two pharmacophores may also increase the degree of these ligands' selectivity for a receptor target. The most obvious example of this scenario is the targeting of heterodimeric receptors, which can exhibit both tissue- and drug-specific properties. Using a bivalent ligand that can target such an entity exhibits a higher degree of selectivity than using the monovalent ligands separately and may even result in functionally selective signaling as well. For example, the bivalent opioid μ agonist/δ antagonist MDAN-21 exhibits reduced tolerance and physical dependency compared with its individual constituent opioid ligand pharmacophores (94). Furthermore, MDAN-21 is 50-fold more potent than morphine and therefore provides proof of principle for the concept of bivalent ligands as tools for the development of analgesics that do not induce tolerance and dependency in patients.

Intriguingly, some examples of bivalent ligands do not show the expected enhancement of affinity (86, 99). In this case, it is necessary to consider both the enthalpic and entropic components of the free-energy change associated with ligand binding, encompassed by the classic equation $\Delta G = \Delta H - T\Delta S$ (where G is the Gibbs free energy, H is the enthalpy, T is a constant temperature, and S is the entropy). Perhaps the most intuitive reason for nonsynergistic affinity would be the bivalent ligand having a linker of insufficient length to allow simultaneous occupation of both binding sites (98). In addition, the linker itself may participate in an undesirable interaction that blunts the potential affinity gain (76). Although a relatively flexible linker increases the likelihood that all ligand-receptor interactions can occur without energetic strain, this can result in unexpected entropic costs (98). Specifically, if the binding of a bivalent ligand is a two-step process, then binding of the second pharmacophore will be unfavorable for conformational entropy because the number of conformations available to the bivalent ligand before complexation will be greater than that following complexation. Ultimately, enthalpy and entropy can have partly compensating effects; if so, then the affinity gain expected from linking pharmacophores is not straightforward to predict. A final issue to consider is the cooperative interaction between the two binding sites of the linked pharmacophores. If the binding of one of the pharmacophores of a bivalent ligand induces a conformation of the remaining binding site that impairs the binding of the second pharmacophore, then the gain in enthalpy of the bivalent ligand will be smaller than the sum of enthalpy gains of the two separate monovalent components.

FROM BIVALENT TO BITOPIC LIGANDS

The growing interest in allosteric modulators of GPCRs has culminated in recent studies seeking to exploit this paradigm within the context of the bivalent ligand approach, that is, to create molecules that explicitly combine defined orthosteric and allosteric pharmacophores in one ligand. Such bivalent compounds have been termed bitopic or dualsteric (100, 101) to explicitly acknowledge the hybrid orthosteric/allosteric nature of the molecule, as opposed to the more general umbrella term bivalent. One may choose to pursue a bitopic ligand approach for numerous reasons. First, the use of a standard allosteric modulator relies on the presence of appropriate endogenous agonist tone, which may not always be the case, e.g., as with neurodegenerative disorders such as Parkinson's and Alzheimer's diseases that involve a loss of neurotransmitter-releasing nerves but not their postsynaptic GPCR targets. Second, the design of a bitopic ligand possessing appropriately paired orthosteric and allosteric fragments can theoretically achieve improvements in affinity, owing to the utilization of two binding sites, and selectivity, owing to the targeting of an allosteric site and/or the promotion of stimulus bias. Importantly, these advantages can be attained through use of a single biologically active molecule.

The first example of a rationally engineered bitopic ligand came from the work of De Amici, Holzgrabe, and colleagues (102), who synthesized hybrid ligands targeting the mAChRs with an orthosteric pharmacophore based on the agonist oxotremorine, which was linked to hexamethonium-derived allosteric modulators. These hybrid molecules did not display improved affinity compared with their individual constituents but did gain subtype selectivity as compared with the parent orthosteric agonist. One reason that the mAChRs represent the best-studied exemplar model of bitopic GPCR ligands is that they have a rich allosteric pharmacology and substantial structural evidence that supports a relatively close apposition of the TM-domain-located orthosteric site with at least one allosteric site comprising more extracellular-facing residues, including some of the extracellular loops (44, 103–105). Accordingly, Steinfeld and colleagues (106) also synthesized a bitopic ligand targeting these regions of the M_2 subtype of mAChR. In this instance, a substantial increase in affinity was noted for the bitopic ligand THRX-160209 (**Figure 6**), compared with either of its orthosteric (3-benzhydryl pyrrolidine) or allosteric (4-aminobenzylpiperidine) moieties (106), as predicted for classic bivalent ligands. Another important outcome from that study was the demonstration that the seven-chain pharmacophore linker itself also promoted an increase in compound affinity, highlighting the importance of including incremental fragments of novel bitopic ligands in control experiments. Most recently, these same researchers have extended this approach to the design of bitopic ligands that appear to target two classes of GPCRs, namely the mAChRs and the β_2 adrenergic receptor (106). In this latter instance, the two pharmacophores that were chosen were an orthosteric antagonist of the mAChRs and an orthosteric agonist of the β_2 receptor, but these were subsequently shown to be allosteric modulators at their nonpreferred receptor (i.e., the mAChR antagonist was an allosteric modulator of the β_2 receptor, and vice versa). Thus, when these pharmacophores were conjugated to form the bivalent ligand THRX-198321 (**Figure 6**), they also yielded, by definition, a bitopic ligand for each receptor.

Observations such as those described above clearly point to the complexities associated with the design of bitopic and bivalent ligands. Empirical approaches to combining pharmacophores with appropriately chosen linkers remain an important part of the process but can also be guided by the application of theoretical models that can describe potential bitopic ligand behaviors. One such model (6, 101, 107) appears in **Figure 7**, which shows that the simplest mass-action description of a bitopic ligand interacting with a receptor in the presence or absence of an orthosteric ligand is an amalgam of the classic orthosteric competitive model and the allosteric ternary complex model. Theoretically, the bitopic ligand can be distributed across a given receptor population in

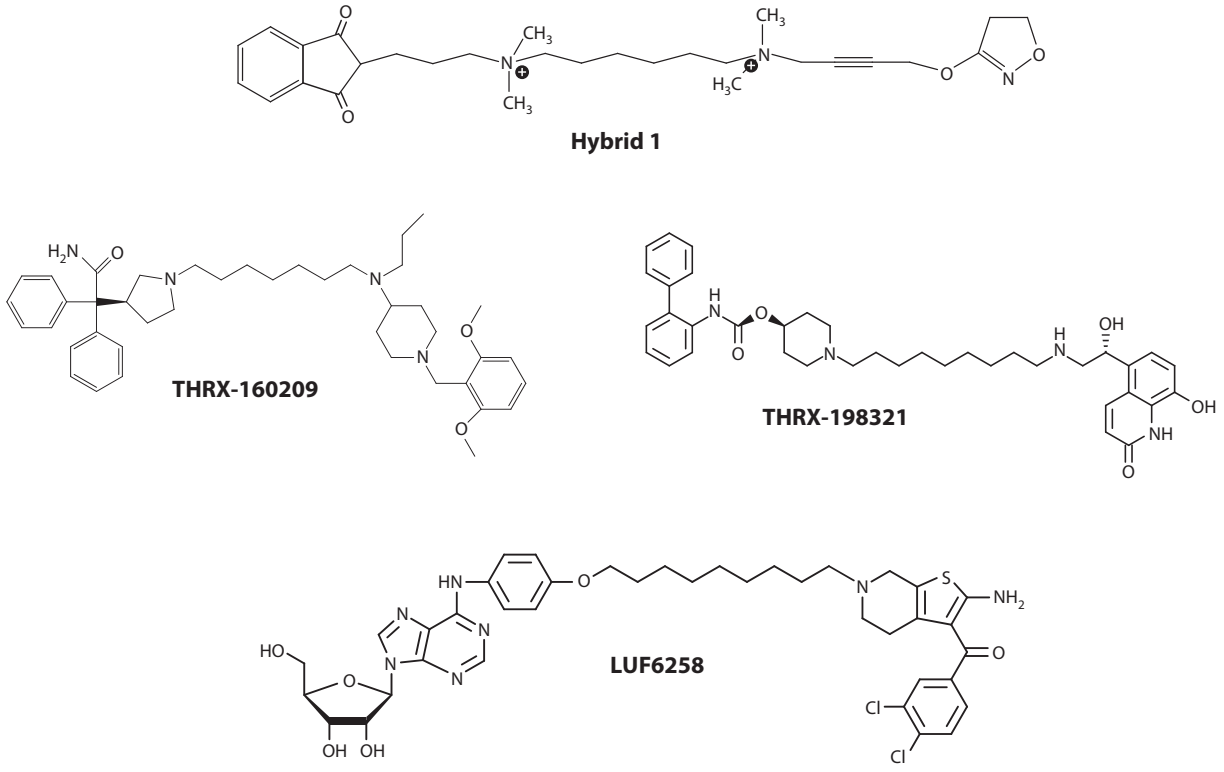

Figure 6

Examples of engineered bitopic ligands of GPCRs. Hybrid 1 and THRX-160209 preferentially target the M_2 mAChR; THRX-198321 targets M_2/M_3 mAChRs and the β_2 adrenergic receptor; LUF6258 targets the adenosine A_1 receptor.

more than one orientation: It can engage both orthosteric and allosteric sites in a bitopic mode, which is indistinguishable from a classic orthosteric binding event, or it can adopt a different pose that recognizes the allosteric site exclusively while allowing the orthosteric site to be exposed for interaction with an orthosteric ligand. The bitopic mode is determined by the strength of the equilibrium dissociation constant $K_{B\text{-ortho}}$, whereas the allosteric mode is determined by the equilibrium constant $K_{B\text{-allo}}$ and by the cooperativity factor α for the interaction with a classic orthosteric ligand. From this model, the fractional occupancy of orthosteric ligand A in the presence of bitopic ligand B is given by the following equation:

$$\rho_A = \frac{[A]}{[A] + K_A \frac{\left(1+[B]\left(\frac{1}{K_{B\text{-ortho}}} + \frac{1}{K_{B\text{-allo}}}\right)\right)}{\left(1+\frac{\alpha[B]}{K_{B\text{-allo}}}\right)}}. \quad 1.$$

This model was utilized in an elegant study by Mohr and colleagues (108) to inform the selection of allosteric and orthosteric building blocks that yielded theoretically predicted profiles of behavior and that, when combined to create hybrid ligands for the M_2 mAChR (e.g., Hybrid 1, **Figure 6**; Hybrid 2, **Figure 8**), also behaved as predicted by the model (**Figure 8**). The study thus provided additional validation that the mode of action of these hybrids was bitopic. An important property of these compounds was the achievement of subtype-selective and stimulus-biased agonism (108, 109).

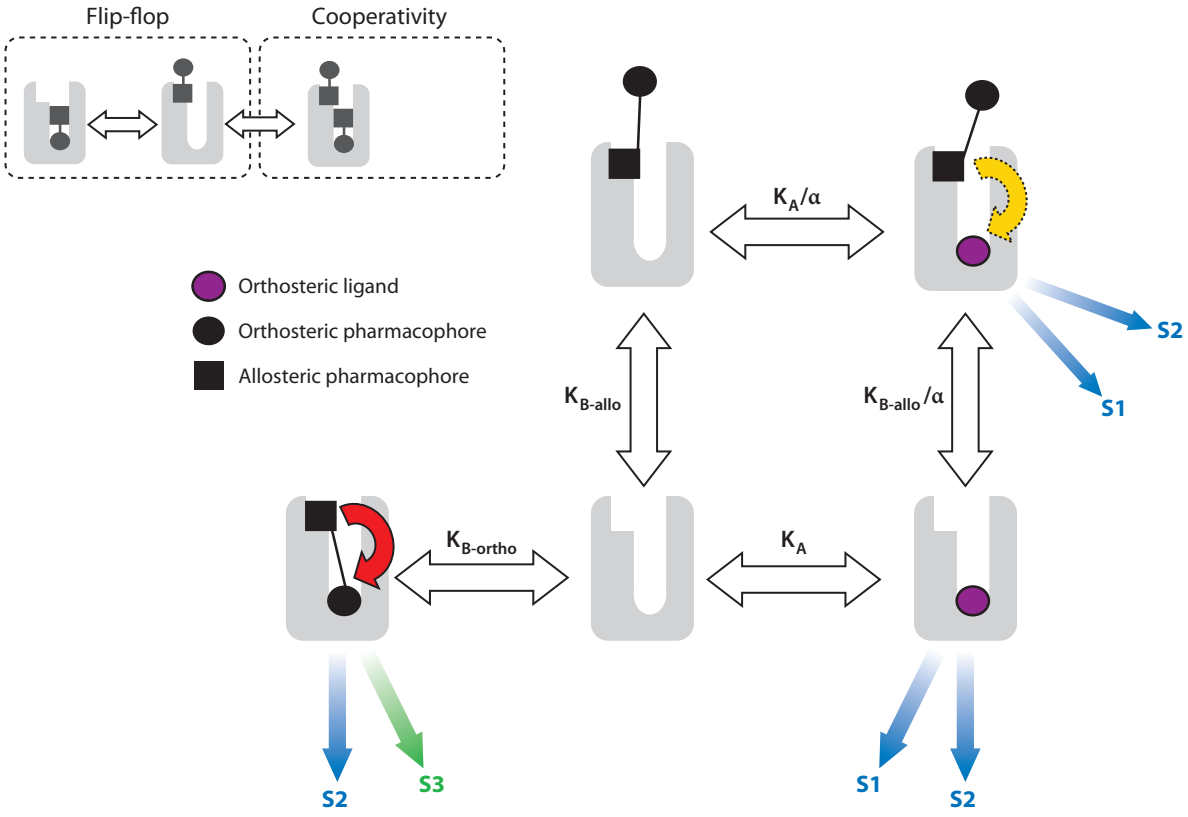

Figure 7

Bitopic mechanisms may display features associated with both competitive and allosteric mass-action schemes. In the main figure, the purple circle denotes the orthosteric ligand, whereas the black circle and square denote the orthosteric and allosteric pharmacophores, respectively. The scheme also illustrates the potential for differential signaling (pathways denoted by the letter S) that may arise between an orthosteric and a bitopic ligand because of the ability of the allosteric moiety in the latter to direct the efficacy of the orthosteric moiety encoded in the same molecule. The inset illustrates variations of the bitopic theme, whereby the molecule cannot bind concomitantly to both sites but distributes between orthosteric or allosteric orientations (flip-flop mechanism) or displays cooperative binding to interact with both sites simultaneously.

Most recently, IJzerman and colleagues (110) extended the bitopic ligand approach to another GPCR, the adenosine A_1 receptor. In that study, an orthosteric adenosine-derived agonist was combined with the allosteric modulator (2-amino-4,5-dimethyl-3-thienyl)-[3-(trifluoromethyl)phenyl]methanone via a flexible 9-carbon linker to yield the bitopic ligand LUF6258 (**Figure 6**). Because the allosteric pharmacophore was an enhancer of the orthosteric agonist pharmacophore, researchers anticipated a substantial increase in potency given that both ligands preferred an active receptor state. Surprisingly, however, the expected gain in affinity was not observed; indeed, the potency of the bitopic ligand was an order of magnitude lower than that of the parent orthosteric agonist (110). It is possible that the flexible carbon linker contributed to a high entropic cost that offset any possible enthalpic gains that may have been achieved by the positive cooperativity between orthosteric and allosteric moieties. Nonetheless, the bitopic ligands demonstrated a reversal of potencies for A_1 receptor–mediated ERK1/2 phosphorylation as compared with those observed in a [^{35}S]GTPγS assay (110), indicating that stimulus bias was engendered by the hybrid molecules.

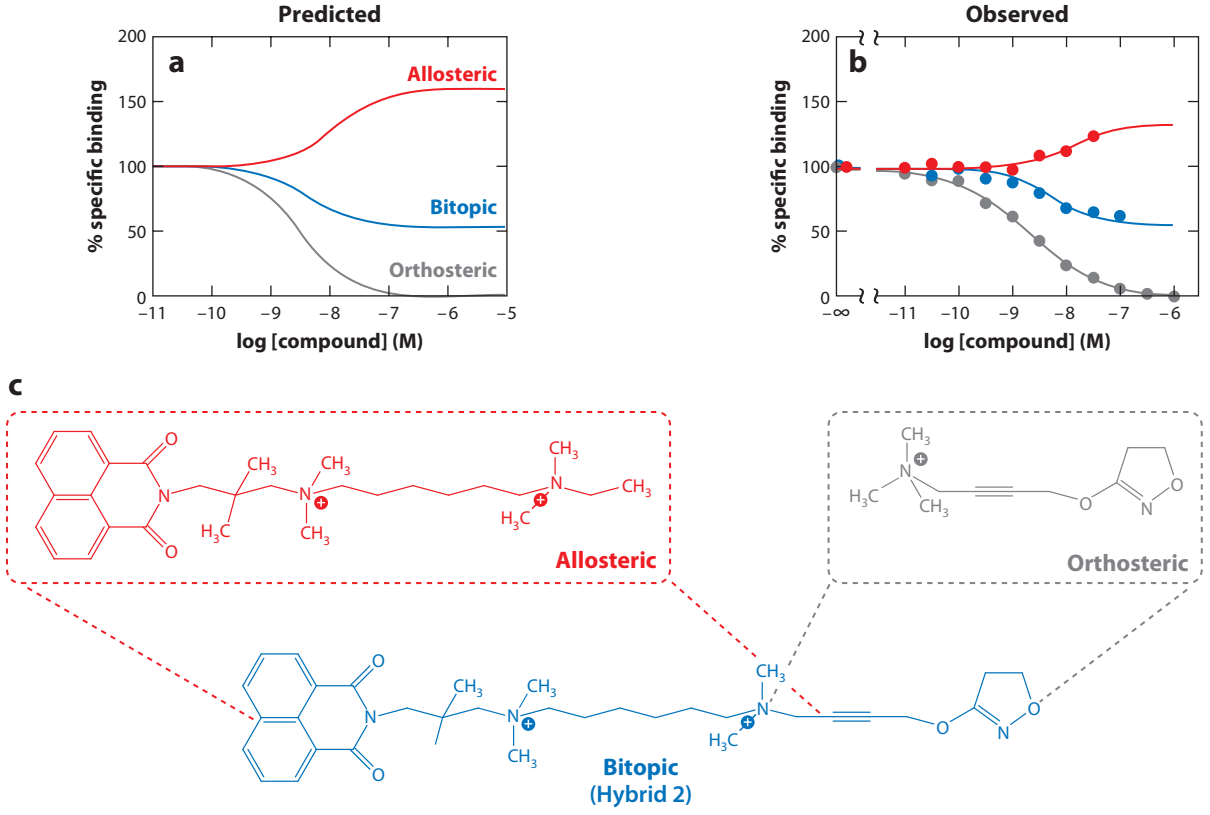

Figure 8

A bitopic model can be used to predict the pharmacology of engineered ligands. To predict the profiles of behavior shown in panel *a*, simulations based on the bitopic model were performed using the following parameters: [A] = K_A = 1 nM; $K_{B\text{-ortho}}$ = 3 nM; $K_{B\text{-allo}}$ = 30 nM; α = 4. Orthosteric and allosteric compounds (panel *c*) approximating this profile of behaviors were used to construct the indicated bitopic ligand. Panel *b* illustrates the observed experimental effects of each of the compounds against the orthosteric antagonist, [^{3}H]N-methylscopolamine, at the M_2 mAChR. Data replotted from Reference 108.

Of course, variations of the scheme shown in **Figure 7** can also be envisaged, for instance, in which the attachment occurs only via the orthosteric site or the allosteric site (perhaps due to a nonoptimal linker length; **Figure 7** inset). This type of flip-flop mechanism (111) would be virtually indistinguishable experimentally from one in which both orthosteric and allosteric sites were concomitantly engaged in the one receptor by the one molecule. An additional possibility is that the bitopic ligand adopts two poses simultaneously in the one receptor, i.e., engaging both orthosteric and allosteric sites (**Figure 7** inset). However, if this were the case, one could expect to see evidence of cooperative binding in the bitopic molecule under appropriate experimental conditions. These types of considerations highlight the need to validate bitopic mechanisms by a variety of experimental approaches. One example, described above, is to use a theoretical model to predict and accommodate the behavior of both the individual pharmacophores and the bitopic ligand. Another method is to use mutagenesis to selectively perturb the orthosteric or allosteric pockets in a GPCR and thus "unmask" predominantly orthosteric or allosteric effects of the bitopic ligand at the mutant receptor. For example, the previously mentioned study by Mohr and

colleagues (108) created M_2 mAChRs with loss-of-function mutations in either the orthosteric site or an extracellular allosteric pocket and found that the bitopic ligand exhibited predominantly allosteric or orthosteric behavior at each mutant, respectively, which is expected if both sites need to be engaged simultaneously at the wild-type receptor. This latter approach, however, requires knowledge of key residues contributing to an allosteric site on the target GPCR. For many GPCRs, including the adenosine A_1 receptor, such knowledge is largely lacking, and alternative approaches to validation of mechanism of action are required. Thus, the study of A_1 bitopic ligands by Narlawar et al. (110) utilized an alternative strategy that assessed the sensitivity of putative bitopic ligand to the coaddition of an excess of monomeric allosteric pharmacophore. An insensitivity to the addition of excess monovalent modulator was observed for bitopic ligands with a linker length greater than 7 carbon atoms long, and this value was thus presumed to represent the minimum length needed for simultaneous occupation of both the orthosteric and allosteric binding sites. In turn, this finding led the authors to suggest a potential role of residues within extracellular loop 2 for the interaction with the allosteric pharmacophore (110).

BITOPIC AGONISM AS AN UNAPPRECIATED PARADIGM UNDERLYING FUNCTIONAL SELECTIVITY

The discovery of bitopic modes of ligand action raises the question of whether other previously described, functionally selective GPCR ligands attain such selectivity (at least in part) through a bitopic mechanism. This was shown to be the case with McN-A-343, a well-known partial agonist of the M_2 mAChR that also displays biased signaling at this receptor type (107, 112, 113). Specifically, by constructing a series of progressively truncated derivatives of this molecule, Valant et al. (107) identified two fragments, tetramethylammonium (TMA) and 3-chlorophenylcarbamate (DDBL-4), that, when combined, recapitulated the pharmacology of the parent molecule (**Figure 9**). On its own, TMA behaved as a high-efficacy orthosteric agonist, whereas DDBL-4 acted as a negative allosteric modulator of TMA signaling efficacy, resulting in a weak partial agonist profile upon coaddition of the two fragments that appeared similar to the profile of the intact McN-A-343 molecule itself. Additionally, mutation of a key residue in the second extracellular loop of the M_2 mAChR to alanine ($Y^{177}A$), which plays a major role in the potency of prototypical allosteric ligands, resulted in a significant reduction of the negative allosteric effect promoted by DDBL-4; the same mutation caused a substantial increase in the signaling efficacy of McN-A-343, presumably because the DDBL-4 component of the molecule could no longer mediate the negative allosteric effect on the TMA component. A molecular model was also proposed for the binding of McN-A-343 at the M_2 mAChR; this model was consistent with a bitopic pose for the molecule (**Figure 10**).

The finding that a previously validated, functionally selective agonist possesses a bitopic mode of action begs the question of whether this applies to other selective agonists. The past few years have witnessed the discovery of novel mAChR agonists that share two common characteristics—namely, that each appears functionally selective for the M_1 mAChR and that each has been suggested to possess an allosteric mode of interaction at this receptor. This list of novel selective agonists includes 4-n-butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl] piperidine hydrogen chloride (AC-42), 1-[3-(butyl-1-piperidinyl)propyl]-3,4-dihydro-2(1H)quinolinone (77-LH-28-1), *N*-desmethylclozapine (NDMC), and 1-[1′-(2-methylbenzyl)-1,4′bipiperidin-4-yl]-1,3-dihydro-2H-benzimidazol-2-one (TBPB) (114–117). However, unambiguous evidence that the agonistic properties of these molecules arise from a purely allosteric mode of interaction is lacking. Although they possess some structural features distinct from those of prototypical orthosteric mAChR-targeting ligands, they also share some similarities. The key experimental

TMA: tetramethylammonium

AC-42: 4-n-butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl] piperidine hydrogen chloride

DDBL-4: 3-chlorophenylcarbamate

77-LH-28-1: 1-[3-(butyl-1-piperidinyl)propyl]-3,4-dihydro-2(1H)quinolinone

NDMC: *N*-desmethylclozapine

TBPB: 1-[1′-(2-methylbenzyl)-1,4′bipiperidin-4-yl]-1,3-dihydro-2H-benzimidazol-2-one

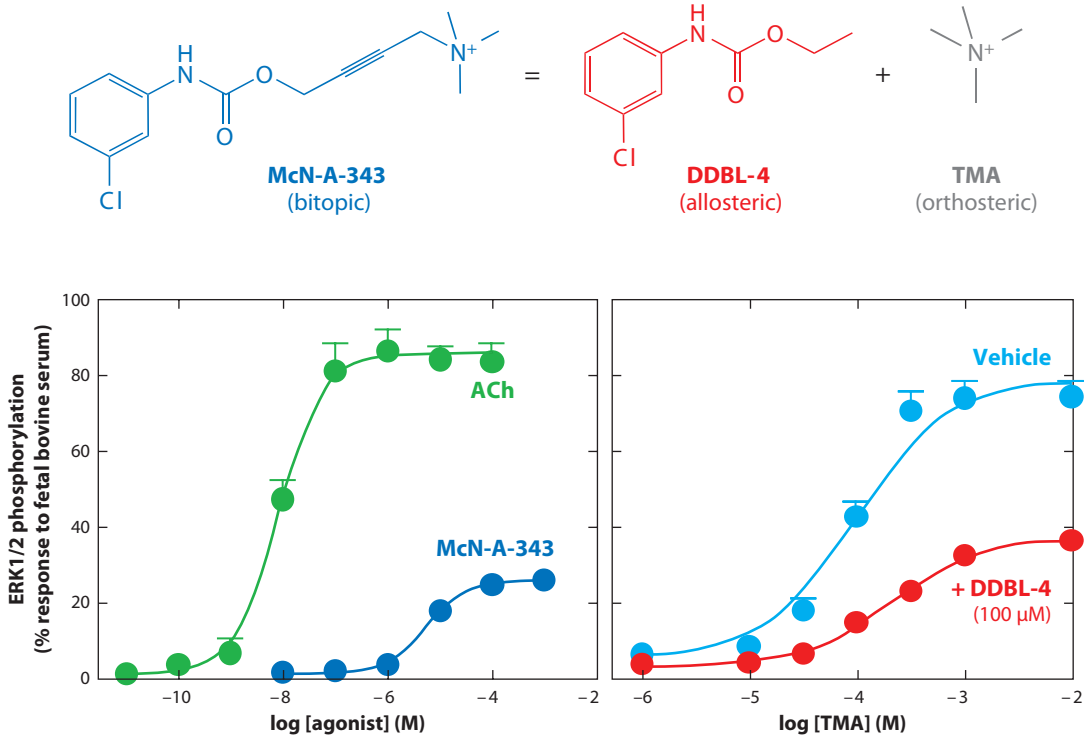

Figure 9

McN-A-343 is a bitopic agonist at the M_2 mAChR. Combination of the orthosteric agonist TMA with the negative allosteric modulator DDBL-4 recapitulates the pharmacology of the parent molecule, McN-A-343, in an assay of M_2 mAChR-mediated ERK1/2 phosphorylation, suggesting a bitopic mechanism as the basis for McN-A-343 partial agonism at this receptor subtype. Data replotted from Reference 108. Abbreviations: ACh, acetylcholine; DDBL-4, 3-chlorophenylcarbamate; ERK1/2, extracellular signal-regulated kinases 1 and 2; mAChR, muscarinic acetylcholine receptor; TMA, tetramethylammonium.

evidence that these compounds can interact allosterically at the mAChRs comes from their ability to retard the dissociation rate of the orthosteric antagonist [^{3}H]*N*-methylscopolamine (118–120), and/or from different patterns of response of the agonists to mutations of the orthosteric site, when compared with ACh-like agonists (36, 117, 120, 121). However, both of these approaches have their own limitations when it comes to confirming a purely allosteric mode of action. Radioligand dissociation rate studies monitor interactions on a receptor that has been preequilibrated with orthosteric ligand; these experiments can reveal that a ligand may adopt a (secondary) allosteric binding mode if the orthosteric site is already occupied, but they cannot be used alone to conclude that an agonist will adopt an allosteric binding mode at the unoccupied receptor. The ligands may adopt a bitopic mode instead in this latter instance, hence activating the receptor via the orthosteric site. In terms of mutagenesis studies, differential agonist sensitivity to specific mutations can certainly be indicative of a different mode of binding but may not necessarily be proof of interaction with an entirely topographically distinct region from the orthosteric site owing to the difficulty in interpreting direct versus indirect effects of receptor mutation.

As shown in **Figure 10**, a recent study of the binding mode of 77-LH-28-1 at the M_1 mAChR suggested an "extended" binding pose that not only encompasses key TM regions implicated in orthosteric binding but also reaches up toward the extracellular domains (119). This is reminiscent

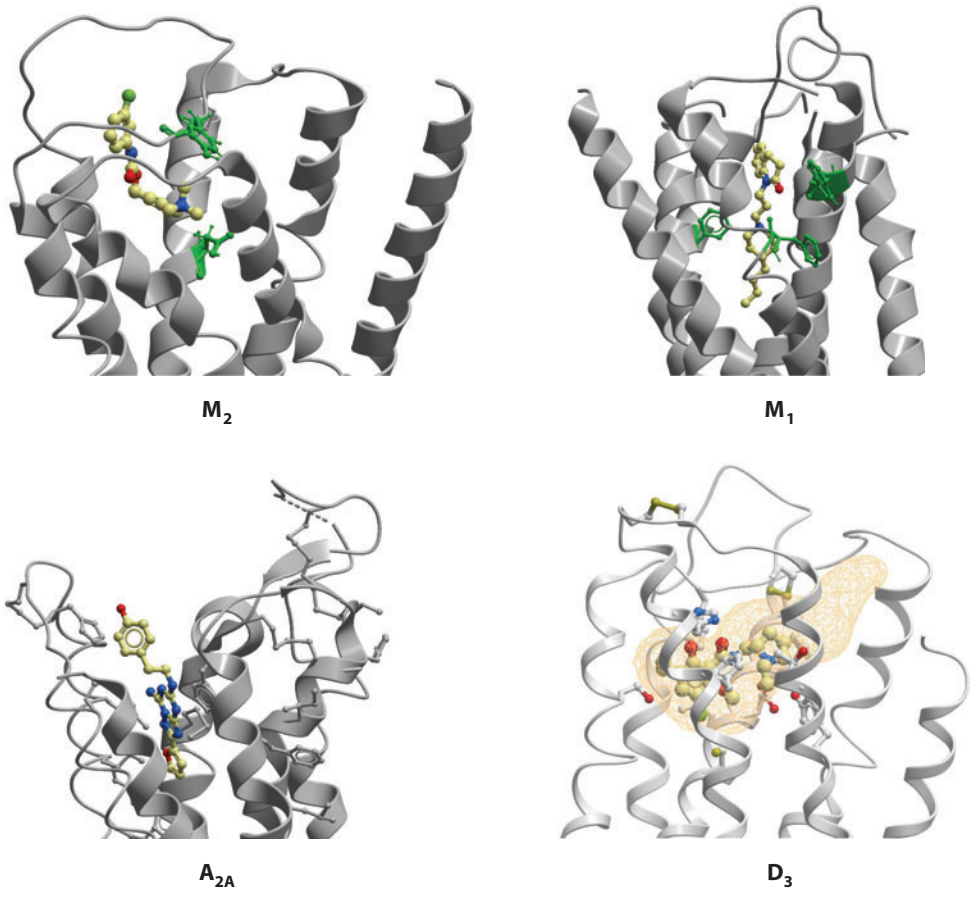

Figure 10

Bitopic binding modes may be more common than currently appreciated. Extended binding poses have been predicted for the functionally selective agonists McN-A-343 (107) and 1-[3-(butyl-1-piperidinyl)propyl]-3, 4-dihydro-2(1H)quinolinone (77-LH-28-1) (119); residues highlighted in green have been implicated in the pharmacology of the indicated ligands through mutagenesis. Solution of the crystal structure of the inactive state of the adenosine A_{2A} receptor also identified an extended binding pose for the inverse agonist ZM241385 (16). Finally, the recent determination of the crystal structure of the dopamine D_3 receptor bound to the inverse agonist eticlopride also identified an extended binding cavity (*orange mesh*) that may provide space for the binding of bitopic ligands (14).

of the proposed binding mode of McN-A-343 at the M_2 mAChR. Interestingly, the publication of high-resolution GPCR crystal structures also reveals novel structural features that may be indicative of the potential for bitopic ligand binding modes. For instance, the pose adopted by the inverse agonist ZM241385 in the inactive state of the adenosine A_{2A} receptor extends perpendicular to the plane of the membrane (**Figure 10**) and makes key contact with regions in the extracellular loops as well as in the TM domains (16). In addition, the recent solution of the structure of the dopamine D_3 receptor bound to the inverse agonist eticlopride revealed a "second" binding pocket extending out from the classic orthosteric domain (**Figure 10**) that may provide additional points of contact for extended ligands, which may prove to be bitopic (14).

CONCLUSIONS

The concept of bitopic orthosteric/allosteric ligands is a new one, having emerged only in the past few years. However, the principles behind the design of such ligands are the same as those applied to the generation of more traditional bivalent ligands. In both instances, the promise lies in the generation of chemical biological tools that can display novel modes of selectivity and/or improved affinity for a target receptor and, thus, allow for new insights to be gained into the biology of GPCRs. Ideally, some bitopic ligands may also prove to be useful drug leads, although the increase in the size of such molecules may pose "druggability" challenges. Clearly, there is much more work ahead for the field in this regard, but the promise of sculpting target selectivity and pathway selectivity by exploiting the best of both (orthosteric and allosteric) worlds is an exciting one.

SUMMARY POINTS

1. GPCRs are the largest class of drug targets in the genome. Despite a common structural architecture, these receptors display a remarkable diversity in the nature of ligands that they can recognize and intracellular effectors with which they interact, indicating a substantial degree of conformational flexibility.
2. The dynamic nature of GPCR conformational changes is being increasingly exploited through the discovery of allosteric modulators, which bind to sites that are topographically distinct from the endogenous agonist (orthosteric) binding site, and biased agonists, which promote unique signaling profiles via a given receptor to the relative exclusion of other pathways. These two phenomena reflect a common underlying mechanism, and it is now acknowledged that allosteric ligands can engender biased signaling in the actions of coadministered orthosteric ligands.
3. Numerous previous studies aimed at designing more potent and/or more selective ligands targeting GPCRs and their associated pathways exploited the message-address concept. This concept views drug design in terms of the synthesis of moieties that target a conserved region of the target responsible for signal transduction (message) and moieties that target less conserved regions (address), with the latter leading to enhanced selectivity. An important outcome of this approach was the joining of two distinct pharmacophores via an appropriately spaced linker to yield what are termed bivalent GPCR ligands. Such ligands have proven useful in achieving higher affinity for target GPCRs or better selectivity—in some instances owing to the fact that they can target dimeric receptor complexes.
4. The most recent modification of the bivalent ligand approach has been the linking of defined orthosteric ligands and allosteric modulators to yield what are known as bitopic ligands. These ligands offer the potential of achieving selectivity by virtue of targeting less conserved allosteric regions on GPCRs while ensuring activation via concomitant interaction with the orthosteric site. Furthermore, bitopic engagement of a GPCR can yield biased signaling via the selection of unique receptor conformation(s).
5. Important challenges to the design of bitopic ligands are the need to validate the mechanism of action using complementary experimental approaches and the need to control for possible effects of the linker moiety that is used to join the orthosteric and allosteric pharmacophores.

6. It is possible that previously identified biased agonists of GPCRs may also act via a bitopic mechanism; studies using fragments of functionally selective ligands, receptor mutagenesis, or X-ray crystallography provide support for bitopic GPCR ligand binding poses. This suggests that the phenomenon of bitopic ligands may be more widespread than currently appreciated.

FUTURE ISSUES

1. Why do some rationally designed bitopic ligands not show the expected gains in affinity at their target GPCRs?
2. How can a bitopic mechanism of action be unambiguously demonstrated?
3. Do other functionally selective compounds achieve their selectivity through a bitopic mechanism?
4. How many GPCRs can be targeted using a bitopic approach?
5. What is the practical limit in the design of bitopic molecules with regard to retaining drug-like characteristics?

DISCLOSURE STATEMENT

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