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Discovery, Synthesis, and Molecular Pharmacology of Selective Positive Allosteric Modulators of the δ-Opioid Receptor

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Abstract

Allosteric modulators of G protein-coupled receptors (GPCRs) have a number of potential advantages compared to agonists or antagonists that bind to the orthosteric site of the receptor. These include the potential or receptor selectivity, maintenance of the temporal and spatial fidelity of signaling in vivo, the ceiling effect of the allosteric cooperativity which may prevent overdose issues, and engendering bias by differentially modulating distinct signaling pathways. Here we describe the discovery, synthesis, and molecular pharmacology of δ-opioid receptor-selective positive allosteric modulators (δPAMs). These δPAMs increase the affinity and/or efficacy of the orthosteric agonists leu-enkephalin, SNC80 and TAN67, as measured by receptor binding, G

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Supporting Information: Specific properties of the 15 compounds listed in Table 1 that were most likely to predict the experimentally determined EC_{50} values at either δ or μ opioid receptors are revealed from multivariante statistical analysis. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.5b00007.

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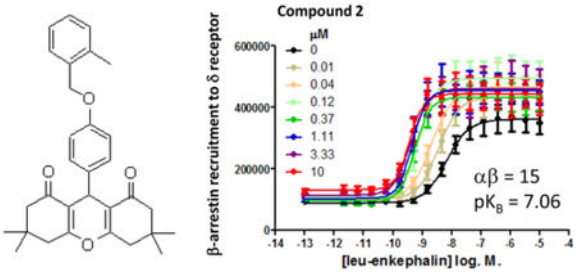
protein activation, β-arrestin recruitment, adenylyl cyclase inhibition, and extracellular signal-regulated kinases (ERK) activation. As such, these compounds are useful pharmacological tools to probe the molecular pharmacology of the δ receptor and to explore the therapeutic potential of δ PAMs in diseases such as chronic pain and depression.

Graphical abstract



Introduction

The δ-opioid receptor is a seven transmembrane domain (7TMD) receptor that belongs to the class A family of G protein-coupled receptors (GPCRs). Agonists of the δ receptor have been shown to be antinociceptive especially in chronic pain models[1] and to have potential as antidepressant agents.[2] The possible dual effects of δ receptor agonists to alleviate chronic pain and mitigate emotional disorders provide a particularly attractive therapeutic strategy because of the high level of comorbidity between chronic pain and depression. However, agonists acting directly at the δ receptor can show proconvulsant effects in animal models, including non-human primates. Indeed, it has been proposed that these seizurogenic properties of δ receptor agonists may be responsible for their antidepressant-like activity analogous to electroconvulsive therapy.[3] On the other hand, slowing the rate of administration of the δ receptor agonist SNC80 reduces seizurogenic activity but has no effect on anti-depressant-like effects.[4] Also, some δ receptor agonists (e.g., ADL5859) show no seizures in rat or mouse models.[5] These and other findings suggest that the convulsive properties of δ receptor agonists can be separated from their antidepressant-like effects.[6–8]

Allosteric modulators for GPCRs bind to a site on the receptor that is topographically distinct from the site that binds the orthosteric (or endogenous) agonist. Positive allosteric modulators (PAMs) increase the affinity and/or efficacy of bound orthosteric agonist ligands. The operational model of allosterism allows the quantification of allosteric effects, and as such, it can estimate the binding affinity of the allosteric ligand to the free receptor (pK_B), the allosteric cooperativity factor ($\alpha\beta$), as well as any intrinsic agonist efficacy (τ_B) of the allosteric ligand. PAMs that have little or no intrinsic efficacy (τ_B) but modulate the orthosteric agonist response have a number of advantages over orthosteric ligands.[9–11] In particular, these PAMs can theoretically maintain the temporal and spatial fidelity of endogenous receptor activation in vivo. The allosteric modulator binds to the target receptor but remains effectively silent until the endogenous orthosteric agonist is presented to the receptor. Therefore, PAMs can amplify the effect of endogenous signaling molecules without disrupting normal physiological regulation of receptor activation and might

therefore be expected to exhibit superior efficacy and side effect profiles compared to traditional orthosteric agonists. Studies with δ receptor selective ligands, or utilizing a genetic deletion of the δ receptor,[1] suggest that native opioid peptide signaling at the δ receptor mediates an increase in pain threshold in models of chronic pain and modulates mood states in rodent models.[12] Therefore, positive allosteric modulation of the δ receptor should enhance responses to the endogenous agonist peptides and thereby be therapeutically efficacious. In addition, the finite nature of the agonist potency shift (defined by the allosteric cooperativity factor), which saturates when the allosteric site is fully occupied, may increase the safety margin between therapeutic effect and possible side effects associated with overactivation of the target receptor. Finally, and pertinent to the δ-receptor system which is known to exhibit ligand-biased signaling,[13] PAMs can modulate the signaling bias of receptor activation toward desired pathways or engender bias from previously unbiased ligands. [14, 15] Thus, δ PAMs may provide a greater therapeutic window between pain relieving and antidepressant-like effects and proconvulsive activity, compared with traditional δ receptor orthosteric agonists.

In this study we report the synthesis and structure–activity relationships (SAR) of the first described δ PAMs. One of the most potent compounds identified, 3,3,6,6-tetramethyl-9-(4-((2-methylbenzyl)oxy)phenyl)-3,4,5,6,7,9-hexahydro-1*H*-xanthene-1,8(2*H*)-dione (**2**, BMS-986187), was further characterized in radioligand binding assays and using a range of cellular functional assays. **2** was shown to positively modulate orthosteric agonist binding affinity and functional potency at the δ receptor and enhance the efficacy of the partial agonist TAN67.

Results

Discovery and Structure–Activity Relationship (SAR) of δ Receptor PAMs

The δ PAM chemotype was identified from a high throughput screen (HTS) using a β-arrestin recruitment assay in a PathHunter U2OS cell line coexpressing μ and δ receptors (U2OS-OPRM1D1) (DiscoveRx, Fremont, CA).[16, 17] The screen was executed in PAM mode by measuring activity in the presence of an EC_{10} concentration of both endomorphin I (a μ-receptor-selective agonist) and leu-enkephalin which in this assay and cell line was a relatively selective agonist for the δ receptor.[18] Typically, when using HTS approaches to identify PAMs, an $EC_{20–40}$ concentration of orthosteric agonist is used.[19] However, in this HTS the sum of the two EC_{10} concentrations of agonists offered a compromise between the detection of both μ and δ receptor PAMs and the ability to maintain the overall signal window so that lower efficacy partial agonists could also be detected. Follow-up in vitro testing to determine structural features necessary for PAM activity was performed utilizing CHO-PathHunter cell lines (CHO-OPRD1 and CHO-OPRM1) obtained from DiscoveRx. Concentration-response curves (CRCs) for HTS hits were determined both in agonist mode (in the absence of orthosteric agonist) to determine agonist activity of the test compounds, and in PAM mode (in the presence of an EC_{20} concentration of orthosteric agonist) to determine allosteric modulator activity using the β-arrestin recruitment assays. Compound 7 (Table 1) was identified as a δ PAM, producing a robust potentiation of the response to an EC_{20} concentration of leu-enkephalin.

As shown in Scheme 1, we synthesized a series of close analogs of 7 to optimize δ PAM potency and selectivity. None of the compounds exhibited significant agonist activity in a β-arrestin recruitment assay, but all of the compounds produced measurable PAM activity at the δ receptor. **1** with an unsubstituted benzyl ring acted as a δ PAM with an EC_{50} value of 0.2 μM and showed 30-fold selectivity in the β-arrestin recruitment assay compared with PAM activity at the μ receptor. Introduction of a methyl group in various positions around the phenyl ring (**2**–**4**) suggested that ortho substitution increased δ receptor PAM activity by an order of magnitude, with minimal effect on μ receptor PAM activity, while meta and para substitution did not significantly affect δ or μ receptor PAM activity. The corresponding ortho-F analog **5** was not significantly more active than **1**, suggesting that the increased δ receptor activity with the *o*-methyl was due to a steric rather than an electronic effect. Similarly, the meta- and para-F analogs **6** and **7** or the ortho-Cl analog **8** did not afford an increase in δ receptor activity. Introduction of a second Cl group in the meta position (**9**) provided a modest improvement in δ receptor activity while maintaining selectivity. A more pronounced effect was observed with the ortho-Br analog **10** which produced equipotent PAM activity to **2** at the δ receptor but no observable PAM activity at the μ receptor, suggesting that 9 - (4-((2-bromobenzyl)oxy)phenyl)-3,3,6,6-tetramethyl-3,4,5,6,7,9-hexahydro-1*H*-xanthene-1,8(2*H*)-dione (**10**, BMS-986188) is the most δ receptor-selective analog we have identified to date. The effect of ortho substitution on δ receptor PAM potency and selectivity appears to be restricted to small substituents. As shown with analogs **11**–**15**, larger ortho sub-stituents did not improve δ PAM activity and had no effect on selectivity. Similarly, more drastic changes to the chemotype, such as increasing the chain length between the ether oxygen and the phenyl ring, or replacement of the benzyl ether with a phenyl amide, yielded a significant loss in δ receptor PAM activity (data not shown). The most potent δ PAM identified was **2**, which in the presence of an EC_{20} of leu-enkephalin produced a β-arrestin response with an average EC_{50} of 33 nM in CHO-OPRD1 cells (Table 1). Representative agonist and PAM mode CRCs for **2** at the μ and δ receptor are shown in Figure 1. In this example, **2** produced little or no activity in agonist mode, but in PAM mode (in the presence of an EC_{20} of leu-enkephalin (in CHO-OPRD1 cells) or endomorphin 1 (in CHO-OPRM1 cells)) produced a response with an EC_{50} of 48 nM in CHO-OPRD1 cells and 2 μM in CHO-OPRM1 cells.

Multivariate statistical analysis of a relatively large number of physicochemical properties calculated for the 15 compounds listed in Table 1 (see Methods and Materials for details) revealed specific properties of the 15 compounds that were most likely to predict EC_{50} values at either δ (Table S3) or μ (Table S4) opioid receptors in line with experimental data. Specifically, the total solvent accessible surface area (SASA, in square angstroms using a probe with a 1.4 Å radius), the π (carbon and attached hydrogen) component of the SASA (PISA), and the parameterized model number 3 (PM3) calculated ionization potential (IP.ev.) allow fitting of the predicted EC_{50} values at the δ opioid receptor (Figure S1). Similarly, the molecular weight of the molecule (mol.MW), the van der Waals surface area of polar nitrogen and oxygen atoms and carbonyl carbon atoms (PSA), and the number of nitrogen and oxygen atoms (X.N and O) were identified as the best predictors for EC_{50} values at the μ opioid receptor (Figure S2). An estimation of the δ/μ selectivity based on the ratio of the predicted EC_{50} values at the δ and μ opioid receptors revealed a definite

separation between compounds with a selectivity above or below a 33-fold cutoff (Figure S3).

Binding Characterization of 2

2 (at concentrations up to 30 μM) does not inhibit binding of the orthosteric antagonist ^{3}H-diprenorphine (DPN) to CHO-hDOPr cell membranes, suggesting that **2** is acting at an allosteric site to produce agonist and PAM activity (Figure 2A). However, in competition binding experiments 10 μM **2** increased the affinity of the orthosteric agonists, leu-enkephalin (Figure 2B), SNC80 (Figure 2C), and TAN67 (Figure 2D) to displace ^{3}H-DPN. This suggests that **2** is an affinity modulator (the α component of the cooperativity factor) in the system tested (Table 2). The affinity shift with the partial agonist TAN67 is less than that seen with the full agonists leu-enkephalin and SNC80 (Table 2).

Functional Characterization of 2

The PAM activity of **2** was further characterized in four different functional assays. In the CHO-OPRD1 PathHunter cells, **2** effects on leu-enkephalin potency and efficacy were studied in both β-arrestin recruitment assays and inhibition of forskolin-stimulated cAMP accumulation assays. Unlike the U2OS cell lines used in the HTS, where forskolin was relatively ineffective at stimulating adenylyl cyclase activity, the recombinant CHO PathHunter cell lines allowed us to investigate both β-arrestin recruitment and inhibition of forskolin-stimulated cAMP accumulation in the same cell line. In the β-arrestin recruitment assay, **2** alone (up to 10 μM) produced only marginal agonist activity ($\sim$10% of a maximal response to leu-enkephalin) but produced a robust 18-fold increase in the potency of leu-enkephalin (Figure 3A). A small increase in the maximal response to leu-enkephalin with **2**, relative to leu-enkephalin alone, was also observed. This suggests that **2** is a PAM with little or no intrinsic efficacy in this system. In contrast, in the inhibition of forskolin-stimulated cAMP assay, **2** alone produced robust activity resulting in full inhibition of cAMP accumulation at concentrations above 3 μM (Figure 3B). At lower concentrations, **2** increased the potency of leu-enkephalin. At a 370 nM concentration of **2** (the highest concentration at which a potency for leu-enkephalin could be determined) the potency of leu-enkephalin was increased by 56-fold. Similar findings were observed using the small molecule orthosteric agonist SNC80 in these two assays (Table 3).

Similar to the findings in the cAMP functional assay, **2** was also shown to be a PAM in [^{35}S]GTPγS binding (Figure 4A) and in ERK1/2 phosphorylation (Figure 4B) in CHO-hDOPr cells, showing agonist activity at higher concentrations and increases in the potency of orthosteric agonist at lower concentrations. No agonist activity to **2** was observed in the parental CHO cells (lacking the δ receptor) in ERK1/2 phosphorylation or in parental CHO cells in inhibition of cAMP accumulation assays (data not shown). **2** increased the potency of leu-enkephalin by 16-fold in the [^{35}S]GTPγS binding assay in CHO-hDOPr membranes and by 8-fold in the ERK1/2 phosphorylation assay in CHO-hDOPr cells. Similar experiments were performed replacing leu-enkephalin with the orthosteric agonists SNC80 and the partial agonist TAN67. By use of an operational model of allosterism[20] (see Methods and Materials), composite cooperativity ($\alpha\beta$) values and pK_B values (denoting the equilibrium dissociation binding constant for **2** at the δ receptor in the absence of orthosteric

agonist, i.e., at the free receptor) were determined for **2** across these different assays and with different orthosteric agonists (Table 3).

The mean ± SEM pK_B across all the assays for **2** was 6.02 ± 0.16 (~1 μM). One would expect that the pK_B values should be the same across all the cell lines, functional assays, and orthosteric agonists used, since the pK_B represents the binding affinity of **2** to the free receptor. Two way ANOVA with multiple comparison test of the pK_B values in Table 3 showed no significant difference between the different orthosteric agonist ligands used in the same functional assay. For SNC80 and TAN67 there were also no significant differences in pK_B values across the different functional pathways tested. However, for leu-enkephalin there were significant differences in the pK_B values between β-arrestin recruitment and [^{35}S]GTPγS binding ($p < 0.01$) and between β-arrestin recruitment and cAMP inhibition ($p < 0.001$).

Discussion

By use of a β-arrestin recruitment assay, the SAR of a δ PAM chemotype identified from HTS was explored, resulting in identification of compounds (**1–15**) with little or no agonist activity but which produced PAM activity at the δ and μ receptor. To compare the allosteric activity of the compounds, we used increasing concentrations with a single (EC_{20}) concentration of orthosteric agonist and analyzed the EC_{50} and Y_{max} values of the functional curves produced. Although the compounds exhibited a range of Y_{max} values in PAM mode (Table 1) which can correlate with the allosteric cooperativity, the large proportion of the analogs tested exhibited efficacy close to or above 100% limiting the usefulness of the Y_{max} parameter for selecting compounds for further study. Instead, potency of the PAM response was used and selectivity was determined using potency ratios between the PAM responses at the δ receptor compared to the μ receptor. While this procedure is useful for selecting δ receptor selective PAM candidates to pursue, one must bear in mind that different orthosteric agonist ligands were used in the PAM mode assays: leu-enkephalin for the δ receptor, and endomorphin I for the μ receptor. Since we currently know little about the possible probe dependence of these PAM compounds at the δ and μ receptor, we cannot necessarily assume that the reported selectivity will be the same with different orthosteric probe ligands.

The selected data set used for multivariate statistical analysis do not allow for thorough cross-validation of the presented linear models, but our results suggest initial physicochemical properties that can be used as searching criteria for additional compounds with potential PAM activity at δ and μ opioid receptors. **2** was selected for further characterization, since it had the highest PAM mode potency at the δ receptor and showed 100-fold selectivity compared to the μ receptor.

The multivariate statistical analysis initially suggested that the compounds may not be readily soluble in aqueous buffer at concentrations in the micromolar range. Also, nephelometry data (not shown) suggest that **2** and **10** show particulate matter in phosphate buffered saline solution at concentrations above 1 μM. When nephelometry was repeated using the specific buffer used for the β-arrestin recruitment assays (HBSS + 25 mM HEPES

and 10% FBS) in Table 1, **2** and **10** produced particulate matter above 3 μM. While the majority of responses to **2** in cells expressing the δ receptor were maximal at 1 μM (and therefore, within the solubility window predicted for **2**), the μ receptor responses (e.g., see Figure 1) also showed sigmoidal responses (i.e., the responses were not biphasic) up to 30 μM **2**, suggesting that solubility was not an issue in these assays in the specific buffers used. However, compound solubility should be an important consideration in further studies and optimization of this chemical series.

From competition binding studies, **2** did not affect ^{3}H-DPN binding to the δ receptor but increased the affinity of orthosteric agonists, suggesting that **2** does not bind to the orthosteric site of the δ receptor but can increase the affinity of orthosteric agonists binding to the receptor (α cooperativity). The precise mechanism for this cooperativity remains unknown. However, in this context it is tempting to make comparisons to recently discovered PAMs of the μ opioid receptor.[21] The μ receptor PAM 2-(3-bromo-4-methoxyphenyl)-3-((4-chlorophenyl)sulfonyl)thiazolidine (**16**, BMS-986122) has been found to differentially increase the affinity of various orthosteric agonists, and the magnitude of the affinity increase (α value) produced by **16** correlates with the intrinsic activity of the orthosteric ligand used.[22] The mechanism by which **16** induces this affinity modulation is suggested to be via reducing the affinity of Na^+ for its binding site on the μ receptor. The precise binding site for **16** on the μ receptor has not been clearly established, and it is unknown whether the δ receptor PAMs described here bind to an analogous binding site on the δ receptor or act via a similar mechanism. However, several analogs of **16** were found to exhibit weak activity at δ receptors, and most of the δ receptor PAMs described here also exhibit some degree of activity at μ receptors. Therefore, it is possible that these δ receptor PAMs may be binding to a site on the δ receptor that is analogous to the **16** binding site on the μ receptor and may work through a similar mechanism. The reduced affinity shift observed with **2** for the partial agonist TAN67 compared with the agonists with higher intrinsic activity, leu-enkephalin and SNC80 (Figure 2, Table 2), is consistent with this hypothesis. It will be interesting to determine whether these δ PAMs reduce the affinity of Na^+ for its binding site on the δ receptor. Sodium ions are known to stabilize a lower affinity state of the δ receptor, and the molecular basis for allosteric Na^+ control of opioid receptor signaling has been elucidated recently.[23, 24]

While TAN67 was a partial agonist in the CHO-hDOPr cell line for [^{35}S]GTPγS binding giving 84% of maximal SNC80 response, it had even less intrinsic activity in a C6-DOPr cell line at 41% of maximal SNC80 response (data not shown). In the presence of **2** (300 nM), the maximal stimulation by TAN67 was increased to 67% of maximal SNC80 response. This suggests that **2** has some allosteric efficacy cooperativity (β), as well as the affinity cooperativity (α) observed above.

In all of the functional assays, **2** acted as a PAM, increasing the potency of the response to orthosteric agonists. No activity was observed in functional assays when **2** was added alone in CHO-parental cells (lacking the recombinant δ opioid receptor) in either the ERK activation assay or cAMP assay (data not shown). However, in cells expressing the δ receptor, **2** (when added alone) produced significant activity in cAMP inhibition,

[^{35}S]GTPγS binding, and ERK activation assays, suggesting that this activity is due to intrinsic efficacy of **2** at the τ receptor. Thus, **2** is a PAM-agonist in these systems. In cells expressing the δ receptor, **2** showed little to no agonist activity in the β-arrestin recruitment assay, which measures an event proximal to receptor activation with limited signal amplification. The difference in observed agonist activity for **2** between the β-arrestin recruitment assay and the cAMP assay (Figure 3) is likely reflective of a higher level of signal amplification and thus a higher receptor reserve in the cAMP assay compared to the β-arrestin recruitment assay in the same cell line.[26, 27] Phosphorylation of the receptor by G protein-coupled receptor kinases (GRKs) is thought to be a prerequisite for β-arrestin recruitment.[28] It would be interesting to see how **2** impacts δ receptor phosphorylation by GRKs and consequently desensitization and internalization of the receptor.

Calculation of pK_B values for **2** across the various functional assays with leu-enkephalin, SNC80, and TAN67, using the allosteric operational model, showed some variability. These differences in pK_B values may result from the allosteric effect not reaching a plateau or ceiling. This could reflect that the allosteric effect was submaximal at concentrations below those at which full agonism was observed with **2** or that the highest concentrations of **2** used did not cause the allosteric EC_{50} shift to reach its ceiling. This can make accurate assessment of the allosteric parameters more difficult to estimate in the model. Other variables, including the use of different cell lines or use of a tagged receptor (in the case of the PathHunter CHO-OPRD1 cell line), may also contribute to the variability of values obtained in the model.

The fact that we observed PAM effects with **2** at concentrations lower than those which produced agonist effects is entirely consistent with the allosteric ternary complex model because the former effects (PAM effects) are observed in the presence of orthosteric agonist, and hence the affinity of the modulator for the receptor is higher, whereas the latter effects (agonist effects) reflect the actions of the modulator at the free receptor and thus require higher concentrations to achieve the same level of fractional occupancy. Therefore, a PAM with a large cooperativity factor ($\alpha\beta$) can exhibit functional activity that is far more potent than its K_B value. This has potential implications for PAM drug discovery programs, suggesting that it is important to track functional PAM activity rather than K_B values when designing assays to support SAR. Additionally, this suggests that assays assessing target engagement may dramatically underestimate the relevant receptor occupancy of a PAM, since the affinity of the PAM (and therefore its fractional receptor occupancy) will be much higher at sites where orthosteric agonist is present. While such sites may represent only a small fraction of the receptor population in vivo, they nonetheless represent the relevant receptor population, since positive allosteric modulation can only occur when and where orthosteric agonist is bound.

Despite the complexities discussed above, all available data suggest that **2** is a δ PAM or a δ PAM-agonist. In future studies, it will be important to confirm the activity of **2** and its analogs in cells or tissues natively expressing δ receptors. Further, it will be of significant interest to determine whether compounds such as **2** also exhibit direct agonist activity in native systems expressing endogenous levels of δ receptors. PAMs devoid of intrinsic agonist activity could theoretically have therapeutic advantages over PAM-agonists,

particularly in the maintenance of the temporal and spatial fidelity of endogenous receptor activation in vivo, as they would effectively be silent when bound to the receptor until orthosteric (endogenous) agonist is presented to the receptor. A key issue will be the determination of these effects in vivo. We intend to evaluate the in vivo activity of **2** and its analogs in models of acute and chronic pain,[29] migraine,[30] depression,[31] and convulsive activity[3] which is a known liability of δ opioid receptor agonists that has limited the pursuit of δ receptor agonists as potential therapeutics.

In summary, we have identified and characterized δ receptor-selective PAMs including our lead compound **2**. Further studies are planned to assess probe dependence and signaling bias for these PAMs using a variety of orthosteric opioid receptor ligands and functional assays. Additional research is also ongoing to determine if this new class of compounds could represent a viable approach to develop new medicines for chronic pain, depression, and other therapeutic indications.

Methods and Materials

Chemistry

Analogs were purchased from external vendors (**1, 3–5, 7**) or synthesized according to Scheme 1 (**2, 6, 8–15**). All purchased and newly synthesized analogs provided analytical data consistent with their assigned structures and were >95% pure based on LCMS.

Synthesis of Intermediate A (Scheme 1)

To a solution of 4-hydroxybenzaldehyde (1.5 g, 12.28 mmol) in 2-propanol (35 mL) were added 5,5-dimethylcyclohexane-1,3-dione (3.44 g, 24.57 mmol) and H_2SO_4 (98%, 0.098 mL, 1.842 mmol). The reaction mixture was refluxed for 1.5 h in an oil bath and then cooled to room temperature, forming a white precipitate. After filtration, 3 g of 9-(4-hydroxyphenyl)-3,3,6,6-tetramethyl-3,4,5,6,7,9-hexahydro-1*H*-xanthene-1,8(2*H*)-dione was obtained in 65% yield (98% purity by LCMS analysis). ^{1}H NMR (400 MHz, CD_3Cl) δ 7.09 (d, J = 8.6 Hz, 2H), 6.56 (d, J = 8.6 Hz, 2H), 4.67 (s, 1H), 2.46 (s, 4H), 2.23 (s, 2H), 2.21 (s, 2H), 1.10 (s, 6H), 1.00 (s, 6H); ESI-MS m/z = 367.08 $[M + H]^+$.

Synthesis of Analogs 1-15

General Procedure—To a solution of 9-(4-hydroxyphenyl)-3,3,6,6-tetramethyl-3,4,5,6,7,9-hexahydro-1*H*-xanthene-1,8(2*H*)-dione (100 μmol, 36.6 mg) in DMF (1.2 mL) were added $ArCH_2Br$ (200 μmol) and Cs_2CO_3 (65.2 mg, 200 μmol). The reaction mixture was stirred at room temperature overnight. Then 10 μL of the reaction solution was taken, dissolved in MeOH (0.2 mL), and analyzed by LCMS. The LCMS showed that the reaction was complete and the desired product as a major peak was found. The product was purified via preparative LC/MS with the following conditions. Column: XBridge C18, 19 mm × 200 mm, 5-μm particles. Mobile phase A: 5:95 acetonitrile/water with 10 mM ammonium acetate. Mobile phase B: 95:5 acetonitrile/water with 10 mM ammonium acetate. Gradient: 70–100% B over 15 min, then a 5 min hold at 100% B. Flow: 20 mL/min. Fractions containing the desired product were combined and dried via centrifugal evaporation.

Two analytical LC/MS injections were used to determine the final purity. Injection 1 conditions were the following. Column: Waters BEH C18, 2.0 mm × 50 mm, 1.7 μm particles. Mobile phase A: 5:95 acetonitrile/water with 10 mM ammonium acetate. Mobile phase B: 95:5 acetonitrile/water with 10 mM ammonium acetate. Temperature: 50 °C. Gradient: 0% B, 0–100% B over 3 min, then a 0.5 min hold at 100% B. Flow: 1 mL/min. Detection: UV at 220 nm.

Injection 2 conditions were the following. Column: Waters BEH C18, 2.0 mm × 50 mm, 1.7 μm particles. Mobile phase A: 5:95 methanol/water with 10 mM ammonium acetate. Mobile phase B: 95:5 methanol/water with 10 mM ammonium acetate. Temperature: 50 °C. Gradient: 0% B, 0–100% B over 3 min, then a 0.5 min hold at 100% B. Flow: 0.5 mL/min. Detection: UV at 220 nm.

Proton NMR was acquired in deuterated $CDCl_3$ or DMSO.

3,3,6,6-Tetramethyl-9-(4-((2-methylbenzyl)oxy)phenyl)-3,4,5,6,7,9-hexahydro-1H-xanthene-1,8(2H)-dione (2, BMS-986187)—^{1}H NMR (400 MHz, chloroform-d) δ 7.51–7.35 (m, 2H), 7.26–7.18 (m, 4H), 6.89 (dd, J = 14.2, 8.6 Hz, 2H), 5.05 (s, 2H), 4.72 (s, 1H), 2.49 (d, J = 5.9 Hz, 4H), 2.38 (d, J = 7.8 Hz, 4H), 2.27–2.21 (m, 3H), 1.16–1.10 (m, 6H), 1.07–1.00 (m, 6H). HRMS: calcd $C_{31}H_{35}O_4$, 471.2530; found, 471.2538

9-(4-((2-Bromobenzyl)oxy)phenyl)-3,3,6,6-tetramethyl-3,4,5,6,7,9-hexahydro-1H-xanthene-1,8(2H)-dione (10, BMS-986188)—The yield of the product was 20.6 mg, and its estimated purity by LCMS analysis was 100%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.67 (d, J = 7.7 Hz, 1H), 7.56 (d, J = 7.3 Hz, 1H), 7.42 (t, J = 7.5 Hz, 1H), 7.31 (t, J = 7.3 Hz, 1H), 7.10 (d, J = 8.1 Hz, 2H), 6.88 (d, J = 8.4 Hz, 2H), 5.04 (s, 2H), 4.48 (s, 1H), 2.54 (d, J = 11.4 Hz, 4H), 2.26 (d, J = 16.1 Hz, 2H), 2.09 (d, J = 16.1 Hz, 2H), 1.04 (s, 6H), 0.91 (s, 6H). HRMS: calcd $C_{30}H_{32}O_4Br$, 535.1478; found, 535.1478.

Calculation and Analysis of Physicochemical Properties

The Schrodinger Suite 2014-4 was used throughout. The two-dimensional (2D) sketcher tool of Maestro 10.0 was used to draw 2D structures of each compound listed in Table 1. These 15 2D structures were imported as 3D structures into LigPrep, version 3.2, and their protonation state was assigned at physiological pH using Epik, version 3.0.[32] Fifty-two physicochemical properties were calculated using QikProp, version 4.2. Among them, 38 properties (see Tables S1 and S2) displayed nonzero variance across the 15 ligands of Table 1 and were therefore used for multivariate statistical analysis.

The bayesglm function in the “arm” R package was used to build a Bayesian linear regression model for the relationship between the calculated physicochemical properties of the 15 compounds in Table 1 and their measured EC_{50} values at δ- or μ-opioid receptors. Specifically, each linear model was built so that $P = \Sigma C_i X_i + C_0$, where P is the EC_{50} value to be predicted, C_0 is the interception, and X_i and C_i are the calculated property and associated regression coefficient, respectively. We arbitrarily assigned a numerical value of 100 to EC_{50} values listed as “>10” in Table 1. To better discriminate the most potent compounds, we used a logarithmic scale for EC_{50}. All models containing combinations of

up to three properties (without interaction terms) were estimated, and the best linear models for δ-opioid (see Table S3 and Figure S1) and μ-opioid (see Table S4 and Figure S2) receptors were selected based on the Akaike information criterion (AIC). Following prediction of −log EC_{50} values at δ- or μ-opioid receptors, the δ/μ selectivity was estimated as the difference of $-\Delta \log EC_{50} = -\log[EC_{50}(\delta)/EC_{50}(\mu)]$ (see Figure S3).

Cell Lines

Chinese hamster ovary (CHO) PathHunter cells expressing enzyme acceptor (EA) tagged β-arrestin **2** and either ProLink (PK) tagged δ receptor (CHO-OPRD1) or PK-tagged μ receptor (CHO-OPRM1) were from DiscoveRx (Fremont, CA). PathHunter is a trademark of DiscoveRx. Cells were grown in F-12 media (Invitrogen 11765), containing Hyclone FBS 10%, Hygromycin 300 μg/mL (Invitrogen 10687), G418 800 μg/mL (Invitrogen 10131) and maintained at 37 °C in a humidified incubator containing 5% CO_2. These cells were used for β-arrestin recruitment assays and inhibition of forskolin-stimulated cAMP accumulation assays described below.

FlpIn CHO cells (Invitrogen, Carlsbad, CA, USA) were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and maintained at 37 °C in a humidified incubator containing 5% CO_2. FlpIn CHO cells were transfected with the pOG44 vector encoding Flp recombinase and the pDEST vector encoding the human δ receptor (hDOPr) at a ratio of 9:1 using polyethylenimine as transfection reagent. At 24 h after transfection the cells (CHO-hDOPr) were subcultured and the medium was supplemented with 700 μg/mL HygroGold as selection agent. Cells were grown and maintained in DMEM containing 20 mM HEPES, 5% fetal bovine serum, and 200 μg/mL Hygromycin-B. Cells were maintained at 37 °C in a humidified incubator containing 5% CO_2. These cells were used for ERK phosphorylation assays, and membranes derived from these cells were used for [^{35}S]GTPγS binding and ^{3}H DPN binding studies as described below.

Materials

PathHunter detection reagents were from DiscoveRx (Fremont, CA). Cell culture media and supplements were from Life Technologies (Carlsbad, CA). Lance-Ultra cAMP detection reagents, Surefire ERK assay reagents, [^{3}H]diprenorphine (DPN), and [^{35}S]GTPγS (guanosine-5′-*O*-(3-thio)triphosphate) were from PerkinElmer Life Sciences (Cambridge, MA). Endomorphin I and TAN67 were obtained from Tocris. All other chemicals, unless otherwise specified, were purchased from Sigma (St. Louis, MO).

PathHunterβ-Arrestin Assay

Confluent flasks of CHO-OPRM1 and CHO-OPRD1 cells were harvested with TrypLE Express and resuspended in F-12 media supplemented with 10% FBS and 25 mM HEPES, at a density of 6.67 × 10 cells/mL and plated (3 μL/well) into white solid TC-treated 1536-well plates (Corning, NY). Plates were incubated overnight at 37 °C in a 5% CO_2 humidified incubator. The next day, compounds (40 nL of 100 × final concentration in 100% DMSO) were added to cell plates 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), or assay buffer containing a low concentration (~4 × EC_{20}) of orthosteric agonist (PAM mode), was added to assay plates. The orthosteric agonists used are described in the Results and Discussion. Plates were covered with a lid and incubated at room temperature for 90 min. Incubations were terminated by the addition of 2 μL of PathHunter Reagent (DiscoveRx). One hour later luminescence was detected using a Viewlux imaging plate reader (PerkinElmer).

Inhibition of Forskolin-Stimulated cAMP Accumulation Assays

CHO-OPRD1 cells were grown to confluence (as described above). Cells were harvested and resuspended at 1 × 10 cells/mL in assay buffer (HBSS + 25 mM HEPES, +0.05% BSA). 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 (Labcyte, CA) followed by a 1 μL addition of cells (2000 cells/well) to all wells. Next, 1 μL of either assay buffer (for agonist mode) or assay buffer containing a 3 × EC_{20} concentration of orthosteric agonist (PAM mode) was added. Finally, 1 μL of 3 × forskolin (2 μM final) was added. Plates were lidded and incubated for 45 min at rt. Incubations were terminated by the addition of Lance-Ultra cAMP detection reagent (PerkinElmer) (1.5 μL of Eu-cryptate-labeled cAMP tracer in lysis buffer, followed by 1.5 μL of U-light conjugated anti-cAMP antibody in lysis buffer). After a 1 h incubation at room temperature, time-resolved fluorescence (TRF) was detected on a Viewlux or Envision plate reader (PerkinElmer) with excitation at 337 nm and emission reads at 615 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 δ-opioid receptor-selective PAMs in the CHO-OPRD1 cAMP assay, using curve-shift assays, were performed as described above using orthosteric agonists described in the Results and Discussion.

Membrane Preparation

Confluent cells were rinsed with phosphate buffered saline and then detached using harvesting buffer (0.68 mM EDTA, 0.15 M NaCl, 20 mM HEPES, pH 7.4). Cells were pelleted by centrifugation at 300*g* for 3 min, followed by resuspension in cold 50 mM Tris-HCl buffer, pH 7.4. Pellet was rehomogenized using a Tissue Tearor and then centrifuged at 20 000*g* for 20 min at 4 °C. The supernatant was discarded, and the process was repeated for an additional rehomogenization and centrifugation. The supernatant was discarded, and the final pellet was resuspended in 50 mM Tris-HCl and flash-frozen in aliquots using liquid nitrogen. Aliquots were kept at −80 °C until assays. Protein concentration was determined using BCA protein assay with bovine serum albumin as the standard.

Radioligand Binding Assay

Cell membranes (as prepared above, 10 μg/well) were incubated in the following mixture for 90 min at 25 °C: assay buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 10 μM GTPγS), various concentrations of orthosteric and allosteric ligand, and 0.35-0.45 nM [^{3}H]DPN. Nonspecific binding was determined in the presence of 10 μM naloxone. Reactions were terminated by rapid filtration through glass microfiber GF/C

filters (Whatman) using a Brandell harvester and washed three times using cold 50 mM Tris-HCl buffer. Filters were dried in a 50 °C oven, and radioactivity was measured by liquid scintillation counting with EcoLume liquid scintillation cocktail (MP Biomedicals) in a Wallac 1450 MicroBeta counter (PerkinElmer).

[^{35}S]GTPγS Assay

CHO-hDOPr cell membranes (as prepared above, 10 μg/well) were incubated for 1 h at 30 °C in buffer comprising 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 5 mM $MgCl_2$, 100 mM NaCl, 0.1 nM [^{35}S]GTPγS, and 30 μM GDP (guanosine 5-diphosphate) in a final volume of 200 μL. Orthosteric and allosteric ligands were also included, with SNC80 used as the maximal standard and assay buffer used to assess basal [^{35}S]GTPγS binding. The reaction was terminated by filtration through glass microfiber GF/C filters (Whatman) using a Brandell harvester. The filters were rinsed, dried, and radioactivity was counted by liquid scintillation counting using EcoLume liquid scintillation cocktail (MP Biomedicals) in a Wallac 1450 McroBeta counter (PerkinElmer).

ERK1/2 Phosphorylation Assay

hDOPr FlpIn CHO cells (CHO-hDOPr) were seeded into 96-well plates at a density of 50 000 cells/well. After 5–7 h, cells were washed with phosphate buffered saline (PBS) and incubated overnight in serum-free DMEM. Initially, time-course experiments were conducted at least twice for each ligand to determine the time required to maximally promote ERK1/2 phosphorylation via the δ-receptor. Concentration–response experiments were performed for the orthosteric ligands in the absence or presence of increasing concentrations of the allosteric modulator at 37 °C. Stimulation of the cells was terminated by removal of the media and the addition of 100 μL of SureFire lysis buffer (PerkinElmer) to each well. The plate was shaken for 5 min at room temperature before transferring 5 μL of the lysates to a white 384-well Proxiplate (PerkinElmer). Then 8 μL of a 240:1440:7:7 mixture of Surefire activation buffer/Surefire reaction buffer/Alphascreen acceptor beads/Alphascreen donor beads was added to the samples and incubated in the dark at 37 °C for 1.5 h. Plates were read using a Fusion plate reader (PerkinElmer).

Data Analysis

For all experiments data were analyzed and EC_{50} or K_i values determined using nonlinear regression analysis to fit a logistic equation using GraphPad Prism, version 6 (GraphPad, San Diego, CA). pK_B and $\alpha\beta$ values were determined using the "operational model of allosterism"[20] (see eq 1), using Graphpad Prism, version 6.

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

Within this model, E is the pharmacological effect, K_A and K_B denote the equilibrium binding constants for the orthosteric ligand A and the allosteric ligand B at the receptor. The binding cooperativity factor α represents the effect of the allosteric ligand on orthosteric agonist binding affinity and vice versa. An activation cooperativity factor β denotes the

effect the allosteric ligand has on orthosteric agonist efficacy. Agonism constants τ_A and τ_B represent the intrinsic activity of the orthosteric agonist and any intrinsic activity of the allosteric ligand, respectively, which is dependent on the cell context and receptor expression level of the cell system and intrinsic efficacy of the ligands used. The remaining parameters E_m and n denote the maximal response of the system and the slope, respectively.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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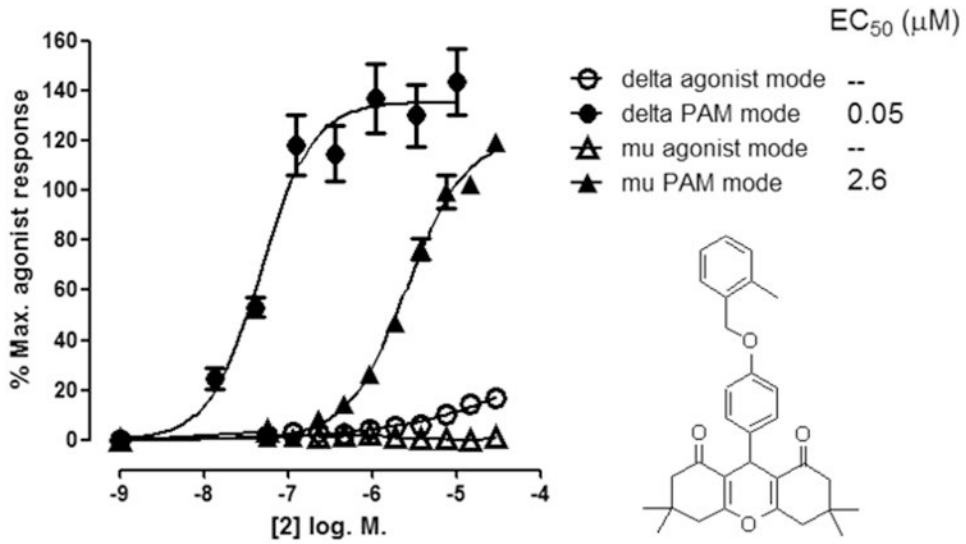


Figure 1.

β-Arrestin recruitment response to 2 in agonist mode (in the absence of orthosteric agonist) and in PAM mode (in the presence of an EC_{20} of orthosteric agonist) in PathHunter cells expressing δ receptors (CHO-OPRD1) and μ receptors (CHO-OPRM1). For CHO-OPRD1 cells the orthosteric agonist was leu-enkephalin, and for CHO-OPRM1 cells the orthosteric agonist was endomorphin I. In PAM mode, the EC_{20} response of orthosteric agonist was normalized to 0%. 100% represents the response to a maximally effective concentration of orthosteric agonist. Data are presented as the mean ± SEM, $n = 4$.

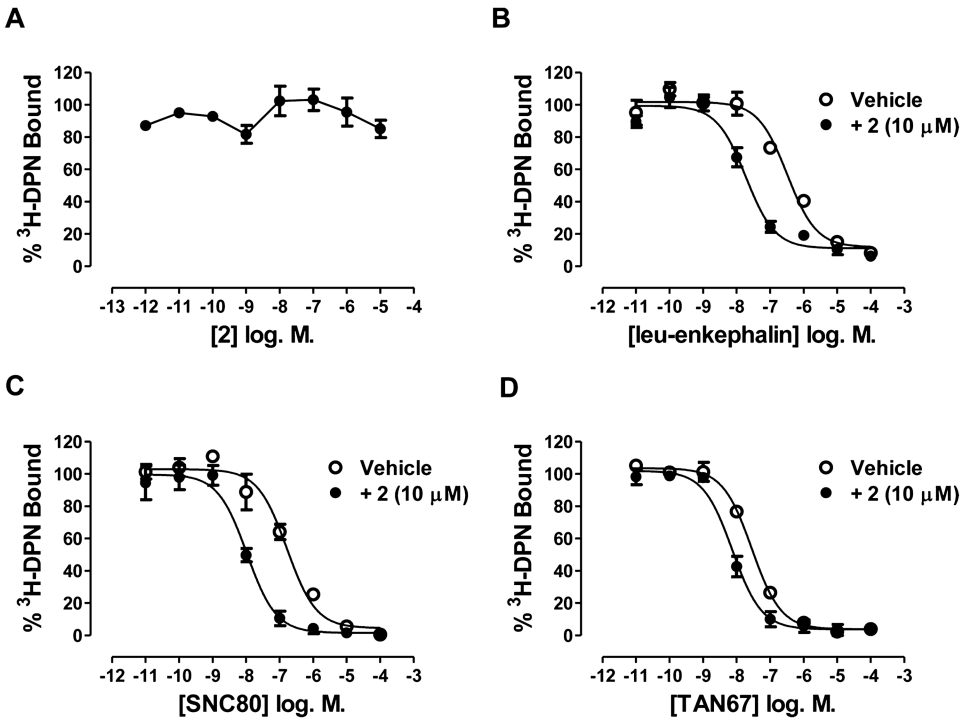


Figure 2.

Effect of **2** on ^{3}H-diprenorphine (DPN) binding (A) and the effect of 10 μM **2** on leu-enkephalin (B), SNC80 (C), and TAN67 (D) competition binding curves in CHO-hDOPr membranes. K_i values are shown in Table 2. Data are presented as the mean ± SEM of three experiments.

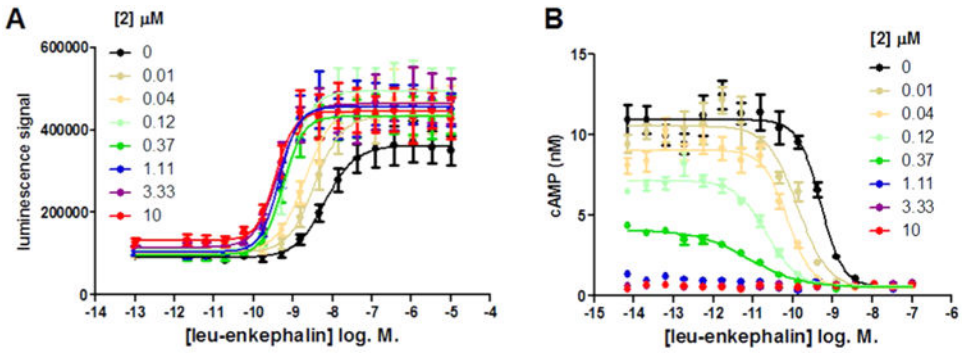


Figure 3.

Effect of increasing concentrations of **2** on leu-enkephalin concentration–response curves in *β*-arrestin recruitment (A) and in inhibition of forskolin-stimulated cAMP accumulation (B) in CHO-OPRD1 cells. Data are presented as the mean ± SEM of four experiments. Data were fitted to the operational model of allosterism (see Table 3).

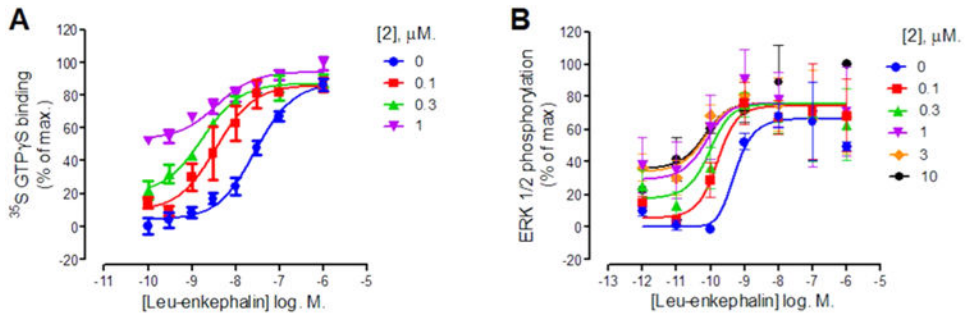
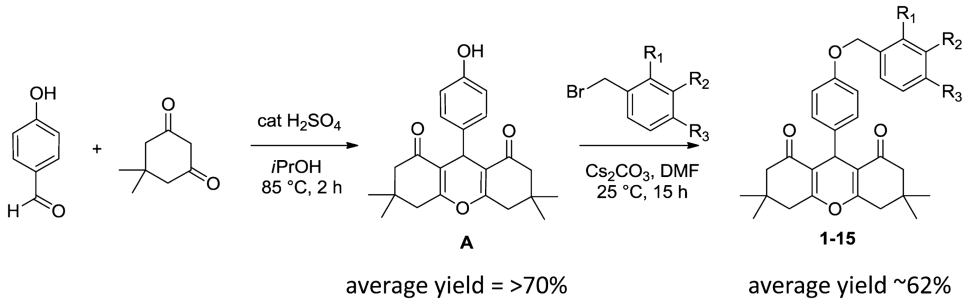


Figure 4.

Effect of increasing concentrations of **2** on leu-enkephalin concentration–response curves in [^{35}S]GTPγS binding in CHO-hDOPr membranes (A) and in pERK in CHO-hDOPr cells (B). In the [^{35}S]GTPγS binding assay, 0% and 100% represent the basal response and the maximal response produced, respectively. In the pERK assay, 0% represents basal activity in serum-free media and 100% represents the pERK response in the presence of 10% serum. Data are presented as the mean ± SEM, n = 3–7. Data were fitted to the operational model of allosterism (see Table 3).



Scheme 1.

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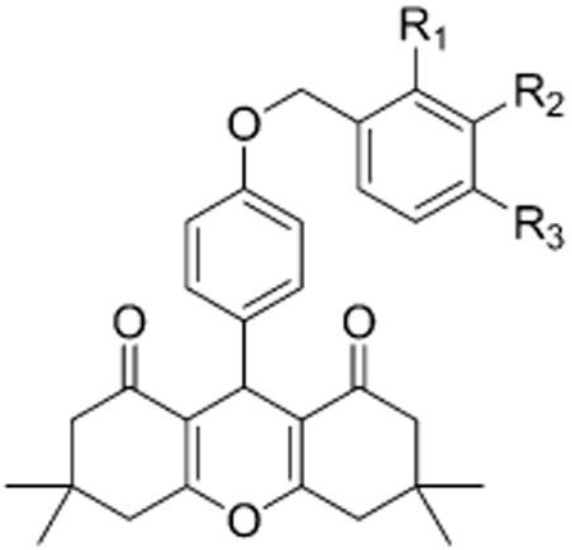
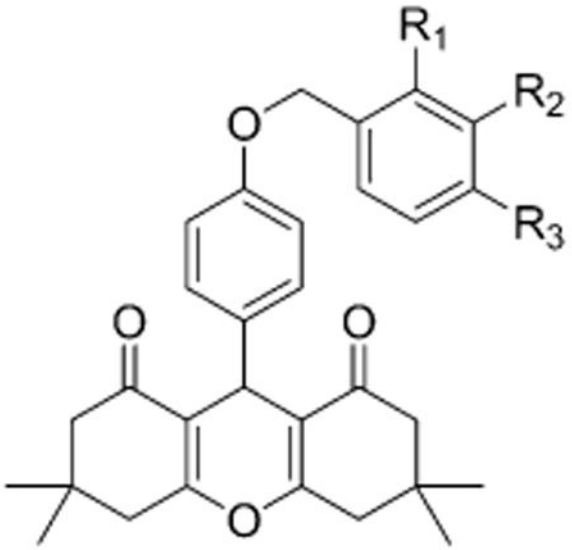
Table 1

Structure-Activity Relationship of the δ-PAM Chemotype in PathHunter CHO-OPRD1 and CHO-OPRM1 Cells in a β-Arrestin Recruitment Assay[a]



				EC_{50} (μM) (% Y_{max})		
compd	**R_1**	**R_2**	**R_3**	**δ**	**μ**	**selectivity μ/δ**
1	H	H	H	0.2 (126)	6 (130)	30
2 (BMS-986187)	CH_3	H	H	0.03 (124)	3 (121)	100
3	H	CH_3	H	0.2 (136)	3 (120)	15
4	H	H	CH_3	0.3 (92)	4 (65)	13
5	F	H	H	0.1 (120)	5 (107)	50
6	H	F	H	1 (136)	7 (182)	7
7	H	H	F	0.1 (95)	2 (72)	20
8	Cl	H	H	0.3 (68)	>10 (>100)	>33
9	Cl	Cl	H	0.1 (116)	>10 (>87)	>100



				EC$_{50}$ (μM) (% Y_{max})		
compd	**R$_1$**	**R$_2$**	**R$_3$**	**δ**	**μ**	**selectivity μ/δ**
10 (BMS-986188)	Br	H	H	0.05 (58)	>10 (>20)	>200
11	$OCHF_2$	H	H	0.2 (109)	3 (80)	15
12	OCF_3	H	H	0.3 (114)	2 (111)	7
13	$S0_2CH_3$	H	H	0.9 (102)	5 (42)	6
14	CH_2OH	H	H	1 (92)	10 (112)	10
15	CF_3	H	H	>10 (>40)	>10 (>20)	

[a] No activity was observed in agonist mode (in the absence of orthosteric agonist (data not shown)). In PAM mode (in the presence of an EC_{20} of leu-enkephalin for OPRD1 cells or an EC_{20} of endomorphin I for OPRM1 cells), robust responses were observed. The mean EC_{50} values, Y_{max} values, and potency ratio of δ receptor activity/μ receptor activity in PAM mode are reported in the table (n = 3).

Table 2

Effect of 2 (10 μM) on Orthosteric Agonist Competition Binding K_i Values in CHO-hDOPr Cell Membranes[a]

	K_i (nM) (95% CI (nM))		
ligand	**with vehicle**	**with 2 (10 μM)**	**affinity ratio (vehicle K_i)/(2 K_i)**
leu-enkephalin	221 (119–324)	7(3–12)	32
SNC80	71 (20–122)	5(3–7)	14
TAN67	10 (7–14)	3 (0.2–5.8)	3

[a] **2** had no effect on ^{3}H-DPN binding (see Figure 2) but increased the affinity of orthosteric agonist competition binding curves. Data are presented as the mean ± SEM of three experiments.

Table 3

Allosteric Parameters for 2 at the δ Receptor[a]

	leu-enkephalin				SNC80				TAN67	
	β-arr	**GTPγS**	**cAMP**	**pERK**	**β-arr**	**GTPγS**	**cAMP**	**pERK**	**GTPγS**	**pERK**
$\log\tau_A$	028 ± 0.04	094 ± 006	2.8 ± 0.03	025 ± 016	0.66 ± 0.08	0.56 ± 0.10	0.86 ± 0.03	1.06 ± 0.15	0.67 ± 0.09	0.22 ± 0.07
$\log\tau_B$	−0.73 ± 0.15	0.36 ± 0.17	1.45 ± 0.27	−0.16 ± 0.15	−1.06 ± 0.11	0.29 ± 0.20	1.01 ± 0.02	−0.04 ± 015	0.45 ± 0.07	0.11 ± 0.08
pK_A	7.90**	6.6**	6.7*	9.13**	6.38 ± 0.11	7.7*	7.7*	7.7*	7.6*	8.4**
pK_B	7.06 ±0.11	5.85 ± 0.23	5.45 ± 0.02	6.13 ± 0.30	6.45 ± 0.05	6.23 ± 0.32	5.52 ± 0.02	5.66 ± 0.30	6.0***	5.80 ± 0.32
log $\alpha\beta$ ($\alpha\beta$)	1.18 ± 0.07 (15)	1.67 ± 0.21 (47)	2.80 ± 0.08 (631)	0.99 ± 0.28 (10)	1.33 ± 0.05 (21)	1.00 ± 0.30 (10)	2.11 ± 0.07 (129)	0.89 ± 0.29 (8)	1.11 ± 0.19 (13)	1.41 ± 0.29 (26)

[a] Values for affinity, efficacy, and allosteric cooperativity for orthosteric ligands and **2** are derived from the operational model of allosterism. Three different orthosteric agonists were used (leu-enkephalin, SNC80, and TAN67), across up to four functional assays (β-arrestin recruitment, [^{35}S]GTPγS binding, cAMP inhibition, and pERK). In the model τ_A and τ_B represent the efficacy of the orthosteric agonist and allosteric modulator, respectively; pK_A and pK_B represent the binding affinity of the orthosteric agonist and the allosteric modulator, respectively, to the free receptor; and $\alpha\beta$ represents the composite allosteric cooperativity factor. Data are presented as the mean ± SEM of three to seven experiments.

* pK_A is fixed to its equilibrium binding affinity, as ligand is a full agonist in all end points tested.

** pK_A of leu-enkephalin and TAN67 in end points where they are partial agonists was obtained from fitting their CRC to the operational model of agonism to obtain a functional affinity in each end point tested.

*** the pK_B for TAN67 in [^{35}S]GTPγS binding had to be fixed to the average of the pK_B obtained from Leu-enk and SNC80 as neither allosteric agonism or potentiation reached a limit.