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Themed Section: Opioids: New Pathways to Functional Selectivity

REVIEW

Positive allosteric modulators of the μ-opioid receptor: a novel approach for future pain medications

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Morphine and other agonists of the μ-opioid receptor are used clinically for acute and chronic pain relief and are considered to be the gold standard for pain medication. However, these opioids also have significant side effects, which are also mediated via activation of the μ-opioid receptor. Since the latter half of the twentieth century, researchers have sought to tease apart the mechanisms underlying analgesia, tolerance and dependence, with the hope of designing drugs with fewer side effects. These efforts have revolved around the design of orthosteric agonists with differing pharmacokinetic properties and/or selectivity profiles for the different opioid receptor types. Recently, μ-opioid receptor-positive allosteric modulators (μ-PAMs) were identified, which bind to a (allosteric) site on the μ-opioid receptor separate from the orthosteric site that binds an endogenous agonist. These allosteric modulators have little or no detectable functional activity when bound to the receptor in the absence of orthosteric agonist, but can potentiate the activity of bound orthosteric agonist, seen as an increase in apparent potency and/or efficacy of the orthosteric agonist. In this review, we describe the potential advantages that a μ-PAM approach might bring to the design of novel therapeutics for pain that may lack the side effects currently associated with opioid therapy.

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Abbreviations
C6-μ cells, C6 glioma cells expressing rat μ-opioid receptors; DAMGO, [D-Ala^2, N-MePhe^4, Gly-ol^5]-enkephalin; μ-PAM, μ-opioid receptor positive allosteric modulator; NAM, negative allosteric modulator; PAM, positive allosteric modulator; SAM, silent allosteric modulator ; U2OS cells, human osteosarcoma cells

Introduction

Pain and opioid analgesics

Pain is the most common ailment for which people seek medical attention. Chronic pain is a problem for millions of patients and can be disabling, interfering with day-to-day functions both at home and in the workplace. Costs in the United States from healthcare expenditure and lost work time due to pain are estimated at $100 billion/year (Melnikova, 2010).

Opioid receptors are key targets in the management of pain (Przewlocki and Przewlocka, 2001; Vallejo *et al.*, 2011). Drug therapies derived from morphine, its derivatives and other small molecules induce pain relief by acting as agonists at opioid receptors, particularly the μ-opioid receptor (Alexander *et al.*, 2013). Morphine-induced analgesia is lost in mice lacking the μ-opioid receptor gene (Matthes *et al.*, 1996). Opioid drugs can produce serious side effects, including respiratory suppression, constipation, allodynia, tolerance, dependence and withdrawal symptoms, as well as rewarding effects and abuse potential (Przewlocki and Przewlocka, 2001; McNicol *et al.*, 2003). All of these effects are reversed by μ-opioid receptor antagonists and absent in μ-opioid receptor-knockout animals (Matthes *et al.*, 1996), showing that they are mediated through activation of the μ-opioid receptor.

Since the early 1990s, there has been a significant increase in the use of opiate analgesics for non-cancer chronic pain,

partly due to the belief that opiate dependence and addiction liability had previously been overstated (Juurlink and Dhalla, 2012). However, this has led to a substantial increase in patients with opiate dependence and addiction. The increased presence of opiates in the household has also led to higher abuse, both accidental and intentional, leading to increased admissions to hospitals for treatment (Woodcock, 2009). Thus, physicians walk a tightrope balancing act in an attempt to achieve both effective pain management and drug safety.

The 'holy grail' of opioid research has been, and continues to be, the identification of drugs that can produce the beneficial analgesic effects of opiates without the development of tolerance or other side effects, including their clear abuse liability. Over the past several decades, many opioid ligands have been synthesized, with varying affinities for the opioid receptor types, and varying pharmacokinetic properties. Combinations of these ligands have also been used (Snyder and Pasternak, 2003; Corbett *et al.*, 2006; Lambert, 2008). However, these efforts have not yet yielded dramatic improvements in the availability of pain medications with fewer side effects.

Opioid receptors

Opioid receptors are categorized within the Class A family of GPCRs. Four opioid receptor types exist; μ-opioid receptors, κ-opioid receptors, δ-opioid receptors and NOP receptors (also known as ORL1) (Alexander *et al.*, 2013; Cox *et al.*, 2015). These receptors were cloned in the 1990s (Evans *et al.*, 1992; Kieffer *et al.*, 1992; Chen *et al.*, 1993; Yasuda *et al.*, 1993; Mollereau *et al.*, 1994; Raynor *et al.*, 1994), and their crystal structures have recently been elucidated (Granier *et al.*, 2012; Manglik *et al.*, 2012; Thompson *et al.*, 2012; Wu *et al.*, 2012). The opioid receptors share about 60% amino acid identity (mainly within the transmembrane domains) and signal through the Gi/o family of heterotrimeric G proteins, resulting in inhibition of adenylate cyclase (AC), modulation of ion channel activity (via G protein βγ subunits), and transcriptional changes in the cell (Waldhoer *et al.*, 2004). There is also evidence for activation of non-G protein-mediated pathways via β-arrestin (Bohn *et al.*, 1999).

The endogenous ligands for the opioid receptors are peptides derived from large precursors and include the enkephalins, endorphins and dynorphins, which have selective affinities for each of the three main opioid receptor types (Janecka *et al.*, 2004) but very low affinity for the NOP receptor. The endomorphins (Zadina *et al.*, 1997) are considered putative endogenous μ-opioid receptor ligands. The endogenous peptide for the NOP receptor is nociceptin/orphanin FQ peptide (Meunier *et al.*, 1995; Reinscheid *et al.*, 1995), which has no affinity for μ-, κ- or δ-opioid receptors.

Opiate physical dependence correlates closely with the development of opiate tolerance (Way *et al.*, 1969), suggesting that they may share common mechanisms. Tolerance can be defined as a reduced response to repeated administration of the same dose of drug, or put another way, increased doses of drug are required to produce the same magnitude of response. There have been considerable studies investigating the underlying mechanisms that result in opioid tolerance and dependence, which have been reviewed elsewhere (Bailey and Connor, 2005; Sadee *et al.*, 2005; Bian *et al.*, 2012; Whistler, 2012; Williams *et al.*, 2013). Hypotheses include μ-opioid receptor phosphorylation and desensitization, receptor internalization/down-regulation, and up-regulation of AC. It has been suggested that the intracellular β-arrestin-2 protein is significantly involved in agonist-mediated development of tolerance, based on the fact that β-arrestin-2 knockout mice have enhanced analgesic effects in response to morphine and lower levels of receptor desensitization, and other unwanted side effects (Bohn *et al.*, 1999). However, despite several decades of research, the mechanistic understanding of how tolerance develops is still relatively poorly understood.

Orthosteric and allosteric ligands

Before we introduce the concept of allosteric modulators, it is beneficial to start with orthosteric ligand interactions with GPCRs. Orthosteric ligands bind to the same site on the receptor that recognizes an endogenous agonist – in the case of the opioid receptors these are the opioid peptides. GPCRs exist in multiple conformational states, but for simplicity we will only refer to two, an inactive (R) conformation and an active (R*) conformation. Orthosteric agonists bind with higher affinity to R*, thus driving the receptor equilibrium from R towards R* to give a high R*/R ratio. Based on the intrinsic activity of a given agonist, the agonist can be full (eliciting a maximal achievable response in that system) or partial (where the elicited response is less than that of a full agonist despite full occupancy of all the available receptor binding sites). This can be explained by a reduced ability of partial agonists to differentiate R and R*, thereby producing a lesser equilibrium shift towards R* than full agonists and/or an ability to induce a different active conformation of the receptor (R^+), which produces less activation of effectors (e.g. G proteins) compared with R* (Tota and Schimerlik, 1990). The phenomenon of biased agonism (Kenakin, 2011) confirms the existence of multiple active conformations of the receptor, but the simple R and R* model is clearly useful because it leads to predictions that are supported by experimental evidence. For example, high-efficacy agonists show a greater binding affinity shift (from high affinity to low affinity) in the presence of guanine nucleotides, compared with lower efficacy agonists (Evans *et al.*, 1985; Emmerson *et al.*, 1996).

The demonstration of constitutive GPCR activity (Costa and Herz, 1989) indicated that receptors could form the R* state and activate G proteins even in the absence of ligand. Ligands termed 'inverse agonists' bind with higher affinity to the R conformation of the receptor, thus driving the receptor equilibrium from R* towards R and inhibiting constitutive activity of the receptor. Neutral antagonists show no preference for binding to the R or R* state and therefore do not affect the equilibrium of receptor conformations, but compete with orthosteric agonists for the orthosteric binding site. The ability to detect constitutive activity in recombinant systems expressing high levels of receptors suggests that most compounds thought to be neutral antagonists may show some preference for R or R*, and are either very weak efficacy agonists or weak efficacy inverse agonists.

It has become increasingly evident that certain ligands can bind to sites on GPCRs that are separate (allosteric) from the orthosteric site. The term 'allosteric' from the Greek 'other site' was first coined in a journal title 50 years ago by Monod, Changeux and Jacob (Monod *et al.*, 1963), followed 2 years later by the Monod, Wyman and Changeux model (Monod *et al.*, 1965) which describes a two-state model where proteins can exist spontaneously in two conformations, an active and inactive state. Orthosteric and allosteric ligands binding to their respective (non-overlapping) binding sites can stabilize one receptor state at the expense of the other. The effects observed from interactions between the orthosteric and allosteric ligands, binding to the protein, were termed the 'allosteric interactions'.

The concept of allostery was first applied to GPCRs with the development of the ternary complex model (De Lean *et al.*, 1980), which described the interactions between agonist, receptor and G protein, where the G protein can be considered as the allosteric modulator, binding at the intracellular side of the receptor. At around the same time, an introduction to the allosteric ternary complex model for GPCRs was also described based on the observed effects of gallamine on muscarinic receptors, which led to the conclusion that gallamine binds to a site distinct from other muscarinic agonists and antagonists (Clark and Mitchelson, 1976; Stockton *et al.*, 1983). Further modifications to these models to account for receptor constitutive activity led to the extended ternary complex model (Samama *et al.*, 1993) and the more thermodynamically complete cubic ternary complex model (Weiss *et al.*, 1996), which applies specifically to two states of the receptor and their interactions with G proteins. The allosteric two-state model (Hall, 2000) looks very similar to the cubic ternary complex model but substitutes G protein (G) with allosteric ligand (B), and applies more directly to orthosteric and allosteric ligands interacting with active and inactive conformations of the receptor. For a comprehensive review, see Christopoulos and Kenakin, 2002.

From a drug discovery perspective, the aim is to first identify and then to monitor the structure activity relationship of allosteric compounds using functional assays. An operational model has been developed based on the allosteric binding models of Ehlert (Ehlert, 1988) and the Black & Leff operational model of agonism (Black and Leff, 1983) that tracks the allosteric cooperativity factors (αβ). The final derivation of this operational model is shown in Scheme 1 as presented by Leach and colleagues (Leach *et al.*, 2007).

$$E = \frac{Em(\tau_A[A](K_B + \alpha\beta[B]) + \tau_B[B]K_A)^n}{\left(([A]K_B + K_AK_B + K_A[B] + \alpha[A][B])^n + (\tau_A[A](K_B + \alpha\beta[B]) + \tau_B[B]K_A)^n\right)} \quad (1)$$

Within this model, E is the pharmacological effect, K_A and K_B denote the equilibrium binding constants for the orthosteric ligand, A, and the allosteric ligand, B, at the receptor. The binding cooperativity factor, α, represents the effect of the allosteric ligand on orthosteric agonist binding affinity, and *vice versa*. An activation cooperativity factor, β, denotes the effect the allosteric ligand has on orthosteric agonist efficacy. Agonism constants τ_A and τ_B represent the intrinsic activity of the orthosteric agonist and any intrinsic activity of the allosteric ligand, respectively, which is dependent on the cell context and receptor expression level of the cell system, and intrinsic efficacy of the ligands used. The remaining parameters, Em and n, denote the maximal response of the system, and the slope, respectively. A simplified cartoon representing components of the operational model and how they apply to the various modes of allosteric modulation observed is shown in Figure 1.

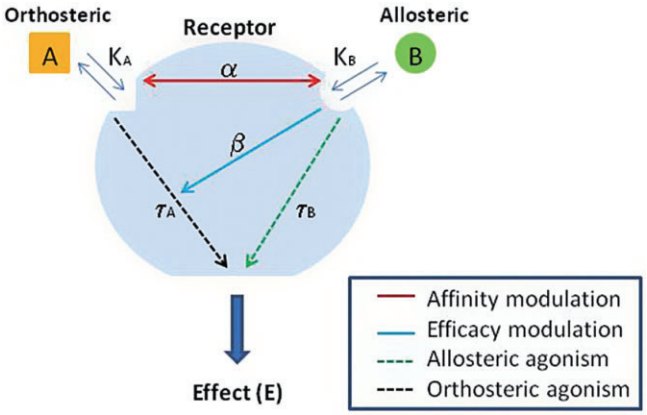

Figure 1

Modes of allosteric modulation. Allosteric ligands (B) bind to a topographically distinct site on the receptor compared with the orthosteric agonist (A), and can modulate orthosteric agonist binding affinity (α), orthosteric agonist efficacy (β), and may have intrinsic agonist activity (τ_B). Cartoon is a modified figure from Conn *et al.*, 2009a.

These parameters lead to the multiple 'flavours' of allosteric ligands that can be observed. Allosteric agonists that can activate the receptor even in the absence of an orthosteric agonist, have τ_B activity, leading to functional efficacy that appears similar to an orthosteric agonist. Allosteric inverse agonists bind to an allosteric site and inhibit the constitutive activity of the receptor in the absence of orthosteric ligand. However, allosteric modulators may have very weak or undetectable intrinsic efficacy when they bind to the receptor, but can positively or negatively modulate (via α and/or β) the binding affinity and/or efficacy of the orthosteric agonist when it binds to the receptor. Compounds with combined cooperativity factor (αβ) values > 1 are considered positive allosteric modulators (PAMs) and result in increased apparent potency and/or efficacy of the orthosteric agonist response. This is typically manifest as leftward shifts in the concentration-response curve for the orthosteric agonist in the presence of the PAM. Systems with spare receptors 'receptor reserve' exhibit leftward shifts in the orthosteric agonist concentration-response curve with increases in α or β. In these cases, one cannot discern the contribution of α or β from the functional assay, but only the combined cooperativity effect (αβ). The magnitude of these leftward shifts increases with increasing PAM concentration, until the PAM effect saturates when the allosteric sites are fully occupied. Therefore, beyond this concentration of PAM there is no further leftward shift in the agonist concentration-response curve. The maximal 'fold-shift' in agonist potency is equal to the cooperativity factor (αβ), and the concentration of PAM which induces a half-maximal leftward fold-shift of the orthosteric agonist potency is termed the apparent K_B

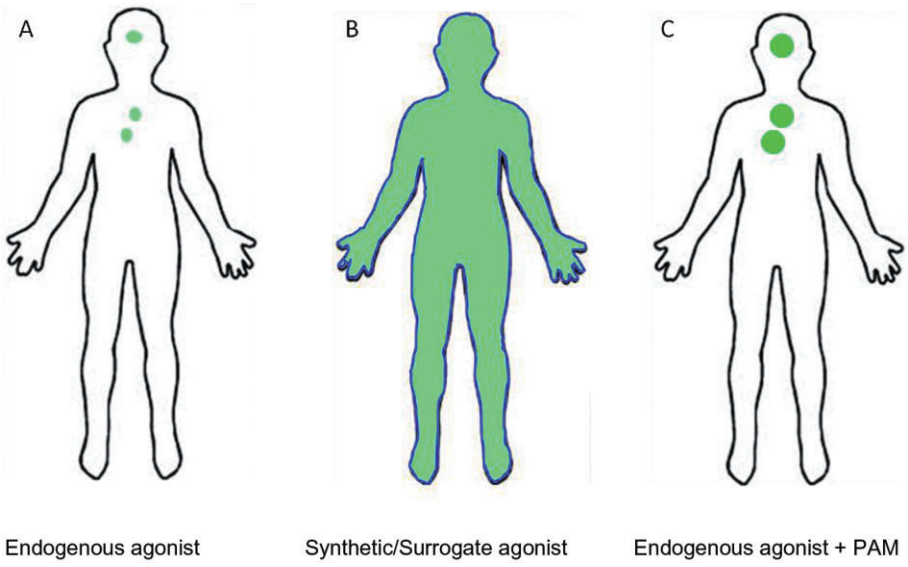

Figure 2

PAMs maintain spatial fidelity of native signalling. Endogenous opioid agonist is released at locations in the brain or spinal cord where it is required, maintaining the spatial fidelity of native signalling (A). Exogenous agonist is distributed and can activate target receptors throughout the body. This may lead to 'on target' side effects (B). PAMs can enhance the effects of endogenous agonists while still maintaining the spatial fidelity of native signalling (C).

(equilibrium binding constant for the PAM), or more colloquially the 'shifty$_{50}$' (Hendricson *et al.*, 2012). It is fairly common for allosteric ligands to have a combination of both allosteric agonist (τ_B) and PAM ($\alpha\beta$) activities depending on the cellular system, and the assay used to monitor functional activity. In these cases, direct agonism is typically seen at significantly higher concentrations of the allosteric ligand than are required for PAM activity (Burford *et al.*, 2011).

Negative allosteric modulators (NAMs) have combined $\alpha\beta$ values <1, resulting in a reduction in the potency and/or efficacy of the orthosteric agonist response. Compounds that bind to the allosteric site with very weak or no PAM or NAM activity are essentially neutral allosteric ligands or silent allosteric modulators (SAMs). These SAMs act as competitive antagonists at the allosteric site, and are therefore useful for characterizing the site of action of identified PAMs and NAMs.

The classification of ligands as agonists, partial agonists, neutral antagonists, inverse agonists, allosteric agonists, PAMs, NAMs and SAMs is dependent on the cellular system evaluated, and the particular aspect of signalling being explored. Also, for allosteric ligands, the allosteric cooperativity can be different depending on which particular orthosteric agonist (probe) is used (Jager *et al.*, 2007; Koole *et al.*, 2010). This is referred to as probe dependence. Therefore, defining a specific compound as a PAM or a NAM should only be done in the context of the cellular system, the agonist probe and the assay used.

Moreover, the situation is even more complex. For example, with homo- and hetero-oligomers (Gomes *et al.*, 2004; Gupta *et al.*, 2010; Costantino *et al.*, 2012; Stockton and Devi, 2012) the partnering receptor can be considered the allosteric modulator (Gomes *et al.*, 2004) causing conformational changes in the target receptor that may affect orthosteric agonist affinity and/or efficacy, as well as possible signalling bias. It is reasonable to assume both orthosteric and allosteric ligands that bind to one receptor in the complex will alter this allosteric interaction between GPCRs.

Allosteric ligands have several potential advantages over traditional orthosteric ligands as drugs (Christopoulos and Kenakin, 2002; Leach *et al.*, 2007; May *et al.*, 2007; Conn *et al.*, 2009a; Burford *et al.*, 2011; Keov *et al.*, 2011; Langmead, 2012). Because they do not bind to highly conserved orthosteric binding pockets, allosteric ligands can exhibit greater receptor selectivity. Additionally, PAMs have key potential advantages over orthosteric agonist drugs: PAMs can increase the amplitude while maintaining the spatial and temporal fidelity, and the physiological regulation, of native signalling patterns – something that orthosteric agonist drugs cannot come close to doing. These key features of PAMs are illustrated in Figures 2 and 3, and discussed below.

Discovery of μ-opioid receptor positive allosteric modulators (μ-PAMs)

Recently our group discovered μ-PAMs, which to our knowledge are the first PAMs described in the literature for this receptor (Burford *et al.*, 2013). Two negative allosteric modulators of opioid receptors have been described previously. Cannabidiol (a cannabinoid CB_1 receptor agonist) has been shown to be a negative allosteric modulator of agonist binding to μ- and δ-opioid receptors (Kathmann *et al.*, 2006). Salvinorin-A is a potent hallucinogenic κ-opioid receptor

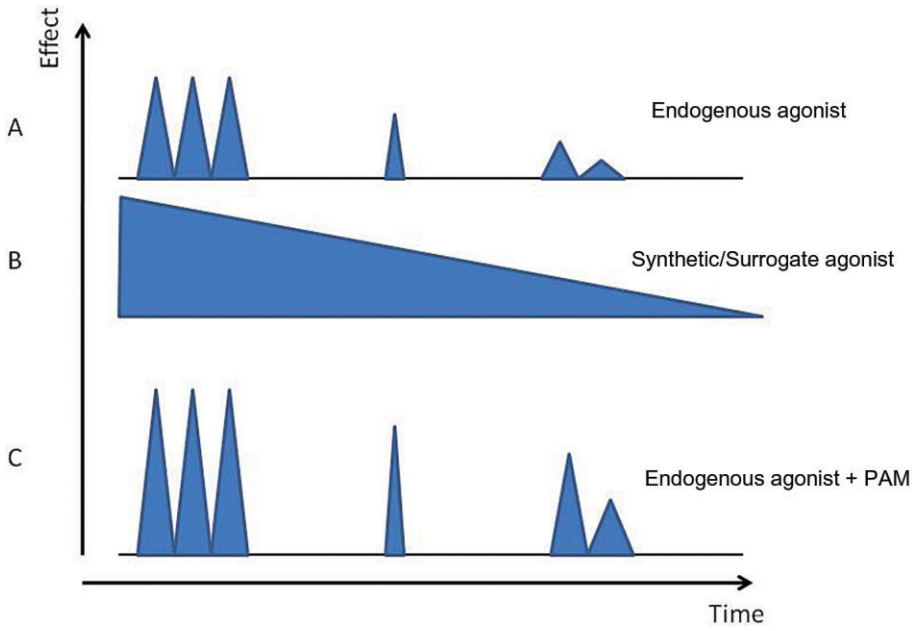

Figure 3

PAMs maintain temporal fidelity of native signalling. Endogenous agonist can be released and cleared or metabolized quickly, leading to signalling effects that have temporal fidelity (A). Exogenous agonist occupies receptors constantly, leading to effects that last until the drug is cleared or metabolized (B). PAMs can enhance the effects of endogenous agonists while still maintaining the temporal fidelity of native signalling (C).

agonist (Sheffler and Roth, 2003), but has also been shown to be a negative allosteric modulator of the μ-opioid receptor, although with ~100-fold weaker potency than its activity at κ-opioid receptor (Rothman *et al.*, 2007). μ-PAMs were identified in a high-throughput screen using a β-arrestin recruitment assay (PathHunter technology, DiscoveRx Corp., Fremont, CA, USA) (Bassoni *et al.*, 2012) in human osteosarcoma cells (U2OS) cells expressing μ-opioid receptors. In this assay, compounds were tested alone (agonist detection mode) or in the presence of a low (approximately EC_{10}) concentration of the μ-opioid receptor agonist, endomorphin-I (PAM detection mode). Concentration–response curves of the screening hits were evaluated in U2OS cells expressing μ-opioid receptors (U2OS-OPRM1 cells) and in U2OS cells expressing δ-opioid receptors (U2OS-OPRD1 cells) in the β-arrestin assay, in both agonist and PAM detection modes. Two of the compounds identified (BMS-986121 and BMS-986122) showed no agonist activity, were selective for μ- over δ-opioid receptors, and produced a sevenfold leftward shift ($\alpha\beta = 7$) in the potency of endomorphin-I in the β-arrestin assay in U2OS-OPRM1 cells.

These PAMs were further evaluated in an inhibition of forskolin-stimulated cAMP assay in CHO cells expressing μ-opioid receptors. In this assay, the PAMs produced leftward shifts in the potency of endomorphin-I as well as two other μ-opioid receptor agonists, leu-enkephalin and morphine. Interestingly, both PAMs showed some direct agonist activity in this assay format (τ_B in Figure 1), although at much weaker potencies than were observed for PAM activity.

Further confirmation that these compounds were μ-PAMs came from ligand binding studies and studies with a radiolabelled, poorly hydrolysed analogue of GTP ([^{35}S]-GTPγS) using membranes from C6 glioma cells expressing rat μ-opioid receptors (C6-μ cells) and mouse brain homogenates. Binding studies in C6-μ cell membranes showed that while the affinity of radiolabelled antagonist [^{3}H]-diprenorphine was unaffected by the μ-PAMs, competition of [^{3}H]-diprenorphine binding with the selective full agonist [D-Ala2, N-MePhe4, Gly-ol5]-enkephalin (DAMGO) showed that the μ-PAMs increased the affinity of DAMGO by sixfold, suggesting that these μ-PAMs act, at least in part, by increasing the affinity of the orthosteric agonist binding to the receptor (α in Figure 1). However, in [^{35}S]-GTPγS binding studies, under conditions where morphine and endomorphin-I were shown to be partial agonists compared with DAMGO, the μ-PAMs were shown to enhance the maximal response of these partial agonists, suggesting that they also can positively modulate the efficacy of responses to agonists (β in Figure 1).

Compounds similar in structure to BMS-986122 were tested in the β-arrestin recruitment assay resulting in some interesting structure activity relationships. Small changes in structure resulted in greatly reduced μ-PAM activity, although the EC_{50} of the responses were similar. It was subsequently shown that some of these compounds were SAMs, binding to the allosteric site but having no detectable effect in modulating the activity of the orthosteric agonist. However, the SAMs could block the activity of the μ-PAM, BMS-986122. Some of the BMS-986122 analogues also showed some δ-opioid receptor PAM activity, suggesting that μ- and δ-opioid receptors may share a similar allosteric site, and that selectivity between μ- and δ-opioid receptors can be engineered into the compounds.

Key features of PAMs compared with orthosteric agonists

Receptor selectivity

Receptors binding the same native agonist(s) necessarily exhibit high homology at the orthosteric agonist binding site. Thus, the identification of orthosteric ligands with selectivity between these related receptors can be difficult. This has posed major challenges for drug discovery programmes, where often one particular receptor type or subtype is the desired therapeutic target, but activity at related receptors can lead to undesired side effects. Well-known examples include the metabotropic glutamate receptors, muscarinic receptors and adenosine receptors, for which selective orthosteric ligands have remained elusive throughout decades of research. In contrast, allosteric sites on GPCRs do not bind the native ligand, and therefore are not under the same evolutionary constraint as orthosteric sites. Presumably because of an increased diversity at allosteric binding pockets, it has been possible to identify several highly selective allosteric agonists and PAMs for the notoriously difficult receptor targets listed above (Bruns and Fergus, 1990; Gasparini *et al.*, 2002; Birdsall and Lazareno, 2005; Gao *et al.*, 2005; Conn *et al.*, 2009b).

For opioid receptors, orthosteric agonist selectivity between the receptor types has largely been achieved through decades of medicinal chemistry programmes. However, allosteric agonists and PAMs may offer new structural scaffolds to further improve receptor type selectivity.

Because of the lack of evolutionary constraint imposed upon allosteric sites, allosteric ligands may be species-selective as well as receptor-selective. This can pose serious issues for drug development where a compound active at receptors in mice or rats may have no activity at the human orthologue, or *vice versa*. Therefore, activity of allosteric ligands at receptor orthologues should be determined early in the drug discovery programme. For the μ-PAMs discovered by our group, we saw no species selectivity between human, rat or mouse orthologues of the μ-opioid receptor.

Maintenance of temporal and spatial fidelity

Another advantage of PAMs is that they can maintain the temporal and spatial activity of receptor signalling *in vivo*. This is illustrated in Figures 2 and 3.

Neuronal signals are closely regulated within the nervous system with a high degree of temporal and spatial precision. When an orthosteric agonist drug is added systemically, it has two major disadvantages. Firstly, it is available throughout the body and not just at the specific location where it is needed. This leads to activation of target receptors in other areas of the brain and in other tissues, which can be detrimental to the therapeutic potential of the drug (Figure 2). Secondly, the added drug activates all the receptors throughout the body for an extended period of time. Usually, neurotransmitter release is pulsatile in nature and quickly removed between bursts of activity. Continuous exposure to an orthosteric agonist drug for extended periods of time may lead to receptor desensitization and tachyphylaxis, as well as toxic side effects mediated by long-term exposure of drug at the receptor (Figure 3).

These disadvantages of orthosteric drugs may be overcome with PAM drugs, where activity of an endogenously released orthosteric agonist are enhanced by the PAM, with the PAM having no effect at the receptor when the receptor is not bound with endogenous agonist. Such drugs would maintain the native temporal and spatial activity of the receptor in response to endogenous agonist.

Based on the pharmacological principle above, one can clearly envisage one potential way that μ-PAMs could provide an advantage over current orthosteric opiate analgesic therapy: μ-PAMs could produce analgesia by enhancing the activity of the endogenous opioid peptide ligands in pain-mediating pathways of the central and peripheral nervous system. In this way, the temporal and spatial activity of the endogenous opioid peptides would be preserved, and side effects resulting from continuous and indiscriminate activation of opioid receptors may be averted. This hypothesis raises several key questions: Does significant endogenous opioid signalling occur physiologically (i.e. is there enough endogenous opioid signals to amplify)? Does this endogenous signalling increase under conditions of injury, or chronic inflammatory or neuropathic pain? Are such increases spatially and/or temporally specific? Evidence for an endogenous peptide agonist-induced tone for μ-opioid receptor activity does exist. For example, inhibition of enkephalinases, which break down endogenous opioid peptides, results in antinociception in animal models of inflammatory and neuropathic pain (Roques *et al.*, 2012). Similarly, naloxone, a μ-opioid receptor antagonist, increased pain perception when administered to post-operative patients who were not taking exogenous opiates, suggesting the endogenous opioid peptides produced a basal analgesic tone (Levine *et al.*, 1978). Recently, opioid receptor antagonists were also shown to increase hyperalgesia in acute and chronic inflammatory pain models in mice that had not been treated with exogenous opioids (Corder *et al.*, 2013). The authors suggested that initial release of endogenous opioids leads to constitutive activation of the μ-opioid receptor, resulting in long-term endogenous analgesia.

The development of μ-PAMs will allow researchers to test whether, when administered alone, they will have efficacy in pain relief models, and whether the side effect profiles may be better compared with current opiate therapy. Of particular interest is whether tolerance and dependence can be avoided with a μ-PAM therapeutic. As the receptors would not be activated all the time by an exogenous agonist, one can hypothesize there will be less tolerance and dependence liability.

A second potential therapeutic utility for μ-PAMs can be envisaged: It is possible that administration of a low dose of opiate with a μ-PAM may also provide therapeutic benefit but with fewer side effects. The combination of a lower dose of opiate enhanced by a μ-PAM might slow or reduce the development of tolerance, which results from long-term exposure to opiates. There is precedence for this behaviour at the $GABA_B$ receptor. The potency of GABA to inhibit forskolin-induced cAMP formation in recombinant cells decreased after exposure to a saturating GABA concentration, but not after a combination of a low GABA concentration and the PAM GS39783, which activated the receptor to the same extent (Gjoni and Urwyler, 2008). The authors suggested that

GS39783 has a lower propensity to develop tolerance due to less receptor desensitization than classical agonists. It will be interesting to see whether a low dose of morphine combined with μ-PAM can produce similar levels of pain relief as a high dose of morphine, but with fewer tolerance and dependence liabilities.

Most of the untoward side effects of opiates (e.g. respiratory depression, constipation) are mediated through μ-opioid receptors, and there is no *a priori* reason to assume that μ-PAMs would not potentiate these unwanted effects of opiates as well as their desired therapeutic effects. However, perhaps the on-target side effects might be minimized by using reduced concentrations of morphine.

Finite shifts in orthosteric agonist potency with increasing concentrations of PAM

Modulation of orthosteric agonist responses by PAMs or NAMs is finite. As modulator concentrations reach the point where the allosteric binding sites on all available receptors are occupied, then no additional change in orthosteric agonist functional potency or efficacy is observed, even when the concentration of PAM or NAM is increased further. Therefore, allosteric modulators can be designed and selected based on their ability to produce a defined 'fold-shift' in functional potency of the orthosteric agonist. The main advantage of this is that PAMs with a defined fold-shift of agonist potency may reduce toxicity or avoid overdosing of the patient.

This would clearly be a potential benefit for the use of μ-PAMs where overdose with opiate drugs is a serious issue, resulting in many deaths. In many of these cases, the need to take more drug to overcome receptor tolerance issues compounds the problem.

Probe dependence

Another important aspect of allostery is the fact that the level or appearance of allosteric modulation can depend upon the orthosteric agonist ligand used, as described above. This has important consequences. Firstly, when evaluating compounds as PAMs, one should use, whenever possible, the endogenous ligand. This can add a level of complexity to a drug discovery programme when multiple endogenous ligands exist. Additionally, it is of note that previously inactive or weak potency metabolites of the endogenous ligand may show significant activity in the presence of a PAM. Therefore, probe dependence is an important consideration when evaluating the therapeutic potential of a given PAM (Wootten *et al.*, 2012).

Opioid receptors have multiple endogenous peptide agonist ligands. So it will be important to establish how each of these ligands is modulated by PAMs. Firstly, the selectivity of the PAM for each of the opioid receptors should be determined. If a PAM is found to be μ-opioid receptor-selective, one must also consider whether peptide agonists that are more κ-opioid receptor-selective but with some μ-opioid receptor activity (e.g. dynorphin-A) become more active at the μ-opioid receptor, and what consequences that has on the various pathways controlled by endogenous peptides. Similarly, metabolites of these peptides, which may not have much affinity/efficacy for opioid receptors, may produce significant activity in the presence of an opioid receptor PAM. Metabolism of morphine and other opiates also produce metabolites which are inactive at the μ-opioid receptor. However, one must ensure that these metabolites in the presence of a μ-PAM do not produce significant activity at the receptor, and if they do, one must determine the consequences.

Ligand-biased signalling and biased modulation

Historically, receptor pharmacology has been thought of in relatively simplistic terms, where ligands bind to and activate a receptor leading to a defined cascade of signalling pathways within the cell. However, over the past decade, research has convincingly shown that ligands acting at the same receptor can activate different signalling pathways, with each ligand producing subtly different changes in conformations of the receptor when they are bound. This feature, commonly called signalling bias or functional selectivity, has greatly increased our understanding of receptor pharmacology and revolutionized approaches to drug discovery (Kenakin, 2011; Whalen *et al.*, 2011; Kenakin and Christopoulos, 2013). The possibility of identifying small molecule orthosteric agonist ligands that can preferentially activate certain signalling pathways and not others offers the potential to discriminate between therapeutically beneficial pathways and unwanted side effect pathways even when the side effects are mediated by the target receptor, as is the case for the μ-opioid receptor.

Recently, there has been a great deal of interest in signalling bias with various opioid receptor ligands, and ligand bias has been observed with respect to agonist-mediated phosphorylation and internalization of the μ-opioid receptor, inhibition of cAMP accumulation, ion channel activity, β-arrestin recruitment responses and other non-canonical signalling pathways (Burford *et al.*, 1998; Mailman, 2007; Violin and Lefkowitz, 2007; Rivero *et al.*, 2010; McPherson *et al.*, 2012; Pradhan *et al.*, 2012; Rives *et al.*, 2012). Based on observations from β-arrestin-2 knockout mice that β-arrestins serve as negative modulators of analgesia, and positive modulators of some μ-opioid receptor-related side effects (including tolerance) (Bohn *et al.*, 1999; 2000), it has been hypothesized that opioid agonists with bias toward the G protein-mediated pathways and away from the β-arrestin-mediated pathways may be beneficial in separating the analgesic effects from the side effects. Indeed, Trevena have recently identified a G protein-biased agonist of the μ-opioid receptor, TRV130, which is reported to be a potent analgesic but with reduced gastrointestinal and respiratory dysfunctional effects compared with morphine (Dewire *et al.*, 2013).

Very recently, phosphorylation of the μ-opioid receptor at Tyr^{336} by Src has been shown to serve as the trigger for conversion of a classical Gi/Go-coupled receptor into a receptor tyrosine kinase-like entity, resulting in a non-canonical pathway leading to increased activation of AC even after the original Gi/Go signals are blunted (Zhang *et al.*, 2013).

Above, we have described the potential advantages of ligand bias signalling with respect to orthosteric agonists at the μ-opioid receptor. However, it is conceivable that a PAM may change the active conformation of the receptor in the presence of agonist, thus changing the signalling cascade to be biased towards one pathway and away from another. This 'biased modulation' has been observed for many GPCRs and

these have been recently reviewed elsewhere (Koole *et al.*, 2010; Keov *et al.*, 2011; Davey *et al.*, 2012; Kenakin and Christopoulos, 2013; Wootten *et al.*, 2013).

Conclusions

The discovery and characterization of μ-PAMs has opened up a new and exciting avenue to explore not only novel pain therapeutics at the μ-opioid receptor, but also therapeutics for conditions, such as mood disorders, for which there is mounting evidence that opioid receptors present viable therapeutic targets (Lambert, 2008; Hegadoren *et al.*, 2009; Lutz and Kieffer, 2013). With the design of improved μ-PAMs with higher affinity for the receptor and better pharmacokinetic, pharmacodynamic, and safety profiles, it will be possible to assess whether μ-PAMs have efficacy in models of pain relief and other medical conditions either when administered alone, thereby modulating endogenous opioid pathways, or in combination with lower concentrations of exogenous opiates, such as morphine. In either scenario, it will be important to know if beneficial actions are enhanced, while sparing tolerance, dependence and other side effects associated with current opioid therapies.

Nevertheless, there are also a number of challenges for any drug discovery programme seeking allosteric modulators of opioid receptors. Due to the probe-dependent nature of allosteric modulation and the non-selectivity of several of the endogenous opioid peptides and opioid drugs, the activity of opioid PAMs will need to be assessed across opioid receptor types and with a variety of endogenous and other orthosteric agonists, including potentially active metabolites (Wootten *et al.*, 2012). An additional complication is that some chemical scaffolds, including the μ-PAMs we have described (Burford *et al.*, 2013) can switch function from PAMs to NAMs or SAMs with only small changes in structure (Melancon *et al.*, 2012). However, there is no doubt that the inherent advantages of PAMs, especially their maintenance of temporal and spatial signalling fidelity and promise of biased modulation, in addition to the potential to use lower doses of opioid drugs, will guide research over the next few years. This will ascertain whether μ-PAMs might represent the 'holy grail' of opioid research, developing powerful analgesic drugs devoid of the side effects associated with morphine.

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