

THEMED SECTION: MOLECULAR PHARMACOLOGY OF G PROTEIN-COUPLED RECEPTORS

REVIEW

Rational design of dualsteric GPCR ligands: quests and promise

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Dualsteric ligands represent a novel mode of targeting G protein-coupled receptors (GPCRs). These compounds attach simultaneously to both, the orthosteric transmitter binding site and an additional allosteric binding area of a receptor protein. This approach allows the exploitation of favourable characteristics of the orthosteric and the allosteric site by a single ligand molecule. The orthosteric interaction provides high affinity binding and activation of receptors. The allosteric interaction yields receptor subtype-selectivity and, in addition, may modulate both, efficacy and intracellular signalling pathway activation. Insight into the spatial arrangement of the orthosteric and the allosteric site is far advanced in the muscarinic acetylcholine receptor, and the design of dualsteric muscarinic agonists has now been accomplished. Using the muscarinic receptor as a paradigm, this review summarizes the way from suggestive evidence for an orthosteric/allosteric overlap binding to the rational design and experimental validation of dualsteric ligands. As allosteric interactions are increasingly described for GPCRs and as insight into the spatial geometry of ligand/GPCR-complexes is growing impressively, the rational design of dualsteric drugs is a promising new approach to achieve fine-tuned GPCR-modulation.

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Abbreviations: ABA, alkane-bis-ammonio; GPCR, G protein-coupled receptor; M-receptor, muscarinic acetylcholine receptor; TMA, tetramethylammonium

The promise

The superfamily of G protein-coupled receptors (GPCRs) has high relevance with regard to both, current drug treatment and new drug discovery (Chung *et al.*, 2008; Heilker *et al.*, 2009). A traditional approach in the design of new ligands is to target the receptor binding site of the endogenous messenger com-

pound. This site is classified as 'orthosteric' site. Ligands that compete with the messenger compound for this site are named orthosteric ligands. It is increasingly recognized that GPCRs may contain additional, 'allosteric' binding sites that allow for simultaneous receptor binding of both, an orthosteric and an allosteric ligand (for recent reviews see Gao and Jacobson, 2006; May *et al.*, 2007a; Conn *et al.*, 2009; De Amici *et al.*, 2009). Such ternary complex formation allows for novel qualities of drug action such as an enhancement of binding and action of the endogenous receptor activator. In addition, allosteric ligands typically display selectivity for receptor subtypes. Whereas the orthosteric site of the endogenous

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messenger molecule is usually conserved between the receptor subtypes, allosteric receptor areas may reveal considerable heterogeneity with respect to amino acid sequence and spatial conformation. Yet, allosteric ligands may not reach the level of affinity that is seen with ligands of the orthosteric site. Recently, a novel approach in drug design has been proposed that should allow the exploitation of the above-mentioned favourable consequences that arise from addressing both the orthosteric and the allosteric site of a receptor protein (Disingrini *et al.*, 2006; Steinfeld *et al.*, 2007). As a proof of concept, a new type of receptor activator has been introduced that is constructed to utilize simultaneously orthosteric and allosteric receptor areas to achieve both, receptor activation and selectivity with respect to receptor subtype binding and intracellular signalling pathway activation (Antony *et al.*, 2009). This novel topography of drug/receptor-interaction has been referred to as dualsteric binding. Terms also used in this context are 'bitopic orthosteric/allosteric' (Tahtaoui *et al.*, 2004; Valant *et al.*, 2008) and 'multivalent orthosteric/allosteric' (Steinfeld *et al.*, 2007). For a brief nomenclature we suggest 'dualsteric' to address concomitant orthosteric/allosteric site occupancy as well as the ligands that display this property. The rational design of dualsteric compounds requires detailed insight into the three-dimensional structure of the receptor protein of interest and into the topography of orthosteric and allosteric ligand binding. With the increasing number of crystallized drug/GPCR-complexes such insight will soon increase (Rosenbaum *et al.*, 2009). Although not having at hand crystallized complexes until now, knowledge about the topography of ligand binding is well advanced in the field of muscarinic acetylcholine receptors (M-receptors). There are five M-receptor subtypes that differ in tissue distribution, G protein-coupling preference and physiological function (Wess *et al.*, 2007; Alexander *et al.*, 2008). The M₂ subtype was the first GPCR to be recognized as being sensitive to allosteric modulation (Lüllmann *et al.*, 1969; Clark and Mitchelson, 1976). Since then, intensive efforts were spent by various groups to gain insight into the molecular determinants governing allosteric/orthosteric interactions at muscarinic receptors. Meanwhile muscarinic receptors have evolved as prototypes for the investigation of allosteric GPCR modulation (for review see Birdsall and Lazareno, 2005; De Amici *et al.*, 2009). In the following we will review how the growing insight into the molecular events underlying muscarinic allosteric/orthosteric receptor interactions paved the way for the rational design of dualsteric ligands that activate muscarinic receptors in a subtype- and signalling pathway-selective fashion. We will discuss experimental approaches to validate that the intended dualsteric receptor targeting is in fact attained. In this context, aspects of GPCR structure and function will be mentioned that await clarification before a fully advanced rational design of dualsteric ligands can be achieved that promises high affinity and subtype-selectivity in conjunction with well adjusted efficacy and signalling pathway selectivity.

A settled quest: spatial vicinity between the allosteric and orthosteric site

The best-studied allosteric site of M-receptors is located in the entrance of the ligand binding pocket that harbours the

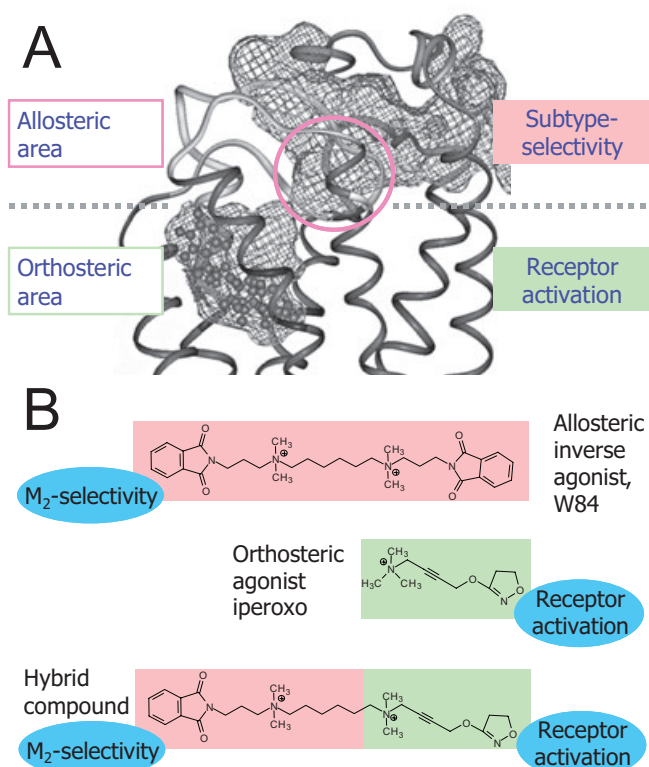


Figure 1 'Blue-print' for the design of dualsteric receptor activators with subtype-selectivity. (A) Side view on the upper part of the muscarinic M₂ acetylcholine receptor. The free volume of the ligand binding pocket is lined by the grid structure. The model is based on the crystal structure of bovine rhodopsin in the inactive state. An inverse agonist (*N*-methylscopolamine) is docked into the orthosteric site to stabilize an inactive receptor conformation in the molecular dynamics simulation (modified from Voigtländer *et al.*, 2003). The circle marks the allosteric core region which harbours non-conserved subtype-selectivity providing amino acids. (B) Design concept for subtype-selective allosteric/orthosteric hybrid agonists. The allosteric building block is derived from the alkane-bis-ammonio (ABA)-type compound W84, iperoxo (agonist d) is the orthosteric building block, the resulting hybrid compound is 1d.

orthosteric receptor binding site further down in the region of the transmembrane helices (Figure 1A). Worth mentioning, there is evidence for more than one allosteric site on muscarinic receptors (for an overview about nomenclature and ligands see Alexander *et al.*, 2008). First evidence for an allosteric binding topography arose from kinetic binding studies using radiolabelled orthosteric antagonists (Stockton *et al.*, 1983; Nedoma *et al.*, 1986; Jepsen *et al.*, 1988; Potter *et al.*, 1989). Muscarinic allosteric ligands typically retard the dissociation and the association of orthosteric radioligands. It was suggested that the allosteric site is located extracellular to the orthosteric site (Jakubík and Tuček, 1994). Allosteric inhibition of orthosteric ligand association results from allosteric ligand binding to the orthosterically free receptor. Inhibition of orthosteric ligand dissociation is a consequence of ternary complex formation, i.e. allosteric agent binding to the orthosterically liganded receptor. Noteworthy, in other GPCRs such as the α_2 -adrenergic receptor (Leppik *et al.*, 1998) orthosteric ligand dissociation is accelerated by allosteric modulators. The allosteric inhibitive actions on muscarinic orthosteric

ligand association and dissociation often differ, as the binding affinity of the allosteric ligands is sensitive to the differences in conformation between the free and the orthosterically liganded receptor. Depending on the affinity ratio or, in other words, the relative extent by which orthosteric ligand association and dissociation are inhibited, the allosteric ligand may change equilibrium binding of the orthosteric ligand:

Association inhibition > dissociation inhibition → decrease of orthoster binding

Association inhibition < dissociation inhibition → increase of orthoster binding

Association inhibition = dissociation inhibition → unchanged orthoster binding

This is the kinetic basis of negative, positive and neutral cooperativity which means a reciprocal inhibiting, enhancing or neutral action on the equilibrium binding between allosteric and orthosteric ligand at muscarinic receptors (Mohr *et al.*, 2003).

The location of the allosteric site on the receptor protein was narrowed down by making use of the subtype selective receptor interaction that is a characteristic of muscarinic allosteric ligands (e.g. Ellis *et al.*, 1991; Lee and El-Fakahany, 1991). This results from a less well conserved amino acid sequence between the muscarinic receptor subtypes in their extracellular regions. Chimeric receptor constructs helped to identify relevant receptor areas (Ellis *et al.*, 1993), point mutated receptors allowed to identify key amino acids for allosteric subtype selectivity. These are contained in the second outer loop (EDGE sequence, ¹⁷²Glu-¹⁷³Asp-¹⁷⁴Gly-¹⁷⁵Glu and ¹⁷⁷Tyr in M₂; Leppik *et al.*, 1994; Voigtländer *et al.*, 2003; Huang *et al.*, 2005) and in the region of the end of the third outer loop/beginning of transmembrane helix 7 [TM7 (Gnagey *et al.*, 1999; Krejčí and Tuček, 2001; Jakubík *et al.*, 2005), in particular M₂⁴²³Thr (Buller *et al.*, 2002), position 7.36 according to Ballesteros and Weinstein, 1995]. Subtype-independent allosteric base-line affinity is provided by Trp 7.35 that is conserved among the five muscarinic subtypes and located in close vicinity to M₂¹⁷⁷Tyr (Prilla *et al.*, 2006). Furthermore, the conserved disulfide bridge between transmembrane helix TM3 and M₂¹⁷⁶Cys of the second outer loop is relevant for allosteric and orthosteric ligand binding (Avlani *et al.*, 2007; Huang and Ellis, 2007). Modelling of the [³H]NMS-bound receptor and allosteric docking simulations revealed that the above-mentioned receptor epitopes are clustered in the so-called allosteric core region that is located at the bottom of the allosteric binding area next to the orthosteric site (Figure 1A; Jöhren and Höltje, 2002; Voigtländer *et al.*, 2003; Prilla *et al.*, 2006). This insight into the allosteric/orthosteric topography provides the rational fundament for the design of dualsteric ligands. The concept is to fuse an orthosteric ligand intended to activate the receptor with an appropriate allosteric anchor to achieve muscarinic subtype-selectivity (Figure 1B).

A closer look at binding modes

When the binding topography of a ligand is to be classified, some additional aspects are worth to be considered with respect to orthosteric, allosteric and dualsteric binding.

Orthosteric binding in muscarinic receptors requires that the ligand has to pass the allosteric region which may modulate both, access to and egress from the orthosteric site. Worth mentioning, the rather fast binding kinetics of [³H]N-methylscopolamine observed in the M₂ subtype are slowed down without a relevant change of the binding affinity of [³H]NMS, when amino acids of the M₂ allosteric core region are replaced by their corresponding counterparts from the M₅-subtype. The M₅ receptor subtype is characterized by very slow [³H]NMS binding kinetics, which are speeded up when the corresponding allosteric M₂ amino acids are introduced (Buller *et al.*, 2002; Prilla *et al.*, 2006). The findings suggest that orthosteric ligands interact with epitopes of the extracellular allosteric site on their way to and from the orthosteric site.

The model of the NMS-bound M₂ receptor (Figure 1A) is characterized by a narrow junction between the orthosteric and the allosteric area. Other conformations will have to occur allowing for sufficient free volume that is required for the passage of bulky inverse agonists such as N-methylscopolamine. In addition, with respect to the receptor conformation engaged in the binding of orthosteric agonists, there is strong evidence for a major conformational change of the allosteric/orthosteric junction in the active agonist-bound receptor protein (Grossmüller *et al.*, 2006; Jäger *et al.*, 2007).

Allosteric binding of a receptor ligand is experimentally obvious, when the equilibrium binding of an orthosteric probe is increased. If orthosteric radioligand binding is decreased by a test compound, allosteric binding is typically proven experimentally by an allosteric modulation of orthosteric radioligand dissociation. As can be taken intuitively from Figure 1A, muscarinic allosteric agents typically inhibit the dissociation of orthosteric radio-antagonists and radio-agonists (Stockton *et al.*, 1983; Nedoma *et al.*, 1986; Jepsen *et al.*, 1988; Gnagey and Ellis, 1996; Kostenis and Mohr, 1996; Grossmüller *et al.*, 2006).

Noteworthy, if a test compound has revealed an effect on orthosteric radioligand dissociation, this finding does not necessarily indicate that the test compound adopts the same binding topography in the orthosterically free receptor. Under this condition the ligand might slip down into the orthosteric orientation. For instance, even the classical orthosteric antagonist atropine is found to induce an allosteric inhibition of [³H]NMS-dissociation but only at excessive concentrations (Waelbroeck *et al.*, 1992; Tränkle *et al.*, 1996). Regarding the interaction of allosteric agents with the free receptor, an orthosteric radioligand association binding assay can hardly discriminate between an allosteric or an orthosteric binding topography of the test compound under investigation; both topographies would result in an inhibition of radioligand association. Additional features of allosteric agent binding such as the pronounced sensitivity of typical muscarinic allosteric agents on the ionic composition of the assay buffer (Schröter *et al.*, 2000) or the dependence of allosteric binding affinity on allosterically *versus* orthosterically point mutated receptors may help to clarify the issue (Antony *et al.*, 2009).

For archetypal allosteric agents such as alcuronium, galamine and the alkane-bis-ammonio (ABA)-type compounds, there is no evidence that they move down into the orthosteric location in orthosterically free receptors (Schröter *et al.*,

2000). Binding affinities of the ABA-type compounds W84 (Figure 1B) and naphmethonium do not respond to mutation of the orthosteric $M_2^{104}\text{Tyr}$ (3.33) which is located next to $M_2^{103}\text{Asp}$ (3.32) which is an orthosteric epitope of key importance for the binding of acetylcholine and other conventional orthosteric agonists and antagonists (Antony *et al.*, 2009). Another experimental evidence for purely allosteric binding in free receptors comes from the ABA-type allosteric antagonist radioligand [^3H]dimethyl-W84. Compared with orthosteric antagonist ligands such as [^3H]N-methylscopolamine, the allosteric radioligand has dramatically faster receptor dissociation kinetics (Tränkle *et al.*, 2003). This observation is in line with a superficial location in the allosteric binding site (Figure 1A).

Dualsteric binding is defined as *simultaneous orthosteric/allosteric overlap binding of a receptor ligand*. Other terms having been used to address such binding topography are 'bitopic orthosteric/allosteric' or 'multivalent orthosteric/allosteric'. This binding mode has to be distinguished from ternary complex formation which means simultaneous binding of two independent molecules (different or identical) to the allosteric and the orthosteric site respectively. In addition, dualsteric binding has to be distinguished from a multiple binding mode, i.e. ligand binding that switches in a 'flip-flop'-like fashion between two or more receptor topographies, in our case for

instance between a purely allosteric and a purely orthosteric binding. Such 'flip-flop'-binding might be worthwhile to consider in the case of small ligand molecules with comparably low binding affinity (i.e. ligands that have a poor fit to the receptor protein) – in contrast to voluminous high affinity ligands that are likely to have a fixed binding topography.

Muscarinic ligands heralding an allosteric/orthosteric binding topography

Initial experimental evidence that suggested an allosteric/orthosteric overlap binding emerged from a deeper mechanistic study of compounds that were developed in academic and industrial laboratories as subtype-selective muscarinic antagonists. With the advent of the antiulcer drug and M_1 -preferring muscarinic antagonist pirenzepine it became clear that the population of muscarinic receptors is heterogeneous (Hammer *et al.*, 1980). Subsequently five subtypes of the muscarinic receptors were identified (Kubo *et al.*, 1986; Bonner *et al.*, 1987).

Methoctramine was introduced by Melchiorre and coworkers who developed polymethylene tetraamines as subtype-selective muscarinic receptor probes. Methoctramine (Figure 2) is an antagonist with pronounced preference for the

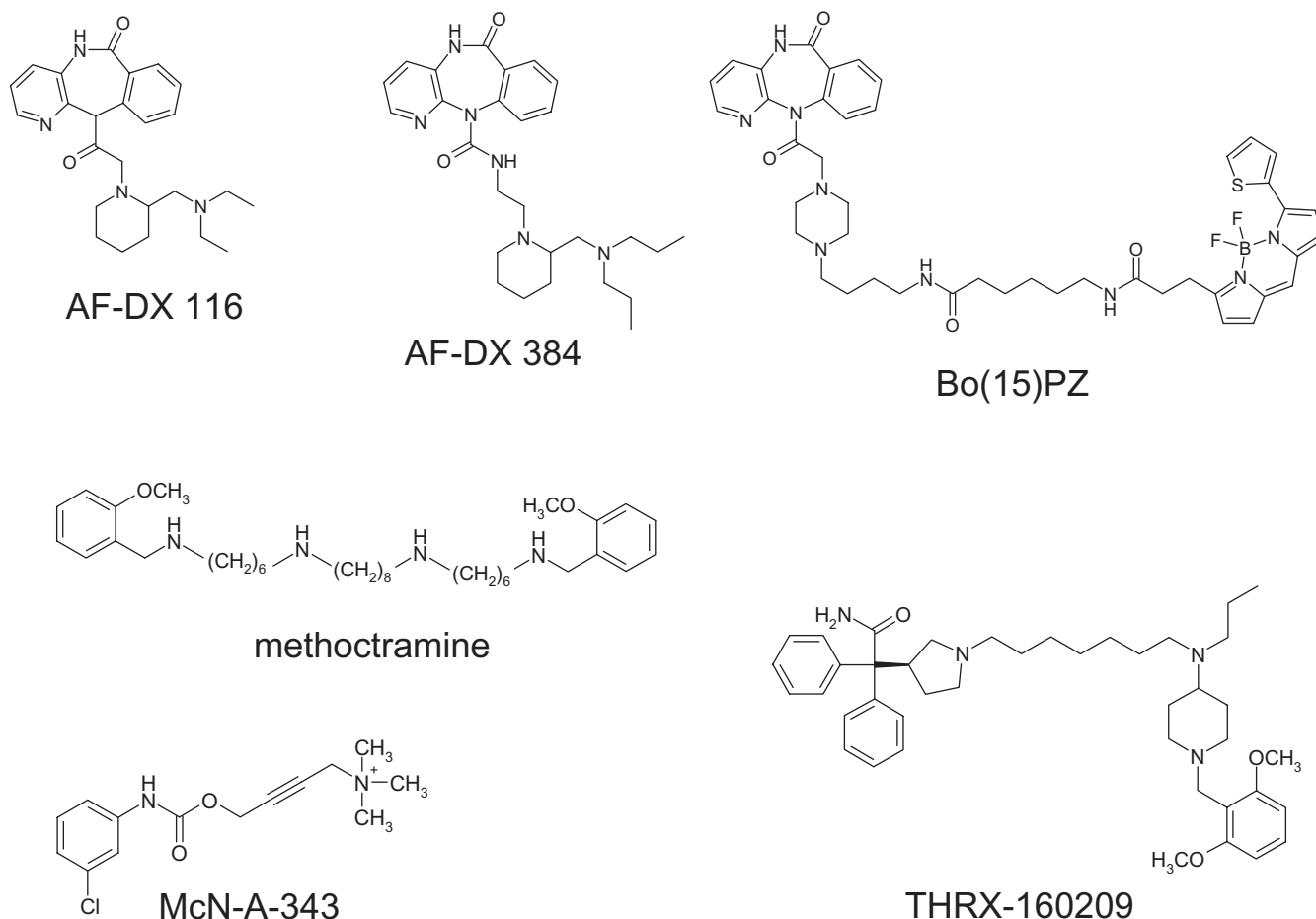


Figure 2 Structures of ligands with a suggested allosteric/orthosteric binding mode at muscarinic receptors.

M₂ subtype (Melchiorre *et al.*, 1987a; b). Studies in living tissue preparations point to a competitive type of receptor interaction with orthosteric agonists over a wide concentration range of methoctramine. In addition, at very high concentrations (100-fold higher than its affinity constant) methoctramine was found to inhibit orthosteric radioantagonist dissociation – a hallmark of allosterism (Giraldo *et al.*, 1988; Roffel *et al.*, 1989). Melchiorre *et al.* (1989) proposed that methoctramine binds to both the orthosteric and allosteric site of the M₂ receptor, capturing affinity from a non-conserved cluster of negatively charged allosteric amino acids (EDGE-sequence, ¹⁷²Glu-¹⁷³Asp-¹⁷⁴Gly-¹⁷⁵Glu). When the orthosteric site is blocked by a radioligand, the allosteric interaction is still possible, but with a lower affinity. In other words, methoctramine may switch from an allosteric/orthosteric high affinity binding to a purely allosteric low affinity binding. Receptor mutagenesis findings did not allow to decide whether methoctramine binds exclusively to the allosteric site or to both the orthosteric and the allosteric site (Matsui *et al.*, 1995; Ellis and Seidenberg, 2000).

AF-DX 116 and AF-DX 384 (Figure 2) are M₂-preferring antagonists with a selectivity profile of M₂ greater than M₁ greater than M₃ (Eberlein *et al.*, 1989; Engel *et al.*, 1989). Displacement of radiolabelled [³H]AF-DX 384 by atropine and AF-DX 116 was compatible with a competitive interaction (Entzeroth and Mayer, 1990). For AF-DX 116 (otenzepad) evidence for an allosteric interaction was found by Lee and El-Fakahany (1991) who applied radioligand binding in cloned muscarinic receptor subtypes and showed that M₂ preferring antagonists including AF-DX 116 allosterically inhibit dissociation of the orthosteric [³H]N-methylscopolamine. Yet, ternary complex formation with [³H]NMS-occupied receptors requires high concentrations that by far exceed those that inhibit equilibrium binding of [³H]NMS. Noteworthy, both actions reveal the same order of subtype selectivity M₂ > M₁ > M₃. The authors conclude that competitive displacement may be mediated via the primary (orthosteric) receptor binding site, whereas the inhibition of radioligand dissociation is the consequence of interaction with the secondary (allosteric) site of the receptor. Evidence for an allosteric component of AF-DX 116 was also provided by Lanzafame *et al.* (2001) who studied its interaction with allosteric and orthosteric antagonists in guinea pig atria and in radioligand binding assays.

For AF-DX 384, combination experiments in paced guinea pig atria suggested that the ABA-type allosteric antagonist modulator W84 binds with considerably lower affinity to M₂ receptors that are liganded with AF-DX 384 compared with the conventional orthosteric antagonist N-methylscopolamine (NMS). In line with this, allosteric inhibition by W84 of the dissociation of [³H]NMS was much more pronounced than was dissociation of [³H]AF-DX 384. In addition, at high concentrations, purely allosteric binding of AF-DX 384 to [³H]NMS-liganded M₂ receptors was shown. It was concluded that M₂ receptor binding of AF-DX 384 extends beyond the orthosteric site and partially includes the allosteric site of the M₂ receptor protein, thereby utilizing allosteric epitopes not being conserved among the muscarinic receptor subtypes (Tränkle *et al.*, 1998). Use of stepwise shortened derivatives of W84 as probes suggested that the overlap

of AF-DX 384 with the M₂ allosteric site extends to about half of the area used by W84 (Mohr *et al.*, 2004), i.e. part of AF-DX 384 would point from the orthosteric site into the allosteric core region (cf. Figure 1). A W84-derived building block was fused with AF-DX 384 hoping that this might further increase the allosteric overlap area of AF-DX 384. However, the resulting compound appeared to bind purely in the allosteric mode instead of the intended orthosteric/allosteric location. It was concluded that the AF-DX 384/W84-hybrid was hindered by its W84-building block from properly diving into the orthosteric binding pocket. Alternatively, the ABA-building block was introduced into the AF-DX 384 molecule at an unfavourable position not facing the allosteric site.

Taken together, the binding topography of the M₂-preferring antagonists of the methoctramine- and AF-DX-type has not been fully elucidated until now. When the above-mentioned studies were carried out, allosteric key amino acids had not been identified. Now, that the close spatial vicinity between the orthosteric binding pocket and the allosteric core region of the M₂ receptor has emerged (Figure 1A), the hypothesis of an orthosteric/allosteric overlap is revitalized with the orthosteric binding providing high affinity and the allosteric binding providing M₂-selectivity.

Fluorescent pirenzepine derivatives

Ilien and coworkers (Tahtaoui *et al.*, 2004) prepared fluorescent derivatives of the M₁ antagonist pirenzepine. Fluorophores such as bodipy were linked with the antagonist through linkers of varying chain length. To check for simultaneous orthosteric/allosteric binding, dissociation from M₁ receptors of the derivatives was measured in the absence and presence of brucine which is an allosteric ligand (Jakubik *et al.*, 1997; Lazareno *et al.*, 1998). Brucine inhibited the dissociation of fluorescent derivatives with a linker shorter than 12 atoms, indicating additional binding of brucine to the ligand receptor complex. Fluorescent derivatives with a chain length of 15 (Bo(15)PZ, Figure 2) and longer, however, were resistant to the allosteric action of brucine. This finding suggests that the area of receptor binding of these derivatives includes the allosteric domain where brucine binds. Taken that pirenzepine binds to the orthosteric site, the brucine-resistant derivatives were classified as 'potential bitopic ligands'.

M₂-preferring antagonist THRX-160209

Steinfeld and coworkers (2007) introduced a high-affinity, M₂ subtype-selective muscarinic antagonist, THRX-160209 (Figure 2), and provided experimental evidence for a 'multivalent' orthosteric/allosteric receptor binding. This compound displays high affinity for the M₂ subtype (pK_i = 9.5). This affinity is far higher than that of the purely allosteric binding mode (pEC_{50,diss} = 6.5) which was measured in M₂ receptors the orthosteric site of which was blocked by [³H]NMS. The THRX-compound is composed of a 3-benzydryl pyrrolidine moiety (putative orthosteric building block, non-selective) that is linked by a seven

carbon-chain with a 4-aminobenzylpiperidine motif (putative allosteric building block, M₂-preferring). Three lines of evidence suggest a simultaneous orthosteric/allosteric binding. First, the binding affinity of the THRX-compound (pK_i = 9.5) is higher than the binding affinity of each of the individual building blocks (pK_i-values of 5.4 and 5.7 respectively). However, the gain in affinity cannot exclusively be related to the building blocks as the heptamethylene linker appears to contribute to THRX-binding. Second, the rank order of affinities is typical for an allosteric interaction (M₂ > M₄ > M₁ > M₃ > M₅). Third, the receptor binding is sensitive to orthosteric and allosteric probes. This check included use of a [³H]THRX-radioligand, which allowed to directly follow receptor dissociation kinetics by an 'infinite dilution assay'. A second, slow component of [³H]THRX-dissociation which is thought to represent bivalent orthosteric/allosteric binding was found to be sensitive to the individual building blocks and to other orthosteric and allosteric receptor ligands.

M₂ partial agonist McN-A-343

Christopoulos and coworkers provided evidence for an allosteric/orthosteric overlap binding of the partial agonist McN-A-343 in M₂ receptors (Valant *et al.*, 2008). The mechanism of action of McN-A-343 has been studied for decades (Roszkowski, 1961; Fozard and Muscholl, 1971; Birdsall *et al.*, 1983; Lambrecht *et al.*, 1993; May *et al.*, 2007b). There is evidence compatible with an orthosteric receptor interaction but an allosteric agonism has also been proposed. The ingenuity of the most recent experimental approach was to consider McN-A-343 (Figure 2) as a molecule containing two building blocks, i.e. tetramethylammonium (TMA) bromide, which is a full muscarinic agonist, and a chlorophenylcarbamate-type building block (Valant *et al.*, 2008). The latter type of agent was shown, first, to act as (weakly potent) positive allosteric modulator on [³H]NMS-binding at the M₂ receptor and, second, to reduce the intrinsic efficacies and the affinities of TMA and acetylcholine in an EK1/2-phosphorylation assay. Mutation of M₂¹⁷⁷Tyr, an allosteric core amino acid (Voigtländer *et al.*, 2003), was shown to increase the efficacy of McN-A-343 and to weaken the action of the chlorophenylcarbamate-type building blocks with respect to both allosteric inhibition of [³H]NMS-dissociation and diminution of TMA- and acetylcholine-intrinsic efficacy. Docking of McN-A-343 in a M₂ receptor model was compatible with an allosteric location of the chlorophenylcarbamate-type building block and with an orthosteric interaction of the TMA building block with the orthosteric M₂¹⁰³Asp. Taken together the findings of Valant *et al.* (2008) suggest that the chlorophenylcarbamate moiety is a built-in allosteric quencher of an orthosterically mediated intrinsic efficacy of McN-A-343.

This hypothesis is in line with findings that were obtained by Disingrini *et al.* (2006) who used the concept displayed in Figure 1B for the design of allosteric/orthosteric ligands and who encountered a loss of orthosteric full agonist efficacy upon fusion with allosteric inverse agonistic building blocks (see below).

Design of dualsteric agonists

For most of the compounds being introduced in the preceding chapter, evidence for the proposed allosteric/orthosteric receptor binding topography came by serendipity, none of these compounds was designed from pre-existing orthosteric and allosteric building blocks. In addition, none of these compounds has efficacy for appreciable M₂ receptor activation. The dualsteric compounds described in this review are the first to emerge from a rational design concept aiming at ligands that encompass orthosteric and allosteric functionalities, i.e. high affinity orthosteric receptor activation and allosteric M₂ subtype-selectivity (Figure 1B). In these ligands, oxotremorine M-related receptor activators serve as orthosteric building blocks. On their own these agonists are devoid of receptor subtype selectivity (Figure 3, compounds a to d upper panel). These orthosteric building blocks are connected, by fusing the common TMA head, with fragments of ABA-type compounds such as the truncated W84 derivative (1 in Figure 3). W84 and its shortened fragments are subtype-selective for M₂ (Figure 3; Antony *et al.*, 2009). In isolated tissue preparations that serve as M₁, M₂ and M₃ models (rabbit vas deferens, guinea pig atrium, guinea pig ileum), all hybrid compounds lose over-all potency, but gain subtype-selectivity. However, compounds 1a, 1b and 1c have almost no intrinsic efficacy. These allosteric/orthosteric hybrids behave as antagonists in the M₁ and M₂ model and, at best, as very weak partial agonists in the M₃ model (Figure 3, lower panel; Disingrini *et al.*, 2006). In membranes from CHO-cells overexpressing human M₂ receptors, however, partial agonist-like G protein activation is observed with some of the hybrid compounds (Disingrini *et al.*, 2006). Therefore, the latter already combine receptor subtype selectivity with weak (but physiologically irrelevant) partial agonism. Compound 1d, which includes the extremely potent agonist building block iperoxo d, acts as a full agonist in the M₁, the M₂ and the M₃ model. With this and a closely related compound, which contains an allosteric building block from the ABA-type compound naphmethonium (cf. Antony *et al.*, 2009), the intended M₂-preferring agonism is achieved. A true dualsteric binding topography is to be validated by various experimental approaches. In particular it is necessary to exclude that the compounds bind in a purely allosteric topography, as activation of muscarinic receptors from the allosteric site has been shown for various subtypes (for review De Amici *et al.*, in press). The M₂ subtype, for instance, is activated by autoantibodies that bind to the second outer loop (e.g. Sterin-Borda *et al.*, 1991; Goin *et al.*, 1997; Hernandez *et al.*, 2008). Therefore, the proposed allosteric/orthosteric binding topography has been verified (Figure 4; for details see Antony *et al.*, 2009). In brief, binding experiments using an orthosteric antagonist radioligand prove that binding of hybrid 1d is sensitive to both, allosteric and orthosteric M₂ receptor mutations. In addition, the mutation-induced changes of cooperativity between the hybrids and an orthosteric radioligand are in line with the predictions of a model for dualsteric ligand binding introduced by May *et al.* (2007a). Furthermore, the hybrid agonist is sensitive to the orthosteric antagonist atropine which eliminates the hybrid's intrinsic efficacy, while still allowing for hybrid binding in the purely allosteric mode. The allosteric

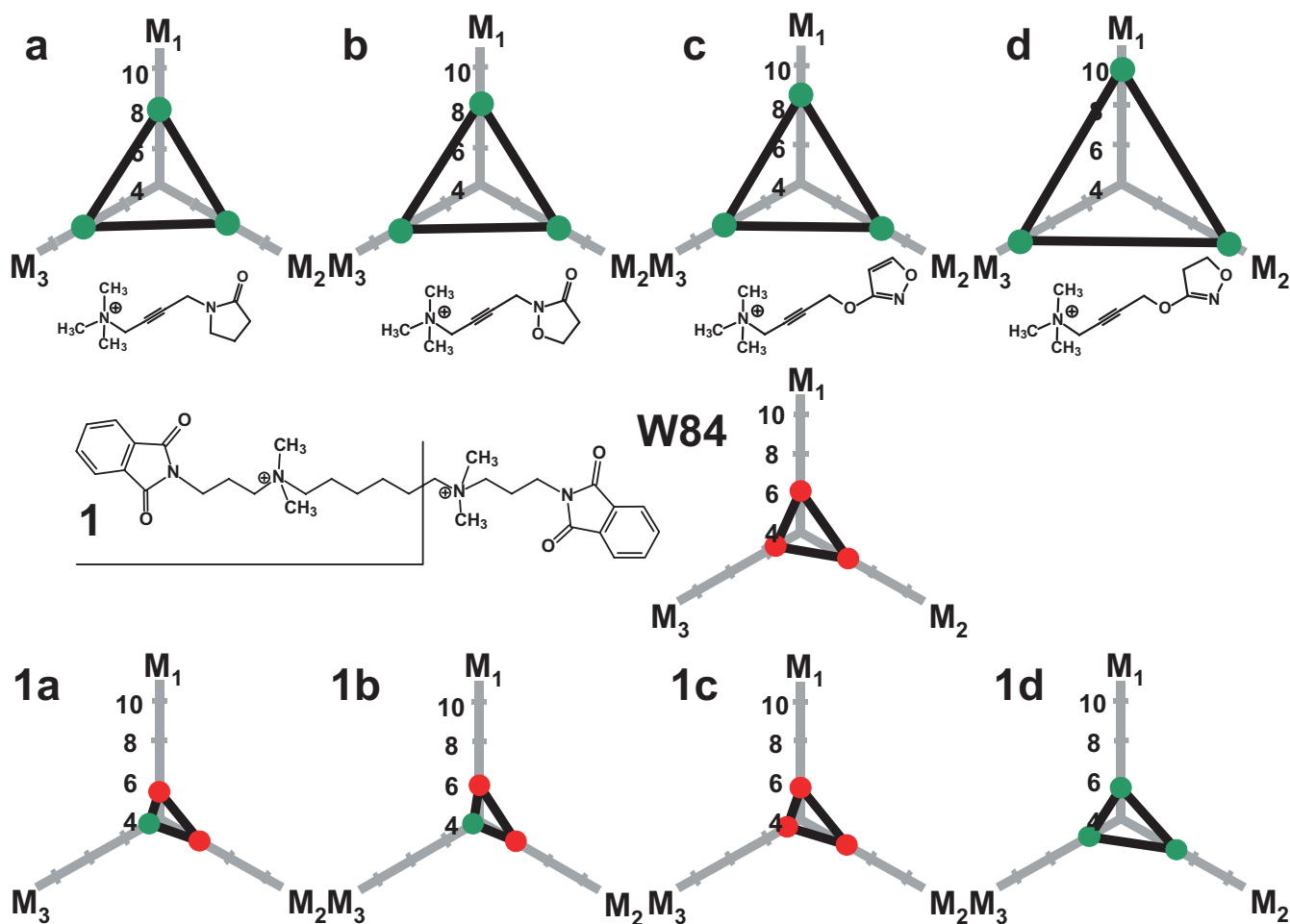


Figure 3 Potency, efficacy and muscarinic subtype-selectivity of building blocks and resulting allosteric/orthosteric hybrid compounds. Potency is indicated as minus log EC_{50} and minus log K_b in the case of agonism and antagonism respectively, for the action of orthosteric agonists a–d (upper panel), allosteric inverse agonist W84 (middle panel) and hybrids 1a–1d (lower panel) in isolated organ models for M_1 (rabbit vas deferens), M_2 (guinea pig left atrium), M_3 (guinea pig ileum). Hybrids consist of the fragment 1 from W84 and the respective agonist molecules a–d. Efficacy is indicated by dot colour – green: agonist activity, red: antagonist activity. Values for a–d are taken from Dallanoce *et al.* (1999), for W84 from Tränkle *et al.*, 1998, for 1a–1c from Disingrini *et al.* (2006) and for 1d from Antony *et al.* (2009).

antagonist W84, however, interacts with the hybrid in a competitive-type antagonism (Antony *et al.*, 2009). A docking simulation shows that the receptor may well accommodate the hybrid in the orthosteric/allosteric binding topography, with the orthosteric agonist moiety being positioned in the orthosteric site and the allosteric building block in the allosteric core region (Antony *et al.*, 2009). Taken together, the allosteric/orthosteric ligand design translates into a dualsteric receptor binding. In addition, the dualsteric binding mode goes along with biased signalling. While common M_2 receptor activating agonists may trigger the G_s pathway in addition to the G_i pathway, the hybrid compound is devoid of G_s activating properties (Antony *et al.*, 2009; Kebig *et al.*, 2009).

Thermodynamic aspects of dualsteric receptor interactions

Here we consider dualsteric binding as a special case of polyvalent interaction (Mammen *et al.*, 1998) in order to predict

which affinity can be expected for the dualsteric interaction relative to the orthosteric and allosteric binding component. In the simplest case, the total free energy change ΔG_{dual} should be approximately the sum of the free energy changes of the individual components ΔG_{ortho} and ΔG_{allo} together with an entropic correction. The free energy change corresponds to receptor binding affinity according to $\Delta G = -RT \ln(K)$, where K is the association constant of ligand binding, R is the gas constant and T is temperature in Kelvin. Therefore, the expected affinity of dualsteric binding K_{dual} should be near the product of the component affinities K_{ortho} and K_{allo} . However, additional aspects have to be considered which may contribute to a less than expected binding affinity. These aspects shall be discussed in the light of the enthalpic and the entropic component of the free energy change $\Delta G = \Delta H - T\Delta S$.

- **Negative cooperativity:** The enthalpy change ΔH_{dual} of the dualsteric interaction will be smaller than the sum of ΔH_{ortho} and ΔH_{allo} , if binding of one of the building blocks imprints a conformation on the receptor protein that

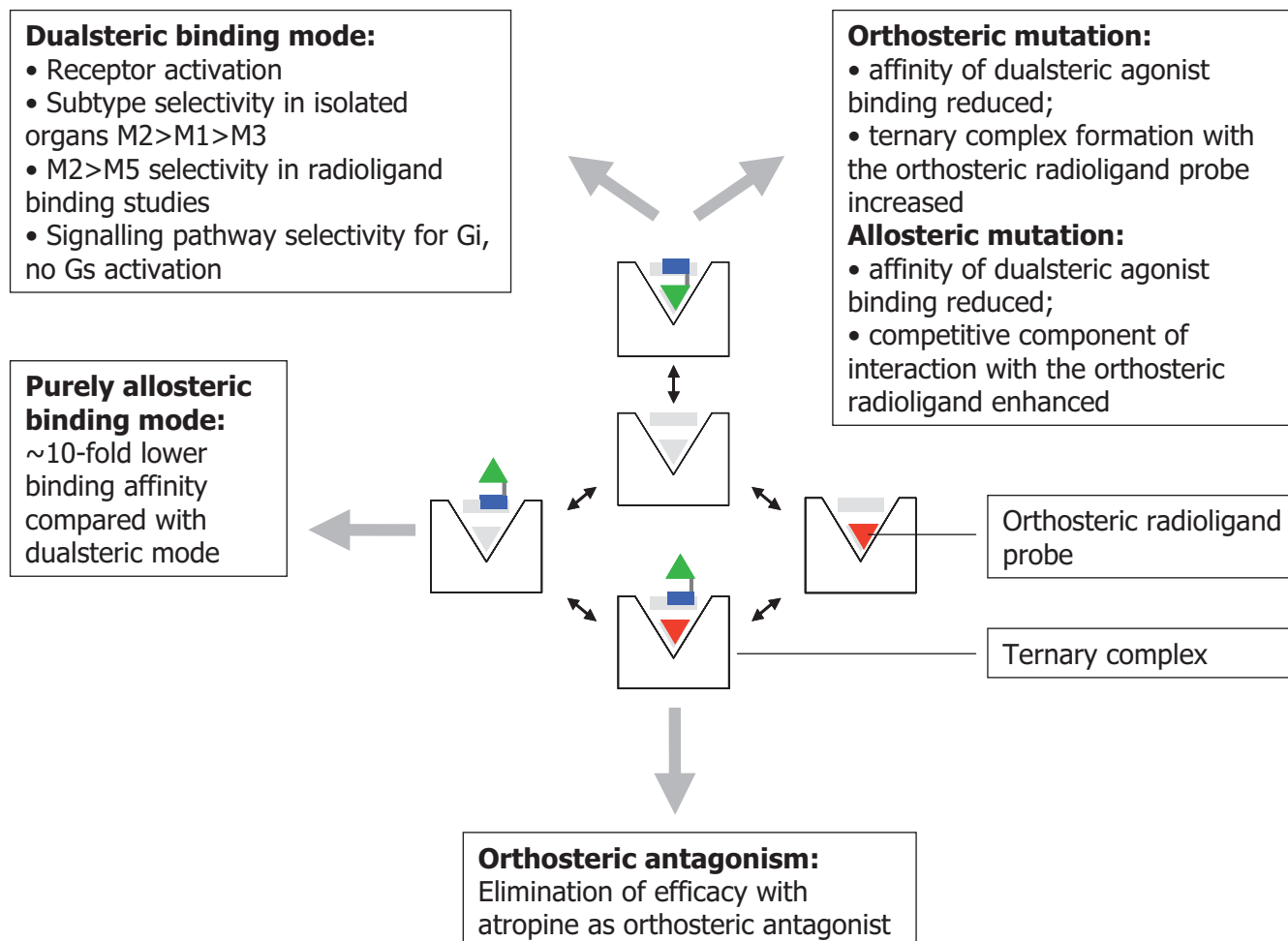


Figure 4 Binding modes of muscarinic dualsteric agonists in the absence and presence of an orthosteric radioligand probe – related experimental evidence and functional consequences. Centre: a symbolized muscarinic receptor contains in its binding cavity the inner orthosteric and the outer allosteric binding region (grey shaded). The dualsteric ligand such as compound 1d (Figure 3) consists of an orthosteric agonist building block (green triangle) which is connected by a linker with an allosteric building block (blue rectangle); orthosteric probe: red triangle. Boxes, right and bottom: experimental evidence for dualsteric binding arising from radioligand binding assays in wild-type and point-mutated human M₂ muscarinic receptors (right box) and from G protein-activation experiments ([³⁵S]GTPγS-assay, bottom box). Boxes, left: functional consequences related to dualsteric binding arising from radioligand binding assays, G protein-activation assays, label-free signalling pathway analysis by dynamic mass redistribution and organ-bath experiments. For details see Antony *et al.* (2009) and Kebig *et al.* (2009).

impairs binding of the other building block. As will be discussed below, negative cooperativity is likely to play a role for the receptor interaction of the dualsteric ligands shown in Figure 3.

- **Non-complementary spatial geometry** between receptor and ligand: enthalpies ΔH_{ortho} and ΔH_{allo} will be less than additive, if the linker between the building blocks of the ligand does not fit the distance between the orthosteric and the allosteric receptor site. Structure-activity-relationships addressing the linker chain length of the dualsteric ligands would help to clarify this issue.
- **Entropic cost:** With respect to *translational and rotational entropy* (movement through space and freedom for rotation), linkage of two independent ligands will reduce the entropic cost of receptor binding. With respect to the total entropic cost of binding, however, *conformational entropy* is important, in particular with respect to the linker (Mammen *et al.*, 1998). Bivalent ligands containing a flex-

ible linker will cause a considerable entropic cost when the molecule is fixed on the receptor surface. Therefore, the highly flexible hexamethylene linker of the dualsteric agonists may diminish binding affinity because of entropic reasons. On the other hand, linker flexibility may be beneficial for spatial geometry reasons (see above). Worth mentioning, the linker itself may have a ligand function and thereby contribute to binding enthalpy.

An unsettled quest: rational tailoring of dualsteric ligands for active receptors

There are two issues in the pharmacological characteristics of the ABA-/iperoxo-type hybrid ligands that await clarification on the receptor level before a fully rational design can be achieved. First, when an additional anchor for receptor

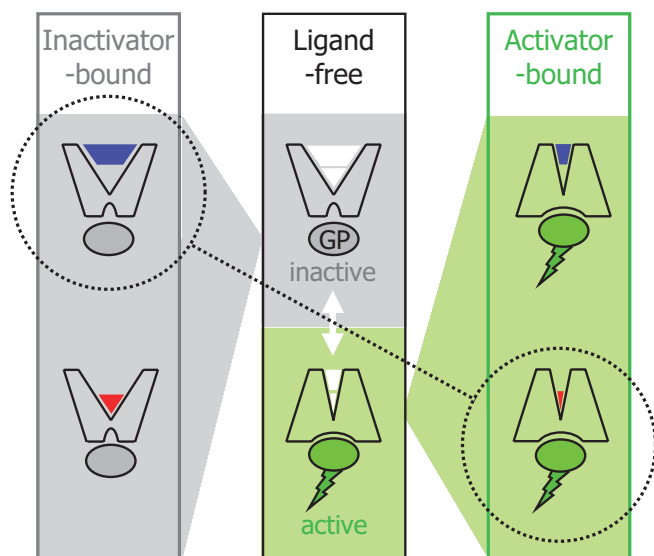


Figure 5 'Functional misfit' of dualsteric ligands which consist of an orthosteric agonist building block and an allosteric inverse agonist building block. Middle panel: the ligand-free receptor may switch spontaneously from the inactive (top) into the active (conformation) which stimulates a G protein (GP); the inactive-to-active conformational transition includes the orthosteric and the allosteric binding site (symbolized as white areas in the ligand binding pocket of the receptor protein). The inactive conformation (left panel) is stabilized by inverse agonist ligands that may bind either to the allosteric (left panel, top) or to the orthosteric site (left panel, bottom). Likewise, the active conformation (right panel) can be stabilized by appropriate allosteric and orthosteric ligands. The building blocks of the hybrid compounds shown in Figure 3 prefer functionally opposing receptor conformations (stippled line).

binding is introduced into a receptor ligand, one would expect receptor binding affinity to be considerably increased (see above). However, relative to their respective parent agonists, all hybrids lose potency and affinity (Figure 3). For instance, in radioligand binding experiments the M_2 -receptor affinity of the orthosteric building block iperoxo was $pK \sim 9$, the affinity of one half of the ABA-type ligand W84 was $pK \sim 4.5$, the measured affinity of the resulting dualsteric '1d' (Figure 3) was only $pK \sim 8$ (Antony *et al.*, 2009; table 1 and Supplemental figure 1 therein), whereas the expected affinity would amount to $pK \sim 13.5$. Second, hybrid formation results in a dramatic loss of intrinsic efficacy relative to the agonist building block (Figure 3 – agents 1a, 1b and 1c), except for 1d, which is derived from the 'superagonist' iperoxo (compound d).

As delineated above, possible reasons for a less than expected level of affinity are negative cooperativity, non-complementary spatial geometry and entropic cost. The concept of a 'functional misfit of building blocks' addresses negative cooperativity (Figure 5). This hypothesis is based on the evidence that GPCR activation involves a global conformational change of the receptor protein including the allosteric site. The rearrangement of the intracellular loop region required for G protein-activation is accompanied by a corresponding conformational transition of the extracellular loop region (for review e.g. Schwartz *et al.*, 2006; Hoffmann *et al.*, 2008; Wess *et al.*, 2008). In fact, there is experimental

evidence that the allosteric site of muscarinic receptors undergoes a pronounced conformational rearrangement, depending on whether the orthosteric site is liganded by either a receptor activator or an inactivator (Grossmüller *et al.*, 2006). For instance, allosteric ligand binding which is promoted by bound orthosteric antagonists (positive cooperative interaction) is strongly reduced when the receptor is bound by orthosteric agonists (negative cooperative interaction; Jäger *et al.*, 2007). In line with this it has been shown that the inactive-to-active transition involves a functional and most probably spatial reorientation of the allosteric core epitope $M_2^{422}Trp$ (7.35). In a 'Janus-like' fashion this amino acid provides affinity for allosteric antagonist binding in the inactive receptor and for orthosteric agonist binding in the active receptor (Jäger *et al.*, 2007). The dualsteric agents described here are composed of building blocks that stabilize functionally opposite receptor conformations: the oxotremorine M-like compounds are orthosteric agonists, the ABA-ligands are allosteric inverse agonists (Figure 5). As individual compounds they would diminish each other's binding affinity in a negative cooperative fashion. Therefore, this 'built-in' functional misfit of building blocks is likely to contribute to the loss of affinity observed with the dualsteric agonists compared with their respective agonist building blocks.

In contrast to the loss of affinity, the clear loss of intrinsic efficacy seen with hybrids 1a, 1b and 1c is unlikely to be solely explained by the concept of functional misfit of building blocks. There is ample evidence that allosteric inverse agonist binding does not diminish maximum receptor activation by orthosteric *full* agonists (Zahn *et al.*, 2002; Jäger *et al.*, 2007). Worth mentioning, however, allosteric inverse agonists may reduce *partial* agonist efficacy at muscarinic M_2 receptors (Zahn *et al.*, 2002; Jäger *et al.*, 2007). In any case, the agonist building blocks of the dualsteric ligands are derived from oxotremorine M which is a full muscarinic agonist. Therefore, the loss of intrinsic efficacy observed upon hybrid formation with oxotremorine M-like building blocks can hardly be explained by a 'built-in' negative activation cooperativity between the dualsteric building blocks. Instead, non-complementary spatial geometry (spatial misfit) may play a role. Intriguingly, only the 'super-high-affinity' iperoxo building block endowed the hybrids with intrinsic efficacy. Its extremely high affinity probably reflects an optimum fit to the orthosteric site of the active receptor. Studies carried out by Kobilka and coworkers in β -adrenergic receptors suggest that agonist-induced receptor activation is a multi-step process that involves more than one receptor conformation (Kobilka and Deupi, 2007). Instead a spectrum of agonist-bound conformations might occur which differ in efficacy reaching from fully active over partially active to inactive. In the light of this concept it can be imagined that an allosteric anchor which is not at an optimal distance from the orthosteric building block might put a strain on the orthosteric agonist building block. In the case of a less tightly fitting agonist building block the strain might move it out of the optimal location within the orthosteric site, thereby favouring less active or even inactive receptor conformations. Due to its tight fit, iperoxo seems to be a 'high fidelity' receptor activator that is resistant to strain imposed by an allosteric anchor. This issue requires more insight into how the

inactive-to-active transition of the receptor affects the spatial orientation of allosteric key epitopes relative to the orthosteric site. With respect to dualsteric ligands this implies that the length of the linker chain may have a function for intrinsic efficacy. In addition, a relaxation of strain by optimizing linker length would increase affinity of dualsteric agent binding.

Taken together, the suspected functional and spatial misfit in the dualsteric binding mode might contribute to the finding that dualsteric binding affinity is only about 10-fold higher than the affinity of purely allosteric binding (Figure 4).

Conclusion

Rationally designed dualsteric GPCR agonists allow to exploit favourable characteristics of orthosteric and allosteric receptor sites simultaneously. In a single molecular entity, orthosteric receptor activation may be linked with allosteric subtype-selectivity and intracellular signalling pathway selectivity. The choice of building blocks allows to fine-tune intrinsic efficacy and, possibly, routes of signalling pathway activation. In addition to a potential therapeutic use, systematically varied dualsteric molecules may serve as valuable research tools for a label-free approach to gain deeper insight into the geometry of activation-related conformational changes of GPCRs.

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Conflict of interest

The authors state no conflict of interest.

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