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Declarations under Rule 4.17:

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[Continued on next page]

(54) Title: POSITIVE ALLOSTERIC MODULATORS AND SILENT ALLOSTERIC MODULATORS OF THE OPIOID RECEPTOR

(57) Abstract: Disclosed are positive allosteric modulators (PAMs) and silent allosteric modulators (SAMs) for mu (μ)-opioid receptors that may be useful for the treatment of pain, either alone or in combination with orthosteric opioid receptor agonists. Methods for treating pain and modulating mu (μ)-opioid receptors are also disclosed.

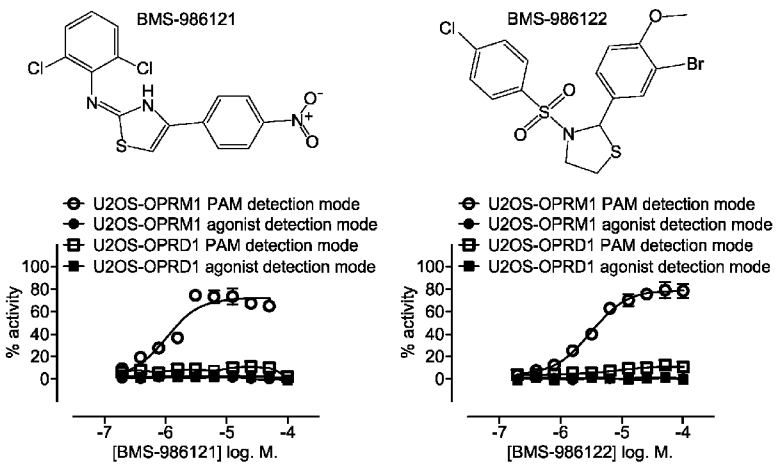

FIG. 1

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POSITIVE ALLOSTERIC MODULATORS AND SILENT ALLOSTERIC MODULATORS OF THE OPIOID RECEPTOR

CROSS REFERENCE TO RELATED APPLICATION

This application claims the priority of U.S. Provisional Application Serial No. 61/748,946 filed January 4, 2013 which is herein incorporated by reference.

DESCRIPTION OF THE INVENTION

A Bibliography is appended hereto that contains references (1) through (34), each of which is incorporated by reference herein.

The invention is specifically described herein with respect to two compounds, i.e., BMS-986121 and BMS-986122 (shown in Fig. 1), which are presented for purposes of exemplification.

The application of the invention is not intended to be limited in scope to these two compounds. Instead, the application of the invention is intended to cover any compounds which function to provide the desirable aspects of the invention. In particular, compounds to which the invention may be applicable include any compounds which function to bind to the opioid receptors and enhance the binding affinity or efficacy (or both) of an orthosteric agonist.

The superfamily of G protein-coupled receptors (GPCRs), comprises plasma membrane spanning proteins that transduce signals via heterotrimeric G proteins on the inner surface of the plasma membrane leading to intracellular signaling cascades involved in many aspects of cellular function (1). The cell surface location, tissue distribution, and diversity of these GPCRs make them ideal targets for drug intervention. Indeed, about 30% of marketed drugs target specific GPCR activity (1, 2).

Opioid receptors are members of the Class A family of GPCRs. Four opioid receptor types exist (mu, delta, kappa, and ORL1) which share about 60% amino acid

identity (mainly in the transmembrane domains) and signal through the Gi/o family of heterotrimeric G proteins, resulting in inhibition of adenylyl cyclase, modulation of ion channel activity, and transcriptional changes in the cell (3). Opioid receptors (and many other GPCRs) can also signal through non-G-protein mediated pathways, one of which is initiated by β-arrestin recruitment to the receptor. β-arrestin is involved in receptor desensitization and internalization/recycling (4, 5). Opioid receptors are key targets in the management of pain and morphine and its derivatives induce pain relief by acting as agonists at opioid receptors, especially the mu-opioid receptor (6, 7). Opioid receptors have been extensively studied because of the need for better pain control while trying to reduce or eliminate adverse side effects. These side effects include tolerance, respiratory suppression, constipation, allodynia, and dependence (3, 8).

To overcome these side effects, studies have focused on developing more selective agonists, which target one specific opioid receptor type over the others, or partial agonists (which have reduced efficacy compared to full agonists), or agonists used together in combination therapy (9, 10). However, these diverse approaches have a single commonality in that they target the orthosteric (endogenous) agonist binding site of the receptor. A different approach that has been used successfully with other GPCRs is the discovery and development of allosteric ligands, which can have specific advantages over their orthosteric counterparts.

Allosteric ligands for a GPCR bind to a site on the receptor that is distinct from the site that binds the orthosteric (or endogenous) agonist (11, 12). An allosteric modulator (AM) can exhibit a range of activities at the target protein. Positive allosteric modulators (PAMs) may have no intrinsic efficacy, but when they bind to the receptor enhance the binding affinity or efficacy (or both) of the orthosteric agonist. Negative allosteric modulators (NAMs) have no intrinsic efficacy, but when they bind to the receptor inhibit the binding affinity or efficacy (or both) of the orthosteric agonist. Silent allosteric modulators (SAMs), also known as neutral allosteric ligands (NALs), bind to the receptor but have no effect on orthosteric agonist affinity or efficacy. However, SAMs can act as competitive antagonists at the same allosteric site, blocking PAM or NAM

activity. Although not particularly useful from a therapeutic standpoint, SAMs can be effective tools to show that presumed PAM or NAM effects are receptor mediated. Finally, allosteric agonists can bind and produce direct agonist activation of the receptor even in the absence of orthosteric agonist.

Allosteric ligands have the potential to exhibit greater selectivity between subtypes of GPCRs in the same family compared with orthosteric ligands. This has been demonstrated for some GPCRs including metabotropic glutamate receptors, adenosine receptors and muscarinic receptors (13-17). This increased selectivity is hypothesized to be based on the evolutionary constraint placed on the orthosteric site between closely related receptor subtypes that bind the same endogenous ligand. This evolutionary constraint may not be required for allosteric sites.

While highly selective orthosteric agonist ligands exist for opioid receptors, additional advantages of PAMs provide intriguing opportunities for opioid receptor PAMs as potential therapeutics in pain management.

PAMs, unlike allosteric agonists, may have no effect when they bind to the receptor in the absence of orthosteric agonist. Therefore, the modulation occurs only when an orthosteric agonist is bound to the receptor. *In vivo*, this leads to preservation of the temporal and spatial characteristics of cell signaling; this is important, especially for signaling in the complex neuronal networks in the brain and enteric nervous system. Additionally, by preserving the temporal aspects of native receptor signaling, PAMs may avoid receptor down-regulation and other compensatory mechanisms that are triggered by sustained receptor activation produced by exogenous orthosteric agonists. Therefore, opioid receptor PAMs could be expected to produce less tolerance and dependence than exogenous orthosteric agonists. Here, we describe the discovery and characterization of mu-opioid receptor PAMs and SAMs. A high-throughput screen (HTS) was developed and executed using a β-arrestin recruitment assay. Mu-selective PAMs resulting from the HTS were shown to be active in both β-arrestin recruitment assays, and in G-protein mediated signaling assays (inhibition of adenylyl cyclase activity

and [^{35}S]GTPγS binding). These studies are the first to describe the existence of mu-selective PAMs and SAMs implicating positive allostery as a potential novel avenue for the discovery of tightly regulated pain therapeutics.

Potential opioid receptor ligands were identified from an HTS campaign using the PathHunter® enzyme complementation assay technology (DiscoveRx, CA) (18). In this system an N-terminal deletion mutant of β-galactosidase, termed enzyme acceptor (EA), is fused to the C-terminus of stably expressed β-arrestin 2 in U2OS cells. A mutated amino-terminal fragment of β-galactosidase, termed ProLink™ (PK), is fused to the carboxyl terminus of the OPRM1 receptor recombinantly expressed in these cells (U2OS-OPRM1). Binding of arrestin to the activated mu-opioid receptor results in a complementation of the enzyme and reconstitution of enzyme activity. Thus, complemented enzyme activity can be used as a measure of the recruitment of arrestin to the mu-opioid receptor. The HTS campaign was performed in the presence of a low (20 nM, ~EC_{10}) concentration of the mu-selective orthosteric agonist, endomorphin-I, to identify both agonists and positive allosteric modulators (PAMs). Two compounds were identified as potential mu-opioid receptor PAMs (mu-PAMs). These compounds have been designated as BMS-986121 and BMS-986122, and their structures are shown in Fig. 1A and B. Neither BMS-986121 nor BMS-986122 produced significant β-arrestin recruitment on their own (agonist detection mode), but both compounds significantly augmented the β-arrestin recruitment response produced by a low concentration of endomorphin-I (PAM detection mode) (Fig. 1C and D). In PAM detection mode, BMS-986121 increased β-arrestin recruitment by 20 nM endomorphin-I to E_{max} (95%CI) of 72 (66-78) % of the response evoked by a maximally effective (1 uM) concentration of endomorphin-I, with an EC_{50} (95%CI) of 1.0 (0.7-1.4) uM. BMS-986122 produced a similar PAM detection mode response increasing the effect of the low concentration of endomorphin-I to 79 (73-84) % of the maximal endomorphin-I response with an EC_{50} of 3.0 (2.3-3.8) uM.

To test the specificity of the response, the compounds were examined in a similar assay in U2OS PathHunter® cells expressing PK-tagged delta opioid receptors

(U2OS-OPRD1). Neither compound had any significant effect in the absence (agonist detection mode) or the presence (PAM detection mode) of a ~EC_{10} (0.4 nM) of the delta agonist leu-enkephalin (Fig. 1C and D). These results suggest that the effects of BMS-986121 and BMS-986122 are mediated through activation of mu-opioid receptors and that the compounds are selective for mu- over delta-opioid receptors.

To further assess mu-PAM activity BMS-986121 and BMS-986122 were tested in three functional assays, β-arrestin recruitment, inhibition of adenylyl cyclase activity, and G protein activation using [^{35}S]GTPγS binding. Compounds were also assessed in receptor binding assays.

Concentration-response curves (CRCs) for endomorphin-I-mediated recruitment of β-arrestin were generated in the absence or presence of varying concentrations of each mu-PAM. BMS-986121 (Fig. 2A) or BMS-986122 (Fig. 2B) produced concentration-dependent and saturable leftward shifts in the potency of endomorphin-I. BMS-986121 produced a maximal 9-fold increase in the potency of endomorphin-I. The concentration of BMS-986121 that produced a half-maximal leftward shift was 1.7 uM. BMS-986122 produced a maximal 8-fold increase in the potency of endomorphin-I, with a half maximal leftward shift value of 4.9 uM (Fig. 2C).

Opioid receptors inhibit adenylyl cyclase via $G\alpha_{i/o}$ proteins (19). In order to assess the effects of the mu-PAMs on this signaling pathway, their effects were measured in a cAMP accumulation assay. The cAMP inhibition responses produced by opioid agonists in the U20S PathHunter® cells were small and inadequate for robust measurement. Therefore, a Chinese hamster ovary (CHO) cell line recombinantly expressing human mu opioid receptors (CHO-mu) was used for these experiments. In this cell line, mu-opioid receptor agonists produce robust and reproducible inhibition of forskolin (1 uM)-stimulated cAMP accumulation. Endomorphin-I produced a 17-fold reduction in cAMP accumulation with an EC_{50} of 76 (60-96) pM (Fig. S1). BMS-986121 and BMS-986122 significantly increased the inhibition of forskolin-stimulated adenylyl cyclase activity produced by a ~EC_{10} (30 pM) concentration of endomorphin-I in CHO-mu

cells (Fig. 3A and B). BMS-986121 and BMS-986122 both afforded potentiation with EC_{50} values of 2.2 (1.7-2.8) and 8.9 (6.1-13.1) uM respectively. The maximal inhibition produced by endomorphin-I in the presence of the PAMs was similar to that of a maximal concentration (10 nM) of endomorphin-I alone. In this assay both mu-PAMs also exhibited some intrinsic agonist activity causing inhibition of cAMP accumulation in the absence of any orthosteric agonist (Fig. 3A and B). The low efficacy agonist activity of BMS-986121 (EC_{50} of 14 (2-100) uM; E_{max} of 35 (6-63) %) was not always reproducible and on some occasions, was too low to determine a fit of the concentration response data. BMS-986122 agonist activity (EC_{50} of 41 (20-86) uM; E_{max} of 60 (24-95) %) was more apparent. The agonist activity of BMS-986121 and BMS-986122 was only seen at concentrations above those required to produce significant potentiation of an endomorphin-I response, and both agonist responses failed to reach the maximal effect of endomorphin-I.

The discrepancy between the agonist activity of the PAMs seen in this assay and the lack of agonist activity seen in the β-arrestin recruitment assay in U2OS-OPRM1 cells may be due to differences in apparent receptor reserve for the two assays and/or cell-lines. In the recombinant CHO cells, endomorphin-I is ~1000-fold more potent for inhibition of forskolin-stimulated cAMP accumulation in comparison with arrestin recruitment in U2OS PathHunter® cells, suggesting that significant levels of receptor reserve are present in the cAMP assay compared with the arrestin assay. It has been shown previously that PAMs can exhibit agonist activity (albeit at higher concentrations than those seen for PAM activity) in recombinant cells expressing high levels of GPCR protein (20). Indeed, it has been suggested that PAMs are aspiring allosteric agonists, and the degree of agonist efficacy observed depends largely on the sensitivity of the system and assay used to detect signals (21).

Next, the mu-PAMs were characterized in G protein activation [^{35}S]GTPγS binding studies in membranes from C6 glioma cells stably expressing recombinant mu-opioid receptors (C6mu) (22). Agonist-stimulated [^{35}S]GTPγS binding was determined after a 5 min. incubation to capture the initial rate of G protein activation which can

differentiate partial agonists from full agonists in this cell line. The mu-opioid receptor agonist DAMGO at 10 uM produced a 250% stimulation of [^{35}S]GTPγS binding above basal activity, with an EC_{50} of 222 (179-274) nM. BMS-986121 (10 uM) resulted in a 4-fold leftward shift in the DAMGO CRC (EC_{50} of 57 (37-89) nM) (Fig. 4A). BMS-986122 (10 uM) resulted in the DAMGO CRC shifting leftwards by 7-fold (EC_{50} of 32 (25-40) nM) (Fig. 4B). No significant agonist activity was detected for either of the PAM compounds in this assay.

Mu-opioid receptor ligand binding was determined in the presence of 100 mM NaCl and 10 uM GTPγS. In saturation binding studies the mu-PAM, BMS-986122, did not affect [^{3}H]diprenorphine binding affinity (K_d in the presence of vehicle was 0.27 (0.21-0.32) nM; K_d in the presence of BMS-986122 (10 uM) was 0.35 (0.18-0.51) nM) but significantly increased the affinity of DAMGO by 6-fold in competition studies with 0.2-0.3 nM [^{3}H]diprenorphine binding (K_i in the presence of vehicle was 340 (208-552) nM; K_i in the presence of BMS-986122 (10 uM) was 56 (41-76) nM) (Fig. 4E; Fig. S2A; Table S1). These data suggest that BMS-986122 is, at least in part, a positive affinity modulator of the mu-opioid receptor for the orthosteric agonist DAMGO.

Enhancement of the maximal response to a partial agonist in the [^{35}S]GTPγS binding assay would suggest that the mu-PAMs are able to modulate observed efficacy in this system. Morphine produced an E_{max} of 42 (38-45) % of the response induced by 30 uM DAMGO after 5 min. incubation with [^{35}S]GTPγS, with an EC_{50} of 110 (71-171) nM (Fig. 4C and D). This confirms that morphine is a partial agonist in this assay system relative to DAMGO (22). BMS-986121 increased morphine potency by 2.5-fold (EC_{50} of 45 (29-68) nM) (Fig. 4C). The potency of morphine was shifted to the left 3-fold in the presence of 10 uM BMS-986122 (EC_{50} 38 (24-61) nM) (Fig. 4D). The maximal effect (E_{max}) of morphine compared to DAMGO was increased by BMS-986121 (72 (67-78) %) (Fig. 4C) and by BMS-986122 (74 (68-81) %) (Fig. 4D). These data confirm that BMS-986121 and BMS-986122 can positively modulate the observed efficacy, measured as an increase in maximal response of the partial agonist morphine in this system.

The previous experiments used heterologous cell systems expressing high concentrations of receptors. To determine whether mu-PAM activity can be observed in native tissues, DAMGO-stimulated [^{35}S]GTPγS binding in membranes from mouse brain was assessed (Fig. 4F). The potency of DAMGO to stimulate [^{35}S]GTPγS binding (EC_{50} of 458 (245-856) nM) was shifted to the left 4.5-fold in the presence of BMS-986122 (EC_{50} of 101 (56-183) nM). No agonist activity was observed with BMS-986122. Therefore, mu PAM activity can also be observed for DAMGO-mediated G protein activation in membranes from a physiologically relevant tissue with endogenous levels of receptor and G-protein.

A number of close analogs of BMS-986122 were tested in the β-arrestin recruitment assay in order to explore the structure-activity relationship (SAR) of the chemical series. Of the 15 analogs tested, 13 showed at least some PAM activity at the mu receptor. None of these compounds showed agonist activity at the mu-opioid receptor (Fig. S3). However, 5 of the analogs did show some low efficacy agonist or PAM activity at the delta receptor (Fig. S3), suggesting that modifications to this chemotype may alter the selectivity for mu-opioid vs. delta-opioid receptors. Modifications to the structure of BMS-986122 affected the compounds' PAM activity in U2OS-OPRM1 cells (Fig. S3; Table S2). For the most part, the analogs examined retain similar potencies relative to BMS-986122 (EC_{50} values in the low μM range). However, minor changes to the structure of BMS-986122 led to a pronounced reduction in the E_{max} values observed in PAM detection mode. This can be inferred to correspond to a decrease in the maximum leftward shift in endomorphin-I potency that can be produced by a compound. Of the analogs tested, none exhibited greater PAM activity than the original screening hit BMS-986122.

It has been observed that allosteric modulators of GPCRs can often exhibit "activity switching" within a chemical series: minor modifications in the chemical structure can change a compound from a PAM to a negative (NAM) or silent (SAM) allosteric modulator (23). The absence of observed PAM efficacy in 2 of the analogs may be due to loss of binding affinity, or functional switching from PAMs to NAMs or SAMs.

Therefore, two of these BMS-986122 analogs (designated BMS-986123 and BMS-986124) were assessed for their ability to inhibit orthosteric agonist activity (in a NAM detection mode assay), or for their ability to inhibit BMS-986122 PAM activity (a SAM detection mode assay) in the β-arrestin recruitment assay in U2OS-OPRM1 cells and in the [^{35}S]GTPγS assay in C6mu cells. Neither compound significantly inhibited an EC_{80} concentration of endomorphin-I (300nM) (Fig. S4) suggesting that they are not NAMs or orthosteric antagonists. However, both compounds were able to inhibit the PAM response to 12.5 uM (~EC_{80}) BMS-986122 in U2OS-OPRM1 cells in the presence of 30 nM (~EC_{20}) endomorphin-I (Fig. 5A). Calculated K_b values (the inhibition constant for a competitive antagonist which at equilibrium would occupy 50% of the receptors in the absence of agonist) for SAM activity of BMS-986123 and BMS-986124 were 1 uM and 2 uM, respectively (Table S2). In a separate set of experiments, DAMGO potency to stimulate [^{35}S]GTPγS binding in C6mu cell membranes (EC_{50} of 224 (167-300) nM) was again enhanced 8-fold in the presence of BMS-986122 (10 uM) (EC_{50} of 29 (22-38) nM) (Fig. 5B, Fig. S5). Co-addition of BMS-986122 (10 uM) with BMS-986124 (50 uM), resulted in an inhibition of the PAM effect, with DAMGO potency enhanced less than 2-fold (EC_{50} of 128 (97-168) nM) compared to the DAMGO potency in the presence of the vehicle control (Fig. 5B; Fig. S5). Together these data confirm that the PAM effects of BMS-986122 can be antagonized by BMS-986124 and strongly suggest that BMS-986123 and BMS-986124 are mu-opioid receptor SAMs (mu-SAMs), competitive antagonists at the allosteric site to which BMS-986122 binds.

The mu-SAM, BMS-986123, produced a small (~2-fold) but significant decrease in [^{3}H]diprenorphine binding affinity (Fig. S2) but had no significant effect on DAMGO binding affinity (Table S1). The potency of DAMGO or morphine in the [^{35}S]GTPγS assay was not significantly increased by BMS-986123 or BMS-986124 (Fig. S6 and S7) at 10 uM, although both SAMs increased morphine E_{max} to a small degree (Fig. S7). No significant agonist activity was detected for either of the SAM compounds in this assay.

Probe dependence (the ability of an allosteric modulator to modulate one orthosteric agonist but not another) is a striking characteristic of some allosteric

modulators (24). BMS-986121 (100 uM) produced leftward shifts in the potency of endomorphin-I (4.3-fold), morphine (6.5-fold), and leu-enkephalin (4.5-fold), in inhibition of forskolin-stimulated cAMP accumulation assays in CHO-mu cells (Fig. S8). Taken together with the DAMGO and morphine data sets from the [^{35}S]GTPγS binding studies and the β-arrestin data, there is no current evidence to suggest strong probe dependence as BMS-986121 produced similar potentiation of peptide agonist- and small molecule agonist-evoked responses.

As noted above, PAMs (unlike allosteric agonists) generally only modulate the activity of the receptor when an orthosteric agonist is bound, maintaining the temporal and spatial aspects of cell signaling *in vivo*. Therefore, PAMs have a striking advantage over their orthosteric agonist counterparts. With traditional agonist ligands, the receptor is turned on for long periods (based on the dosing regime), often resulting in adverse effects, such as desensitization of the receptor response or receptor-mediated side-effects caused by long-term stimulation. In the case of opioid receptors, long-term dosing with opiates leads to the development of tolerance and dependence, as well as other acute receptor-mediated side-effects such as, respiratory suppression, constipation and allodynia (3, 8). We have determined that the mu-PAMs described here can positively modulate mu-opioid receptor responses to the endogenous agonists endomorphin-I and leu-enkephalin. It will be important to determine whether mu-PAMs can produce antinociceptive effects when administered alone *in vivo*, potentiating responses to endogenous opioid agonists. Evidence for a basal tone of mu-opioid receptor activation does exist. For example, inhibition of enkephalinases which breakdown endogenous opioid peptides results in antinociception in animal models of inflammatory and neuropathic pain (25). In addition, the opioid receptor antagonist naloxone increased pain perception when administered to post-operative patients who were not taking exogenous opiates, suggesting that endogenous opioid peptides produce a basal analgesic tone, which can be reduced by naloxone (26).

Another advantage of PAMs is their ability to shift the potency of orthosteric agonist to the left by a finite amount. For example, the analogs of BMS-986122 (Table

S2) showed a differential ability to shift the potency of orthosteric agonist to the left, resulting in different E_{max} values when the compounds were co-administered with a low dose of endomorphin-I. Drug development programs can take advantage of this finite potency shift to improve safety by designing PAMs that cannot exceed the required level of effect.

Opioid receptor tolerance and dependence results from prolonged exposure to opiates resulting in changes in cell function leading to the requirement for increased doses of agonist to mediate the same analgesic effect. One can predict that a lower dose of morphine administered together with a mu-PAM, might produce the same functional response as a higher dose of morphine alone and so may spare the development of tolerance. It will be important to determine whether the combination of agonist with PAM leads to a reduction in desensitization and/or tolerance and dependence *vs.* agonist alone. There is some precedence for this with GABA-B receptors. The GABA-B receptor PAM, GS39783, when combined with a low dose of agonist, produced the same level of functional response as a higher dose of GABA-B agonist, yet produced less GABA-B receptor desensitization (27). This may suggest that co-administration of lower doses of opiates with a mu-PAM, may discriminate between the therapeutic analgesic properties of opiates, and their tolerance and dependence liabilities. In addition, there is the possibility that a mu-PAM may bias an orthosteric agonist response away from signaling pathways that mediate tolerance and dependence and other unwanted effects in favor of signaling pathways that mediate a therapeutic response as observed in other systems (24, 27, 28).

In this patent application we have specifically described the discovery and characterization of two mu-opioid receptor-selective PAMs. The BMS-986122 chemotype showed chemical tractability from structure activity relationship studies and also led to the identification of mu-opioid receptor SAMs. To our knowledge these are the first opioid receptor PAMs and SAMs to be described in the literature. The two PAMs show potentiation of orthosteric agonist-mediated β-arrestin recruitment, adenylyl cyclase inhibition, and G protein activation. BMS-986122 potentiates DAMGO-

mediated [^{35}S]GTPγS binding in mouse brain membranes and appears to be, at least in part, a positive affinity modulator of the mu-opioid receptor for DAMGO binding. These studies provide proof-of-concept for the development of novel opioid allosteric modulators which may have therapeutic potential in chronic pain management with improved side-effects and reduced tolerance and dependence liabilities.

Some of the aspects of the invention are described below.

In one aspect of the invention there is provided a method of screening to identify mu-opioid receptor positive allosteric modulators comprising the steps of:

(i) adding a positive allosteric modulator test compound and a low concentration of a mu-selective orthosteric agonist to cells either alone or in conjunction with the silent allosteric compound represented by Formula I (BMS-986123);

(ii) measuring the effect of said mu-selective orthosteric agonist and said test compound on said cells either alone or in the presence of said Formula I compound; and

(iii) identifying said test compound as being a positive allosteric modulator if said compound of Formula I shows competitive binding with said test compound as evidenced by a decrease in the positive allosteric agonist activity of said test compound.

Preferably, the low concentration of a mu-selective orthosteric agonist is selected from the group consisting of:

(a) less than or equal to about the calculated EC80 in said cells;

(b) less than or equal to about the calculated EC70 in said cells;

(c) less than or equal to about the calculated EC60 in said cells;

(d) less than or equal to about the calculated EC50 in said cells;

(e) less than or equal to about the calculated EC40 in said cells;

(f) less than or equal to about the calculated EC30 in said cells;

(g) less than or equal to about the calculated EC20 in said cells;

(h) less than or equal to about the calculated EC10 in said cells.

Preferably, the cells are U2OS cells, CHO cells or C6 glioma cells.

In another aspect of the invention, there is provided a method of screening to identify mu-opioid receptor negative allosteric modulators comprising the steps of:

(i) adding a negative allosteric modulator test compound and a high concentration of a mu-selective orthosteric agonist to cells either alone or in conjunction with the silent allosteric compound represented by Formula I (BMS-986123);

(ii) measuring the effect of said mu-selective orthosteric agonist and said test compound on said cells either alone or in the presence of said Formula I compound; and

(iii) identifying said test compound as being a negative allosteric modulator if said compound of Formula I shows competitive binding with said test compound as evidenced by a decrease in the negative allosteric agonist activity of said test compound.

Preferably, the low concentration of a mu-selective orthosteric agonist is selected from the group consisting of:

(a) greater than or equal to about the calculated EC10 in said cells;

(b) greater than or equal to about the calculated EC20 in said cells;

(c) greater than or equal to about the calculated EC30 in said cells;

(d) greater than or equal to about the calculated EC40 in said cells;

(e) greater than or equal to about the calculated EC50 in said cells;

(f) greater than or equal to about the calculated EC60 in said cells;

(g) greater than or equal to about the calculated EC70 in said cells;

(h) greater than or equal to about the calculated EC80 in said cells;

(i) greater than or equal to about the calculated EC90 in said cells; and

(j) greater than or equal to about the calculated EC100 in said cells.

Preferably, the cells recombinantly express both an N-terminal deletion mutant of β-galactosidase fused to the C-terminus of β-arrestin 2, in addition to a mutated amino-terminal fragment of β-galactosidase fused to the C-terminus of OPRM1.

Preferably, the mu-selective orthosteric agonist is endomorphin-I.

Preferably, the cells recombinantly express human mu opioid receptor (CHO-mu).

Preferably, the cells recombinantly express human mu opioid receptor (C6-mu).

Preferably, the mu-selective orthosteric agonist is DAMGO.

In another aspect of the invention, there is provided a method to confirm a positive allosteric modulator test compound has mu-opioid receptor positive allosteric modulators comprising the steps of:

(i) adding a positive allosteric modulator test compound and a low concentration of a mu-selective orthosteric agonist to cells either alone or in conjunction with the silent allosteric compound represented by Formula I (BMS-986123);

(ii) measuring the effect of said mu-selective orthosteric agonist and said test compound on said cells either alone or in the presence of said Formula I compound; and

(iii) conforming said test compound has positive allosteric modulating activity if said compound of Formula I shows competitive binding with said test compound as evidenced by a decrease in the positive allosteric agonist activity of said test compound.

Preferably, the low concentration of a mu-selective orthosteric agonist is selected from the group consisting of:

(a) less than or equal to about the calculated EC80 in said cells;

(b) less than or equal to about the calculated EC70 in said cells;

(c) less than or equal to about the calculated EC60 in said cells;

(d) less than or equal to about the calculated EC50 in said cells;

(e) less than or equal to about the calculated EC40 in said cells;

(f) less than or equal to about the calculated EC30 in said cells;

(g) less than or equal to about the calculated EC20 in said cells;

(h) less than or equal to about the calculated EC10 in said cells.

In another aspect of the invention there is provided a method to confirm a negative allosteric modulator test compound has mu-opioid receptor negative allosteric modulators comprising the steps:

(i) adding a negative allosteric modulator test compound and a high concentration of a mu-selective orthosteric agonist to cells either alone or in conjunction with the silent allosteric compound represented by Formula I (BMS-986123);

(ii) measuring the effect of said mu-selective orthosteric agonist and said test compound on said cells either alone or in the presence of said Formula I compound; and

(iii) confirming said test compound has a negative allosteric modulator if said compound of Formula I shows competitive binding with said test compound as evidenced by a decrease in the negative allosteric agonist activity of said test compound.

Preferably, the low concentration of a mu-selective orthosteric agonist is selected from the group consisting of:

(a) greater than or equal to about the calculated EC10 in said cells;

(b) greater than or equal to about the calculated EC20 in said cells;

(c) greater than or equal to about the calculated EC30 in said cells;

(d) greater than or equal to about the calculated EC40 in said cells;

(e) greater than or equal to about the calculated EC50 in said cells;

(f) greater than or equal to about the calculated EC60 in said cells;

(g) greater than or equal to about the calculated EC70 in said cells;

(h) greater than or equal to about the calculated EC80 in said cells;

(i) greater than or equal to about the calculated EC90 in said cells; and

(j) greater than or equal to about the calculated EC100 in said cells.

Preferably, the cells are U2OS cells, CHO cells or C6 glioma cells.

Preferably, the cells recombinantly express both an N-terminal deletion mutant of β-galactosidase fused to the C-terminus of β-arrestin 2, in addition to a mutated amino-terminal fragment of β-galactosidase fused to the C-terminus of OPRM1.

Preferably, the mu-selective orthosteric agonist is endomorphin-I.

Preferably, the cells recombinantly express human mu opioid receptor (CHO-mu).

Preferably, the cells recombinantly express human mu opioid receptor (C6-mu).

Preferably, the mu-selective orthosteric agonist is DAMGO.

In another aspect of the invention, there is provided a method of treating pain in a patient in need thereof comprising administering to the patient a compound which is a positive allosteric modulator for the mu-opioid receptor.

Preferably, the compound is selective for mu-opioid receptors over delta-opioid receptors.

Preferably, the compound is effective to provide augmentation of at least one mu-opioid receptor function selected from G protein activation, inhibition of adenylyl cyclase activity, or b-arrestin recruitment.

In another aspect of the invention, there is provided a method of treating pain in a patient in need thereof comprising administering to the patient a compound which is a positive allosteric modulator for the mu-opioid receptor in combination with another compound which is an orthosteric agonist for the mu-opioid receptor.

Preferably, the compound which is a positive allosteric modulator for the mu-opioid receptor is selective for mu-opioid receptors over delta-opioid receptors

Preferably, the compound which is a positive allosteric modulator for the mu-opioid receptor is effective to provide augmentation of at least one mu-opioid receptor function selected from G protein activation, inhibition of adenylyl cyclase activity, or β-arrestin recruitment.

In another aspect of the invention, there is provided a method of modulating the mu-opioid receptor comprising contacting the receptor with a compound that is effective to provide an increase in the receptor function in the presence of orthosteric exogenous or endogenous agonist.

Preferably, the increase in receptor function is observed in maximal effect, potency, or both.

Although the invention has been described with respect to specific aspects, it is intended that the claims made herein shall not be limited to the specific aspects described and that the claims will be entitled to the full scope provided under the law.

Certain of the Materials and Methods used in the experiments described above were as follows.

Reagents and Cells.

PathHunter® β-arrestin U2OS cells engineered to express either Prolink/Enzyme Donor (PK)-tagged OPRM1 (mu-opioid) receptors or Prolink/Enzyme Donor (PK)-tagged OPRD1

(delta opioid) receptors, were from DiscoveRx (Fremont, CA). Chinese Hamster Ovary cells (CHO-K1) expressing recombinant mu opioid receptors (CHO-mu) were from PerkinElmer (Waltham, MA). C6 glioma cells stably expressing recombinant mu opioid receptors (C6mu) were developed as previously described (29). Cell culture media and supplements were from Life Technologies™ (Carlsbad, CA). HTRF® cAMP detection reagents were from Cisbio (Cambridge, MA). PathHunter® detection reagents, were from DiscoveRx™ (Freemont, CA). Morphine sulfate, leu-enkephalin, β-endorphin, and all other non-opioid ligand biochemical reagents were from Sigma-Aldrich® (St. Louis, MO). [^{35}S]GTPγS and [^{3}H]diprenorphine were from PerkinElmer. All other opioid ligands were from Tocris (Ellisville, MO).

β-Arrestin Recruitment Assay

The β-arrestin recruitment assay was performed in U2OS-OPRM1 and U2OS-OPRD1 cell suspensions, according to DiscoveRx established protocols (see SI Methods).

Inhibition of Forskolin-Stimulated cAMP Accumulation Assays.

Inhibition of forskolin-stimulated cAMP accumulation was conducted in CHO-mu cell suspensions using the CisBio HTRF cAMP detection kit with established protocols (see SI Methods).

Cell Membrane Homogenates

C6 glioma cells stably expressing rat MOR (C6mu) were grown and cell membranes were prepared as previously described (30). For mouse brain membranes, mice were euthanized by cervical dislocation. Whole brain tissue, minus cerebellum was removed, immediately chilled in ice-cold 50 mM Tris-HCl, pH 7.4, and membrane homogenates were prepared as described previously (31). Final membrane pellets were resuspended in 50 mM Tris-HCl, pH 7.4, aliquoted and stored at -80^{o}C. Protein content was determined using the method of Bradford (32).

[^{35}S]GTPγS Binding Assay

[^{35}S]GTPγS binding in membranes was conducted as described previously (22) (see SI Methods).

[^{3}H]Diprenorphine Saturation Binding Studies

[^{3}H]Diprenorphine saturation binding studies were performed as previously described (33). Briefly, membranes (5 ug) were incubated with 0-4 nM [^{3}H]diprenorphine and 10 uM modulator with or without 10 uM naloxone in 100 mM NaCl, 10 uM GTPγS, 5 mM $MgCl_2$ and 50 mM Tris-HCl, pH 7.4 for 80 min. at room temp. Samples were quickly filtered through glass-fiber filter mats as described for [^{35}S]GTPγS binding in SI Methods.

[^{3}H]Diprenorphine Competition Binding Studies

[^{3}H]Diprenorphine competition binding studies were performed as previously described (34). Membranes (10 ug) were incubated with 0.2 nM [^{3}H]diprenorphine and 0-10 uM DAMGO with or without 10 uM modulator and/or 10 uM naloxone in 100 mM NaCl, 10 uM GTPγS, 5 mM $MgCl_2$ and 50 mM Tris-HCl, pH 7.4 for 60 min. at room temp. Samples were quickly filtered through glass-fiber filter mats as described for [^{35}S]GTPγS binding in SI Methods.

Concentration response data were fit to a logistic equation (eq.1) using non-linear regression analysis to provide estimates of Ymin (Bottom), Ymax (Top), potency (EC_{50}) and slope factor (Hill slope), using GraphPad Prism™ 5.01 (sigmoidal dose response with variable slope).

$$Y=Bottom + (Top-Bottom)/(1+10^{((LogEC_{50}-X)*HillSlope)}) \quad (eq.1)$$

Where P values are described, data were analyzed by 2-way ANOVA with a Bonferroni Post-Test using GraphPad Prism™ 5.01.

β-Arrestin Recruitment Assay

PathHunter® U2OS-OPRM1 and U2OS-OPRD1 cells were grown in modified Eagle medium (MEM) containing 10% fetal bovine serum (FBS), 500 ug/ml G418, and 250 ug/ml hygromycin. Cells were grown to confluence in cell culture Nunc triple-layer flasks

(Thermo Fisher Scientific), harvested with TrypLE™ Express, and resuspended in assay buffer (Hanks Buffered Salt Solution (HBSS) + 25 mM HEPES, 100 IU/ml penicillin, 100 ug/ml streptomycin , 0.05% BSA at 1 x 10^6 cells / ml. Compounds (20 nl of 100x final concentration in 100% DMSO) were added to white, non-treated 1536-well plates (Corning, NY) by acoustic dispense using an Echo-550 (Labcyte, Sunnyvale, CA) from Echo-qualified 1536-well source plates (Labcyte). Next, 1 ul of assay buffer (agonist detection mode), or assay buffer containing a low concentration (~EC_{10}) of orthosteric agonist (PAM detection mode), or assay buffer containing a ~EC_{80} concentration of orthosteric agonist (antagonist/NAM detection mode), were added to assay plates. The orthosteric agonists used and their final concentrations are described in the Results. Finally, 1 ul of cells (1000 cells / well) in assay buffer were added to the wells to initiate the incubation period. Plates were lidded and incubated at room temperature for 90 min. Incubations were terminated by the addition of 1 ul PathHunter® Reagent. One hour later luminescence was detected using a Viewlux® imaging plate reader (PerkinElmer). Additional characterization of certain mu-selective PAMs in the β-arrestin assay were performed essentially as described above using the various orthosteric agonist ligands and cell lines described in the Results.

Inhibition of Forskolin-Stimulated cAMP Accumulation Assays.

CHO cells expressing recombinant human mu-opioid receptor (CHO-mu) were grown to confluence in F12 media containing 10% FBS, 100 IU/ml penicillin, 100 ug/ml streptomycin and 400 ug/ml G418 in T-175 tissue culture flasks (Corning) and harvested with TripLE™ Express. Cells were pelleted by centrifugation and resuspended in assay buffer at 6.67 x 10^5 cells / ml.

Compounds (30 nl of 100 x final concentration in 100% DMSO) were added to 1536-well white solid non-treated plates by acoustic dispense using an Echo-550. Next, 1.5 ul of assay buffer containing 1 mM IBMX and 2x forskolin (1 uM final), without (agonist detection mode) or with (PAM detection mode) 2x endomorphin-I (30 pM final, a ~EC_{10} concentration) were added to the plates. Finally, cells (1.5 ul / well) were added to begin the incubation. Plates were incubated at room temperature for 30 min. followed

by the addition of Cisbio HTRF® dynamic cAMP detection reagent (1.5 ul of D2-labelled cAMP tracer in lysis buffer, followed by 1.5 ul of Eu-cryptate conjugated anti-cAMP antibody in lysis buffer). After a 1 hr. 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 was then converted to cAMP (nM) based on a standard curve for cAMP (replacing the cell addition step) run at the same time and under identical conditions to the assay.

Characterization of mu-selective PAMs in the CHO-mu cAMP assay, using curve-shift assays and probe dependence assays, were performed as described above, using orthosteric agonists and modulators described in the Results.

[^{35}S]GTPγS Binding Assay

Membranes were diluted with 50 mM Tris-HCl, pH 7.4 and pre-incubated with assay buffer containing GDP (1 volume membrane + 2 volumes of 2x assay buffer) for 30 min. at room temp. in a shaking water bath. Then 150 ul membrane/assay buffer mix was added to wells containing 50 ul drugs and [^{35}S]GTPγS (final concentrations: 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 0.1 mM dithiothreitol, 30-100 uM GDP, 0.1 nM [^{35}S]GTPγS, 0-30 uM DAMGO or morphine, 10 uM modulator or DMSO to achieve 2% DMSO final concentration and either 15 ug membrane protein/well for C6mu cell membranes or 10 ug membrane protein/well for mouse brain membranes). After incubation for an additional 5 min. at room temp. samples were quickly filtered through glass-fiber filter mats using a Brandel cell harvester and rinsed 5 times with ice-cold wash buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$). Filter mats were dried, scintillation cocktail was added, and radioactivity retained on the filters was counted in a Wallac MicroBeta (Perkin Elmer).

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1

Chemical structure and effect of mu-PAMs on β-arrestin recruitment. Chemical structures for two apparent mu-opioid receptor selective positive allosteric modulators (mu-PAMs), designated as BMS-986121 (A) and BMS-986122 (B), were identified from a high-throughput β-arrestin recruitment screen. BMS-986121 (C) and BMS-986122 (D) were assayed at varying concentrations in agonist detection mode (with compound alone) and in PAM detection mode (compound in the presence of a low concentration (~EC_{10}) of orthosteric agonist). For mu-opioid receptor expressing cells (U2OS-OPRM1) endomorphin-I (20 nM) was the orthosteric agonist used, and for delta-opioid receptor expressing cells (U2OS-OPRD1) leu-enkephalin (0.4 nM) was the orthosteric agonist used. Data are represented as mean ± s.e.m. of three experiments. In agonist detection mode, 0 and 100% activity represent basal activity and an E_{max} of endomorphin-I (in U2OS-OPRM1, a 6-fold signal) or leu-enkephalin (in U2OS-OPRD1, a 4-fold signal), respectively. In PAM detection mode, 0% activity is normalized to the low concentration (~EC_{10}) of orthosteric agonist (endomorphin-I in U2OS-OPRM1 cells, and leu-enkephalin in U2OS-OPRD1 cells). 100% activity represents the response to an E_{max} concentration of these respective agonists.

Fig. 2

Effect of mu-PAMs BMS-986121 and BMS-986122 on endomorphin-I stimulated β-arrestin recruitment in U2OS-OPRM1 cells. Both BMS-986121 (A) and BMS-986122 (B), produced concentration-dependent leftward shifts in the β-arrestin recruitment response to the agonist endomorphin-I. Calculated EC_{50} values (nM) for endomorphin-I at each concentration of compound are shown in each figure legend. The fold leftward shift in EC_{50} values for endomorphin-I in the presence of increasing concentrations of PAM compound is presented (C). Data are represented as mean +/- s.e.m. of 4 experiments.

Fig. 3

Effect of mu-PAMs on inhibition of forskolin-stimulated cAMP accumulation in CHO-mu cells. Both BMS-986121 (A) and BMS-986122 (B) increased the effect of a low (~EC_{10}; 30 pM) concentration of endomorphin-I (PAM detection mode) in a concentration-dependent manner. However, both compounds also showed some agonist activity above basal activity when added alone (agonist detection mode). For agonist detection mode, 0% activity represents vehicle (basal) activity. For PAM detection mode, 0% is normalized to the response to a ~EC_{10} (30 pM) concentration of endomorphin-I. The 100% response represents the response to an Emax. concentration of endomorphin-I (10 nM) in both agonist and PAM detection modes. Data are represented as mean ± s.e.m. of three experiments.

Fig. 4

Effect of mu-PAMs on mu-opioid agonist stimulated [^{35}S]GTPγS binding in membranes from C6mu cells and mouse brain, and DAMGO binding affinity in C6mu cell membranes. [^{35}S]GTPγS binding in C6mu membranes was determined as described in the Methods and Materials. The EC_{50} of DAMGO to stimulate [^{35}S]GTPγS binding was shifted to the left 4-fold in the presence of 10 uM of the mu-PAM BMS-986121 (A). BMS-986122 increased the DAMGO potency 7-fold (B). The maximal stimulation by DAMGO was not affected by BMS-986122 or BMS-986121. The mu-PAMs did not significantly affect basal values (vehicle control basal = 3.2 ± 0.2 fmol bound/mg protein). The EC_{50} of morphine to stimulate [^{35}S]GTPγS binding was shifted to the left 2.5-fold in the presence of 10 uM BMS-986121 (C). BMS-986122 (10 uM) increased the morphine potency 3-fold (D). The maximal effect of morphine compared to DAMGO was increased by BMS-986121 (C) and BMS-986122 (D). BMS-986122 (10uM) produced a 6-fold leftward shift in DAMGO affinity in DAMGO competition binding studies with [^{3}H]diprenorphine (E), but had no effect on [^{3}H]diprenorphine binding affinity (Fig. S2, Table S1). The EC_{50} of DAMGO to stimulate [^{35}S]GTPγS binding in membranes from mouse brain was shifted to the left 4.5-fold in the presence of 10 uM BMS-986122 (F). Basal [^{35}S]GTPγS binding (4.8 ± 0.4 fmol bound/mg protein) was not affected by 10 uM BMS-986122. Shown are the combined mean ± s.e.m. data from 3-7 separate assays, each performed in duplicate.

Fig. 5

Characterization of functional SAMs in the β-arrestin recruitment assay and in DAMGO-mediated [^{35}S]GTPγS binding. (A) BMS-986123 and BMS-986124 inhibited PAM responses to a ~EC_{80} concentration of BMS-986122 (12.5 uM) plus a ~EC_{20} concentration of endomorphin-I (30nM) (SAM detection mode) in the β-arrestin recruitment assay in U2OS-OPRM1 cells. 100% activity represents the activity of the combined BMS-986122 plus endomorphin-I, and 0% activity represents the activity of the ~EC_{20} concentration of endomorphin-I alone. Graphs show the mean ± s.e.m. of three experiments. BMS-986123 and BMS-986124 showed no activity in agonist or PAM detection modes in either U2OS-OPRM1 cells or U2OS-OPRD1 cells (Fig. S3). Similarly, these two compounds showed no NAM/antagonist activity (in the presence of a ~EC_{80} (300 nM) concentration of endomorphin-I) in U2OS-OPRM1 cells (Fig. S4). (B) DAMGO potency to stimulate [^{35}S]GTPγS binding in C6mu membranes was increased 8-fold in the presence of the mu-PAM BMS-986122 (10 uM). Co-incubation of the SAM BMS-986124 (50 uM) with BMS-986122 (10 uM) resulted in only a 2-fold increase in potency for DAMGO suggesting that BMS-986124 can antagonize the BMS-986122 PAM effect. Shown are the combined mean ± s.e.m. data from 3-7 separate assays, each performed in duplicate. EC_{50} values were compared by Student's t-test using GraphPad Prism. (**) represents $p < 0.01$. Concentration response curves for DAMGO-stimulated [^{35}S]GTPγS binding under the various conditions are shown in Fig. S5.

Fig. S1

Effect of endomorphin-I on inhibition of 1 uM forskolin-stimulated cAMP accumulation in CHO-mu cells. Endomorphin-I induced a 17-fold reduction in forskolin-stimulated cAMP accumulation with an EC_{50} of 76 (60-96) pM. Data are represented as the mean ± s.e.m. of three experiments.

Fig. S2

Effect of the mu-PAM, BMS-986122, and the SAM, BMS-986123, on [^{3}H]diprenorphine saturation binding in membranes from C6mu cells. BMS-986122 (A) had no significant

effect on [^{3}H]diprenorphine binding affinity but induced a 6-fold increase in the affinity of DAMGO in competition binding studies (see Fig. 5C, Table S1). BMS-986123 (B) produced a small (~2-fold) but significant decrease in [^{3}H]diprenorphine affinity, but had no significant effect on DAMGO affinity (see Table S1). Data are represented as the mean ± s.e.m. of 3-7 experiments.

Fig. S3

Activity of BMS-986122 and 18 analogs in a β-arrestin recruitment assay in U2OS-OPRD1 and U2OS-OPRM1 cells. Compounds were tested in U2OS-OPRD1 cells in agonist detection mode (in the absence of leu-enkephalin) and PAM detection mode (in the presence of an ~EC_{10} concentration of leu-enkephalin). Compounds were also tested in U2OS-OPRM1 cells in agonist detection mode (in the absence of endomorphin-I) and PAM detection mode (in the presence of an ~EC_{10} concentration of endomorphin-I). Finally, compounds that exhibited no agonist or PAM activity in U2OS-OPRM1 cells were tested in SAM detection mode (in the presence of an ~EC_{20} concentration of endomorphin-I plus an ~EC_{80} of the PAM BMS-986122). Graphical curve fit data are representative of three combined experiments. EC_{50} and E_{max} values are represented in Table S2. For agonist detection mode, 0% and 100% activity represent basal activity and an Emax concentration of orthosteric agonist, respectively. For PAM detection mode, 0% activity represents the response to a low (~EC_{10}) concentration of agonist alone, and 100% activity represents the response to an E_{max} concentration of agonist. For SAM detection mode, 0% inhibition represents the response to a low (~EC_{20}) concentration of endomorphin-I combined with an ~EC_{80} concentration of the mu-PAM BMS-986122. 100% inhibition represents the response to a low (~EC_{20}) concentration of endomorphin-I alone.

Fig. S4

Effect of BMS-986123 and BMS-986124 on β-arrestin response to an ~EC_{80} concentration of endomorphin-I (antagonist/NAM detection mode) in U2OS-OPRM1 cells. BMS-986123 and BMS-986124 had no significant effect on endomorphin-I (300 nM) mediated β-arrestin activity in U2OS-OPRM1 cells. 100% activity is normalized to

the response to endomorphin-I (300 nM) alone. 0% activity represents basal activity. Data are represented as the mean + s.e.m. of three experiments.

Fig. S5

Effect of BMS-986124 on BMS-986122-mediated PAM activity to DAMGO-stimulated [^{35}S]GTPγS binding in C6mu cell membranes. DAMGO-stimulated [^{35}S]GTPγS binding potency in C6mu membranes was increased 8-fold in the presence of the mu-PAM BMS-986122 (10 uM) (A). DAMGO-stimulated [^{35}S]GTPγS binding potency was not affected by incubation with 50uM BMS-986124 (B). Co-incubation of BMS-986124 (50 uM) with BMS-986122 (10 uM) resulted in a rightward shift in DAMGO potency when compared to incubation with BMS-986122 alone (C). The potency of DAMGO, in the presence of both BMS-986122 and BMS-986124 was shifted leftward by only 2-fold compared with DAMGO potency in the presence of the vehicle control. These data suggest that BMS-986124 can antagonize the BMS-986122 PAM effect. Shown are the combined mean ± s.e.m. data from 3-7 separate assays, each performed in duplicate. EC_{50} values are given in the Results & Discussion section and in Fig. 5B.

Fig. S6

Effect of the SAMs BMS-986123 and BMS-986124 on DAMGO-stimulated [^{35}S]GTPγS binding above basal activity in membranes from C6mu cells. DAMGO potency (EC_{50} of 222 (179-274) nM) was not significantly affected by BMS-986123 (A) (EC_{50} of 321 239-432) nM) or BMS-986124 (B) (EC_{50} of 223 (150-331) nM. The maximal stimulation by DAMGO (control max=232 (223-242) %, was not affected by BMS-986124 (243 (224-262) %. Maximal stimulation was decreased slightly by BMS-986123 (206 (193-218) %. The modulators did not significantly affect the basal values (vehicle control basal=3.2 ± 0.2 fmol bound/mg protein). Shown are the combined data from 3-7 separate assays, each performed in duplicate.

Fig. S7

Effect of the SAMs BMS-986123 and BMS-986124 on morphine-stimulated [^{35}S]GTPγS binding in membranes from C6mu cells. The EC_{50} of morphine to stimulate [^{35}S]GTPγS

binding (110 (71-171) nM) was not significantly affected by BMS-986123 (A) (140 (67-293)nM; 67-293) nM), but was decreased by BMS-986124 (B) (245 (161-372) nM). The maximal effect of morphine compared to DAMGO (30 uM) (control max=42 (38-45) %) was increased to a small degree by BMS-986123 (62 (52-73) %) and BMS-986124 (58 (53-64) %). Shown are the combined data from 3-7 separate assays, each performed in duplicate.

Fig. S8

Effect of mu-PAM BMS-986121 on inhibition of forskolin-stimulated cAMP accumulation, mediated by different orthosteric agonists, in CHO-mu cells. BMS-986121 (100 uM) produced leftward shifts in agonist potency for each of the three orthosteric ligands used ((**A**) endomorphin-I = 4-fold, (**B**) morphine = 6.5-fold, and (**C**) leu-enkephalin = 4.5-fold)). EC_{50} values for the agonists at each BMS-986121 concentration are shown in the legend. Data represent the mean $\pm$ s.e.m. of 3 experiments.

Table S1

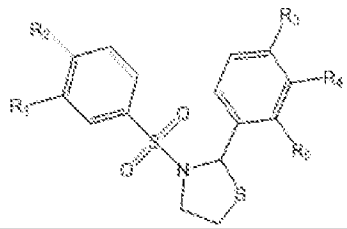
Effect of the mu-PAM, BMS-986122, and the SAM, BMS-986123, on [^{3}H]diprenorphine saturation binding and DAMGO competition binding in membranes from C6mu cells.

Compound added (10 uM)	[^{3}H]diprenorphine Kd (nM) mean (95%CI)	DAMGO Ki (nM) mean (95%CI)
Vehicle control	0.27 (0.21-0.32)	340 (208-552)
BMS-986122	0.35 (0.18-0.51)	56 (41-76)
BMS-986123	0.71 (0.57-0.86)	270 (179-406)

Table S2

Structure activity relationship of BMS-986122 and analogs tested in the β-arrestin recruitment assay in U2OS-OPRM1 cells, in PAM detection mode. Compounds

exhibiting PAM activity were described based on their efficacy as Full, Strong, Moderate, or Weak PAMs. 0 % activity represents the response to a low (~EC_{10}) concentration of endomorphin-I alone and 100 % activity represents the response to an E_{max} concentration of endomorphin-I. The 2 compounds that showed no PAM activity were additionally tested in SAM detection mode (inhibition of BMS-986122 (~EC_{80}) response in the presence of a low concentration (~EC_{20}) of endomorphin-I) where the compounds were shown to inhibit the BMS-986122 response (Fig. S3). Calculated K_b values are provided from IC_{50} values. Concentration response curves for the SAM compounds, BMS-986123 and BMS-986124, are shown in Fig. 5A. Data are represented as the mean of 3 experiments.



Substance	R1	R2	R3	R4	R5	β-arrestin PAM Detection Mode (% E_{max})	β-arrestin PAM detection mode (EC_{50}, uM)	Description	β-arrestin SAM Detection Mode (K_b, uM)
BMS-986122	H	Cl	OMe	Br	H	79	3	Full PAM (ref)	
Analog 1	H	Nitro	OMe	Br	H	53	1	Moderate PAM	
Analog 2	H	Me	OMe	Br	H	18	4	Weak PAM	
Analog 3	H	OMe	OMe	Br	H	16	6	Weak PAM	
Analog 4	Me	H	OMe	Br	H	20	9	Weak PAM	
Analog 5	H	Cl	OMe	Nitro	H	45	2	Moderate PAM	
Analog 6	H	Br	OMe	Nitro	H	45	1	Moderate PAM	
Analog 7 (BMS-986123)	H	Me	OMe	Nitro	H	_	_	SAM	1
Analog 8	H	Br	OMe	OMe	H	18	14	Weak PAM	
Analog 9	H	Br	OMe	H	H	44	7	Moderate PAM	
Analog 10	H	H	OMe	H	H	17	23	Weak PAM	
Analog 11	H	Cl	H	Cl	H	65	5	Strong PAM	
Analog 12	H	Cl	Br	H	H	26	6	Weak PAM	
Analog 13 (BMS-986124)	H	Cl	Br	H	OMe	_	_	SAM	2
Analog 14	H	OMe	Br	H	H	3	7	Weak PAM	
Analog 15	H	OMe	OMe	H	H	9	37	Weak PAM	

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CLAIMS

1. A method of screening to identify mu-opioid receptor positive allosteric modulators comprising the steps of:

(i) adding a positive allosteric modulator test compound and a low concentration of a mu-selective orthosteric agonist to cells either alone or in conjunction with the silent allosteric compound represented by Formula I (BMS-986123);

(ii) measuring the effect of said mu-selective orthosteric agonist and said test compound on said cells either alone or in the presence of said Formula I compound; and

(iii) identifying said test compound as being a positive allosteric modulator if said compound of Formula I shows competitive binding with said test compound as evidenced by a decrease in the positive allosteric agonist activity of said test compound.

2. The method according to Claim 1, wherein the low concentration of a mu-selective orthosteric agonist is selected from the group consisting of:

(a) less than or equal to about the calculated EC80 in said cells;

(b) less than or equal to about the calculated EC70 in said cells;

(c) less than or equal to about the calculated EC60 in said cells;

(d) less than or equal to about the calculated EC50 in said cells;

(e) less than or equal to about the calculated EC40 in said cells;

(f) less than or equal to about the calculated EC30 in said cells;

(g) less than or equal to about the calculated EC20 in said cells;

(h) less than or equal to about the calculated EC10 in said cells.

3. A method of screening to identify mu-opioid receptor negative allosteric modulators comprising the steps of:

(i) adding a negative allosteric modulator test compound and a high concentration of a mu-selective orthosteric agonist to cells either alone or

in conjunction with the silent allosteric compound represented by Formula I (BMS-986123);

(ii) measuring the effect of said mu-selective orthosteric agonist and said test compound on said cells either alone or in the presence of said Formula I compound; and

(iii) identifying said test compound as being a negative allosteric modulator if said compound of Formula I shows competitive binding with said test compound as evidenced by a decrease in the negative allosteric agonist activity of said test compound.

4. The method according to Claim 3, wherein the low concentration of a mu-selective orthosteric agonist is selected from the group consisting of:

(a) greater than or equal to about the calculated EC10 in said cells;

(b) greater than or equal to about the calculated EC20 in said cells;

(c) greater than or equal to about the calculated EC30 in said cells;

(d) greater than or equal to about the calculated EC40 in said cells;

(e) greater than or equal to about the calculated EC50 in said cells;

(f) greater than or equal to about the calculated EC60 in said cells;

(g) greater than or equal to about the calculated EC70 in said cells;

(h) greater than or equal to about the calculated EC80 in said cells;

(i) greater than or equal to about the calculated EC90 in said cells; and

(j) greater than or equal to about the calculated EC100 in said cells.

5. The method according to Claim 1, wherein said cells are U2OS cells.

6. The method according to Claim 5 wherein said cells recombinantly express both an N-terminal deletion mutant of β-galactosidase fused to the C-terminus of β-arrestin 2, in addition to a mutated amino-terminal fragment of β-galactosidase fused to the C-terminus of OPRM1.

7. The method according to Claim 6 wherein said mu-selective orthosteric agonist is endomorphin-I.

8. The method according to Claim 1, wherein said cells are CHO cells.

9. The method according to Claim 8 wherein said cells recombinantly express human mu opioid receptor (CHO-mu).

10. The method according to Claim 9 wherein said mu-selective orthosteric agonist is endomorphin-I.

11. The method according to Claim 1, wherein said cells are C6 glioma cells.

12. The method according to Claim 11 wherein said cells recombinantly express human mu opioid receptor (C6-mu).

13. The method according to Claim 12 wherein said mu-selective orthosteric agonist is DAMGO.

14. A method to confirm a positive allosteric modulator test compound has mu-opioid receptor positive allosteric modulator activity comprising the steps of:

(i) adding a positive allosteric modulator test compound and a low concentration of a mu-selective orthosteric agonist to cells either alone or in conjunction with the silent allosteric compound represented by Formula I (BMS-986123);

(ii) measuring the effect of said mu-selective orthosteric agonist and said test compound on said cells either alone or in the presence of said Formula I compound; and

(iii) confirming said test compound has positive allosteric modulating activity if said compound of Formula I shows competitive binding with said test

compound as evidenced by a decrease in the positive allosteric agonist activity of said test compound.

15. The method according to Claim 14, wherein the low concentration of a mu-selective orthosteric agonist is selected from the group consisting of:

(a) less than or equal to about the calculated EC80 in said cells;

(b) less than or equal to about the calculated EC70 in said cells;

(c) less than or equal to about the calculated EC60 in said cells;

(d) less than or equal to about the calculated EC50 in said cells;

(e) less than or equal to about the calculated EC40 in said cells;

(f) less than or equal to about the calculated EC30 in said cells;

(g) less than or equal to about the calculated EC20 in said cells;

(h) less than or equal to about the calculated EC10 in said cells.

16. A method to confirm a negative allosteric modulator test compound has mu-opioid receptor negative allosteric modulators comprising the steps:

(i) adding a negative allosteric modulator test compound and a high concentration of a mu-selective orthosteric agonist to cells either alone or in conjunction with the silent allosteric compound represented by Formula I (BMS-986123);

(ii) measuring the effect of said mu-selective orthosteric agonist and said test compound on said cells either alone or in the presence of said Formula I compound; and

(iii) comfirming said test compound has a negative allosteric modulator if said compound of Formula I shows competitive binding with said test compound as evidenced by a decrease in the negative allosteric agonist activity of said test compound.

17. The method according to Claim 16, wherein the low concentration of a mu-selective orthosteric agonist is selected from the group consisting of:

(a) greater than or equal to about the calculated EC10 in said cells;

(b) greater than or equal to about the calculated EC20 in said cells;

(c) greater than or equal to about the calculated EC30 in said cells;

(d) greater than or equal to about the calculated EC40 in said cells;

(e) greater than or equal to about the calculated EC50 in said cells;

(f) greater than or equal to about the calculated EC60 in said cells;

(g) greater than or equal to about the calculated EC70 in said cells;

(h) greater than or equal to about the calculated EC80 in said cells;

(i) greater than or equal to about the calculated EC90 in said cells; and

(j) greater than or equal to about the calculated EC100 in said cells.

18. The method according to Claim 14, wherein said cells are U2OS cells.

19. The method according to Claim 18 wherein said cells recombinantly express both an N-terminal deletion mutant of β-galactosidase fused to the C-terminus of β-arrestin 2, in addition to a mutated amino-terminal fragment of β-galactosidase fused to the C-terminus of OPRM1.

20. The method according to Claim 19 wherein said mu-selective orthosteric agonist is endomorphin-I.

21. The method according to Claim 14, wherein said cells are CHO cells.

22. The method according to Claim 21 wherein said cells recombinantly express human mu opioid receptor (CHO-mu).

23. The method according to Claim 22 wherein said mu-selective orthosteric agonist is endomorphin-I.

24. The method according to Claim 14, wherein said cells are C6 glioma cells.

25. The method according to Claim 24 wherein said cells recombinantly express human mu opioid receptor (C6-mu).

26. The method according to Claim 25 wherein said mu-selective orthosteric agonist is DAMGO.

27. A method of treating pain in a patient in need thereof comprising administering to the patient a compound which is a positive allosteric modulator for the mu-opioid receptor.

28. A method of treating pain in a patient in need thereof comprising administering to the patient a compound which is a positive allosteric modulator for the mu-opioid receptor in combination with another compound which is an orthosteric agonist for the mu-opioid receptor.

29. The method of claim 27 wherein the compound is selective for mu-opioid receptors over delta-opioid receptors

30. The method of claim 28 wherein the compound which is a positive allosteric modulator for the mu-opioid receptor is selective for mu-opioid receptors over delta-opioid receptors

31. The method of claim 27 wherein the compound is effective to provide augmentation of at least one mu-opioid receptor function selected from G protein activation, inhibition of adenylyl cyclase activity, or b-arrestin recruitment.

32. The method of claim 28 wherein the compound which is a positive allosteric modulator for the mu-opioid receptor is effective to provide augmentation of at least one mu-opioid receptor function selected from G protein activation, inhibition of adenylyl cyclase activity, or -arrestin recruitment.

33. A method of modulating the mu-opioid receptor comprising contacting the receptor with a compound that is effective to provide an increase in the receptor function in the presence of orthosteric exogenous or endogenous agonist.

34. The method of claim 33 wherein the increase in receptor function is observed in maximal effect, potency, or both.

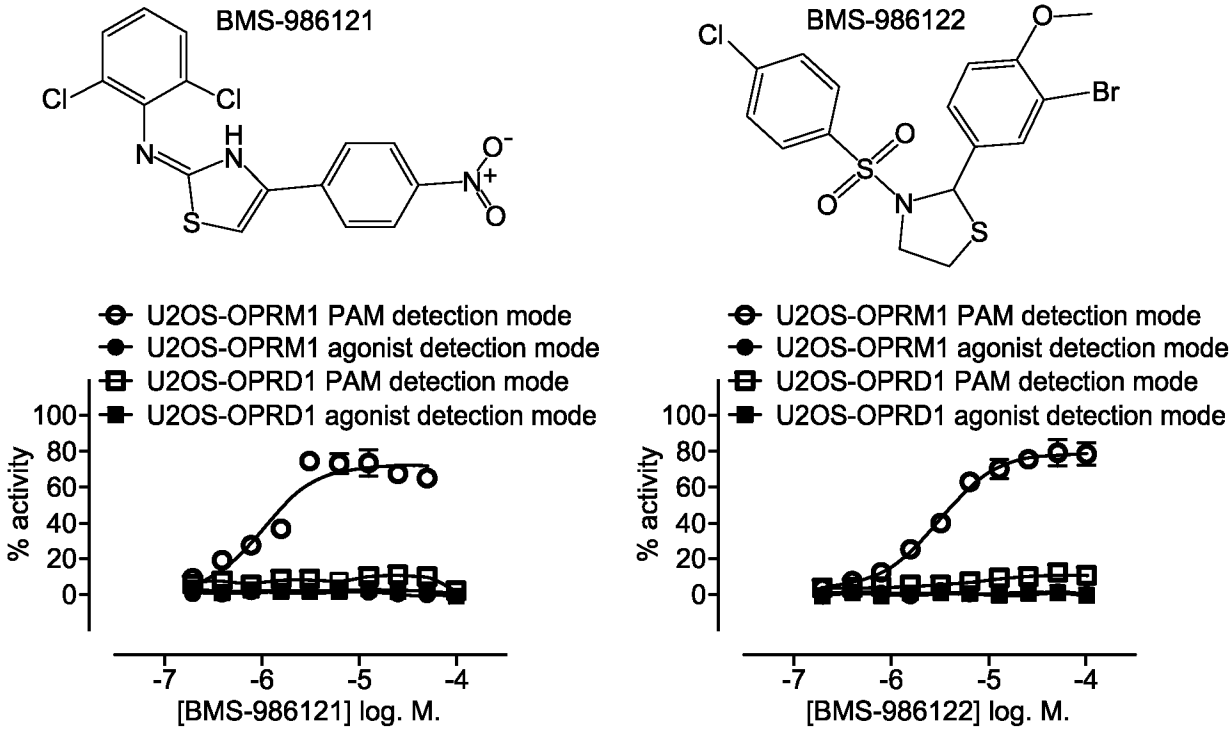

BMS-986121
Cl
Cl
H
N
N
S
O-
N+
O
BMS-986122
Cl
O
Br
O
S
O
N
S
U2OS-OPRM1 PAM detection mode
U2OS-OPRM1 agonist detection mode
U2OS-OPRD1 PAM detection mode
U2OS-OPRD1 agonist detection mode
% activity
100
80
60
40
20
0
-7
-6
-5
-4
[BMS-986121] log. M.
[BMS-986122] log. M.

FIG. 1

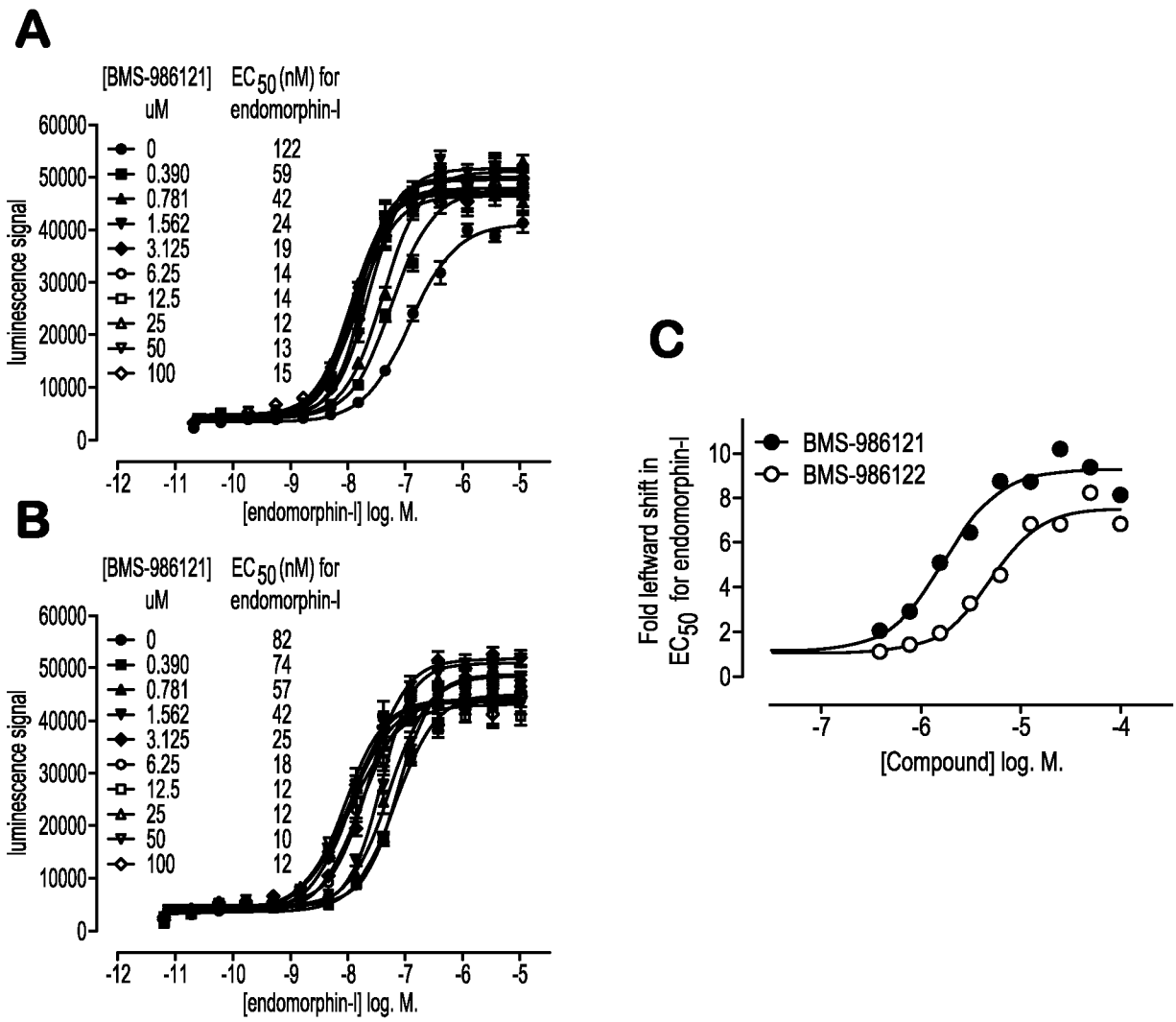

A
[BMS-986121] uM
EC50 (nM) for endomorphin-I
0 122
0.390 59
0.781 42
1.562 24
3.125 19
6.25 14
12.5 14
25 12
50 13
100 15
luminescence signal
60000
50000
40000
30000
20000
10000
0
-12 -11 -10 -9 -8 -7 -6 -5
[endomorphin-I] log. M.
B
[BMS-986121] uM
EC50 (nM) for endomorphin-I
0 82
0.390 74
0.781 57
1.562 42
3.125 25
6.25 18
12.5 12
25 12
50 10
100 12
luminescence signal
60000
50000
40000
30000
20000
10000
0
-12 -11 -10 -9 -8 -7 -6 -5
[endomorphin-I] log. M.
C
BMS-986121
BMS-986122
Fold leftward shift in EC50 for endomorphin-I
10
8
6
4
2
0
-7 -6 -5 -4
[Compound] log. M.

FIG. 2

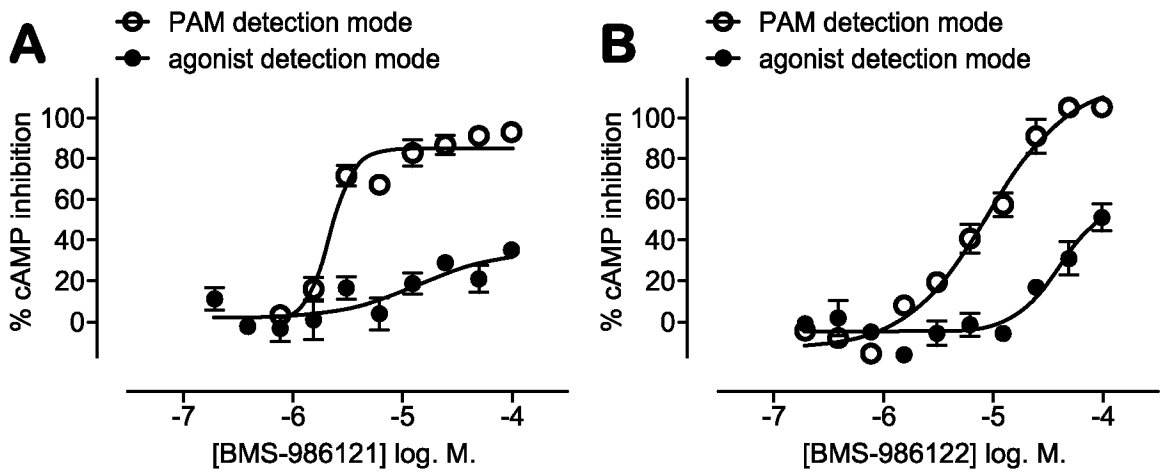

A
PAM detection mode
agonist detection mode
% cAMP inhibition
100
80
60
40
20
0
-7
-6
-5
-4
[BMS-986121] log. M.
B
PAM detection mode
agonist detection mode
% cAMP inhibition
100
80
60
40
20
0
-7
-6
-5
-4
[BMS-986122] log. M.

FIG. 3

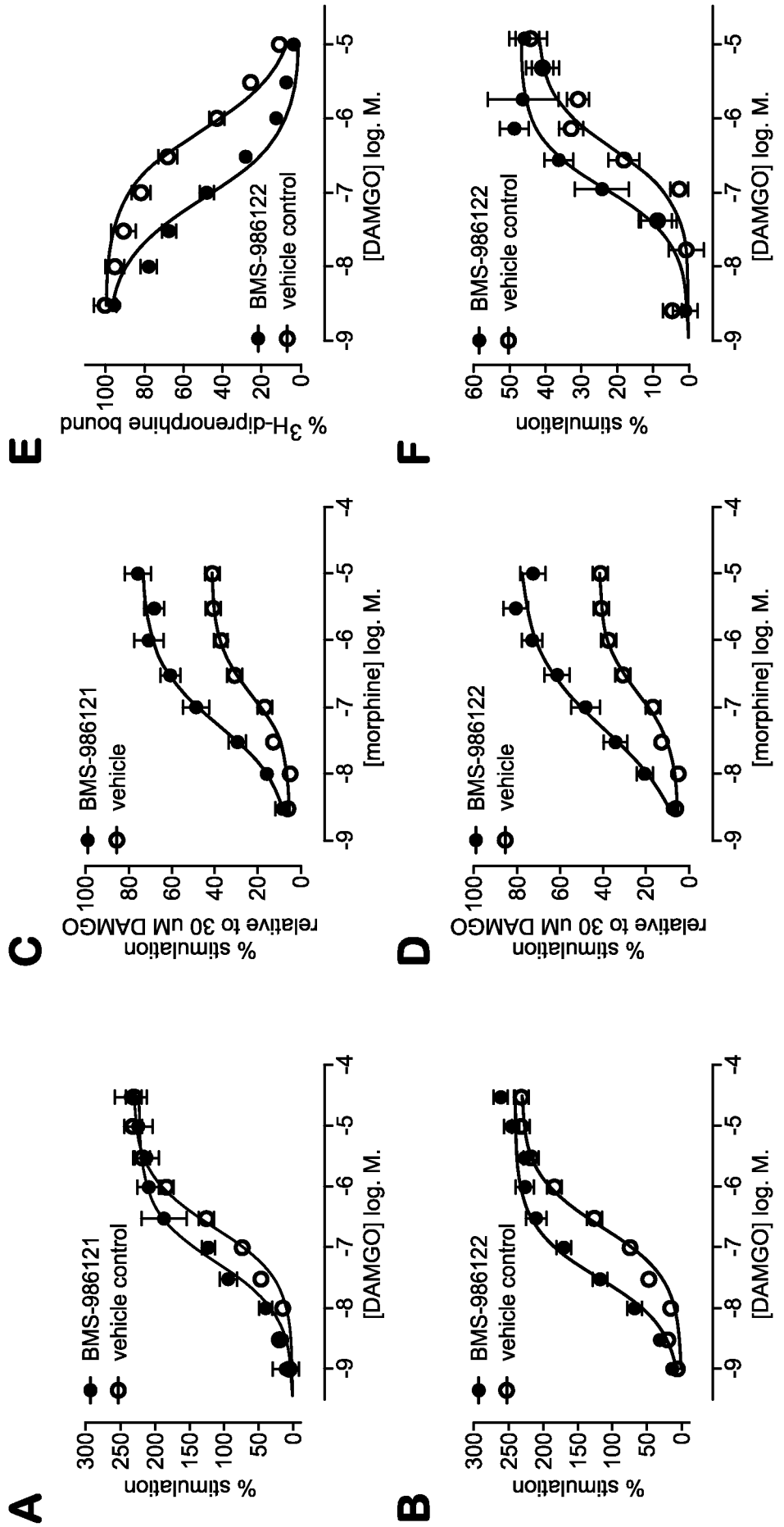

A
% stimulation
BMS-986121
vehicle control
[DAMGO] log. M.
B
% stimulation
BMS-986122
vehicle control
[DAMGO] log. M.
C
% stimulation relative to 30 uM DAMGO
BMS-986121
vehicle
[morphine] log. M.
D
% stimulation relative to 30 uM DAMGO
BMS-986122
vehicle
[morphine] log. M.
E
% 3H-diprenorphine bound
BMS-986122
vehicle control
[DAMGO] log. M.
F
% stimulation
BMS-986122
vehicle control
[DAMGO] log. M.

FIG. 4

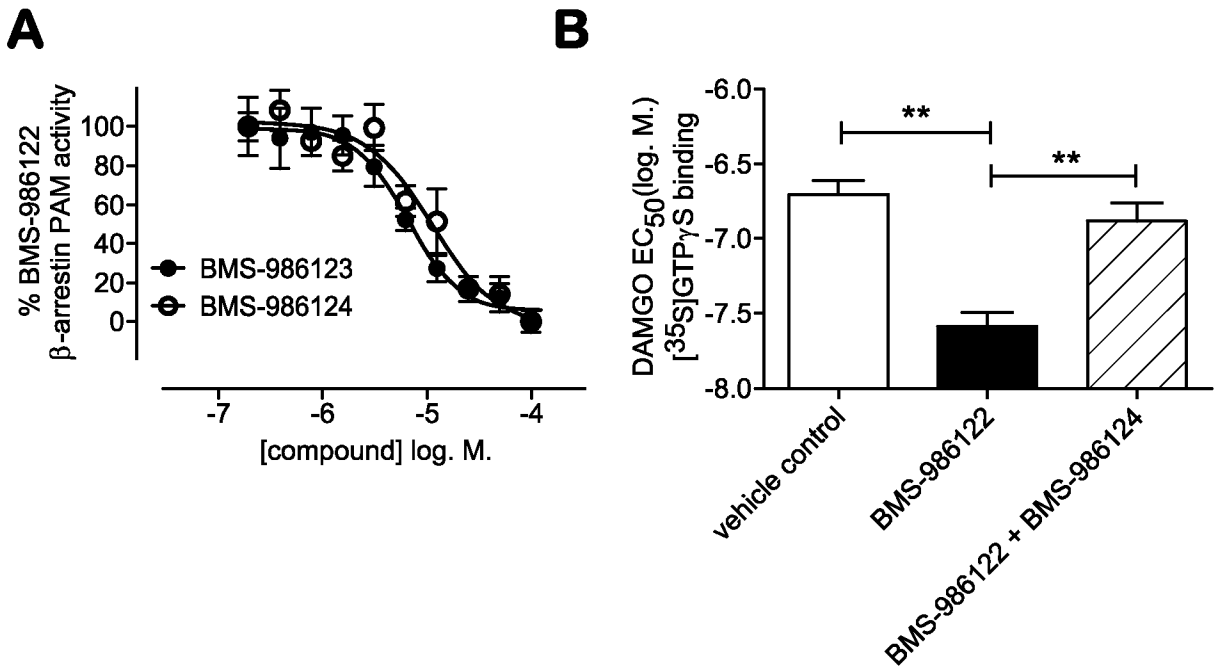

A
% BMS-986122
β-arrestin PAM activity
100
80
60
40
20
0
BMS-986123
BMS-986124
-7
-6
-5
-4
[compound] log. M.
B
DAMGO EC50(log. M.)
[35S]GTPγS binding
-6.0
-6.5
-7.0
-7.5
-8.0
**
**
vehicle control
BMS-986122
BMS-986122 + BMS-986124

FIG. 5

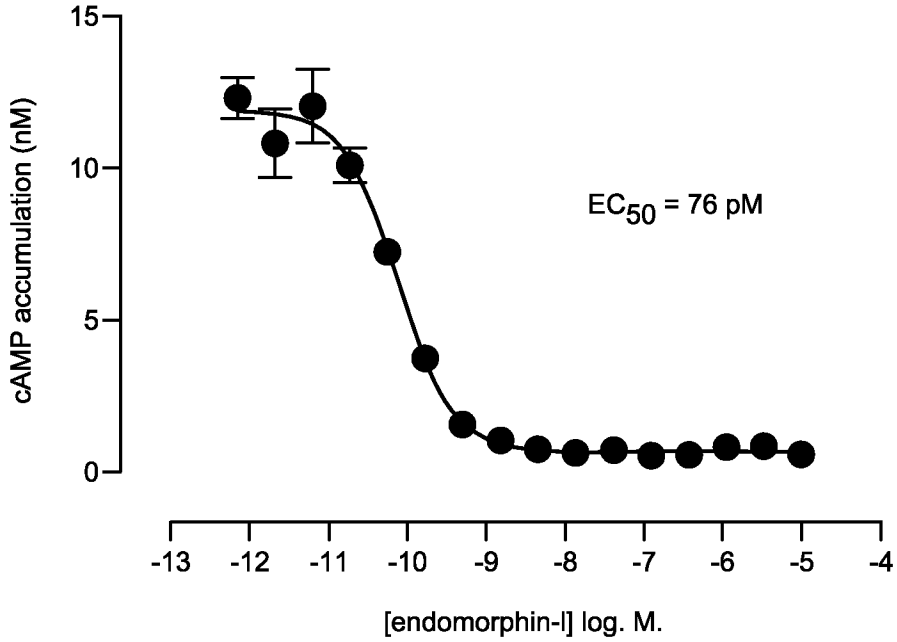

15
10
5
0
cAMP accumulation (nM)
EC50 = 76 pM
-13
-12
-11
-10
-9
-8
-7
-6
-5
-4
[endomorphin-I] log. M.

FIG. S1

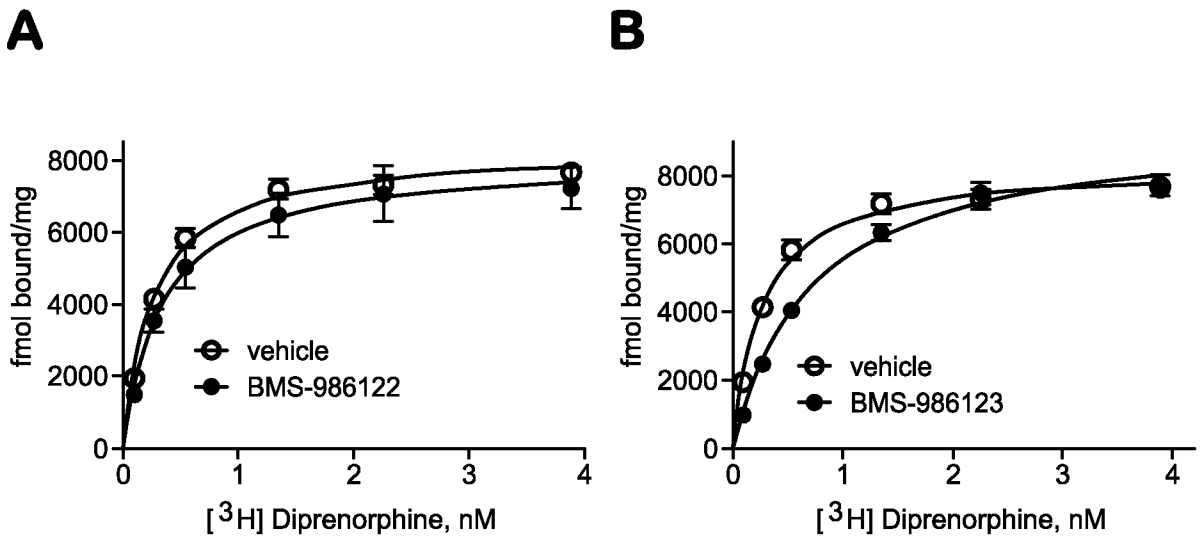

A
B
fmol bound/mg
8000
6000
4000
2000
0
0
1
2
3
4
[3H] Diprenorphine, nM
vehicle
BMS-986122
BMS-986123

FIG. S2

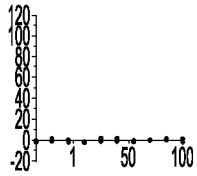
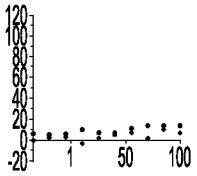
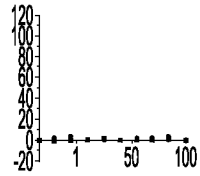
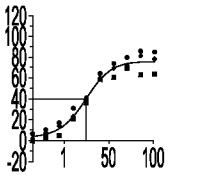
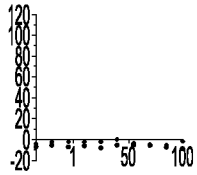
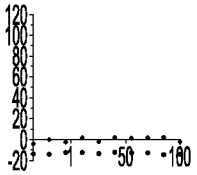
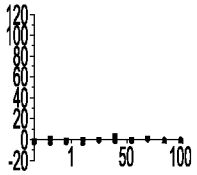
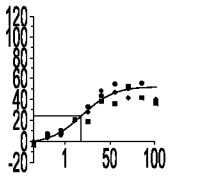
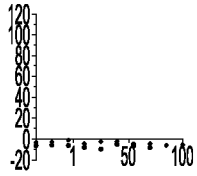
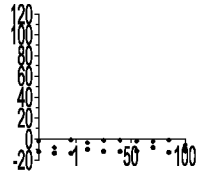
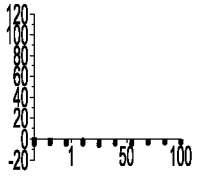
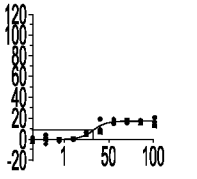
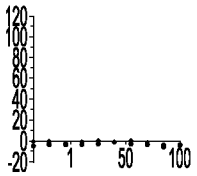
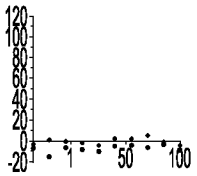
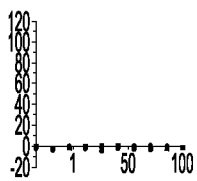
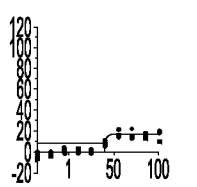
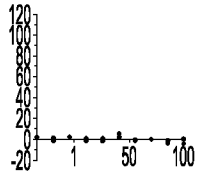
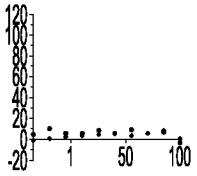
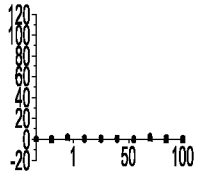
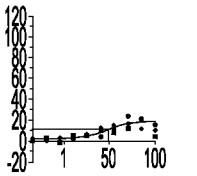
	delta agonist mode	delta PAM mode	mu agonist mode	mu PAM mode	mu SAM mode
	% activity	% activity	% activity	% activity	% activity
Subtance	U2OS-OPRD1	U2OS-OPRD1	U2OS-OPRM1	U2OS-OPRM1	U2OS-OPRM1
BMS-986122					
Analog 1					
Analog 2					
Analog 3					
Analog 4					

FIG. S3

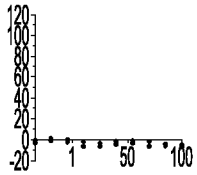
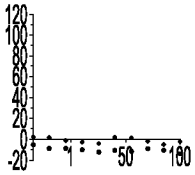
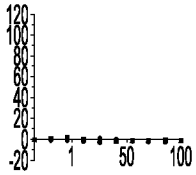
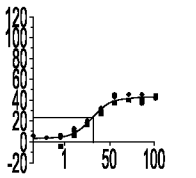
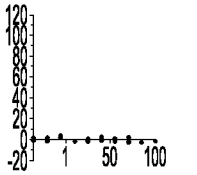
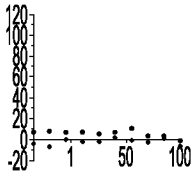
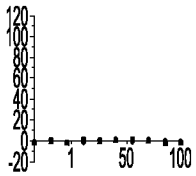
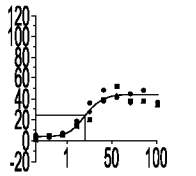
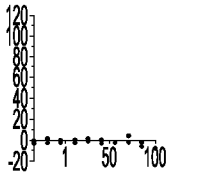
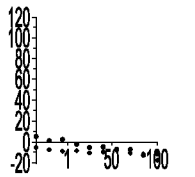
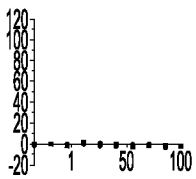
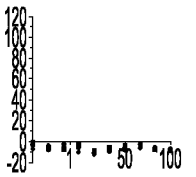
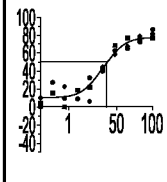
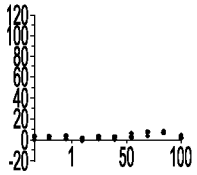
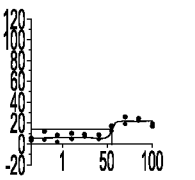
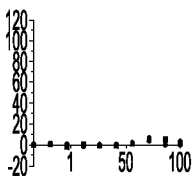
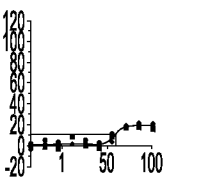
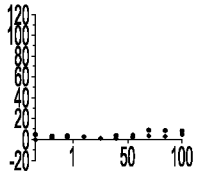
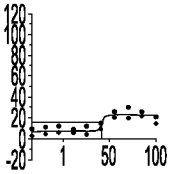
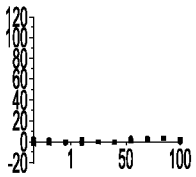
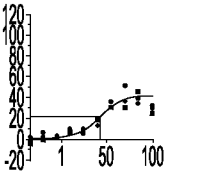
	delta agonist mode	delta PAM mode	mu agonist mode	mu PAM mode	mu SAM mode
	% activity	% activity	% activity	% activity	% activity
Subtance	U2OS-OPRD1	U2OS-OPRD1	U2OS-OPRM1	U2OS-OPRM1	U2OS-OPRM1
Analog 5					
Analog 6					
Analog 7 (BMS-986123)					
Analog 8					
Analog 9					

FIG. S3

continued

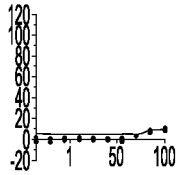
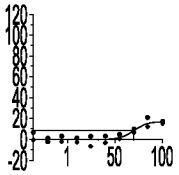
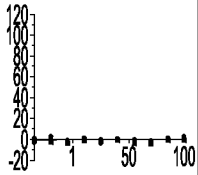
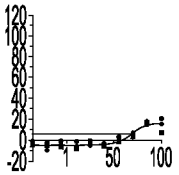
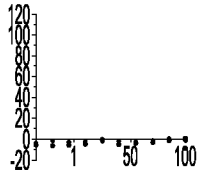
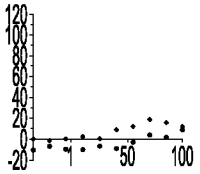
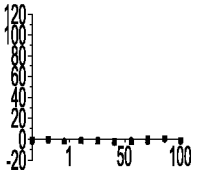
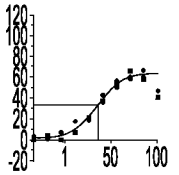
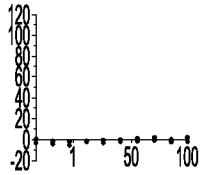
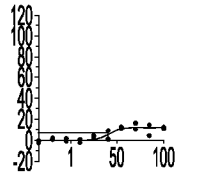
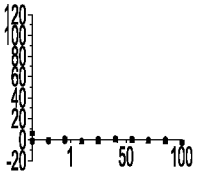
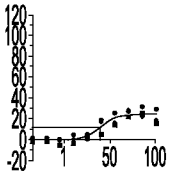
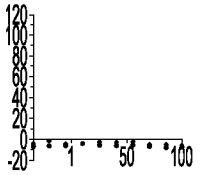
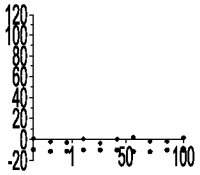
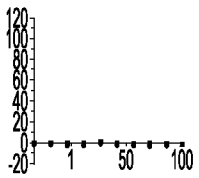
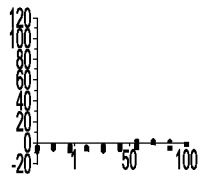
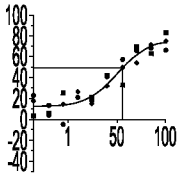
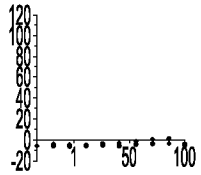
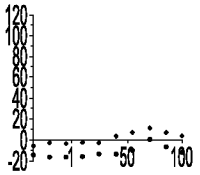
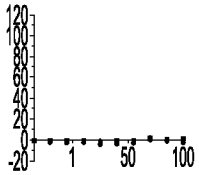
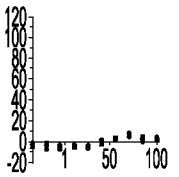
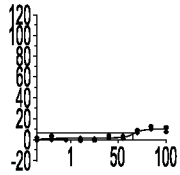
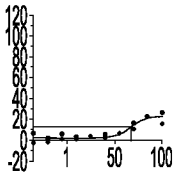
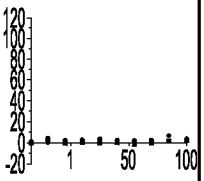
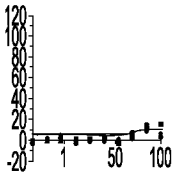
	delta agonist mode	delta PAM mode	mu agonist mode	mu PAM mode	mu SAM mode
	% activity	% activity	% activity	% activity	% activity
Subtance	U2OS-OPRD1	U2OS-OPRD1	U2OS-OPRM1	U2OS-OPRM1	U2OS-OPRM1
Analog 10					
Analog 11					
Analog 12					
Analog 13 (BMS-986124)					
Analog 14					
Analog 15					

FIG. S3

continued

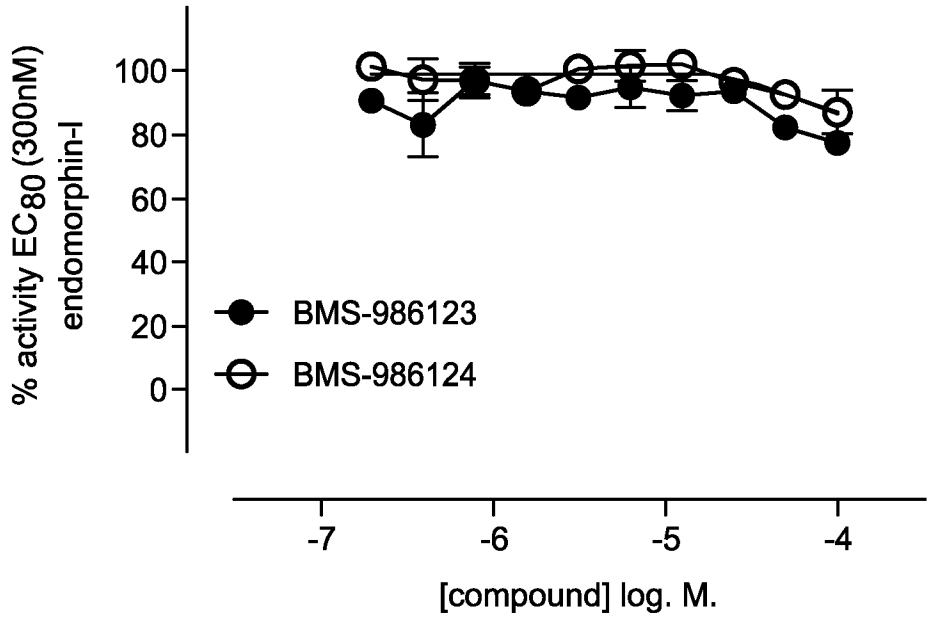

% activity EC80 (300nM) endomorphin-I
100
80
60
40
20
0
BMS-986123
BMS-986124
-7
-6
-5
-4
[compound] log. M.

FIG. S4

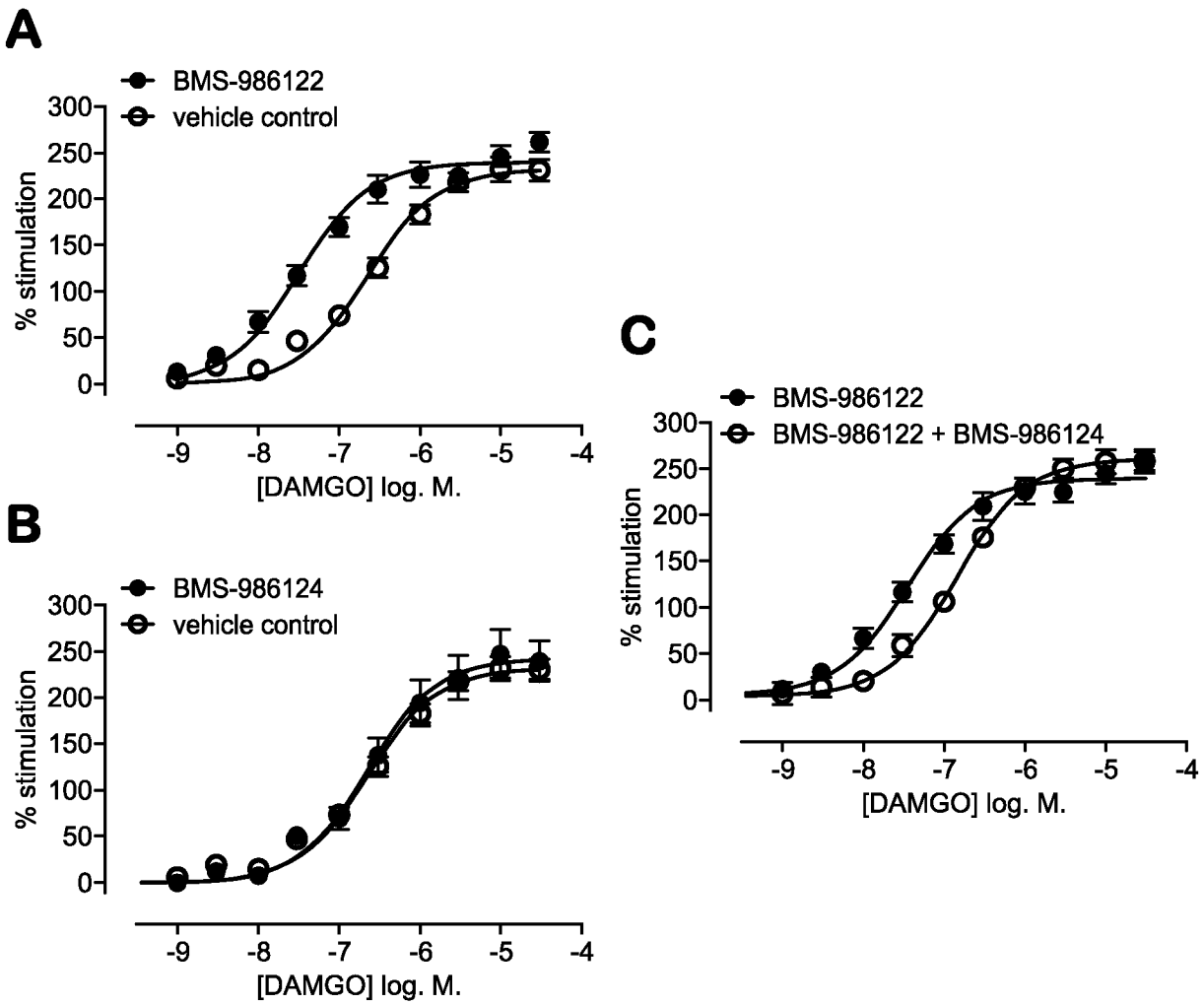

A
BMS-986122
vehicle control
% stimulation
300
250
200
150
100
50
0
-9
-8
-7
-6
-5
-4
[DAMGO] log. M.
B
BMS-986124
vehicle control
% stimulation
300
250
200
150
100
50
0
-9
-8
-7
-6
-5
-4
[DAMGO] log. M.
C
BMS-986122
BMS-986122 + BMS-986124
% stimulation
300
250
200
150
100
50
0
-9
-8
-7
-6
-5
-4
[DAMGO] log. M.

FIG. S5

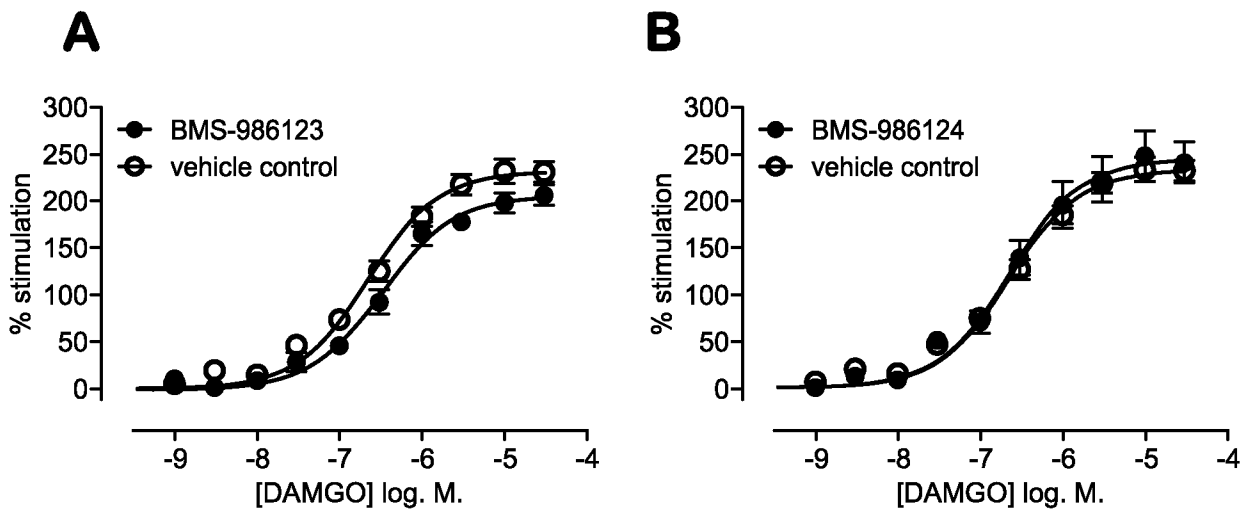

A
B
BMS-986123
vehicle control
BMS-986124
vehicle control
% stimulation
300
250
200
150
100
50
0
-9
-8
-7
-6
-5
-4
[DAMGO] log. M.

FIG. S6

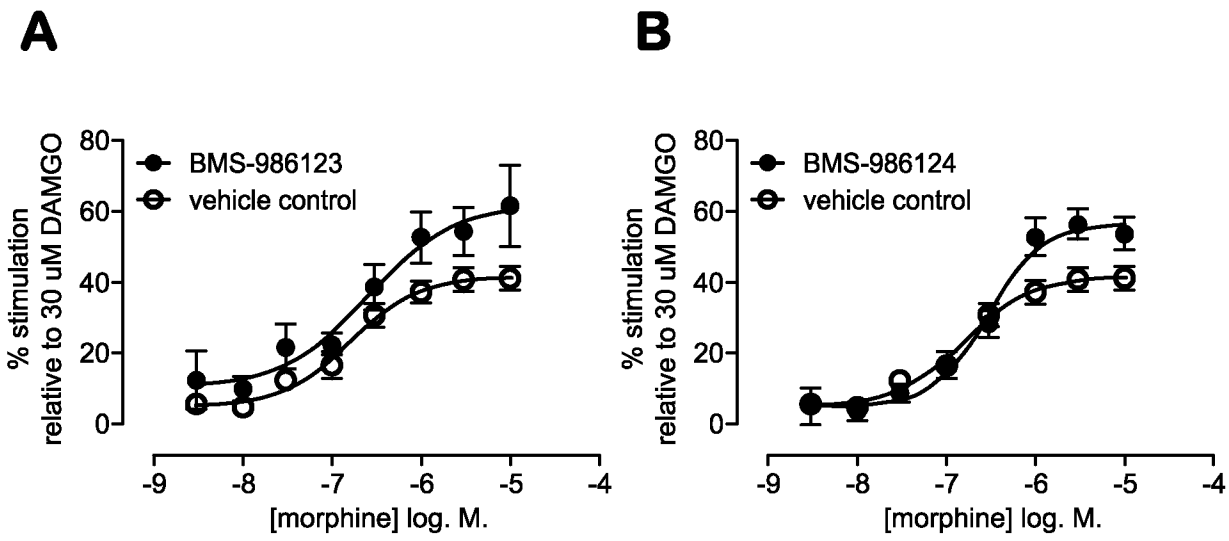
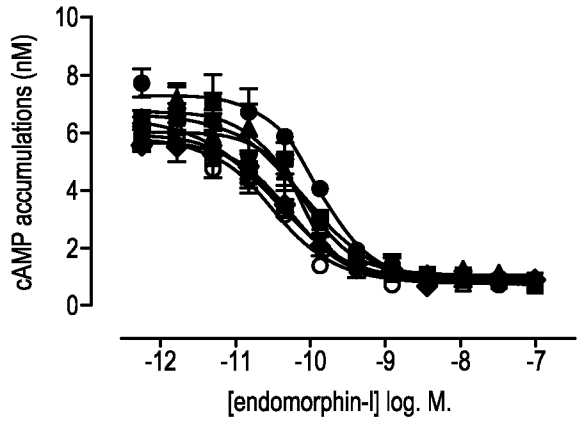

A
B
% stimulation relative to 30 uM DAMGO
80
60
40
20
0
BMS-986123
vehicle control
-9
-8
-7
-6
-5
-4
[morphine] log. M.
BMS-986124
vehicle control

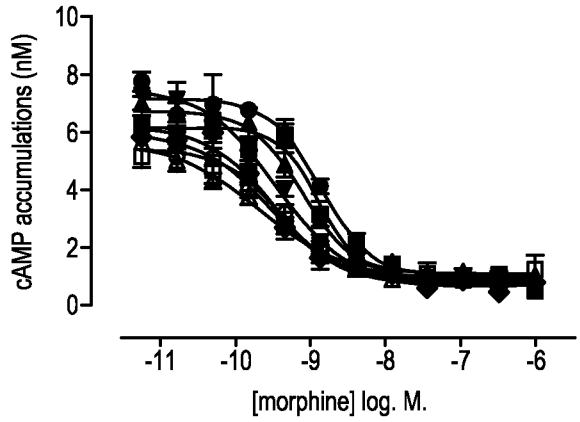
FIG. S7

A



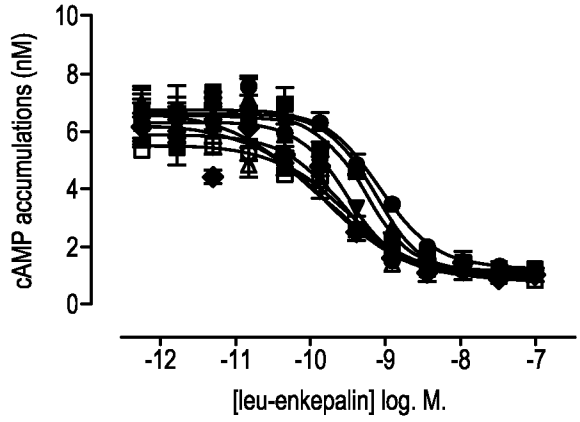
[BMS-986121] uM	EC_{50} (nM) for endomorphin-I
0	0.13
0.14	0.09
0.41	0.07
1.23	0.08
3.7	0.05
11	0.03
33	0.04
100	0.03

B



[BMS-986121] uM	EC_{50} (nM) for morphine
0	1.43
0.14	0.33
0.41	0.78
1.23	0.34
3.7	0.31
11	0.30
33	0.38
100	0.22

C



[BMS-986121] uM	EC_{50} (nM) for leu-enkephalin
0	0.09
0.14	0.05
0.41	0.07
1.23	0.03
3.7	0.03
11	0.01
33	0.03
100	0.02

FIG. S8

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2013/077018

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/566
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MARKUS KATHMANN ET AL: "Cannabidiol is an allosteric modulator at mu and delta-opioid receptors", 1 February 2006 (2006-02-01), NAUNYN-SCHMIEDEBERG'S ARCHIVES OF PHARMACOLOGY, SPRINGER, BERLIN, DE, PAGE(S) 354 - 361, XP019326119, ISSN: 1432-1912 the whole document ----- -/--	1-26

[X] Further documents are listed in the continuation of Box C.

[ ] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 March 2014	11/04/2014

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Lüdemann, Susanna

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/077018

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 27-34
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2013/077018

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	R. B. ROTHMAN ET AL: "Salvinorin A: Allosteric Interactions at the -Opioid Receptor", JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS, vol. 320, no. 2, 13 July 2006 (2006-07-13) , pages 801-810, XP055107592, ISSN: 0022-3565, DOI: 10.1124/jpet.106.113167 the whole document ----	1-26
A	STEPHAN SCHANN ET AL: "Chemical Switch of a Metabotropic Glutamate Receptor 2 Silent Allosteric Modulator into Dual Metabotropic Glutamate Receptor 2/3 Negative/Positive Allosteric Modulators", JOURNAL OF MEDICINAL CHEMISTRY, vol. 53, no. 24, 23 December 2010 (2010-12-23), pages 8775-8779, XP055107628, ISSN: 0022-2623, DOI: 10.1021/jm101069m the whole document ----	1-26
T	N. T. BURFORD ET AL: "Discovery of positive allosteric modulators and silent allosteric modulators of the -opioid receptor", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, vol. 110, no. 26, 25 June 2013 (2013-06-25), pages 10830-10835, XP055107237, ISSN: 0027-8424, DOI: 10.1073/pnas.1300393110 the whole document ----	

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.2

Claims Nos.: 27-34

Present claims 27-34 encompass compounds defined only by their desired function, contrary to the requirements of clarity of Article 6 PCT, because the result-to-be-achieved type of definition does not allow the scope of the claim to be ascertained. The fact that any compound could be screened does not overcome this objection, as the skilled person would not have knowledge beforehand as to whether it would fall within the scope claimed. No compounds with the desired function have been disclosed in the description or the examples. Undue experimentation would be required to screen compounds randomly.
The non-compliance with the substantive provisions is to such an extent that no meaningful search of claims 27-34 could be carried out at all (Article 17(2) PCT).
Furthermore claims 27-34 relate to subject-matter according to R. 39.1 (iv) PCT, i.e. methods for the treatment of the human or animal body by therapy.

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guidelines C-IV, 7.2), should the problems which led to the Article 17(2) declaration be overcome.