Pharmacological Properties of Bivalent Ligands Containing Butorphan Linked to Nalbuphine, Naltrexone, and Naloxone at μ, δ, and κ Opioid Receptors

Xuemei Peng,[†] Brian I. Knapp,[‡] Jean M. Bidlack,[‡] and John L. Neumeyer*[,†]

Alcohol and Drug Abuse Research Center, McLean Hospital, Harvard Medical School, 115 Mill Street, Belmont, Massachusetts 02478, and Department of Pharmacology and Physiology, School of Medicine and Dentistry, University of Rochester, Rochester, New York 14642

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Our investigation of bivalent ligands at μ, δ, and κ opioid receptors is focused on the preparation of ligands containing κ agonist and μ agonist/antagonist pharmacophores at one end joined by a chain containing the μ antagonist pharmacophores (naltrexone, naloxone, or nalbuphine) at the other end. These ligands were evaluated in vitro by their binding affinity at μ, δ, and κ opioid receptors and their relative efficacy in the [^{35}S]GTPγS assay.

Introduction

The heterodimerization of G-protein-coupled receptors has important implications because it represents another mechanism that could modulate receptor function and suggests additional targets for drug development.[1−3] There is now an increasing realization that activity at a single receptor is insufficient for modulating multiple targets for the treatment of a range of disorders.[4] Bivalent ligands have been developed for a variety of G-protein-coupled receptor targets including opioid,[5,6] adrenergic,[7] dopamine,[8] serotonin,[9] and muscarinic receptors[10] and also enzymes such as butyrylcholinesterase.[11] The methodical combination of pharmacophores from selective ligands that act on specific targets (receptors) is an important technique used for the generation of bivalent ligands. There is the possibility that the development of bivalent ligands in the opioid field that bridges the gap between binding sites on dimerized receptors will lead to a new generation of analgesic drugs that may not cause physical dependence or tolerance with chronic use.[12]

Previous reports from our laboratories indicated that the mixed action of the κ/μ agonist butorphan[a] (**1**) has a more promising profile of activity than the κ agonist/μ antagonist cyclorphan.[13,14] This finding led to the synthesis of a series of homobivalent ligands incorporating **1** as the pharmacophore connected by linking spacers of varying lengths.[15,16] It was observed that the affinity of these ligands was sensitive to the character and length of the spacer. The homobivalent ligand **9** containing **1** at both ends of the 10-carbon linking ester chain (Figure 1) ($K_i = 0.09$ nM at μ; $K_i = 0.049$ nM at κ) was the most potent ligand in this series.[15,16] In the course of the synthesis of a series of heterobivalent ligands containing **1** at one end and other pharmacophores at the other end of the linker, we also found that the stereochemistry of the pharmacophores, the N-substituents of the pharmacophore, ester linkages, and the spacer lengths were crucial factors for optimum interactions of such ligands at opioid receptor binding sites.[17] The spacer length for these compounds was dictated by the peak potency that was observed when the sebacoyl ester (10-carbon) unit was incorporated into the molecule. Multiple ligand **10** (Figure 1) derived from the linkage of a δ selective peptide antagonist Dmt-Tic (2′6′-dimethyl-L-tyrosine-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid) and a μ/k morphinan agonist **1** through a two-methylene spacer was found to maintain exactly the same characteristics as the two reference compounds.[18]

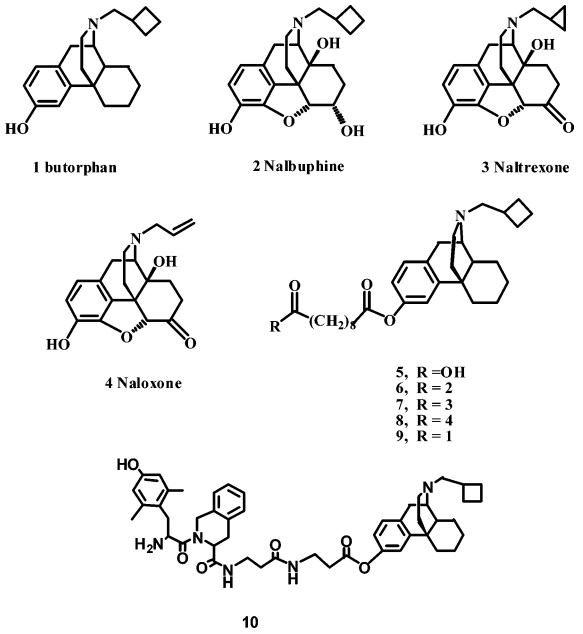

Figure 1. Structures of opioids and bivalent ligands.

Portoghese et al. has also reported a range of homo- and heterodimeric ligands with varying linker lengths designed to investigate pharmacodynamic and organizational features of opioid receptors.[19] For example, recently reported heterodimeric ligands containing δ antagonist (naltrindole) and κ_1 agonist (ICI-199,441) pharmacophores joined by variable-length oligoglycyl-based linkers were demonstrated to possess significantly greater potency and selectivity compared to their monomer congeners, providing further evidence for the opioid receptor hetero-oligomerization phenomena.[20]

To further investigate opioid bivalent ligands containing pharmacophores that have established $\kappa/\mu/\delta$ affinity, a combination of agonist and antagonist pharmacophores was employed

* To whom correspondence should be addressed. Phone: 617-855-3388. Fax: 617-855-2519. E-mail: Neumeyer@mclean.harvard.edu.
[†] Harvard Medical School.
[‡] University of Rochester.

[a] Abbreviations: butorphan, (−)-3-hydroxy-*N*-cyclobutylmethylmorphinan; U-50488, *trans*-(1*S*,2*S*)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]benzeneacetamide; U-69593, (+)-(5α,7α,8β)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4,5]dec-8-yl]benzeneacetamide; DAMGO, [D-Ala^2, *N*-Me-Phe^4,Gly-ol^5]enkephalin.

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Table 1. K_i for the Inhibition of μ, δ, and κ Opioid Binding to CHO Membrane by Heterodimeric Opioids

	$K_i \pm$ SEM (nM)			selectivity	
compd	[^{3}H]DAMGO (μ)	[^{3}H]U-69593 (κ)	[^{3}H]naltrindole (δ)	κ/μ	κ/δ
1 (butorphan)	0.23 ± 0.01	0.079 ± 0.003	5.9 ± 0.6	3	70
2 (nalbuphine)	0.89 ± 0.02	2.2 ± 0.01	240 ± 18	0.4	109
3 (naltrexone)	0.23 ± 0.05	0.25 ± 0.02	38 ± 3	1	152
4 (naloxone)	0.79 ± 0.02	1.1 ± 0.03	76 ± 2	1	69
5[a]	0.71 ± 0.02	0.29 ± 0.02	18 ± 1	2	62
6	0.46 ± 0.02	0.34 ± 0.008	28 ± 4	1	82
7	0.29 ± 0.0007	0.12 ± 0.002	15 ± 2	2	125
8	0.43 ± 0.001	0.13 ± 0.002	39 ± 6	3	300
9[a]	0.09 ± 0.004	0.05 ± 0.001	4.2 ± 0.4	2	84

[a] These compounds were reported as compounds **8** and **18** in ref 17.

Table 2. Agonist and Antagonist Properties of Compounds in Stimulating [^{35}S]GTPγS Binding Mediated by the κ Opioid Receptor[a]

compd	pharmacological properties	E_{max} (% maximal stimulation)	EC_{50} (nM)	I_{max} (% maximal inhibition)	IC_{50} (nM)
(−)-U-50488	agonist	110 ± 2	46 ± 16		
1 (butorphan)	agonist	80 ± 7	1.3 ± 0.4		
2 (nalbuphine)	agonist	81 ± 4	27 ± 3	NI	NI
3 (naltrexone)	antagonist	10 ± 2	NA	67 ± 4	200 ± 13
4 (naloxone)	agonist/antagonist	25 ± 2	10 ± 1	55 ± 3	320 ± 2
5	agonist	75 ± 1	28 ± 0.4	NI	
6	agonist/antagonist	67 ± 2	3.2 ± 0.4	39 ± 3	600 ± 170
7	agonist	76 ± 5	3.4 ± 0.9	NI	NI
8	agonist	86 ± 7	1.6 ± 0.2	NI	NI

[a] Membranes from CHO cells that stably expressed only the κ opioid receptor were incubated with varying concentrations of the compounds. The stimulation of [^{35}S]GTPγS binding was measured as described in the Experimental Section. To determine the antagonist properties of a compound, membranes were incubated with 100 nM κ agonist U-50488 in the presence of varying concentrations of the compound. The I_{max} is the maximal percent inhibition obtained with the compound. The IC_{50} is the concentration of compound needed to produce half-maximal inhibition. NI = no inhibition. NA = not applicable. Dashed lines indicate that the compound was not tested for antagonist properties because of its high E_{max}.

in the design of bivalent ligands for exploring the interaction between receptors. Here, we report the synthesis of three heterodimeric ligands derived from the linkage via a 10-carbon spacer of the μ antagonists nalbuphine (**2**), naltrexone (**3**), or naloxone (**4**) and a μ/κ agonist butorphan (**1**).

Chemistry

The heterodimeric ligands **6**−**8** were prepared by condensing the acid **5** with **2**, **3**, or **4** in the presence of DCC and DMAP as previously reported (Figure 1).[17]

Pharmacological Results and Discussion

Affinity and Selectivity of the Synthesized Ligands. All the novel heterodimer ligands were evaluated for their affinity at and selectivity for μ, δ, and κ human opioid receptors with Chinese hamster ovary (CHO) cell membranes stably expressing one of the human opioid receptors. The data are summarized in Table 1. For comparison purposes, opioid binding affinity data for **1**−**4** are included in Table 1. The monovalent ligand **5** and the homobivalent ligand **9** reported previously[17] were also included in order to evaluate the contribution of the spacer itself or the pharmacophores to binding.

Heterodimeric compounds such as **6** (**1** combined with **2**), **7** (**1** combined with **3**), and **8** (**1** combined with **4**) with a 10-carbon linking ester displayed slightly better affinity at μ (around 2-fold) compared to the monovalent ligand **5**. Compounds **7** and **8** showed lightly better affinity at the κ receptor (~2-fold), while **6** retained same affinity at κ, but all had lower affinity than **1**. From the data shown in Table 1, the heterodimer **6** showed increased affinities at μ (K_i = 0.46 nM) and a 6-fold increase (K_i = 0.34 nM) at κ receptors compared to **2**, while the affinity at the δ receptor was an average of the two monomeric ligands **1** and **2**. Similarly, the heterodimer **8** (containing **1** at one end and **4** at the other) displayed a 2-fold increase at μ (K_i = 0.43 nM) and a 10-fold increase at κ receptors (K_i = 0.13 nM) as well as a 2-fold increase at the δ receptor compared to **4**. It is interesting to note that **7** displayed almost identical affinities at all three opioid receptors as the monomer naltrexone.

Efficacy of Selected Ligands. To characterize the relative efficacy of the ligands, **1**, **2**, and the monovalent ligand **5** were selected for the [^{35}S]GTPγS assay. Table 2 shows the agonist and antagonist properties of the ligands in stimulating [^{35}S]GTPγS binding mediated by the κ opioid receptor. Ligand **6** produced similar maximal stimulation of [^{35}S]GTPγS binding (E_{max}) comparable to that of **1** and **2** but less than that of selective agonist U-50488. The EC_{50} of this ligand is slightly higher than **1** but much lower than **2**. Contrasted to the parent compounds **1** and **2**, ligand **6** can inhibit U-50488-stimulated [^{35}S]GTPγS binding although it had a high IC_{50}, which suggests that this ligand was a κ agonist/antagonist.

Ligand **7** produced similar maximal stimulation of [^{35}S]GTPγS binding (E_{max}) compared to that of **1**, but it was higher than that of **3**. Contrasted with the parent compound **3**, ligand **7** did not inhibit U-50488-stimulated [^{35}S]GTPγS, suggesting that this ligand was a κ agonist.

The agonist and antagonist properties of these ligands in stimulating [^{35}S]GTPγS binding mediated by the μ opioid receptor are shown in Table 3. Ligand **6** produced minimal stimulation of [^{35}S]GTPγS binding mediated by the μ receptor, while it produced complete inhibition (I_{max}) of the DAMGO stimulated [^{35}S]GTPγS binding comparable to that of **1** and **2**. These data indicate that ligand **6** is a μ antagonist. Ligand **7** produced similar maximal stimulation of [^{35}S]GTPγS binding (E_{max}) and maximal inhibition (I_{max}) of the DAMGO stimulated [^{35}S]GTPγS binding mediated by μ receptor comparable to that of **1** while producing higher maximal stimulation of [^{35}S]GTPγS binding (E_{max}) and lower maximal inhibition (I_{max}) of the

Table 3. Agonist and Antagonist Properties of Compounds in Stimulating [^{35}S]GTPγS Binding Mediated by the μ Opioid Receptor[a]

compd	pharmacological properties	E_{max} (% maximal stimulation)	EC_{50} (nM)	I_{max} (% maximal inhibition)	IC_{50} (nM)
DAMGO	agonist	120 ± 12	110 ± 9		
1 (butorphan)	agonist/antagonist	50 ± 3	1.6 ± 0.2	50 ± 3	20 ± 3
2 (nalbuphine)	agonist/antagonist	47 ± 3	14 ± 3	74 ± 1	110 ± 21
3 (naltrexone)	antagonist	6.7 ± 2	NA	79 ± 1	17 ± 5
4 (naloxone)	antagonist	13 ± 1	NA	92 ± 2	23 ± 2
5	agonist	110 ± 8	3.0 ± 0.6	NI	NI
6	antagonist	5.1 ± 2	NA	94 ± 1	18 ± 6
7	agonist/antagonist	43 ± 1	4.4 ± 0.3	50 ± 2	160 ± 44
8	agonist/antagonist	34 ± 1	2.0 ± 0.5	73 ± 1	25 ± 2

[a] Membranes from CHO cells that stably expressed only the μ opioid receptor were incubated with varying concentrations of the compounds. The stimulation of [^{35}S]GTPγS binding was measured as described in the Experimental Section. EC_{50} values were the concentration of compound needed to produce 50% of the E_{max}. When the E_{max} value was 30% or lower, it was not possible to calculate an EC_{50}. To determine the antagonist properties of a compound, membranes were incubated with 200 nM μ agonist DAMGO in the presence of varying concentrations of the compound. The I_{max} is the maximal percent inhibition obtained with the compound. The IC_{50} is the concentration of compound needed to produce half-maximal inhibition. NI = no inhibition. NA = not applicable. Dashed lines indicate that the compound was not tested.

DAMGO-stimulated [^{35}S]GTPγS binding mediated by μ receptor comparable to that of **3**. The data indicate that ligand **7** is a μ agonist/antagonist.

Conclusions

Heterodimeric ligands were synthesized containing κ agonist and μ agonist/antagonist pharmacophores at one end joined by a 10-carbon chain containing μ antagonists pharmacophores (naltrexone, naloxone, and nalbuphine) at the other end. These ligands were evaluated in vitro by their binding affinity at opioid receptors. Ligands **6**−**8** displayed slightly better or retained the same affinity at κ and μ receptors compared to the monovalent ligands **1**−**5**. Ligands **6**−**8** showed reduced affinity at the δ receptor compared to the monovalent ligands **1** and **5**. Functional assays showed that ligand **6** was a κ agonist/antagonist and μ antagonist while ligand **7** was a κ agonist and μ agonist/antagonist.

A possible explanation for the lower affinity at the κ receptor displayed by ligand **5** (**1** with alkyl side chain) in comparison to butorphan **1** would be that the side chain in **5** hinders robust binding of the ligand at the κ and μ receptor sites. Similarly the higher affinity at the κ receptor for the bivalent ligands **6**−**8** containing **1** (a high-affinity κ receptor ligand), a 10-carbon linking chain, and a μ antagonist ligand such as nalbuphine, naltrexone, or naloxone could be attributed to the higher binding affinity of **1** at the κ site and the μ antagonist (nalbuphine, naltrexone, or naloxone) at the μ receptor site.

These ligands retained or displayed better affinity at κ, μ, and δ receptors compared to the reference compounds. These heterodimeric ligands could serve as probes of the opioid receptor oligomerization phenomena and represent a useful starting point in the synthesis of a new generation of ligands endowed with analgesic effects with minor tolerance and dependence. Potential medications for cocaine abuse requiring κ agonist and μ antagonist[13,21] require further pharmacological studies to confirm these observations.

Experimental Section

Melting points were determined on a Thomas-Hoover capillary tube apparatus and are reported uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC300 spectrometer using tetramethylsilane as an internal reference. Element analyses, performed by Atlantic Microlabs, Atlanta, GA, were within 0.4% of theoretical values. Analytical thin-layer chromatography (TLC) was carried out on 0.2 mm Kieselgel 60F 254 silica gel plastic sheets (EM Science, Newark, NJ). Flash chromatography was used for the routine purification of reaction products. The column output was monitored by TLC.

General Procedure for the Preparation of Ligands 6−8. Acid **5** (0.6 mmol) and an appropriate opioid (0.5 mmol) were dissolved in anhydrous dichloromethane (15 mL) under nitrogen. A catalytic amount of 4-dimethylaminopyridine was added, followed by *N*,*N*′-dicyclohexylcarbodiimide (0.6 mmol). The solution mixture was stirred at room temperature overnight, the solid was filtered off, and the crude product was purified by column chromatography on silica gel (EtOAc/Et_3N, 100:1) to afford the corresponding bivalent ligands.

(5α,6α)-17-(Cyclobutylmethyl)-6,14-dihydroxy-4,5-epoxymorphinan-3-yl-17-(cyclobutylmethyl)morphinan-3-yl Sebacoylate (6). Colorless oil (40.4%). ^{1}H NMR (300 Hz, $CDCl_3$): δ 7.10 (d, J=8.4 Hz, 1H), 6.92 (d, J = 2.1 Hz, 1H), 6.85 (dd, J = 8.1, 2.1 Hz, 1H), 6.78 (d, J = 8.1 Hz, 1H), 6.65 (dd, J = 8.1, 2.4 Hz, 1H), 4.64 (d, J = 5.1 Hz, 1H), 4.60 (d, J = 4.8 Hz, 1H), 4.17−4.08 (m, 2H), 3.12 (d, J = 18.9 Hz, 1H), 3.02 (d, J = 18.9 Hz, 1H), 2.85−1.05 (m, 59H). ^{13}C NMR (75 Hz, $CDCl_3$): δ 172.3, 171.5, 149.2, 148.5, 141.9, 135.1, 132.9, 131.3, 130.7, 128.4, 121.5, 118.7, 118.4, 118.0, 91.6, 69.9, 66.5, 62.9, 61.4, 60.5, 55.8, 46.1, 45.6, 44.8, 43.6, 41.7, 37.7, 36.5, 34.8, 34.3, 33.8, 33.6, 32.6, 32.0, 30.8, 28.9, 28.8, 27.7, 26.8, 26.69, 26.65, 26.4, 26.3, 24.8, 24.7, 24.3, 23.9, 23.3, 22.0, 18.7, 18.6, 14.1. Anal. ($C_{52}H_{70}N_2O_7$·0.5H_2O) C, H, N.

17-(Cyclopropylmethyl)morphinan-3-yl(5α)-17-(cyclopropylmethyl)-14-hydroxy-6-oxo-4,5-epoxymorphiana-3-yl Sebacoylate (7). Colorless oil (48.4%). ^{1}H NMR (300 Hz, $CDCl_3$): δ 7.10 (d, J = 8.1 Hz, 1H), 6.92 (d, J = 2.1 Hz, 1H), 6.85 (dd, J = 8.1, 3 Hz, 2H), 6.68 (d, J = 8.4 Hz, 1H), 4.69 (s, 1H), 3.21 (d, J = 5.7 Hz, 1H), 3.12−0.84 (m, 55H), 0.57 (d, J = 7.5 Hz, 2H), 0.16 (d, J = 4.8 Hz, 2H). ^{13}C NMR (75 Hz, $CDCl_3$): δ 207.6, 172.4, 171.3, 149.2, 147.7, 142.0, 135.2, 132.6, 130.09, 130.07, 128.4, 122.8, 119.2, 118.5, 118.1, 90.6, 70.0, 61.9, 61.5, 59.2, 55.8, 50.6, 45.6, 44.9, 41.8, 37.7, 36.5, 36.0, 34.9, 34.4, 33.9, 31.2, 30.7, 29.03, 29.00, 28.9, 27.8, 26.7, 26.5, 24.8, 24.7, 24.4, 22.9, 22.1, 18.8, 9.3, 4.0, 3.8. Anal. ($C_{51}H_{66}N_2O_7$·1.5H_2O·2HCl) C, H, N.

(5α)-17-Allyl-14-hydroxy-6-oxo-4,5-epoxymorphinan-3-yl-17-(cyclobutylmethyl)morphinan-3-yl Sebacoylate (8). Pink solid (22.9%). ^{1}H NMR (300 Hz, $CDCl_3$): δ 7.10 (d, J = 8.1 Hz, 1H), 6.92 (s, J = 2.1 Hz, 1H), 6.85 (dd, J = 8.1,1.5 Hz, 2H), 6.70 (d, J = 8.4 Hz, 1H), 5.86−5.75 (m, 1H), 5.26−5.17 (m, 2H), 4.69 (s, 1H), 3.17−2.82 (m, 6H), 2.65−0.93 (m, 49H). ^{13}C NMR (75 Hz, $CDCl_3$): δ 183.6, 172.4, 171.3, 149.2, 147.7, 142.0, 135.1, 134.9, 132.6, 130.0, 129.9, 128.4, 122.9, 119.2, 118.4, 118.2, 118.1, 90.5, 70.1, 62.0, 61.4, 57.6, 55.8, 50.5, 45.6, 44.7, 43.1, 41.6, 37.7, 36.5, 36.0, 34.8, 34.3, 33.9, 32.7, 31.1, 30.5, 29.0, 28.9, 27.9, 27.8, 26.7, 26.5, 24.7, 24.6, 24.4, 24.0, 23.0, 22.1, 18.8. Anal. ($C_{50}H_{64}N_2O_7$·2H_2O) C, H, N.

Opioid Binding to the Human μ, δ, and κ Opioid Receptors. CHO cells stably transfected with the human κ opioid receptor (hKOR-CHO), δ-opioid receptor (hDOR-CHO), and the μ-opioid receptor (hMOR-CHO) were obtained from Drs. Larry Toll (SRI International, Palo Alto, CA) and George Uhl (NIDA Intramural Program, Bethesda, MD), respectively. The cells were grown in

100 mm dishes in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin−streptomycin (10 000 units/mL) at 37 °C in a 5% CO_2 atmosphere. The affinity and selectivity of the compounds for the multiple opioid receptors were determined by incubating the membranes with radiolabeled ligands and 12 different concentrations of the compounds at 25 °C in a final volume of 1 mL of 50 mM Tris-HCl, pH 7.5. Incubation times of 60 min were used for the μ-selective peptide [^{3}H]DAMGO and the κ-selective ligand [^{3}H]U-69593. A 3 h incubation was used with the δ-selective antagonist [^{3}H]naltrindole.

[^{35}S]GTPγS Binding Studies To Measure Coupling to G Proteins. Membranes from CHO cells stably expressing the human κ or μ opioid receptor were used in the experiments. Cells were scraped from tissue culture plates and then centrifuged at 1000*g* for 10 min at 4 °C. The cells were resuspended in phosphate-buffered saline, pH 7.4, containing 0.04% EDTA. After centrifugation at 1000*g* for 10 min at 4 °C, the cell pellet was resuspended in membrane buffer, which consisted of 50 mM Tris-HCl, 3 mM $MgCl_2$, and 1 mM EGTA, pH 7.4. The membranes were homogenized by with a Dounce homogenizer, followed by centrifugation at 40000*g* for 20 min at 4 °C. The membrane pellet was resuspended in membrane buffer, and the centrifugation step was repeated. The membranes were then resuspended in assay buffer, which consisted of 50 mM Tris-HCl, 3 mM $MgCl_2$, 100 mM NaCl, and 0.2 mM EGTA, pH 7.4. The protein concentration was determined by the Bradford assay using bovine serum albumin as the standard. The membranes were frozen at −80 °C until use.

CHO cell membranes expressing the human κ opioid receptor (15 μg of protein per tube) or μ opioid receptor (7.5 μg of protein per tube) were incubated with 12 different concentrations of the agonist in assay buffer for 60 min at 30 °C in a final volume of 0.5 mL. The reaction mixture contained 3 μM GDP and 80 pmol of [^{35}S]GTPγS. Basal activity was determined in the presence of 3 μM GDP and in the absence of an agonist, and nonspecific binding was determined in the presence of 10 μM unlabeled GTPγS. Then the membranes were filtered onto glass fiber filters by vacuum filtration, followed by three washes with 3 mL of ice-cold 50 mM Tris-HCl, pH 7.5. Samples were counted in 2 mL of Ecoscint A scintillation fluid. Data represent the percent of agonist stimulation [^{35}S]GTPγS binding over the basal activity, defined as {[(specific binding)/(basal binding)] × 100} − 100. All experiments were repeated at least three times and were performed in triplicate. To determine antagonist activity of a compound at the μ opioid receptors, CHO membranes expressing the μ opioid receptor were incubated with the compound in the presence of 200 nM of the agonist DAMGO. To determine antagonist activity of a compound at the κ opioid receptors, CHO membranes expressing the κ opioid receptor were incubated with the compound in the presence of 100 nM of the κ agonist U-50488.

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Supporting Information Available: Results from elemental analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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