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LETTER

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Characterization of 6α- and 6β-*N*-Heterocyclic Substituted Naltrexamine Derivatives as Novel Leads to Development of Mu Opioid Receptor Selective Antagonists

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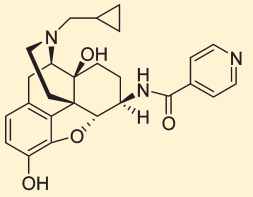
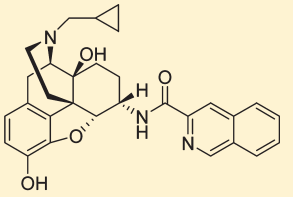
ABSTRACT: As important pharmacological probes, highly selective opioid receptor antagonists are essential in opioid receptor structural characterization and opioid agonist functional studies. At present, a nonpeptidyl, highly selective, and reversible mu opioid receptor antagonist is still not available. Among a series of novel naltrexamine derivatives that have been designed and synthesized following molecular modeling studies, two compounds, NAP and NAQ, were identified as leads based on the results of in vitro and in vivo pharmacological assays. Both of them displayed high binding affinity and selectivity to the mu opioid receptor. Further pharmacokinetic and functional characterization revealed that NAP seems to be a peripheral nervous system agent while NAQ seems to be a central one. Such characteristics provide two distinguished potential application routes for these two agents and their derivatives. These results also supported our hypothesis that they may serve as leads to develop more potent and selective antagonists for the mu opioid receptor.



NAP



NAQ

KEYWORDS: Mu opioid receptor, selective antagonist, NAP, NAQ

Opioid receptor selective antagonists are important pharmacological probes to study the structure−function relationship of each opioid receptor.[1−3] It has been demonstrated that, for many clinically available opiates, not only their analgesic function but also their side effects (such as addiction and abuse liability, respiratory depression, and tolerance) are primarily due to their interaction with the mu opioid receptor (MOR).[4−6] Yet the lack of a nonpeptidyl, highly selective, and potent MOR antagonist limits our understanding of the structure−function relationship of MOR. Currently available antagonists for the MOR carry certain characteristics that limit their application (Figure 1). For example, cyprodime[7] only possesses a moderate selectivity for the MOR over the delta opioid receptor (DOR) and kappa opioid receptor (KOR) (K_i value ratios are kappa/mu ≈ 45, delta/mu ≈ 40) with much lower affinity for the MOR than naloxone and naltrexone.[8] β-FNA, clocinnamox, and other irreversible antagonists for the MOR[9−11] bind covalently with the receptor, which largely limits their utility. Some currently available conformation-constrained peptides, for example, CTOP and CTAP, are highly selective and reversible MOR antagonists. They are relatively metabolically stable and have been used to target the MOR in in vitro and in vivo studies, while their limited bioavailability when administered peripherally hindered their potential medical applications.[12−19] Because the utility of antagonists as pharmacological tools requires both in vitro and in vivo activity, nonpeptide ligands are still preferred due to their ability to penetrate the central nervous system (CNS) and lesser vulnerability to metabolic inactivation compared to the peptide agents. Therefore, the development of a nonpeptidyl, potent, selective, and reversible antagonist for the MOR is highly desirable.

Recently, based on the "message-address concept" and molecular modeling studies, a series of 6α- and 6β-*N*-heterocyclic substituted naltrexamine derivatives were designed, synthesized, and characterized.[20] Among them, NAP and NAQ (Figure 1) seemed to be promising leads as MOR selective antagonists. NAP displayed high binding affinity for the MOR at K_i = 0.37 nM with over 700-fold selectivity for the MOR over the DOR and more than 150-fold selectivity over the KOR. The binding affinity of NAQ to MOR was 0.55 nM with over 200-fold selectivity for the MOR over the DOR and approximately 50-fold selectivity over the KOR. Meanwhile they were both low efficacy MOR agonists compared with DAMGO in the ^{35}S-GTP[γS]-binding assay with MOR expressing CHO cell lines.

To confirm ligand selectivity of NAP and NAQ in neuronal tissue and to avoid sole reliance on data from transfected cell lines, the ^{35}S-GTP[γS]-binding assay in membranes prepared

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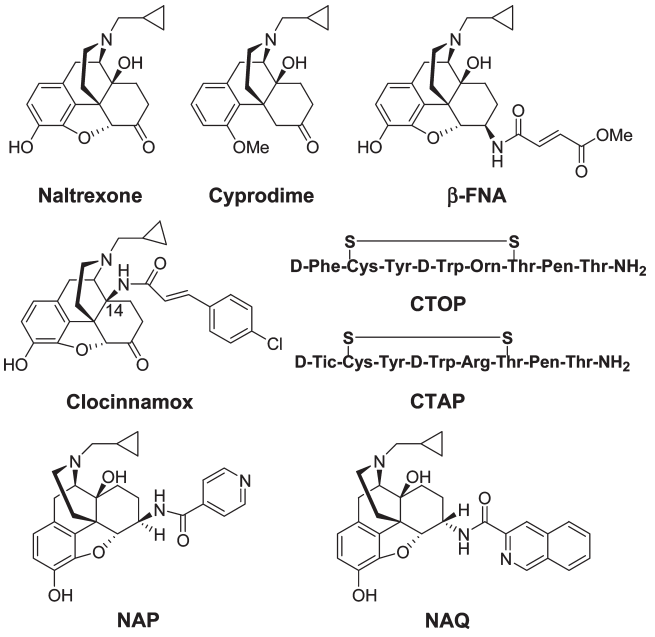

Figure 1. Chemical structures of known MOR antagonists, NAP, and NAQ.

Table 1. E_{max}, EC_{50}, and K_i Values of NAP and NAQ from ^{35}S-GTP[γS]-Binding Assays in Rat Thalamus[a]

compd	K_i (nM) ± SEM vs DAMGO (10 μM)	EC_{50} (nM)	E_{max} (% stim)	E_{max} (% of 10 μM DAMGO)
NAP	4.8 ± 1.6	54.6 ± 36.9	9.5 ± 1.2	16.7 ± 2.0
NAQ	3.5 ± 2.2	4.2 ± 1.8	8.7 ± 0.7	15.5 ± 1.8

[a] Thalamic membranes were incubated with 30 μM GDP, 0.1 nM ^{35}S-GTP[γS], and varying concentrations of NAP or NAQ in the absence (EC_{50}, E_{max}) or presence (K_i) of 10 μM DAMGO. Data are mean values ± SEM (n = 3).

from rat thalamus, which expresses mostly the MOR, was conducted. Both NAP and NAQ acted as potent antagonists to inhibit DAMGO-stimulated ^{35}S-GTP[γS]-binding, as shown by the low nanomolar K_i values obtained when varying concentrations of these leads were coincubated with DAMGO (Table 1). However, these K_i values for DAMGO antagonism by NAP and NAQ in thalamus (4.8 and 3.5 nM, respectively) were approximately 6-fold greater than the previously reported MOR binding K_i values from MOR-expressing CHO cells (0.37 and 0.55 nM, respectively).[20] This discrepancy could be due to the different assay conditions, such as the presence of sodium and guanine nucleotides in the ^{35}S-GTP[γS]-binding assay, which would be expected to modestly inhibit partial agonist affinity. Indeed, the EC_{50} value for NAQ-stimulated ^{35}S-GTP[γS] binding (4.2 nM) was similar to its K_i value to inhibit DAMGO-stimulated ^{35}S-GTP[γS] binding in this tissue, although the EC_{50} value of NAP (54.6 nM) was approximately 10-fold greater. However, it is difficult to obtain accurate EC_{50} values for these compounds in thalamus due to the very low level of stimulation produced by the compounds alone. Accordingly, when incubated alone (in the absence of DAMGO), both lead compounds produced low levels of stimulation (~15% of DAMGO), indicating that they are

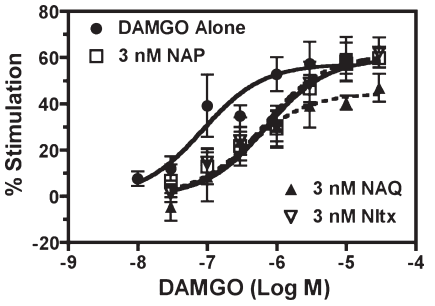

Figure 2. Competitive antagonist functional assay of naltrexone, NAP, and NAQ against DAMGO.

Table 2. Competitive Antagonism of DAMGO-Stimulated ^{35}S-GTP[γS] Binding by Naltrexone (NTX), NAP, and NAQ in Low MOR-Expressing CHO Cells[a]

compd	DAMGO EC_{50} (nM)	DAMGO E_{max} (% stim)	EC_{50} ratio	K_e (nM)
DAMGO alone	159.5 ± 53.5	59.1 ± 7.6	N/A	N/A
DAMGO + NTX	1049 ± 510	61.7 ± 4.6	9.0 ± 2.8	0.56 ± 0.14
DAMGO + NAP	1450 ± 878	62.0 ± 3.2	7.2 ± 2.0	0.83 ± 0.25
DAMGO + NAQ	846 ± 448	46.6 ± 2.6	5.6 ± 1.3	0.59 ± 0.12

[a] Cell membranes were incubated with 10 μM GDP, 0.1 nM ^{35}S-GTP[γS], and varying concentrations of DAMGO in the absence or presence of 3 nM naltrexone (NTX), NAP, or NAQ. Data are mean values ± SEM (n = 5−7).

partial agonists of low relative efficacy in thalamus. These results are consistent with the ones previously observed in an MOR-transfected CHO cell line,[20] and confirm that these lead compounds are low efficacy partial agonists of the MOR.

To further characterize the competitive antagonist property of both leads, a functional assay was conducted to determine the ability of these compounds to competitively right-shift the DAMGO concentration-effect curve. For these experiments, a CHO cell line engineered to express relatively low levels of the MOR (0.4 pmol per mg of membrane protein was used),[21] so that NAP and NAQ would act as pure antagonists and readily allow determination of parallel rightward shifts of the DAMGO curve. This MOR expression level was similar to a previously reported MOR B_{max} values (0.2−0.4 pmol/mg) from multiple regions of mouse brain.[22] When incubated with membranes from these cells in the absence of DAMGO, neither NAP nor NAQ produced any stimulation of ^{35}S-GTP[γS] binding at ligand concentrations up to 100 nM (data not shown). A concentration of 3 nM NAP and NAQ was selected for co-incubation with DAMGO and compared to the effect of 3 nM naltrexone as a positive control. The results are summarized in Figure 2 and show that both NAP and NAQ acted as competitive antagonists against DAMGO, similarly to naltrexone. Although NAQ seemed to reduce the DAMGO E_{max} value slightly, suggesting a noncompetitive component, the DAMGO E_{max} values obtained in the presence of any of the three antagonists were not significantly different from that of DAMGO alone, as determined by ANOVA with posthoc Dunnett's test (Table 2). The calculated K_e values for NAP and NAQ (0.83 and 0.59 nM, respectively) were similar to that of naltrexone (0.56 nM) and to previously reported MOR binding K_i values for these compounds (0.37 and 0.55 nM, respectively). These results indicate

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Table 3. E_{max} and EC_{50} Values of SNC80, NAP, and NAQ in DOR-Expressing CHO Cells[a]

compd	EC_{50} (nM)	E_{max} (% stim)	E_{max} (% of 3 μM SNC80)
SNC80	12.2 ± 2.6	395.2 ± 17.7	100 ± 4.5
NAP	15.2 ± 15.2	36.6 ± 11.6	10.2 ± 3.1
NAQ	98.6 ± 23.7	189.0 ± 25.9	53.5 ± 5.4

[a] Cell membranes were incubated with 20 μM GDP, 0.1 nM ^{35}S-GTP[γS], and varying concentrations of SNC80, NAP, or NAQ. Data are mean values ± SEM (n = 3).

Table 4. E_{max} and EC_{50} Values of U50,488H, NAP, and NAQ in KOR-Expressing CHO Cells[a]

compd	EC_{50} (nM)	E_{max} (% Stim)	% max of U50,488H
U50,488H	33.3 ± 8.7	173.2 ± 18.1	100.0 ± 10.5
NAP	28.8 ± 14.4	79.5 ± 8.7	45.5 ± 4.4
NAQ	10.9 ± 7.9	23.6 ± 5.3	13.1 ± 2.0

[a] Cell membranes were incubated with 20 μM GDP, 0.1 nM ^{35}S-GTP[γS], and varying concentrations of SNC80, NAP, or NAQ. Data are mean values ± SEM (n = 3).

that NAP and NAQ can act as competitive, high affinity MOR antagonists.

While both compounds acted as MOR-selective ligands, one of the concerns was whether they would act as potent agonists of the DOR and/or KOR with high efficacy, which would limit their usefulness. From the results of our studies (Tables 3 and 4), NAP acted as a partial agonist of the DOR with low efficacy and a partial agonist of the KOR with moderate efficacy but low potency. NAQ acted as a partial agonist of the DOR with moderate efficacy and low potency and as a partial agonist of the KOR with low efficacy. It is interesting that NAP showed somewhat higher DOR and KOR potency than predicted from receptor binding assay results. It is possible that this ligand binds with higher affinity under the conditions of the ^{35}S-GTP[γS]-binding assay (e.g., with sodium and guanine nucleotides present). In general, these results show that both leads have low to moderate efficacy at the DOR and KOR, and this information is encouraging to our next generation of molecular design.

Primarily the in vivo pharmacological evaluation of the NAP and NAQ was focused on acute antinociceptive agonistic and antagonistic effects in the tail immersion test in mice. It was noticed that both NAP and NAQ acted as potent antagonists in the in vivo tests without any significant agonist activity even at the very high dose of 100 mg/kg. As antagonists, their potency (NAP AD_{50} was 4.98 mg/kg and NAQ was 0.46 mg/kg) was lower than that of naloxone (at 0.05 mg/kg).[20] Apparently, this is not consistent with their in vitro high potency.

To understand the discrepancy of NAP and NAQ between their in vitro and in vivo properties, a preliminary pharmacokinetic permeability study was conducted (Figure 3). The apparent permeability of NAP was significantly lower than that of NAQ and naltrexone. When NAP was tested in the presence of P-glycoprotein selective substrate GF120918 in the same system, its apparent permeability improved dramatically and was similar to that of naltrexone. This suggested that NAP may be a substrate of P-glycoprotein, thus failing to penetrate the blood-brain barrier as well as naltrexone, thereby decreasing its

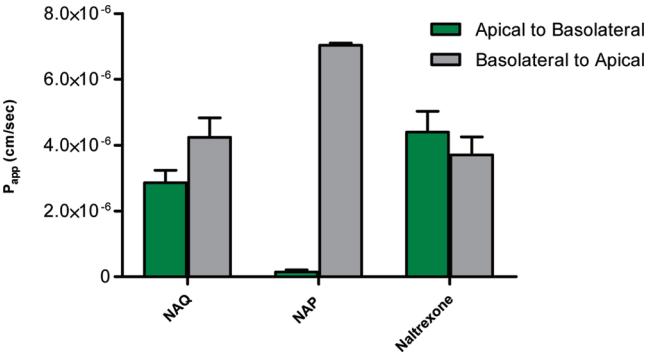

Figure 3. Bidirectional transport of NAP, NAQ, and naltrexone in Caco-2 cells.

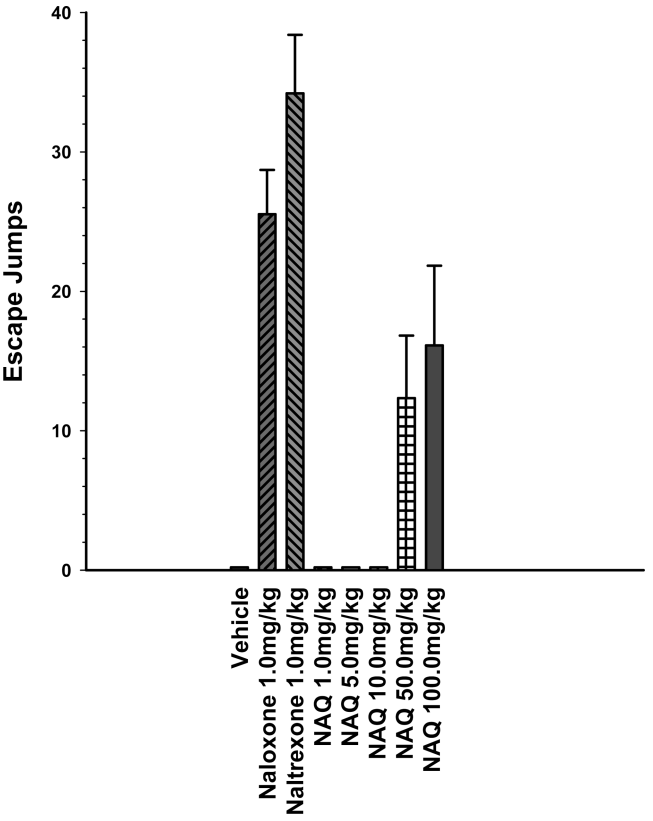

Figure 4. NAQ antagonism in withdrawal assay in chronic morphine exposed mice: Escape jumps.

apparent CNS activity in the in vivo assays. As a result, NAP might be applied as a lead to develop peripheral MOR selective antagonists.

To further characterize the CNS activity of NAQ, a comparative opioid withdrawal precipitation study was conducted with this lead compound. As shown in Figures 4 and 5, NAQ exhibits an interesting profile compared with the well-known opioid antagonists, naloxone and naltrexone. In morphine pelleted mice, NAQ (10 mg/kg) did not precipitate jumps and only modestly precipitated wet-dog shakes at a dose 10 times higher than that of naloxone or naltrexone. Moreover, even at a 100-fold greater dose than that of naloxone or naltrexone, NAQ (100 mg/kg) did not exhibit full activity to precipitate these opiate withdrawal signs. Interestingly, a similar phenomenon was observed

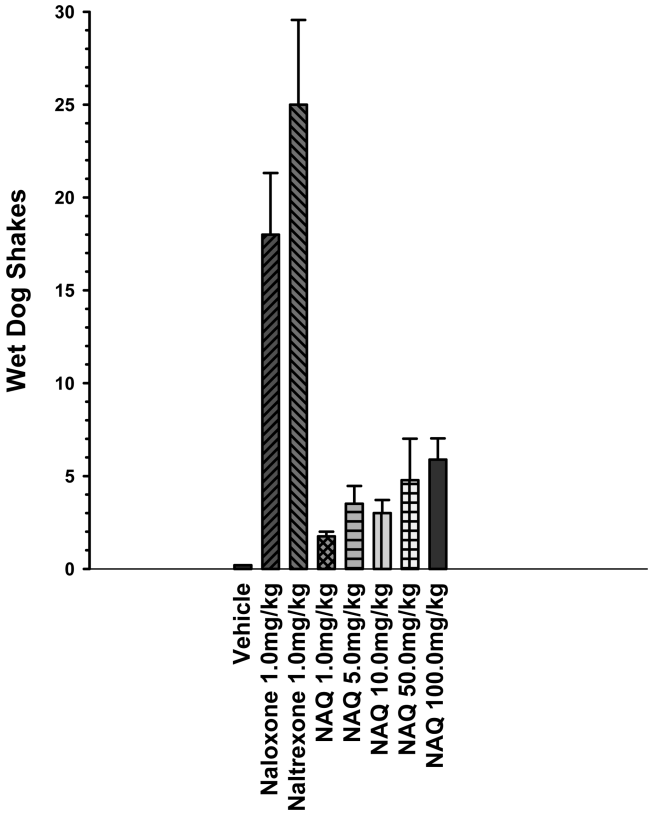

Figure 5. NAQ antagonism in withdrawal assay in chronic morphine exposed mice: Wet-dog shakes.

previously with 6β-naltrexol,[23] although the substitution on the 6-position of the naltrexone skeleton is totally different between these two ligands. The present results show that NAQ can act as a low efficacy partial agonist or antagonist depending on MOR expression level or tissue type, which along with potential pharmacokinetic differences might explain its reduced potency to induce withdrawal relative to naloxone and naltrexone. The reduction of withdrawal effects associated with NAQ indicated that NAQ could be used as a lead to develop MOR antagonists with some advantages to treat opioid overdose, abuse and addiction.

In summary, based on pharmacological study results, two novel ligands have been characterized as MOR selective ligands with low efficacy at the mu opioid receptor. They both seem to be promising leads to further develop highly potent MOR-selective antagonists. Their differential pharmacological and pharmacokinetic profiles may substantiate their potential application either in treating opioid receptor related peripheral or CNS system diseases.

METHODS

Chemistry. The compounds were synthesized following the procedures in ref 20.

Pharmacology. *^{35}S-GTP[γS]-Binding Assays in Rat Thalamus Membranes.* The ^{35}S-GTP[γS]-binding assay was conducted in rat thalamus membranes (30 μg protein). Varying concentrations of NAP or NAQ were incubated with 30 μM GDP and 0.1 nM ^{35}S-GTP[γS] in assay buffer (50 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 0.2 mM EGTA and 100 mM NaCl) in the presence and absence of 3 μM DAMGO for 2 h at 30 °C. Nonspecific binding was determined with 10 μM unlabeled GTP[γS]. Some samples included DAMGO (3 μM) alone as a maximally effective concentration of a full agonist of the mu opioid receptor, for determination of relative efficacy values. The incubation was terminated by rapid vacuum filtration through GF/B glass fiber filters. Bound radioactivity was determined by liquid scintillation spectrophotometry. Additional methodological details of the assay were described previously (ref 21).

^{35}S-GTP[γS]-Binding Assays in the Low MOR-Expressing CHO Cell Line. Cell membranes (10 μg protein) were incubated with 10 μM GDP and 0.1 nM ^{35}S-GTP[γS] in assay buffer in the presence and absence of varying concentrations of DAMGO with and without naltrexone (NTX), NAP, or NAQ for 90 min at 30 °C. Nonspecific binding was determined with 10 μM unlabeled GTP[γS]. The reaction was terminated and bound radioactivity determined as described above and in ref 21.

^{35}S-GTP[γS]-Binding Assays in DOR- or KOR-Expressing CHO Cell Lines. Cell membranes (10 μg protein) were incubated with 20 μM GDP and 0.1 nM ^{35}S-GTP[γS] in assay buffer in the presence and absence of varying concentrations of NAP, NAQ, or SNC80 (DOR) or U50,488H (KOR) for 90 min at 30 °C. Nonspecific binding was determined with 10 μM unlabeled GTP[γS]. The reaction was terminated and bound radioactivity determined as described above and in ref 21.

Data Analysis of ^{35}S-GTP[γS]-Binding Assays. All samples were assayed in triplicate and repeated at least twice for a total of $\geq$3 independent determinations. Results are reported as mean $\pm$ SEM. Concentration-effect curves were fit by nonlinear regression to a one-site binding model, using GraphPad Prism software, to determine EC_{50} and E_{max} values. IC_{50} values were obtained from Hill plots, analyzed by linear regression using Microsoft Excel software. K_i values were determined from IC_{50} values using a modification of the Cheng−Prusoff equation: $K_i = IC_{50}/1 + ([L]/EC_{50})$, where [L] is the concentration of DAMGO and EC_{50} is the concentration of DAMGO at which half-maximal stimulation of ^{35}S-GTP[γS] binding was achieved. K_e values were determined using the equation K_e = [Ant]/DR-1, where [Ant] is the concentration of antagonist and DR is the ratio of the DAMGO EC_{50} of values in the presence and absence of antagonist. Inferential statistics were applied where appropriate by performing ANOVA with posthoc Dunnett's test, using GraphPad Prism software.

NAQ Antagonism in Withdrawal Assay in Chronic Morphine Exposed Mice: Escape Jumps and Wet-Dog Shakes. *Animals.* Male Swiss Webster mice (Harlan Laboratories, Indianapolis, IN) weighing 25−30 g were housed six to a cage in animal care quarters and maintained at 22 $\pm$ 2 °C on a 12 h light-dark cycle. Food and water were available ad libitum. The mice were brought to a test room (22 $\pm$ 2 °C, 12 h light-dark cycle), weighed, and marked for identification and then allowed 18 h to recover from transport and handling. Protocols and procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Commonwealth University Medical Center and comply with the recommendations of the IASP (International Association for the Study of Pain).

Surgical Implantation of Morphine Pellets. Mice were anesthetized with 2.5% isoflurane (Baxter, Deerfield, IL) before shaving the hair around the base of the neck. Adequate anesthesia was noted by the absence of the righting-reflex and lack of response to toe-pinch, according to IACUC guidelines. The skin was scrubbed with 10% povidone iodine (General Medical Corp., Prichard, WV) and rinsed with alcohol before making a 1 cm horizontal incision at the base of the neck. The underlying subcutaneous space toward the dorsal flanks was loosened using sterile hemostats. Maintenance of a stringent aseptic surgical field minimized any potential contamination of the pellet, incision, and subcutaneous space. A 75 mg morphine pellet was inserted in the space before closing the site with Clay Adams Brand, MikRon AutoClip 9 mm wound clips (Becton Dickinson and Co., Sparks, MD) and again applying iodine to the surface. The animals were allowed to recover in their home cages where they remained throughout the experiment.

Antagonist-Precipitated Withdrawal. Withdrawal was precipitated in mice at 72 h from pellet implantation with naloxone (1.0 mg/kg, s.c.), naltrexone (1.0 mg/kg, s.c.), NAQ (1.0−100.0 mg/kg s.c.), or NAP (0.50−100.0 mg/kg s.c.) and was scored according to the method described by Vaupel et al. (1997).[24] Mice were allowed for 30 min to habituate to an open-topped, square, clear Plexiglas observation chamber (26 × 26 × 26 cm^3) with lines partitioning the bottom into quadrants then given antagonist. Withdrawal commenced within 1 min after antagonist administration. Escape jumps and wet-dog shakes were quantified by counting their occurrences over 20 min.

Drugs and Chemicals. The 75 mg morphine pellets were obtained from the National Institute on Drug Abuse (NIDA), Bethesda, MD. Naloxone and naltrexone were obtained from Sigma Life Science (St. Louis, MO), and NAP and NAQ were synthesized in one of our laboratories. All antagonists were dissolved in pyrogen-free isotonic saline (Baxter Healthcare, Deerfield, IL).

Experimental Design and Statistical Analysis. Morphine-pelleted mice were placed into groups of 4−8 mice per group. Groups were administered saline (control), naloxone, naltrexone, or NAQ or NAP at designated doses according to body weights (g). After counting withdrawal signs, mice were euthanized by CO_2 according to AVMA Guidelines on Euthanasia (2007).

Data are expressed as mean values ± SEM. Analysis of variance (ANOVA) followed by the post hoc Dunnett test was performed to assess significance using the Instat 3.0 software (GraphPad Software, San Diego, CA). The withdrawal signs of jumping and wet -dog shakes were counted and subjected to two-factor ANOVA.

Pharmacokinetics. *Bidirectional Transport of NAP, NAQ, and naltrxeone in Caco-2 Cells.* Caco-2 cells (passages 39−45; ATCC, Manassas, VA) were cultured and plated on Transwell filters, and directional permeability studies were performed as previously described.[1] Briefly, cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and supplemented with penicillin/streptomycin and nonessential amino acids for 21−25 days after seeding on 12 mm 0.4 μm #3460 Transwell-Clear inserts (Fisher Scientific). Drug solutions in Hank's balanced salt solution were added to either the apical or basolateral chambers, with sampling up to 2 h. Samples were mixed with acetonitrile (25 μL) and centrifuged. Separation was performed by HPLC using an Alltima HP C18 column (3 μm, 4.6 × 100 mm; Alltech, Deerfield, IL) and gradient elution (aqueous 0.05% trifluoroacetic acid and acetonitrile, from 90:10 to 50:50) with detection by UV absorbance at 232 nm.

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Author Contributions

Y.Z. conceived and overlooked the project and drafted and finalized the manuscript. G.L. and Y.Y. conducted the chemical synthesis of the ligands studied. H.H. conducted in vitro pharmacological study. D.E.S. designed, supervised, and analyzed the in vitro pharmacological study. D.L.S. and P.K. conducted some in vivo pharmacological study. K.L.S. and W.L.D. designed and overlooked the in vivo pharmacological study. P.M. conducted the pharmacokinetic study. P.M.G. designed the pharmacokinetic study. D.E.S., K.L.S., W.L.D., and P.M.G. helped revise the manuscript.

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DISCLOSURE

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute on Drug Abuse or the National Institutes of Health.

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