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Asymmetric Synthesis and in Vitro and in Vivo Activity of Tetrahydroquinolines Featuring a Diverse Set of Polar Substitutions at the 6 Position as Mixed-Efficacy μ Opioid Receptor/ δ Opioid **Receptor Ligands**

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Supporting Information



ABSTRACT: We previously reported a small series of mixed-efficacy μ opioid receptor (MOR) agonist/ δ opioid receptor (DOR) antagonist peptidomimetics featuring a tetrahydroquinoline scaffold and showed the promise of this series as effective analgesics after intraperitoneal administration in mice. We report here an expanded structure-activity relationship study of the pendant region of these compounds and focus in particular on the incorporation of heteroatoms into this side chain. These analogues provide new insight into the binding requirements for this scaffold at MOR, DOR, and the κ opioid receptor (KOR), and several of them (10j, 10k, 10m, and 10n) significantly improve upon the overall MOR agonist/DOR antagonist profile of our previous compounds. In vivo data for 10j, 10k, 10m, and 10n are also reported and show the antinociceptive potency and duration of action of compounds 10j and 10m to be comparable to those of morphine.

KEYWORDS: Opioid, mixed efficacy, intraperitoneal, dependence, tolerance, tetrahydroquinoline

Opioid analgesics have long been the standard for the treatment of severe pain. Unfortunately, the use of opioids can lead to the development of a number of undesirable side effects, such as respiratory depression, constipation, and perhaps most problematically, dependence and tolerance.¹ There is therefore a great unmet need to develop agents that act as potent analgesics but without the development of these side effects.^{2,3}

There is a growing body of evidence to suggest that the δ opioid receptor (DOR) plays a significant role in the modulation of the side effects related to the chronic use of opioid analgesics. Although the analgesic effects of traditional opioid agents such as morphine are associated with stimulation of the μ opioid receptor (MOR), the coadministration of DOR antagonists has been shown to maintain the desired

antinociceptive activity but with a reduced side-effect profile compared with a MOR agonist alone.⁴⁻⁷

Because of these findings, our group and others have sought to develop bifunctional ligands that bind to both MOR and DOR but stimulate only MOR. Several classes of compounds have been employed in this pursuit, including peptides,⁸ pseudopeptides,^{9,10} and small molecules.^{11,12} The MOR/DOR bivalent ligands developed by Portoghese and colleagues have been demonstrated to be effective analgesics with a diminished tolerance profile (Figure 1d),¹³ and recently the MOR/DOR heteromer-biased agonist CYM51010 was also shown to display

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Figure 1. Bifunctional MOR/DOR ligands. Compound a is from ref 17.

Scheme 1. Synthesis of Intermediates 4a-c,f-o^a



"Reagents and conditions: (a) 3-bromopropionyl chloride, K_2CO_3 , DCM, r.t.; (b) NaOtBu, DMF, r.t.; (c) TfOH, DCE, r.t.; (d) (Boc)₂O, DMAP, DIPEA, DCM, reflux; (e) NBS, benzoyl peroxide, CCl₄, reflux; (f) boronic acid or pinacol ester, Pd(dppf)Cl₂, K₂CO₃, acetone, water, 100 °C with microwave irradiation; (g) secondary amine, K₂CO₃, DMF, r.t.

reduced antinociceptive tolerance compared with morphine (Figure 1c).¹⁴ MOR agonist/DOR antagonist compounds are also being developed clinically for the treatment of irritable bowel syndrome.¹⁵ We have recently reported two MOR agonist/DOR antagonist compounds that are effective and bioavailable analgesics: a glycosylated cyclic pentapeptide¹⁶ and a small molecule with a tetrahydroquinoline (THQ) core (Figure 1a).^{17,18} Additionally, UMB425, a small-molecule MOR agonist/DOR antagonist derived from thebaine, was reported to display analgesia after subcutaneous administration with reduced tolerance compared with morphine (Figure 1b).¹⁹

The compounds reported in this paper build on the limited initial structure-activity relationship (SAR) study done on our bioavailable THQ lead compound (S)-2-amino-N-((R)-6benzyl-1,2,3,4-tetrahydroquinolin-4-yl)-3-(4-hydroxy-2,6dimethylphenyl)propanamide (a in Figure 1a). Initially, bulky hydrophobic modifications to the side chain at position R_1 (see Table 1) were examined,¹⁷ as these substitutions were directly comparable to a previously reported series of MOR/DOR cyclic peptides 20,21 and were hypothesized to interact favorably with residues in the MOR active pocket to act as full MOR agonists while simultaneously behaving as DOR antagonists. These initial compounds were highly lipophilic, which is desirable for blood-brain barrier penetration but less than optimal for both aqueous solubility and metabolic stability.²² We therefore explored a variety of polar side chains on this scaffold for the purpose of improving these parameters and further probing the chemical space in this region of each of the

receptors. Simple heteroatom replacements not only had profound effects on receptor selectivity but also led to compounds that improved upon the in vivo profile of our initial THQ analogue. Compounds **10**j and **10m** both produced a maximum antinociceptive response in the mouse warm water tail withdrawal (WWTW) assay, and both improved significantly upon the duration of action of our lead peptidomimetic. These findings highlight the promise of this scaffold in vivo and show that our SAR strategy focusing on polar side chains was effective for improving bioavailability. In vitro data for both binding affinity and efficacy are presented for all three opioid receptor types (MOR, DOR, and the κ opioid receptor (KOR)).

RESULTS AND DISCUSSION

Chemistry. The synthesis of compounds 10a-c,f-o began with the acylation of *p*-toluidine with 3-bromopropionyl chloride (Scheme 1). The resulting alkyl bromide then underwent an intramolecular cyclization followed by triflic acid-mediated β -lactam rearrangement to give ketone 1,^{23,24} which was subsequently Boc-protected on the THQ nitrogen to give **2**. Ketone **2** was then brominated on the aryl methyl group as described previously²⁵ to give **3**, onto which could be added the pendant of choice, through either Suzuki coupling (4a,f-i) or substitution with the appropriate secondary amine (4b,c,j-o). It is important to note that **3** could be synthesized on a multigram scale and that all substitutions on this intermediate were high-yielding. For the synthesis of compounds **10d** and

Scheme 2. Synthesis of Intermediates 4d and 4e^a



"Reagents and conditions: (a) 3-bromopropionyl chloride, K₂CO₃, DCM, r.t.; (b) NaOtBu, DMF, r.t.; (c) TfOH, DCE, r.t.; (d) (Boc)₂O, DMAP, DIPEA, DCM, reflux.

Scheme 3. Final Steps in the Synthesis of $10a-o^{a}$



"Reagents and conditions: (h) (R)-(+)-2-methyl-2-propanesulfinamide, Ti(OEt)₄, THF, reflux; (i) NaBH₄, THF; (j) conc. HCl, 1,4-dioxane, r.t.; (k) Boc-L-Dmt, PyBOP, DIPEA, HOBt-Cl, DMF, r.t.; (l) TFA, DCM, r.t.; (m) (Ac)₂O, pyridine, r.t..

10e, commercially available para-substituted anilines were carried forward in a similar manner as in the synthesis of compound **2** to give intermediates **4d** and **4e** (Scheme 2).

Ketones **4a**–**o** were converted to the corresponding imines with (*R*)-(+)-2-methyl-2-propanesulfinamide and Ti(OEt)₄ and could then be reduced asymmetrically with NaBH₄ in situ to give *tert*-butanesulfinyl-protected amines **9a**–**o** as single diastereomers (Scheme 3), as previously described for analogous scaffolds.^{26,27} Deprotection with concentrated HCl gave the corresponding primary, enantiomerically pure (*R*)amines as HCl salts. The stereochemistry of the HCl salts was verified by X-ray crystallography of 6-benzyl-1-(*tert*-butoxycarbonyl)-1,2,3,4-tetrahydroquinolin-4-aminium chloride, which was prepared by an identical synthetic route (Figure 2).

Boc-protected L-2,6-dimethyltyrosine (Boc-L-Dmt) could then be coupled to the chiral HCl salt, and subsequent deprotection with trifluoroacetic acid (TFA) afforded 10a-1(Scheme 3). The analogues were then purified by reversedphase (RP) HPLC to provide enough material for in vitro and in vivo pharmacological evaluation (~5-10 mg). In the case of the N-acetylated analogues 10m-o, Boc deprotection of the THQ nitrogen was performed prior to coupling to Boc-L-Dmt. After the amide coupling, the acetyl group was introduced by stirring the crude material in excess pyridine/acetic anhydride (1:1) overnight, followed by a second Boc deprotection and RP-HPLC purification. The TFA content of the final analogues



Figure 2. Crystal structure of (*R*)-6-benzyl-1-(*tert*-butoxycarbonyl)-1,2,3,4-tetrahydroquinolin-4-aminium chloride.

was estimated by ¹⁹F NMR analysis as described previously using a fluorinated analogue of **10m** (see the Supporting Information) and was found to be approximately 2.5 TFA molecules per molecule of final compound.²⁸

SAR Studies. In previous reports, we described a mixedefficacy MOR agonist/DOR antagonist opioid peptidomimetic featuring a THQ scaffold and a benzyl pendant at ring position $6^{17,18}$ (a in Figure 1a). This compound was shown to be an

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effective analgesic in the WWTW assay after intraperitoneal administration, with a duration of action slightly shorter than that of morphine.¹⁷ The initial SAR study done on this lead compound was focused on several additional hydrophobic, aromatic substitutions at the 6 position, including 1methylnaphthyl, 2-methylnaphthyl, 2-methylindanyl, and ethylphenyl. The side chains of these four compounds were chosen to mirror modifications made in a peptide series upon which the peptidomimetic scaffold was based,²⁰ and as expected, modifications featuring a more extended pendant (2-methylnaphthyl, 2-methylindanyl, ethylphenyl) were compatible with the larger DOR inactive binding pocket but not the smaller DOR active pocket, explaining the observed low efficacy at DOR. While these compounds displayed the desired MOR agonist/DOR antagonist efficacy profile, their binding profile was not optimal. The MOR affinity for all four compounds was at least an order of magnitude higher than the DOR affinity, and the 2-methylnaphthyl compound showed an over 2 orders of magnitude preference for MOR. Ligands with more balanced binding affinities at MOR and DOR would provide a better starting point for further development of this type of mixedefficacy opioid ligand.^{9,30} Additionally, although we showed that an extended hydrophobic pendant translates to low DOR efficacy, changes in the electronic characteristics and polarity of the pendant were left unexplored.

To begin our expanded SAR, we first replaced the phenyl pendant of our lead compound (Figure 1a) with a 3-pyridine (10a; Table 1). We observed not only a slight loss in binding affinity (K_i) at both MOR and DOR (Table 1) but also a significant loss in MOR efficacy (EC50) and potency (as maximal % stimulation) (Table 2) (see Methods for details of the in vitro assays). Although 10a adopts a similar conformation as our lead compound in the MOR active site, this loss in MOR binding and efficacy can be attributed to loss of hydrophobic contacts in this region of the receptor binding pocket (see Figure 3). Although this analogue did not improve upon the MOR agonist/DOR antagonist profile of our previous compounds, we were intrigued by the drastic consequences that a simple change in pendant electronics had on both the binding and efficacy and wished to explore this further. Compared with 10a and our lead compound, replacement with piperidine in analogue 10b widened the binding affinity preference for MOR over DOR even further, although this compound behaved as a moderately potent full agonist at MOR, improving upon the MOR efficacy profile of 10a. Expansion of the piperidine ring in 10b to azepane (10c) resulted in improved binding at DOR and KOR. In contrast, the morpholine analogue 10d displayed diminished binding affinities at DOR and KOR and also decreased potency at MOR compared with 10b. We next turned our attention to smaller aromatic systems, including 1,2,4-triazole (10e) and 3-furan (10f). While the overall binding profile of 10f was comparable to those for the previous substitutions, 10e displayed a marked loss in binding affinity for MOR and KOR and displayed no efficacy at MOR.

In the initial series,¹⁷ the 2-methylnaphthyl modification resulted in the highest MOR efficacy, but the MOR/DOR binding balance favored MOR by over 2 orders of magnitude. To see whether changes in the electronics of the naphthyl system could improve DOR binding while maintaining low DOR efficacy, we first synthesized quinoline analogue **10g**. Interestingly, the binding affinities of 6-quinoline analogue **10g** at all three receptors were considerably lower than those of the previous bicyclic analogues. This finding suggests that both an

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$\underset{H_{1}}{\overset{H_{2}}{\longrightarrow}} \overset{\overset{H_{2}}{\overset{H_{2}}{\longrightarrow}}}{\overset{H_{2}}{\longrightarrow}} _{OH} \qquad \qquad Binding, K_{i} \left(nM\right)$							
Compound	R ₁	R ₂	MOR	DOR	KOR		
(Fig 1a)		H	0.22±0.02 ^b	9.4±0.8 ^b	68 ± 2^b	3.7	
10a	, ∠	Н	0.66±0.08	17±4	66±8	2.2	
10b	's'	Н	0.3±0.1	120±29	29±9	2.7	
10c	N N	Н	0.15±0.02	61±9	3.6±0.7	3.3	
10d	م مند 0	Н	0.6±0.1	140±67	170±32	1.4	
10e	^N . _N .≵ N ∛	Н	3.1±0.6	50±14	450±14	0.75	
10f	\sim	Н	0.8±0.2	18±6	20±3	2.9	
10g		Н	2.1±0.6	23±5	120±21	3.6	
10h		Н	0.10±0.02	1.5±0.2	16±4	3.6	
10i		Н	0.12±0.01	4.3±0.8	21±2	3.7	
10j		Н	0.15±0.08	15±5	2±1	3.1	
10k		Н	0.03±0.01	3.1±0.2	2.2±0.4	3.6	
101		Н	0.15±0.01	4.8±0.9	37±8	4.1	
10m		Acetyl	0.19±0.1	0.89±0.2	0.78±0.1	3.2	
10n	N N	Acetyl	0.32±0.09	2.6±0.2	7±3	2.7	
100		Acetyl	0.8±0.2	2±1	15±6	3.7	

Table 1. Opioid Receptor Binding Affinities and clogP Values for Analogues $10a-o^a$

^{*a*}Binding affinities (K_i) were obtained by competitive displacement of [³H]diprenorphine in membrane preparations expressing either MOR, DOR, or KOR. All values are mean \pm standard error of the mean (SEM) of three separate assays performed in duplicate. ^{*b*}Data from ref 17. ^{*c*}Calculated using ChemBioDraw Ultra, version 14.0.

extended pendant and pendant electronic characteristics are important for maintaining binding at DOR for this series. Using our previously published models of interactions of opioid ligands with the active states of the three receptors,^{20,21} we docked **10g** into the MOR active binding pocket. The quinoline nitrogen of **10g** was found to extend much deeper into the hydrophobic pocket of the MOR active pocket, disrupting important contacts with hydrophobic residues W133, V143, and I144, as shown in Figure 3.

These initial data suggested that superior MOR efficacy (and low DOR efficacy) may result from a fused-ring pendant in which the six-membered non-heteroatom-containing aromatic moiety is located in the position most distal from the THQ

Table 2. Opioid Receptor Efficacies for Analogues 10a-o^a

	EC_{50} (nM)			% stimulation			
compound	MOR	DOR	KOR	MOR	DOR	KOR	
a (Fig. 1)	1.6 ± 0.3^{b}	110 ± 6^{b}	540 ± 72^{b}	81 ± 2^{b}	16 ± 2^{b}	22 ± 2^{b}	
10a	93 ± 20	dns	dns	37 ± 7	dns	dns	
10b	9 ± 1	dns	dns	73 ± 8	dns	dns	
10c	25 ± 11	dns	dns	52 ± 2	dns	dns	
10d	60 ± 2	dns	dns	82 ± 2	dns	dns	
10e	dns	dns	dns	dns	dns	dns	
10f	72 ± 24	dns	>1000	18 ± 2	dns	>40	
10g	23 ± 13	dns	dns	34 ± 6	dns	dns	
10h	2.2 ± 0.9	dns	dns	84 ± 6	dns	dns	
10i	14 ± 3	dns	dns	36 ± 3	dns	dns	
10j	3 ± 1	dns	15 ± 9	96 ± 4	dns	14 ± 2	
10k	0.4 ± 0.1	dns	90 ± 65	105 ± 6	dns	25 ± 4	
101	2.0 ± 0.5	dns	600 ± 400	56 ± 2	dns	14 ± 1	
10m	6 ± 2	dns	160 ± 36	91 ± 8	dns	46 ± 5	
10n	0.9 ± 0.4	dns	400 ± 130	118 ± 5	dns	32 ± 1	
100	40 ± 20	dns	>2000	72 ± 3	dns	>20	

^{*a*}Efficacy data were obtained using agonist-induced stimulation of [35 S]GTP γ S binding in membrane preparations expressing either MOR, DOR, or KOR. Potencies are represented as EC₅₀ (nM) and efficacies as percent maximal stimulation relative to the standard agonist DAMGO (MOR), DPDPE (DOR), or U69,593 (KOR) at 10 μ M. All values are expressed as the mean \pm SEM of three separate assays performed in duplicate. dns: does not stimulate. ^{*b*}Data from ref 17.



Figure 3. Docking of 10g in the MOR active site. Key hydrophobic contacts (I144, V143, W133) are highlighted in red.

core. To test this hypothesis, analogues 10h-l were synthesized. 10h showed high efficacy at MOR and approximately 10-fold improved DOR binding compared with the lead compound and 10g. 10j and 10k both behaved as potent full MOR agonists that improved upon the efficacy of our original lead with no efficacy at DOR. On the other hand, the MOR efficacy was reduced in the case of 3,4-(methylenedioxy)phenyl analogue 10i. This is again consistent with the observation that distal electron rich substitutions adversely affect the MOR efficacy. Reduction of the aromatic ring of 10k to give decahydroisoquinoline analogue 10l maintained a comparable but slightly inferior in vitro profile compared with 10k. While 10j and 10k showed potent stimulation at MOR (while exhibiting no efficacy at DOR), we still wished to improve the binding affinities of each at DOR. We reasoned that the THQ aniline was synthetically accessible and amenable to substitutions and would be the next logical site for diversification. Preliminary studies on related analogues (to be published in due course) suggested that N-acetylation at the

THQ core improves DOR affinity without increasing DOR efficacy, so we likewise explored the effect of an acetyl substituent here, giving the final analogues **10m–o**. We were pleased to see not only that this modification improved DOR binding relative to the unacetylated counterpart compounds (**10j–1**) but also that **10m** showed similarly high affinity for MOR and DOR and, interestingly, for KOR as well. As expected from its high binding affinity and lack of stimulation of [35 S]GTP γ S binding, **10m** acted as an antagonist of DPDPE. **10m** afforded a 7.8-fold rightward shift in the agonist concentration–response curve for DPDPE, giving an antagonist affinity constant (K_e) of 4.6 nM for **10m**.

An overlay of **10k** docked into the active sites of all three receptors is shown in Figure 4. The compound fits nicely into the MOR active site but clashes with M199 and L125 in the DOR active site. It is interesting to note that **10k** and **10m**, both featuring the 1,2,3,4-tetrahydroisoquinoline (THIQ) pendant, behave as partial KOR agonists. As shown in Figure



Figure 4. Overlay of 10k in the MOR, DOR, and KOR active sites. Gray, yellow, and purple residues correspond to MOR, DOR, and KOR.

4, 10k fits in the KOR active site but clashes slightly with I294 (and thus displays lower efficacy compared with MOR). Additionally, the THIQ nitrogen of 10k is positioned to make a polar contact with Y312, a residue unique to the KOR binding pocket at this position, which may account for the high affinity of 10k and 10m for KOR. The MOR agonist/KOR agonist mixed-efficacy profile has shown promise as a treatment for drug dependence, specifically cocaine addiction, 31-33 and an additional SAR study on MOR/KOR agonist peptides has recently been reported.³⁴ Further substitutions on the THIO pendant will have to be explored to fully optimize this profile, particularly for the purpose of improved potency at KOR.

In Vivo Studies. On the basis of their favorable in vitro profiles, compounds 10j, 10k, 10m, and 10n were chosen for in vivo studies. The effects of 10j, 10k, 10m, and 10n were compared with those of the lead compound by two-way analysis of variance with Tukey's multiple comparisons post hoc test. A significant interaction (F(12,76) = 8.7, p < 0.0001)and significant main effects of dose (F(3,76) = 82.7, p <(0.0001) and compound (F(4,76) = 24.6, p < 0.0001) were found. In the mouse WWTW assay (Figure 5), our benzyl



Figure 5. Cumulative antinociceptive dose-response curves for compounds 10j, 10k, 10m, and 10n in the mouse WWTW assay after intraperitoneal administration (n = 3-6). Data are plotted as mean ± SEM.

pendant lead compound and compounds 10j and 10m were fully efficacious and produced dose-dependent increases in latency to tail flick, with 3.2 mg/kg (at least p < 0.05) and 10 mg/kg (p < 0.001) significantly increasing the latency time compared with the baseline. 10m was not statistically different from the lead compound, but 10j produced slightly higher tail flick latencies at 3.2 mg/kg (p < 0.001) and 10 mg/kg (p <0.05) compared with the lead compound. It is interesting to note that 10k (which lacks only the N-acetyl group of 10m) and 10n (which is the N-acetylated counterpart of 10j) did not significantly increase the tail flick latency above baseline levels up to a dose of 10 mg/kg. To determine the duration of action of compounds 10j and 10m, tail withdrawal latencies were measured at intervals following the administration of the cumulative 10 mg/kg dose (Figure 6). Compounds 10j and 10m showed a full antinociceptive response for 200 min before returning to baseline. Compared with the lead compound (Figure 1a), these compounds both displayed a much longer duration of action after intraperitoneal injection.

In an effort to explain the unpredictable in vivo results for these structurally similar analogues, compounds 10j, 10k, and 10m were screened to determine their plasma stabilities, and all



Figure 6. Time courses of antinociceptive response for compounds 10j and 10m in the mouse WWTW assay after intraperitoneal administration of a cumulative dose of 10 mg/kg.

time (min)

were found to be completely stable after 30 min at 37 °C (99.6, 100.3, and 95.3 mean % remaining, respectively, after 30 min compared with the positive control eucatropine (43.2%)). There also appears to be no correlation between the predicted clogP values for these analogues (Table 1) and the observed in vivo data. While the origin of the disparate in vivo results is unclear, differences in first-pass metabolism after intraperitoneal administration³⁵ may be a contributing factor. To this end, additional synthetic and pharmacokinetic studies will need to be performed to shed light on how these subtle structural differences can have such a profound effect on the bioavailability of this scaffold.

CONCLUSIONS

latency to tail flick (sec)

We have described a series of opioid peptidomimetics with chemically diverse substitutions at the 6 position of the THQ scaffold and have shown that changes in both the steric and electronic characteristics of the pendant can have profound impacts on receptor selectivity for both binding and efficacy. Intermediate 3, which is relatively simple to synthesize and can be produced on a multigram scale, represents a valuable building block for the expedient synthesis of a wide range of opioid small molecules and makes possible the incorporation of diverse and readily available side chains (through either Suzuki coupling or S_N2 substitution) that have not yet been explored in traditional opioid ligands. Compounds 10j and 10m also display promise in vivo, with efficacy and duration of action comparable to those of morphine, and improve upon the duration of action of our original lead.

METHODS

General Synthetic Methods. All of the reagents and solvents were obtained from commercial sources and used without additional purification. Reactions were carried out in anhydrous solvents under an inert atmosphere unless otherwise specified. Suzuki couplings were performed in a Discover S-class (CEM) microwave reactor in a closed vessel with a maximum power input of 300 W. Flash column chromatography was carried out using P60 silica gel (230-400 mesh). Purification of final compounds was performed using a Waters semipreparative HPLC instrument with a Vydac protein and peptide C18 RP column, using a linear gradient of 10% solvent B (0.1% TFA in acetonitrile) in solvent A (0.1% TFA in water) to 60% solvent B in solvent A at a rate of 1% per minute. UV absorbance was monitored at 230 nm. The purities of synthesized compounds were determined on a Waters Alliance 2690 analytical HPLC instrument with a Vydac protein and peptide C18 RP column, using a linear gradient of 0%

solvent B in solvent A to 45% solvent B in solvent A in 45 min, measuring the UV absorbance at 230 nm. The purities of the final compounds used for testing were \geq 95% as determined by HPLC. ¹H NMR, ¹³C NMR, and ¹⁹F NMR data were obtained on either a 400 or 500 MHz Varian instrument. In CDCl₃, the chemical shifts were referenced to tetramethylsilane (TMS). If the TMS peak was not visible in the ¹³C NMR spectrum, the chemical shifts were referenced to the solvent peak (δ 77.16). Samples in CD₃OD were unreferenced. Mass spectral analysis was performed using an Agilent 6130 LC–MS instrument in positive mode.

In Vitro Assays. Binding affinity (K_i) was measured by the competitive displacement of [³H]diprenorphine (a nonselective opioid antagonist) in C6 cells stably expressing MOR or DOR or in Chinese hamster ovary (CHO) cells stably expressing KOR. In vitro potencies (EC_{50}) and efficacies (as maximal % stimulation) were obtained by agonist-stimulated [³⁵S]GTP γ S binding in the same cell types using previously described protocols.^{21,29,36} To determine the DOR antagonist activity of **10m**, a concentration–response curve for DPDPE was obtained using the [³⁵S]GTP γ S binding assay in C6 cells expressing DOR in the presence or absence of 30 nM compound **10m**, as previously described.²¹ The ratio of EC₅₀ values of DPDPE in the presence and absence of **10m** was determined to provide the dose ratio. The antagonist affinity constant K_e for **10m** was calculated using the equation $K_e = [10m]/(DR - 1)$, where DR is the dose ratio of agonist in the presence and absence of **10m**. All of the concentration–response curves were analyzed using GraphPad Prism (La Jolla, CA).

Animals. Adult male C57BL/6 mice, purchased from Harlan Laboratories (Indianapolis, IN, USA) and weighing between 20 and 30 g at 8–16 weeks old, were used for the described experiments. Mice were group-housed and had free access to food and water at all times. Experiments were conducted in the housing room, which was maintained on a 12 h light/dark cycle (with lights on at 0700). Each mouse was used only once, and experiments were conducted between 9 am and 5 pm. Studies were performed in accordance with the University of Michigan Committee on the Use and Care of Animals and the Guide for the Care and Use of Laboratory Animals.³⁷

Antinociception. Each compound was dissolved in sterile saline and administered by intraperitoneal injection in a volume of 10 mL/kg of body weight. Antinociceptive effects were evaluated in the WWTW assay. Tail withdrawal latencies were determined by briefly placing a mouse into a plastic, cylindrical restrainer and putting 2–3 cm of the tail tip into a water bath maintained at 50 °C. The latency to tail withdrawal or rapidly flicking the tail back and forth was recorded with a maximum cutoff time of 20 s. If the mouse did not remove its tail by the cutoff time, the experimenter removed its tail from the water to prevent tissue damage.

Acute antinociceptive effects were determined using a cumulative dosing procedure. Each animal received an intraperitoneal injection of saline, and then 30 min later the baseline withdrawal latencies (3-6 s) were recorded. Following baseline determinations, increasing cumulative doses of the test compound were given intraperitoneally at 30 min intervals. Thirty minutes after each injection, the tail withdrawal latency was measured as described above.

Plasma Stability. Plasma stability was assessed by Quintara Discovery (San Francisco, CA). Mouse plasma (K2 EDTA) was obtained from BioreclamationIVT. The assay was carried out in 96-well microtiter plates. Compounds were diluted to 200 μ M in DMSO and then spiked into the plasma. After mixing, samples were immediately aliquoted into three 96-well plates. The *t* = 0 plate was quenched immediately. The other two plates were incubated at 37 °C. Reaction mixtures (20 μ L) contained a test compound final concentration of 1 μ M. The extent of metabolism was calculated as the disappearance of the test compound compared with the 0 min control reaction incubations. Eucatropine was included as a positive control to verify the assay performance.

At each of the time points, 150 μ L of quench solution (100% acetonitrile with 0.1% formic acid) with internal standard was transferred to each well. Plates were sealed, vortexed, and centrifuged at 4 °C for 15 min at 4000 rpm. The supernatant was transferred to fresh plates for LC–MS/MS analysis.

All of the samples were analyzed by LC–MS/MS using an AB Sciex API 4000 instrument coupled to a Shimadzu LC-20AD LC pump system. Analytical samples were separated using a Waters Atlantis T3 dC18 RP-HPLC column (10 mm \times 2.1 mm) at a flow rate of 0.5 mL/ min. The mobile phase consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in 100% acetonitrile (solvent B).

ASSOCIATED CONTENT

Supporting Information

Experimental procedures, ¹H and ¹³C NMR data, MS (EI) and HPLC data, X-ray crystallography data, and copies of NMR spectra. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/ acschemneuro.Sb00100.

AUTHOR INFORMATION

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Author Contributions

Synthesis was carried out by A.M.B. The research project was designed by A.M.B., H.I.M., and J.R.T. In vitro assays were carried out by N.W.G. In vivo studies were designed by J.P.A. and E.M.J. and carried out by J.P.A.

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Notes

The authors declare no competing financial interest.

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