Journal of Biomolecular Screening
2014, Vol. 19(9) 1255–1265

DOI: 10.1177/1087057114542975
jbx.sagepub.com
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Identification of Selective Agonists and Positive Allosteric Modulators for μ- and δ-Opioid Receptors from a Single High-Throughput Screen

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Abstract

Hetero-oligomeric complexes of G protein–coupled receptors (GPCRs) may represent novel therapeutic targets exhibiting different pharmacology and tissue- or cell-specific site of action compared with receptor monomers or homo-oligomers. An ideal tool for validating this concept pharmacologically would be a hetero-oligomer selective ligand. We set out to develop and execute a 1536-well high-throughput screen of over 1 million compounds to detect potential hetero-oligomer selective ligands using a β-arrestin recruitment assay in U2OS cells coexpressing recombinant μ- and δ-opioid receptors. Hetero-oligomer selective ligands may bind to orthosteric or allosteric sites, and we might anticipate that the formation of hetero-oligomers may provide novel allosteric binding pockets for ligand binding. Therefore, our goal was to execute the screen in such a way as to identify positive allosteric modulators (PAMs) as well as agonists for μ, δ, and hetero-oligomeric receptors. While no hetero-oligomer selective ligands were identified (based on our selection criteria), this single screen did identify numerous μ- and δ-selective agonists and PAMs as well as nonselective agonists and PAMs. To our knowledge, these are the first μ- and δ-opioid receptor PAMs described in the literature.

Keywords

opioid receptor, positive allosteric modulator, high-throughput screen, GPCR, β-arrestin

Introduction

Opioid receptors are one of the most studied families of G protein–coupled receptors (GPCRs) since they are the target receptors for opiates such as morphine, which are widely used for chronic pain management, and represent a multibillion dollar market for the pharmaceutical industry.[1] These opioid ligands also can have significant side effects, including tolerance and abuse liabilities.[2,3] Research in this area is focused on developing ligands that can maintain pain relief while reducing these adverse effects.

Over the past decade, evidence has been accumulating that the three main types of opioid receptors—μ-, δ-, and κ-opioid receptors—may dimerize or form hetero-oligomers, thereby creating potential novel therapeutic targets that exhibit distinct pharmacology from monomers or homo-oligomers and may display a tissue- or even cell-specific site of action.[4,5] Hetero-oligomer–selective ligands would be extremely useful tools to pharmacologically decipher how hetero-oligomeric forms of the receptor may benefit pain management and reduce the side effects associated with opiate ligands.

Hetero-oligomer–selective ligands may bind at either orthosteric or allosteric binding sites on the two individual protomers or at novel allosteric sites produced only when the two receptors form a hetero-oligomeric complex. Thus, it would be beneficial when screening for hetero-oligomer–selective ligands to be able to identify allosteric as well as orthosteric ligands.

Allosteric ligands can have intrinsic efficacy at the receptor (allosteric agonists), or they can modulate the functional activity of orthosteric agonists by modulating the affinity and/or efficacy of the orthosteric agonist. This modulation can lead to enhanced activity of the orthosteric agonist (in the case of positive allosteric modulators or PAMs),

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Received Mar 18, 2014, and in revised form May 16, 2014. Accepted for publication Jun 17, 2014.

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typically resulting in leftward shifts in the functional potency of the orthosteric agonist.[6,7] Negative allosteric modulators (NAMs) reduce orthosteric agonist functional responses, resulting in rightward shifts in agonist potency and/or reduction in efficacy.

We selected the PathHunter β-arrestin recruitment system from DiscoveRx (Freemont, CA)[8,9] that could be configured to detect agonists, PAMs, and hetero-oligomer–selective agonists and PAMs at μ- and δ-opioid receptors from a single primary screening assay. In this system, an engineered cell line was developed expressing both recombinant μ- and δ-opioid receptors. This assay detects β-arrestin-2 recruitment to μ-opioid receptors using enzyme complementation. We also showed that these cells are capable of detecting δ-opioid receptor–specific ligands by transactivation of μ-opioid receptors. Although we did not discover any obvious hetero-oligomer–selective ligands (based on our selection criteria), we did discover novel μ- and δ-opioid receptor–selective agonists and PAMs. Two of the μ-selective PAMs were further characterized in terms of their ability to modulate orthosteric agonist-stimulated arrestin and G protein–mediated responses.[10] To our knowledge, these are the first opioid receptor–selective PAMs described in the literature and represent novel tools for opioid receptor research.

Materials and Methods

U2OS PathHunter cells expressing enzyme acceptor (EA)–tagged β-arrestin-2 and either ProLink (PK)–tagged μ-opioid receptor (U2OS-OPRM1), PK-tagged δ-opioid receptor (U2OS-OPRD1), or PK-tagged μ- and native δ-opioid receptors (U2OS-OPRM1/D1), as well as PathHunter detection reagents, were from DiscoveRx. Chinese hamster ovary (CHO) cells expressing recombinant μ-opioid receptors (CHO-μ) were from PerkinElmer (Waltham, MA). Cell culture media and supplements were from Life Technologies (Carlsbad, CA). HTRF cAMP detection reagents were from Cisbio (Cambridge, MA). Bovine serum albumin (BSA), morphine sulfate, leu-enkephalin, β-endorphin, forskolin, and IBMX were from Sigma-Aldrich (St. Louis, MO). All other opioid ligands were from Tocris (Ellisville, MO).

Cell Line Validation

PathHunter U2OS-OPRM1, U2OS-OPRD1, and U2OS-OPRM1/D1 cells were grown in modified Eagle's medium (MEM) containing 10% fetal bovine serum (FBS), 500 μg/mL G418, and 250 μg/mL hygromycin. Cells were grown to confluence in cell culture Nunc triple-layer flasks (Thermo Fisher Scientific (Roskilde, Denmark), harvested with TrypLE Express (Life Technologies, Carlsbad, CA), and resuspended in assay buffer (Hank's buffered salt solution [HBSS] + 25 mM HEPES, 100 IU/mL penicillin, 100 μg/mL streptomycin, 0.05% BSA at 1×10^6 cells/mL).

Compounds (20 nL of 100× final concentration in 100% DMSO) were added to white, nontreated 1536-well plates (Corning, New York, NY) by acoustic dispense using an Echo-550 (Labcyte, Sunnyvale, CA) from Echo-qualified 1536-well source plates (Labcyte). Next, 1 μL of assay buffer (agonist mode), assay buffer containing a low concentration (~EC_{10}) of orthosteric agonist (PAM mode), or assay buffer containing an ~EC_{80} concentration of orthosteric agonist (antagonist mode) was added to assay plates. The orthosteric agonists used are described in the Results. Finally, 1 μL 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 μL PathHunter Reagent. One hour later, luminescence was detected using a Viewlux imaging plate reader (PerkinElmer).

Additional characterization of certain μ-opioid receptor–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 and Discussion.

Inhibition of Forskolin-Stimulated cAMP Accumulation Assays

CHO cells expressing recombinant human μ-opioid receptors (CHO-μ) were grown to confluence in F12 media containing 10% FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 400 μg/mL G418 in T-175 tissue culture flasks (Corning) and harvested with TrypLE Express. Cells were pelleted by centrifugation and resuspended in assay buffer at 6.67×10^5 cells/mL.

Compounds (30 nL of 100× final concentration in 100% DMSO) were added to 1536-well white solid NT plates by acoustic dispense using an Echo-550. Next, 1.5 μL of assay buffer containing 1 mM IBMX and 2× forskolin (1 μM final), without (agonist mode) or with (PAM mode) 2× endomorphin-I (30 pM final, which was an ~EC_{10} concentration), was added to the plates. Finally, cells (1.5 μL/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 μL of D2-labeled cAMP tracer in lysis buffer, followed by 1.5 μL of Eu-cryptate–conjugated anti-cAMP antibody in lysis buffer). After a 1-h incubation at room temperature, time-resolved fluorescence was detected on a Viewlux or Envision plate reader (PerkinElmer) with excitation at 337 nm and emission reads at 615 nm and 665 nm. The ratiometric data (665 nm read/615 nm read)*10,000 were then converted to cAMP (nM) based on a standard curve for cAMP (replacing the cell addition step) run at the same time and under identical conditions to the assay.

Characterization of μ-opioid receptor–selective PAMs in the CHO-μ cAMP assay, using curve-shift assays, were performed as described above using orthosteric agonists described in the Results and Discussion.

Data Analysis

Concentration-response data were fit to a logistic equation using nonlinear regression analysis to provide estimates of Y_{min} (bottom), Y_{max} (top), potency (EC_{50}), and slope factor (Hill slope), using GraphPad Prism 5.01 (sigmoidal dose response with variable slope; GraphPad Software, La Jolla, CA). For Hill slope comparisons, one-way analysis of variance (ANOVA) was used followed by Tukey's posttest, with significance set at $p < 0.05$.

The curve shift assays were analyzed using an allosteric ternary complex model (GraphPad Prism 5.01 dose response—special-allosteric EC_{50} shift) to determine log Kb and the cooperativity factor (α) of the PAMs.

Results and Discussion

Enzyme Fragment Complementation β-Arrestin Recruitment Assay

To design a system that could detect hetero-oligomer–selective compounds, as well as receptor protomer compounds, we began with a PathHunter arrestin assay[8,9] expressing recombinant μ-opioid receptors in U2OS cells (U2OS-OPRM1). In this system, a mutated amino-terminal fragment of β-galactosidase, termed *ProLink* (PK), was fused to the carboxyl terminus of the μ-opioid receptor, and an N-terminal deletion mutant of β-galactosidase, termed *enzyme acceptor* (EA), was fused to the C-terminus of the β-arrestin molecule. Binding of arrestin to the activated μ-opioid receptor resulted 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 μ-opioid receptors. Upon expression of recombinant native δ-opioid receptors into this system (U2OS-OPRM1/D1), the cells responded to δ-opioid receptor–specific agonists as well. This effect was specific to δ-opioid receptors, as expression of eight other recombinant GPCR targets in the U2OS-OPRM1 cell line failed to produce a signal when stimulated (data not shown). These results suggest that activation of δ-opioid receptors causes arrestin recruitment to μ-opioid receptors through a specific transactivation mechanism that may be due to the formation of μ/δ-opioid receptor hetero-oligomers. There is considerable evidence from the literature that μ- and δ-opioid receptors can form hetero-oligomers.[4,5,11]

Pharmacological Characterization of Cell Lines

First, it was important to validate this cell line to see if it produced a robust signal in a miniaturized 1536-well high-throughput screening (HTS) format. To differentiate the activities of ligands at μ/δ-opioid receptor hetero-oligomers versus the individual monomers (or homo-oligomers), it was also necessary to validate the PathHunter U2OS cells expressing recombinant PK-tagged μ-opioid receptor (U2OS-OPRM1) alone and PathHunter U2OS cells expressing recombinant PK-tagged δ-opioid receptor (U2OS-OPRD1) alone. Results of concentration-response assays to a variety of nonselective as well as μ- and δ-opioid receptor–selective agonists are shown in **Figure 1A–C** and **Table 1**. Endomorphin-I (a μ-opioid receptor–selective agonist) produced a robust response in U2OS-OPRM1 and OPRM1/D1 cells and no response in U2OS-OPRD1 cells. Similarly, the δ-opioid receptor–selective agonists deltorphin-II and SNC80 produced robust responses in U2OS-OPRD1 cells but no responses in U2OS-OPRM1 cells. However, both μ- and δ-opioid receptor–selective agonists produced similar, maximal responses in the U2OS-OPRM1/D1 cell line, even though only μ-opioid receptors have the PK tag attached. This confirms that δ-opioid receptor–selective agonists can "transactivate" the μ-opioid PK-tagged receptor, suggesting that μ- and δ-opioid receptors form a hetero-oligomeric complex in this cell line.

Interestingly, DAMGO and morphine (both μ-opioid receptor–selective agonists) showed reduced efficacy relative to endomorphin-I in U2OS-OPRM1 cells, suggesting that they are partial agonists in this system. This reduced efficacy was also observed in the U2OS-OPRM1/D1 cell line. This is not unexpected for morphine, which is reported to only weakly recruit β-arrestin.[12] However, the finding that DAMGO appears to be a partial agonist in this system is unexpected, since DAMGO appears to be a full agonist in G protein–mediated signaling in studies from the literature.[13] The Hill slope for morphine in the U2OS-OPRM1/D1 cells was significantly lower than for DAMGO and deltorphin-II. Morphine can activate both μ- and δ-opioid receptors (but with higher potency at μ-opioid receptors), and the lower Hill slope may represent the combination of activity of morphine at both the μ- and δ-opioid receptors in this cell line.

Leu-enkephalin and β-endorphin, considered relatively nonselective agonists for μ- and δ-opioid receptors,[14] produced more potent responses (885-fold and 30-fold, respectively) in the U2OS-OPRD1 cell line compared with the U2OS-OPRM1 cell line. Nickolls and colleagues[15] showed that pretreatment of U2OS-OPRM1 cells with increasing concentrations of the irreversible binding ligand β-FNA resulted in a reduction in ^{3}H-diprenorphine binding sites. Increasing concentration of β-FNA pretreatment resulted in a reduction in efficacy of μ-opioid receptor agonists in the β-arrestin recruitment assay but had no effect on agonist potencies. This suggests that the arrestin recruitment assay lacks a receptor reserve, and functional potencies of ligands in this assay reflect binding affinities.[15] The data therefore suggest that leu-enkephalin and β-endorphin have a higher affinity for δ- than for μ-opioid receptors.

δ-Opioid receptor–selective ligands were less potent (about 3-fold) in the U2OS-OPRM1/D1 cell line compared

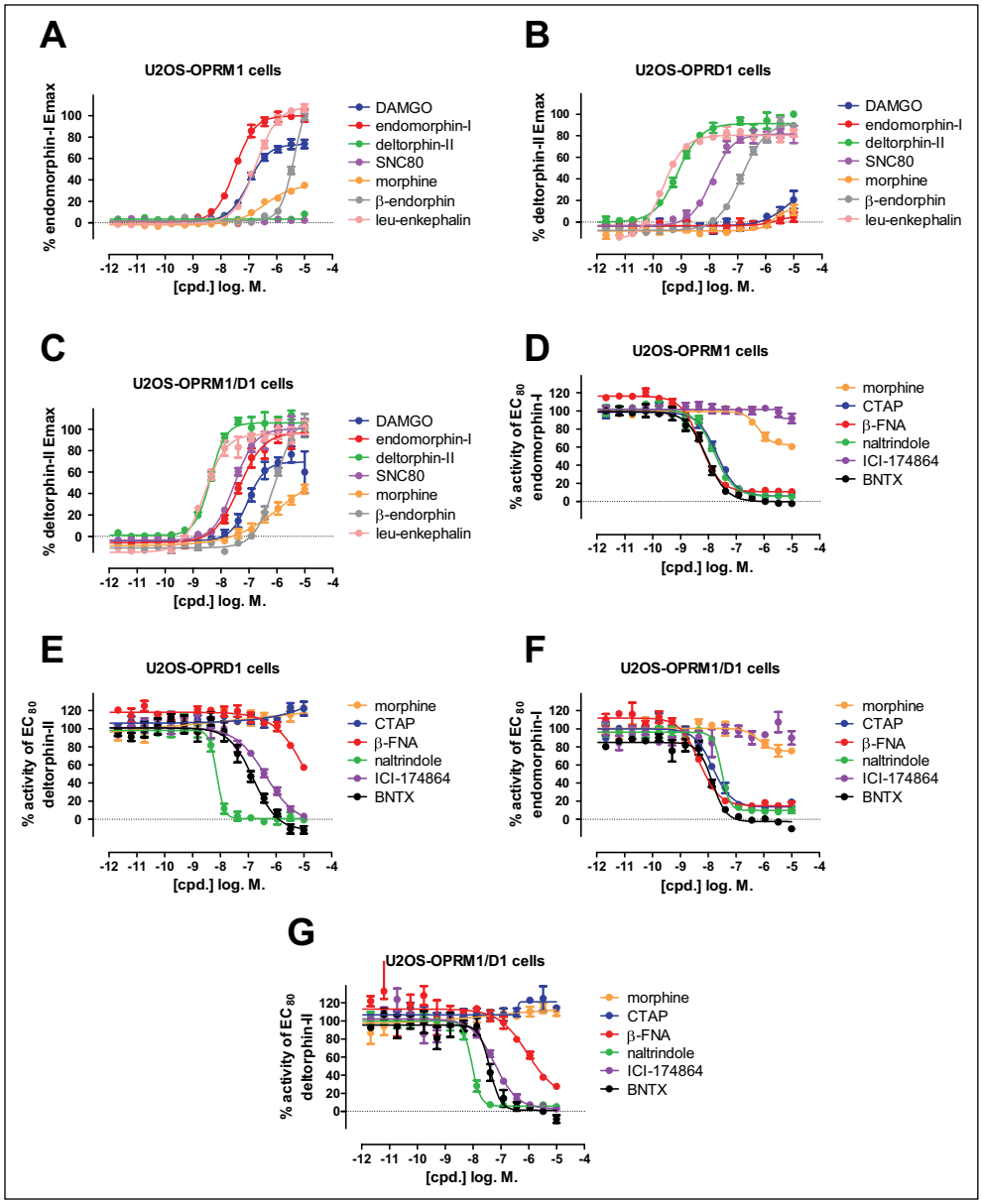

Figure 1. Characterization of opioid receptor agonists and antagonists in the PathHunter β-arrestin recruitment assay in U2OS-OPRM1 (**A**, **D**), OPRD1 (**B**, **E**), and OPRM1/D1 (**C**, **F**, **G**) cells. Data are expressed as a % of endomorphin-I E_{max} response (**A**) and % of deltorphin-II E_{max} response (**B**, **C**). Antagonist data are expressed as a % activity of an EC_{80} endomorphin-I response (**D**, **F**) and as % activity of a deltorphin-II EC_{80} response (**E**, **G**). Data are the mean + SEM of three experiments. EC_{50}s, IC_{50}s, E_{max} values, and Hill slopes are reported in **Tables 1** and **2**.

with U2OS-OPRD1 cells, while μ-opioid receptor–selective agonists had potencies that were not significantly different between U2OS-OPRM1 and U2OS-OPRM1/D1 cells. There are a number of possibilities to explain this. First, the level of expression of δ-opioid receptors in U2OS-OPRD1 and U2OS-OPRM1/D1 may be different, and the rightward shift in concentration-response curves (CRCs) in U2OS-OPRM1/D1 may signify a reduction in receptor reserve compared with U2OS-OPRD1. However, Gomes et al.[16] showed that the U2OS-OPRD1 cell line has fewer ^{3}H-deltorphin-II binding sites (3.7 pmoles/mg protein) than in the U2OS-OPRM1/D1 cell line (6 pmoles/mg protein), suggesting that δ-opioid receptor expression in U2OS-OPRD1 cells is less than in U2OS-OPRM1/D1 cells. Also, on the basis of the observations of Nickolls et al.,[15] the potencies of agonist ligands in this assay are thought to reflect their binding affinities (i.e., there is no receptor reserve). A second possibility is that since the response in U2OS-OPRM1/D1 cells comes from transactivation of only the PK-tagged μ-opioid receptors, the potency difference may indicate that only a fraction of the expressed δ-opioid receptors is capable of transactivating μ-opioid receptors. Again, this would seem unlikely if the assay system is thought to mimic the binding affinity (i.e., it has no receptor reserve). Gomes et al.[16] showed that the U2OS-OPRM1/D1 cell line has 0.3 pmoles/mg protein of ^{3}H-DAMGO binding sites compared with 0.6 pmoles/mg protein in U2OS-OPRM1 cells. This suggests that δ-opioid receptors are

Table 1. Characterization of Opioid Receptor Agonists in the PathHunter β-Arrestin Recruitment Assay in U2OS-OPRM1, U2OS-OPRD1, and U2OS-OPRM1/D1 Cells.

	U2OS-OPRM1 Cells			U2OS-OPRD1 Cells			U2OS-OPRM1/D1 Cells		
Ligand	EC_{50} (95% CI), nM	E_{max} (95% CI), %[a]	Hill Slope (95% CI)	EC_{50} (95% CI), nM	E_{max} (95% CI), %[b]	Hill Slope (95% CI)	EC_{50} (95% CI), nM	E_{max} (95% CI), %[b]	Hill Slope (95% CI)
DAMGO	98 (83–116)	73 (70–76)	1.2 (1.0–1.5)		NA		92 (60–140)	69 (61–77)	1.4 (0.7–2.1)
Endomorphin-I	32 (28–37)	100 (97–103)	1.3 (1.1–1.5)		NA		44 (33–60)	97 (91–103)	1.0 (0.7–1.2)
Deltorphin-II		NA		0.7 (0.6–1.0)	91 (88–95)	1.0 (0.8–1.3)	4 (3–5)	106 (102–110)	1.6 (1.1–2.0)
SNC80		NA		12 (10–15)	82 (78–85)	1.2 (0.9–1.4)	30 (24–38)	100 (96–105)	1.2 (0.9–1.4)
Morphine	367 (249–540)	36 (32–40)	0.8 (0.6–1.1)		NA		1433 (127–16160)	64 (25–102)	0.5 (0.2–0.7)
β-Endorphin	4239 (3461–5491)	120 (105–136)	1.8 (1.4–2.1)	140 (116–169)	92 (88–97)	1.0 (0.8–1.2)	905 (721–1135)	114 (104–124)	1.2 (0.9–1.4)
Leu-enkephalin	177 (158–197)	108 (105–111)	1.1 (1.0–1.2)	0.2 (0.2–0.3)	81 (79–82)	1.1 (1.0–1.3)	3 (2–3)	96 (91–100)	1.1 (0.9–1.4)

Data are expressed as the mean and 95% confidence interval (CI) of three experiments. Graphical representations of the data are shown in **Figure 1**. NA, not active at 1 μM.
[a]Relative to 10 μM endomorphin-I response.
[b]Relative to 10 μM deltorphin-II response.

expressed at a much higher level compared with μ-opioid receptors in U2OS-OPRM1/D1 cells. However, this does not tell us what fraction of δ-opioid receptors is involved in transactivating the PK-tagged μ-opioid receptors in U2OS-OPRM1/D1 cells. The third possibility is that these δ-opioid receptor agonists are less potent when δ-opioid receptors form hetero-oligomers with μ-opioid receptors. Without additional studies, it is difficult to conclude which (if any) of these scenarios is correct.

Characterization of the cell lines with antagonists was performed in the presence of an ~EC_{80} concentration of either endomorphin-I (100 nM) (in U2OS-OPRM1 and U2OS-OPRM1/D1 cells) or deltorphin-II (10 nM) (in U2OS-OPRD1 and U2OS-OPRM1/D1 cells) (**Fig. 1D–G** and **Table 2**). None of the antagonists reduced activity below basal activity in the arrestin assay, suggesting that there was either no detectable constitutive activity of these receptors or that none of the antagonist ligands has inverse agonist activity in this system (data not shown). Morphine, a μ-opioid receptor–selective partial agonist, produced partial antagonism of the response to endomorphin-I in U2OS-OPRM1 and U2OS-OPRM1/D1 cells. The μ-opioid receptor–selective antagonist CTAP was a potent inhibitor of endomorphin-I responses in U2OS-OPRM1 and U2OS-OPRM1/D1 cells but had no inhibitory activity on deltorphin-II–stimulated responses in U2OS-OPRD1 and U2OS-OPRM1/D1 cells. Conversely, the δ-opioid receptor–selective antagonist ICI-174864 inhibited deltorphin-II–stimulated responses but not endomorphin-I–stimulated responses. These data suggest that a δ-opioid receptor–selective competitive antagonist cannot "transinhibit" the μ-opioid receptor responses to endomorphin-I in the coexpressing U2OS-OPRM1/D1 cell line. Indeed, the profile of antagonist responses to endomorphin-I–stimulated activity in U2OS-OPRM1 and U2OS-OPRM1/D1 cells and to deltorphin-II–stimulated activity in U2OS-OPRD1 and U2OS-OPRM1/D1 cells is similar. These findings differ from the observations of Gomes et al.,[16] who reported that $TIPP_{\psi}$ (a δ-opioid receptor–selective antagonist) could inhibit arrestin recruitment mediated by the μ-opioid receptor–selective agonist, DAMGO, and CTOP (a μ-opioid receptor–selective antagonist) could inhibit arrestin recruitment mediated by the δ-opioid receptor–selective agonist, deltorphin-II, only in the cell line coexpressing μ- and δ-opioid receptors. These differences may be explained by the use of different agonist and antagonist ligands in our study and/or differences in assay method. For instance, in the current study, we used suspensions of cells with a room temperature incubation, whereas in the Gomes et al.[16] study, adherent cells were used with a 37 °C incubation. However, there is no evidence to suggest that these assay differences can change inverse agonist activity to neutral antagonism. The literature suggests that the antagonists used here are neutral antagonists. It would be interesting to see what an inverse agonist might do in this system (i.e., could an inverse agonist

Table 2. Characterization of Opioid Receptor Antagonists in the PathHunter β-Arrestin Recruitment Assay in U2OS-OPRM1, U2OS-OPRD1, and U2OS-OPRM1/D1 Cells.

Ligand	U2OS-OPRM1 cells with ~EC_{80} endomorphin-I	U2OS-OPRD1 cells with ~EC_{80} deltorphin-II	U2OS-OPRM1/D1 cells with ~EC_{80} endomorphin-I	U2OS-OPRM1/D1 cells with ~EC_{80} deltorphin-II
	IC_{50} (95%CI) (nM) E_{max} (95%CI) (% inhib.)[1] *Hill slope (95%CI)*	IC_{50} (95%CI) (nM) E_{max} (95%CI) (% inhib.)[2] *Hill slope (95%CI)*	IC_{50} (95%CI) (nM) E_{max} (95%CI) (% inhib.)[1] *Hill slope (95%CI)*	IC_{50} (95%CI) (nM) E_{max} (95%CI) (% inhib.)[2] *Hill slope (95%CI)*
Morphine	611 (420-891) 38 (33-43) *−1.9 (−2.9 to -0.8)*	NA	657 (256-1688) 25 (16-33) *−1.7 (−4.0 to +0.6)*	NA
CTAP	20 (17-25) 96 (91-98) *−1.3 (−1.5 to −1.0)*	NA	15 (11-20) 86 (81-91) *−1.3 (−1.8 to −0.8)*	NA
β-FNA	5 (5-6) 89 (87-91) *−1.2 (−1.4 to −1.1)*	NA	5 (4-6) 85 (81-90) *−1.2 (−1.6 to −0.9)*	889 (191-4138) 86 (39-130) *−0.8 (−1.5 to −0.2)*
Naltrindole	16 (13-20) 96 (91-98) *−1.2 (−1.5 to −1.0)*	7 (6-9) 99 (95-104) *−3.3 (−4.5 to −2.1)*	31 (27-35) 90 (87-93) *−3.0 (−4.0 to −2.0)*	9 (8-11) 94 (91-98) *−2.7 (−3.6 to −1.9)*
ICI-174864	NA	417 (208-832) 104 (85-124) *−0.8 (−1.1 to −0.5)*	NA	61 (39-93) 99 (88-107) −1.1 (−1.5 to −0.6)
BNTX	9 (7-11) 100 (96-104) *−1.2 (−1.5 to −0.9)*	154 (96-248) 112 (99-125) *−1.0 (−1.4 to −0.6)*	14 (11-18) 103 (98-107) *−1.7 (−2.4 to −1.1)*	40 (29-54) 99 (91-107) *−2.3 (−4.0 to −0.6)*

1 = relative to 10 μM endomorphin-I response; 2 = relative to 10 μM deltorphin-II response; NA = not active at 1 μM (poor curve fit determination).

of the δ-opioid receptor "transinhibit" μ-opioid receptor response to endomorphin-I in U2OS-OPRM1/D1 cells).

Antagonism of deltorphin-II in U2OS-OPRM1/D1 cells was more potent than in U2OS-OPRD1 cells for β-FNA, ICI-174864, and BNTX. However, since deltorphin-II was about 5-fold more potent in U2OS-OPRD1 cells than in U2OS-OPRM1/D1 cells, and the same concentration of deltorphin-II was used in both cell lines in the antagonist studies, these IC_{50} value differences may be due to differences in deltorphin-II stimulation levels between the cell lines. Naltrindole inhibition curves produced notably steeper slopes in the U2OS-OPRD1 and U2OS-OPRM1/D1 cells compared with responses in the U2OS-OPRM1 cells. However, we have no explanation for this observation.

HTS Campaign

The observation that activation of μ- and δ-opioid receptors in U2OS-OPRM1/D1 cells yields similar maximal responses allowed us to develop and execute an HTS campaign to identify both agonists and PAMs in the same screen. This required using a relatively low concentration (~EC_{10}) of both endomorphin-I (7.5 nM) to activate μ-opioid receptors and leu-enkephalin (3 nM) to activate δ-opioid receptors. The concentration of leu-enkephalin used in the U2OS-OPRM1/D1 cells produced no activity in the U2OS-OPRM1 cells, suggesting that all of the activity seen with leu-enkephalin at this concentration is mediated through δ-opioid receptors. **Figure 2** shows an example and description of a primary screening plate heat map. The screen was conducted in 1536-well plates, and over 1 million compounds were tested.

Quality control plates containing CRCs of reference agonists were placed at the beginning and end of each day's screening run to monitor the consistency of the pharmacology. Generally, the screen performed well with a signal to background (S/B) ratio of 2 to 2.5 and Z′ of 0.4 to 0.7.

After the primary screen was completed, hits were selected using a cutoff of >25% activity response relative to an E_{max} concentration of endomorphin-I. These hits (15,837 compounds) were retested in the same assay at the original 20-μM concentration used in the primary screen and again at 2.5 μM, each in triplicate plates. At the 20-μM concentration, 7748 compounds produced a mean activity of >25% activity (a 49% confirmation rate). Since the follow-up of hits involved multiple repetitions in multiple cell lines, we decided to pursue only the more potent hits. A cutoff of >20% activity at the 2.5-μM concentration was selected (1041 compounds) and reordered for CRCs.

Hit Assessment

To determine whether the hits were agonists or PAMs and whether they were μ- or δ-opioid receptor selective or nonselective, the compound CRCs were run in multiple cell

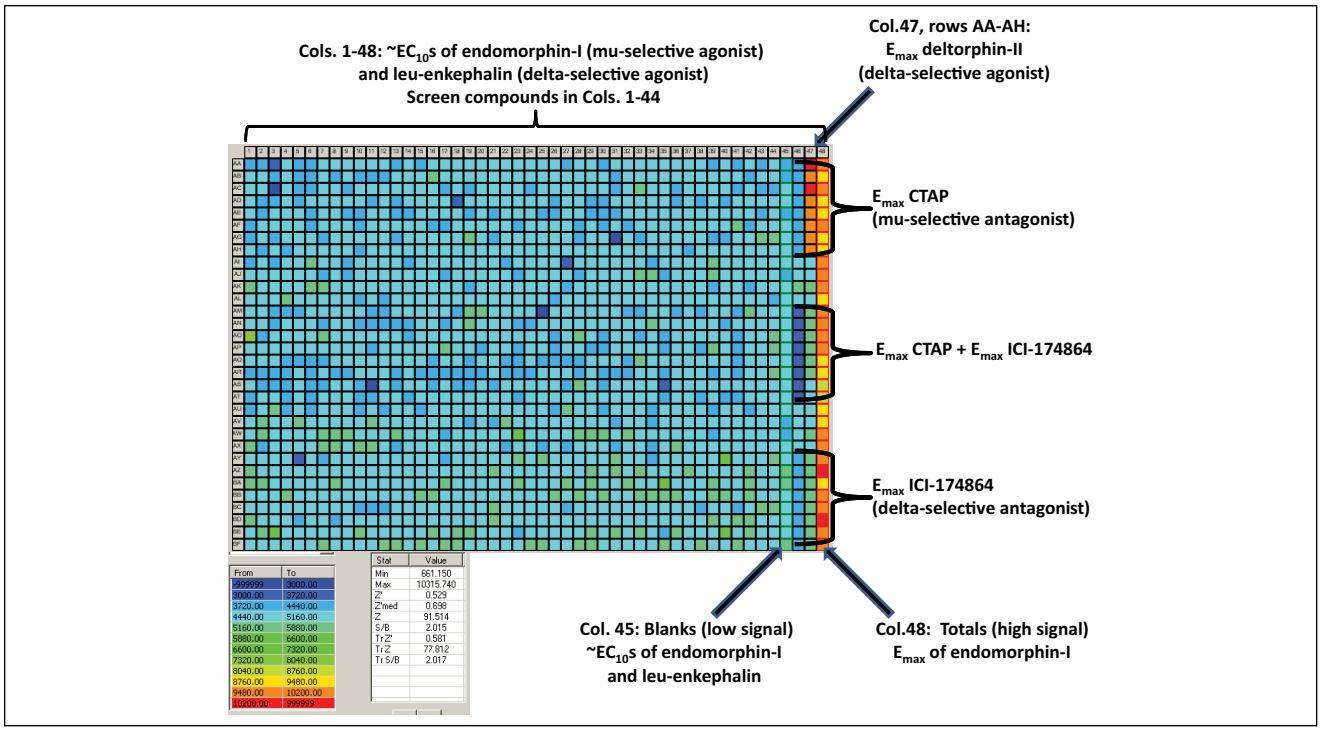

Figure 2. Example of heat map activity of a primary screen plate for the high-throughput screen. The screen was conducted in 1536-well plates with compounds (20 nL in 100% DMSO) in each well of columns 1 to 44. Column 45 contained an equal volume of DMSO. Column 46 was divided into three sections, with each containing CTAP, ICI-174864, and both antagonists together, at an E_{max} concentration (20 nL in 100% DMSO). The top wells of column 47 contained a maximal concentration of deltorphin-II (20 nL in 100% DMSO), and column 48 contained an E_{max} concentration of endomorphin-I (20 nL in 100% DMSO). A mixture of an ~EC_{10} of endomorphin-I and ~EC_{10} of leu-enkephalin was added to the whole plate. Finally, U2OS-OPRM1/D1 cells were added to the whole plate. Columns 45 and 48 provided low and high signal, respectively, for Z′ determination. The three sections of column 46 allowed us to determine the relative stimulation level of the cells with the mixture of endomorphin-I and leu-enkephalin, and thus the level of μ- and δ-opioid receptor stimulation, since the responses were inhibited by the selective antagonists. The deltorphin-II response in column 47 allowed us to monitor δ-opioid receptor maximal activity relative to μ-opioid receptor activity, which was monitored in column 48.

lines (U2OS-OPRM1/D1, U2OS-OPRM1, and U2OS-OPRD1) and formats (agonist and PAM modes), including dual PAM mode (in the presence of an EC_{10} of both endomorphin-I and leu-enkephalin) in the U2OS-OPRM1/D1 cells. By using a 1536-well ECHO-master source plate and dispensing 20 nL/well by acoustic dispensing, we were able to run all of the CRCs, for all the assays, in triplicate, from one source plate of compounds containing 5.5 μL of compound/well.

By visual inspection of all the CRC graphs, we were able to categorize the compounds as agonists, PAMs, or ago-PAMs and whether they were μ- or δ-opioid receptor selective or nonselective. Our selectivity criteria were set at a 5-fold or greater change in potency for a compound between the different cell lines. Changes in E_{max} (between the different cell lines) were not taken into account. While none of the compounds showed a potency preference for the μ/δ hetero-oligomer over the individual protomers, both μ- and δ-opioid receptor–selective agonists and PAMs were identified from the HTS. The lack of hetero-oligomeric receptor–selective agonists or PAMs identified in the screen suggests that these ligands may not be abundant. However, there is precedent for hetero-oligomer–selective ligands. One such agonist ligand, 6′GNTI, has been described[17] with some selectivity for κ/δ hetero-oligomers. However, the use of this tool is complicated because it is also a potent partial agonist of κ-opioid receptors for G protein–mediated signaling and an antagonist of κ-opioid receptor–mediated β-arrestin signaling.[18] Recently, Gomes et al.[16] performed a similar HTS in agonist detection mode using these same cell lines and reported the discovery of heterodimer-selective agonists, including CYM51010. It should be noted that CYM51010 would have been unlikely to be identified as heteromer selective in the current HTS because its selectivity for receptor heteromers versus homomers (just under 5-fold difference in potency in cells expressing μ- and δ-opioid receptors vs. μ-opioid receptors alone) would not have passed the selectivity criterion used for this study.

Although the selectivity of CYM51010 for heteromers is relatively modest, CYM51010 was also found to exhibit a ~2-fold increase in E_{max} in cells coexpressing μ- and δ-opioid receptors compared with cells expressing morphine alone, and heteromer-mediated activity of CYM51010 was confirmed using a heterodimer-selective antibody both in vitro and in vivo.[16] It will be of great interest to the field to see if chemical optimization of the CYM51010 series can result in compounds showing even greater selectivity of action at heteromers versus homomers.

The strength of the HTS described in the current article lies in its ability to detect PAMs as well as agonists. An example of a μ-opioid receptor–selective PAM discovered in the screen is BMS-986121. This compound showed no agonist activity in any of the cell lines. However, in the presence of a low concentration of endomorphin-I (mu PAM mode), the compound showed increased activity in the U2OS-OPRM1/D1 and U2OS-OPRM1 cell lines with an EC_{50} of 2 μM. No increased activity was observed with a low concentration of leu-enkephalin either in U2OS-OPRM1/D1 or U2OS-OPRD1 cells, suggesting that this compound is a μ-opioid receptor–selective PAM.

Opioid receptors couple to the Gi/o family of heterotrimeric G proteins.[19] To assess the activities of μ-opioid receptor–selective PAMs on G protein–mediated signaling, we sought to develop a cAMP assay to characterize these compounds. We found that the U2OS PathHunter cells did not produce particularly robust signals in the cAMP accumulation assay in the presence of reference opioid agonists (data not shown). This was also observed by Nickolls et al.,[15] who showed that [^{35}S]GTPγS binding and inhibition of forskolin-stimulated cAMP accumulation exhibited Z′ values of 0.21 and 0.13, respectively, in U2OS-OPRM1 cells. But they did proceed to generate data under those conditions. A robust inhibition of forskolin-stimulated cAMP assay was established by our group in CHO cells expressing recombinant μ-opioid receptors (CHO-μ). The pharmacological profile of several opioid receptor agonists in CHO-μ cells is shown in **Figure 3**. The reference compounds gave much more potent cAMP responses in CHO-μ cells than for β-arrestin recruitment in the U2OS-OPRM1 cells. This could be due to amplification of signal at the level of the second-messenger cascade, resulting in an increased apparent receptor reserve. As described earlier, potency in the β-arrestin recruitment assay may more closely resemble the binding affinity of compounds,[15] explaining the rightward shift in potency seen in this assay relative to adenylate cyclase inhibition. However, looking at two different assays in two different cell backgrounds, one cannot draw many conclusions from these data. It would be interesting to compare β-arrestin and cAMP responses in the same cell background as this may allow conclusions to be drawn about biased signaling of these ligands.

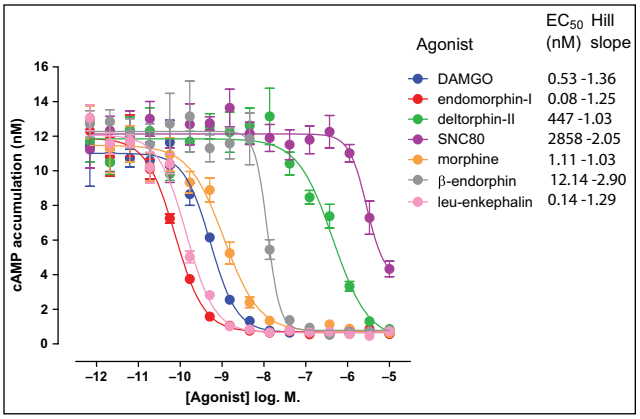

Figure 3. Characterization of reference opioid receptor agonists in inhibition of forskolin-stimulated cAMP accumulation assays in CHO-μ cells. Mean EC_{50} values (nM) and Hill slopes are shown next to each agonist ligand in the legend.

μ-Opioid Receptor–Selective PAM Characterization

Next, we used two of the μ-opioid receptor–selective PAMs discovered from the HTS campaign to see if they could produce leftward shifts in orthosteric agonist potencies in both the β-arrestin assay (in U2OS-OPRM1 cells) and the inhibition of forskolin-stimulated cAMP assay (in CHO-μ cells). Both compounds, designated here as BMS-986121 and BMS-986122 (**Fig. 4C,D**), produced leftward shifts in the EC_{50} for endomorphin-I (~7-fold) in the β-arrestin recruitment assay (**Fig. 4A,B**). No agonism was observed with either compound. The EC_{50} concentration of these two compounds required to shift the potency of endomorphin-I by half of its full potential, which has been termed "shifty 50,"[20] was about 2 μM for each compound. In the cAMP assay, BMS-986121 and BMS-986122 produced ~6-fold leftward shifts in the EC_{50} for endomorphin-I (**Fig. 4E,F**), although this required a higher concentration of the compounds (shifty-50s ~10 μM). Interestingly, a low level of agonist activity was detected at the highest concentrations of BMS-986121 used. BMS-986122 also showed significant agonist activity, seen as a reduction in forskolin-stimulated cAMP accumulation even in the absence of endomorphin-I (**Fig. 4F**). The agonist response to BMS-986122 was not observed in a CHO cell line that did not express the recombinant μ-opioid receptor (data not shown), suggesting that the BMS-986122 agonist response is μ-opioid receptor mediated. Therefore, BMS-986122 and, to a lesser extent, BMS-986121 appear to be ago-PAMs in the cAMP assay. It has been reported that pure PAMs can exhibit agonist activity, particularly in cell systems where the receptor is overexpressed.[21] The additional receptor reserve may increase the level of agonism shown by the allosteric modulator. This would be consistent with our

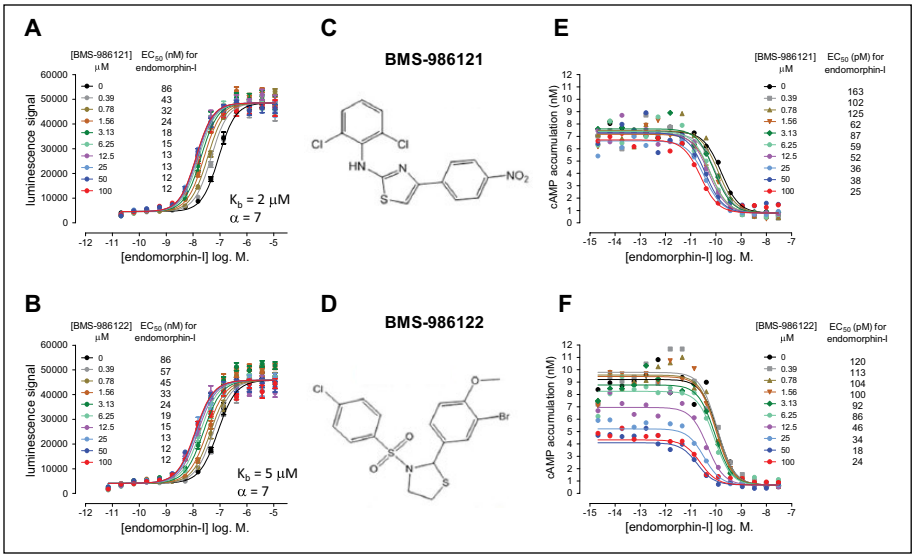

Figure 4. Characterization of the ability of two μ-opioid receptor–selective positive allosteric modulators, BMS-986121 (**A**, **C**, **E**) and BMS-986122 (**B**, **D**, **F**), to shift the potency of endomorphin-I to the left in a β-arrestin recruitment assay in U2OS-OPRM1 cells (**A**, **B**) and in an inhibition of forskolin-stimulated cAMP accumulation assay in CHO-μ cells (**E**, **F**). Mean EC_{50} (nM) values for endomorphin-I at each concentration of compound are shown in the legend. Data are represented as the mean + SEM of four experiments for the β-arrestin assays (**A**, **B**) and the mean (shown without error bars for clarity) of four experiments for the cAMP assay (**E**, **F**).

findings, where the cAMP system showed considerably more apparent receptor reserve than the β-arrestin system. Additional studies have shown that the agonist activity of BMS-986122 cannot be detected in mouse cortical membranes in [^{35}S]GTPγS binding studies. However, BMS-986122 did increase the potency of DAMGO-stimulated [^{35}S]GTPγS binding in those studies.[10]

Possible Implications of PAMs for Opioid Receptor Research

There are several potential benefits of allosteric ligands over their orthosteric counterparts for GPCR-based drug discovery. The first is that they can provide subtype selectivity where this selectivity has not been possible for ligands that bind to the orthosteric site.[22,23] This appears to be less of an issue for opioid receptors, since highly selective orthosteric agonists and antagonists exist for μ-, δ-, and κ-opioid receptors.[3] Second, allosteric modulators may have no efficacy on their own but modify responses to the levels of endogenous agonist binding to the receptor. Therefore, allosteric modulators can enhance or inhibit responses to endogenous agonist while still maintaining the temporal and spatial control of cell signaling by the endogenous agonist. This benefit may be of particular interest for researchers working on opioid receptor signaling, where chronic treatment with exogenous orthosteric opiates, such as morphine, can lead to significant side effects as well as tolerance and dependence liabilities. Studies with enkephalinase inhibitors suggest that modulating the activity of endogenous opioid receptor ligands may offer therapeutic benefit. Enkephalinase inhibitors have shown analgesic effects in animal models of inflammatory and neuropathic pain.[24] Similarly, naloxone (an opioid receptor antagonist) administered to postoperative patients who were not taking exogenous opioid medication resulted in increased pain perception, suggesting that endogenous opioid peptides produce a basal analgesic tone.[25] It will be interesting to see whether administration of opioid receptor PAMs alone can enhance this physiological tone. Third, the level to which PAMs increase the functional potency of an orthosteric agonist is limited, as the allosteric sites becomes fully occupied. This results in a finite leftward shift in the CRC of an orthosteric agonist as the PAM concentration is increased, providing a "ceiling effect." This ceiling effect may provide benefit in the form of safety in overdose.

Another relatively new concept in the field of GPCR research has been the discovery that different agonist ligands can cause different conformational changes in the same receptor, leading to functional selectivity or biased signaling.[26] Indeed, for the μ-opioid receptor, morphine is biased against β-arrestin–mediated desensitization and receptor internalization,[12,27] while endomorphins appear to show bias toward β-arrestin recruitment.[28,29] Recently, a G protein–biased μ-opioid receptor agonist was described that causes potent analgesia but with fewer side effects such as respiratory suppression and constipation.[30] Allosteric ligands have also been shown to be biased and may alter the second-messenger pathway that is stimulated by agonist, alter the level of G protein–mediated versus β-arrestin–mediated signaling, or change the kinetics of receptor internalization and recycling.[31,32] One example is the $GABA_B$ receptor PAM, GS39783, which, when combined with a low dose of agonist, prevented $GABA_B$ receptor desensitization that was observed with a higher concentration of agonist alone, despite producing an equivalent level of functional activity.[32] One can envision that opioid receptor PAMs could also be coadministered with exogenous opioid

agonists. In this scenario, the same functional responses may be expected by using a lower dose of agonist in combination with a PAM. However, if the PAM exhibited signaling bias toward analgesia and away from tolerance and dependence liabilities, it would be a valuable therapeutic for pain management. It will be important to follow up on these μ-opioid receptor–selective PAMs to determine if they have activity in vivo either on their own (potentiating stimulation by endogenously produced opioid agonist peptides) or in combination with opioid agonists.

In summary, we developed and executed a single HTS assay that can identify both μ- and δ-opioid receptor–selective agonists and PAMs and has the potential to identify hetero-oligomeric receptor–selective agonists or PAMs. Coexpression of different receptors in PathHunter cells, where only one receptor is tagged with the PK sequence, could be used to determine which receptors may hetero-oligomerize and lead to transactivation. The current study also suggests that expression of two (or more) PK-tagged receptors within the same cell line may allow discovery groups to screen for agonists at multiple targets within a single high-throughput screen, as long as the basal activity and maximal efficacy of activation of each receptor in the system were similar.

To our knowledge, the discovery of μ- and δ-opioid receptor–selective PAMs has not been previously reported in the literature. These compounds exhibit PAM activity in both arrestin-mediated and G protein–mediated signaling pathways. Further characterization of the μ-opioid receptor–selective PAMs is ongoing. This discovery may have important implications for therapeutic strategies for chronic pain, substance abuse, and depression indications.

Acknowledgments

We thank Shilpa Madari, Adriana Motyl, Joshua Prentiss, and Dr. Sukhanya Jayachandra for supplying cellular reagents for this screening campaign; Qumber Jafri for assay development support; and Dr. John Watson for critical reading of the manuscript.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The authors received no financial support for the research, authorship, and/or publication of this article.

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