



Published in final edited form as:

J Med Chem. 2012 January 26; 55(2): 670–677. doi:10.1021/jm200902v.

Opioid activity of spinally selective analogues of *N*-Naphthoyl- β -naltrexamine (NNTA) in HEK-293 cells and mice

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Abstract

Using the selective mu-kappa agonist, NNTA **1**, as the prototype ligand, a series of closely related naphthalene analogues were synthesized to study the chemical space around the naphthalene moiety in an effort to evaluate how receptor selectivity is affected by chemical modification. Nine analogues (**2–10**) of compound **1** were synthesized and tested on HEK-293 cells expressing homomeric and heteromeric opioid receptors, and in the mouse tail-flick assay. It was found that a small change in structure produces profound changes in selectivity in this series. This is exemplified by the discovery that introduction of a 6-fluoro group transforms **1** from a selective mu-kappa heteromeric receptor agonist to a delta-preferring agonist **7**. The *in vivo* studies reveal that many of the ligands are more potent spinally than supraspinally and devoid of tolerance.

Introduction

Morphine and other opiates derived from opium have been employed as analgesics for well over a century. A feature common to these analgesics is that they produce side effects that include tolerance, physical dependence, constipation and respiratory depression. These ligands produce their effects through interaction with opioid receptors that are class A members of the G protein-coupled receptor (GPCR) superfamily^{1, 2}.

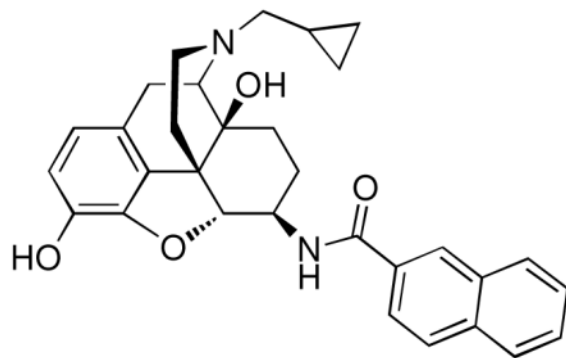
The three major classes of opioid receptors known to mediate antinociception upon activation are designated mu (MOP), delta (DOP), and kappa (KOP)². Since the establishment of these receptor types through binding, pharmacology, and cloning, selective opioid ligands have played an important role in deconvoluting the effects of opioid ligands acting at multiple opioid receptors³. This classification was based primarily on the assumption that opioid receptors exist as monomers⁴.

The concept of mu, delta, and kappa receptors as monomers was first challenged nearly three decades ago through studies with bivalent ligands that could bridge putative homomeric opioid receptors^{5, 6}. Later studies in cultured cells revealed that class A–C GPCRs can exist as homodimers or heteromers^{7–9}. This was followed by the discovery of mu-delta, kappa-delta, and mu-kappa heteromers^{10–13}.

The existence of opioid receptor heteromers suggests a more complex level of action and regulation than the traditional view of monomeric receptor pharmacology. For example, morphine, fentanyl, and methadone, traditionally viewed as mu-selective analgesics, have been reported to be more potent in activating mu-delta heteromers in cultured cells¹⁴. Moreover, mu-delta heteromers also appear to be the principal target for producing antinociception in monkeys¹⁵.

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Molecular tools that target opioid heteromers have been developed in an effort to sort out the effects mediated by such receptors. These have included ligands that selectively target mu-delta, mu-kappa, and delta-kappa heteromers. The recently reported, spinally selective mu-kappa agonist, *N*-naphthoyl- β -naltrexamine **1** (NNTA)¹⁶, selectively activates mu-kappa heteromers in HEK-293 cells and it produces exceptionally potent antinociception upon intrathecal (i.t.) administration in mice. Given the ~100-fold greater spinal potency of **1** than by the intracerebroventricular (i.c.v.) route, it is believed that the mu-kappa heteromers that mediate antinociception are localized in the spinal cord rather than supraspinally. Significantly, no tolerance was produced i.t., and only marginal tolerance was observed i.c.v. This profile prompted us to synthesize and evaluate derivatives related to ligand **1** in order to explore the structural requirements for selectivity.



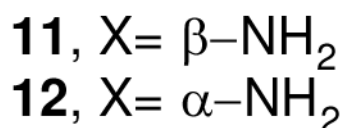
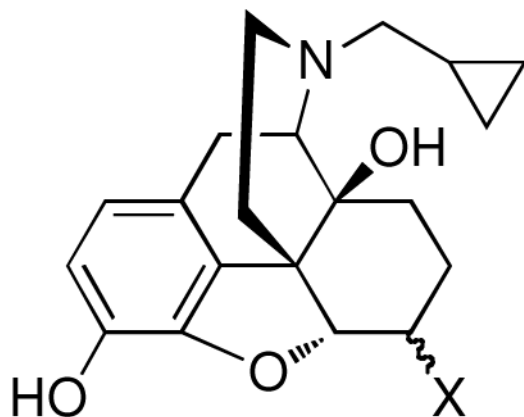
1 (NNTA)

Chemistry

The series was designed based on conservative structural changes in the amide moiety. In this regard, the key question that we wished to address was the structural requirements for the mu-kappa selectivity of **1**. Consequently, all members (**2–10**) of the series contain a naltrexamine opioid pharmacophore linked through an amide moiety to a substituted naphthalene or a heterocycle isosteric with naphthalene.

β -Naltrexamine **11** was employed as the opioid pharmacophore for all members of the series except **2**, which was derived from α -naltrexamine **12**, in order to evaluate the stereochemical role of the amide moiety in conferring selectivity. Both α - and β -naltrexamine were derived from naltrexone¹⁷.

The general procedure for synthesis of analogues **2–10** involved coupling of naltrexamines **11** or **12** in presence of benzotriazol-1-yl-oxy-tris-(dimethylamino)phosphonium hexafluorophosphate (BOP) and diisopropylethylamine (DIPEA) in dichloromethane (DCM)¹⁸. The target compounds **2–10** were thus obtained in moderate to high yields (58–84%) after chromatographic purification.



Biological Results

Intracellular Ca release studies

The target compounds were tested for agonist activity using an intracellular calcium release assay. Briefly, HEK-293 cells were transfected with a chimeric $\Delta 6$ -G _{α qi4-myR protein¹⁵ employed to measure intracellular Ca⁺² ion release upon receptor activation.¹ Cells stably expressing the chimeric protein were selected from transiently transfected cells in zeocin containing media (DMEM+10% fetal bovine serum+1% penicillin/streptomycin+0.1 μ g/ml zeocin). Opioid receptors were transiently transfected using different combinations of DNA for heteromers (μ -delta, μ -kappa, kappa-delta) or for singly expressing homomers (μ , delta, kappa). Intracellular calcium release was measured using a FLIPR calcium kit (Molecular Devices) in a FlexStation3 apparatus. For each compound, concentration-response profiles were established by measuring the fluorescence for 90 seconds after addition of the compound and determining the peak effect (maximum-minimum). A dose-response curve was plotted with the change in Relative Fluorescence Units (Δ RFU) vs. concentration (Table 1). Because the efficacy of **1** was substantially higher than other members of the series, the RFU scale for activity of **2–10** is expanded to 2.5x (0–1000) that of the ligand of reference **1**(0–2500).}

The selectivity profile of **1** is displayed as the structurally related reference compound for the closely related series of compounds. Its 6 α -epimer **2** was synthesized in order to evaluate the role of the C-6 stereocenter in the activation of μ - κ heteromers. It is noteworthy that its selectivity profile differs substantially from that of **1**, in that μ -kappa receptor activation has been greatly reduced. Other opioid receptors also showed reduced activation. These data led us to explore only close structural modifications in the 6 β -series.

The activity of the N-methyl analogue **3**²⁰ differed significantly from that of compound **1** in that activation of μ -kappa receptors was lost. There was an apparent small increase in activation at delta receptors in the higher concentration range. Conformational differences of the naphthyl moiety induced by the N-methyl group is one of several possibilities that could contribute to loss of μ -kappa selectivity.

Regioisomer **4** activated both mu-kappa and delta-kappa heteromers to the same degree, but the mu-kappa component was ~3-fold less effective than **1**. The curves for mu-kappa, mu-delta, and mu receptors were grouped together and have lower RFU values than the kappa-containing heteromers. No activation of delta receptors is apparent.

Various substituents were introduced at the 3'- and 6'-position of the naphthoyl group. Introduction of a 6'-methoxy (**5**) resulted in a loss of efficacy, suggesting that an electron-donating group in that position is not well tolerated. However, the same substituent in the 3'-position (**6**) afforded moderate delta selectivity. As the 3'-methoxy group could engage in intramolecular hydrogen bonding with the carboxamide NH, the delta selectivity might arise by restricting the naphthyl moiety to a conformation that differs from that of **5**.

Interestingly, the congener with a 6'-fluoro group (**7**) was delta-selective and exhibited considerably greater agonist potency and efficacy for delta receptors than **6**. That the activity of **7** is substantially more potent than **5** is consistent with the idea that an electron withdrawing group favors delta receptor activation in view of the inactivity of the 6'-methoxy analogue.

We also explored heterocyclic analogues (**8–10**) of **1** whose aromatic rings are isosteric with naphthalene. They included quinazoline **8** and isomeric quinolines **9** and **10**. All of these analogues were inactive at delta receptors, but possessed nonselective opioid activity at heteromeric and homomeric receptors in the nanomolar range. In this regard, **8** and **9** had similar profiles with higher efficacy at mu-delta and mu-kappa receptors.

Pharmacological evaluation in mice

The *in vivo* profiles of compounds **2–10** were evaluated using the mouse tail-flick assay after the ligands were administered by the i.c.v and/or i.t routes^{21–23} (Table 2). Tolerance was measured only for selected compounds displaying a full agonist profile i.c.v and/or i.t by comparing the ED_{80–90} dose measured on day one to the same dose measured 24 hours later on the same mice.

The 6 α -stereoisomer **2** and N-Me analogue **3** possessed partial agonist antinociceptive activity when administered by both routes. These data were supported by their low efficacy in the calcium mobilization assay as shown previously (Table 1).

Compound **4** displayed an i.t potency similar to the parent compound (20.98 pmol/mouse versus 18.7 pmol for **1** but it did not exhibit a full agonist profile when given supraspinally. The exceptional spinal activity was accompanied by tolerance 24 hours after i.t. administration.

The negative effect of a 6'-methoxy group (**5**) on efficacy, as revealed in the calcium mobilization assay, was supported by its i.t and i.c.v partial agonist profile. The activity of the 3'-isomer **6** was greater than that of **5** when injected spinally (ED₅₀=115 pmol/mouse) or supraspinally (ED₅₀= 312 pmol) and consistent with the cell-based results. No i.t tolerance was apparent, while some tolerance was observed after i.c.v injection.

The 6'-fluoro analogue **7** was found to be a fairly potent agonist by the i.t route, with an ED₅₀ of 81.1 pmol. No tolerance was observed. Because **7** was found to be delta-selective in the calcium release assay, it was further evaluated using the selective antagonists, nor-BNI²⁴ (kappa) and NTI²⁵ (delta), and in mu receptor knock-out mice (Figure 1). After i.t administration of **7**, NTI produced a 26-fold shift consistent with the delta selectivity observed in the cell-based assay. The finding that its activity was also antagonized by nor-BNI may be due to antagonism of the combined agonist effect mediated by kappa, kappa-

delta, and kappa-mu receptors, given that **7** activates these receptor systems in the nM range as shown in the calcium release assay. Such antagonism by nor-BNI may be mediated through a negative allosteric effect mediated via the kappa receptor, as suggested previously²⁶. Thus, nor-BNI may antagonize a mu or delta receptor in a heteromer through via an associated kappa protomer in vivo. This is consistent with the decreased antinociception of **7** in the mu-opioid receptors knock-out mice due to absence of mu-delta heteromers,

The quinoxaline analogue **8** was found to be a potent agonist by both i.c.v and i.t routes (ED_{50} 50.76 pmol and 757.2 pmol, respectively), with 15-fold greater spinal potency. It is noteworthy that **8** exhibited no apparent tolerance by either route of administration. Quinoline analogue **9** also exhibited full i.t. and i.c.v. agonist activity without tolerance, but it was substantially less potent than that of **8**. Interestingly, compound **4**, the isosteric naphthalene analogue of **9**, was exceptionally potent i.t., but displayed tolerance. The isomeric quinoline analogue **10** behaved as a partial agonist when administered spinally or supraspinally.

Discussion

Using the selective mu-kappa agonist **1**, as the prototype ligand, a series of closely related naphthalene analogues were synthesized to study the chemical space around the naphthalene moiety in an effort to evaluate how receptor selectivity is affected by chemical modification. Nine analogues (**2–10**) were synthesized and tested on HEK-293 cells expressing homomeric and heteromeric opioid receptors, and in the mouse tail-flick assay.

Since **1** is derived from 6β -naltrexamine, we first explored the effect of configuration at the C6 chiral center by the synthesis of **2**. As it appeared from the cell-based data that **2** gave a profound reduction in the activation of opioid receptors, particularly mu-kappa, relative to that of **1**, we decided to focus on ligands in the 6β -series.

In this regard we first evaluated the effect of substitution on the 6β nitrogen atom with compound **3**, as we suspected a *N*-methyl group would alter the rotational conformational preference of the naphthalene moiety. Once again, the activation of heteromeric and homomeric receptors was greatly reduced, suggesting that NH hydrogen bonding with the receptor(s) and/or conformational factors might be involved.

Interestingly, the 1-naphthyl analogue **4** exhibited improved Δ RFU values for activation of heteromeric mu-kappa and delta-kappa receptors relative to compound **3**. As both of these heteromers have been reported to be potently activated in the spinal cord of rodents, it is not surprising that **4** produces high i.t. antinociception. Other receptors were activated to a lesser degree, and delta showed no activation.

The effect of substitution on the naphthyl group had a profound effect on selectivity and potency. Thus, a 6'-OMe substituent (**5**) greatly reduced activation of all homomers and heteromers, whereas its 3'-regioisomer (**6**) selectively activated delta receptors. These results are consistent with several possible explanations that include an unfavorable electronic or steric effect by the 6'-OMe in **5** and a favorable effect on the conformation of the naphthyl group of **6** due to possible intramolecular H-bonding between 6β -NH and the 3'-OMe.

The 6'-fluoro-substituted analogue **7** appeared to be more delta-selective than **6**. One possible explanation could be due to the electron withdrawing effect of the 6'-fluoro group,

in view of the electron donating 6'-OMe of **5** having the opposite effect on activity. It is noteworthy that **7** did not produce tolerance and was highly potent in mice by the i.t. route.

The quinoxaline analogue **8** and quinoline analogues (**9**, **10**) were essentially nonselective agonists in HEK-293 cells. The trend seems to support greater activation for mu-delta and mu-kappa. Interestingly, these ligands failed to activate delta receptors.

Of the nine analogues tested in mice, five (**4**, **6–9**) were highly potent full agonists after i.t. administration. Among these, only compound **4** exhibited tolerance. Analogues **2**, **3**, **5**, and **10** produced partial agonism by this route. Partial agonism among members of the series was observed more frequently upon i.c.v. administration, and in this regard **2–5**, **7**, **10** produced this effect. The only members of the series that were full agonists both i.c.v. and i.t. were compounds **6**, **8**, **9**. It is noteworthy that i.t. administration resulted in greater potency than by the i.c.v. route when the ED₅₀ values could be determined after both i.t. and i.c.v. administration.

Conclusions

Judging from the calcium release studies for compounds **2–10** in HEK-293 cells, the high selectivity of **1** for activation of heteromeric mu-kappa receptors appears to be unique in this series. Intriguingly, the 6-fluoro analogue **7** appeared to selectively activate homomeric delta receptors in vitro and it induced potent i.t. antinociception without tolerance in mice. The finding that a small change in structure produces a profound change in selectivity exemplifies the complexity of the structure activity relationships in this series. The complex profiles of these compounds in activating the six combinations of opioid receptors makes it difficult to come to any firm conclusions regarding the relationship of receptor activation to potency and tolerance in vivo. Nevertheless, based on the i.t. and i.c.v. data, most of the analogues possess potent antinociceptive activity that arises from the selective activation of spinal opioid receptors.

Experimental section

All commercial reagents and anhydrous solvents were purchased from vendors and were used without further purification or distillation. Analytical thin-layer chromatography (TLC) was performed on EM Science silica gel 60 F₂₅₄ (0.25 mm). Compounds were visualized by UV light. Flash column chromatography was performed on Fisher Scientific silica gel (230 – 400 mesh).

Melting points were determined on a Thomas-Hoover melting point apparatus and are uncorrected. ¹H NMR spectra and ¹³C NMR spectra were taken on a Bruker Avance 400 MHz instruments and calibrated using an internal reference. Chemical shifts are expressed in ppm and coupling constants (J) are in hertz (Hz). Peak multiplicities are abbreviated: broad, br; singlet, s; doublet, d; triplet, t; and multiplet, m. ESI mode mass spectra were recorded on a BrukerBioTOF II mass spectrometer. Elemental analyses were performed by M-H-W Laboratories, Phoenix, AZ. Analytical data confirmed the purity of the products was 95%.

General procedure for the amidation of β-naltrexamine with a carboxylic acid

β-naltrexamine (100 mg, 0.29 mmol), heterocyclic carboxylic acid (0.58 mmol) and BOP (258 mg, 0.58 mmol) were dissolved in DCM (5 mL). To this solution, DIPEA (150 mL, 0.81 mmol) was added and the mixture was stirred at room temperature for 3 to 16 hours. The solution was concentrated under reduced pressure and the residue was taken up in MeOH (5 mL), and K₂CO₃ was added (300 mg). After 1 hour at room temperature, the

mixture was concentrated to dryness. The final crude was purified by SiO₂ chromatography to afford the target compound.

The title compound was then subsequently converted into the HCl salt for biological testing.

17-Cyclopropylmethyl-3,14β-dihydroxy-4,5α-epoxy-6α-(2'-

naphthyl)acetamidomorphinan (2)—Compound **2** was prepared according to the general procedure described above; combining α-naltrexamine (100 mg, 0.29 mmol), 2-naphthoic acid (117 mg, 0.58 mmol); BOP (258 mg, 0.58 mmol), and DIPEA (150 μL, 0.81 mmol) followed by basic solvolysis with K₂CO₃ gave the target compound which was purified by flash chromatography [EA/hexanes; 70/30], and then recrystallized from an acetone/hexanes mixture to provide **2** as a white solid (116 mg, 81%).

¹H NMR (DMSO-*d*₆) δ: 0.13 (m, 2H); 0.49 (m, 2H); 0.88 (m, 1H); 1.37–1.69 (m, 4H); 2.14–2.37 (m, 4H); 2.96–3.08 (m, 2H); 4.54 (m, 1H); 4.69 (d, 1H, *J*_{H5-H6} = 3.8 Hz); 4.92 (bs, 1H, OH-14); 6.48 (d, 1H, *J*_{H1-H2} = 8.1 Hz); 6.59 (d, 1H, *J*_{H2-H1} = 8.1 Hz); 7.59–7.63 (m, 2H); 7.93–8.04 (m, 4H); 8.10 (d, 1H amide, *J* = 7.7 Hz); 8.47 (s, 1H); 8.89 (bs, 1H, OH-3); ¹³C NMR (DMSO-*d*₆) δ: 3.40, 3.81, 9.13, 19.98, 22.31, 29.44, 33.53, 42.78, 46.52, 46.65, 58.75, 61.36, 69.23, 88.27, 117.22, 118.49, 124.40, 124.85, 126.67, 127.51, 127.58 (x2), 127.74, 128.76, 130.68, 131.88, 132.05, 134.06, 137.97, 145.82, 165.91. mp = 232–234 °C. Anal. Calcd. for C₃₁H₃₃ClN₂O₄: C, 69.85; H, 6.24; N, 5.26. Found: C, 69.98; H, 6.13; N, 5.32. ESI-TOF MS calculated for C₃₁H₃₂N₂O₄, *m/z* 496.236, found 519.303 (M+Na)⁺

17-Cyclopropylmethyl-3,14β-dihydroxy-4,5α-epoxy-6β- (1'-

naphthyl)acetamidomorphinan (4)—Compound **4** was prepared according to the general procedure described above; combining β-naltrexamine (100 mg, 0.29 mmol), 1-naphthoic acid (117 mg, 0.58 mmol); BOP (258 mg, 0.58 mmol), and DIPEA (150 μL, 0.81 mmol) followed by basic solvolysis with K₂CO₃ gave the target compound which was purified by flash chromatography [EA/hexanes (75/25)], and then recrystallized from an acetone/hexanes mixture to provide **4** as a white solid (120 mg, 84%).

¹H NMR (CDCl₃) δ: 0.11 (m, 2H); 0.52 (m, 2H); 0.81 (m, 1H); 1.47 (m, 2H); 1.62–1.75 (m, 2H); 1.91 (m, 1H); 2.17 (m, 2H); 2.35 (m, 2H); 2.61 (m, 2H); 3.01 (d, 1H, *J* = Hz); 3.08 (m, 1H); 4.18 (m, 1H); 4.50 (d, 1H, *J*_{H5-H6} = 7.8 Hz); 6.55 (d, 1H, *J*_{H1-H2} = 8.1 Hz); 6.72 (d, 1H, *J*_{H2-H1} = 8.1 Hz); 6.87 (d, 1H, *J* = 8.9 Hz); 7.38 (m, 1H); 7.48–7.53 (m, 2H); 7.58 (Dd, 1H, *J* = 7 Hz, *J* = 1.2 Hz); 7.79–7.92 (m, 2H), 8.32 (m, 1H); ¹³C NMR (CDCl₃) δ: 3.77, 3.97, 9.38, 22.64, 23.91, 31.23, 31.45, 43.96, 47.54, 51.00, 59.26, 62.26, 70.14, 93.40, 117.83, 119.19, 124.54, 124.72, 125.04, 125.42, 126.36, 127.12, 128.27, 130.10, 130.58, 130.86, 133.65, 134.44, 139.82, 142.97, 169.46; mp = 224–226 °C. Anal. Calcd. for C₃₁H₃₃ClN₂O₄: C, 74.98; H, 6.50; N, 5.64. Found: C, 75.27; H, 6.57; N, 5.74. ESI-TOF MS calculated for C₃₁H₃₂N₂O₄, *m/z* 496.236, found 497.139 (M+H)⁺, 993.260 (2xM+H)⁺

17-Cyclopropylmethyl-3,14β-dihydroxy-4,5α-epoxy-6β- (6'-methoxy-2'-

naphthyl)acetamidomorphinan (5)—Compound **5** was prepared according to the general procedure described above; combining β-naltrexamine (100 mg, 0.29 mmol), 6-methoxy-2-naphthoic acid (117 mg, 0.58 mmol); BOP (258 mg, 0.58 mmol), and DIPEA (150 μL, 0.81 mmol) followed by basic solvolysis with K₂CO₃ gave the target compound which was purified by flash chromatography [EA/hexanes; 75/25], and then recrystallized from an acetone/hexanes mixture to provide **5** as a white solid (114 mg, 75%).

¹H NMR (DMSO-*d*₆) δ: 0.15 (m, 2H); 0.50 (m, 2H); 0.88 (m, 1H); 1.23–1.62 (m, 4H); 1.82–1.91 (m, 2H); 2.08 (m, 1H); 2.27 (m, 2H); 2.36 (m, 2H); 3.03 (m, 2H); 3.75 (m, 1H); 3.90 (s, 3H); 4.77 (d, 1H, *J*_{H5-H6} = 7.1 Hz); 4.91 (bs, 1H, OH-14); 6.57–6.60 (m, 2H); 7.23

(Dd, 1H, J= 9 Hz, J= 2.4 Hz); 7.38 (d, 1H, J= 1.9 Hz); 7.86–7.94 (m, 3H), 8.42 (s, 1H); 8.72 (D, 1H amide, J_{NH-H6} = 8.1 Hz); 9.04 (bs, 1H, OH-3); ^{13}C NMR (DMSO- d_6) δ : 3.27, 3.45, 9.03, 22.32, 24.46, 29.89, 31.14, 38.81, 41.66, 45.71, 51.47, 55.27, 61.17, 69.63, 90.48, 105.82, 117.20, 118.76, 119.33, 123.45, 124.61, 126.57, 127.30, 127.42, 127.55, 129.28, 130.37, 135.70, 140.60, 142.11, 158.50, 165.67. mp= 199–201 °C. Anal. Calcd. for $C_{32}H_{35}ClN_2O_5$: C, 68.25; H, 6.27; N, 4.97. Found: C, 68.38; H, 6.13; N, 5.04. ESI-TOF MS calculated for $C_{32}H_{34}N_2O_5$; m/z 526.247, found 527.298 (M+H)⁺

17-Cyclopropylmethyl-3,14 β -dihydroxy-4,5 α -epoxy-6 β - (3'-methoxy-2'-naphthyl)acetamido]morphinan (6)—Compound **6** was prepared according to the general procedure described above; combining β -naltrexamine (100 mg, 0.29 mmol), 6-methoxy-2-naphthoic acid (117 mg, 0.58 mmol); BOP (258 mg, 0.58 mmol), and DIPEA (150 μ L, 0.81 mmol) followed by basic solvolysis with K_2CO_3 gave the target compound which was purified by flash chromatography [EA/hexanes; 75/25], and then recrystallized from an acetone/hexanes mixture to provide **6** as a white solid (119 mg, 78%).

1H NMR (DMSO- d_6) δ : 0.12 (m, 2H); 0.47 (m, 2H); 0.85 (m, 1H); 1.26–1.66 (m, 4H); 1.81–1.90 (m, 2H); 2.08 (m, 1H); 2.27 (m, 2H); 2.36 (m, 2H); 3.03 (m, 2H); 3.75 (m, 1H); 3.90 (s, 3H); 4.77 (d, 1H, J_{H5-H6} = 7.1 Hz); 4.91 (bs, 1H, OH-14); 6.57–6.60 (m, 2H); 7.23 (Dd, 1H, J= 9 Hz, J= 2.4 Hz); 7.38 (d, 1H, J= 1.9 Hz); 7.86–7.94 (m, 3H), 8.23 (s, 1H); 8.50 (D, 1H amide, J_{NH-H6} = 8.1 Hz); 9.04 (bs, 1H, OH-3); ^{13}C NMR (DMSO- d_6) δ : 3.45, 3.71, 9.17, 22.17, 24.30, 29.97, 30.36, 41.23, 45.67, 47.13, 51.56, 55.83, 61.72, 69.63, 90.63, 106.46, 115.91, 117.96, 124.20, 125.30, 126.33, 127.41, 127.66, 127.77, 128.17, 128.34, 130.66, 134.94, 140.43, 142.17, 154.41, 164.73. mp= 215–26 °C. Anal. Calcd. for $C_{32}H_{35}ClN_2O_5$: C, 68.25; H, 6.27; N, 4.97. Found: C, 68.47; H, 6.21; N, 5.02. ESI-TOF MS calculated for $C_{32}H_{34}N_2O_5$, m/z 526.247, found 527.301(M+H)⁺

17-Cyclopropylmethyl-3,14 β -dihydroxy-4,5 α -epoxy-6 β - (6'-fluoro-2'-naphthyl)acetamido]morphinan (7)—Compound **7** was prepared according to the general procedure described above; combining β -naltrexamine (100 mg, 0.29 mmol), 6-fluoro-2-naphthoic acid (110 mg, 0.58 mmol); BOP (258 mg, 0.58 mmol), and DIPEA (150 μ L, 0.81 mmol) followed by basic solvolysis with K_2CO_3 gave the target compound which was purified by flash chromatography [EA/hexanes; 80/20], and then recrystallized from an acetone/hexanes mixture to provide **7** as a white solid (118 mg, 79%).

1H NMR (CD_3OD) δ : 0.23 (m, 2H); 0.59 (m, 2H); 0.94 (m, 1H); 1.47–1.79 (m, 4H); 2.03 (m, 1H); 2.17–2.35 (m, 2H); 2.44–2.52 (m, 2H); 2.70–2.76 (m, 2H); 3.13–3.23 (m, 2H); 4.01 (m, 1H); 4.72 (d, 1H, J = 7.7 Hz); 6.63 (d, 1H, J = 8.1 Hz); 6.68 (d, 1H, J = 8.1 Hz); 7.42 (td, 1H, J = 2.5 Hz, J = 9.8 Hz); 7.62 (dd, 1H, J = 2.4 Hz, J = 9.9 Hz); 7.93–7.99 (m, 2H); 8.08 (m, 1H), 8.45 (s, 1H); ^{13}C NMR (CD_3OD) δ : 4.14, 4.58, 10.23, 23.61, 25.59, 31.34, 31.42, 43.86, 49.01, 53.49, 60.19, 63.80, 71.82, 93.04, 111.69, 111.90, 117.93, 118.18, 118.67, 120.13, 126.10 (x2), 128.70, 128.91, 131.12, 132.55, 132.88, 134.05, 140.47, 143.74, 169.76; ^{19}F NMR (CD_3OD) δ : -114.03; mp= 189–190 °C. Anal. Calcd. for $C_{31}H_{32}ClFN_2O_5$: C, 67.57; H, 5.85; N, 5.08. Found: C, 67.91; H, 5.85; N, 5.17. ESI-TOF MS calculated for $C_{31}H_{31}FN_2O_5$, m/z 514.227, found 515.300 (M+H)⁺

17-Cyclopropylmethyl-3,14 β -dihydroxy-4,5 α -epoxy-6 β - (2'-quinoxalyl)acetamido]morphinan (8)—Compound **8** was prepared according to the general procedure described above; combining β -naltrexamine (100 mg, 0.29 mmol), 2-quinoxalinecarboxylic acid (101 mg, 0.58 mmol); BOP (258 mg, 0.58 mmol), and DIPEA (150 μ L, 0.81 mmol) followed by basic solvolysis with K_2CO_3 gave the target compound which was purified by flash chromatography [EA/hexanes (75/25)], and then recrystallized from an acetone/hexanes mixture to provide **8** as a white solid (110 mg, 77%).

^1H NMR (DMSO- d_6) δ : 0.01 (m, 2H); 0.37 (m, 2H); 0.72 (m, 1H); 1.28 (m, 1H); 1.49 (m, 2H); 1.85 (m, 2H); 2.05 (m, 2H); 2.25 (m, 2H); 2.55 (m, 2H); 2.93–3.02 (m, 2H); 4.00 (m, 1H); 4.37 (d, 1H, $J_{\text{H5-H6}} = 7.8$ Hz); 6.46 (d, 1H, $J_{\text{H1-H2}} = 8.1$ Hz); 6.55 (d, 1H, $J_{\text{H2-H1}} = 8.1$ Hz); 7.31–7.36 (m, 3H); 7.44 (m, 1H); 7.81 (d, 1H, $J = 8.3$ Hz); 7.89 (d, 2H, $J = 8.2$ Hz); 8.15 (d, 1H, $J = 8.7$ Hz); 8.39 (s, 1H,); ^{13}C NMR (DMSO- d_6) δ : 3.52, 3.64, 9.36, 24.40, 25.26, 30.06, 30.27, 43.6, 47.43, 52.67, 58.37, 61.75, 69.56, 90.22, 110.87, 115.64, 118.31, 119.08, 126.54, 129.07, 129.38, 131.26, 139.76, 142.08, 142.84, 143.66, 144.36, 156.54, 162.88. mp= 177–180°C. Anal. Calcd. for $\text{C}_{29}\text{H}_{31}\text{ClN}_4\text{O}_4$: C. 65.10; H. 5.84; N. 10.47. Found: C. 64.81; H. 5.81; N. 10.55. ESI-TOF MS calculated for $\text{C}_{29}\text{H}_{30}\text{N}_4\text{O}_4$, m/z 498.227, found 499.121 (M+H) $^+$, 1019.221 (2xM+Na) $^+$

17-Cyclopropylmethyl-3,14 β -dihydroxy-4,5 α -epoxy-6 β - (1'-

isoquinolyl)acetamido]morphinan (9)—Compound **9** was prepared according to the general procedure described above; combining β -naltrexamine (100 mg, 0.29 mmol), isoquinoline-1-carboxylic acid (100.4 mg, 0.58 mmol); BOP (258 mg, 0.58 mmol), and DIPEA (150 μL , 0.81 mmol) followed by basic solvolysis with K_2CO_3 gave the target compound which was purified by flash chromatography [EA/hexanes (90/10)], and then recrystallized from an acetone/hexanes mixture to provide **9** as a white solid (90 mg, 63%).

^1H NMR (CDCl_3) δ : 0.13 (m, 2H); 0.53 (m, 2H); 0.85 (m, 1H); 1.17–1.68 (m, 4H); 1.83–2.04 (m, 3H); 2.18 (m, 2H); 2.25 (m, 2H); 2.38 (m, 2H); 2.61–2.67 (m, 2H); 4.06 (m, 1H); 4.60 (d, 1H, $J_{\text{H5-H6}} = 7.8$ Hz); 6.58 (d, 1H, $J_{\text{H1-H2}} = 8.1$ Hz); 6.73 (d, 1H, $J_{\text{H2-H1}} = 8.1$ Hz); 7.58–7.78 (m, 4H); 8.39 (d, 1H, $J = 5.5$ Hz); 8.58 (d, 1H, $J = 9.1$ Hz), 9.49 (d, 1H amide, $J_{\text{NH}} = 8.2$ Hz); ^{13}C NMR (CDCl_3) δ : 3.79, 3.97, 9.44, 22.70, 24.54, 30.27, 30.57, 44.11, 47.84, 51.34, 59.23, 62.37, 70.21, 93.82, 117.73, 119.17, 124.36, 124.44, 126.69, 126.92, 127.76, 128.56, 130.38, 131.15, 137.32, 140.06, 140.12, 142.56, 147.93, 165.89; mp= 152–154 °C. Anal. Calcd. for $\text{C}_{30}\text{H}_{32}\text{ClN}_3\text{O}_4$: C. 67.47; H. 6.04; N. 7.87. Found: C. 67.11; H. 6.08; N. 7.93. ESI-TOF MS calculated for $\text{C}_{30}\text{H}_{31}\text{N}_3\text{O}_4$, m/z 497.231, found 498.226 (M+H) $^+$, 1017.425 (2xM+Na) $^+$

17-Cyclopropylmethyl-3,14 β -dihydroxy-4,5 α -epoxy-6 β - (5'-

quinolyl)acetamido]morphinan (10)—Compound **10** was prepared according to the general procedure described above; combining β -naltrexamine (100 mg, 0.29 mmol), 5-quinoline-1-carboxylic acid (100.4 mg, 0.58 mmol); BOP (258 mg, 0.58 mmol), and DIPEA (150 μL , 0.81 mmol) followed by basic solvolysis with K_2CO_3 gave the target compound which was purified by flash chromatography [4%MeOH/DCM], and then recrystallized from an acetone/hexanes mixture to provide **10** as a white solid (83 mg, 58%).

^1H NMR (CD_3OD) δ : 0.18 (m, 2H); 0.56 (m, 2H); 0.91 (m, 1H); 1.45 (m, 1H); 1.58–1.65 (m, 2H); 1.84 (m, 1H); 2.00 (m, 1H); 2.13–2.36 (m, 2H); 2.43–2.51 (m, 2H); 2.69–2.73 (m, 2H); 3.10–3.20 (m, 2H); 3.99 (m, 1H); 4.59 (d, 1H, $J_{\text{H5-H6}} = 7.8$ Hz); 6.62 (d, 1H, $J_{\text{H1-H2}} = 8.1$ Hz); 6.69 (d, 1H, $J_{\text{H2-H1}} = 8.1$ Hz); 7.62 (m, 1H); 7.82–7.85 (m, 2H); 8.14 (t, 1H, $J = 5.3$ Hz); 8.78 (d, 1H, $J = 9.5$ Hz); 8.90 (dd, 1H, $J = 1.6$ Hz, $J = 4.2$ Hz); ^{13}C NMR (CD_3OD) δ : 4.15, 4.54, 10.13, 23.59, 25.66, 31.44, 31.71, 45.44, 48.78, 53.54, 60.20, 63.75, 71.80, 92.95, 118.61, 120.18, 123.25, 125.36, 127.02, 127.24, 130.18, 131.60, 132.54, 136.16, 136.27, 142.08; 143.73, 148.69, 151.74, 170.64. mp= 165–167 °C. Anal. Calcd. for $\text{C}_{30}\text{H}_{32}\text{ClN}_3\text{O}_4$: C. 67.47; H. 6.04; N. 7.87. Found: C. 67.82; H. 6.09; N. 7.79. ESI-TOF MS calculated for $\text{C}_{30}\text{H}_{31}\text{N}_3\text{O}_4$, m/z 497.231, found 498.133 (M+H) $^+$

Materials and Methods

Intracellular calcium release

To determine the functional selectivity of the above ligands, a modified intracellular calcium release assay was employed. As activation of opioid receptors does not regulate signaling mechanisms that cause calcium release because they are coupled to Gi/o, a chimeric G-protein (D6-Gqi4-myr), was employed. The construction of this chimera has been reported¹⁹. Human embryonic kidney cells (HEK-293) with stably expressing human Giq protein were grown at 37 °C and 10% CO₂ in Dulbecco's modified medium (GIBCO) using zeocin as the antibiotic for selecting cells expressing the Gi G-protein.

HEK-293 cells containing the various opioid receptors were created by transiently transfecting opioid receptor DNA in OptiMEM medium (Invitrogen) at a concentration of 200 ng/20,000 cells using Lipofectamine 2000 (Invitrogen, Carlsbad CA). Twenty-four hours later, 20,000 cells/well were seeded into 96-well black plates (Corning Inc.). The FLIPR Calcium Explorer kit (Molecular devices) was used for the assay. On the third day, cells were incubated with a Ca⁺⁺ chelating dye from the kit and incubated for 60 minutes at 37°C. The plates were then assayed in a FlexStation 3 apparatus (Molecular Devices) using a range of concentrations of the ligand tested. The response was measured as the change in Relative Fluorescence Units (Δ RFU = RFU_{max}-RFU_{min}) and the time of the response was measured in seconds. Following a 30 second equilibration period, the ligand was added to each well of the plate. RFUs were measured continuously for 60 seconds following the addition of the ligand (the response before calcium ion reuptake mechanisms bring the Ca²⁺ ion concentration back to basal levels). The Δ RFU was computed for each concentration, which was plotted as a concentration response curve and analyzed to obtain the IC₅₀ value using nonlinear regression. The peak effect was also recorded. At least three independent replications with four internal replicates were used to evaluate each ligand in each of the cell types.

Animals

Male ICR-CD1 mice (17 – 25g; Harlan, Madison, WI) employed in the testing were housed in groups of 8 in a temperature- and humidity-controlled environment with unlimited access to food and water. They were maintained on a 12 h light/dark cycle. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Minnesota (Minneapolis, MN).

Antinociceptive Testing

The tail flick assay for antinociception described by D'Amour²¹ and Smith and modified by Dewey *et Al.* was employed²². For the measurement, of the tail-flick latency, mice were held gently with the tail positioned in the apparatus (Tail Flick Analgesia Meter, Columbus Instruments, Columbus, Ohio) for radiant heat stimulus. The tail-flick response was elicited by applying radiant heat to the dorsal side of the tail. The intensity of the heat is set so that the mouse flicks its tail within 2 to 3 s. The test latency is measured before drug treatment (control) and again after the drug treatment (test) at the peak time of the compound, a 10 s maximum cut-off time is used to prevent damage to the tail. Antinociception is quantified according to the method of Harris and Pierson²³ as the percent maximal possible effect (%MPE): $\%MPE = (\text{Test} - \text{Control}) / (10 - \text{Control}) \times 100$.

At least three groups of eight to ten mice are used for each dose response curve, and each mouse is used only once. ED₅₀ values with 95% confidence intervals (C.I.) are computed with GraphPad Prism 4 by using nonlinear regression methods.

Agonism

All the compounds were dissolved in 10% DMSO and then diluted to less than 1% DMSO in the test solutions. Controls when given either i.c.v. or i.t. with 1% or less DMSO do not show any antinociception. All compounds were administered in a 5- μ l volume in conscious mice according to the method of Haley and McCormick²⁷ for i.c.v and Hylden and Wilcox²⁸ for i.t. injections. A time course study (10, 20, 30 and 60 min) were used to determine the peak antinociception. Administration of ligands and antagonist were timed so that they peaked at the same time (nor-BNI (2.5 nmol/mouse – 20 minute peak time), NTI (5 nmol/mouse – 20 minute peak time)). Potency ratios (test ED₅₀/control ED₅₀) were deemed significant if their 95% confidence intervals do not overlap.

Acute tolerance was measured on select compounds by comparing the initial ED₈₀₋₉₀ dose to the same dose measured 24 hours later on the same mouse.

Knock-out Studies

MOR-KO mice (male and female, 13–15 weeks old) were grouped with equal numbers of male and females so that there were eight mice per group. Each mouse was only used once. The MOR-KO mice were injected i.t. with the ED₈₀₋₉₀ of the given agonist to see if there was a change in the %MPE. The point was considered significant if the 95% confidence intervals did not overlap.

Acknowledgments

We thank Dr. Evi Kostenis for providing us with the cDNA for $\Delta 6$ -G α_{qi4} -myr chimeric G α subunit, and Dr. Sabita Roy for the MOR-KO mice. This research was supported by National Institute of Health research grant DA01533.

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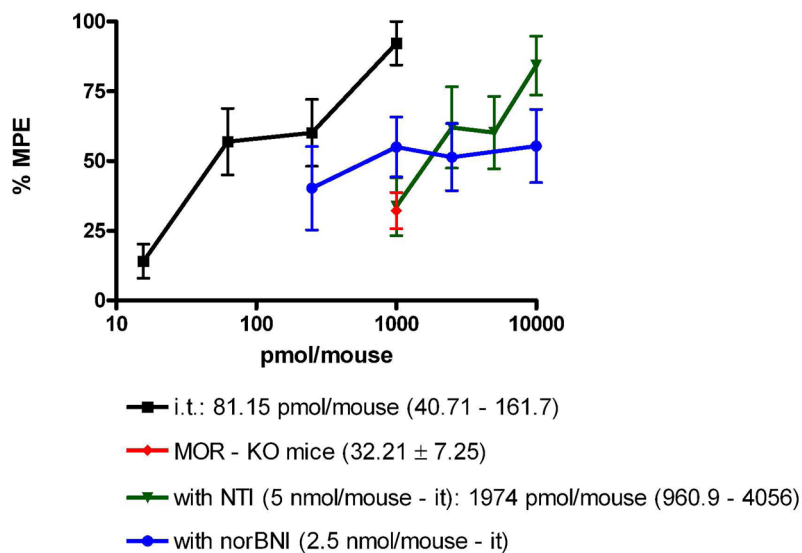
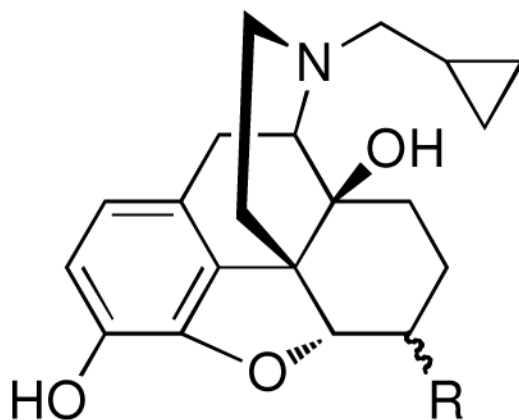
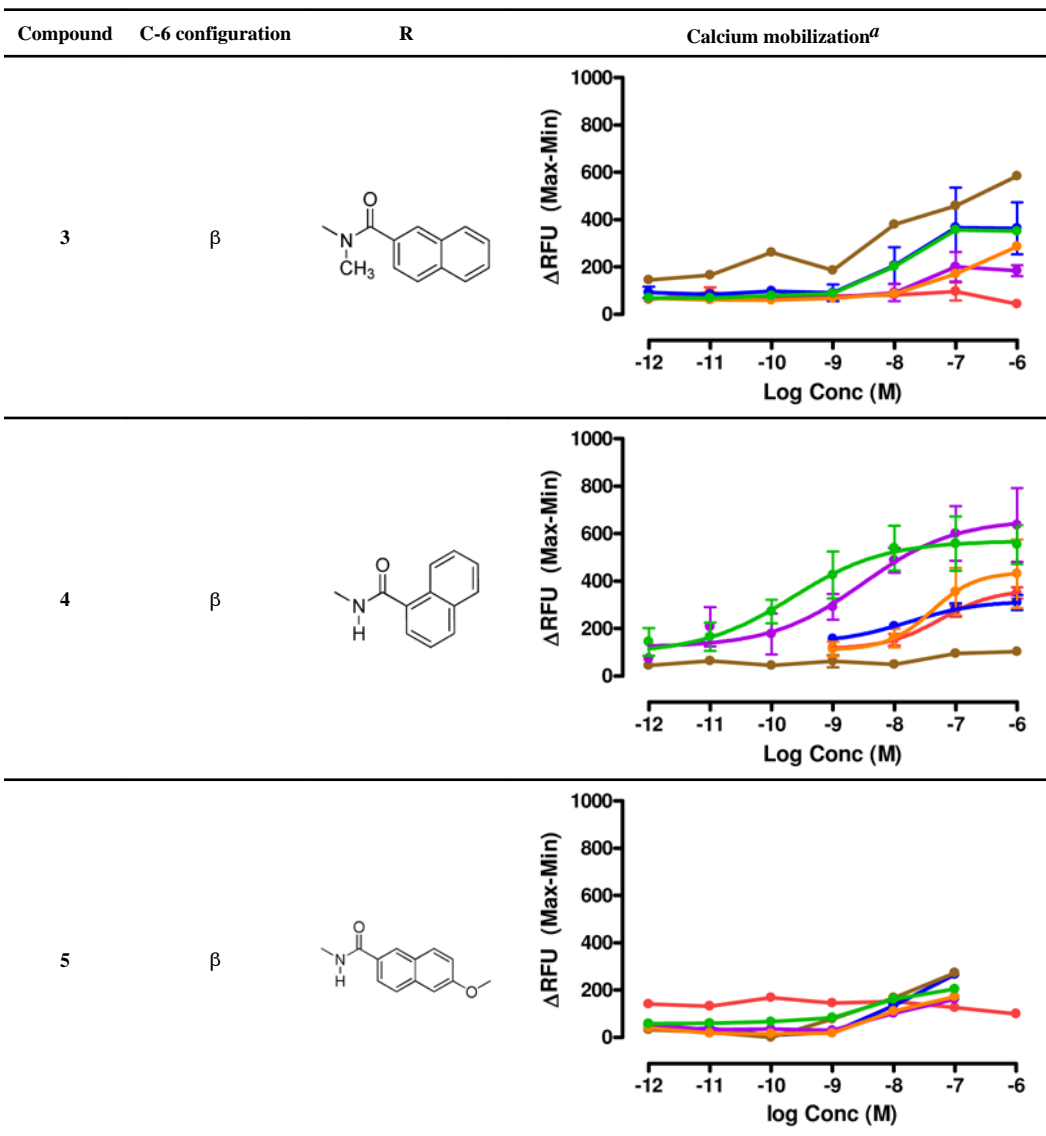
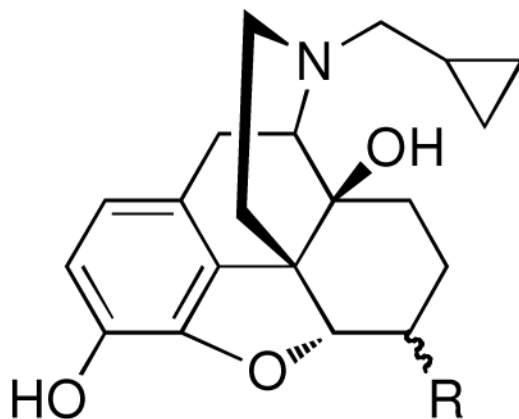


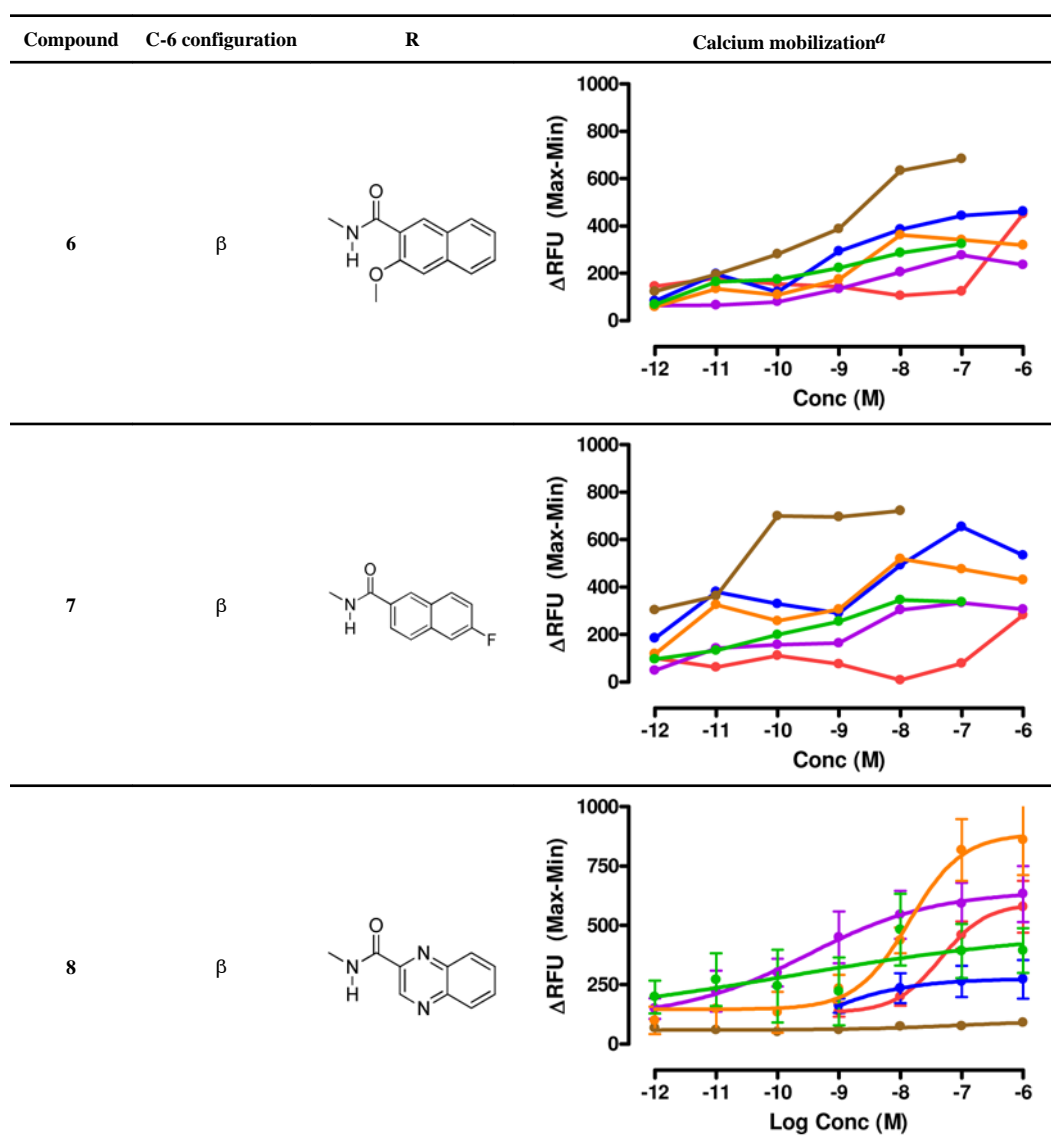
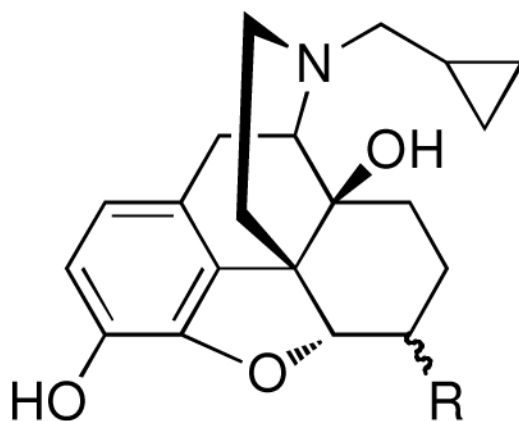
Figure 1. Antinociception of compound **7** after treatment with opioid antagonists naltrindole (NTI) and norbinorphimine (norBNI) after i.t administration in wild-type mice and in mu opioid receptor knockout (MOR-KO) mice.

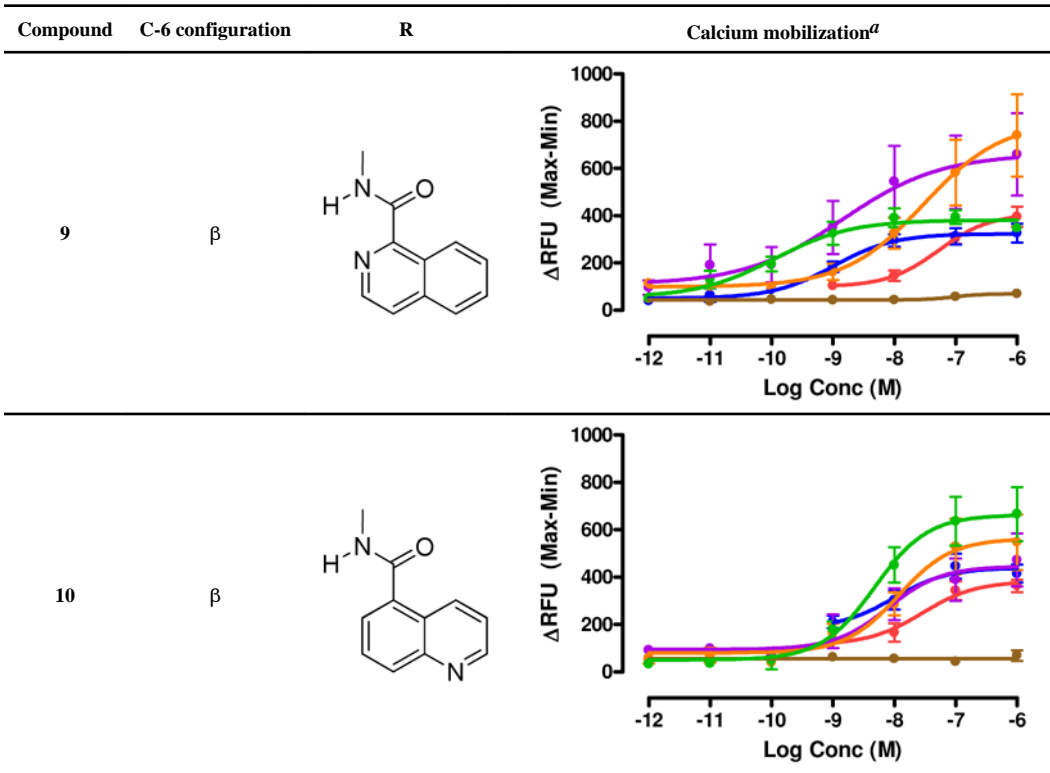
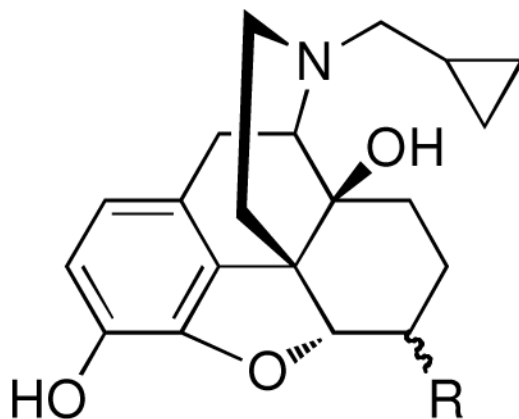
Table 1

Intracellular Ca²⁺ release profiles at multiple opioid receptors.

Compound	C-6 configuration	R	Calcium mobilization ^a
1 ^b (NNTA)	β		
2	α		







^aData are mean \pm SEM ($n=3-8$). RFU, Relative Fluorescence Unit.

^b(1) data from ref 16.

Table 2Antinociceptive activity and tolerance of **2–10** after i.c.v. or i.t. administration in mice.

Compound ^a	Injection site	ED ₅₀ pmol/mouse (95% C.I.) or	24 hour tolerance ^b
1 (NNTA)	i.t.	18.7 (10.3 – 32.8)	No
	i.c.v.	2060 (1090 – 3270)	Yes [†]
2	i.t.	partial agonist ^c	-
	i.c.v.	partial agonist	-
3	i.t.	partial agonist	-
	i.c.v.	partial agonist	-
4	i.t.	20.9 (13.5 – 32.7)	Yes
	i.c.v.	partial agonist	-
5	i.t.	partial agonist	-
	i.c.v.	partial agonist	-
6	i.t.	115.1 (95.5 – 138.7)	No
	i.c.v.	312.4 (168.6 – 578.7)	Yes
7	i.t.	81.1 (40.7 – 161.7)	No
	i.c.v.	partial agonist	-
8	i.t.	50.8 (30.0 – 95.6)	No
	i.c.v.	757.2 (561.7 – 1021)	No
9	i.t.	1408 (931 – 2128)	No
	i.c.v.	2070 (1490 – 2876)	No
10	i.t.	partial agonist	-
	i.c.v.	partial agonist	-

^aPeak times for the dose response curves were as follows for i.t. **1, 5, 6, 8** all 5 min; **7, 9** both 20 min and for i.c.v. **5, 9** both 10 min; NNTA, 20 min.

^b24 hour tolerance was calculated using the highest dose of the dose-response curve on day 1 and repeated on day 2. If there was no significant difference between the two days the animals were said to be not tolerant.

^cPartial agonist is defined as when the maximum %MPE was < 60%