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Pharmacological Evidence for a Putative Conserved Allosteric Site on Opioid Receptors

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Running Title: Evidence for a conserved opioid receptor allosteric site

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Non-standard abbreviations: δ-OR (delta opioid receptor), κ-OR (kappa opioid receptor), μ-OR (mu opioid receptor), AC (adenylate cyclase), CHO (Chinese Hamster Ovary), DPN (diprenorphine), GDP (guanosine 5'-diphosphate), GTPγS (guanosine-5'-O-(3-thio)triphosphate), GPCR (G protein coupled receptor), NOPR (nociceptin receptor), PAM (positive allosteric modulator), SAM (silent allosteric modulator), TM (transmembrane)

Abstract

Allosteric modulators of G protein-coupled receptors (GPCRs), including opioid receptors, have been proposed as possible therapeutic agents with enhanced selectivity. BMS-986122 is a positive allosteric modulator (PAM) of the mu-opioid receptor (μ-OR). BMS-986187 is a structurally distinct PAM for the delta-opioid receptor (δ-OR) that has been reported to show 100-fold selectivity in promoting δ-OR over μ-OR agonism. Here we use ligand binding and second messenger assays to show that BMS-986187 is actually an effective PAM at μ-OR and at the kappa opioid receptor (κ-OR), but is ineffective at the nociceptin receptor (NOPR). The affinity of BMS-986187 for δ-ORs and κ-ORs is approximately 20-30 fold higher than for μ-ORs, determined using an allosteric ternary complex model. Moreover, we provide evidence, using a silent allosteric modulator as an allosteric antagonist, that BMS-986187 and BMS-986122 bind to a similar region on all three traditional opioid receptor types (μ-OR, δ-OR and κ-OR). In contrast to the dogma surrounding allosteric modulators the results indicate a possible conserved allosteric binding site across the opioid receptor family that is able to accommodate structurally diverse molecules. The findings have implications for the development of selective allosteric modulators.

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Introduction

Allosteric modulation of G protein-coupled receptors (GPCRs) is a promising new avenue to develop safer drugs. With this aim in mind we have discovered positive allosteric modulators (PAMs) of both mu (μ-OR) and delta (δ-OR) opioid receptors. These allosteric modulators (Burford *et al.*, 2013, 2015; Livingston and Traynor, 2014; Bisignano *et al.*, 2015) bind to a site on the receptor distinct from the orthosteric site for endogenous opioid peptides and traditional opiate analgesics drugs to enhance the affinity and/or efficacy of various orthosteric ligands in an agonist-dependent manner, described as probe dependence. BMS-986122 (Fig 1) is selective for μ-OR and has no detectable activity at the closely related δ-OR (Burford *et al.*, 2013) while the δ-OR PAM, BMS-986187 (Fig 1), possesses a 100-higher potency at δ-OR over μ-OR (Burford *et al.*, 2015).

The selectivity of the PAMs for μ-OR over δ-OR is not surprising as enhanced selectivity is one of the touted benefits of allosteric modulation of GPCRs over traditional orthosteric activation. Ligand-binding domains were evolved and maintained to bind endogenous ligands, whereas sites for small allosteric ligands face less evolutionary pressure to be conserved. Therefore, even closely related receptors of the same family that have very similar orthosteric binding pockets can have different allosteric binding sites (for review see (Conn *et al.*, 2009)). For example, the μ- and δ-ORs share over 64% sequence identity and are nearly identical in the transmembrane (TM) domains (Stevens, 2009), with highly conserved orthosteric binding pockets as determined from multiple inactive-state crystal structures (Lin *et al.*, 2009; Manglik *et al.*, 2012; Fenalti *et al.*, 2014) as well as an active-state structure of μ-OR (Huang *et al.*, 2015). In addition, the opioid peptides Leu- and Met-enkephalin are endogenous ligands for both receptors and the signaling pathways downstream of the receptors are very similar (Chen *et al.*,

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1993). On the other hand the allosteric ligands discovered to date for the two receptors are highly structurally distinct (Fig 1).

In previous work we have demonstrated the action of BMS-986122 at μ-OR involves a disruption with the binding of Na^+ ions (Livingston and Traynor, 2014) that modulate the activity of many class A GPCRs, including the opioid receptor family (for review see (Katritch *et al.*, 2014)). Na^+ binds at a well-described site within the 7-TM bundle and contributes to stabilization of the receptor in an inactive state with reduced affinity for agonists (Pert *et al.*, 1973; Pert and Snyder, 1974; Liu *et al.*, 2012). We previously hypothesized (Livingston and Traynor, 2014) that allosteric disruption of Na^+ binding may be a common mechanism for PAMs of class A GPCRs.

Although BMS-986187 possesses selectivity for δ-OR over μ-OR, it does appear to have some PAM activity at activity at μ-OR when endomorphin 1 is used as the orthosteric ligand (Burford *et al.*, 2015). This and the structural and functional similarities between the μ-OR and δ-OR led us to examine the action of BMS-986187 at μ-OR and to test the hypothesis of a general mechanism of action of allosteric modulation of GPCRs by studying whether the binding of the δ-OR-PAM BMS-986187 and Na^+ ions are mutually incompatible at both δ-OR and μ-OR. Finally, we asked whether the allosteric sites on the two receptors were similar using the silent allosteric modulator BMS-986124 which acts as an antagonist at the allosteric site in μ-OR (Burford *et al.*, 2013) and also investigated if these modulators have activity at the closely related kappa opioid receptor (κ-OR) and nociceptin receptor (NOPR).

Overall, our findings confirm that the selective δ-PAM BMS-986187, has PAM activity at μ-OR and we further show this compound is also a PAM at κ-OR, but not at NOPR. In addition, we find that the μ-PAM BMS-986122 is a silent allosteric modulator at δ-OR and κ-

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OR. Together, these data suggest that the allosteric binding sites on μ-OR, δ-OR, and κ-OR may be conserved. The results have ramifications for identification of the allosteric site(s) and for drug development of allosteric modulators for the opioid receptors.

Materials and Methods

Materials: [^{3}H]Diprenorphine (DPN) and GTPγ^{35}S ((guanosine-5'-O(3-thio)triphosphate), were from PerkinElmer Life Sciences. All tissue culture medium, penicillin-streptomycin, geneticin (G148), trypsin, and fetal bovine serum were from Invitrogen (Carlsbad, CA). DAMGO, naloxone, and morphine sulfate were from Sigma-Aldrich. PathHunter detection reagents were from DiscoveRx (Freemont, CA). Lance-Ultra cAMP detection reagents were from PerkinElmer Life Sciences (Cambridge, MA). BMS-986122, BMS-986124, and BMS-986187 (structures in Fig 1) were synthesized or obtained as previously described (Burford *et al.*, 2013, 2015). Methadone was from the Opioid Basic Research Center at the University of Michigan. All other chemicals, unless otherwise specified, were purchased from Sigma (St. Louis, MO).

Cell Lines and Membrane Preparation: The generation and maintenance of C6 rat glioma cells stably transfected with rat mu opioid receptor (μ-OR) or rat delta opioid receptor (δ-OR) were performed as described (Clark *et al.*, 2008). Chinese Hamster Ovary (CHO) cells expressing human κ-OR, HEK293T cells expressing human ORL1 and Flp-In^{TM} CHO expressing human δ-OR (CHO-δ) were generated and maintained Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum, in the presence of 0.4 mg/ml G418 and 0.1% penicillin/streptomycin. Cell membranes were prepared for binding assays as described(Livingston and Traynor, 2014). Briefly, cells were grown to confluence and washed twice with 37 ºC phosphate-buffered saline

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(pH 7.4). Cells were detached in harvesting buffer (20 mM HEPES, 150 mM NaCl, 0.68 mM EDTA, pH7.4) and pelleted by centrifugation at 200 x g for 3 min at room temperature. The pellet was resuspended in ice-cold 50 mM Tris (pH 7.4) and homogenized using a Tissue Tearor (Dremel). This homogenate was centrifuged at 20000 x g at 4 ºC for 20 min. The pellet was then resuspended, homogenized, and centrifuged once more. The final pellet was resuspended in 50 mM Tris (pH 7.4) using a glass dounce homogenizer and aliquots were flash frozen in liquid nitrogen. Aliquots were stored at -80 ºC until use. Protein concentration was determined using BCA quantification method with bovine serum albumin as the standard.

CHO PathHunter cells expressing enzyme acceptor (EA)-tagged β-arrestin-2 and ProLink (PK)-tagged μ-OR receptor (CHO-μ) were from DiscoveRx (Freemont, CA). Cells were grown in F-12 media (Invitrogen 11765), containing Hyclone FBS 10%, Hygromycin 300 μg/mL, Geneticin (G418) 800 μg/mL and maintained at 37 °C in a humidified incubator containing 5% CO_2. These cells were used for β-arrestin-2 recruitment assays and inhibition of forskolin-stimulated cAMP accumulation assays described below.

Radioligand Binding Assays: Ligand binding assays were performed using the cell membrane homogenates described above. Competition binding assays were performed as previously described (Clark *et al*., 2003). Briefly, ^{3}H-DPN; (0.2-0.3 nM) was incubated in assay buffer (50 mM Tris pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 10 μM GTPγS) with 10 μg membrane protein, orthosteric ligand, and allosteric ligand (or vehicle) to promote a low affinity state of the receptor. In some experiments the κ-OR ligand ^{3}H-U69,583 was examined in Tris-HCl buffer (pH 7.4) only. Nonspecific binding was determined in the presence of 10 μM naloxone. Assays were incubated at room temperature for 75-90 min to reach equilibrium and then terminated and counted as described (Livingston and Traynor, 2014). Three independent

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experiments, each done in duplicate, were performed and the values were pooled to generate the mean curve as displayed in the figures.

GTPγ^{35}S Assays: GTPγ^{35}S binding experiments were performed as described (Traynor and Nahorski, 1995) using cell membrane homogenates prepared as described above. Briefly, 10 µg aliquots of membrane protein were incubated for 1 h at 25 ºC in buffer [50 mM Tris pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA] with 0.1 nM GTPγ^{35}S 30 µM GDP (guanosine 5'-diphosphate), orthosteric ligand, and allosteric ligand (or vehicle). An internal standard at 10 µM (DAMGO [([D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin)] for µ-OR, SNC80 for δ-OR, U69,593 for κ-OR, and nociceptin for NOPR) was used to define maximal activation, water or vehicle defined basal binding. The assays were terminated and counted as described (Livingston and Traynor, 2014). Three independent experiments, each done in duplicate, were performed and the values were pooled to generate a mean curve as displayed in the figures.

PathHunter β-Arrestin-2 Assay: Confluent flasks of CHO-µ cells were harvested with TrypLE^{TM} Express, and resuspended in F-12 media supplemented with 10 % FBS and 25 mM HEPES, at a density of 6.67 x 10^5 cells /mL and plated (3 µL / well) into white solid TC-treated 1536-well plates (Corning, NY). Plates were incubated overnight at 37^{o}C in a 5% CO_2 humidified incubator. The next day, increasing concentrations of BMS-986187 (40 nL of 100 x final concentration in 100% DMSO) were added to separate rows of the assay plates by acoustic dispense using an Echo-550 (Labcyte, Sunnyvale, CA) from Echo-qualified 1536-well source plates (Labcyte). Next, 1 µL of increasing concentrations of DAMGO (4 x final concentration in assay buffer) were added to separate columns of the assay plates containing cells. Plates were covered with a lid and incubated at room temperature for 90 min. Incubations were terminated

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by the addition of 2 µL PathHunter Reagent (DiscoveRx). Luminescence was measured 1 h later using a Viewlux imaging plate reader (PerkinElmer).

Inhibition of Forskolin-Stimulated cAMP Accumulation Assays: CHO-µ cells were grown to confluence then harvested and resuspended at 10^6 cells / mL in assay buffer (HBSS + 25 mM HEPES, + 0.05% BSA). Increasing concentrations of BMS-986187 (30 nL of 100 x final concentration in 100% DMSO) were added to separate rows of 1536-well white solid NT plates by acoustic dispense using an Echo-550 (Labcyte, CA). Next, 1 µl of increasing concentrations of DAMGO (at 3 x final concentration in assay buffer) were added to separate columns of the plates. Then, 1 µL of cells (1000 cells / well) were added to all wells followed by 1 µL of forskolin (3 x final concentration in assay buffer). Plates were lidded and incubated for 45 min at room temperature. Incubations were terminated by the addition of Lance-Ultra cAMP detection reagent (Perkin Elmer) (1.5 µL of Eu-cryptate-labelled cAMP tracer in lysis buffer, followed by 1.5 µL of U-light conjugated anti-cAMP antibody in lysis buffer). After a 1 h incubation at room temperature, time-resolved fluorescence (TRF) was detected on a Viewlux or Envision plate reader (PerkinElmer) with excitation at 337 nm and emission reads at 615 nm and 665 nm. The ratiometric data (665 nm read/615 nm read)*10,000 were then converted to cAMP (nM) based on a standard curve for cAMP run at the same time under identical conditions.

Data Analysis and reporting: Data were analyzed using GraphPad Prism version 6 (GraphPad, San Diego, CA, USA). Orthosteric ligand affinity (K_i) values and potency (EC_{50}) values were determined using nonlinear regression, with basal and maximal values constrained and are presented as mean ± SEM or with 95 % confidence limits. Functional cooperativity values ($\alpha\beta$) and the affinities of allosteric modulators for the orthosteric agonist-unoccupied receptor (K_B) were obtained using the allosteric ternary complex model (Christopoulos and Kenakin, 2002;

Leach et al., 2007) or a derivation of the allosteric ternary complex model (Leach et al., 2010). Sample size was predetermined to be a minimum of three independent experiments. None of the functional assays were blinded to investigators and no data was excluded.

Results

Activity of the δ-PAM, BMS-986187, at μ-OR

BMS-986187 is reported to have a 100-fold selectivity for δ-OR as compared to μ-OR, although initial data on BMS-986187 suggested that the compound is an efficacious, yet low affinity PAM at μ-OR (Burford et al., 2015). To confirm the PAM activity of BMS-986187 at μ-OR, we studied the effects of BMS-986187 using cell membrane preparations from C6 rat glioma cells stably expressing rat μOR (C6μ, Lee *et al.*, 1999). Since we have previously shown that modulators act by allosterically displacing Na^+ ions from inactive μ-OR, binding was measured by competition assay using the orthosteric antagonist [^{3}H]-diprenorphine ([^{3}H]-DPN), in the presence of Na^+ ions and GTPγS to uncouple heterotrimeric G protein and so stablize inactive (R) conformations of the receptor (Livingston and Traynor, 2014). We verified that BMS-986187 does not compete with [^{3}H]-DPN, (Fig 2a) for the orthosteric site on μOR, or stimulate GTPγ^{35}S binding (Fig 2b). Conversely, BMS-986187 significantly increased the binding affinity (K_i) of agonists for μ-OR, consistent with a positive allosteric ligand. As such, in the presence of 10 μM BMS-986187 the affinity of the μ-OR agonist DAMGO was enhanced 11-fold from 724 nM to 63 nM, while the affinity of methadone was increased 24-fold from 603 nM to 25 nM. In contrast, the affinity of morphine, a partial agonist, was enhanced a non-significant 3-fold, from 229 nM to 71 nM (Fig 2, Table 1).

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The allosteric modulatory actions of BMS-986187 at μ-OR were then investigated in three signaling assays. BMS-986187 (1 μM) significantly enhanced the potency of DAMGO by 6-fold from 91 nM to 16 nM, methadone by 20-fold from 203 nM to 10 nM, but shifted morphine only a non-significant 3-fold from120 nM to 38 nM in the GTPγ^{35}S binding assay in membranes from C6μ cells. There was also a significant increase in the maximal effect of the partial agonist morphine, from 70% to 90% of the DAMGO response (Fig 2, Table 1). The larger shift seen with methadone allowed us to repeat the assay in the presence of increasing concentrations of BMS-986187 to generate of a limited series of concentration-response curves (Fig 3) that, when analyzed using a derivation of the allosteric ternary complex model (Leach et al., 2010; see methods), resulted in a pK_B value, representing the affinity of BMS-986187 for the unoccupied μ-OR, of 5.23 ± 0.19 and a log αβ value of 1.16 ± 0.13 for the cooperativity between methadone and BMS-986187.

We then used high throughput methods to generate a more comprehensive series of concentration-effect curves to study the action of the modulator on adenylate cyclase (AC) inhibition and β-arrestin-2 recruitment, that enabled us to determine binding affinity and allosteric cooperativity using the full operational model of allostery (Leach et al., 2007, see methods). Using inhibition of AC in CHO-μ cells as a downstream measure of Gi/o signaling BMS-986187 concentration-dependently enhanced the potency of DAMGO by 10-fold (EC_{50} from 66 pM to 7 pM (Fig 4a). These data afforded a pK_B value for BMS-986187 of 4.78 ± 0.15 and a log αβ value for cooperativity of 1.57 ± 0.17. Analysis using the simplified operational model (Leach et al., 2010) gave the same result (pK_B = 4.83 ± 0.15; logαβ = 1.52 ± 0.17). When recruitment of β-arrestin-2 was measured as a signaling output in the same cells, BMS-986187 was also observed to concentration-dependently enhance the potency of DAMGO (EC_{50} from

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170 nM to 3 nM) representing a 58-fold shift in DAMGO potency (Fig 4b). Analyses of these data afforded a pK_B value for BMS-986187 of 4.61 ± 0.06 (Fig 4) and a log αβ value of cooperativity of 2.1 ± 0.05.

It should be noted that the coupling efficiency of the AC assay is much higher than in the β-arrestin-2 or GTPγ^{35}S assays as evidenced by the 2500-fold higher potency of DAMGO in the former assay. Because of this high coupling efficiency, BMS-986187 alone was seen to inhibit adenylate cyclase (Fig 4c). In comparison, no agonist activity was observed in the GTPγ^{35}S assay (Fig 2b) or the β-arrestin-2 assay (Fig 4d). This direct agonist activity in the AC assay at μ-OR is not due to an action of BMS-986187 at the orthosteric site on μ-OR as shown by the ^{3}H-DPN displacement assays (Fig 2a) and so the compound should be designated as an "ago-PAM" (Christopoulos et al., 2014) . This is reminiscent of its activity at the δ-OR where it is capable of G protein activation, AC inhibition, and mitogen-activated protein kinase activation in the absence of orthosteric agonist, in addition to allosterically enhancing orthosteric agonist affinity and activity (Burford *et al.*, 2015). However, the ago-PAM efficacy of BMS-986187 at μ-OR, is low, such that this can only be observed when measured using the more amplified AC signaling output in cells overexpressing μ-OR receptors.

BMS-986187 acts to disrupt Na^+ binding at both μ-OR and δ-OR

Na^+ ion binding is highly coordinated and contributes to stabilization of inactive receptor (R; Fenalti *et al.*, 2014; Katritch *et al.*, 2014). Disruption of Na^+-coordination leads to an increased level of active-state μ-OR (R*) and therefore a reduction in the binding affinity of orthosteric agonists (Pert *et al.*, 1973; Pert and Snyder, 1974). We have previously demonstrated that the binding of the μ-PAM, BMS-986122, and Na^+ ions are incompatible leading BMS-986122 to

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promote R* (Livingston and Traynor, 2014). Here we show that the potency (EC_{50}) of Na^+ ions to inhibit the binding of the μ-OR agonist DAMGO to membranes from C6μ cells was reduced 5-fold in the presence of BMS-986187 (from 3.8 (0.6-20) mM to 20.2 (12-34) mM, Fig 5a), although due to small window in the control group the effect did not reach significance. Likewise, at the δ-OR expressed in CHO cells there was a negative relationship between Na^+ ions and BMS-986187 such that BMS-986187 caused a concentration-dependent 4-fold rightward shift in the NaCl concentration-response curve (from 45 (35-59) mM to 172 (100-300) mM) to inhibit the constitutive receptor-mediated GTPγ^{35}S binding that occurs in Na^+ free buffer (Szekeres and Traynor, 1997; Fig 5b).

BMS-986122 and BMS-986187 are competitive at μ-OR and δ-OR

Although our initial hypothesis predicted that BMS-986122 and BMS-986187 have distinct binding sites on μ-OR based on their diverse structural features (Fig 1), the fact that they show a similar pattern of probe dependence in their interaction with full versus partial agonists versus antagonists and a similar interaction with Na^+ ions could indicate they actually engage μOR at the same allosteric site(s). To evaluate this, we used the μ-silent allosteric modulator (μ-SAM) BMS-986124, a close analogue of BMS-986122 (Fig 1), which has been previously reported to block BMS-986122 action at μ-OR while having no effects alone (Burford *et al.*, 2013). We asked if BMS-986124 could inhibit the action of BMS-986187 at μ-OR and even at δ-OR.

BMS-986187, at 10 μM, produced a 6-fold increase in the potency of DAMGO to stimulate GTPγ^{35}S binding in C6μ cell membranes (from 91 nM to 16 nM; Table 1). The addition of the μ-SAM increased the EC_{50} of DAMGO (to 59.4 (47.5 – 74.2) nM) such that the

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PAM effects of BMS-986187 were no longer significant (Fig 6A). Using the same assay in membranes from C6δ cells BMS-986187 (300 nM) displayed no agonism alone but enhanced the potency of the orthosteric agonist Leu-Enkephalin to stimulate GTPγ^{35}S by 6-fold (from 378 (287-498) nM to 65.4 (44.9-95.4) nM). This effect was reversed in the presence of BMS-986124 (217 (154-295) nM) (Fig 6B). Furthermore, since BMS-986124 blocks the action of BMS-986122 at μ-OR and BMS-986187 at δ-OR, we predicted BMS-986122, because of its structural similarity to BMS-986124 (Fig 1) might actually bind to δ-OR, but lack allosteric efficacy. In a separate set of experiments in C6δ cell membranes the presence of BMS-986187 (300 nM) increased the potency of Leu-Enkephalin (from 715 (548-933) nM to 57.7 (42.1-79.0) nM) but this was decreased by the addition of 30 μM BMS-986122 (to 176 (136-228) nM) (Fig 6C). The fact that we observed only partial reversal may be explained by the finding that BMS-986122 might not be entirely silent at δ-OR. For example, in a separate experiment the compound did slightly enhance the potency of Leu-Enkephalin (from 667 (525-848 nM) to 293 (234-374) nM; Fig 6D). BMS-986187 is an ago-PAM at δ-OR and alone will stimulate GTPγ^{35}S binding *via* the allosteric site. Both the μ-SAM (BMS-986124) and the μ-PAM (BMS986122) were able to inhibit the ago-PAM activity of BMS-986187 (Fig 6E).

BMS-986187 is a positive allosteric modulator of κ-OR

Since BMS-986187 has activity at both μ-OR and δ-OR, we sought to determine if it also had activity at the kappa opioid receptor (κ-OR) since these three receptors share a high degree of homology as well as endogenous ligands. In CHO cells expressing the human κ-OR, GTPγ^{35}S binding stimulated by the κ-OR preferring peptide dynorphin A(1-17) was enhanced 10-fold from 15 nM to 1.0 nM) in the presence of 10 μM BMS-986187 with no change in the maximal

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response (Fig 7, Table 1). Similarly, the activities of the κ-OR agonist U69,593, the non-basic nitrogen containing agonist Salvinorin A, and the partial agonist DPN (Traynor *et al.*, 1987) were enhanced in the presence of 10 μM BMS-986187. The potency of U69,593 was shifted 14-fold from 881 nM to 64 nM and the potency of Salvinorin A was shifted by 12-fold from 322 nM to 26 nM while the maximal responses to both compounds was unaltered (Fig 7, Table 1). In contrast, the potency of the partial agonist DPN was not significantly shifted (46 nM to 8.9 nM) but the maximal stimulation was enhanced from 31 % to 56 % of that seen with U69,593 (Fig 7, Table 1). Analysis of the concentration-response curves for the stimulation of GTPγ^{35}S binding by U69,593 in the presence of BMS-986187 (Fig 7B) using the simplified operational model (Leach et al., 2010) afforded a pK_B value for the modulator of 6.22 ± 0.19 and a log αβ value of 1.11 ± 0.13.

We could not investigate the effect of BMS-986187 using binding assays with ^{3}H-DPN as the tracer ligand at κ-OR because DPN has agonist activity at this receptor and this action is sensitive to BMS-986187 (Fig 7D). Consequently we studied the effects of the modulators on the binding of ^{3}H-U69,593. This was examined in Tris-buffers in the absence of added Na^+ ions and GTP. Even so BMS-986187 enhanced the affinity of ^{3}H-U69,593 to κ-OR from K_D = 0.9 ± 0.2 nM to 0.3 ± 0.1 nM (p = 0.03; Fig 7E, Table 1) without a change in the Bmax. The effect is small because receptors will mostly exist in the R* state under these conditions. Neither BMS-986122 nor BMS-986124 had an effect on ^{3}H-U69,593 binding alone, but BMS-986124 prevented the BMS-986187-mediated enhancement of binding (Fig 7F), suggesting that it is also a κ-SAM.

BMS-986122 and BMS-986187 have no detectable PAM activity at NOPR

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The nociceptin receptor (NOPR) is now recognized as a fourth member of the opioid receptor family (Cox *et al*., 2015), and like the other family members is coupled to $G\alpha_{i/o}$ proteins. Using HEK293T cells expressing NOPR, concentration-response curves of the abilities of the endogenous ligand nociceptin and the small molecule synthetic agonist R064-6198 to stimulate GTPγ^{35}S binding were determined in the presence or absence of BMS-986187 (Fig 8, Table 1). Neither the potency nor maximal effect of either NOPR ligand was altered.

Discussion

Our results show the δ-PAM BMS-986187 is also a μ-PAM and κ-PAM, providing the first description of a κ-PAM. Moreover, the μ-PAM BMS-986122 is a SAM, or allosteric antagonist, at δ-OR and the μ-SAM BMS-986124 is an allosteric antagonist at μ-OR, δ-OR, and κ-OR. In addition, the probe dependence of BMS-986187 at the μ-OR and κ-OR is the same as seen with BMS-986122 at the μ-OR (Livingston and Traynor, 2014) and BMS-986187 at the δ-OR (Burford *et al*., 2015). In particular, there is an increase in ligand affinity and potency for full agonists but an increase in the maximal effect for the partial agonists, morphine at μ-OR and diprenorphine at the κ-OR, with little change in their affinity or potency. Finally, the mechanism of action of the PAMs at μ-OR and δ-OR involves destabilization of the inactive Na^+-bound receptor. Thus, it appears that the allosteric ligand binding site on the μ-, δ-, and κ-OR is similar enough to recognize the same ligands and interact with the orthosteric site but also sufficiently promiscuous to accommodate different chemical scaffolds as exemplified by BMS-986122 and BMS-986187 (Fig 1). The findings provide pharmacological evidence for a possible conserved allosteric site across the three naloxone-sensitive members of the opioid receptor family.

The average affinity of BMS-986187 for orthosteric-agonist-free μ-OR (pK_B) determined from the GTPγ^{35}S, β-arrestin-2 and AC assays is 4.75 ± 0.07. In contrast the pK_B at the κ-OR

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derived from GTPγ^{35}S data in 6.22, similar to the reported average pK_B value for BMS-986187 at δ-OR (6.02; Burford et al., 2015). Since the pK_B for BMS-986187 is independent of the ligand occupying the orthosteric site we can compare these values to show that BMS-986187 is approximately 20-30 fold selective for binding to δ-OR and κ-OR over μ-OR. The cooperativity factor (log αβ) between DAMGO and BMS-986187 at μ-OR for the GTPγ^{35}S and adenylate cyclase assays is 1.57, whereas there is a greater cooperativity in the β-arrestin-2 assay (logαβ = 2.1). The cooperativity factor (log αβ) between U69,593 and BMS-986187 at κ-OR derived from GTPγ^{35}S assay data is 1.1. This compares with full agonists (Leu-enkephalin and SNC80 respectively) at the δ-OR that afforded log αβ values of 1.18 and 1.33 in the β-arrestin-2 assay, 1.67 and 1.0 in the [^{35}S]GTPγS assay and 2.8 and 2.1 in the AC assay (Burford *et al*., 2015). Although it is not possible to directly compare these values because of the different orthosteric ligands used, the similarity between log αβ values at μ-OR, δ-OR and κ-OR would suggest that the preference of BMS-986187 for the δ-OR and κ-OR is mainly due to its higher binding affinity. It is worthy of note that the co-operativity values at μ-OR are greater for β-arrestin-2 than AC while the opposite is true at the δ-OR.

Previously a 100-fold preference was reported for the ability of BMS-986187 to act as a PAM at the δ-OR compared to the μ-OR. This selectivity was determined by comparison of potency ratios for the compounds as PAMs at the μ-OR using endomorphin-1 and the δ-OR using Leu-enkephalin (Burford *et al.*, 2015) and this could well vary depending on the probes used, a facet that is complicated by the many endogenous ligands for the opioid receptor family. On the other hand, the affinity values reported here are for the agonist unoccupied receptor, and do not depend on the orthosteric ligand used, thus the discrimination obtained is the actual selectivity for BMS-986187 between the μ-OR and the κ- or δ-OR.

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The apparent lack of selectivity of the allosteric modulator between κ- and δ-OR and the low level of selectivity for these receptors over μ-OR is counter to the many observations across other GPCRs that allosteric modulators can selectively target closely related receptors that traditional orthosteric ligands have been unable to achieve, for review see (May *et al.*, 2007; Keov *et al.*, 2011). The idea of non-selectivity at allosteric sites has been previously seen with the muscarinic acetylcholine receptor (mAchRs) family of five receptors that, like the opioid receptors, share a very high level of homology. The allosteric modulator C_7/3-phth acts at all subtypes of mAchRs, although it has the highest affinity for the M_2R mAChR (Christopoulos *et al.*, 1999) and LY2033298 is a PAM at both M_2R and M_4R mAChRs (Chan *et al.*, 2008; Valant *et al.*, 2012). Indeed, recent crystallographic studies with mAchRs have found that the allosteric sites of these family members are quite homologous (Thal *et al.*, 2016), giving a rational explanation for the difficulty in developing selective allosteric ligands for these receptors as well as orthosteric ligands. However, selectivity may arise from the cooperativity of allosteric ligands with orthosteric sites and their ligands. For example, an allosteric ligand may bind different receptors, but only show cooperativity with the orthosteric site of one receptor and this may, or may not, be dependent on the orthosteric ligand.

One of the most pressing questions to better understand the mechanism of the allosteric modulators and for the rational design of modulators is the identification of their binding site(s) on the opioid receptors. Comparisons of two recent and independent molecular dynamics simulations suggest that the binding of BMS-986122 at μ-OR (Bartuzi *et al.*, 2016) and BMS-986187 at δ-OR (Shang *et al.*, 2016) rely upon the same residues at the top of TM domains 2 and 7 (Livingston and Traynor, 2017). Indeed, the striking similarity in theoretical binding pockets for these ligands at two different opioid receptors supports our pharmacological evidence of a

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conserved site. In the proposed region Tyr 2.64, His 7.36 and Ile 7.39 (numbers refer to Ballesteros-Weinstein generic numbering scheme; (Ballesteros and Weinstein, 1995) are conserved across μ-OR, δ-OR, and κ-OR. In particular, there may be an important role for Tyr 2.64, which is seen to undergo large conformational changes upon μ-OR receptor activation when comparing the inactive and active crystal structures of this receptor (Manglik *et al.*, 2012; Huang *et al.*, 2015). It is also worth noting that Tyr 7.35 in κ-OR, an amino acid implicated in the putative allosteric sites on δ-OR and μ-OR (Bartuzi *et al.*, 2016; Shang *et al.*, 2016), has been shown to be involved in hydrogen bond formation with the orthosteric antagonist JD-Tic (Wu *et al.*, 2012) which could indicate that JD-Tic is bitopic and reaches into the allosteric site of κ-OR. This residue 7.35, as a Trp or Tyr, is crucial for the binding and function of allosteric modulators and agonist-mediated conformational changes at a number of GPCRs (reviewed in Livingston and Traynor, 2017), including M1 (Abdul-Ridha *et al.*, 2014), M2 (Jäger *et al.*, 2007; Haga *et al.*, 2012; Dror *et al.*, 2013), M4 receptors (Thal *et al.*, 2016), β2AR and M2R (DeVree *et al.*, 2016).

The allosteric site for BMS-986122 and BMS-986187 does not appear to be conserved on NOPR as we failed to see allosteric activity of the ligands. There are differences in amino-acid composition when comparing the reported putative allosteric sites in μ-OR and δ-OR with the same region of the NOPR. This is not surprising as NOPR shares the least amount of homology with the other opioid receptors, and classically has not been characterized as an opioid receptor due to its inability to bind the antagonist naloxone (Cox *et al.*, 2015). The orthosteric binding sites of the four opioid receptors are generally conserved, but with one striking difference (reviewed in Cox, 2013). In μ-OR, δ-OR, and κ-OR a His in TM6 is joined by a two water molecule bridge to the phenolic hydroxyl on Tyr^1 of the opioid peptides or on the aromatic ring

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of small molecule opioids, including naloxone. In NOPR this His is replaced by Gln and so does not form the water bridge linking to the phenolic hydroxyl. Instead, NOPR ligands have a Phe in position 1 that is suggested to interact with a Tyr in TM3 via π–stacking (Thompson *et al.*, 2012). This difference could account for the lack of cooperativity of NOPR orthosteric agonists with BMS-986122 and BMS-986187. Nevertheless, it cannot be ruled out that these allosteric ligands might be modulators of NOPR but have much lower affinity so that higher concentrations are required or, due to probe dependence, may exhibit cooperativity with ligands other than Ro64-6198 and the endogenous ligand nociceptin that we examined. For example, the CCR5 receptor negative allosteric modulator aplaviroc fully prevents the binding of the chemokine CCR3 while having almost no effect on CCL5 binding (Watson *et al.*, 2005). In addition, BMS-986187 and BMS-986122 might be SAMs at NOPR but without a known PAM for NOPR, testing this is not currently possible.

There are very few examples reported of endogenous allosteric modulators for GPCRs (for review see van der Westhuizen *et al.*, 2015) including dynorphin A, a putative allosteric modulator of the M_2 mAChR (Hu and El-Fakahany, 1993), glutathione, an allosteric ligand for the calcium sensing receptor (Wang *et al.*, 2006; Broadhead *et al.*, 2011), as well as lipoxin A4 which has been proposed as an endogenous cannabinoid receptor modulator (Duarte *et al.*, 2012). Since evidence presented suggests the three classic members of the opioid family of receptors share a similar allosteric binding site, it is tempting to speculate that this may be caused by evolutionary pressure from an endogenous allosteric modulator that binds the opioid receptors, though this may be vestigal.

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Authorship Contributions

Participated in research design: Livingston and Traynor.

Conducted experiments: Livingston, Stanczyk, Burford, Alt

Contributed new reagents or analytic tools: Alt and Burford.

Performed data analysis: Livingston, Canals and Traynor.

Wrote the manuscript: Livingston and Traynor.

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Footnotes

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Table 1: Effects of BMS-986187 on ligand affinity and [^{35}S]GTPγS binding at μ, δ, κ and NOP receptors

	Ki (nM)		Potency (GTPγ^{35}S)(nM)		E max (% standard)	
	Control	**BMS-986187**	**Control**	**BMS-986187**	**Control**	**BMS-986187**
μ-OR[a]						
DAMGO	724 (562-912)	63 (13-316)*	91 (55-154)	16 (6-38)*	111 (103-119)	110 (105-115)
Methadone	603 (288-1259)	25 (19-32)*	203 (121-284)	10 (3-24)*	105 (98-111)	107 (102-111)
Morphine	229 (178-302)	71 (43-120)*	120 (85-166)	38 (10-151)	70 (67-74)	90 (83-97)*
δ-OR[b]						
Leu-Enkephalin	221 (119-324)	7 (3-12)*	28 (18-42)	3.6 (1.6-8.2)*	87 (79-95)	94 (89-100)
SNC80	71 (20-122)	5 (3-7)*	NT	NT	NT	NT
TAN67	10 (7-14)	3 (0.2-5.8)*	NT	NT	NT	NT
κ-OR[c]						
U69,593[d]	0.9 (0.3-1.6)	0.3 (0.2-0.5)[#]	881 (480-1601)	64 (35-121)*	105 (82-128)	104 (89-118)
Dynorphin A	NT	NT	15 (11-19)	1.0 (0.5-2)*	100 (95-105)	95 (86-104)
Salvinorin A	NT	NT	322 (221-471)	25.9 (11-60)*	110 (97-122)	116 (101-131)
Diprenorphine	NT	NT	46 (25-37)	8.9 (1.9-26)	31 (25-37)	56 (47-67)*
NOPR[e]						
Nociceptin	NT	NT	2.6 (2.0-4)	2.1 (1.5-3)	102 (94-110)	105 (100-111)
Ro-64-6198	NT	NT	13 (8.0-19)	18 (7.8-45)	109 (102-115)	108 (93-123)

a. In C6 rat glioma cells expressing rat μ-OR; b. Data taken from Burford et al. 2015 using; c. In CHO cells expressing human κ-OR; d. Determined by saturation binding in Tris-buffer; e. HEK293 cells expressing human NOPR; NT = not tested.

Values in parentheses represent 95% confidence intervals

*Significantly different from control conditions (non-overlapping confidence intervals)

[#] p = 0.03 compared to control (paired t -test)

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Figure Legends

Fig. 1. Structure of (A) BMS-986187, (B) BMS-986122, and (C) BMS-986124

Fig 2. BMS-986187 enhances the affinity, potency, and/or maximal stimulation of several opioid ligands at μ-OR expressed in C6 cell membranes. (A) BMS-986187 alone did not displace ^{3}H-DPN from the orthosteric site or (B) stimulate GTPγ^{35}S binding The presence of 10 μM BMS-986187 (filled symbols) increased the binding affinity and potency or maximal effect to stimulate GTPγ^{35}S for DAMGO (C,D), methadone (E,F) and morphine (G, H) compared to control conditions (open symbols). Data are presented as percent ^{3}H-DPN bound for the binding data or % stimulation of a maximal concentration (10 μM) of the full agonist DAMGO for the GTPγ^{35}S data. Nonlinear regression analysis using GraphPad Prism 6.01 fit all curves to one-site. Data shown are means ± SEM of 3 (^{3}H-DPN) or 4 (GTPγ^{35}S) independent experiments each performed in duplicate.

Fig. 3. BMS-986187 has a concentration-dependent effect on the potency of methadone to activate G protein. Stimulation of GTPγ^{35}S binding in C6μ cell membranes by methadone was performed in the presence of increasing concentrations (0.3-10 μM) of BMS-986187. Data were analyzed using a modified allosteric ternary complex model as described in the methods. Points shown are means ± SEM of 3 independent experiments each in duplicate.

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Fig. 4. Effects of BMS-986187 on opioid-mediated adenylate cyclase (AC) inhibition and β-arrestin-2 recruitment in CHO-μ cells. (A) concentration-response curves for the full μ-OR agonist DAMGO to inhibit AC in the absence or presence of increasing concentrations of BMS-986187. (B) concentration-response curves of DAMGO to recruit β-arrestin-2 were performed in the absence or presence of increasing concentrations of BMS-986187 as listed in panel A. (C) and (D) effect of BMS-986187 on inhibition of AC or recruitment of β-arrestin-2 in the absence of orthosteric agonist. Data were analyzed using GraphPad Prism 6.01 and points shown are means ± SEM of 3 independent experiments, each done in quadruplicate. Data in (A) and (B) were analyzed using an allosteric ternary complex model as described in the methods.

Fig. 5. Antagonism between Na^+ ions and BMS-986187. (A) The displacement by DAMGO of ^{3}H-DPN binding to μ-OR is inhibited by Na^+ ions (open circles). BMS-986187 (10 μM) enhances the binding of DAMGO and reduces the potency of Na^+ ions to inhibit DAMGO binding (closed circles). (B) The ability of increasing concentrations of NaCl to decrease constitutive δ-OR-mediated GTPγ^{35}S binding in the absence (open circles) or presence (filled circles) of increasing concentrations of BMS-986187 was measured in membranes from CHO-δ cells. Data were analyzed GraphPad Prism 6.01 and points shown are means ± SEM of 3 independent experiments, each in duplicate.

Fig 6. Interactions between BMS-986187 and related allosteric modulators. (A) The ability of 10 μM BMS-986187 to enhance the potency of DAMGO-mediated GTPγ^{35}S stimulation at the μ-OR in C6μ cell membranes was measured in the presence or absence of 30 μM of the μ-SAM

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BMS-986124. (B) In C6δ cell membranes the effect of 300 nM BMS-986187 to enhance the potency of Leu-Enkephalin-mediated GTPγ^{35}S stimulation was measured in the presence or absence of the μ-SAM 30 μM BMS-986124. (C) The ability of 300 nM BMS-986187 to enhance the potency of Leu-Enkephalin-mediated GTPγ^{35}S stimulation at δ-OR was measured in the presence or absence of 30 μM of the μ-PAM BMS-986122. (D) 30 μM BMS-986122 has a small effect on the potency of Leu-Enkephalin to activate G protein. (E) BMS-986122 and BMS-986124 inhibit the ago-PAM action of BMS-986187 to stimulate GTPγ^{35}S binding to CHOδ cell membranes. *BMS-986187-mediated stimulation of GTPγ^{35}S binding is different from the vehicle control and significantly inhibited by BMS-986122 or BMS-986122 (ANOVA with Tukey post-hoc test). Data shown are mean ± SEM from 3 experiments in duplicate. Potency values were obtained from fitting the data by linear regression with Hill slopes of unity using GraphPad Prism 6.01.

Fig. 7. BMS-986187 is a PAM at κ-OR. (A) Using CHO cells expressing κ-OR, the ability of dynorphin A (1-17) to active G protein as measured by the GTPγ^{35}S binding assay was determined in the absence or presence of 10μM BMS-986187. Using the same cell membranes, concentration-response curves were generated for (B) U69,593, (C) Salvinorin A and (D) DPN in the presence of vehicle or 10 μM BMS-986187. (E) Representative experiment (of 4 each in duplicate) showing the affinity but not maximal binding of ^{3}H-U69,593 was enhanced in the presence of BMS-986187. (F) BMS-986122 or BMS-986124 did not alter the binding of ^{3}H-U69,593 but blocked the PAM effect of BMS-986187. *Analysis by ANOVA followed by

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Tukey post-hoc showed significant differences between BMS-986187 alone and all other conditions.

Fig. 8. BMS-986187 (10 μM) failed to alter the potency of the NOPR agonists (A) nociceptin or (B) Ro-64-6198 to stimulate GTPγ^{35}S binding in membranes from HEK293 cells stably expressing human NOPR. Data shown are means ± SEM from 4 experiments in duplicate fitted by linear regression with Hill slopes of unity using GraphPad Prism 6.01.

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Fig 1

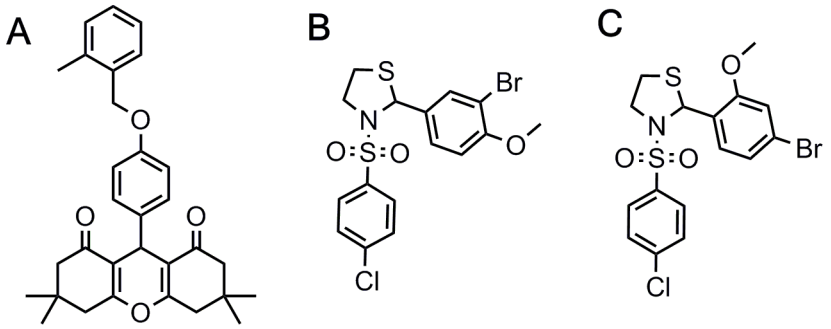

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Fig 2

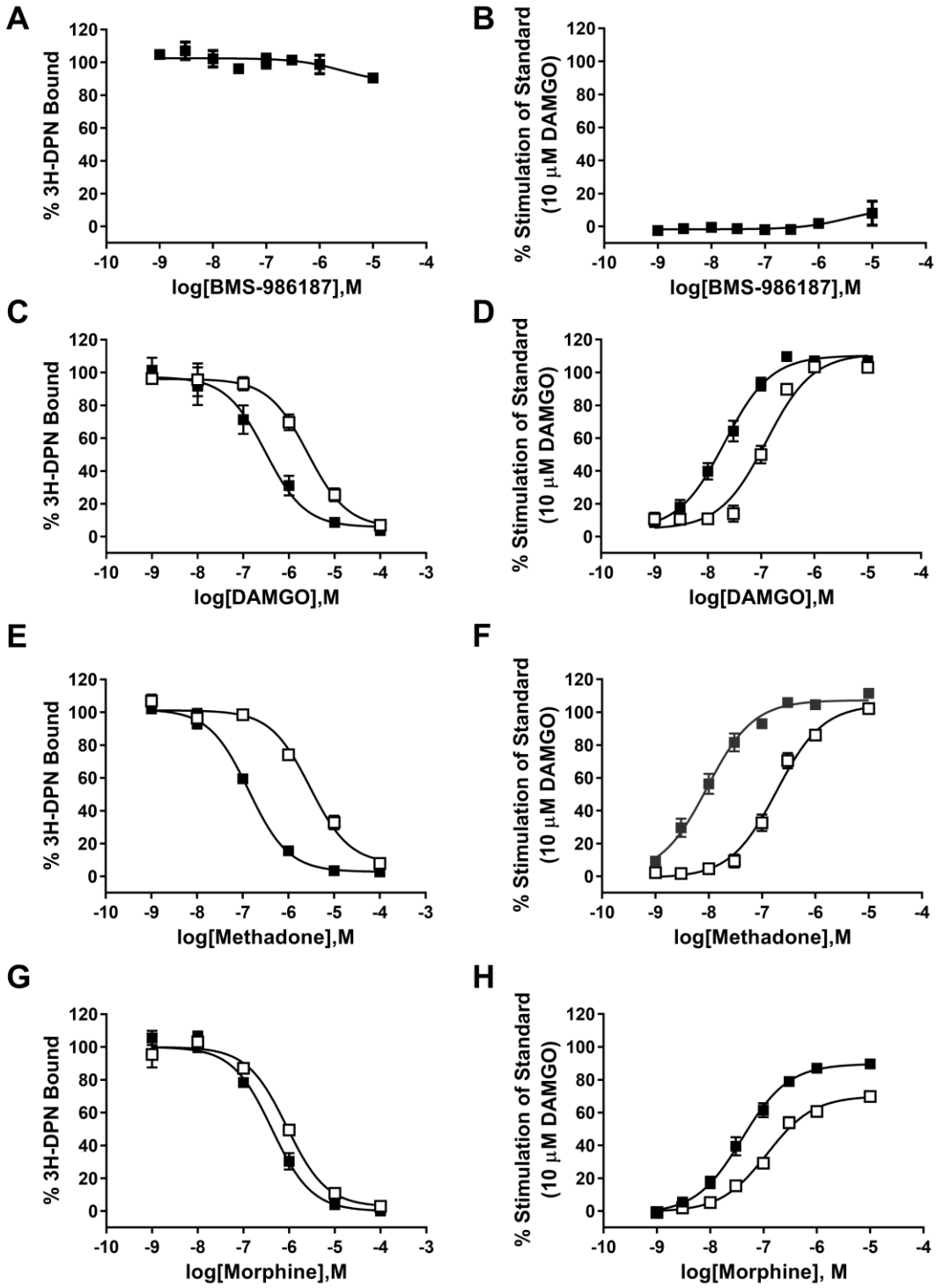

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Fig 3

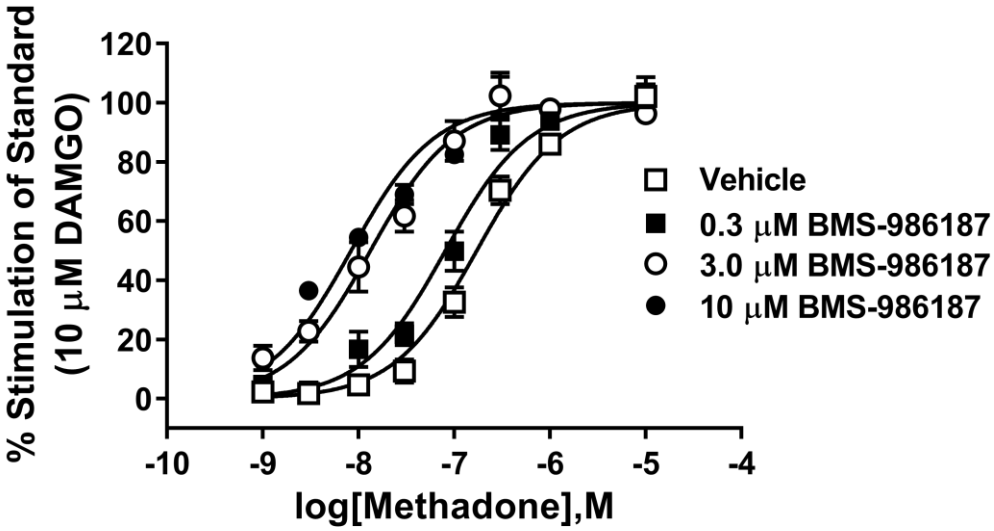

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Fig 4

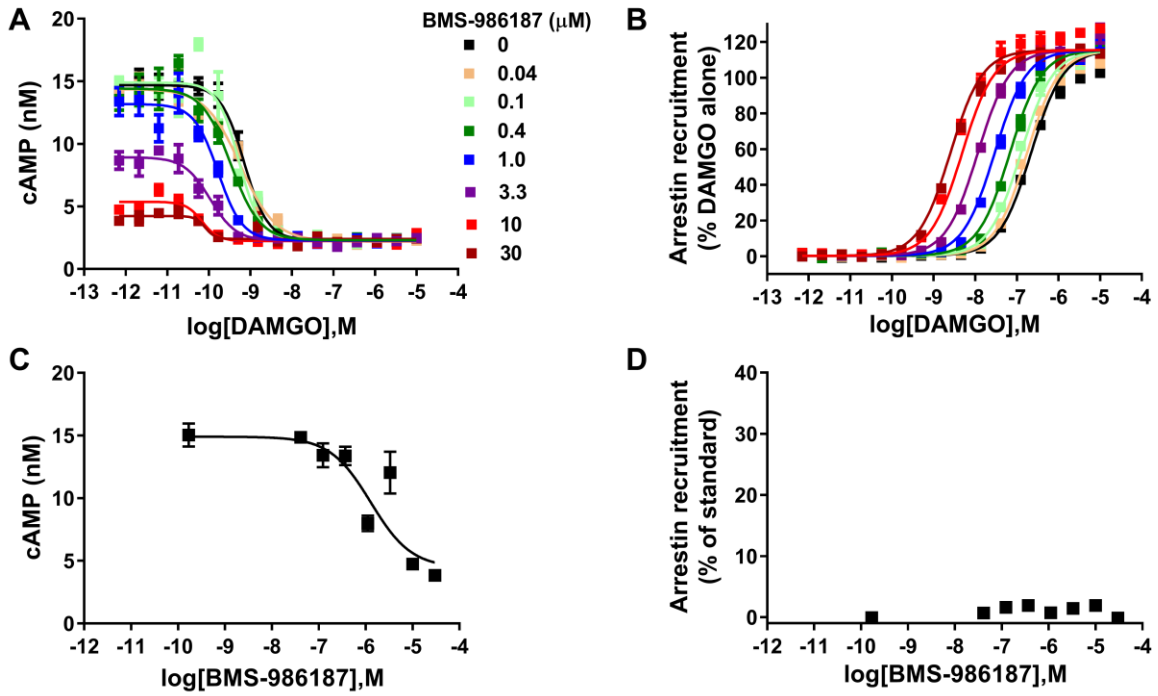

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Fig 5

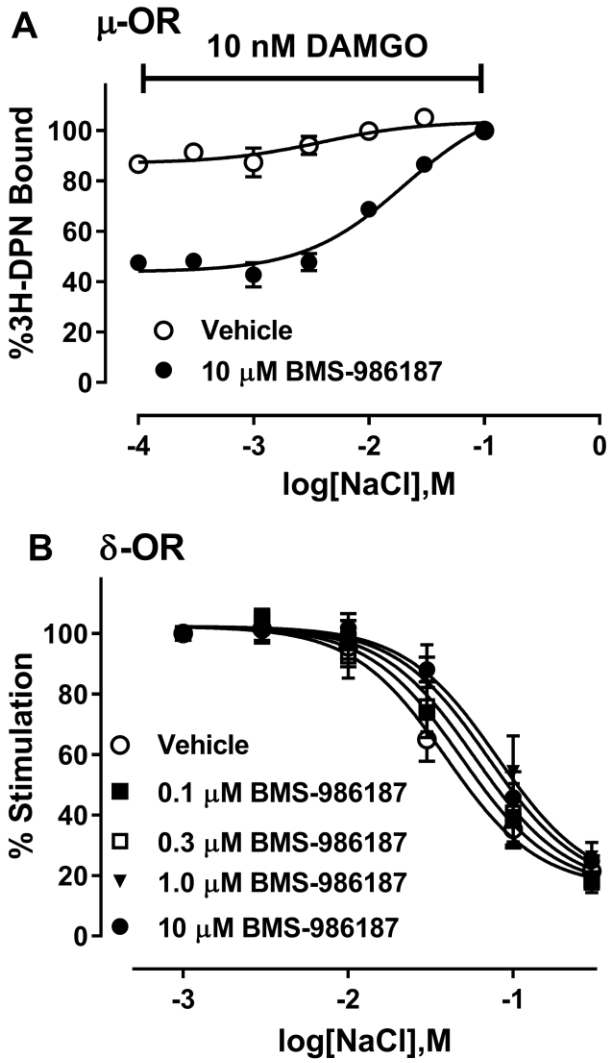

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Fig 6

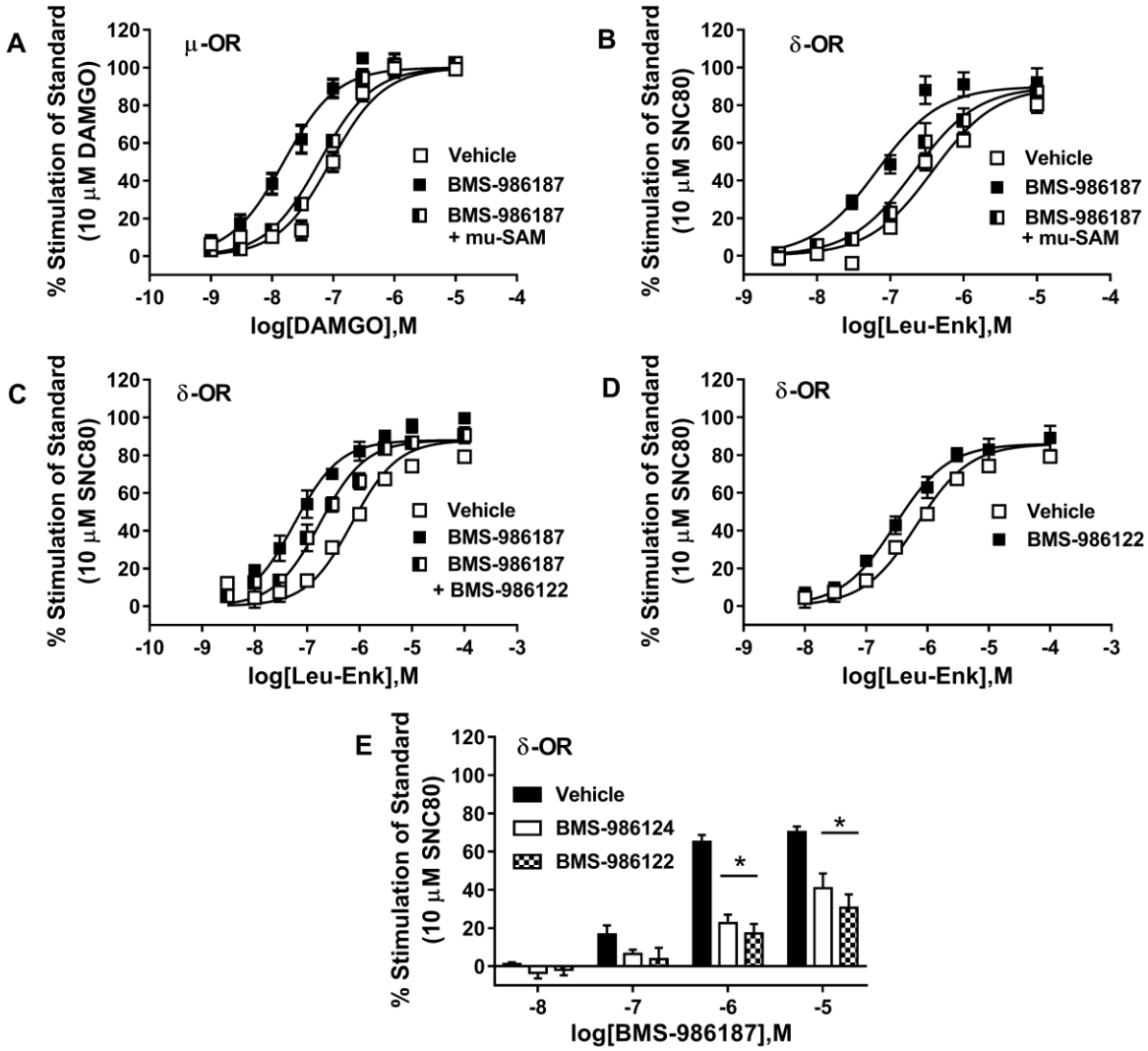

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Fig 7

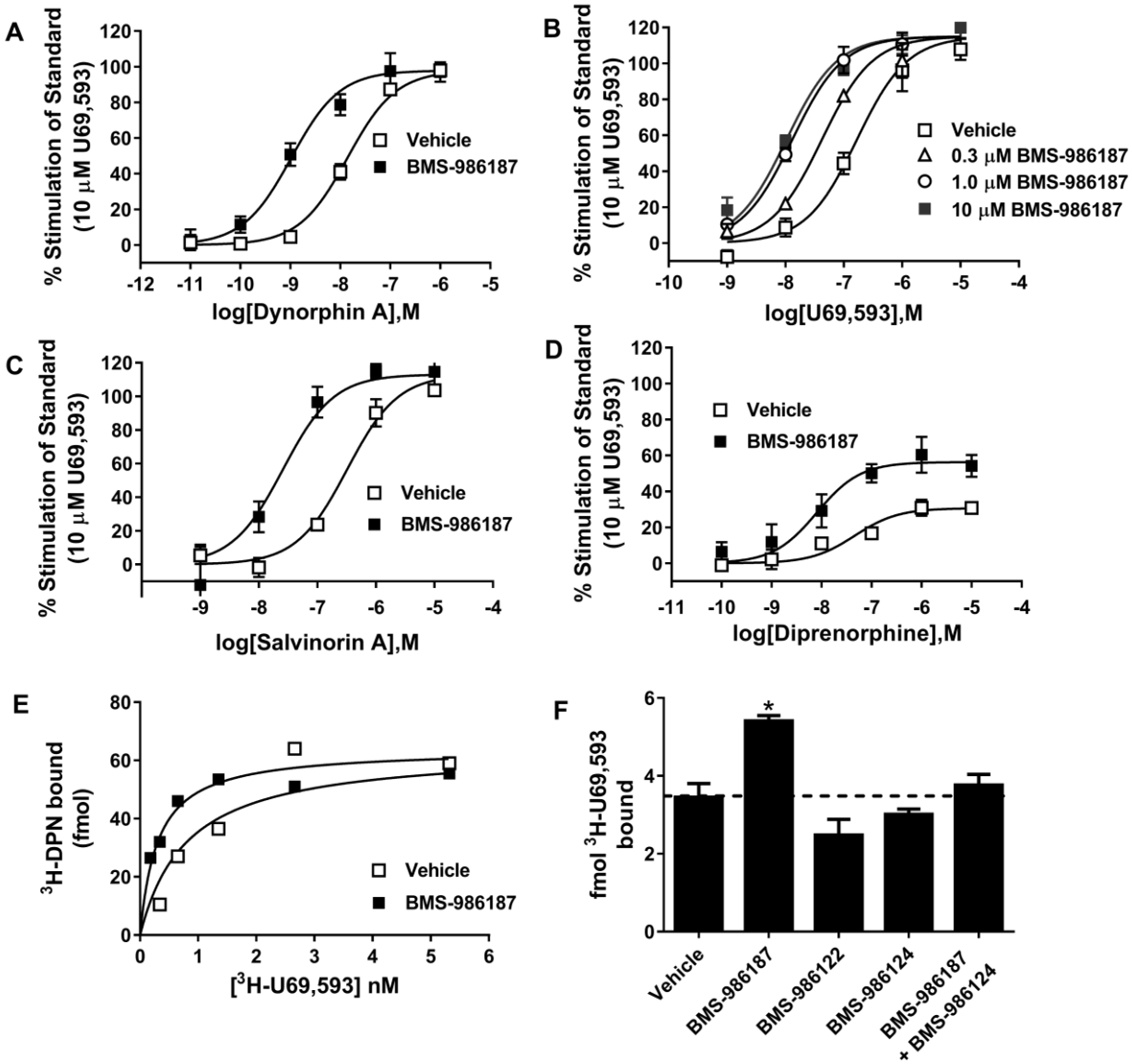

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Fig 8