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REVIEW ARTICLE

Allostery at opioid receptors: modulation with small molecule ligands

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Opioid receptors are 7-transmembrane domain receptors that couple to heterotrimeric G proteins. The endogenous ligands for opioid receptors are peptides which bind to the orthosteric site on the receptors. The μ-opioid receptor is the target for opioid analgesics, while the δ-opioid receptor has been suggested as a target for pain management, migraine and depression. Similarly, κ-opioid receptors are involved in pain and depression and nociceptin receptors in pain and mood behaviours. However, exogenous orthosteric ligands for opioid receptors cause a myriad of on-target side effects. Recently, selective allosteric ligands for μ- and δ-opioid receptors have been described. These compounds bind to a site on the receptor distinct from the orthosteric site. Occupation of this allosteric site leads to modulation of orthosteric ligand binding affinity and/or efficacy. Allosteric modulators may be positive, negative or silent (neutral) (PAMs, NAMs or SAMs respectively). PAMs may have *in vivo* activity by enhancing the activity of exogenous drugs or endogenous opioid peptides. Enhancing endogenous opioid peptide activity maintains the temporal and spatial distribution of these molecules but improves, and potentially qualitatively changes, activity at their cognate receptors which could limit side effects compared with traditional opioid drugs. In this review, we describe the rationale and promise for the development of allosteric modulators for opioid receptors, the discovery of selective allosteric modulators, the identification of potential allosteric sites on opioid receptors and the mode of action of the modulators.

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Abbreviations

GPCR, G proteins coupled receptor; MD, Molecular dynamics; NAM, Negative allosteric modulator; PAM, Positive allosteric modulator; Pdb, Protein data bank; SAM, Silent allosteric modulator; TM, Transmembrane

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Introduction

The role of the **μ-opioid receptor** (Alexander *et al.*, 2015a), in the modulation of pain makes this receptor one of the most pharmacologically targeted GPCRs in modern medicine and μ-receptor agonists such as **morphine** and **oxycodone** are invaluable in the clinic. As a result, the number of prescriptions written for opioid analgesics is rising rapidly. However, while activation of μ-receptors provides pain relief, it also results in a wide range of unwanted effects including constipation and life-threatening respiratory depression, as well as rewarding effects that lead to addiction liability (Matthes *et al.*, 1996). This in turn has led to the current opioid abuse epidemic in the UK, USA and other countries. In the USA specifically, there has been a fourfold increase in the number of deaths from licit and illicit opioids since 1999 (Volkow, 2014; Heron, 2016). In addition, the effectiveness of traditional opioid drugs such as morphine in the management of neuropathic pain is controversial (Smith *et al.*, 2012; McNicol *et al.*, 2013). Consequently, there remains an unmet need for safer efficacious analgesics that circumvent the issues associated with activation of μ-receptors by opioid drugs.

Alternative approaches have included the development of compounds acting at other members of the **opioid receptor** (OR) family (Alexander *et al.*, 2015a), particularly **δ-receptor** and **κ- receptor** agonists, as well as compounds with activity at more than one opioid receptor. While δ-receptor agonists suffer from a lack of efficacious analgesia and proconvulsive effects, they may be effective as antidepressants (Jutkiewicz, 2006) and in migraine treatment (Charles and Pradhan, 2016). Additionally, κ-receptor agonists possess analgesic properties (Chavkin, 2011) but are linked with dysphoria (Pfeiffer *et al.*, 1986) and are pro-depressant to the extent that κ-receptor antagonists may find use in the management of depression (Shippenberg, 2009; Chavkin, 2011; Lalanne *et al.*, 2014). Finally, agonists for the **nociceptin (NOP) receptor** (Alexander *et al.*, 2015a) may be analgesic or pro-nociceptive, depending on the circumstances (Lambert, 2008). Selective δ-, κ- and NOP receptor compounds have not successfully found their way into the clinic, although mixed μ-/δ- receptor (Harland *et al.*, 2015) and mixed μ- /NOP receptor compounds (Toll, 2013) show promise.

Despite best efforts, the μ-receptor system remains the most efficacious target for the treatment of pain. In this review, we discuss allosteric modulators as a novel way to harness the analgesic efficacy of these receptors and the potentially beneficial therapeutic actions of other opioid receptors. This article focuses on small molecule (low molecular weight) exogenous ligands as allosteric modulators of the μ-receptors. In addition, allosteric modulation of the μ-receptor (and other GPCRs) *via* receptor heteromers has also been proposed. For information on this aspect, the reader is referred to reviews by Fujita *et al.* (2015) and Ferré *et al.* (2014).

Allosteric ligands of opioid receptors as potential therapeutic agents

Morphine and other traditional opioid drugs act at the orthosteric site on the opioid receptors, defined as the site for the **endogenous opioid peptides**, including **Leu-** and **Met-enkephalin**, **β-endorphin** and the **dynorphins**. Opioid receptors, like all 7-transmembrane domain (7-TM) GPCRs, are allosteric proteins. The simplest idea of 7-TM domain receptor action can be explained by the Monod–Wyman–Changeux two-state allostery model (Monod *et al.*, 1965) where receptors are distributed into inactive (R) and active (R*) conformations that exist in equilibrium. These do not represent individual conformations but rather ensembles of R and R* states (Kenakin, 2013). R* states are distinguished from R states by an ability to bind agonists with high affinity and activate heterotrimeric G proteins, triggering downstream intracellular signalling pathways. The conformational state of a GPCR, including the opioid receptors, is controlled not only by agonist occupying the orthosteric site but also by endogenous substances acting at other sites on the receptor. These include sodium ions (Pert and Snyder, 1974; Simon and Groth, 1975; Yabaluri and Medzihradsky, 1997; Fenalti *et al.*, 2014; Shang *et al.*, 2014) and interacting proteins, especially heterotrimeric G proteins that stabilize the R* state (DeVree *et al.*, 2016). Furthermore, there is evidence that the lipid environment regulates GPCR function. In particular, cholesterol modulates the function of both μ- and δ-receptors (Xu *et al.*, 2006; Levitt *et al.*, 2009; Zheng *et al.*, 2012) as well as other GPCRs, probably by a combination of direct actions at a conserved motif on the receptors and membrane effects (see Oates and Watts, 2011).

A burgeoning field in drug discovery at GPCRs is the development of small molecule allosteric modulators that bind to druggable pockets on receptors separate from the orthosteric sites. These spatially distinct allosteric sites are defined by the ability of molecules binding at these sites to regulate the activity of molecules binding at the orthosteric site (Figure 1). Allosteric modulators can alter affinity, potency and efficacy of orthosteric ligands. Positive allosteric modulators (PAMs) improve the activity of orthosteric ligands. Negative allosteric modulators (NAMs) do the opposite, and SAMs or silent allosteric modulators occupy the site without activity and as such act as antagonists to PAMs and NAMs. Ideally, PAMs and NAMs would enhance or inhibit respectively the affinity and/or efficacy of an orthosteric ligand while failing to directly activate or inhibit the receptor on its own. However, some compounds may have direct agonist activity; such compounds are known as 'ago-PAMs' (Figure 1). Allosteric activity is dependent on the binding affinity (K_B) of the modulator and the allosteric cooperativity (αβ) which describes the ability of the modulator to change the affinity and/or efficacy of an orthosteric ligand (Figure 1; Christopoulos and Kenakin, 2002; Melancon *et al.*, 2012; Christopoulos, 2014). Allosteric modulators also have differing effects depending on the orthosteric ligand, a phenomenon called 'probe dependence'. It is thought that allosteric modulators provide better selectivity and could provide better therapeutic indexes with fewer side effects. For more on this topic, see (Christopoulos and Kenakin, 2002; Christopoulos, 2014; Christopoulos *et al.*, 2014; Burford *et al.*, 2015a).

A prime example of the potential power of allosteric modulators is PAM activity at the μ-receptor (μ-PAM). Such a compound could serve to increase the potency and/or efficacy of opioid drugs like morphine and lower the dose

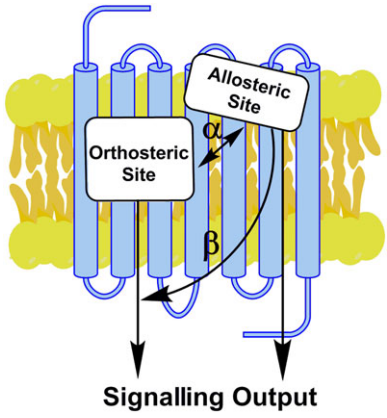

Figure 1

Small molecule allosteric modulation at GPCRs. Allosteric ligands bind to a site distinct from the orthosteric site to modulate orthosteric agonist affinity and/or efficacy. α is the co-operativity factor between the two sites and represents the degree of an enhancement by a PAM (if a value > 1) or reduction by a NAM (if a value < 1) of the affinity of the orthosteric ligand. β is the modulation factor and describes allosteric modulation of orthosteric ligand efficacy. β will have a value > 1 for a PAM or < 1 for a NAM. Allosteric modulators may activate intracellular messengers directly as "ago-PAMs" (modified from Conn *et al.*, 2009).

requirement. Perhaps more importantly, a μ-PAM can be predicted to enhance the activity of endogenous opioid peptides which are elevated during stress and in pain states (Hughes, 1983). This activity would be confined to μ-receptors that have access to released endogenous opioids at specific times and so maintain their spatial as well as temporal selectivity pattern. This is in sharp contrast to traditional opioid agonists which activate μ-receptors across many tissues with limitations set only by pharmacokinetic parameters. There is evidence that such an approach would be feasible since non-selectively increasing opioid peptide levels by blocking enzymes responsible for their degradation with inhibitors of **enkephalinase** (neutral endopeptidase; NEP) provides preclinical analgesia (Roques *et al.*, 2012), but not constipation (Noble *et al.*, 2008), respiratory depression (Boudinot *et al.*, 2001), antinociceptive tolerance (Noble *et al.*, 1992b) or abuse liability (Noble *et al.*, 1992a; Valverde *et al.*, 1996).

An additional potential advantage of using small molecule allosteric modulators for the opioid receptors is to introduce a signalling bias downstream of the receptors. Biased agonism is the preferential activation of one signalling pathway over another and has been demonstrated at μ-receptors between β-arrestin recruitment and G protein activation (McPherson *et al.*, 2010; Thompson *et al.*, 2015). The goal of biased signalling is to activate pathways downstream of opioid receptors responsible for the beneficial effects (e.g. pain relieving in the case of the μ-receptors) without activating pathways producing undesirable effects. For example, the β-arrestin pathway has been implicated in the constipatory and respiratory depressive actions of opioids (Raehal and Bohn, 2011), and newly developed biased ligands including **oliceridine (TRV130**; Dewire *et al.*, 2013) and **PZM21** (Manglik *et al.*, 2016) avoid activation of this pathway. It is tempting to speculate that the μ-receptor occupied by a PAM might behave as a novel receptor, compared with an unoccupied μ-receptor and so be envisaged to signal differently. Similarly, introducing bias at δ-receptors could promote antidepressant actions over proconvulsant actions and at κ-receptors could enhance analgesia at the expense of dysphoria. For a more comprehensive discussion of the potential benefits of opioid PAMs as therapeutic agents, see Burford *et al.*, (2015a).

Discovery of small molecule allosteric modulators of opioid receptors

The BMS series of compounds

The first selective positive allosteric modulators of μ-receptors, **BMS-986121** and **BMS-986122**, were identified in 2013 (Table 1; Figure 2; Burford *et al.*, 2013) using a high-throughput screen (HTS) monitoring for ability to enhance

Table 1

Confirmed or putative small molecule allosteric modulators of opioid receptors[a]

	μ-OR	δ-OR	κ-OR	References
Salvinorin A	x	–	–	Rothman *et al.*, 2007
Cannabidiol	x	x	–	Vaysse *et al.*, 1987; Kathmann *et al.*, 2006
THC	x	x	–	Vaysse *et al.*, 1987; Kathmann *et al.*, 2006
BMS-986122	x	–	–	Burford *et al.*, 2013; Livingston and Traynor, 2014
BMS-986121	x	–	–	Burford *et al.*, 2013
BMS-986124	–	–	–	Burford *et al.*, 2013
BMS-986187	x	x	–	Burford *et al.*, 2015b
MS1	x	–	–	Bisignano *et al.*, 2015
Ignavine	x	–	–	Ohbuchi *et al.*, 2016
SCH-202676	x	x	x	Fawzi *et al.*, 2001 (but see Göblyös *et al.*, 2005; Lewandowicz *et al.*, 2006)

[a]To date, no modulators have been identified for NOP receptors.

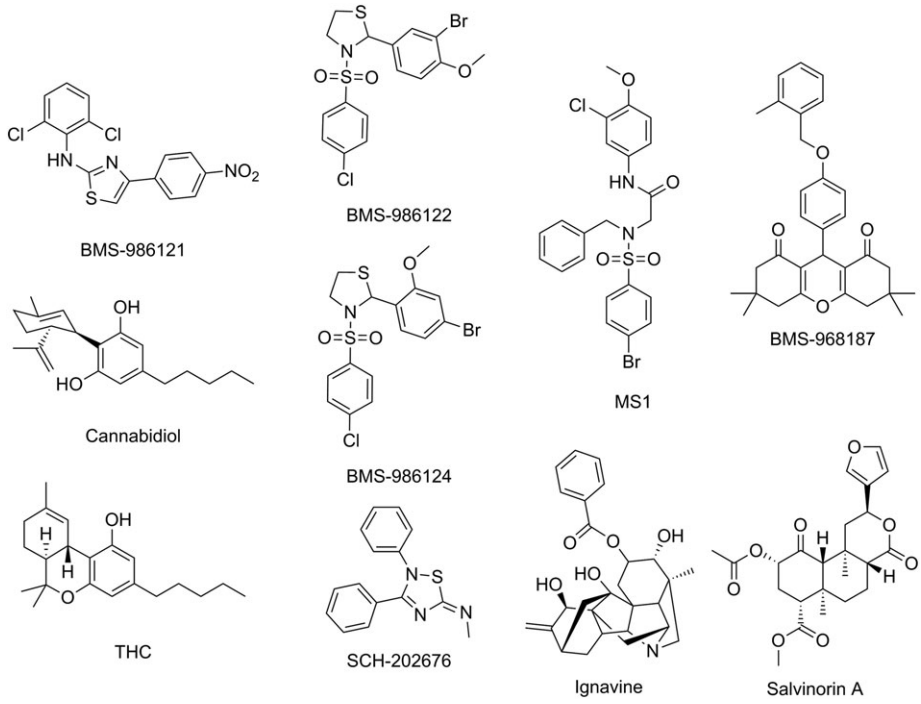

Figure 2

Structures of known or putative allosteric modulators of opioid receptors discussed in the text. The Figure shows compounds discovered by high-throughput screening that exhibit PAM (BMS-986122 and BMS-986121) or SAM (BMS-986124) activity at μ-receptors plus the similarly structured MS1 which was identified by chemoinformatic analysis. BMS-986187 is a δ-PAM discovered by high-throughput screening. Other compounds that have been suggested as modulators include the natural products cannabidiol, THC, ignavine and salvinorin A as well as the low MW compound SCH-202676.

a low concentration of the putative endogenous μ-receptor agonist, **endomorphin-1**, to recruit β-arrestin to μ-receptors. The HTS methodology has been described in detail (Burford *et al.*, 2014; Bertekap *et al.*, 2015). Further studies with BMS-986122 showed that it can enhance the affinity and/or efficacy of various opioid agonists, including opioid peptides Leu- and Met-enkephalin, β-endorphin as well as endomorphin-1. Along with BMS-986122, a number of structurally similar μ-PAMs were identified, plus SAMs such as **BMS-986124** (Figure 2). BMS-986122 exhibits dramatic probe dependence in that its effects are reliant on the ligand occupying the orthosteric site. For agonists such as **methadone**, **DAMGO** and the endogenous opioid peptides, BMS-986122 enhances the potency and affinity, while for morphine and **nalbuphine**, it enhances agonist efficacy with no alteration in the affinity. There is no effect on the binding of antagonists (Livingston and Traynor, 2014). This is discussed in more detail later under 'Mechanism of allostery at opioid receptors' in the subsection 'Role of orthosteric ligand and Na^+ ions' as it points to a potential explanation for the action of the modulators. BMS-986122 does not have PAM activity at δ- receptors, a fact which has been taken into account in structure–activity studies.

The structure–activity relationships of the BMS series of μ-receptor allosteric modulators published so far is unclear. Subtle changes have profound effects on defining a compound as a PAM or a SAM (compare BMS-986122 and BMS-986124 in Figure 2). No NAMs have been described in this series. With this in mind, Bisignano *et al.* (2015) searched the eMolecules (www.emolecules.com) and ZINC (Irwin *et al.*, 2012) databases for structural analogues. Of the compounds identified, 28 were evaluated in the β-arrestin recruitment assay, 14 were found to be PAMs and 12 were identified as SAMs. None of the compounds had higher affinity than the original molecules, although one compound, MS1 (Table 1; Figure 2), was chosen for a more extensive study. MS1 did not bind to the μ-receptor orthosteric site but improved the affinity of methadone and the potency of methadone to activate heterotrimeric G proteins. Surprisingly, neither the affinity nor potency to activate G proteins was enhanced for **endomorphin-1** or DAMGO, in spite of the fact that MS1 was discovered using endomorphin-1 as the orthosteric probe. This anomaly could be due to the fact that endomorphins may be β-arrestin-biased molecules (McPherson *et al.*, 2010). On the other hand, the conflicting probe dependence may be explained by the relatively weak allosteric cooperativity of MS1, even against methadone which thus far is the most sensitive orthosteric ligand to allosteric modulation (Livingston and Traynor, 2014).

Using the β-arrestin recruitment HTS assay (Burford *et al.*, 2014; Bertekap *et al.*, 2015) allosteric modulators of the closely related δ-receptor have been discovered (Burford *et al.*, 2015b). These compounds are structurally dissimilar to BMS-986122 being tetramethyl substituted hexahydro-xanthine-1,8-diones. The lead compound BMS-986187 (Table 1; Figure 2) is effective as a δ-PAM in the <100 nM

range, while showing 100-fold weaker PAM activity at the μ-receptor. The high potency of BMS-986187 is somewhat surprising given its affinity for the unoccupied receptor (K_B) of approximately 1 μM. On the other hand, this demonstrates that allostery is bidirectional and so the orthosteric agonist enhances PAM affinity and also highlights the importance of efficacy interactions (β) as well as affinity interactions (α). Indeed, BMS-986187 is able to stimulate signalling downstream of δ- receptors even in the absence of orthosteric agonist. However, the compound does not bind to the orthosteric site, as determined by its inability to displace **^{3}H-diprenorphine**, and so, it is activating the receptor through its allosteric site. Consequently, it is designated as an 'ago-PAM' (Figure 1). Like the μ-PAMs, BMS-986187 also exhibited probe dependence when tested on a limited number of compounds with a greatest effect on the affinity of the peptide Leu-enkephalin (32-fold shift) and smaller shifts for **SNC80** (14-fold shift) and Tan-67 (threefold shift). BMS-986187 acted as a δ-PAM for several downstream measures including the [^{35}S]GTPγS binding assay, inhibition of adenylate cyclase, recruitment of β-arrestin and phosphorylation of ERK1/2 for all three of these ligands (Burford *et al.*, 2015b). Moreover, BMS-986187 has been demonstrated to potentiate endogenous opioid signalling at δ- receptors in intercalated cells that modulate output from the amygdala (Winters *et al.*, 2017).

Other putative allosteric modulators

In addition to the small molecules described above, other putative, structurally unrelated modulators of ORs have been described (Table 1; Figure 2).

The cannabinoids (CBs) **Δ^9-tetrahydrocannabinol** (THC) and **cannabidiol** (CBD) (Figure 2) were suggested many years ago (Vaysse *et al.*, 1987) to be NAMs of both μ- and δ- receptors. This assertion was based on the ability of the CBs to fully inhibit ^{3}H-orthosteric agonist binding to the μ-receptors and δ- receptors in rat brain membranes, in a non-competitive manner, by reducing the Bmax but not altering ligand affinity. To avoid the caveat that the compounds might be working *via* **CB receptors** in the rat brain membranes and so acting indirectly through receptor–receptor interactions, the authors showed THC to be just as effective at solubilized, partially purified μ-receptors This also suggests the effect was on the receptor itself or a closely associated lipid, but not due to a non-specific effect on the lipid bilayer. In support of this CB receptor-independent effect on μ-receptors, several other CBs displayed a wide variety of activities that were not correlated with their activity at CB receptors. For instance, levonantradol and dextronatradol were equiactive at inhibiting binding of the μ-receptor orthosteric agonist ^{3}H-**dihydromorphine** (DHM) to rat brain membranes but showed 100-fold difference in behavioural potencies as CBs (Johnson *et al.*, 1981). Also, 11-hydroxy-THC displayed comparable CB potency to THC but had less than 20% of the activity of THC at displacing ^{3}H-DHM. Later kinetic experiments comparing the effect of THC and CBD at μ- and δ- receptors in rat cortical membranes (Kathmann *et al.*, 2006) were claimed to support the idea of the CBs as allosteric modulators by demonstrating that CBD and THC at high concentrations (30-100 μM) increased the dissociation rate for the μ-receptor agonist ^{3}H-DAMGO in the presence of a high concentration of naloxone. Similar but much smaller shifts in the dissociation rate of the antagonist **^{3}H-naltrindole** (NTI) from δ- receptors were seen. On the other hand, both THC and CBD alone enhanced ^{3}H-DAMGO and ^{3}H-NTI dissociation and also displaced DAMGO binding in competition assays, giving affinities in the 10 μM range, albeit with a reported Hill slope ~ 1.5, though only CBD inhibited ^{3}H-NTI binding. Moreover, no functional studies of allosterism have been reported. Thus, it cannot be ruled out that the CBs at these very high concentrations are acting non-specifically or even binding to the orthosteric site rather than acting as true allosteric modulators. The **CB_1 receptor** antagonist **rimonabant** shows a similar profile (Kathmann *et al.*, 2006), and this compound has been reported to have an affinity at μ-receptors of 650 nM and to be an antagonist at these receptors *in vivo* and *in vitro* (Seely *et al.*, 2012). The concentrations of the CBs acting at opioid receptors are much higher than their affinity for the CB_1 receptor, suggesting that activity at opioid receptors is not likely to play a role in the *in vivo* activities of the CBs.

The neoclerodanediterpene **salvinorin A** (Sal A; Figure 2) is a selective κ-receptor agonist which lacks a positively charged nitrogen atom for interaction with the conserved Asp in TM-III of the κ-receptor (Roth *et al.*, 2002). Based on the observation that Sal A has a weak ability to compete with orthosteric ligands at μ-receptors, Rothman and colleagues examined the compound as a possible allosteric modulator of this receptor (Rothman *et al.*, 2007). Their data suggested that Sal A might be a negative allosteric modulator of μ-receptors, based on its ability to only partly inhibit binding of the agonists ^{3}H-DAMGO or ^{125}I-[IOXY] or the antagonist ^{3}H-diprenorphine to the orthosteric site of the receptor in both μ-receptor expressing CHO cells and rat brain membranes. Binding experiments showed that Sal A decreased the affinity of the orthosteric ligands by twofold to threefold, reduced Bmax values and had complex effects on ligand dissociation. In the [^{35}S]GTPγS assay, which measures μ-receptor activation of heterotrimeric G proteins, Sal A decreased both the potency (EC_{50}) and Bmax for DAMGO. The concentrations of Sal A used in these experiments were in the high μM range, much higher than the affinity of Sal A for the κ-receptor (~4 nM; Roth *et al.*, 2002). This will make *in vivo* studies challenging, although a study in κ- receptor knockout animals might be informative.

Ignavine (Figure 2) is a diterpene alkaloid isolated from the plant *Aconitum japonica* (Saito *et al.*, 1982; Ohbuchi *et al.*, 2016). There is evidence that the 'processed aconite tuber' has analgesic activity mediated by κ-receptors, although the specific κ-receptor agonist has not been isolated (Ohbuchi *et al.*, 2016). Ignavine itself gives a biphasic antinociceptive dose–response curve in the mouse tail-flick and tail pressure tests. The title of a recent publication (Ohbuchi *et al.*, 2016) states ignavine is a 'novel allosteric modulator of the μ-receptor'. This claim is based on the finding that the compound both enhances and inhibits the activity of the μ-receptor orthosteric agonist DAMGO to inhibit cAMP accumulation and to cause internalization of μ-receptors in HEK 293 cells depending on the ignavine concentration (1 or 10 μM respectively). However, binding

studies reported in the same publication indicate that the compound fully displaces ^{3}H-diprenorphine from the orthosteric site of the μ-receptor in an apparently competitive manner and docking studies suggest that the compound binds at the orthosteric site. Thus, this compound would seem to be incorrectly identified as an allosteric modulator but may have other actions at μ-receptors, for example, as a μ-receptor partial agonist.

Finally, a thiazolidine compound, SCH-202676 (Figure 2), has been claimed to be a non-specific allosteric modulator of many GPCRs including the μ-, δ- and κ-receptors (Fawzi *et al.*, 2001). However, this compound covalently binds to GPCRs by sulfhydryl bond formation and so is not a true allosteric modulator (Göblyös *et al.*, 2005; Lewandowicz *et al.*, 2006).

The above evidence suggests certain CBs and Sal A are NAMs of μ-receptors. A negative modulator, unless it can be specially targeted at reducing the side effects of orthosteric μ-receptor agonists, for example, by introducing a bias into downstream signalling as discussed above, may not make a useful clinical compound. Nonetheless, it will be important to re-evaluate these putative modulators (as well as ignavine) of opioid receptors, using more rigorous analysis methods for the determination of allostery (Melancon *et al.*, 2012; Christopoulos, 2014), as these natural products could provide scaffolds for the future design of modulators.

Mechanism of allostery at opioid receptors

Allosteric binding site(s) on opioid receptors

There is no definitive structural work that accurately defines the nature and location of allosteric sites on the opioid receptors. However, there have been several attempts to identify site(s) on μ- and δ-receptors by computational methods using docking and molecular dynamics (MD) simulations.

Using molecular docking, Bartuzi *et al.* (2016) obtained several poses for BMS-986122 within the μ-receptor although two had very similar orientations and interaction energies. These data indicated an allosteric site involving amino acids above the orthosteric binding pocket and towards the extracellular surface in TM domains II and VII (Figure 3). At the δ-receptor, Shang *et al.* (2016) using metadynamic calculations (Schneider *et al.*, 2015) of the δ- receptor bound to the orthosteric ligand SNC80 and in a water-lipid environment found two metastable binding poses for BMS-986187 occupying the same site that was in close proximity to the orthosteric site but, as in the μ-receptor, towards the extracellular surface (Figure 3). Both metastable states formed direct polar, water-mediated polar, hydrophobic and/or aromatic interactions with amino acids residues in TM domains I, II and VII, with several residues specific to a particular pose. Mutational studies of several amino acid residues in the putative site affected either the binding of the modulator and/or the degree of cooperativity between the modulator and the othosteric ligand, therefore giving some credence to this as an allosteric site (Shang *et al.*, 2016), although with the caveat that mutagenesis can alter orthosteric ligand affinity and basal activity of the receptor, thus providing confounds.

MD simulations of an active μ-receptor homology model in complex with $G\alpha_s$ protein in a raft-like membrane suggested a common binding pocket for lipophilic modulators CBD and THC at the top of TM domains I, II and VI (Bartuzi *et al.*, 2015). Cannabinoids occupying this site appear to oppose the action of agonists by moving the TM domains closer together towards an inactive receptor state. In addition, a second site for Sal A at μ-receptors that overlapped with the binding site for DAMGO was suggested, possibly explaining its NAM activity.

Overall, computational evidence suggests the μ- and δ-receptors are predicted to have similarly positioned allosteric sites (Figure 3). It is worth noting that this putative site is correspondingly situated to the allosteric site on the

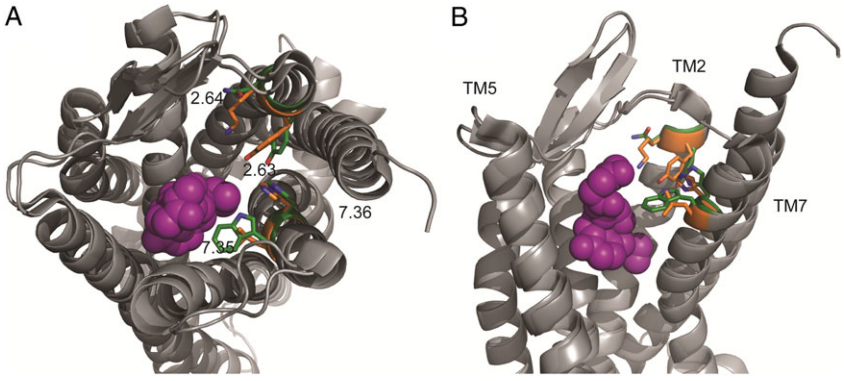

Figure 3

Theoretical binding site for BMS-986122 on both μ- and δ-receptors. Inactive state μ-receptors (pdb 4DKL; Huang *et al.*, 2015) and inactive state δ- receptors (pdb 4N6H; Fenalti *et al.*, 2014) were aligned. The residues proposed (Bartuzi *et al.*, 2016; Shang *et al.*, 2016) to be involved in allosteric ligand binding are highlighted in green for the μ-receptor and orange for the δ- receptor. (A) view of aligned receptors from the extracellular side, (B) side view. The orthosteric site is shown occupied by the irreversible μ-receptor antagonist β-funaltrexamine (purple). Extracellular loop 2 and TM6 have been removed from image B for clarity.

muscarinic receptors including M_1(Abdul-Ridha *et al.*, 2014), M_2 (Jäger *et al.*, 2007; Haga *et al.*, 2012; Dror *et al.*, 2013) and M_4 receptors (Thal *et al.*, 2016) and the site for the modulator **maraviroc** on the **CCR5** chemokine receptor (Tan *et al.*, 2013), suggesting that this region of class A GPCRs could be a common site for allosterism.

Role of orthosteric ligand and Na^+ ions

Na^+ ions play a major role in stabilizing inactive R states of 7-TM domain receptors. This was first shown by the ability of NaCl to inhibit the binding of orthosteric agonists to μ-receptors while having no effect on orthosteric antagonist binding (Pert *et al.*, 1973), due to a shift in equilibrium to inactive R conformational states. Thus, Na^+ can be considered an endogenous NAM of 7-TM receptors. Current understanding of the mechanisms by which Na^+ stabilizes R is better appreciated due to recent high-resolution crystallographic work performed first with the **adenosine A_{2A} receptor** (Liu *et al.*, 2012) and later with other receptors including the δ-receptor (Fenalti *et al.*, 2014). The binding site of Na^+ is conserved across many GPCRs, including all the opioid receptors. This site is located in the middle of the 7-TM bundle and involves coordination with an aspartate residue in TM II (Asp 2.50) and a Ser residue in TM II (Ser 3.39) plus a number of highly organized water molecules across TM domains II, III, VI and VII (Katritch *et al.*, 2014). Importantly, the Na^+ ion is absent in the structures of active GPCRs including μ-receptors (Huang *et al.*, 2015) because movement of the TM domains upon receptor activation provides insufficient space for the Na^+ ion and its associated water molecules.

The Monod–Wyman–Changeux two-state model has been applied to describe the action of small molecule allosteric modulators of muscarinic receptors (Canals *et al.*, 2012) and of the μ-receptor (Livingston and Traynor, 2014) where they act to promote formation of R*. There is evidence that the allosteric activity of BMS-986122 at the μ-receptor is related to the negative modulatory activity of Na^+ ions (Livingston and Traynor, 2014). The degree of allosteric activity of BMS-986122 is dependent on the orthosteric probe, such that antagonists are insensitive and agonists are generally highly sensitive in line with their efficacy. There is a strong inverse correlation between the sensitivity of a μ-receptor agonist to Na^+ ions and the sensitivity of the same ligand to positive allosteric modulation by BMS-986122, with methadone being the orthosteric ligand most sensitive to μ-PAM activity (Figure 4). Moreover, the action of BMS-986122 antagonizes the ability of Na^+ ions to inhibit agonist binding such that BMS-986122 and Na^+ ions oppose each other's action. As BMS-986122 is selective for μ- over δ-receptors while the Na^+ binding site is conserved, we have proposed a model in which the μ-PAM binds at a distinct site from Na^+ to allosterically disrupt the binding of Na^+ (Figure 5). In support of this, ligands that target the Na^+ binding site on GPCRs, such as amiloride, are not selective amongst GPCRs that are sensitive to Na^+ ions (Gao and Ijzerman, 2000; Hoare *et al.*, 2000; Schetz and Sibley, 2001; Heitman *et al.*, 2008). It is notable that 'superagonists' at μ-receptors such as **BU72** and **etorphine** do not fit this pattern. These compounds are insensitive to the actions of the modulators (Livingston and Traynor, 2014) and much less affected by Na^+ ions (Lee *et al.*, 1999).

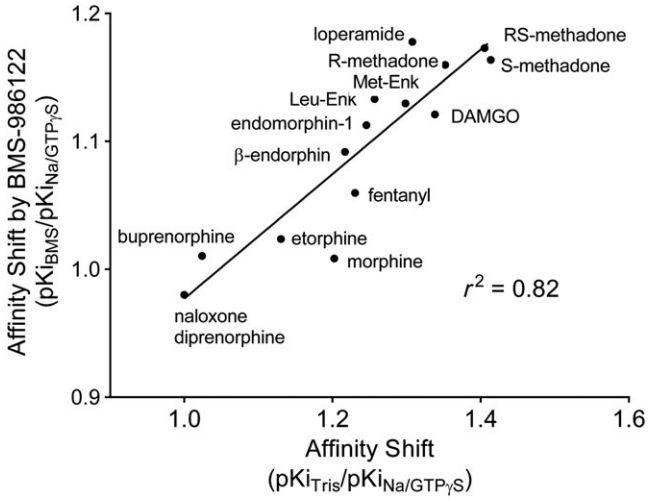

Figure 4

Relationship between the effect of the μ-PAM, BMS-986122 and the action of Na^+ ions plus guanine nucleotide on the binding affinity of opioid ligands to the orthosteric site on μ-receptors. The abscissa represents a reduction in affinity values (as a shift ratio) for each opioid ligand in the presence of Na^+ ions and guanine nucleotide. The ordinate represents the increase in affinity (as a shift ratio) in the presence of Na^+ ions and guanine nucleotide in the presence of BMS-986122 (adapted from Livingston and Traynor, 2014).

Following publication of the experimental data discussed above, Bartuzi and colleagues (Bartuzi *et al.*, 2016) performed principal component analysis of μ-receptors in a native membrane environment. Their calculations showed that BMS-986122 bound to a putative allosteric site (see above) and interacted with a Trp at the top of TM VII (Trp 7.35) to alter the conformation of this TM domain resulting in stabilization of the binding of the orthosteric ligand methadone as determined by its interaction with Asp 3.32 in the orthosteric pocket and destabilization of Na^+ ion binding as measured by the distance of this ion from Asp 2.50. Similarly, recent MD simulations of the **galanin receptor** identified a potential allosteric site involving TM domains 2 and 3 and extracellular loops 1 and 2 that the authors propose could disrupt Na^+ binding (Hui *et al.*, 2016).

It should be understood that the putative binding sites used for these calculations are defined by docking procedures and may not represent the true allosteric sites. Nonetheless, there are multiple binding sites on μ-receptors that allosterically communicate, including the orthosteric site, allosteric site for BMS-986122, the Na^+ binding site and the G protein-binding site (Figure 5). The interplay between the sites differs depending on the orthosteric ligand. As Na^+ regulates a number of GPCRs, and the Na^+ site is highly conserved and MD simulations suggest that allosteric sites are similarly situated close to the orthosteric binding pocket, this may be a common mechanism of action for small molecule allosteric modulation across GPCRs.

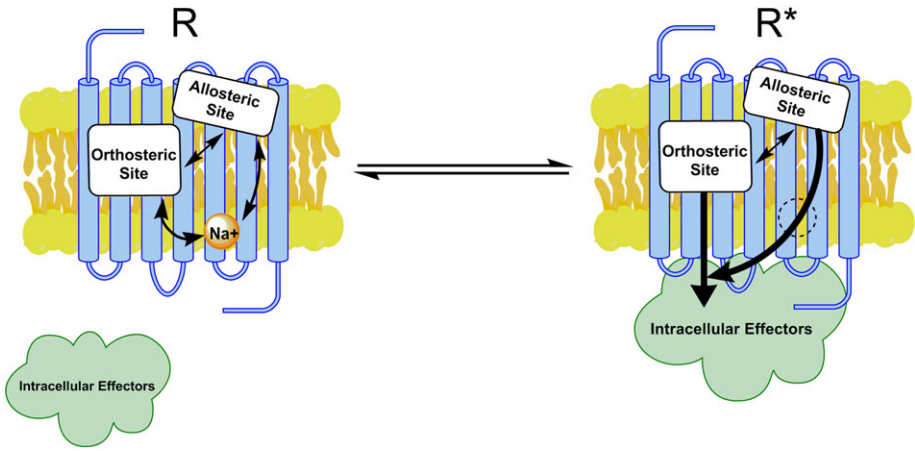

Figure 5

Allosteric interactions within μ-receptors. Inactive receptor (R) contains a Na^+ ion. Orthosteric agonist captures an active state (R*) that does not contain Na^+ ion (dotted circle) and allows for receptor interaction with intracellular signalling proteins (e.g. heterotrimeric G protein or β-arrestin). It is proposed that the μ-PAM improves the affinity and potency of the orthosteric agonist by its incompatibility with Na^+ binding, thereby promoting a state that more readily binds and responds to agonist.

Conclusions

Selective allosteric modulators of the μ- and δ- receptors have been described, but there are no specific modulators published to date for the κ- or the NOP receptors. Knowledge of allosteric modulation of opioid receptors is still in its infancy. However, we know that the μ- and δ- receptor PAMs show a marked probe dependence that appears to relate to the efficacy of the probe (the ligand occupying the orthosteric site) and to the sensitivity of the probe to Na^+ ions that stabilize inactive R states of the receptors. At least for the μ- and δ- receptors, proof-of-principle for *in vivo* efficacy of allosteric modulators is needed. This will require the development of more potent and drug-like molecules. Although some ideas about structural requirements and identity of the allosteric site on opioid receptors have been developed using computational methods, the field will benefit immensely from confirmation of the location and nature of allosteric binding site(s) and the conduit by which occupation of this site leads to dissociation of the bound Na^+ ion and formation of R*. This will come from biophysical methods such as hydrogen–deuterium exchange mass spectrometry, NMR and X-ray crystallography. Given the recent success in crystallizing GPCRs, including crystal structures of muscarinic receptors bound to allosteric modulators, this information should soon be available, allowing for the rational design of a new generation of allosteric modulators acting at opioid receptors.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.*, 2015a,b).

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Conflict of interest

The authors declare no conflicts of interest.

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