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6β-N-Heterocyclic Substituted Naltrexamine Derivative BNAP: A Peripherally Selective Mixed MOR/KOR Ligand

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Abstract

The 6β-N-heterocyclic naltrexamine derivative, NAP, has been demonstrated to be a peripherally selective mu opioid receptor modulator. To further improve peripheral selectivity of this highly potent ligand, its pyridal ring was quaterinized with benzyl bromide to produce BNAP. In radioligand binding assay, the Ki of BNAP for MOR was 0.76 ± 0.09 nM and was >900 fold more selective for MOR than DOR. The Ki for KOR was 3.46 ± 0.05 nM. In [^{35}S]GTPγS ligand stimulated assay, BNAP showed low agonist efficacy with 14.6% of the maximum response of DAMGO with an EC_{50} of 4.84 ± 0.6 nM. However, unlike its parent compound NAP, BNAP displayed partial agonist activity at KOR with % maximum response at 45.9 ± 1.7% of U50,488H. BNAP did not reverse morphine-induced antinociception when administered subcutaneously but did antagonize when administered intracerebroventricularly. BNAP antagonized morphine-induced contractions of the circular muscle in mice colon. BNAP inhibition of field-stimulated contractions in longitudinal muscle strips for the guinea-pig ileum were also blocked by nor-BNI, a kappa opioid receptor antagonist. BNAP induced inhibition of acetic acid induced abdominal stretching in chronic morphine treated mice. These findings suggest that BNAP is a dual MOR antagonist/KOR agonist and may have functional use in irritable bowel patients.

Keywords

periphery selectivity; mixed opioid ligand; irritable bowel syndrome; visceral pain; opioid induced constipation

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Supporting Information Results of analytic parameters for BNAP including ^{1}H NMR, 13C NMR, MS, IR and HPLC as Supporting Information.

Author Contribution Y.Z. and H.I.A conceived and oversaw the project. Y.Z., H.I.A., D.E.S., and W.L.D. designed the experiments. D.A.W. conducted the chemical synthesis. D.A.W. and Y. Zheng drafted the manuscript. Y.Y. conducted radioligand binding assays under supervision of D.E.S. S.A.Z. finished the modeling studies under supervision of Y.Z. D.A.W., B.G.D., D.L.S., and K.L.S conducted organ bath and animal studies. H.I.A. and Y.Z. analyzed the data and discussed the results. Y.Z. and H.I.A. revised the manuscript.

INTRODUCTION

Opioids are the most commonly prescribed medications for the treatment of malignant and non-malignant pain. Despite their proven analgesic efficacy, significant side effects such as addiction, nausea, dizziness, urinary retention, and constipation limit the clinical utility of these drugs, particularly with chronic use.[1] Among these side effects, opioid-induced constipation (OIC) is one of the most common and distressing. It is estimated that up to 40% of patients on opioid treatment experience OIC.[2] OIC is often so debilitating that patients forego opioid treatment and suffer with their pain. The analgesic effects of opioids are primarily facilitated through activation of mu opioid receptors (MORs) located on neurons within the central and peripheral nervous system. Within the enteric nervous system, activation of MORs mediate the constipating effects of opioids.[3] Propulsion within the gastrointestinal (GI) tract is the consequence of the concerted actions of circular muscles and longitudinal muscles that grind and propel the food bolus forward. Each of these actions is controlled by neurons of the myenteric plexus. Inhibition of neurotransmitter release is a primary mechanism by which MORs inhibit peristalsis.

Treatment of OIC is a significant challenge as laxative therapy is often ineffective.[4] Peripheral MOR antagonists have been recently developed that can prevent or reverse OIC.[5] Methylnaltrexone (MNTX, **2**, Figure 1) and Alvimopan (**3,** Figure 1) are two peripherally selective MOR antagonists approved by the FDA for the treatment of OIC.[6] However, MNTX suffers from low activity at producing spontaneous bowel movements and prolonged use of Alvimopan increases the risk of myocardial infarction.[7] Therefore, the development of peripherally selective MOR antagonists would be of great benefit to patients suffering from OIC. Such compounds will also aid in further elucidating the role of MORs in OIC and other gastrointestinal neuropathies.

We have previously identified the 6β-N-heterocyclic substituted naltrexamine derivative NAP (**4,** Figure 1) as a novel MOR antagonist with peripheral selectivity and a 300 fold greater potency than methylnaltrexone.[7] Further studies in the pharmacology of NAP demonstrated that it has mixed partial agonist and antagonist activity, with a bias towards antagonism of the β-arrestin2 pathway.[8] Given these promising results we sought to improve the peripheral selectivity of NAP via structural modifications and examine the pharmacology of the new derivative. This report describes our efforts towards peripheral selectivity through selective alkylation of the pyridal nitrogen of NAP and characterizing its pharmacological profile within the gastrointestinal tract.

RESLTS AND DISCUSSION

It is generally agreed that activation of MORs on the enteric neurons of the GI tract play a critical role in the development of OIC. In this study, we designed and synthesized a peripherally selective 6β-N-heterocyclic naltrexamine derivative and investigated its pharmacology in vitro and in vivo as a selective MOR antagonist. BNAP was synthesized in one step via alkylation with benzyl bromide in high yield and purity. Having synthesized and structurally characterized BNAP, radioligand binding and receptor functional studies were carried out at MOR, KOR and DOR to determine the impact of the benzyl group on binding

affinity, selectivity, and functional activity at each of the three opioid receptors. The binding affinity and functional activity for BNAP and NAP at each receptor was determined as previously described[9] and the results are shown in Table 1. BNAP maintained sub-nanomolar affinity for MOR with a Ki = 0.76 ± 0.09 nM compared with that of the parent compound NAP (Ki = 0.37 ± 0.07). BNAP showed a >900 fold selectivity for the MOR over the DOR. Interestingly its selectivity over the KOR was less than the parent compound NAP. Ligand-stimulated [^{35}S]GTPγS binding was then utilized to determine the relative efficacy of BNAP to activate MOR, DOR and KOR. At the MOR, BNAP showed low agonist efficacy with a response only 14.6 ± 0.9% of the maximum response for the full agonist DAMGO (NAP, 22.72 ± 0.84%), with an EC_{50} value of 4.84 ± 0.60 nM (NAP, 1.14 ± 0.38 nM). Similarly at the DOR, BNAP behaved as a low efficacy agonist with a % maximum response at 9.2 ± 2.2% (NAP, 10.2 ± 3.1%) when compared to the full agonist SNC80, and also low potency to activate the DOR (EC_{50} = 107.6 ± 69.0 nM; NAP, 15.2 ± 15.2 nM). In conjunction with the lower selectivity of BNAP at KOR, BNAP also displayed partial agonist activity at KOR with a % maximum response at 45.9 ± 1.7% when compared to the full agonist U50,488H (NAP = 45.5 ± 4.4%). The potency for BNAP relative to NAP was greater at KOR, with an EC_{50} value of 2.9 ± 1.1 nM compared to 28.8 ± 14.4 nM for NAP. Interestingly, despite the 4.6-fold lower binding affinity of BNAP for the KOR relative to the MOR, this compound had approximately equal potency to activate these two receptors in the functional assay. Compared to NAP, the selectivity profile for BNAP was changed from MOR selective to MOR/KOR dually selective.

To help understand possible reasons for the observed changes in selectivity of BNAP over the parent compound, docking studies about BNAP in the MOR, DOR and KOR antagonist-bound crystal structures were undertaken. As seen in Figure 2 the binding pocket for the morphinan skeleton of BNAP across the three opioid receptors was highly conserved. However, differences in the residues surrounding the alkylated pyridine provided insight into potential changes in affinity and selectivity. Within the MOR, Glu229 may interact with the positively charged nitrogen atom of the pyridyl ring via ionic interactions. Within the KOR and DOR Asp223 and Asp210 occupy these positions respectively. The shorter length of the aspartate residue would not allow for such stabilizing ionic interactions. However, in KOR plausible hydrogen bonding interactions between the pyridyl nitrogen and Tyr219 could provide similar stabilization as that seen in MOR. These studies showed that the binding pocket of MOR, DOR, and KOR can accommodate the added benzyl ring; however, the introduction of the positive charge on the pyridyl nitrogen may be responsible for the reduced MOR/KOR selectivity due to the now possible hydrogen bonding interactions within the KOR.

To further examine the pharmacology of BNAP, a combined in vivo and in vitro approach was taken. We first determined the effects of BNAP on morphine (**1**, Figure 1) antinociception following two routes of administration. This set of experiments was designed to give us insight into the potency of BNAP within the CNS by measuring its effect on morphine antinociception in the warm water tail flick assay. As shown in Figure 3 at 10mg/kg BNAP s.c. alone did not produce significant antinociceptive effects whereas morphine dose dependently induced antinociception. BNAP at 10 mg/kg did not

significantly antagonize morphine antinociception in the warm water tail flick assay at any of the morphine doses tested. These results supported our hypothesis that quaternization of the pyridyl nitrogen limited activity of BNAP to the periphery. To further determine if BNAP behaves as an MOR antagonist, BNAP was administered via intracerebroventricular (ICV) injection (thus directly into the CNS) before assessing activity in the warm-water tail-flick assay. Under this experimental paradigm BNAP alone at 1.0 μg/5 μL did not produce significant antinociception when compared to morphine alone (Figure 4). A dose dependent reversal of morphine antinociception was observed with increasing concentrations of BNAP with complete reversal occurring at 0.3 μg/5 μL ICV of BNAP. These results are consistent with the in vitro functional assay data identifying BNAP as an MOR antagonist. These data in conjunction with the subcutaneously administered BNAP experiments supported our hypothesis that quaternization of the pyridyl nitrogen with the benzyl group limits CNS penetration of BNAP. BNAP did not antagonize morphine-induced thermal antinociception when administered subcutaneously, but reversed morphine antinociception when administered via intracerebroventricular (ICV) route. Beyond antagonism of morphine-induced thermal antinociception, another important observation from these studies was lack of thermal antinociception displayed by BNAP alone irrespective of the route of administration. This result was significant as the radioligand binding and in vitro functional studies showed BNAP to have moderate potency and efficacy as a KOR agonist considering the fact that KOR agonists are typically known to have psychotomimetic side effects that accompany their analgesic properties.[10] Further expanded dose response study would be deemed in due course.

Recently, there has been a renewed interest in understanding the regional differences in responsiveness of the rodent GI tract to various drug classes.[11a, 12] Ono et al. have recently suggested that regions more sensitive to the effects of morphine play a key role in the development of OIC.[11a] Radioligand binding and in vitro functional studies showed that NAP and BNAP had comparable affinity and similar efficacy for the MOR in the [^{35}S]GTPγS-binding assay. Thus, as expected, BNAP should demonstrate similar inhibitory potency as that of the parent compound (NAP) in the isolated tissue preparations. One of the approaches used to study the effects of opioid ligands on the GI tract is the examination of isometric tension recordings from isolated tissues preparations, which our laboratory has utilized extensively to study the development of opioid tolerance in the mouse GI tract.[11b, 13] We have previously shown that morphine induced contractions of the circular muscle and work by Ono has suggested that the circular muscle contractions played a key role in the constipation effects of opioids.[11] Therefore we examined the effects of BNAP on morphine induced circular muscle contractions of the mouse colon. As expected, morphine dose dependently induced contractions in both the distal and proximal colon sections (Figure 5A). The proximal colon responded to morphine at concentrations ten times lower than those that produced equal effects in the distal colon, suggesting that this portion of the colon is more sensitive to morphine's effects (Figure 5B). The pD_2 value for morphine in naive tissue in the distal colon was 6.0 ± 0.1 which were consistent with previous findings.[11b] In the proximal colon the morphine pD_2 was 6.8 ± 0.1, nearly ten-fold difference in morphine sensitivity. Having observed these differences in response to morphine in the distal and proximal sections the functional activity of BNAP was examined in both preparations and

compared to the parent compound NAP to determine possible differences in sensitivity to these antagonists. Neither BNAP nor NAP alone at concentrations up to 30 μM induced contractions in the tissue preparations. Accordingly, each antagonist (BNAP or NAP) was evaluated at three concentrations: 1, 10 and 100 nM in both distal and proximal colon preparations. At 1 nM, neither NAP nor BNAP showed significant inhibition of morphine-induced contractions when compared to controls in the distal or proximal colon preparations (data not shown). In the distal colon at 10 nM neither NAP nor BNAP showed significant antagonism of morphine effects (Figure 6A) while at 100 nM significant antagonism of morphine affects were observed for both as noted by the reduction in morphine pD_2 (NAP 5.3 and BNAP 5.2 Figure 6B). In the proximal colon at 10 nM NAP and BNAP showed equal antagonism of morphine reducing the pD_2 for morphine to 5.9 (Figure 6C). Increasing the concentration of NAP and BNAP to 100 nM showed no further reduction of the morphine pD_2 suggesting antagonism was near maximal at 10 nM. In all, at all of the concentrations tested, BNAP and NAP showed comparable activities for antagonizing morphine induced contractions of the mouse distal and proximal colon. We observed that the proximal colon is approximately 10-fold more sensitive to morphine than the distal colon. Regional difference in morphine's effect along the GI tract have been previously demonstrated.[1b]

Having confirmed antagonist activity of BNAP in the circular muscle preparations as well as limited CNS activity, we next examined the effect of BNAP on GI motility in mice treated acutely with morphine using the charcoal gavage and colonic bead expulsion assays (Figure 7). We first examined the effects of BNAP on colonic motility. In naïve mice the average bead expulsion time was 17 min. Following exposure to morphine at 3 mg/kg, bead expulsion time was increased to 75 min. It was anticipated that administration of BNAP would decrease expulsion time. Surprisingly, when administered alone BNAP at 10 mg/kg increased bead expulsion time to 43 min. When administered in the presence of morphine either concurrently or as a pretreatment 5 or 15 min before morphine, no significant antagonism of morphine's effects was observed. Similar observations were observed that BNAP reduced intestinal motility 1.5-fold compared to the 3-fold decrease by morphine when examined in the charcoal gavage intestinal motility assay. Thus unlike the parent compound NAP[7], BNAP was unable to reverse morphine induced inhibition of intestinal motility. These in vivo data were unexpected and seemed contradictory to those observed in the in vitro systems. One possible reason for this difference could be the partial agonist activity of BNAP at the KOR.

The in vivo data described above suggested that: 1) BNAP behaves differently in longitudinal muscle than in circular muscle and 2) the partial agonism of BNAP at KOR may prevent MOR antagonism in vivo. Fichna recently reported that KOR agonist Salvinorin A reduced colonic motility in rodent models.[14] In light of this finding we suspected that the seemingly dichotomous nature of BNAP was the result of its partial agonsim at KOR acting on longitudinal muscle. To test this hypothesis we investigated the effects of BNAP on Electrical Field Stimulated (EFS) contractions in Longitudinal Muscle Myenteric Plexus (LMMP) preparations from the guinea pig ileum. Studies by others and us have shown that MOR agonists inhibited EFS contractions in a dose dependent manner; however, little is known about the effects of KOR agonists on EFS contractions of LMMP

preparations. As shown in Figure 8A, BNAP dose dependently inhibited EFS contractions in LMMP preparations with comparable potency to the selective MOR agonist DAMGO (pIC_{50} = 7.67 ± 0.03, pIC_{50} BNAP = 7.86 ± 0.07). That result in agreement with its KOR partial agonist behavior seen in [^{35}S]GTPγS-binding Assays. To establish the potential role of KOR in this response the tissue was pretreated with the selective KOR antagonist nor-BNI 15 min prior to agonist exposure. Figure 8B showed that nor-BNI at 0.1 μM did not significantly effect DAMGO inhibition of EFS contractions. On the other hand, nor-BNI dose dependently shifted the BNAP concentration-response curve to the right, suggesting the inhibitory effects of BNAP on GI motility as observed in the in vivo studies were in part mediated via activation of KOR (Figure 8C).

As stated previously, there are conflicting reports concerning the effect of KOR agonist on GI motility.[10, 14] In humans, Asimadoline, a peripheral kappa agonist did not alter GI transit.[15] We found that the inhibitory effects of BNAP on longitudinal muscle were blocked by the selective KOR antagonist nor-BNI suggesting that impairment of GI motility by BNAP result at least in part from KOR agonism. Additionally, this study sheds light on the role that the KOR plays in mouse GI motility and our results are consistent with the work of Fichna, which has shown that the full KOR agonist Salvinorin A can reduce GI motility in mouse models.[14] These results were also surprising as BNAP showed no activity in thermal antinocepetion studies when administered via ICV injection. This may suggest significant differences in sensitivity of the KORs expressed in the periphery verses centrally.

Within the past few years a renewed interest has developed in the development of peripherally selective KOR agonists.[16] Such agonists are of interest because they display analgesic activity while avoiding the negative side effects associated with MOR agonists.[10] To further investigate KOR mediated effects of BNAP, a visceral pain model was utilized. Given the mixed MOR/KOR pharmacological profile observed by BNAP, we proposed that this ligand may serve as a new template to design novel analgesics for the treatment for visceral pain without sides affects associated with centrally active KOR agonists. Therefore, the acetic acid writhing assay was used to determine the effect of systemically administered BNAP on nociception by s. c. In this assay, BNAP dose dependently reduced the number of writhes (Figure 9A). We also examined the effects of BNAP in a hypernociceptive model of visceral pain. We have previously shown that mice that were rendered tolerant to the analgesic effects of morphine by 5-day pellet implantations exhibit a two-fold increase in the writhing responses. When tested in this assay BNAP was active at three fold lower concentration (Figure 9B) compared to morphine. Recent evidence suggests that KOR agonists can decrease the overall perception of pain due to colonic distension in irritable bowel syndrome patients. The effects of Asimadomine, a peripherally active kappa agonist in clinical trials, are more pronounced in models of colonic inflammation and may reflect changes/increases in KOR in disease states. Our findings that BNAP significantly reduced abdominal stretches in chronic morphine mice compared to naïve mice supports this hypothesis. In order to further confirm this, we performed the acetic acid writhing test in the presence of the selective KOR antagonist, nor-BNI (Figure 9C). Antagonism of the peripheral KOR effectively blocked the analgesia produced by BNAP in the morphine pelleted animals. We have previously shown that hypernociception occurs in chronic morphine treated mice[17] and may be due to bacterial translocation following chronic

morphine induced breakdown of the epithelial barrier.[18] The kappa-agonist activity results in a compound that reduces visceral pain sensation and in combination with peripheral mu opioid receptor antagonism may have potential use in functional GI disorders such as irritable bowel syndrome.

In summary, we have shown that the introduction of the permanent charge as in BNAP limits its activity to the periphery while maintaining affinity for both the MOR and KOR. Through in vitro and in vivo studies it was determined that BNAP has mixed pharmacology acting as a MOR antagonist and KOR partial agonist. BNAP did not reverse morphine's effects on GI motility, however it was effective in reducing pain responses in the writhing assay. In particular in hypernocicepetion models of visceral pain BNAP was active at concentrations three-fold lower than in morphine naive animals. Together these data show that BNAP can serve as a novel scaffold upon which to design peripherally selective mixed MOR/KOR ligands. Such ligands would be useful in the treatment of irritable bowel syndrome in patients suffering from visceral pain that is accompanied by both constipation and diarrhea.

METHODS

Drugs and Chemicals

Morphine (morphine sulfate pentahydrate salt), was procured from the National Institute of Drug Abuse (NIDA), Bethesda, MD and made into a 10 μM stock solution by dissolving in distilled water which was further diluted to the desired concentrations. NAP was synthesized according to previous reports[19] as the HCl salts and dissolved in distilled water to a stock concentration of 10 μM, which was further diluted with distilled water to make the targeted concentration. 1-Benzyl-17-Cyclopropylmethyl-3,14β-dihydroxy-4,5α-epoxy-6β-[(4'-pyridyl)acetamido]morphinan (BNAP) was synthesized by the reaction of NAP as the free base with benzyl bromide in acetone at room temperature until no more precipitate was formed.

Animals

Male Swiss Webster mice (Harlan Laboratories, Indianapolis, IN) weighing 25 – 30 g were housed 5 to a cage in animal care quarters and maintained at 22 ± 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 ± 2 °C, 12 h light-dark cycle), marked for identification and 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).

Synthesis of BNAP

In an attempt to increase the PNS selectivity of NAP it was hypothesized that quaternization of the pyridal nitrogen would yield a PNS selective derivative while not negatively impacting the antinociceptive potency. Such quaternizations are typically carried out through alkylation with the appropriate alkyl halide. One point of consideration in attempting this alkylation is the presence of the basic nitrogen at the 17 position which could compete for

the alkylating agent and give rise to mixtures of alkylated products. Of the alkylating agents tested, benzyl bromide would ultimately prove to be the most selective under the reaction conditions tried. Alkylation of NAP with benzyl bromide was found to give selective alkylation of the pyridyl nitrogen in a high yield with easy purification (Scheme 1). NAP (1.0 mmol), prepared according to previously described procedures[19] was stirred for 5 days with benzyl bromide (5.0 mmol) in acetone. Then the solid precipitate was filtered off and taken back up in fresh acetone and allowed to stir overnight at room temperature. Filtration gave BNAP as a white solid in analytically pure form. A second crop of BNAP could be obtained if the filtrated was reduced to one-third its volume and allowed to stir for another 5 days. The full characterization of BNAP were available in the Supporting Information.

Competitive Radioligand Binding and Function Studies

The detail of Radioligand Binding and Functution Studies were described before.[19] Chinese hamster ovarian (CHO) cell line was used as monocloned opioid expressed cells. The μ, δ, and κ opioid receptors were labeled by [^{3}H]naloxone, [^{3}H]NTI and [^{3}H]norBNI. The membranes were incubated with the radioligands under different concentrations of the drug in investigation at 30 °C for 1 h. With the absence and presence of 10 μM naltrexone, specific binding was got as the difference in binding obtained. The specific binding of the drugs to the radioligand was got from data using linear regression analysis of Hill plots.

[^{35}S]GTPγS-binding Assays in the Low MOR-expressing CHO Cell Line - Varying concentrations of BNAP or 3 μM DAMGO (standard full agonist at MOR) were incubated with 10 μM GDP and 0.1 nM [^{35}S]GTPγS in assay buffer (50 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 0.2 mM EGTA and 100 mM NaCl) for 90 min at 30 °C. Nonspecific binding was determined with 10 μM unlabeled GTPγS. 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.[9]

[^{35}S]-GTPγS-Binding Assays in DOR- or KOR-Expressing CHO Cell Lines Cell membranes (10 μg protein) were incubated with 10 μM GDP and 0.1 nM [^{35}S]-GTPγS in assay buffer in the presence and absence of varying concentrations of P, 3 μM SNC80 (standard full agonist at DOR) or 3 μM U50,488H (standard full agonist at KOR) for 90 min at 30 °C. Nonspecific binding was determined with 10 μM unlabeled GTPγS. The reaction was terminated and bound radioactivity determined as described above and previously.[9]

Data Analysis of [^{35}S]-GTPγS-Binding Assays

All samples were assayed in triplicate and repeated at least twice for a total of ≥3 independent determinations. Results were reported as mean values ± 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. Binding K_i values were determined from IC_{50} values using the Cheng-Prusoff equation: $K_i = IC_{50}/1 + ([L]/K_D)$, where [L] is the concentration of competitor and K_D is the K_D of the radioligand.

Docking studies

The structure of BNAP was sketched using SYBYL-X 2.0. After energy minimization (10,000 iterations), the Gasteiger–Hückel charges of BNAP were assigned using TAFF. The docking study was conducted via GOLD 5.1 with standard default settings. The defined binding sites included all atoms within 10 Å of the α-carbon atom of $Asp^{3.25}$ for the crystal structures of MOR, KOR and DOR. The best docked solution was selected basing on the fitness scores and the binding orientation of each ligand within the binding cavity. In order to remove clashes and minimize strain energy, the combined receptor–ligand structures were energy-minimized using the parameters described above to optimize the interactions between ligand and receptor within the binding pocket.

Tail Immersion Test

The warm water tail-immersion test was performed according to previously described methods using a water bath with the temperature maintained at 56 ± 0.1°C.[20] Briefly, before giving the mice injections, a baseline (control) latency was determined. Only mice with a control reaction time of 2 to 4 s were used. The average baseline latency for these experiments was 3.0 ± 0.1 s. The test latency after drug treatment was assessed at the appropriate time, and a 10 s maximal cutoff time was imposed to prevent tissue damage. Antinociception was quantified according to established procedures as the percentage of maximum possible effect (%MPE), which was calculated as follows: %MPE = [(test latency - control latency)/(10 - control latency)] × 100.[21] Percentage MPE was calculated for each mouse, using at least six mice per group.

Intracerebroventricular Injections

Intracerebroventricular (ICV) injections were performed as described previously.[22] Mice were anesthetized with 2.5% isoflurane and a horizontal incision was made in the scalp. A needle was inserted to a depth of 3 mm into the lateral ventrical (2 mm rostral and 2 mm lateral at a 45° angle from the bregma). At intervals, 5 μL injections of drug or vehicle were made using this guide insertion to the same depth using a needle with a guard in nonanesthetized animals.[22, 23] Animals underwent the anesthetized surgery in the morning of the experiment and were then injected with drug at intervals indicated in the text without additional anesthesia. Immediately after testing, the animals were euthanized to minimize any type of distress, according to IACUC guidelines.

Preparation of Colon Circular Muscle for Isometric Tension Recordings

Mice were euthanized by cervical dislocation. The colon was dissected, flushed of its contents, and trimmed of mesentery. Segments of the distal colon (approximately 1 cm from anus) and proximal colon (approximately 1.5 cm from distal portion) were removed and placed in a dissecting dish containing Krebs solution (118 mM NaCl, 4.6 mM KCl, 1.3 mM NaH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 11 mM glucose, and 2.5 mM $CaCl_2$) bubbled with 95% O_2 and 5% CO_2. The tissues, 0.5 cm in length, were suspended vertically along the axis of the circular muscle with a metal triangle tied to a hook under 1 g of passive tension in 15 mL siliconized organ baths. The tissues were allowed to equilibrate for 60–90 min prior to drug exposure, with the Krebs solution changed every 10 min for the first 40

min. Prior to administering the selected antagonist a control run was conducted to ensure the preparations were responding to morphine. Tissues not responding to morphine in the control run were discarded. Following the control run tissue samples were washed with krebs every 10 – 15 min for 1.5 – 2.0 hours until values returned to baseline levels. Following this the antagonist was added, allowed to incubate with the tissue for 15 min and a second dose response curve to morphine was conducted. Isometric contractions were recorded by a force transducer (GR-FT03; Radnoti, Monrovia, CA) connected to a personal computer using Acknowledge 382 software (BIO-PAC Systems, Inc., Santa Barbara, CA).

Charcoal Meal Test for Gastrointestinal Transit Analysis

Forty-eight hours before testing, mice were placed in cages with raised mesh wire to suspend them above their bedding and prevent ingestion of feces or bedding. The animals were habituated for 24 h in the presence of food and water and then fasted for 24 h with free access to water as previously reported.[24] This time frame was chosen to deplete the intestine and colon of any feces. To maintain caloric intake and to avoid hypoglycemia, mice had access to a sugar water solution consisting of a final concentration of 5% dextrose for the first 8 h of the fasting period. Mice were treated with either saline (10 μL/g s.c.) or morphine (10 mg/kg s.c.), and 20 min later they were given an oral gavage consisting of 5% aqueous suspension of charcoal in a 10% gum Arabic solution. At 30 min after the administration of the charcoal meal, the mice were euthanized by cervical dislocation, and the small intestine from the jejunum to the cecum was dissected and placed in cold saline to stop peristalsis. The distance traveled by the leading edge of the charcoal meal was measured relative to the total length of the small intestine, and the percentage of intestinal transit for each animal was calculated as percentage transit (charcoal distance)/(small intestinal length) × 100. This is referred to as intestinal transit in the text.

Colonic Bead Expulsion Assay

Mice were habituated and fasted as described above for the gastrointestinal transit analysis. Mice were given an injection of either saline (10 μL/g b.wt.) or morphine (10 mg/kg s.c.). At 20 min postinjection, animals were anesthetized with isoflurane (1 – 2 min) to insert a single 2-mm glass bead into the distal colon at a distance of 3 cm from the anus. Bead insertion was accomplished using a glass rod with a fire- polished end to avoid tissue damage and marked at 3 cm.[25] After bead insertion, mice were placed in individual cages and the time to bead expulsion was monitored. Animals were monitored for a maximum of 2 h unless bead expulsion occurred sooner.

Guinea Pig Longitudinal Muscle Myenteric Plexus Strips for Isometric Tension Recordings

Male albino guinea pigs (350–450 g) were euthanized by asphyxiation with CO_2. A segment of ileum was removed and placed in a dissection bath filled with pre-oxygenated Krebs' solution (118 mM NaCl, 4.6 mM KCl, 1.3 mM NaH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 11 mM glucose, and 2.5 mM $CaCl_2$). Longitudinal Muscle Myenteric Plexus (LMMP) strips were prepared as previously described.[26] Electrical field stimulation (EFS) (50 V, 7.5 Hz, unless stated otherwise) was applied through concentric electrodes over longitudinal muscle or L-type stimulating electrodes over circular muscle strips to produce

neurogenic contractions/relaxations. Drugs were added in single or cumulative doses depending on the assay to determine their inhibitory effects on the neurogenic responses.

Measurement of BNAP antagonism of Acetic Acid Induced Writhing

To determine whether BNAP provides KOR mediated analgesia acetic acid-induced abdominal stretches were counted in mice. Swiss Webster mice were allowed to acclimate in individual testing cages for at least 10 min. Following the acclimation period the vehicle or BNAP was administered via s.c injection at the specified dose. After waiting for 10 min the mice were injected intraperitoneally with 10 mL/kg 0.6% (wt/vol) acetic acid and returned to their observation chambers. After the first 3 min, the number of stretches and abdominal contractions were counted for 15 min. All values shown for abdominal stretching experiments represent separate groups of mice (n = 4–8) as each animal was tested only once and euthanized immediately after testing.

In order to confirm the involvement of the KOR in the analgesia produced by BNAP in the Acetic Acid Induced Writhing test, this test was performed in the presence of the KOR-specific antagonist nor-Binaltorphimine 2HCl (nor-BNI). According to previous literature, nor-BNI antagonist activity reaches optimal selectivity and efficacy in the writhing test at 24 hours post administration.[27, 28] These animals were injected sub-cutaneously with either 10mg/kg nor-BNI or saline 24 hours prior to initiation of the writhing test. The writhing test was then performed as described above (n=5–6).

Data Analysis

Contractile responses after repeated administration of morphine were analyzed by taking the integrated responses between doses. Data are presented as the mean ± S.E.M, values of $P \leq 0.05$ were considered significant. Effective concentration of agonists to produce 50%-maximal response [reported as negative log (EC_{50}) or pD_2] was calculated by nonlinear regression, and data were analyzed by appropriate statistical tools using GraphPad Prism software (Graph-Pad Software Inc.).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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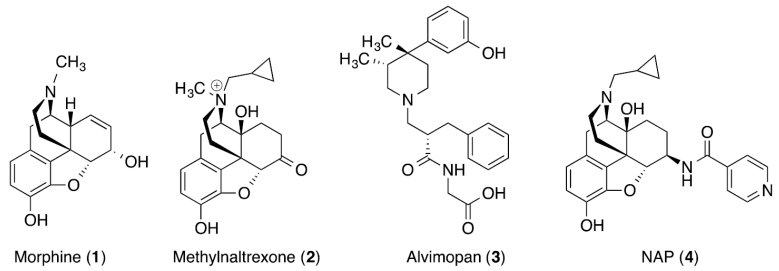


Figure 1.
Morphine and three known peripherally selective mu-opioid receptor antagonists.

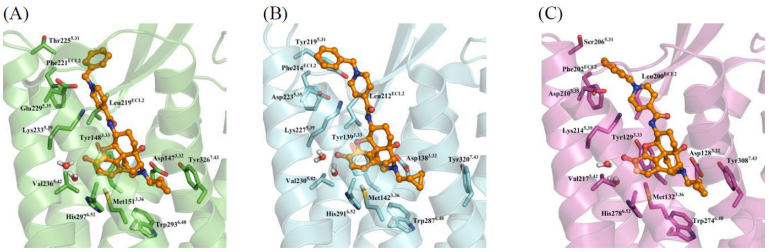


Figure 2.

Ligand docking study of BNAP (orange balls and sticks) in the MOR (A) and KOR (B) and DOR (C) crystal structures.

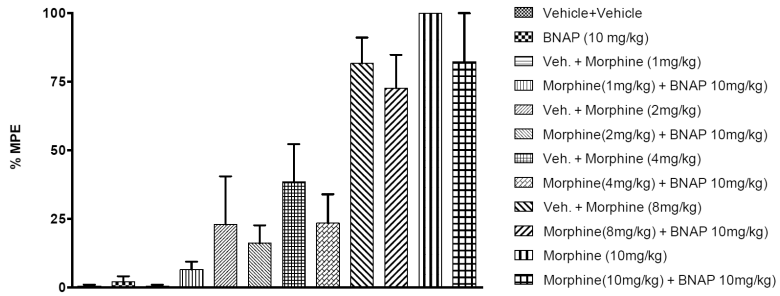


Figure 3.

BNAP effects on morphine antinociception when administered via subcutaneous (s.c) injection. Data point are mean responses ± S.E.M, n = 5.

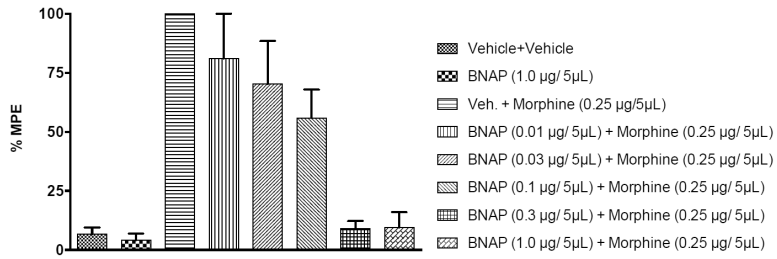


Figure 4.

BNAP effects on morphine antinociception when administered via intracerebroventricular (ICV) injection. Data point are mean responses ± S.E.M, n = 5.

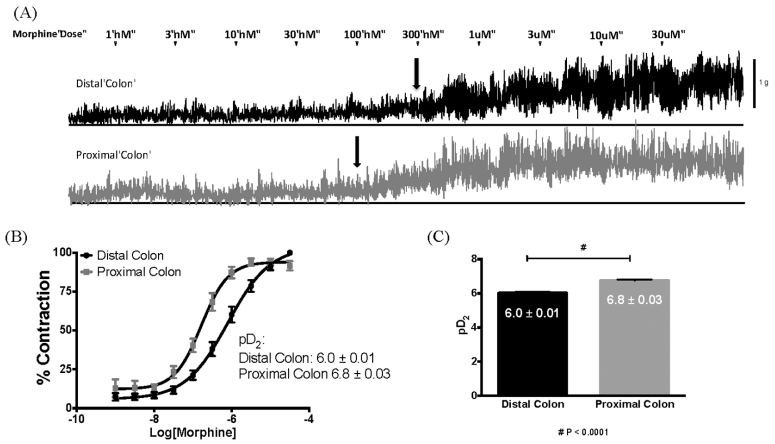


Figure 5.

(A) Raw traces of morphine induced contractions in the mouse distal and proximal colon from the same animal. (B) Graph indicating the concentration dependent stimulation of contractions in response to morphine in the distal and proximal colon. (C) Histogram depicting the >5 fold difference in sensitivity between the distal and proximal colon. (Data points are mean responses ± S.E.M of no less than three independent runs).

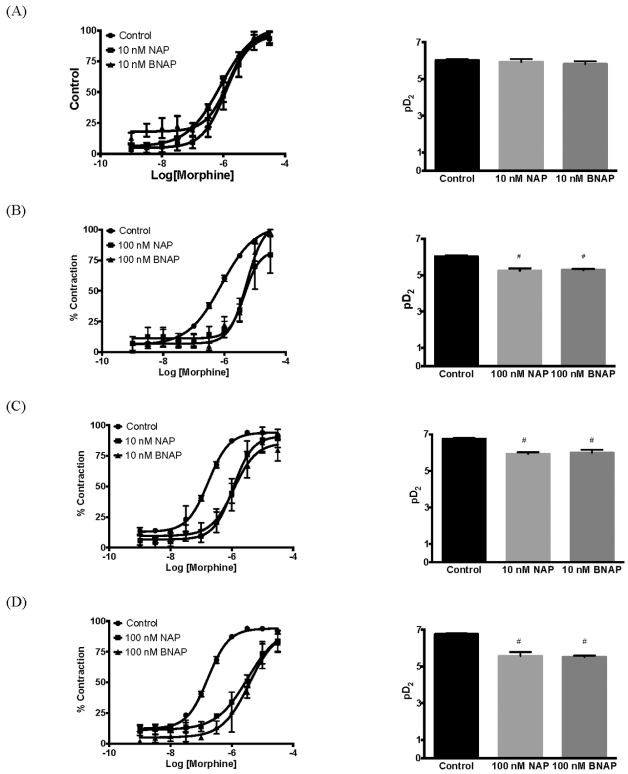


Figure 6.

Morphine dose response curves in the presence of NAP or BNAP in distal (A, B) and proximal (C, D) colon. Data point are mean responses ± S.E.M, n = 4-6.

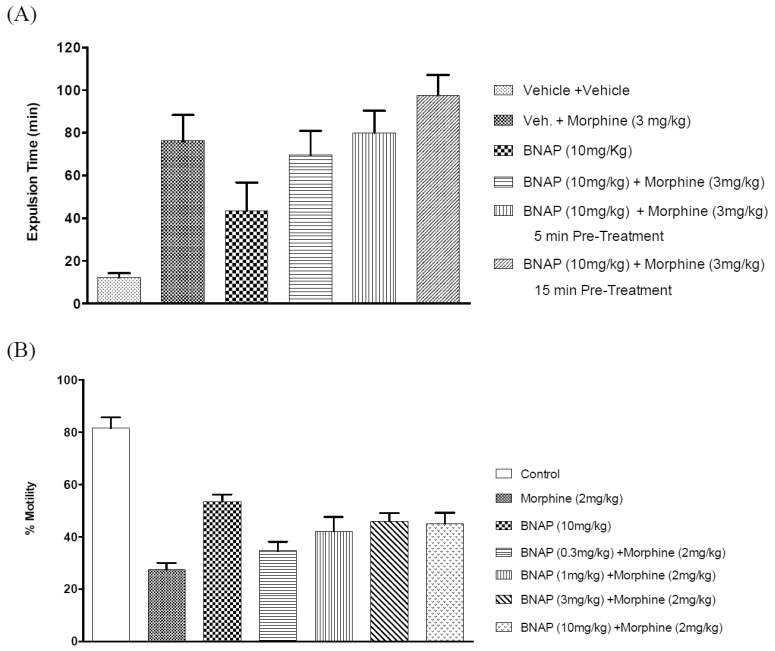


Figure 7.

(A) Effect of Morphine and BNAP on gastrointestinal transit as measured by bead expulsion. (B) Effect of Morphine and BNAP on gastrointestinal transit as measured by charcoal gavage. Data point are mean responses ± S.E.M, n = 6–8.

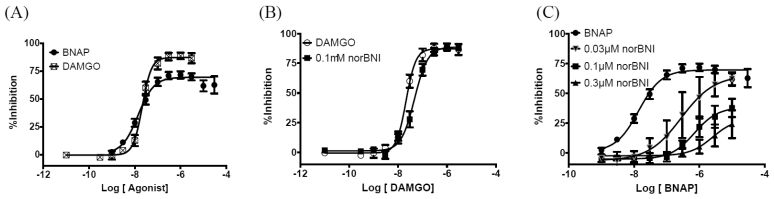


Figure 8.

The inhibition of electrical field stimulated contractions by opioid agonist. (A) Inhibition of EFS by the MOR full agonist DAMGO and the newly prepared KOR partial agonist BNAP. (B) Attempts at blocking the effects of DAMGO with the selective KOR antagonist nor-BNI. No significant change was observed. (C) Blocking of BNAP effects by nor-BNI. The concentration response curve is shifted to the right suggesting antagonism of BNAP by nor-BNI. Data point are mean responses ± S.E.M, n = 4-6.

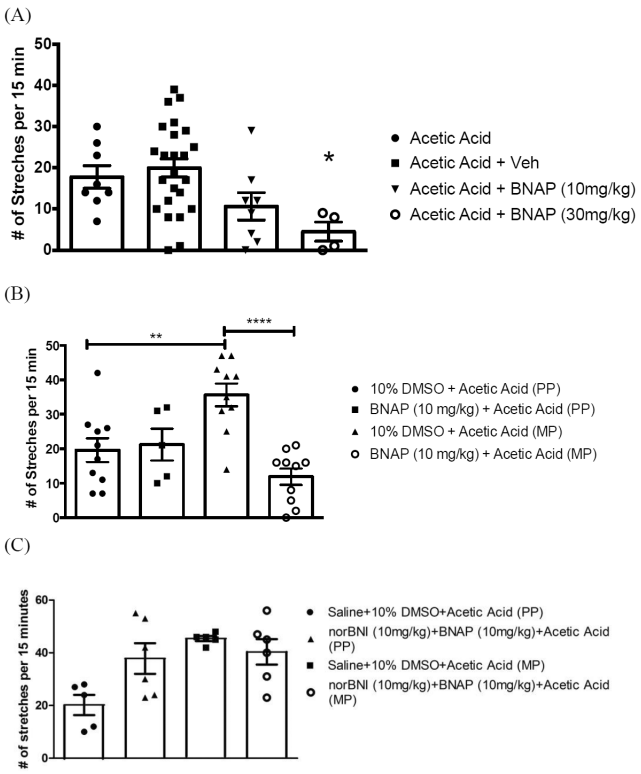
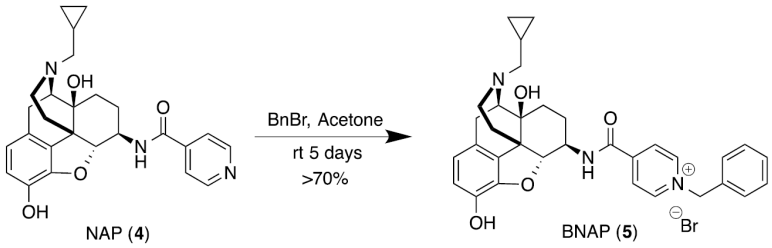


Figure 9.

(A) Analgesic activity of BNAP in the acetic acid induced writhing assay. Data point are mean responses ± S.E.M. (B) Analgesic activity of BNAP in the acetic acid induced writhing assay in mice chronically treated with a 75 mg morphine pellet (MP) or placebo pellet (PP). (C) Prevention of BNAP analgesia in the presence of 10mg/kg nor-BNI.



Scheme 1.
Synthesis of BNAP as a novel peripherally selective opioid ligand.

Table 1

Binding Affinity and ^{35}S-GTP[γS] assay results for BNAP and NAP[9b] at MOR, KOR and DOR. The values are the mean ± SEM of three independent experiments. [^{3}H]Naloxone, [^{3}H]NTI, and [^{3}H]nor-BNI were used to label MOR, DOR, and KOR, respectively, unless otherwise stated. The percentage stimulation to agonist is the E_{max} of the compound compared to that of a full agonist (normalized to 100%): DAMGO for MOR, SNC80 for DOR and U50,488H for KOR.

Compound	Receptor	K_i (nM) ± SEM	Selectivity	EC_{50} (nM)	% max of agonist
BNAP	MOR	0.76 ± 0.09	-	4.8 ± 0.6	14.6 ± 0.9
	KOR	3.46 ± 0.05	4.6 (κ/μ)	2.9 ± 1.1	45.9 ± 1.7
	DOR	722 ± 65	950 (δ/μ)	107.6 ± 69.0	9.2 ± 2.2
NAP	MOR	0.37 ± 0.07	-	1.1 ± 0.4	22.7 ± 0.8
	KOR	60.72 ± 5.58	163 (κ/μ)	28.8 ± 14.4	45.5 ± 4.4
	DOR	277.51 ± 7.97	747 (δ/μ)	15.2 ± 15.2	10.2 ± 3.1