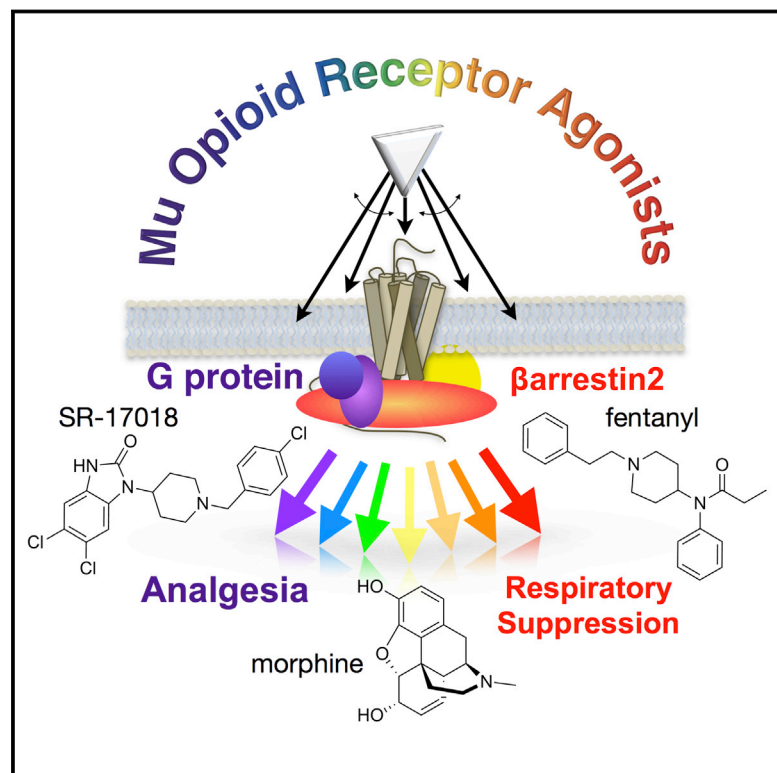


Bias Factor and Therapeutic Window Correlate to Predict Safer Opioid Analgesics

Graphical Abstract



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In Brief

Exploiting ligand bias enables the design of new opioid receptor ligands aimed at reducing side effects.

Highlights

- Mu opioid agonists were made to promote signaling through G proteins or β arrestin2
- Potency was determined for pain relief and respiratory depression in mice
- Fentanyl's narrow safety window correlates with its preference for β arrestin2
- Increasing signaling through G proteins directly improves the therapeutic index



Bias Factor and Therapeutic Window Correlate to Predict Safer Opioid Analgesics

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SUMMARY

Biased agonism has been proposed as a means to separate desirable and adverse drug responses downstream of G protein-coupled receptor (GPCR) targets. Herein, we describe structural features of a series of mu-opioid-receptor (MOR)-selective agonists that preferentially activate receptors to couple to G proteins or to recruit β arrestin proteins. By comparing relative bias for MOR-mediated signaling in each pathway, we demonstrate a strong correlation between the respiratory suppression/antinociception therapeutic window in a series of compounds spanning a wide range of signaling bias. We find that β arrestin-biased compounds, such as fentanyl, are more likely to induce respiratory suppression at weak analgesic doses, while G protein signaling bias broadens the therapeutic window, allowing for antinociception in the absence of respiratory suppression.

INTRODUCTION

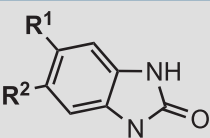
Opioids such as morphine and fentanyl are highly efficacious for the treatment of severe pain (Melnikova, 2010); however, the number of deaths due to overdose caused by respiratory distress have drastically increased over the past decade due to the misuse of prescription and illicit narcotics (Frank and Pollack, 2017; Rudd et al., 2016). Both the analgesic and respiratory suppressive effects of opioids are due the activation of the mu opioid receptor (MOR) (Dahan et al., 2001; Matthes et al., 1996). As a G protein-coupled receptor (GPCR), the MOR also interacts with β arrestins, scaffolding proteins that serve to regulate or facilitate subsequent GPCR signaling. In studies spanning more than a decade, researchers have shown that the interaction between MOR and β arrestin2 may drive many of the unwanted side effects of MOR activation (Bohn et al., 1999; 2000; Bu et al., 2015; Li et al., 2009; Raehal et al., 2011; Raehal and Bohn, 2005). β arrestin2-knockout (KO) mice, for example, display enhanced and prolonged morphine-induced antinociception yet are protected from morphine-

induced respiratory suppression (Bohn et al., 1999; Raehal et al., 2005). These findings suggest that activating the MOR without engaging β arrestin2 regulation may be critically important for developing safer opioid analgesics.

A recent development in pharmacological theory and practice is the concept that the structure of a GPCR ligand may be systematically modified to confer alternative receptor conformations upon binding, with each displaying a unique pattern of activation of intracellular signaling cascades (Rankovic et al., 2016; Urban et al., 2007). This concept of functional selectivity, or biased agonism, can be quantified by comparing drug potency and efficacy in cell-based signaling assays to the performance of a reference agonist (a compound that fully activates the system and thus defines the full potential of what one might hope to measure in the assay). Application of the operational model, described by Black and Leff (1983), allows one to simultaneously compare the relative potency and efficacy of a test agonist to the reference agonist, allowing for normalization within an assay (derivation of the parameter: $\Delta\text{Log } \tau/K_A$). After normalization, the performance of the compound can then be compared to its performance within another assay. The result of the comparison can be calculated as a “bias factor,” which essentially defines the extent of difference in relative agonist activity between two assays (the bias factor is $10^{\Delta\Delta\text{Log } \tau/K_A(\text{assay1}-\text{assay2})}$) (Kenakin et al., 2012). The higher the bias factor, the greater the separation between an agonist’s performance in the two assays relative to the performance of the reference agonist.

Biased agonism at the MOR is a promising avenue for therapeutic development, as late-phase clinical studies are demonstrating encouraging effects of biased agonism in human patients. TRV-130, or Oliceridine, the clinical candidate from Trevena, has shown efficacy in providing pain relief with modest improvement in preventing respiratory events (Singla et al., 2016; Soergel et al., 2014). In early preclinical studies, TRV-130 was shown to be biased toward activating G protein-mediated inhibition of adenylyl cyclase while displaying only marginally detectable signaling in β arrestin2 recruitment assays (DeWire et al., 2013). Applying analysis based on the operational model and taking into account the relative affinity of the agonist, the group assigned a bias factor of three to TRV-130 as an indicator of its preference for the G protein signaling pathway over the β arrestin pathway. Studies in

Table 1. Structural Features Influencing GTP γ S Binding and β Arrestin2 Recruitment

Substituents ^a							GTP γ S binding		β arrestin2 recruitment	
Agonist	Entry	R ¹	R ²	R ³	R ⁶	EC ₅₀	E _{MAX}	EC ₅₀	E _{MAX}	
						nM	%	nM	%	
	DAMGO					33 ± 1.4	100	229 ± 12	100	
	Morphine					64 ± 4.2	81 ± 1	372 ± 20	24 ± 1	
	Sufentanil					1.3 ± 0.18	78 ± 1	1.5 ± 0.6	73 ± 11	
	Fentanyl					43 ± 9.7	80 ± 5	53 ± 6.8	60 ± 2	
	(±) SR-8595	1	H	H	H	Me	102 ± 10	89 ± 7	447 ± 52	67 ± 3.8
	(±) SR-11065	2	H	H	Cl	Me	16 ± 1.2	97 ± 7	253 ± 88	76 ± 8.8
	SR-20382	3	H	H	Cl	H	563 ± 104	62 ± 4	7656 ± 1469	67 ± 9.8
	SR-20437	4	Cl	Cl	H	H	171 ± 24	60 ± 5	1092 ± 273	24 ± 3.6
	SR-17018	5	Cl	Cl	Cl	H	97 ± 13	75 ± 4	>10,000	10 ± 6 ^c
	SR-15099	6	Cl	Cl	Br	H	155 ± 11	81 ± 4	>10,000	3 ± 1 ^c
	(±) SR-14968	7	Cl	Cl	Br	Me	8.9 ± 3.8	92 ± 1	2438 ± 710	64 ± 5
	(±) SR-14969 ^a	8	Cl	Cl	Cl	Me	28 ± 7.7	88 ± 2	2949 ± 789	81 ± 6
	SR-15098 ^a	9	Cl	Cl	Cl	H	179 ± 24	68 ± 4	>10,000	12 ± 5 ^c
	(±) SR-11501	10	H	H	^b	Me	106 ± 9.1	70 ± 1	374 ± 60	59 ± 2

Potency (EC₅₀) and efficacy (E_{MAX}, percent of DAMGO) values from GTP γ S binding assays performed on CHO-hMOR membranes and β arrestin2 recruitment determined by EFC β arrestin2 assay with the U2OS- β arrestin2-hMOR-PathHunter cells. Data are presented as mean ± SEM of three or more assays run in duplicate or triplicate.

See also [Figure S1](#) for chemical synthesis, [Figure S2](#) for counter-screens against other opioid receptors, and [Table S1](#) for binding affinities for the compounds below the dotted line.

^aR⁵ = F

^b(R³ + R⁴) = (-OCH₂CH₂O-); for all other compounds, R⁴ = R⁵ = H

^cPercent of maximum stimulation at the 10 μ concentration is presented rather than E_{MAX}

rodents showed that at certain doses, TRV-130 could induce antinociception in the rodent thermal pain tests without respiratory suppression; however, comparisons between effective dose (ED)₅₀ values and a quantified assessment of therapeutic window was not determined. Clinical trials reveal that the compound serves as a potent analgesic in humans; however, in human patients, the therapeutic window is not as broad as originally anticipated ([Singla et al., 2016](#); [Soergel et al., 2014](#)). The question remains as to whether it is simply enough to have biased signaling or if the degree of bias (i.e., the magnitude of the bias factor) will impact the separation of analgesia and respiratory side effects.

In addition to TRV-130, other recent examples of G protein-biased MOR agonists have emerged in the preclinical literature. While no calculation of the degree of bias that each of these individual compounds possess was provided, promising results were obtained showing that they could induce antinociception with fewer side effects in mice ([Kruegel et al., 2016](#); [Manglik et al., 2016](#)). However, there has not been a comprehensive evaluation of the contribution of the degree of bias to the separation of the therapeutic window, nor have there been reports on agonists that preferentially recruit β arrestin2 over G protein signaling pathways. In this study, we show how agonists can be driven to promote one pathway over another in a series of new but structurally related compounds. We also present preclinical studies in mice that establish a direct correlation of increasing bias factor with an improved separation of therapeutic benefit from respiratory side effects.

RESULTS

Development of MOR Agonists with Functional Selectivity between G Protein Signaling and β Arrestin2 Recruitment

In order to fully address whether the degree of signaling bias affects the therapeutic window, we developed a series of compounds with a piperidine core structure, as shown in [Table 1](#) and [Figure S1](#). While distantly related compounds (e.g., bezitramide) had been shown to be opioid agonists ([Janssen et al., 1971](#)), no studies have emerged to show how substituent modifications may effect bias ([Table 1](#)). As shown with a subset of the series, several of these compounds have high affinity for MOR (0.2–3.0 nM) and are highly selective for MOR over other opioid receptors (kappa opioid receptors [KORs] or delta opioid receptors [DORs]), as shown by radioligand competition binding assays ([Table S1](#)). While some affinity at KORs and DORs was detected, subsequent assays show no functional effect on these receptors for any of the compounds; further, no functional impact on the opioid-like receptor (nociception receptor, NOP) could be detected ([Figure S2](#)). MOR selectivity is therefore conferred, which is a very important consideration when studying biased agonist effects *in vivo*.

Functional activity at human MOR was characterized using cell-based assays designed to measure G protein signaling or β arrestin2 recruitment in comparison to the enkephalin analog, [D-Ala², NMe-Phe⁴, Gly-ol⁵]-enkephalin (DAMGO), as the reference agonist ([Figure 1](#) and [Table 2](#)). Initially, ³⁵S-GTP γ S (GTP,

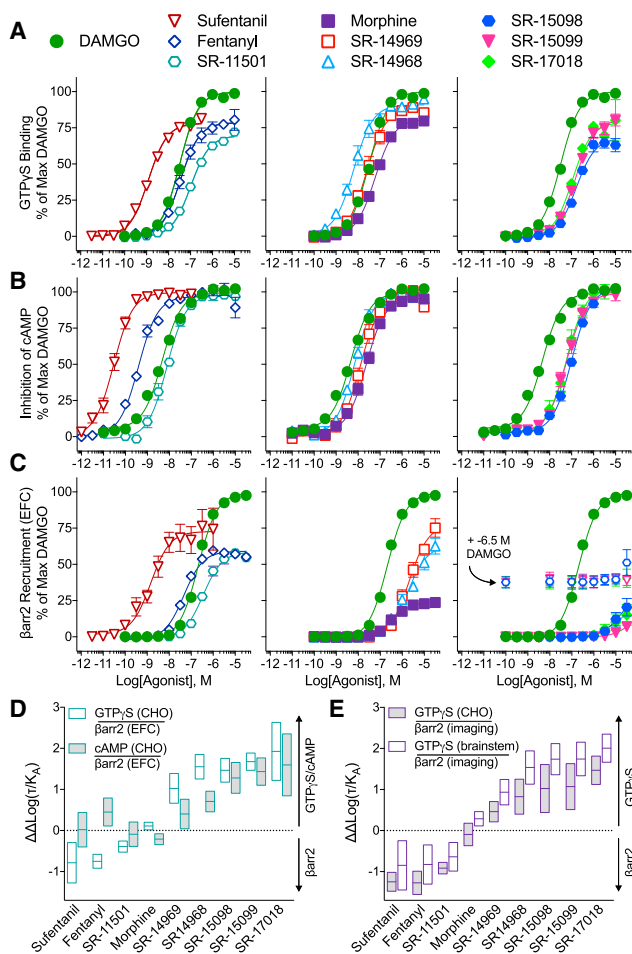


Figure 1. SR Compounds Are Potent Activators of GTP γ S Binding but Have Differential β ARRESTIN2 Signaling Profiles at the Human MOR

(A–C) Cell-based assays assessing (A) stimulation of GTP γ S binding in membranes, (B) inhibition of forskolin-stimulated cAMP accumulation in CHO-hMOR cells, and (C) stimulation of β arrestin2 recruitment in the U2OS- β arrestin-hMOR-PathHunter via the EFC assay. For SR-15098, SR-15099, and SR-17018, β arrestin2 EFC-concentration response curves were also performed in the presence of $10^{-6.5}$ M DAMGO (open symbols) to test for partial agonism. For all three assays, the data were normalized to the % maximal response for DAMGO and are presented as mean \pm SEM of three or more assays run in duplicate or triplicate.

(D and E) The $\Delta\Delta\text{Log}(\tau/K_A)$ bias values with 95% CI for the (D) human MOR and (E) mouse MOR. The G protein signaling was determined by either the GTP γ S binding assay in CHO-hMOR or CHO-mMOR cells or mouse brainstem or by inhibition of forskolin-stimulated cAMP in CHO-hMOR cells. β arrestin2 recruitment to the MOR was determined by the EFC assay in U2OS- β arrestin-hMOR-PathHunter cells for the human receptor and by the β arrestin2-imaging-based assay using the U2OS- β arrestin2-GFP-mMOR cell line for the mouse receptor. In all assays, DAMGO served as the reference agonist.

See also Table 2 for the $\text{Log}(\tau/K_A)$ and $\Delta\Delta\text{Log}(\tau/K_A)$ values with statistical comparison and Figure S3 for the concentration response curves for the mouse MOR assays (cells and brainstem).

guanosine triphosphate) binding assays were performed in membranes from Chinese hamster ovary (CHO)-hMOR cells (Figure 1A), while a commercially available enzyme fragment complementation (EFC) assay was used to assess β arrestin2 recruitment (Figure 1C). The compounds performed as partial or full agonists in the GTP γ S binding assay with a range of potencies between 9–563 nM. Their ability to induce β arrestin2 recruitment to the MOR varied more substantially, with some compounds, such as SR-15098, SR-15099, and SR-17018, revealing no significant efficacy in the β arrestin2 EFC assay until the 10 μ M concentration.

The evaluation of analogs in our series of MOR agonists suggests structural origins for MOR potency that directed further modifications to the molecules to produce compounds with greater G protein signaling potency and less β arrestin2 recruitment. The substituents R¹–R⁶ markedly impact the properties of MOR agonists. As shown in Table 1, the compound SR-8595 (entry 1) lacks substituents on each aryl ring and is a full MOR agonist, showing modest preference toward recruiting β arrestin2 over G protein signaling. Adding a chlorine atom at R³ (entry 2) improves potency for G protein signaling with little effect on β arrestin2 recruitment. Omission of the methyl group at R⁶ (entry 3) erodes potency in both assays. Having chlorine atoms at positions R¹ and R² rather than at R³ (entry 4) modestly improves potency but does not impart separation between the signaling assays. Importantly, when the chlorine (or alternatively bromine) substituents R¹–R³ are used in combination (entries 5–9), a greater separation between G protein signaling and β arrestin2 recruitment is observed in MOR agonists. Compounds with non-halogen substituents at R¹–R⁵, such as SR-11501 (entry 10), which has an ethylenedioxy group spanning the R³ and R⁴ positions, show improved potency in β arrestin2 recruitment relative to G protein signaling.

To quantitatively compare the differences observed between the two signaling assays, the operational model was used to calculate $\Delta\Delta\text{Log}(\tau/K_A)$ values with confidence intervals (CI; bias factor = $10^{\Delta\Delta\text{Log}(\tau/K_A)}$, Table 2). The series of compounds was narrowed to six SR candidates (Table 1, entries 5–10) selected to capture a wide range of bias factors; these were further compared to morphine, fentanyl, and sufentanil as examples of clinically relevant opioid analgesics. We find that fentanyl, sufentanil, and SR-11501 promote bias toward β arrestin2 recruitment in the EFC β arrestin2 assay when compared to GTP γ S binding assay, relative to DAMGO (Figure 1 and Table 2). At the other end of the spectrum, SR-15098, SR-15099, and SR-17018 promote preferential signaling toward GTP γ S binding. Morphine, SR-14968, and SR-14969 fall between these two extremes. Examination of structural features within this series of compounds shows that halogen substituents at R¹, R², R³, and/or R⁵ apparently favor MOR conformations that promote robust GTP γ S binding while disfavoring β arrestin2 signaling (Table 1).

It is important to note that, for an appropriate fit, the operational model requires a reasonable estimate of functional affinity, which cannot be produced in a curve that does not reach a maximum response. The inactivity of SR-15098, 15099, and 17018 in the β arrestin2 EFC assay therefore poses the complication that no reliable potency can be inferred from a flat line. Modifications were hence made to the model based on two

Table 2. SR Compounds Display a Range of Bias at the Human and Mouse MOR

Agonist	Human MOR						
	$\Delta\log(\tau/K_A)$			GTP γ S (CHO)/ β arr2		cAMP (CHO)/ β arr2	
	GTP γ S (CHO)	cAMP (CHO)	β arr2 (EFC)	$\Delta\Delta\log(\tau/K_A)$	Bias	$\Delta\Delta\log(\tau/K_A)$	Bias
DAMGO					1		1
Sufentanil	1.42 \pm 0.01	2.24 \pm 0.10	2.21 \pm 0.16	-0.78 (-1.28 to -0.29) ^b	0.16	0.02 (-0.40 to 0.44)	1.1
Fentanyl	-0.19 \pm 0.08	1.00 \pm 0.13	0.56 \pm 0.04	-0.75 (-0.92 to -0.58) ^d	0.18	0.45 (0.11 to 0.79) ^a	2.8
SR-11501	-0.58 \pm 0.02	-0.28 \pm 0.12	-0.19 \pm 0.06	-0.39 (-0.53 to -0.26) ^c	0.41	-0.09 (-0.39 to 0.21)	0.81
Morphine	-0.35 \pm 0.02	-0.67 \pm 0.07	-0.46 \pm 0.03	0.11 (0.02 to 0.20) ^a	1.3	-0.21 (-0.34 to -0.08) ^b	0.62
SR-14969	0.14 \pm 0.11	-0.48 \pm 0.08	-0.88 \pm 0.12	1.03 (0.66 to 1.39) ^d	11	0.40 (0.04 to 0.76) ^a	2.5
SR-14968	0.70 \pm 0.13	-0.15 \pm 0.09	-0.85 \pm 0.07	1.55 (1.25 to 1.85) ^d	36	0.71 (0.46 to 0.96) ^d	5.1
SR-15098	-0.88 \pm 0.04	-1.04 \pm 0.11	-2.34 \pm 0.11	1.47 (1.18 to 1.75) ^d	29	1.28 (0.91 to 1.66) ^d	19
SR-15099	-0.81 \pm 0.05	-1.05 \pm 0.22	-2.48 \pm 0.06	1.68 (1.47 to 1.89) ^d	47	1.44 (1.10 to 1.77) ^d	27
SR-17018	-0.64 \pm 0.06	-0.92 \pm 0.29	-2.56 \pm 0.20	1.93 (1.22 to 2.63) ^d	85	1.60 (0.84 to 2.35) ^c	40
	Mouse MOR						
	$\Delta\log(\tau/K_A)$			GTP γ S (CHO)/ β arr2		GTP γ S (brain)/ β arr2	
	GTP γ S (CHO)	GTP γ S (brain)	β arr2 (imaging)	$\Delta\Delta\log(\tau/K_A)$	Bias	$\Delta\Delta\log(\tau/K_A)$	Bias
DAMGO					1		1
Sufentanil	1.08 \pm 0.07	1.48 \pm 0.22	2.33 \pm 0.06	-1.25 (-1.48 to -1.02) ^d	0.06	-0.85 (-1.45 to -0.25) ^a	0.14
Fentanyl	-0.44 \pm 0.03	0.003 \pm 0.26	0.83 \pm 0.08	-1.27 (-1.56 to -0.99) ^d	0.05	-0.83 (-1.30 to -0.35) ^b	0.15
SR-11501	-0.68 \pm 0.02	-0.41 \pm 0.12	0.23 \pm 0.05	-0.91 (-1.06 to -0.77) ^d	0.12	-0.64 (-0.99 to -0.29) ^b	0.23
Morphine	-0.42 \pm 0.07	-0.04 \pm 0.05	-0.33 \pm 0.07	-0.09 (-0.37 to 0.18)	0.80	0.29 (0.11 to 0.46) ^b	1.9
SR-14969	-0.06 \pm 0.05	0.41 \pm 0.09	-0.52 \pm 0.09	0.46 (0.21 to 0.71) ^b	2.9	0.93 (0.63 to 1.24) ^c	8.6
SR-14968	0.50 \pm 0.08	1.22 \pm 0.10	-0.32 \pm 0.15	0.83 (0.40 to 1.25) ^b	6.7	1.54 (1.13 to 1.94) ^d	34
SR-15098	-0.94 \pm 0.02	-0.22 \pm 0.05	-1.96 \pm 0.19	1.03 (0.44 to 1.61) ^b	11	1.74 (1.36 to 2.12) ^d	55
SR-15099	-0.92 \pm 0.02	-0.25 \pm 0.08	-1.99 \pm 0.19	1.07 (0.51 to 1.63) ^b	12	1.74 (1.33 to 2.15) ^d	55
SR-17018	-0.86 \pm 0.03	-0.32 \pm 0.08	-2.33 \pm 0.12	1.47 (1.12 to 1.82) ^d	30	2.01 (1.67 to 2.35) ^d	102

Bias factor parameters of MOR agonists are presented across functional assays. GTP γ S binding was determined in membranes from CHO-hMOR or -mMOR cells or from brainstems of C57BL/6J mice. Inhibition of forskolin-stimulated cAMP accumulation was measured in CHO-hMOR cells. β arrestin2 recruitment to the human MOR was determined by the EFC assay performed in the U2OS- β arrestin-hMOR-PathHunter cells and to the mouse MOR by the β arrestin2-eGFP translocation imaging based assay in U2OS- β arrestin2-eGFP-mMOR cells. Data are presented as mean \pm SEM for $\Delta\log(\tau/K_A)$ values and with 95% CI for $\Delta\Delta\log(\tau/K_A)$ values. Assays were run in duplicate or triplicate, $n \geq 3$. $\Delta\log(\tau/K_A)$ values were calculated relative to DAMGO within each individual experiment. $\Delta\Delta\log(\tau/K_A)$ values were then calculated between the indicated assays as described in the methods to propagate error (95% CI).

See also Figures 1 and S3 for the corresponding concentration response curves and Table S2 for corresponding EC₅₀ and E_{MAX} values. Unpaired, two-tailed *t* test versus DAMGO.

^a*p* < 0.05

^b*p* < 0.01

^c*p* < 0.001

^d*p* < 0.0001

observations. First, to ensure that the compounds are not merely weakly efficacious, potent partial agonists, we tested whether they could block a stimulatory dose of DAMGO (a potent, weakly efficacious partial agonist will behave as an antagonist under these conditions and allow for derivation of potency). Previously, we determined that this approach could be used to refine the assessment of bias when conditions of an assay preclude the detection of an effect or give the appearance of “extreme bias” (Stahl et al., 2015). As shown in Figure 1C, the compounds do not compete with DAMGO at the doses tested, suggesting that their potency in the β arrestin2 assay must be greater than 10 μ M. This, taken together with their high affinity for the MOR in the ³H-DAMGO competition binding assays (Table S1), led

us to apply a conservative constraint to the operational model to limit the calculated functional affinity; the $\log(K_A)$ values were constrained to fall between 0 and 10⁻¹⁵ M, and the $\Delta\log(\tau/K_A)$ values, calculated relative to DAMGO, were constrained to be less than 10 (Brust et al., 2016; Stahl et al., 2015).

Since context greatly influences the determination of the numerical bias factor, we opted to evaluate the compounds in multiple systems to determine if the rank order of signaling preference is maintained. As a secondary measure of MOR signaling through inhibitory G proteins, we measured the inhibition of forskolin-stimulated cyclic AMP (cAMP) accumulation in CHO-hMOR cells (Figure 1B and Table S2). G protein signaling bias was preserved for morphine and SR compounds, with the

exception of SR-11501, regardless of whether the GTP γ S binding assay or the cAMP accumulation assay was used. Though the bias factors decreased, ranging from 2.5 to 40 for the cyclase assay compared to 11 to 85 for the GTP γ S binding assay, the rank order remains the same (Figure 1D and Table 2). However, sufentanil and SR-11507 no longer calculate as significantly biased toward β arrestin2 when cyclase inhibition is used the measure of G protein signaling; meanwhile, fentanyl's bias profile switches from being biased against GTP γ S binding to being biased toward inhibition of cAMP accumulation.

Collectively, these findings showcase the utility of bias factors as a means of comparing relative performance of compounds in cell-based assays but also underscore the fact that a calculated bias factor is a function of the cellular context. Therefore, bias factors may serve as guiding parameters, but not as numeric constants that define a ligand in the absence of context. Since context plays an important role in determining relative signaling preference, we asked whether the rank order of bias would be maintained at the mouse MOR since mice would be used for *in vivo* studies. In cells expressing the mouse MOR, agonism in GTP γ S binding assays (Figure S3A) were compared to effects in an imaging-based β arrestin2-EGFP translocation assay (Figure S3C). Although the cell lines, assays, and the species of the receptor changed, the overall bias profiles for these compounds are similar to those measured for human MOR (GTP γ S binding and β arrestin2 EFC) (Figure 1E and Table S2).

Moving directly to the mouse model, agonists were shown to stimulate GTP γ S binding in mouse brainstem, a region rich in pain regulatory neurons. DAMGO maximally stimulated GTP γ S binding with a potency of 400 ± 33 nM; while morphine performed as a partial agonist (41% E_{MAX}) relative to DAMGO, with a potency of 159 ± 19 nM (Figure S3B and Table S2). While most of the agonists performed as nearly full agonists in the cell-line G protein signaling assays, partial agonism (38%–41%) could be more readily observed for some compounds, including fentanyl and sufentanil (32%–33%), in the endogenous environment. The SR compounds showed a range in potency in this assay, with SR-14968 as the most potent (26 ± 2 nM) and SR-11501 as the least potent (396 ± 68 nM) (Table S2). Importantly, MOR-agonist-stimulated GTP γ S binding is absent in brainstem from MOR KO mice (Figure S3D), demonstrating selectivity of the compounds *in vivo*. When a bias factor is calculated using the parameters from the brainstem GTP γ S binding assays compared to the mouse MOR β arrestin2 imaging assay, we find that the rank order bias profiles, while differing in absolute value, are again maintained (Figure 1E and Table S2).

Therefore, regardless of the assay used or the species of receptor, the relative rank order of bias—in reference to DAMGO—is mostly conserved for the compounds: SR-14969 and SR-14968 show moderate bias, while SR-15098, 15099, and 17018 display high bias toward G protein signaling compared to β arrestin2 recruitment (Figure 1D and 1E and Table 2). The $\Delta\Delta\log(\tau/K_A)$ for the β arrestin2-preferring compounds (fentanyl, sufentanil, and SR-11501) are mostly conserved among the assays except for when the inhibition of cAMP accumulation is used as a measure of their ability to promote G protein signaling. Morphine's $\Delta\Delta\log(\tau/K_A)$ values vacil-

late around zero, suggesting that it is relatively unbiased, or balanced, when comparing G protein signaling and β arrestin2 recruitment.

Biased MOR Agonists Are Long Lasting and Brain Penetrant with Systemic Delivery

Since an important question is whether a pharmacological parameter (i.e., bias factor) that is broadly influenced by cellular context can be used to predict response profiles *in vivo*, we set out to compare the physiological effects of the compounds in mice to those induced by fentanyl and morphine. First, we determined that the SR MOR agonists could enter the brain by systemic drug delivery, like fentanyl and morphine. Morphine and the SR compounds were administered intraperitoneally (i.p.) at 6 mg/kg; plasma (Figure 2A) and brain (Figure 2B) levels were determined over 6 hr. The plasma levels for SR-15099 and SR-17018 rise more slowly than for morphine, suggesting a slower absorption rate, and all of the SR compounds peak at the same level in the plasma as in morphine within 30 min of injection. Importantly, all of the SR compounds are present in brain 1 hr following systemic injection, while some of them remain at high levels in the brain and plasma for the duration of the 6-hr period. Fentanyl, known to be very potent and brain penetrant, was injected at 1 mg/kg i.p., and brain levels were determined after 15 min for comparison. As anticipated, fentanyl was quickly cleared from brain and plasma, with no detection after 2 hr (Kalvass et al., 2007). When tested at higher doses, fentanyl and morphine brain levels escalated; however, the levels of the more biased compounds—SR-15098, SR-15099, and SR-17018—appear to have leveled off, indicating that a maximum concentration may have been reached in the brain by 24 mg/kg despite higher dosing (Figure 2C). Plasma protein binding assays determined that the SR compounds are between 90%–95% bound, which allows for an estimation of approximately 200 nM freely circulating compound at 6 mg/kg i.p. (Table S3); notably, this is within range of the EC_{50} values calculated for GTP γ S binding (26–400 nM) in membranes from the mouse brainstem (Table S2). These studies demonstrate that the drugs used *in vivo* have a comparable opportunity to activate MOR in the brain of mice and further informed the dosing used in the behavioral studies.

G Protein Signaling-Biased MOR Agonists Promote Antinociception with Less Respiratory Suppression

Given that the SR compounds are brain penetrant following systemic dosing, we then compared them to morphine and fentanyl in mouse thermal nociception assays. In both the hot plate and warm water tail withdrawal (tail flick) assays, all of the compounds produce antinociception that is on par with morphine and fentanyl (maximum efficacious doses are shown for comparison in Figure 3A). However, when these doses are tested for effects on respiration (% arterial oxygen saturation and breathing frequency), a clear delineation becomes apparent (Figure 3B). Remarkably, the SR compounds with the greater preference for stimulating GTP γ S binding over β arrestin2 recruitment produce the least respiratory suppression compared to an equi-antinociceptive dose of morphine tested.

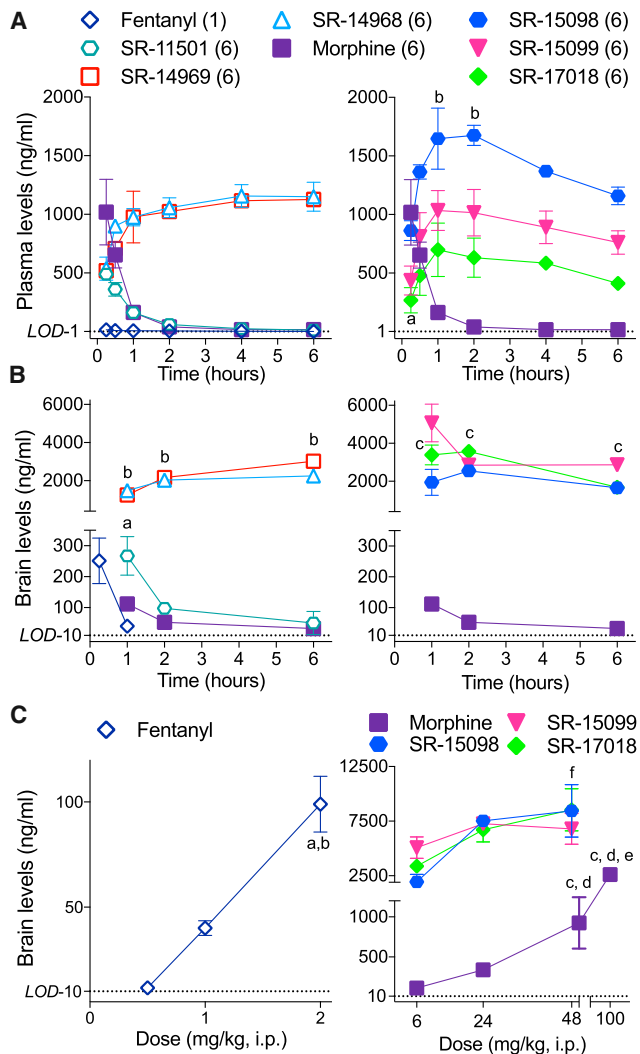


Figure 2. SR Agonists Cross the Blood Brain Barrier and Are Present in Plasma 6 hr after Injection

(A and B) C57BL/6J mice were systemically (*i.p.*) injected with 6 mg/kg of each agonist (or 1 mg/kg for fentanyl) and (A) plasma and (B) brain levels were determined at the indicated time points by LC/MS analysis. (A) While morphine and SR-11501 levels decrease over time, the other SR compounds remain at elevated levels up to 6 hr after injection (Dunnett's multiple comparisons test: morphine [15 min] versus [a] SR-15099 or SR-17018, $p < 0.05$; [b] SR-15098, $p < 0.05$). (B) The SR compounds can be detected in the brain at higher concentrations (which persist 6 hr following treatment) (Dunnett's multiple comparisons test: morphine [1 hr] versus [a] SR-11501, $p < 0.01$; [b] SR-14968 or SR-14969, $p < 0.0001$; [c] SR-15098 or SR-15099 or SR-17018, $p < 0.01$). (C) C57BL/6J mice were administered the indicated dose of compound and brain levels were determined 1 hr after injection (*i.p.*). Increasing the dose of morphine or fentanyl increases the amount of drug in the brain, but there is no difference between the amount of drug in the brain at the 24 and 48 mg/kg doses of the SR compounds tested (one-way ANOVA, followed by Tukey's post hoc analysis within each treatment: $p < 0.05$ when compared to [a] 0.5 mg/kg, [b] 1 mg/kg, [c] 6 mg/kg, [d] 24 mg/kg, or [e] 50 mg/kg; [f] for SR15-098, 6 versus 48 $p < 0.5$). Data are presented as mean \pm SEM of three or more mice. The limits of detection (LOD) are indicated for plasma (1 ng/mL) and brain homogenates (10 ng/mL). See also Table S3 for the plasma protein binding and estimated free plasma concentrations and Figure S4 for the antinociceptive and respiratory responses that correspond to these doses of the drugs.

To fully investigate this apparent separation in therapeutic efficacies between the responses, we performed dose response studies for all of the compounds (Figures S4A–S4D and Table 3). Since the different compounds have different pharmacokinetic properties, we calculated ED_{50} values for each response by analyzing the area under the curve (AUC) for 1 hr following drug treatment in order to capture the peak effect of each drug in each assay, as well as to account for each drug being present in the brain within that time frame. It can be readily seen that in both the hot plate and tail flick assays, all compounds induce dose-dependent antinociception that is very similar, if not more potent, than that produced by morphine. However, as the G protein bias factors increase (as determined from the cellular assays), we begin to see less respiratory suppression at the higher doses. In contrast, fentanyl and SR-11501, which are calculated to be biased toward β arrestin2 recruitment over $GTP\gamma S$ binding, produce robust respiratory suppression at low doses. Additionally, it should be noted that the maximal doses of each of the compounds have no effect in MOR-KO mice demonstrating MOR selectivity for mediating these effects. Further, the same separation between antinociception and respiratory responses can be observed in female mice (Figure S4E).

For the highly G protein signaling-biased compounds—SR-15098, SR-15099, and SR-17018—very little respiratory suppression is detected compared to vehicle treatment even when administered at 48 mg/kg. Since we did not define a maximum response in these animals, we performed the estimation of ED_{50} values for these measures by imposing a maximum threshold (for arterial oxygen saturation, data were normalized to a maximum response threshold of a 70% O_2 and for breath rate, 75 breaths per min). In this manner, we make a very conservative assumption that at high enough doses, the maximum suppression could be obtained and thereby calculate ED_{50} values for SR-15098, SR-15099, and SR-17018 (Table 3).

G Protein Signaling-Biased MOR Agonists Produce Wider Therapeutic Windows

The calculated potencies were then used to generate therapeutic windows by dividing the ED_{50} values obtained in each of the two respiratory measures by the ED_{50} values from the two antinociception assays; a greater value indicates a higher degree of separation between the two responses (*i.e.*, more pain relief with less respiratory suppression) (Table 3). As with the bias factor calculations, there are no absolute values for the therapeutic windows, but there are general trends. For instance, we find that fentanyl and SR-11501, the compounds that show β arrestin2 bias over G protein signaling, are more likely to induce respiratory suppression at lower doses and have very narrow therapeutic windows compared to morphine. The compounds that show bias for G protein signaling over β arrestin2 recruitment prove to have much broader therapeutic windows than morphine. This profile is preserved regardless of which respiratory or antinociception measure is used; for graphical representation, the therapeutic windows relative to morphine are presented in Figure 3C.

Bias Factor Correlates with Therapeutic Window

A linear correlation analysis of the bias factor (hMOR $GTP\gamma S$ binding over β arrestin2 EFC) against the therapeutic window

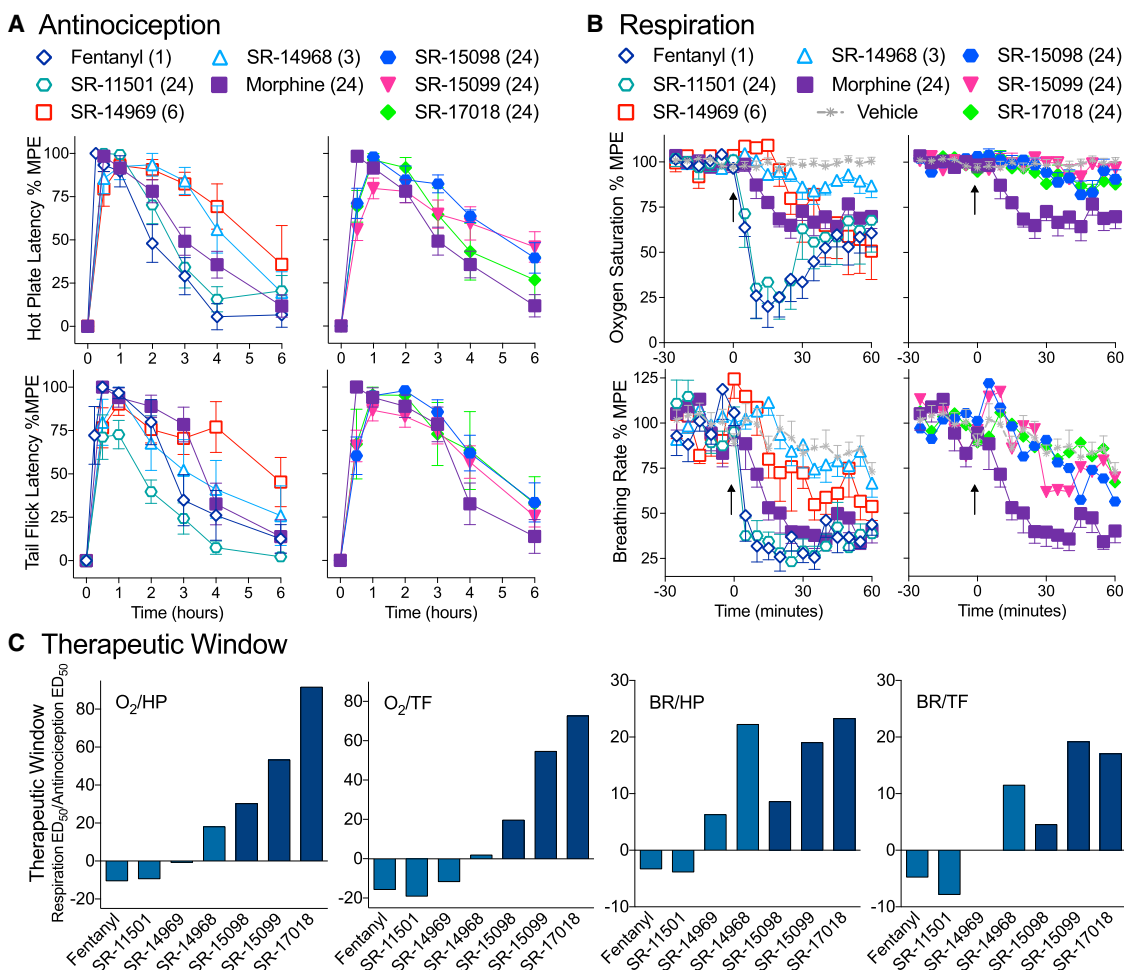


Figure 3. Agonists That Displayed G Protein-Signaling Bias in the Cell-Based Assays Promote Antinociception with Less Respiratory Suppression

(A and B) (A) Antinociceptive responses were measured in male C57BL/6J mice in (top) hot plate (52°C) and (bottom) warm-water tail-flick (49°C) assays over 6 hr at doses (mg/kg, *i.p.*) of compounds that produce antinociceptive responses on par with morphine in male C57BL/6J mice. (B) Respiratory responses were tested at the same doses in male C57BL/6J mice fit with a pulse oximeter to detect (top) % arterial oxygen saturation and (bottom) breath rate changes over 1 hr. The data are presented as mean \pm SEM of the % maximal possible effect (100% maximum possible effect [MPE]), with basal responses determined for each mouse (A) prior to injection or (B) as the average response for 30 min prior to injection (at time 0, arrow) and setting the maximum thresholds at 20 s for hot plate, 30 s for tail flick, 70% for oxygen saturation, and 75 breaths per min for breath rate measures.

(C) Therapeutic windows were calculated by dividing the ED₅₀ values for the respiratory measures (%O₂, arterial oxygen saturation or BR, breath rate) by the ED₅₀ values for the antinociception measures (HP, hot plate or TF, tail flick) presented in Figure S4 and Table 2. To show comparison to morphine, the values for morphine were then subtracted from each compound (morphine therapeutic window = 0).

See also Figure S4 for dose response curves for all the compounds in both the antinociceptive and respiratory assays, as well as single dose in MOR-KO mice and in female mice, Table S4 for the number of mice used in each study, and Table 3 for the calculated ED₅₀ values and therapeutic windows.

(% arterial oxygen saturation and hot plate antinociception) reveals a high coefficient of determination ($R^2 = 0.96$; Figure 4A, left panel), suggesting that increasing bias, as determined in cell-based signaling assays, can be predictive of an improved therapeutic window for analgesia without respiratory suppression in the mouse model. A similar correlation between a compound's bias and its therapeutic window is observed when inhibition of cAMP accumulation is used as the measure of G protein signaling ($R^2 = 0.95$; Figure 4A, right panel). When we compare other cell-based signaling-biased factors, whether they be the

parameters derived from assays using mouse MOR or mouse brainstem, the correlation remains high when compared to the therapeutic windows calculated from hot plate and arterial oxygen saturation potencies ($R^2 > 0.82$; Table S5). In general, the therapeutic windows derived from the tail-flick potencies were less correlative, with R^2 values ranging from 0.64–0.94. In the calculation of bias factors, we noted that fentanyl and SR-11501 produced differently directed bias profiles depending upon the G protein signaling assay used (Figures 1D and 1E); therefore, we performed a correlation analysis on this subset of

Table 3. ED₅₀ Values and Therapeutic Windows for SR Compounds

Agonist	ED ₅₀ (95% CI)				Therapeutic Window			
	Hot Plate	Tail Flick	%O ₂ Saturation	Breath Rate	O ₂ /HP	O ₂ /TF	BR/HP	BR/TF
Fentanyl	0.24 (0.16 to 0.32)	0.14 (0.07 to 0.20)	0.71 (0.60 to 0.85)	0.52 (0.37 to 0.67)	3	5	2	4
SR-11501	4.6 (2.9 to 6.3)	11 (5.6 to 16)	18 (13 to 27)	7.6 (4.6 to 11)	4	2	2	1
Morphine	5.9 (4.4 to 7.5)	3.8 (2.5 to 5.2)	79 (62 to 104)	33 (23 to 42)	13	21	5	9
SR-14969	1.7 (0.88 to 2.5)	2.3 (1.4 to 3.2)	21 (16 to 29)	20 (9.8 to 30)	13	9	12	9
SR-14968	0.44 (0.31 to 0.57)	0.61 (0.32 to 0.89)	14 (11 to 18)	11 (5.6 to 17)	31	23	28	20
SR-15098	12 (9.5 to 15)	13 (8.9 to 18)	538 (276 to 3359)	174 (70 to 277)	44	40	14	13
SR-15099	8.4 (6.2 to 11)	7.4 (4.9 to 9.9)	560 (260 to > 5000)	206 (25 to 386)	67	75	28	28
SR-17018	6.9 (4.7 to 9.1)	7.7 (4.1 to 11.3)	719 (278 to > 5000)	197 (24 to 370)	105	93	29	26

Hot plate (HP), tail flick (TF), % arterial oxygen saturation (O₂) and breath rate (BR, breaths per min) ED₅₀ values (mg/kg) were calculated from the hyperbolic fits (maximum value shared at 100%) of the area-under-the-curve analysis of dose response studies performed in male C57BL6/J mice (Figures S4A–S4D) for the 1-hr period following drug treatment. The ratios of ED₅₀ values for respiratory suppression over antinociception are represented by the therapeutic windows. Mean and 95% CI are provided. % MPE for all assays is defined on the lower limit by the baseline (BL) response and an upper limit (max, 100%) designed to limit the severity of each assay: HP (52°C): BL: 6.78 ± 0.14 s, max: 20 s; TF (49°C): BL: 2.95 ± 0.07 s, max: 30 s; % O₂: BL: 95.11 ± 0.12%, max: 70%; BR: BL: 165.0 ± 0.2, max: 75).

See also Figure S4 for the behavioral dose response curves and Figure 3C for a graphical representation of the therapeutic windows.

compounds, including morphine, to determine which G protein assay is more correlative with the therapeutic window. We find that a bias toward β arrestin2 recruitment over GTP γ S binding, but not cAMP accumulation, highly correlates with a narrow therapeutic window (hot plate/%O₂) compared to morphine ($R^2 = 0.99$ for GTP γ S compared to $R^2 = 0.41$ for cAMP; Figure 4B). As such, for compounds that had more narrow safety margins, the bias factors calculated from comparing the GTP γ S assays to β arrestin2 recruitment were more correlative than those derived from comparing the cAMP accumulation assay to β arrestin2 recruitment.

DISCUSSION

In this study, we have used several cell-based signaling assays to compare a series of structurally related MOR agonists, as well as prescription opiates, for their ability to activate MOR in comparison to a reference compound, DAMGO. Using these data, we normalized their potencies and efficacies with respect to the efficiency of DAMGO in each assay using the classic operational model to determine bias factors (Black and Leff, 1983; Kenakin et al., 2012). We then tested each compound in mice, using conventional antinociception assays and a mouse pulse oximeter to determine *in vivo* efficacies. Upon calculating potency (ED₅₀) values from the mouse studies, we then calculated therapeutic windows by dividing the potencies observed in the respiratory assays by the potencies measured in the pain assays. A comparison between the degree of separation measured in the cell-based G protein signaling and β arrestin2 recruitment assays (bias factors) positively correlates with the ability to separate antinociception and respiratory suppression *in vivo* (increase in the therapeutic window). Using these parameters, we show for the first time that fentanyl induces bias toward promoting β arrestin2 recruitment over inducing GTP γ S binding and that this correlates with an increase propensity for respiratory suppression at low doses. Since the therapeutic window of fentanyl is known to be narrower than morphine in humans, these findings

may be important for understanding fentanyl-related overdose fatalities.

One of the goals of this study was to understand how changing the signaling assay and the cellular context impacts the calculation of bias (while maintaining the reference agonist constant). Interestingly, compounds in our studies that display bias toward β arrestin2 over GTP γ S do not maintain this bias profile when inhibition of cAMP accumulation is measured as a surrogate for G protein signaling. Moreover, we found that the GTP γ S-binding-assay-derived bias factors were more predictive of the calculated therapeutic windows, especially of those for fentanyl and SR-11501, than the cAMP assay-derived bias factors were (Figure 4 and Table S5). Overall, we determined that once we reached bias factors exceeding 10 using the hMOR GTP γ S/ β arrestin2 measures, the type of assay used to subsequently test G protein signaling had little bearing on the confidence in bias calculation. However, as that value approaches zero, we found greater variation in bias calculations when other G protein signaling assays were used (i.e., inhibition of cAMP accumulation).

In the early β arrestin2-KO mouse studies, morphine was shown to induce pronounced antinociception when compared to wild-type (WT) mice (Bohn et al., 1999). In subsequent studies, it was shown that the β arrestin2-KO mice were resistant to morphine-induced respiratory suppression (Raehal et al., 2005). Together, the studies in the KO mice along with the biased agonists strongly support the hypothesis that compounds that do not recruit β arrestin2 may prove to be safer (i.e., have a wider therapeutic window) than current clinical opioids. However, it is important to acknowledge that to date, it has not been directly demonstrated that β arrestins are indeed mediating the respiratory side effects in the mice. Moreover, it also remains to be seen whether bias factors correlate with the expression of other opiate side effects, such as constipation, analgesic tolerance, and the development of physical dependence and addiction, as these physiological responses and adaptations are complex and are resultant of multiple converging biological systems. Going forward, the G protein-biased compounds will be useful

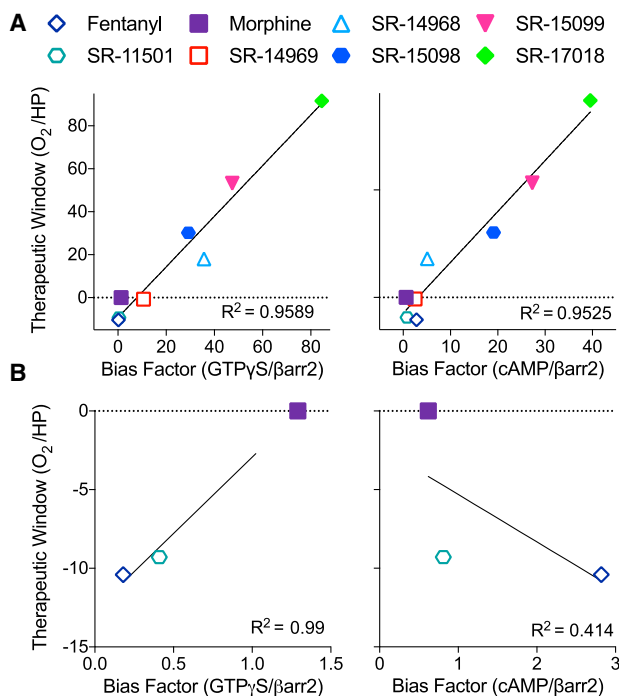


Figure 4. Bias Factors Positively Correlate with Therapeutic Window

(A) The bias factors determined in the hMOR cell lines ($GTP\gamma S$ binding in CHO-hMOR membranes [left] or inhibition of forskolin-stimulated cAMP accumulation in the CHO-hMOR cells [right]) versus β arrestin2 recruitment in the U2OS- β arrestin-hMOR-PathHunter EFC assay) when plotted against the therapeutic windows calculated from the *in vivo* studies (O_2 ED_{50} : % arterial oxygen saturation over HP ED_{50} : hot plate antinociception) produce linear correlations: $GTP\gamma S/\beta arr2$: $R^2 = 0.9589$; $cAMP/\beta arr2$: $R^2 = 0.9525$.

(B) Correlation analysis of compounds from (A) that display bias toward β arrestin2 (i.e., fentanyl and SR-11501) with morphine when plotted against the therapeutic window (HP/O_2) reveals a strong correlation when $GTP\gamma S/\beta arr2$ bias factors ($R^2 = 0.99$) are plotted, but not with $cAMP/\beta arr2$ bias factors ($R^2 = 0.4140$).

See also Table S5 for the correlation analysis between bias factor and therapeutic window for the other bias factors calculated for the compounds (CHO-mMOR and brainstem) and the therapeutic windows for the other behavioral measures (breath rate and tail flick).

for elucidating how MOR signals *in vivo* to promote these effects. Moreover, compounds like fentanyl, sufentanil, and SR-11501 may serve as important tools to challenge the counter-scenario.

Another important aspect of this study is the emphasis that bias factors and therapeutic windows are highly context dependent. The use of the operational model to estimate the degree of separation of functional performance between two cellular assays is an attempt to normalize the contributions of assay variances. The normalization is made possible by use of a reference agonist that serves to define the maximum potential of the receptor to produce a response in that system. Therefore, the correlation plots of response parameters are not to be viewed as a universal scale upon which independently generated values could be plotted. For new compounds to be assessed, a defining dataset should be produced; further, the different assays should all be performed in parallel and including the appropriate reference ag-

onists (those that produce the maximum response in the system) prior to calculation of bias between assays. Bias factors calculated for compounds wherein different mathematical models are used to calculate bias, different assays are run to determine G protein or β arrestin2 signaling potential, or different reference agonists are used could not be appropriately plotted with the data reported here. Similarly, one should not expect to plot therapeutic windows derived from other species, other pain tests, or other side effect measures and infer that a linear correlation would ensue. Such conclusions must be empirically determined.

With these limitations in mind, this study still remains the first of its kind to systematically assess a chemical series of agonists for bias across multiple signaling assays and also to perform thorough analyses of behavioral responses in a dose-dependent manner. The demonstration of a correlation between the bias observed to the width of the therapeutic window is highly encouraging for using these signaling assays to predict favorable outcomes in the mouse models used here. Moreover, we have demonstrated that key regions of the chemical scaffold can be mindfully modified to direct signaling between the different assays in cell culture and that this recapitulates as differences in potencies *in vivo*. Finally, this study introduces a new series of G protein signaling-biased MOR agonists that have the highest degree of separation between respiratory suppression and antinociception in rodent models reported to date. Our hope is that this work may aid the pharmaceutical development of safer alternatives to current opioid therapeutics.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information includes four figures and five tables and can be found with this article online at <https://doi.org/10.1016/j.cell.2017.10.035>.

AUTHOR CONTRIBUTIONS

Conceptualization, L.M.B., C.L.S., and T.D.B. Methodology, L.M.B., T.D.B., M.D.C., C.L.S., N.M.K., N.C.R., K.M.L., and Z.Y. Validation, J.M., N.M.K., and K.M.L. Formal analysis, L.M.B. and C.L.S. Investigation, C.L.S., N.M.K., N.C.R., K.M.L., Z.Y., and J.M. Writing—original draft, L.M.B., T.D.B., C.L.S., and N.M.K. Writing—reviewing and editing, N.C.R. and M.D.C. Visualization, L.M.B., C.L.S., and T.D.B. Supervision, L.M.B. and T.D.B.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
HA Tag Monoclonal Antibody (16B12), Alexa Fluor 594	ThermoFisher Scientific	Cat# A-21288; RRID: AB_2535830
Hoechst 33342, Trihydrochloride, Trihydrate	ThermoFisher Scientific	Cat# H3570
Chemicals, Peptides and Recombinant Proteins		
DAMGO	Tocris	Cat# 1171
DTT	Fisher Scientific	Cat# BP172-5
Fentanyl Citrate	Sigma-Aldrich	Cat# F3886
Forskolin	Sigma-Aldrich	Cat# F6886
GDP, disodium salt	Fisher Scientific	Cat# ICN15121325
[Tyrosyl-3,5- ³ H(N)]-DAMGO	PerkinElmer	Cat# NET902250UC
[Phenyl-3,4- ³ H]-U-69,593	PerkinElmer	Cat# NET952250UC
[15,16- ³ H]-Diprenorphine	PerkinElmer	Cat# NET1121250UC
[³⁵ S]-GTP γ S	PerkinElmer	Cat# NEG030H250UC
Morphine sulfate pentahydrate	NIDA Drug Supply Program	Cat# 9300-001
Naloxone hydrochloride dihydrate	Sigma-Aldrich	Cat# N7758
Naltrindole	Tocris	Cat# 0740
Nociceptin	Tocris	Cat# 0910
NorBNI	Sigma-Aldrich	Cat# N-1771
[Nphe ¹]Nociceptin(1-13)NH ₂	Tocris	Cat#1308
Poly(ethyleneimine) solution (PEI)	Sigma-Aldrich	Cat# 03880
Ro-20-1724	Sigma-Aldrich	Cat# B8279
SNC80	Enzo Life Sciences	Cat# ALX-550-172-M005
Solution D	ThermoFisher Scientific	Cat# K1156
SR-8595	This study	N/A
SR-11065	This study	N/A
SR-11501	This study	N/A
SR-14968	This study	N/A
SR-14969	This study	N/A
SR-15098	This study	N/A
SR-15099	This study	N/A
SR-17018	This study	N/A
SR-20382	This study	N/A
SR-20437	This study	N/A
Sufentanil Citrate	NIDA Drug Supply Program	Cat# 9740-001
U69,593	Sigma-Aldrich	Cat#U103
Critical Commercial Assays		
cAMP HiRange Kit	Cisbio	Cat# 62AM6PEC
LiveBLAzer FRET-B/G loading kit	ThermoFisher Scientific	Cat#K1095
PathHunter Detection Kit	DiscoverRx	Cat# 93-0001
Rapid Equilibrium Dialysis Device	ThermoFisher Scientific	Cat#90006
Experimental Models: Cell Lines		
CHO-K1	ATCC	Cat# CCL-61, RRID:CVCL_0214
CHO-hMOR	R.B. Rothman: (Ananthan et al., 2012)	N/A
CHO-mMOR	This study	N/A

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
CHO-hDOR	L.M. Bohn: (Zhou et al., 2013)	N/A
CHO-hKOR	L.M. Bohn: (Schmid et al., 2013)	N/A
Phoenix-Ampho	ATCC	Cat# SD-3443, RRID:CVCL_H716
U2OS- β arrestin-hMOR PathHunter	DiscoverRx	Cat# 93-0213C3
U2OS- β arrestin2-GFP	L. Barak: Addiction Research GPCR Assay Bank, NIDA	N/A
U2OS- β arrestin2-GFP-mMOR	This study	N/A
U2OS-Tango-hOPRL1- <i>b/a</i>	ThermoFisher Scientific	Cat# K1786
Experimental Models: Organisms/Strains		
Mouse: C57BL/6J	The Jackson Laboratory	RRID:IMSR_JAX:000664
Mouse: MOR-KO: B6.129S2- <i>Oprm1</i> ^{tm1kff} /J	The Jackson Laboratory	RRID:IMSR_JAX:007559
Recombinant DNA		
HA-mMOR-MSCV	L.M.Bohn: (Groer et al., 2011)	N/A
Software and Algorithms		
HCS Studio 2.0 Cell Analysis Software	ThermoFisher Scientific	https://www.thermofisher.com/us/en/home.html
MouseOx Plus Revision 1.5.1b	Starr Life Sciences Corporation	https://www.starlifesciences.com/
Prism v. 6.0h	GraphPad Software	https://www.graphpad.com

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for reagents may be directed to and will be fulfilled by the Lead Contact, Laura M. Bohn (lbohn@scripps.edu). The Scripps Research Institute requires that a material transfer agreement (MTA) be signed for the transfer of materials.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals

Male and female C57BL/6J and male MOR-KO mice were purchased from The Jackson Laboratory and propagated by homozygous breeding in-house. Mice were group housed (3-5 mice per cage) and maintained on a 12-hour light/dark cycle with food and water *ad libitum*. Experiments were performed on naive adult mice between 10-14 weeks of age. Same sex littermates were randomly assigned to experimental groups; males and females were separately tested and their responses are separately reported. Experiments were performed by investigators who were blinded to the treatment assignments. Mice were dosed i.p. at a volume of 10 μ l/g mouse, except all 48 mg/kg injections were dosed at a volume of 20 μ l/g mouse to adjust for compound solubility. The number of mice used in each assay are indicated in Table S4. All mice were used in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals with approval by The Scripps Research Institute Animal Care and Use Committee.

Cell lines

Chinese Hamster Ovary (CHO-K1) and Phoenix-AMPHO cells were purchased from ATCC; U2OS- β arrestin-hMOR PathHunter cells were purchased from DiscoverRx and the U2OS-Tango-hOPRL1-*b/a* cells were purchased from ThermoFisher Scientific. The U2OS- β arrestin2-GFP cells were provided by the Addiction Research GPCR Assay Bank. Based on the other reports, all of the parent cell lines are female (query: <http://web.expasy.org/cellosaurus/>). Receptor levels in the CHO-hMOR, hKOR and hDOR cells have been described previously as well as in the current manuscript (Ananthan et al., 2012; Schmid et al., 2013; Zhou et al., 2013). To make the mMOR lines, the HA (haemagglutinin)-N terminus tagged mMOR was packaged into murine stem cell retroviral particles via the phoenix packaging system and then CHO-K1 and U2OS- β arrestin2-GFP cells were transduced with the particles. A BD FACSARIA3 flow cytometer was used to select for high expressing cells using an anti-HA AlexaFluor 594 conjugate antibody (1:100). All cells lines were cultured according to standard protocols at 37° in the indicated media with 10% fetal bovine serum (FBS) and 1% pen/strep: DMEM/F12—all CHO-K1 lines; DMEM—Phoenix- AMPHO; MEM—all U2OS cell lines. CHO-hMOR, -hKOR and -hDOR cell lines were grown under geneticin selection (500 μ g/ μ l). U2OS mMOR β arrestin2 cells lines were grown under puromycin selection (500 μ g/ μ l). The U2OS- β arrestin-hMOR PathHunter cell line (in which the MOR retains its natural C-terminal tail, tagged with the enzyme fragment) was cultured according to the manufacturer's protocol (DiscoverRx), as was the U2OS-Tango-OPRL1-*b/a* cell line (ThermoFisher Scientific).

METHOD DETAILS

Synthesis of MOR ligands

All reagents and anhydrous solvents were used as obtained from commercial vendors. ^1H NMR spectra were recorded at 400 MHz, with chemical shifts are reported in parts per million (ppm) using an internal standard, CHCl_3 (δ 7.26), MeOH (δ 3.34) or DMSO (δ 2.54). Mass spectra were recorded by ESI Ion trap. Analytical HPLC retention times were measured using reverse phase conditions with a Zorbax 5 micron column, model Eclipse-XDB-C18 80Å (155 × 4.6 mm), column temperature = 40°C, flow rate = 3.00 mL/min. The method incorporates a gradient elution, beginning with 98% H_2O / 2% acetonitrile, each with 0.1% TFA. After 1 min, hydrophobicity was increased to 5% acetonitrile and then linearly in a gradient to 95% acetonitrile over an additional 5 min. Purity assessment (> 95%) was made LC using UV absorbance at multiple wavelengths, typically 215, 254, and 280 nm.

General synthesis methods: the synthesis of all compounds followed the standard methods depicted in Figure S1. Nucleophilic aromatic substitution followed by nitro group reduction (Obase et al., 1983), urea formation (Budzik et al., 2010), standard Boc deprotection, and finally direct alkylation (Lindsley et al., 2005) or reductive amination of an aldehyde (Zhao et al., 2005) or ketone (Patel et al., 2014) gave the indicated SR compounds. The compounds were isolated and characterized in free base form unless indicated (overall yields 15%–40% for 5 steps) and then the compounds were evaluated in all biological and pharmacological assays as their mono mesylate salts.

Representative nucleophilic aromatic substitution procedure, $\text{R}^1 = \text{R}^2 = \text{Cl}$: 1,2-Dichloro-4-fluoro-5-nitrobenzene (0.43 mL, 3.3 mmol) was added to a mixture of *tert*-butyl 4-aminopiperidine-1-carboxylate (0.66 g, 3.3 mmol) and K_2CO_3 (0.50 g, 3.6 mmol) in DMSO (5 mL). The reaction mixture was stirred overnight at room temperature under argon. Water was added and the organic layer extracted with EtOAc, dried over Na_2SO_4 , and concentrated under reduced pressure. Purification was achieved by flash column chromatography on silica gel using a gradient of EtOAc:hexanes as the eluent to give an orange solid (0.79 g, 62% yield). ^1H NMR (400 MHz, CDCl_3) δ 8.29 (s, 1H), 8.01 (d, $J = 7.2$ Hz, 1H), 6.97 (s, 1H), 4.03 (d, $J = 13.6$ Hz, 2H), 3.65–3.56 (m, 1H), 3.05 (td, $J = 12.4, 2.8$ Hz, 2H), 2.05 (dd, $J = 13.0, 3.4$ Hz, 2H), 1.63–1.49 (m, 2H), 1.47 (s, 9H); MS(m/z): [M + H] calc'd for $\text{C}_{16}\text{H}_{21}\text{Cl}_2\text{N}_3\text{O}_4$ is 390.26, found 389.49.

Representative nitro group reduction procedure, $\text{R}^1 = \text{R}^2 = \text{Cl}$: *Tert*-butyl 4-((4,5-dichloro-2-nitrophenyl)amino)piperidine-1-carboxylate (0.79 g, 2.0 mmol) was dissolved in EtOH (40 mL) and a 50% aqueous suspension of Raney nickel (5 mL) was added. Hydrazine hydrate (0.98 mL, 20 mmol) was then added dropwise. The mixture was heated to 45°C and maintained at that temperature for 10 min. The mixture was filtered through a pad of Celite[®] which was washed with MeOH. The filtrate was concentrated under reduced pressure. Purification was achieved by flash column chromatography on silica gel using a gradient of EtOAc: hexanes as the eluent to give the diamine product (0.55 g, 76% yield). ^1H NMR (400 MHz, CD_3OD) δ 6.75 (s, 1H), 6.62 (s, 1H), 4.46 (s, 1H), 4.01 (d, $J = 13.2$ Hz, 2H), 3.36 (tt, $J = 10.0, 4.0$ Hz, 1H), 2.95 (t, $J = 12.2$ Hz, 2H), 2.00 (d, $J = 13.6$ Hz, 2H), 1.45 (s, 9H), 1.36 (qd, $J = 12.0, 4.0$ Hz, 2H); MS(m/z): [M + H] calc'd for $\text{C}_{16}\text{H}_{23}\text{Cl}_2\text{N}_3\text{O}_2$ is 360.28, found 359.58.

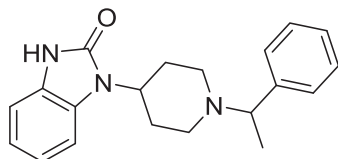
Representative urea formation and Boc removal procedure, $\text{R}^1 = \text{R}^2 = \text{Cl}$: *Tert*-butyl 4-((2-amino-4,5-dichloro-phenyl)amino)piperidine-1-carboxylate (0.55 g, 1.5 mmol) was dissolved in THF (15 mL) under argon. CDI (0.35 g, 2.1 mmol) was added and the reaction mixture was stirred at room temperature overnight. Upon completion, the solvent was removed under reduced pressure and the residue was dissolved in EtOAc. This mixture was washed with 1M $\text{HCl}_{(\text{aq})}$ followed by brine. The organic layer was dried over Na_2SO_4 and the solvent was removed under reduced pressure. Purification was achieved by flash column chromatography on silica gel using a gradient of EtOAc: hexanes as the eluent to give *tert*-butyl 4-(5,6-dichloro-2-oxo-2,3-dihydro-1*H*-benzo[d]imidazol-1-yl)piperidine-1-carboxylate (0.54 g, 91% yield). ^1H NMR (400 MHz, CDCl_3) δ 9.78 (s, 1H), 7.20 (s, 1H), 7.19 (s, 1H), 4.44–4.34 (m, 3H), 2.85 (t, $J = 11.2$ Hz, 2H), 2.26 (qd, $J = 12.6, 4.2$ Hz, 2H), 1.82 (d, $J = 10.8$ Hz, 2H), 1.52 (s, 9H); MS(m/z): [M + H] calc'd for $\text{C}_{17}\text{H}_{21}\text{Cl}_2\text{N}_3\text{O}_3$ is 386.27, found 385.33. This product was dissolved in a 33% solution of TFA in CH_2Cl_2 (4 mL). Upon completion, the solvent was removed under reduced pressure and the residue was dissolved in a minimal amount of water-acetonitrile (1:1). The solution was frozen and was then subjected to lyophilization overnight, giving the amine product as a TFA salt (86% crude yield). ^1H NMR (400 MHz, CDCl_3) δ 7.36 (s, 1H), 7.17 (s, 1H), 4.36 (tt, $J = 12.6, 4.0$ Hz, 1H), 3.27 (d, $J = 12.0$ Hz, 2H), 2.79 (td, $J = 12.2, 2.0$ Hz, 2H), 2.27 (qd, $J = 12.4, 4.0$ Hz, 2H), 1.83 (dd, $J = 12.0, 2.0$ Hz, 2H); MS(m/z): [M + H] calc'd for $\text{C}_{12}\text{H}_{13}\text{Cl}_2\text{N}_3\text{O}$ is 286.16, found 286.12.

Representative reductive amination procedure, $\text{R}^1 = \text{R}^2 = \text{Cl}$, $\text{R}^3 = \text{Br}$: $\text{NaBH}(\text{OAc})_3$ (97 mg, 0.44 mmol) was added to an anhydrous DCE (3 mL) solution of 5,6-Dichloro-1-(piperidin-4-yl)-1,3-dihydro-2*H*-benzo[d]imidazol-2-one (59 mg, 0.15 mmol), and 4-bromobenzaldehyde (85 mg, 0.44 mmol). A few drops of AcOH were added to the solution and the reaction mixture was stirred overnight at room temperature under argon. Upon completion, saturated aq. NaHCO_3 was added to the reaction mixture, which was then diluted with CH_2Cl_2 . The aqueous layer was extracted with CH_2Cl_2 and the combined organic layers were washed with brine, dried over Na_2SO_4 , and concentrated under reduced pressure. Purification was achieved by flash column chromatography on silica gel using with CH_2Cl_2 :MeOH as the eluent to give the desired product SR-15099 (36 mg, 55% yield). Methanesulfonic acid (5.2 μL , 0.08 mmol) was added to a suspension of SR-15099 free base in EtOH (1 mL). The mixture was heated to 60°C for 30 min. The solvent was evaporated under reduced pressure and the residue was dissolved in a minimal amount of water-acetonitrile (1:1). The solution was frozen and subjected to lyophilization overnight, giving 5,6-Dichloro-1-(1-(4-bromobenzyl)piperidin-4-yl)-1,3-dihydro-2*H*-benzo

[d]imidazol-2-one methanesulfonate as a white powder. Analytical data for this tested compound is given later in this section. Note: for reductive amination reactions of methyl ketones rather than aldehydes, the ketone (3 equiv.) and amine was treated with 10 eq. of $\text{Ti}(\text{O}i\text{-Pr})_4$, heated to 60°C , then 8.75 equiv. of NaBH_3CN in ethanol was added. After overnight reaction, the mixture was processed as described above. Added note: the unsubstituted N-benzyl compound SR-20437 was prepared by alkylation rather than by reductive amination. To the amine in minimal DMF was added 1.1 equiv. of benzyl bromide, 1.1 equiv. of K_2CO_3 , 1.0 equiv. of NaI . Heating at 60°C overnight, standard workup, and salt formation gave the desired material in 53% yield.

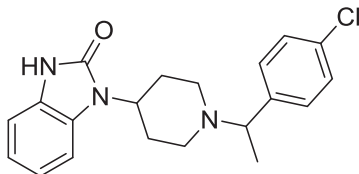
Analytical data for final compounds

SR-8595 (Table S1, entry 1), (\pm)-1-(1-(1-phenylethyl)piperidin-4-yl)-1,3-dihydro-2H-benzo[d]imidazol-2-one



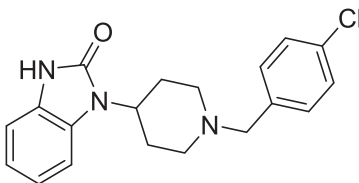
^1H NMR (400 MHz, $(\text{CD}_3)_2\text{SO}$) δ 10.80 (s, 1H), 7.34 (t, J = 3.0 Hz, 4H), 7.26-7.19 (m, 2H), 6.99-6.95 (m, 3H), 4.05 (tt, J = 12.6, 4.0 Hz, 1H), 3.53 (q, J = 6.8 Hz, 1H), 3.10 (d, J = 10.0 Hz, 1H), 2.89 (d, J = 9.6 Hz, 1H), 2.45-2.23 (m, 2H), 2.07 (td, J = 10.4, 2.4 Hz, 1H), 1.97 (td, J = 10.8, 2.0 Hz, 1H), 1.66 (d, J = 10.8 Hz, 1H), 1.58 (d, J = 10.4 Hz, 1H), 1.33 (d, J = 6.8 Hz, 3H); MS(m/z): [M + H] calculated for $\text{C}_{20}\text{H}_{23}\text{N}_3\text{O}$ is 321.42, found 321.96; HPLC t_R = 3.52 min.

SR-11065 (Table S1, entry 2), (\pm)-1-(1-(1-(4-chlorophenyl)ethyl)piperidin-4-yl)-1,3-dihydro-2H-benzo[d]imidazol-2-one



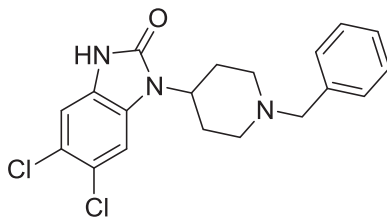
^1H NMR (400 MHz, CDCl_3) δ 7.93 (d, J = 8.0 Hz, 1H), 7.59 (d, J = 8.4 Hz, 2H), 7.46 (d, J = 8.4 Hz, 2H), 7.19 (td, J = 7.6, 1.2 Hz, 1H), 7.09-7.02 (m, 2H), 4.62-4.54 (m, 1H), 4.21-4.18 (m, 1H), 3.89 (d, J = 10.4 Hz, 1H), 3.52 (d, J = 12.8 Hz, 2H), 3.27 (qd, J = 11.6, 4.0 Hz, 1H), 2.78 (q, J = 10.4 Hz, 1H), 2.65 (q, J = 11.2 Hz, 1H), 1.97 (d, J = 6.8 Hz, 3H), 1.93-1.89 (m, 2H); MS(m/z): [M + H] calculated for $\text{C}_{20}\text{H}_{22}\text{ClN}_3\text{O}$ is 355.87, found 355.92; HPLC t_R = 3.76 min.

SR-20382 (Table S1, entry 3), 1-(1-(4-chlorobenzyl)piperidin-4-yl)-1,3-dihydro-2H-benzo[d]imidazol-2-one methanesulfonate



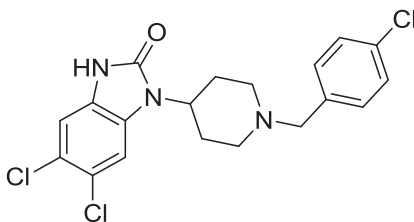
^1H NMR of the mesylate salt (400 MHz, CD_3OD) 7.56-7.54 (m, 4H), 7.29-7.26 (m, 1H), 7.10-7.07 (m, 3H), 4.56 (tt, J = 12.4, 4.0 Hz, 1H), 4.39 (s, 2H), 3.66 (dd, J = 10.6, 1.8 Hz, 2H), 3.27-3.23 (m, 2H), 2.80 (qd, J = 13.4, 3.8 Hz, 2H), 2.72 (s, 3H), 2.09 (d, J = 14.8 Hz, 2H); MS(m/z): [M + H] calculated for $\text{C}_{19}\text{H}_{20}\text{ClN}_3\text{O}$ is 341.84, found 342.02; HPLC t_R = 3.67 min.

SR-20437 (Table S1, entry 4), 5,6-dichloro-1-(1-(benzyl)piperidin-4-yl)-1,3-dihydro-2H-benzo[d]imidazol-2-one methanesulfonate



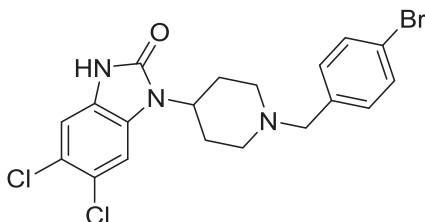
^1H NMR of the mesylate salt (400 MHz, CD_3OD) δ 7.56-7.52 (m, 5H), 7.48 (s, 1H), 7.19 (s, 1H), 4.53 (tt, J = 12.4, 4.0 Hz, 1H), 4.39 (s, 2H), 3.65 (d, J = 12.8 Hz, 2H), 3.28-3.22 (m, 2H), 2.77 (qd, J = 13.4, 4.0 Hz, 2H), 2.72 (s, 3H), 2.08 (d, J = 14.8 Hz, 2H); MS(m/z): [M + H] calculated for $\text{C}_{19}\text{H}_{19}\text{Cl}_2\text{N}_3\text{O}$ is 376.28, found 375.98; HPLC t_R = 3.95 min.

SR-17018 (Table 1; Table S1, entry 5), 5,6-dichloro-1-(1-(4-chlorobenzyl)piperidin-4-yl)-1,3-dihydro-2H-benzo[d]imidazol-2-one methanesulfonate



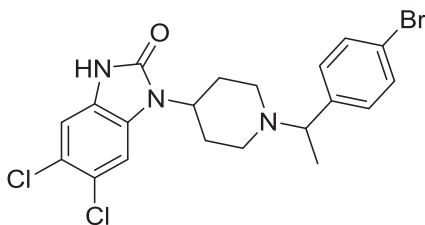
^1H NMR of the mesylate salt (400 MHz, CD_3OD) δ 7.58-7.53 (m, 4H), 7.48 (s, 1H), 7.19 (s, 1H), 4.52 (tt, $J = 12.4, 3.8$ Hz, 1H), 4.38 (s, 2H), 3.65 (d, $J = 12.8$ Hz, 2H), 3.25 (t, $J = 12.2$ Hz, 2H), 2.80-2.69 (m, 5H), 2.08 (d, $J = 13.6$ Hz, 2H); MS(m/z): [M + H] calculated for $\text{C}_{19}\text{H}_{18}\text{Cl}_3\text{N}_3\text{O}$ is 410.72, found 410.01; HPLC $t_{\text{R}} = 4.12$ min.

SR-15099 (Table 1; Table S1, entry 6), 5,6-dichloro-1-(1-(4-bromobenzyl)piperidin-4-yl)-1,3-dihydro-2H-benzo[d]imidazol-2-one methanesulfonate



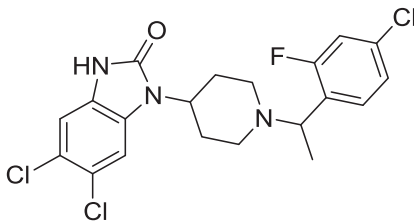
^1H NMR of the mesylate salt (400 MHz, CD_3OD) δ 7.70 (dt, $J = 8.4, 2.1$ Hz, 2H), 7.50 (dd, $J = 6.6, 2.0$ Hz, 3H), 7.19 (s, 1H), 4.53 (tt, $J = 12.4, 4.0$ Hz, 1H), 4.37 (s, 2H), 3.63 (d, $J = 12.8$ Hz, 2H), 3.24 (t, $J = 12.4$ Hz, 2H), 2.79-2.70 (m, 6H), 2.07 (d, $J = 12.4$ Hz, 2H); MS(m/z): [M + H] calculated for $\text{C}_{19}\text{H}_{18}\text{BrCl}_2\text{N}_3\text{O}$ is 455.18, found 456.23; HPLC $t_{\text{R}} = 3.76$ min.

SR-14968 (Table 1; Table S1, entry 7), (\pm)-5,6-dichloro-1-(1-(1-(4-bromophenyl)ethyl)piperidin-4-yl)-1,3-dihydro-2H-benzo[d]imidazol-2-one methanesulfonate



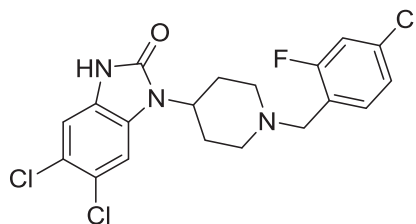
^1H NMR of the mesylate salt (400 MHz, CD_3OD) δ 7.71 (d, $J = 8.4$ Hz, 2H), 7.49 (t, $J = 8.4$ Hz, 3H), 7.19 (s, 1H), 4.57 (q, $J = 6.8$ Hz, 1H), 4.44 (tt, $J = 12.4, 4.0$ Hz, 1H), 3.87 (dd, $J = 11.0, 1.8$ Hz, 1H), 3.49 (dd, $J = 11.2, 2.0$ Hz, 1H), 3.14 (td, $J = 13.0, 2.8$ Hz, 1H), 3.04 (td, $J = 13.0, 2.8$ Hz, 1H), 2.84-2.66 (m, 6H), 2.12-2.02 (m, 2H), 1.80 (d, $J = 6.8$ Hz, 3H); MS(m/z): [M + H] calculated for $\text{C}_{20}\text{H}_{20}\text{BrCl}_2\text{N}_3\text{O}$ is 469.20, found 469.89; HPLC $t_{\text{R}} = 4.00$ min.

SR-14969 (Table 1; Table S1, entry 8), (\pm)-5,6-dichloro-1-(1-(1-(4-chloro-2-fluorophenyl)ethyl)piperidin-4-yl)-1,3-dihydro-2H-benzo[d]imidazol-2-one methanesulfonate



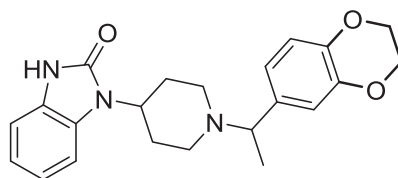
^1H NMR of the mesylate salt (400 MHz, CD_3OD) δ 7.67 (t, $J = 7.8$ Hz, 1H), 7.48-7.43 (m, 3H), 7.19 (s, 1H), 4.93-4.89 (m, 1H), 4.47 (tt, $J = 12.2, 4.0$ Hz, 1H), 3.89 (d, $J = 12.0$ Hz, 1H), 3.60 (d, $J = 10.4$ Hz, 1H), 3.19 (td, $J = 13.0, 2.2$ Hz, 1H), 3.10 (td, $J = 13.0, 2.2$ Hz, 1H), 2.88-2.72 (m, 6H), 2.12-2.04 (m, 2H), 1.83 (d, $J = 6.8$ Hz, 3H); MS(m/z): [M + H] calculated for $\text{C}_{20}\text{H}_{19}\text{Cl}_3\text{FN}_3\text{O}$ is 442.74, found 441.87; HPLC $t_{\text{R}} = 3.94$ min.

SR-15098 (Table 1; Table S1, entry 9) 5,6-dichloro-1-(1-(4-chloro-2-fluorobenzyl)piperidin-4-yl)-1,3-dihydro-2H-benzo[d]imidazol-2-one methanesulfonate



^1H NMR of the mesylate salt (400 MHz, CD_3OD) δ 7.67 (t, J = 8.2 Hz, 1H), 7.50 (s, 1H), 7.43 (qd, J = 7.8, 2.0 Hz, 2H), 7.19 (s, 1H), 4.55 (tt, J = 12.4, 4.0 Hz, 1H), 4.46 (s, 2H), 3.70 (d, J = 12.4 Hz, 2H), 3.36-3.30 (m, 2H), 2.82-2.71 (m, 5H), 2.09 (d, J = 13.6 Hz, 2H); MS(m/z): [M + H] calculated for $\text{C}_{19}\text{H}_{17}\text{Cl}_3\text{FN}_3\text{O}$ is 428.71, found 427.96; HPLC t_{R} = 4.15 min.

SR-11501 (Table 1; Table S1, entry 10), (\pm)-1-(1-(1-(2,3-dihydrobenzo[*b*][1,4]dioxin-6-yl)ethyl)piperidin-4-yl)-1,3-dihydro-2H-benzo[d]imidazol-2-one methanesulfonate



^1H NMR of the mesylate salt (400 MHz, CD_3OD) δ 7.30-7.27 (m, 1H), 7.09-7.06 (m, 4H), 7.01 (dd, J = 8.4, 2.0 Hz, 1H), 6.96 (d, J = 8.4 Hz, 1H), 4.50-4.41 (m, 2H), 4.28 (s, 4H), 3.84 (dt, J = 12.8, 1.9 Hz, 1H), 3.49 (dt, J = 11.8, 1.4 Hz, 1H), 3.14 (td, J = 13.0, 2.8 Hz, 1H), 3.04 (td, J = 13.0, 2.8 Hz, 1H), 2.92-2.72 (m, 6H), 2.12-2.01 (m2H), 1.77 (d, J = 7.2 Hz, 3H); MS(m/z): [M + H] calculated for $\text{C}_{22}\text{H}_{25}\text{N}_3\text{O}_3$ is 379.46, found 379.87; HPLC t_{R} = 4.58 min.

Preparation of drug solutions

For the *in vitro* studies, the reference compounds DAMGO, morphine sulfate and nociceptin were prepared in water as a 10 mM stock and a 10 mM stock of U69,593 was prepared in ethanol. All of the other compounds were prepared in DMSO at concentrations spanning from 32 nM to 10 mM, for dilutions. For all assays, the final DMSO concentration was 1%. For the *in vivo* studies, compounds were dissolved from powder immediately prior to use. Morphine sulfate and the test compounds were prepared in a vehicle of 1:1:8 DMSO: Tween 80: dH_2O . Fentanyl citrate was dissolved in 0.9% saline for the studies in C57BL/6J male mice. For studies where only one dose was tested (females and MOR-KO mice) all compounds were made in the same vehicle to facilitate blinding of drug preparation and experimenter handling. Compounds were administered intraperitoneally (i.p.) at a concentration of 10 μL per gram mouse, except for the 48 mg/kg dose of the test compounds. In this case, the drugs were administered at a volume of 20 μL per gram mouse due to limited solubility. Morphine sulfate and fentanyl citrate dosing is based on the salt weight of the drugs, while the SR compounds dosing is based on the free base weight.

Saturation and competition radioligand binding

Receptor binding assays were performed on CHO-hMOR, CHO-hDOR and CHO-hKOR cell lines as previously described (Groer et al., 2011; Schmid et al., 2013). Cells were serum-starved for 30 min, cells were collected and membrane pellets were prepared by Teflon-on-glass dounce homogenization in membrane buffer containing (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA), followed by centrifugation at 20,000 $\times g$ for 30 min at 4°C. Membranes were resuspended in assay buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl). Binding reactions (200 μL volume) were performed on 10 μg membranes with the appropriate radioligand (MOR, ^3H -DAMGO; KOR, ^3H -U69,593; DOR, ^3H -diprenorphine) for 2 hr at 25°C. For competition experiments, the concentration of each of the radioligands was approximately 1 nM (0.96–1.10 nM ^3H -DAMGO; 1.06–1.19 nM ^3H -U69,593; 0.92–0.98 nM ^3H -diprenorphine). Nonspecific binding was determined in the presence of 10 μM DAMGO (MOR), 10 μM U69,593 (KOR) or 10 μM Naloxone (DOR). Reactions were terminated by filtration through GF/B glass fiber filter plates (PerkinElmer), which had been pre-incubated with 0.1% polyethyleneimine, on a Brandel cell harvester. Radioactivity was counted with Microscint on a TopCount NXT Scintillation Counter (PerkinElmer). Saturation binding assays and hyperbolic curve fitting of specific binding was used to determine radioligand binding affinities and receptor numbers for the CHO cell lines (hMOR, 1.02 ± 0.10 nM for ^3H -DAMGO and 1.58 ± 0.11 pmol/mg; hDOR, 0.70 ± 0.11 nM [^3H]-Diprenorphine and 1.46 ± 0.26 pmol/mg; hKOR, 1.07 ± 0.01 nM [^3H]-U69,593 and 0.71 ± 0.12 pmol/mg).

^{35}S -GTP γS binding to membranes

^{35}S -GTP γS binding was determined in membranes prepared from CHO-hMOR and CHO-mMOR cells and brainstems isolated from adult male C57BL/6J and MOR-KO mice as described previously (Schmid et al., 2013). CHO-hMOR and CHO-mMOR cellular membranes, collected and prepared as described above with in GTP γS binding membrane buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl,

1 mM EDTA). Reactions (200 μ L volume) were performed for 1 hr at 25°C on 10 μ g membranes suspended in assay buffer (50 mM Tris-Cl, pH 7.4, 100 mM NaCl, 5 mM MgCl₂, 1 mM EDTA) with 50 μ M guanosine-5''-diphosphate (GDP) and 0.1 nM ³⁵S-GTP γ S. Reactions were terminated by filtration through GF/B filter plates and radioactivity was counted as described above. For [³⁵S]-GTP γ S binding on brainstems isolated from C57BL/6J and MOR-KO mice, tissues were homogenized by polytronic tissue tearor and membranes were prepared as described above. Binding reactions, containing 2.5 μ g protein, 1 mM dithiothreitol (DTT), 20 μ M GPD and 0.1 nM ³⁵S-GTP γ S, were incubated at room temperature for 2 hrs prior to harvesting. The average vehicle value for the CHO-hMOR membranes was 786 \pm 78 cpm and the average fold over vehicle for DAMGO was 4.6 \pm 0.26. The average vehicle value for the CHO-mMOR cell membranes was 694 \pm 28 cpm and the average fold over vehicle for DAMGO was 5.9 \pm 0.57. The average vehicle for the C57BL/6J brainstem membranes was 657 \pm 62 cpm and the average fold over vehicle for DAMGO was 1.9 \pm 0.03. The average vehicle for the MOR-KO brainstem membranes was 1647 \pm 507 cpm.

cAMP accumulation assay

CHO-hMOR, -hDOR and -hKOR cells were plated at a density of 4,000 cells per well of a 384-well, white-walled, 30 μ L-volume microplate (Greiner Bio-One) in Opti-MEM containing 1% FBS 4 hr prior to assaying. Cells were treated with 20 μ M forskolin, 25 μ M 4-(3-Butoxy-4-methoxybenzyl)imidazolidin-2-one (Ro-20-1724) and increasing concentrations of test compounds for 30 min at 25°C. Inhibition of cAMP was then determined using the Homogeneous Time-Resolved Fluorescence resonance energy transfer (FRET) cAMP HiRange assay by Cisbio (Cisbio-62AM6PEC). Fluorescence was measured at 620 and 665 nm using an Envision Multilabel Reader (PerkinElmer). FRET was calculated by the ratio of 665 nm / 620 nm. The average vehicle ratio for CHO-hMOR cells was 3134 \pm 99 and the average fold over vehicle for DAMGO was 2.2 \pm 0.04. The average vehicle ratio for CHO-hDOR cells was 2962 \pm 181 and the average fold over vehicle for SNC80 was 1.6 \pm 0.04. The average vehicle ratio for CHO-hKOR cells was 2965 \pm 153 and the average fold over vehicle for U69,593 was 1.9 \pm 0.12.

β Arrestin2 recruitment assays

To determine β arrestin2 recruitment to the human MOR a commercial enzyme fragment complementation assay (β -galactosidase) was used. U2OS- β arrestin-hMOR PathHunter cells were plated at a density of 5,000 cells per well of a 384-well, white-walled assay microplate (Greiner Bio-One) in Assay Complete Cell Plating 5 Reagent (DiscoverRx) 16-20 hr prior to measuring the signal. Cells were treated for 90 min with increasing concentrations of test compounds at 37°C and β arrestin2 recruitment was determined using the PathHunter Detection Kit with the β -galactosidase substrate to detect functional β -galactosidas. The resulting increase in luminescence was measured using a SpectraMax M5[®] Microplate Reader (Molecular Devices). The average vehicle for the PathHunter U2OS OPRM1 β arrestin cells was 446 \pm 25 RLU and the average fold over vehicle for DAMGO was 36 \pm 1.

To determine β arrestin2 recruitment to the mMOR, an imaging-based assay as was used (Zhou et al., 2013). U2OS- β arrestin2-GFP-mMOR cells were plated at a density of 5,000 cells per well of a 384-well, black-walled, clear-bottom optical imaging microplate (Brooks) in normal media 16-20 hr prior to assaying. Cells were serum-starved for 1 hr and then treated with increasing concentrations of test compounds for 20 min at 37°C. Cells were fixed with 4% paraformaldehyde (PFA) containing Hoechst nuclear stain at a dilution of 1:1000. β Arrestin 2 translocation was measured using the 20X objective on a CellInsight CX5 High Content Screening Platform (ThermoFisher Scientific). Punctae (normalized to Hoechst stain) were quantified using the Cellomics' Spot Detection BioApplication (ThermoFisher Scientific). The average punctae / Hoechst ratio for vehicle treated U2OS- β arrestin2-GFP-mMOR cells was 2.2 \pm 0.54 and the average fold over vehicle for DAMGO was 61 \pm 13.

To determine whether the compounds have activity at NOP, β arrestin2 recruitment to the receptor was determined in the U2OS-Tango-hOPRL1-*bla* cells. U2OS-Tango-hOPRL1-*bla* cells were plated at a density of 10,000 cells per well of a 384-well, black-walled, clear-bottom assay plate in 32 μ L assay media (DMEM + 10% dialyzed FBS, 0.1 mM NEAA, 25 mM HEPES and 1% pen/strep) 16-20 hr prior to assaying. Cells were treated with increasing concentrations of test compounds for 5 hr at 37°C. NOP activation was determined using the LiveBLazer FRET-B/G loading kit with Solution D (ThermoFisher Scientific), according to the manufacturer's protocol. FRET signal (excitation 409 nm, emissions at 460 nm and 530 nm) was measured using a SpectraMax M5[®] Microplate Reader (Molecular Devices). The average 460/530 ratio vehicle treated U2OS-Tango-hOPRL1-*bla* cells was 0.31 \pm 0.03 and the average fold over vehicle for nociceptin was 7.6 \pm 0.68.

Pharmacokinetics and plasma protein binding

Pharmacokinetic parameters were determined in the C57BL/6J mice by i.p. dosing. Plasma was generated by standard centrifugation techniques, resulting in \sim 10 μ L of plasma that was immediately frozen. For brain collection, mice were sacrificed by cervical dislocation and brains were isolated and flash frozen in liquid nitrogen. Drug levels were determined using a LC (Shimadzu)-tandem mass spectrometry (AB Sciex) operated in positive-ion mode using multiple reaction monitoring methods (Brust et al., 2016). Plasma protein binding for fentanyl and morphine was determined using Rapid Equilibrium Dialysis (RED) devices (ThermoFisher). For the SR compounds, plasma samples (0.5 mL at 0.5 μ M test compound) were prepared and 900 μ L was transferred to a 2 mL polycarbonate ultracentrifuge tube. The sample was centrifuged at 400,000 \times g for two hr using a Beckman Coulter Optima Max ultracentrifuge (130,000 RPM max) with a TLA 120.2 rotor held at 25°C. The centrifuged sample separates into three layers. The protein-rich bottom layer contains most of the albumin and is easily visualized. The top layer is not as easily discerned, but contains a high concentration of lipoproteins. The middle layer (1-2 mm below surface using the described conditions) has very low protein concentrations and can

be used to determine the amount of unbound drug. The percent unbound compound was determined by LC-MS/MS by comparison of the compound concentration in the middle layer of the centrifuged sample to the concentration of a parallel sample that did not undergo centrifugation (Kieltyka et al., 2016).

Antinociception

Antinociceptive responses to thermal stimuli were determined according to previously published protocols (Bohn et al., 1999; Raehal et al., 2011). Basal nociceptive responses were determined by measuring the amount of time until a mouse rapidly flicked its tail when placed into a 49°C water bath (tail flick test) or until it licked or flicked its fore- or hind-paws when placed on a to a 52°C hot plate (hot plate test; Hotplate Analgesia Meter, Columbus Instruments). Baseline response latencies averaged 2.95 ± 0.07 s (tail flick) and 6.17 ± 0.06 s (hot plate) for C57BL/6J male mice, 2.34 ± 0.18 s (tail flick) and 6.78 ± 0.14 s (hot plate) for C57BL/6J female mice and 2.29 ± 0.12 s (tail flick) and 6.54 ± 0.17 s (hot plate) for MOR-KO male mice. Antinociceptive responses were determined at the indicated time points over the course of 6 hr immediately following injection. To minimize tissue damage, maximum response latencies were limited to 30 and 20 s for tail flick and hot plate assays, respectively. Data are presented as “% maximum possible effect” which was calculated by (response latency – baseline) / (maximal response cutoff latency – baseline) * 100.

Respiration

A MouseOx Plus pulse oximeter (Starr Life Sciences Corporation) was used to monitor mouse vital signs following drug treatment. Two days prior to testing, mice were shaved around the neck and habituated to the oximeter collars and 50 mL conical tubes that had been modified to restrain mice during testing. Mice were again habituated to the collars and conical tubes one day prior to testing. On the testing day, mice were fit with collars and returned to the conical tubes and basal vital signs were monitored for 30 min; mice were then immediately injected with drug and vital signs were monitored for an additional hour. Raw data were averaged into 5 min bins. The average baseline responses (average over first 30 min) for C57BL/6J male mice were $95.11 \pm 0.12\%$ (% oxygen saturation) and 165.0 ± 0.2 bpm (breath rate). The average baseline responses for C57BL/6J female mice were $96.30 \pm 0.32\%$ (% oxygen saturation) and 150.1 ± 1.6 bpm (breath rate). The average baseline responses for MOR-KO male mice were $94.14 \pm 0.38\%$ (% oxygen saturation) and 156.6 ± 2.5 bpm (breath rate). Data are presented as “% maximum possible effect” which was calculated by (response – average baseline) / (maximal response cutoff – average baseline) * 100. The maximum responses cutoff for % oxygen saturation and breath rate were set at 70% O₂ and 75 breaths per min (brpm), respectively.

QUANTIFICATION AND STATISTICAL ANALYSIS

Software and analysis

GraphPad Prism software (v. 7.0) was used for data and statistical analyses which are specifically described in the figure legends. All data are presented as mean \pm SEM or 95% confidence intervals, as indicated. For the *in vitro* studies, the compounds were assayed in duplicate or triplicate, with at least 3 independent replicates. Concentration response curves are presented as % of DAMGO, as indicated in the figures, and were fit to a non-linear regression (three parameter) model to determine EC₅₀ and E_{MAX}, with the average of the values from each individual experiment reported. For brainstem GTP γ S binding assays, all studies were performed on brainstem taken from individual mice (one mouse brainstem per n, n \geq 3). For the *in vivo* experiments, the number of animals used in each of the assays is provided in Table S4.

Calculation of signaling bias

For the *in vitro* assays, DAMGO was used as the reference compound in every experiment for normalization. For the calculation of bias, each concentration response curve was fit to the operational model based on the model by Black and Leff (Black and Leff, 1983) and is provided in the Prism software:

$$= \frac{E_{MAX}}{1 + \left(\frac{1 + \frac{A}{10^{\log(K_A)}}}{A \times 10^{\log(\tau/K_A)}} \right)^n}$$

where E_{MAX} is the maximal response, A is the molar concentration of the drug, K_A is the equilibrium dissociation constant, the τ parameter is defined as the agonist efficacy and the $\log(\tau/K_A)$ is the transduction coefficient. For each assay, the E_{MAX} is constrained to be a shared value and the $\log(K_A)$ is constrained so that it must be between zero and 10⁻¹⁵ M to permit convergence of the model. Within each individual experiment, the $\log(\tau/K_A)$ values were calculated for the reference agonist, DAMGO, and the test agonists and then $\Delta\log(\tau/K_A)$ values were calculated with the constraint that they be an absolute less than 10 by the equation:

$$= \log\left(\frac{\tau}{K_A}\right)_{test} - \log\left(\frac{\tau}{K_A}\right)_{DAMGO}$$

The $\Delta\log(\tau/K_A)$ values were fit within each individual experiment and then the values were averaged for to generate a mean $\Delta\log(\tau/K_A)$ and error (SEM), which are provided in Table 2. (This method of determining $\Delta\log(\tau/K_A)$ has been demonstrated to be

normally distributed and, therefore, appropriately estimates the $\Delta\log(\tau/K_A)$ of the test ligand in each population (Stahl et al., 2015.) $\Delta\Delta\log(\tau/K_A)$ values were derived by subtracting the mean $\Delta\log(\tau/K_A)$ produced from multiple independent experiments for assay 2 (Barrestin assay) from the similarly calculated $\Delta\log(\tau/K_A)$ for assay 1 (GTP γ S binding or cAMP inhibition) and is presented as the mean with the 95% confidence intervals. Error is propagated by using Prism v. 7.0 and comparing the two $\Delta\log(\tau/K_A)$ values via an unpaired t test and acquiring the difference between the two values with 95% confidence intervals. Bias factors were calculated by taking the antilog of the $\Delta\Delta\log(\tau/K_A)$ (Griffin et al., 2007; Kenakin et al., 2012):

$$= 10^{\left(\Delta\log\left(\frac{\tau}{K_A}\right)_{\text{assay1}} - \Delta\log\left(\frac{\tau}{K_A}\right)_{\text{assay2}} \right)}$$

Determination of ED₅₀ and Therapeutic Window

For the *in vivo* studies, raw data were converted to the % maximal possible effect, as described in each behavioral method section above. An area under the curve (AUC) analysis was performed using GraphPad Prism, on each individual animal, with the baseline defined as time zero in the antinociception assays, and the mean response during the 30-minute habituation phase in the respiratory assays. Peaks that go below baseline were also considered. For the calculation of the AUC in the antinociception assays, only the data from the first hour after drug treatment was used. The AUC was then normalized to the percent maximal possible response within each assay (75 for the antinociception assays and 5750 for the respiratory assays). The ED₅₀ values and asymmetric 95% confidence intervals were then calculated by fitting the AUC %_{MAX} to a hyperbolic fit, with the maximum constrained to 100%. The therapeutic window was calculated by:

$$= \frac{ED_{50\text{respiration}}}{ED_{50\text{antinociception}}}$$

Supplemental Figures

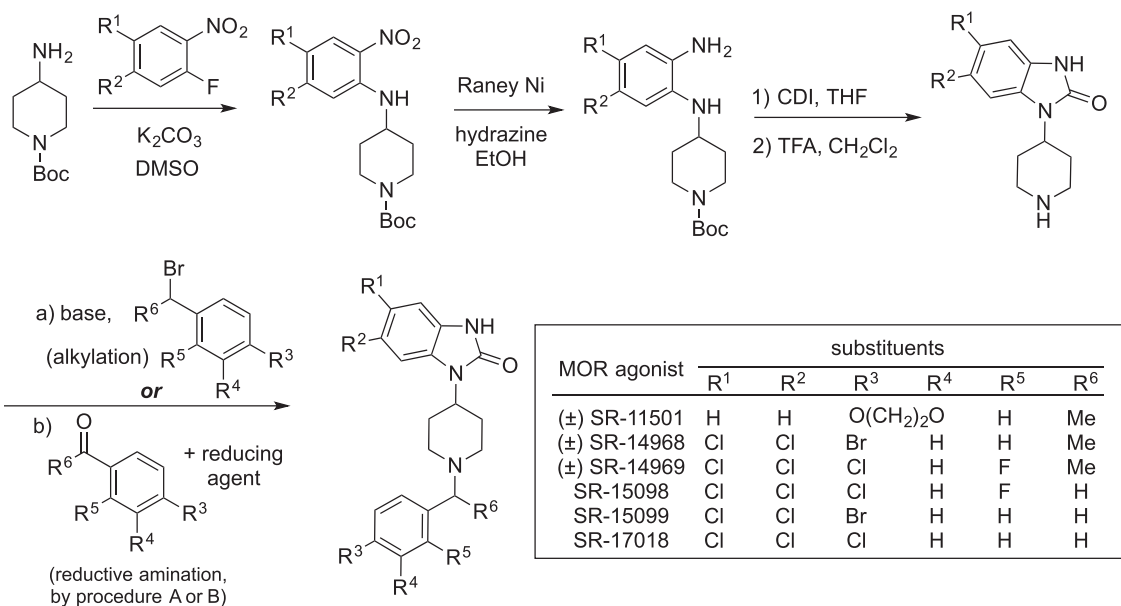


Figure S1. Synthesis of MOR-Selective Ligands, Related to Table 1

General synthesis scheme for novel piperidine MOR agonists. The versatile 5-step route includes a nucleophilic aromatic substitution reaction, nitro reduction, urea formation, Boc group deprotection, and finally a direct alkylation or a reductive amination reaction. Solvent or reagent abbreviations are as follows: dimethyl sulfoxide (DMSO), carbonyl diimidazole (CDI), tetrahydrofuran (THF) and trifluoroacetic acid (TFA).

See also Table 1 for structure activity relationships in this series; Table S1 for binding affinities for MOR over other opioid receptors and Figure S2 for functional profiling at DOR, KOR and NOP.

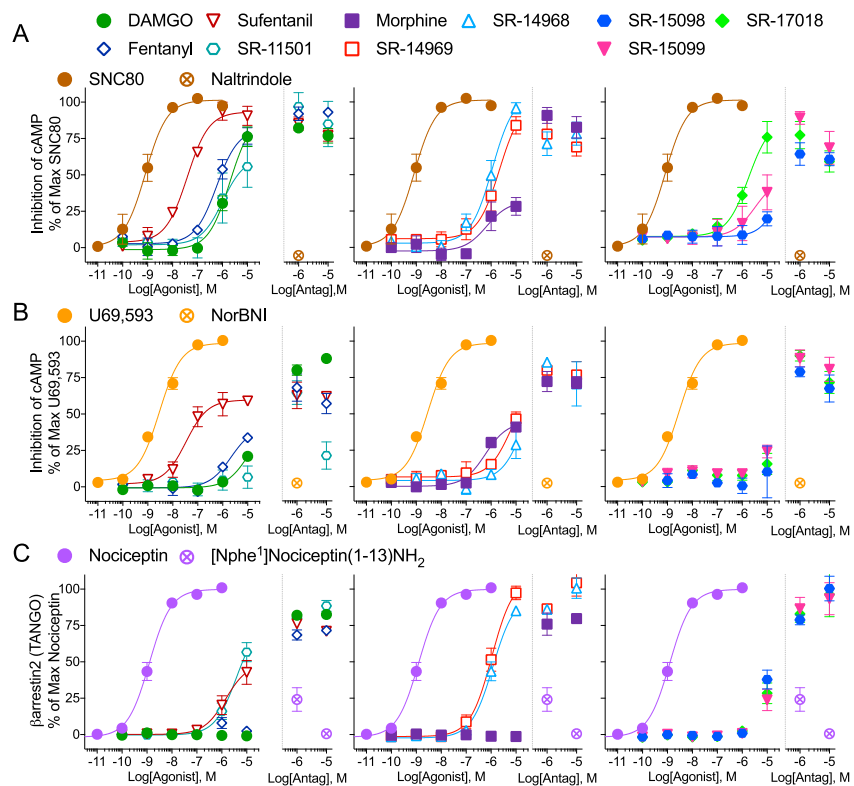


Figure S2. Functional Effects of MOR Agonists at the Human DOR, KOR, and NOP, Related to Table 1

(A and B) The SR compounds are not potent agonists or antagonists in DOR, KOR or NOP functional assays. Data are presented as mean \pm SEM of 3 independent assays, assayed in duplicate. Agonism and antagonism (“antag,” *right side of graph*) of forskolin-stimulated cAMP accumulation were determined in (A) CHO-hDOR or (B) CHO-hKOR cells. The antagonist assays were performed in the presence of 100 nM SNC80 and compared to naltrindole for the DOR and in the presence of 100 nM U69,593 and compared to *nor*-Binaltorphimine (NorBNI) for the KOR.

(C) Agonism and antagonism of β arrestin2 recruitment to NOP was determined by a FRET-based assay in U2OS-Tango-hOPRL1-*bla* cells. The antagonist assays were performed in the presence of 10 nM nociceptin, and compared to the NOP antagonist, [Nphe¹]Nociceptin(1-13)NH₂.

See also Tables 1 and S1 for potency and binding affinities at the human opioid receptors.

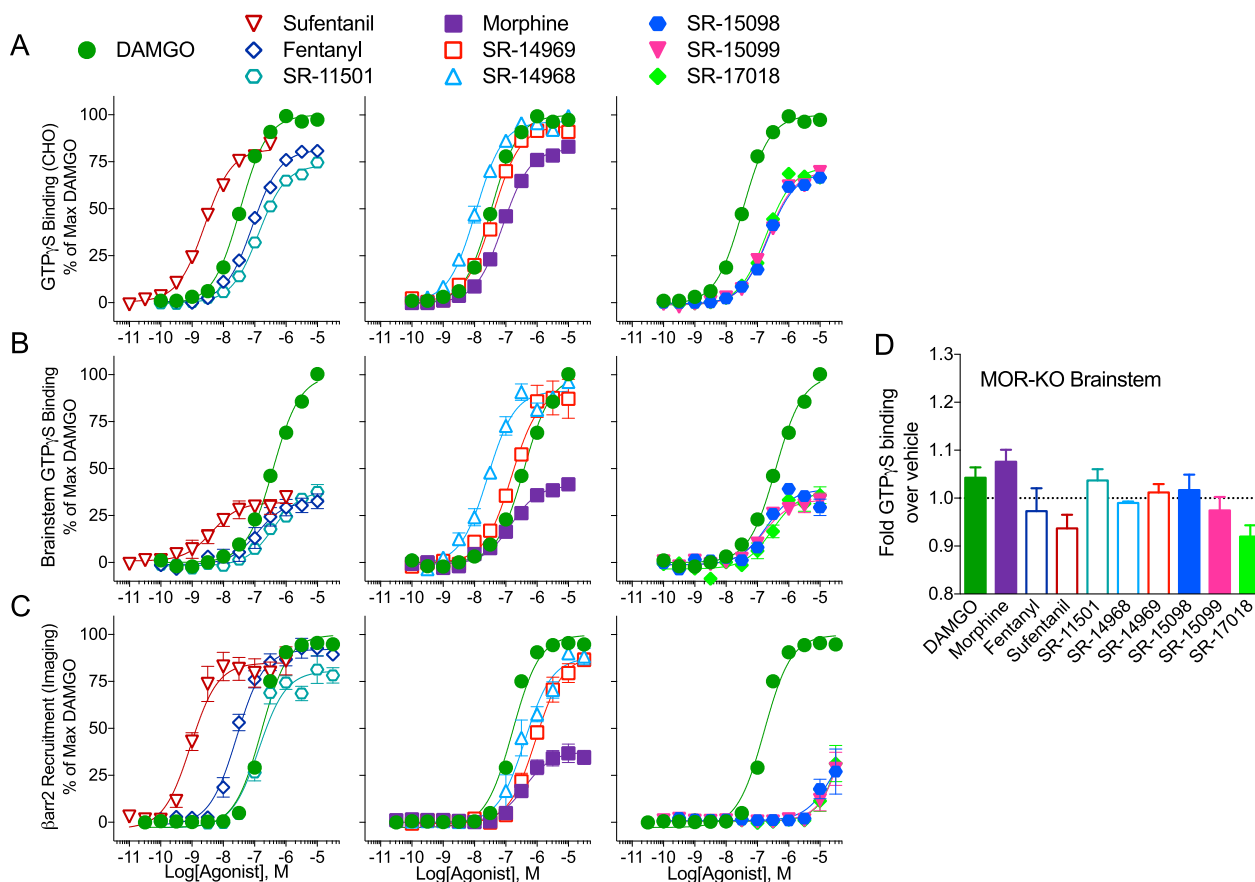


Figure S3. GTP γ S Binding at Mouse MOR Expressed in CHO Cells and Mouse Brainstem Compared to β Arrestin2 Recruitment to Mouse MOR, Related to Figure 1 and Table 2

(A and B) MOR agonist stimulated 35 S-GTP γ S binding in membranes from (A) CHO-mMOR cells or from (B) brainstem membranes from C57BL/6 mice.

(C) β arrestin2 recruitment to the mMOR as determined by an imaging based assay in U2OS- β arrestin2-GFP-mMOR cells. For A-C, the data were normalized to the % maximal response for DAMGO and are presented as mean \pm SEM of 3 or more assays (1 mouse/assay) performed in duplicate or triplicate.

(D) The SR compounds do not stimulate 35 S-GTP γ S binding over vehicle levels in brainstem membranes isolated from MOR-KO mice. The data are presented as fold over vehicle, with the mean \pm SEM of 3 or more assays (1 mouse/assay) assayed in quadruplicate.

See also Tables 2 and S2 for potency, efficacy, and bias factors, and Figure 1E for graphical representation.

Increasing Bias Factor

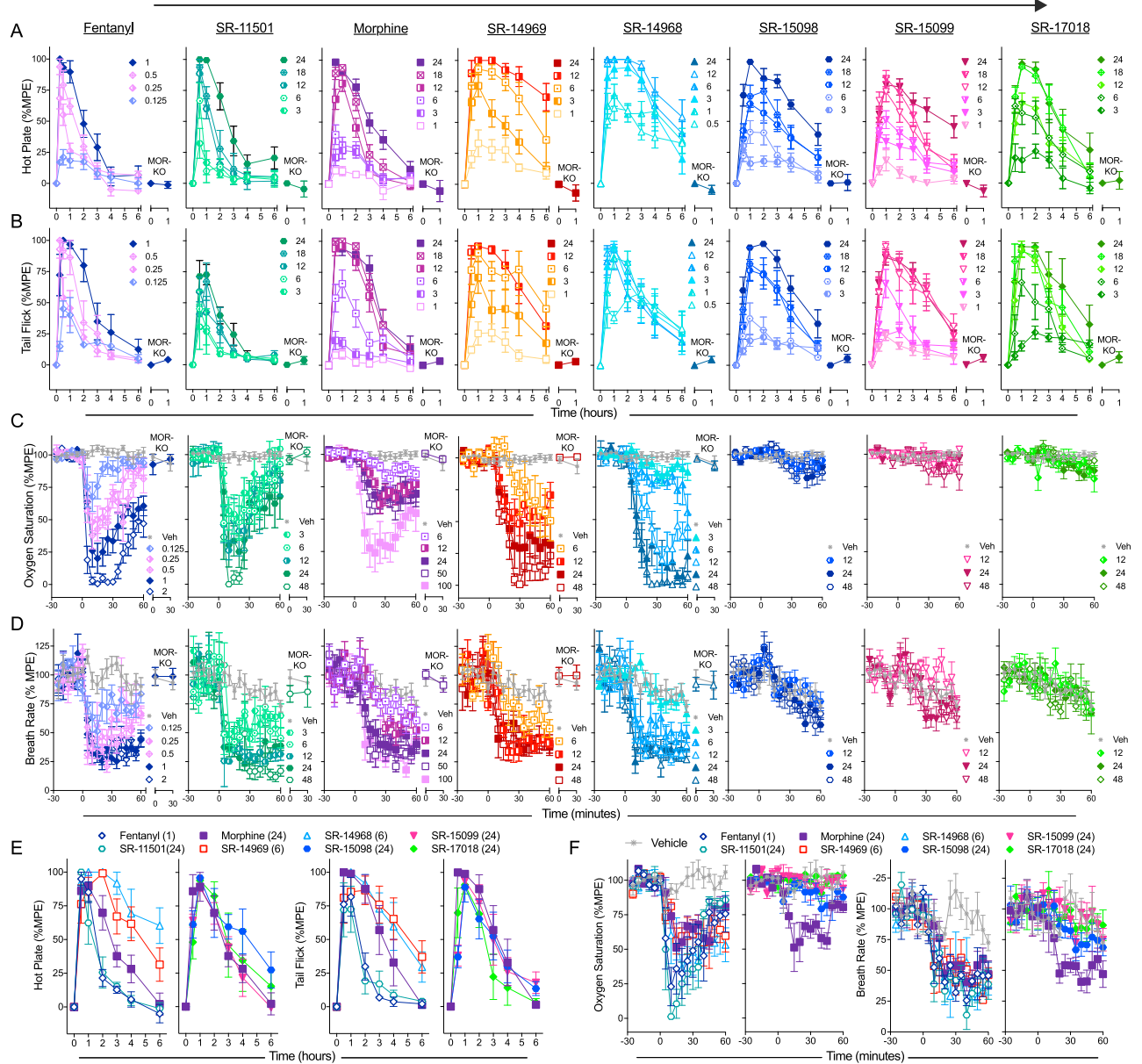


Figure S4. Dose Response for the Fentanyl, Morphine, and the SR Compounds in the Antinociception and Respiratory Assays and Efficacy in Female Mice, Related to Figure 3 and Table 3

(A–F) Antinociception dose response studies (mg/kg, *i.p.* doses in legends) in C57BL/6J male mice were performed for (A) hot plate (52°C) and (B) warm-water (49°C) tail flick assays over time. Respiratory dose response studies (mg/kg, *i.p.* doses in legends) in C57BL/6J male mice fit with a pulse oximeter to detect (C) % arterial oxygen saturation and (D) breath rate changes over time. Basal responses were determined for 30 min prior to injection (at time 0). Female C57BL/6J mice have similar (E) antinociceptive and (F) respiratory responses as their male counterparts at the doses tested (mg/kg *i.p.*, doses in legend), $n = 4$. MOR-KO mice displayed no response to the doses of compounds that produced near maximal effects in C57 mice (within MOR-KO, Student's *t* test: $p > 0.05$). The data are presented as mean \pm S. E. M. of the % maximal possible effect (%MPE), with baselines determined for each mouse and the maximums set at 20 s for hot plate, 30 s for tail flick, 70% for oxygen saturation and 75 breaths per min for breath rate measures.

See also Figure 3C and Table 3 for ED₅₀ values and therapeutic windows and Table S4 for the number of male mice used in each assay.