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Design, Synthesis, and Biological Evaluation of the Third Generation 17-Cyclopropylmethyl-3,14 β -dihydroxy-4,5 α -epoxy-6 β -[(4'-pyridyl)carboxamido]morphinan (NAP) Derivatives as μ/κ Opioid Receptor Dual Selective Ligands

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Abstract

μ opioid receptor (MOR) agonists have been widely applied for treating moderate to severe pain. However, numerous adverse effects have been associated with their application, including opioid-induced constipation (OIC), respiratory depression, and addiction. On the basis of previous work in our laboratory, NAP, a 6 β -N-4'-pyridyl substituted naltrexamine derivative, was identified as a peripheral MOR antagonist that may be used to treat OIC. To further explore its structure–activity relationship, a new series of NAP derivatives were designed, synthesized, and biologically evaluated. Among these derivatives, NFP and NYP significantly antagonized the antinociception effect of morphine. Whereas NAP acted mainly peripherally, its derivatives NFP and NYP actually can act centrally. Furthermore, NFP produced significantly lesser withdrawal symptoms than naloxone at similar doses. These results suggest that NFP has the potential to be a lead compound to treat opioid abuse and addiction.

Graphical Abstract

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Author Contributions

Y. Zhang conceived and oversaw the project and finalized the manuscript. Y. Zheng, D. A. Williams, C. Zou, and B. Peddibhotla conducted the chemical synthesis. Y. Zheng, S. Obeng, and H. Wang drafted the manuscript together. S. Obeng, A. M. Jali, and D. L. Stevens conducted radioligand binding assays and in vivo studies under the supervision of D. E. Selley, W. L. Dewey, and H. I. Akbarali. H. Wang finished the docking studies under the supervision of Y. Zhang.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.8b01158. Molecular formula strings and some data (CSV)

Coordinates information for structure representation (PDB)

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NMR and IR spectra for 4'-pyridylcarboxylic acids prepared in our lab, HPLC spectra for all newly synthesized NAP derivatives, and the shortest distances between atoms on critical amino acid residues and atoms on the ligands of NFP in the MOR, KOR, and DOR (PDF)

The authors declare no competing financial interest.

INTRODUCTION

Opioid receptors belong to the class A rhodopsin-like G-protein-coupled receptor (GPCR) family and may be classified into three main subtypes known as the μ opioid receptor (MOR), κ opioid receptor (KOR), δ opioid receptor (DOR).^{1–4} Among them, MOR is the main pharmacological target for opioid medications, such as morphine.⁵ After agonist binding, the conformation change of the MOR will lead to activation of the receptor, which may further result in opening of inwardly rectifying K^+ (GIRK) channels, inhibition of the voltage-gated Ca^{2+} (VGCC) channels, and inhibition of adenylyl cyclase activity.^{6–9} These changes may further induce a variety of changes in membrane morphology, neurons excitation, neurotransmitters release, and expression of genes through the second messenger systems.¹⁰ The behavioral effects of MOR activation include antinociception and addiction or abuse.^{11,12} Opioid addiction or abuse has become a global epidemic.^{13,14} Moreover, opioid abuse increases the prevalence of diseases such as HIV, tuberculosis, and hepatitis, especially for users who inject these drugs.^{15–19} It is reported that over 33 000 people died due to opioid overdose in the U.S. in 2015,²⁰ which has increased over 14 times in the past 20 years.²¹

Currently, there are two approaches to treat opioid addiction, which are detoxification and maintenance therapy by using opioid receptor agonists,^{22–24} partial agonists,^{25–27} or antagonists.^{28,29} Among them, opioid antagonists have been used as potential opioid addiction treatments for years.^{28,29} The synthesized opioid antagonists include peptide opioids and non-peptide opioids. CTOP (Figure 1) and CTAP (Figure 1) are cyclic peptides that are highly effective opioid antagonists with high MOR selectivity and metabolic stability, but their poor bioavailability limited their development as clinical therapeutics.^{30–32} Compared to peptide opioids, non-peptide opioid antagonists may have advantages of high bioavailability, easily crossing the blood–brain barrier (BBB), and good metabolic stability.³² The classical opioid antagonists used in opioid addiction treatments are naltrexone (Figure 1) and naloxone (Figure 1). As a result of their different half-lives, naloxone is a short-acting medication while naltrexone is used for long-term treatment.^{33,34} As opioid antagonists, they do not carry the side effects related to opioid agonists, such as addiction and respiratory suppression.³⁵ However, high doses of naltrexone and naloxone may induce hepatotoxicity^{36,37} and cardiovascular and pulmonary problems.³⁸ Besides, the interactions between these compounds and the DOR or KOR may induce mood changes^{39,40} or block transmission of neurotransmitters,^{41–43} though more evidence is still needed to further prove them.

The side effects associated with low selectivity MOR antagonists prompt researchers to develop new opioid antagonists. β -Funtaltrexamine (β -FNA, Figure 1), a MOR antagonist, has similar potency to the MOR and KOR.^{44b} Cyprodime (Figure 1), another MOR antagonist, has moderate selectivity to the MOR ($K_{i,\delta/\mu} = 39$ and $K_{i,\kappa/\mu} = 10$).^{45,46} Clocinnamox (Figure 1) and methocinnamox (Figure 1) are potent MOR antagonists but with similar binding affinity to all three opioid receptors.⁴⁷ Meanwhile, it has been reported that activation of the KOR and further dynorphin release have a relationship with mood behaviors, such as drug-related withdrawal, aversion, and relapse induced by stress.⁴⁸ Studies have shown that norBNI ($K_{i,\kappa} = 1.8$ nM) increased the symptoms of opioid withdrawal in rats, while in another study with KOR knockout mice precipitation of opioid withdrawal was not observed.^{49,50a} De Marco et al. studied a new highly selective KOR partial agonist as anodyne with less harmful side effects.^{50b} These studies suggest that KOR probably is a potential target for drug addiction and side effects related to mood behaviors. Collectively, highly selective and potent opioid antagonists are still extremely desirable.

Keeping this in mind, a number of new chemical entities have been designed and synthesized by modifying the structure of naltrexone to achieve highly selective opioid ligands.^{51–53} One such compound from our lab, 17-cyclopropylmethyl-3,14 β -dihydroxy-4,5 α -epoxy-6 β -(4'-pyridylcarboxamido)-morphinan (NAP) (Figure 1), a 6 β -N-4'-pyridyl substituted naltrexamine derivative, was identified as a peripherally selective MOR antagonist ($K_{i,\delta/\mu} \approx 747$, $K_{i,\delta/\kappa} \approx 163$; ED₅₀ = 0.0088 mg/kg).^{54–56} To avoid diarrhea induced at high doses of NAP, the second generation NAP derivatives were designed and synthesized by modifying the C(6)-pyridyl ring system of NAP which was shown to interact with the MOR “address” region. NMP (17-cyclopropylmethyl-3,14 β -dihydroxy-4,5 α -epoxy-6 β -{[4'-(3'-methylpyridyl)]carboxamido}morphinan), a second generation NAP derivative, had improved pharmacological properties compared to NAP since it not only acted as a peripherally selective MOR antagonist but also improved motility of the gastrointestinal tract in vivo.^{57a58} Further molecular modeling studies showed that the methyl group at 3' position on the pyridyl group could reinforce the interactions between NMP and the “address” region of the MOR.⁵⁸ On the basis of these previous study results, we sought to further explore the influence on binding affinity of 3' position on the pyridyl ring of NAP to opioid receptors based on the “message–address” concept.^{57a58} Thus, we designed, synthesized, and biologically evaluated a series of new compounds as the third generation NAP derivatives. Two new compounds, NFP and NYP, were characterized in vitro and in vivo as opioid receptor antagonists. Interestingly, these two compounds were found to be centrally acting with fewer withdrawal effects compared to naloxone, which was contrary to the peripherally acting NAP and NMP.

RESULTS AND DISCUSSION

Molecular Design.

Our molecular modeling study on NAP in the three opioid receptors demonstrated that the C(6)-pyridyl ring of NAP may strengthen the selectivity of NAP for the MOR over the DOR and KOR through aromatic stacking and hydrogen-bonding interactions.⁴⁴ Meanwhile, a molecular dynamics simulation study of NMP in the MOR, DOR, and KOR showed that the

3'-methyl group on the pyridyl ring may reinforce the π - π stacking interaction between the aromatic pyridyl ring of NMP and W318^{7,35} of the MOR, which resulted in the high selectivity of NMP to the MOR over the DOR and KOR.⁵⁸ Further modeling study results indicated that the characteristics of the substituents on the 3' position of pyridyl ring of NAP derivatives, such as steric hindrance, electrostatic, and hydrophobic effects, may have significant effect on the binding affinity and selectivity of NAP derivatives to the three opioid receptors.⁵⁸ Therefore, in designing the third generation NAP derivatives, substitutions with different electronic, bulky, steric, and hydrophobic properties were introduced at the 3' position of pyridyl ring (Figure 2).

Chemistry.

Twelve new NAP derivatives (Table 1) were synthesized. Compounds **1–6** were derivatives with electron-withdrawing groups at the 3' position on the pyridyl ring with a corresponding increment in size of the substituents. Compounds **7–12** were derivatives with electron-donating groups at the 3' position on the pyridyl ring with different bulkiness. For compounds **1–3**, **9**, and **12**, the 3'-substituted pyridylcarboxylic acids were from commercial resources. For compounds **4–8**, **10**, and **11**, the 3'-substituted pyridylcarboxylic acids had to be prepared by ourselves as shown in Scheme 1 (Supporting Information).^{57b–d} In addition, 6 β -naltrexamine was synthesized as reported previously with a yield of 58% (Scheme 2).^{44a} Then the 4'-pyridylcarboxylic acids were coupled with 6 β -naltrexamine by EDCI/HOBt method.^{44,57a} After that, the reaction crudes were treated with K₂CO₃ to get compounds **1–12** with reasonable yields (Scheme 2). All compounds were then transferred to hydrochloride salt and used for in vitro and in vivo studies.

In vitro binding and functional studies were first conducted to evaluate the binding affinity, selectivity, and efficacy of the compounds to the opioid receptors. In vivo studies were further conducted to characterize the behavioral profiles of these compounds.

In Vitro Radioligand Binding and MOR[³⁵S]GTP γ S Functional Studies.

The competition radioligand binding assay was used to determine the binding affinity and selectivity of the new NAP derivatives for the MOR, KOR, and DOR. Chinese hamster ovary (CHO) cell lines expressing mouse monocloned opioid receptors were used for this assay. The MOR was labeled with [³H]naloxone, while the KOR and DOR were both labeled with [³H]diprenorphine.

As shown in Table 1, all the compounds maintained subnanomolar binding affinity to the MOR except compounds **6** and **8** which showed nanomolar binding affinity. Compared to the first and second generation lead compounds, NAP ($K_{i,MOR} = 0.37 \pm 0.07$ nM) and NMP ($K_{i,MOR} = 0.58 \pm 0.25$ nM), most of the third generation derivatives still maintained high binding affinity to the MOR. This indicated that the substituent groups with different electronic properties and sizes at 3' position on the pyridyl ring did not significantly affect the binding affinity of these derivatives to the MOR. However, it is worth noting that esterification of the carboxylic acid at the 3' position (compound **6**) and introduction of an isobutyl group at the 3' position (compound **8**) resulted in a significant reduction in their MOR binding affinity. As mentioned above, the structural features of substituents on the 3'

position of pyridyl ring, such as the steric hindrance, electrostatic, and hydrophobic effects, would significantly affect the binding affinity and selectivity of NAP derivatives to the three opioid receptors.⁵⁸ Compound **6** was an esterification product of compound **5**. After esterification, the original ionic interactions between carboxylic moiety in compound **5** and basic residues in the binding pocket of the MOR would be weakened in compound **6** significantly so that the binding affinity of compound **6** to the MOR was reduced. Furthermore, the isobutyl group on the pyridyl ring of compound **8** may result in steric clashes or unfavorable interactions with hydrophilic residues in the binding pocket that would decrease the binding affinity of compound **8** to the MOR. Interestingly, compared to compound **8**, compounds **9–12** carried even bulkier substituents on the pyridyl ring while still maintaining high binding affinities to the MOR at the subnanomolar level. One possible explanation of such an observation could be that the electrostatic and hydrophobic interactions induced by the much larger substituents on the pyridyl ring (compounds **9–11**) may facilitate the compounds to interact with the binding pocket favorably in the MOR in a different binding mode than that of compound **8**.

The selectivity of these compounds for the MOR over the DOR varied with different substituent groups at 3' position on the pyridyl ring. In general, derivatives with electron-withdrawing groups showed higher selectivity for the MOR over the DOR than derivatives with electron-donating groups. For example, compounds **1–6** showed more than 30-fold selectivity for the MOR over the DOR, especially for compounds **1** (NFP, $\delta/\mu = 435.8$) and **3** ($\delta/\mu = 562.4$). Both compounds had similar selectivity for the MOR over the DOR as NMP ($\delta/\mu = 470$). For the derivatives with electron-donating groups, compounds **7, 9, and 12** showed 50- to 134- fold selectivity for the MOR over the DOR while compounds **8, 10, and 11** showed 14- to 20-fold selectivity for the MOR over the DOR. Thus, it seemed that introduction of bulky and alkyl groups at position 3' on the pyridyl ring may reduce their selectivity for the MOR over the DOR.

On the KOR, there was a significant improvement in the binding affinity of all the compounds compared to NAP and NMP except for compound **8** which showed similar binding affinity to the KOR as of NAP and NMP. Compounds **1–7** and **9–12** maintained one-digit nanomolar or two-digit nanomolar binding affinity to the KOR. Thus, collectively, with substitution at 3' position on the pyridyl ring, the third generation NAP derivatives seemed to act as MOR/KOR dual-selective ligands.

The [³⁵S]GTP γ S functional assay was then conducted to determine the potency and relative efficacy of the synthesized compounds at the MOR. The potency was determined as the EC₅₀ while the efficacy was determined as the $E_{\max}$ relative to DAMGO, a MOR full agonist. From these results, it seemed that all the compounds acted as partial agonists with low to moderate efficacy. Derivatives with electron-withdrawing groups (compounds **1–6**) showed 20–40% MOR stimulation with one-digit nanomolar potency except compound **6**. Meanwhile, derivatives with electron-donating groups (compounds **7–12**) showed more than 40% efficacy with one- or two-digit nanomolar potencies except compound **8** which had the lowest potency.

In Vivo Tail Immersion Assay.

All the newly synthesized NAP derivatives were further assessed for their acute agonistic and/or antagonistic effects using the tail immersion assay in mice as previously reported.^{44,57} The baseline latency (control) was determined before the tested compound was injected into the mice. For the study of agonism, the time of tail immersion was 20 min (time that morphine's antinociceptive effect starts to peak) after the test compound was injected (sc). For the study of antagonism, the test compound was given 5 min before morphine. The tail immersion test was then conducted 20 min after morphine was given. The percent maximum possible effects (%MPE) was calculated for each mouse using at least five mice per drug. Briefly, the derivatives were studied for their ability to produce antinociception or block the antinociception produced by morphine (10 mg/kg). As shown in Figure 3A, none of these new derivatives showed significant antinociception compared to morphine at the same dose (10 mg/kg). However, when they were tested as antagonists at the same dose (Figure 3B, 10 mg/kg), compounds **1** (NFP) and **2** (NYP) antagonized morphine's antinociception effect significantly. The %MPEs of morphine (10 mg/kg) in the presence of NFP and NYP (10 mg/kg) were only $6.2 \pm 2.4\%$ and $7.8 \pm 5.4\%$, respectively. The antagonism of NFP and NYP was shown to be dose-dependent (Figure 4A and Figure 4B, AD₅₀ values of NFP and NYP were 2.82 (1.34–5.94) mg/kg with 95% confidence level (CL) and 1.75 (0.88–3.47) mg/kg with 95% CL, respectively). In contrast to NFP and NYP, other NAP derivatives did not significantly block morphine's antinociception at the tested dose of 10 mg/kg. These results suggested that NFP and NYP had the ability to cross the blood–brain barrier (BBB) and block the analgesic effect of morphine in CNS.

It was intriguing to us that two of the third generation NAP derivatives, NFP and NYP, crossed the BBB and antagonized morphine's antinociception, while NAP was identified as a P-glycoprotein substrate and failed to cross the BBB effectively. Apparently introduction of fluoro and nitrile groups on the pyridyl ring may play an important role in improving their CNS penetration. To be noted, about 5–15% of drugs on the market are fluorinated compounds,^{59,60} and it has been shown that these compounds have improved metabolic stability and physicochemical properties. Meanwhile fluorination seems to enhance the CNS penetration ability of non-CNS drugs and the efflux function of P-glycoprotein.^{61,55} This may help explain why NFP crossed the BBB while NAP did not.⁶¹ In addition, it has been reported that introducing nitrile groups may also enhance metabolic stability and improve hydrogen bonding interactions with residues in the orthosteric binding site of protein targets.⁶² Currently, there are at least 30 drugs containing nitrile groups on the market and more than 20 drug candidates containing nitrile groups under clinical trials.⁶² An example of such a drug is piritramide, an opioid analgesic that crosses the BBB and is used for postoperation pain.⁶³

In Vivo Withdrawal Study.

Opioid antagonists such as naloxone and naltrexone have been applied to reverse the effects of opioid agonists in cases of opioid overdose and in opioid addiction treatments. However, it has been shown that naloxone and naltrexone produce significant withdrawal symptoms when administered, which limits their use in opioid addiction treatments.⁶⁴ Since NFP and NYP were identified as opioid antagonists in vivo, a withdrawal study was conducted using

morphine-pelleted mice to determine whether these two compounds would produce withdrawal effects similar to naloxone. In this study, somatic symptoms of opioid withdrawal (shakes, jumps, and paw tremors) were quantified over a period of 20 min.^{55a} As shown in Figure 5, NFP produced significantly fewer wet dog shakes, jumps, and paw tremors at 10 mg/kg than naloxone at 1 mg/kg. Meanwhile, at much higher doses (50 and 100 mg/kg) NFP produced wet dog shakes (Figure 5A) and paw tremors (Figure 5C) but not significantly different from those of naloxone at 1 mg/kg. More interestingly, at a high dose of 50 mg/kg NFP still produced significantly fewer jumps (Figure 5B) than naloxone at 1 mg/kg. NYP, on the other hand, produced significantly fewer wet dog shakes and jumps at 1 mg/kg than naloxone at 1 mg/kg, but at doses 5 mg/kg and higher it produced wet dog shakes (Figure 6A) and jumps (Figure 6B) similar to naloxone at 1 mg/kg. Also, NYP produced similar paw tremors (Figure 6C) at 5 mg/kg similar to naloxone at 1 mg/kg.

It has been reported that naloxone can antagonize morphine's antinociception effect potently (AD_{50} at 3.1 mg/kg) in a tail flick assay^{55b} while it may induce significant withdraw symptom (e.g., Figures 5 and 6, at 1 mg/kg). Compared to naloxone, NFP can effectively across BBB entering into brain and reverse morphine's antinociception effect at a similar potency level but showed much lesser withdrawal symptoms. These results are very encouraging since they suggest that NFP may have potential application in opioid addiction treatments with more compatible patient compliance profiles compared to naloxone.

We then further characterized NFP's partial agonism and antagonism via concentration-effect curves for stimulation of [³⁵S]GTP γ S binding in mMOR-CHO cells by NFP and DAMGO alone (Figure 7A) in comparison to NFP curves in the presence of an EC_{90} DAMGO concentration (Figure 7B). Naltrexone was examined as a positive control for MOR antagonism (Figure 7B). Relative to DAMGO (normalized $E_{max} = 100 \pm 2.6\%$ and $EC_{50} = 32.5 \pm 1.1$ nM), NFP was a partial agonist with an E_{max} value of $34.9 \pm 1.5\%$ and EC_{50} value of 1.21 ± 0.19 nM, in agreement with our initial screening results (Table 1). In the presence of 0.3 μ M DAMGO, NFP produced a concentration-dependent inhibition of DAMGO-stimulated [³⁵S]GTP γ S binding, achieving maximal inhibition (minimum) at $37.7 \pm 1.5\%$ of the maximal stimulation produced by DAMGO. Similarly, naltrexone produced concentration-dependent inhibition, with maximal inhibition at $12.8 \pm 0.4\%$ of maxima DAMGO stimulation. Functional K_i values were then calculated from these data. NFP and naltrexone inhibited DAMGO-stimulated [³⁵S]GTP γ S binding with K_i values of 0.86 ± 0.09 and 0.67 ± 0.05 nM, similar to their K_i values for MOR binding (Table 1). NFP had a relative efficacy that was approximately 35% of DAMGO and 3-fold that of naltrexone under these experimental conditions.

Modeling Study.

As described above, NFP (Figure 8) showed dual-selective binding affinity to the MOR and KOR and displayed significant selectivity over the DOR ($K_{i,8/\mu} = 435.8$). Meanwhile, NFP acted as an MOR partial agonist with moderate efficacy (Table 1) and a KOR partial agonist with low efficacy ($EC_{50} = 13.62 \pm 1.57$ nM, $26.71 \pm 1.94\%$ max effect of U50,488H) and a DOR antagonist (EC_{50} not determined, $7.22 \pm 1.17\%$ max effect of SNC80). However, the results of its in vivo studies displayed that NFP acted as an effective opioid antagonist in the

CNS. To understand its selectivity profile to the opioid receptors, the antagonist-bound crystal structures of MOR (PDB code 4DKL),⁶⁴ KOR (PDB code 4DJH),⁶⁵ and DOR (PDB code 4EJ4)⁶⁶ were adopted from Protein Data Bank, <http://www.rcsb.org>, and NFP was docked into the three receptors by GOLD 5.4.^{67,68} The binding poses of NFP with the highest CHEM-PLP scores from the docking studies were chosen as the optimal binding poses of the ligand in the MOR, KOR, and DOR.

Similar to the lead compound NAP, NFP was designed and synthesized according to the “message–address” concept where the “message” moiety (epoxymorphinan moiety) was assumed to determine its efficacy and the “address” moiety (pyridyl ring) to contribute to its selectivity (Figure 8).⁵⁸ Comparing the binding poses of NFP in the MOR, KOR, and DOR with those of NAP, we found that the identical “message” moiety of NFP and NAP bound with the same domain of the MOR, KOR, and DOR through similar interactions with the conserved residues in the three receptors.⁵⁸ Basically, the epoxymorphinan moiety formed hydrophobic interactions with the conserved residues M^{3.36}, W^{6.48}, and H^{6.52} (superscript numbers follow the Ballesteros–Weinstein numbering method for GPCRs⁶⁹) and hydrogen bonding interactions with Y^{3.33}. Meanwhile, an ionic interaction was formed between the conserved D^{3.32} and the nitrogen atom at 17-position of the epoxymorphinan moiety of NFP (Figure 9). Collectively, the interactions between the “message” moiety (epoxymorphinan moiety) of NFP and the conserved residues seemed to have no significant influence on the selectivity of NFP to the MOR, KOR, and DOR.

The fluorine atom on the pyridyl ring (“address” moiety) of NFP is a strong electron withdrawing group that would weaken consequence, the nitrogen atom on the pyridyl ring of NFP to keep a proton compared to the case of NAP and NMP.⁵⁸ In consequence, the nitrogen atom on the pyridyl ring of NFP might form electrostatic interactions with the conserved residue E229^{5.35} in the MOR, D223^{5.35} in the KOR, and D210^{5.35} in the DOR. In addition, the pyridyl ring of NFP could also form hydrophobic interactions with the conserved hydrophobic residue L^{ECL2} and F^{ECL2} (Table S1). Thus, the “address” moiety (pyridyl ring) of NFP seemed to bind to the same domain (termed “address” domain) in the MOR, KOR, and DOR. However, several nonconserved residues located at this “address” domain (T218^{ECL2}, T225^{5.31}, and L232^{5.38} in the MOR, S211^{ECL2}, Y219^{5.31}, and M226^{5.38} in the KOR, and M199^{ECL2}, S206^{5.31}, and T213^{5.38} in the DOR, Figure 9) could also form direct interactions with the “address” moiety (pyridyl ring) of NFP.

As the distances shown in Figure 9, the electrostatic interaction between the nitrogen atom on the pyridyl ring of NFP and E229^{5.35} in the MOR was much stronger than those in the NFP/KOR and NFP/DOR complexes, which may help to explain the highest affinity of NFP to the MOR. For the NFP/KOR complex, the nonconserved Y219^{5.31} and S211^{ECL2} could also form hydrogen bond with the nitrogen atom on the pyridyl ring of NFP, which may facilitate the binding of NFP to the KOR. However, for the NFP/DOR complex, no additional interaction being observed between the “address” moiety of NFP and the residues located in the “address” domain of DOR except the possibly weakest electrostatic one from D210^{5.35} (Table S1). Overall, the different interactions between the “address” moiety of NFP and the “address” domain of the MOR, KOR, and DOR helped explain the binding affinity profile observed for NFP in the three receptors.

CONCLUSIONS

On the basis of previous studies from our group, the third generation 6 β -N-4'-pyridyl substituted naltrexamine derivatives were designed, synthesized, and evaluated in both in vitro and in vivo assays. The in vitro competition assays showed that the third generation compounds bearing various substitutions at the 3' position of pyridyl ring carried a different selectivity profile than NAP. In that, the third generation compounds were typically MOR/KOR dual-selective while NAP was highly MOR selective. Most of the new compounds retained their binding affinity to the MOR with subnanomolar level and one-digit nanomolar binding affinity to the KOR. Meanwhile, most of the compounds had low to moderate efficacies at the MOR with one-digit nanomolar potencies. Among these derivatives, NFP and NYP significantly antagonized the antinociception produced by morphine in a dose dependent fashion. The in vivo studies showed that NFP produced significantly fewer withdrawal symptoms than naloxone at similar doses. These findings suggest that NFP may serve as a lead compound to develop novel MOR/KOR dual selective ligand for treating opioid addiction and abuse.

EXPERIMENTAL SECTION

Chemistry.

General Methods.—Reagents were purchased from either Sigma-Aldrich or Alfa Aesar. TLC analyses were carried out on the Analtech Uniplate F254 plates. Chromatographic purification was conducted on silica gel column (230–400 mesh, Merck). ¹H (400 MHz) and ¹³C (100 MHz) nuclear magnetic resonance (NMR) spectra were recorded at ambient temperature with tetramethylsilane as the internal standard on a Varian Mercury 400 MHz NMR spectrometer.

Melting points were recorded with OptiMelt automated melting point system (Fisher Scientific). IR spectra were obtained with Nicolet iS10 instrument (Thermo Scientific). Applied Bio Systems 3200 Q trap with a turbo V source for TurbolonSpray was used for MS analysis. HPLC analysis was performed by a Varian ProStar 210 system on Agilent Microsorb-MV 100–5 C18 (250 mm × 4.6 mm) at 210 nm with water/acetonitrile (25/75, 30/70 or 35/65, 0.01% TFA in water) at 0.8 mL/min over 30 min. The purity of the third generation NAP derivatives was determined by above analytical methods, and their purity was identified as 95%.

General Procedure.—*N*-(3-Dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (2.5 equiv), hydrobenzotriazole (2.5 equiv), 4 Å molecular sieves, and trimethylamine (5 equiv) were added to a solution of the carboxylic acid (2.5 equiv) in DMF on an ice–water bath under N₂ protection. After 30 min, a solution of 6 β -naltrexamine (1 equiv) in DMF was added dropwise. The mixture was kept stirring overnight at room temperature and filtered the next day. The filtrate was then concentrated under reduced pressure to remove the solvent. The residue was dissolved in MeOH, and potassium carbonate (2 equiv) was added to the mixture. The reaction mixture was stirred overnight at room temperature. Next day, the mixture was concentrated and the residue was purified with silica gel column using

methanol/dichloromethane (1% NH₃H₂O) as the solvent system to obtain the target compounds.

17-Cyclopropylmethyl-3,14β-dihydroxy-4,5α-epoxy-6β-[(3'-fluoro-4'-pyridyl)acetamido]morphinan (compound 1, NFP).—Compound 1 was synthesized as shown in the general procedure with a yield of 48%. ¹H NMR (400 MHz, CDCl₃) δ 8.94 (s, 1 H), 8.74 (d, *J* = 8.08 Hz, 1 H), 8.58 (m, 1 H), 8.43 (m, 1 H), 7.46 (t, *J* = 5.6 Hz, 1H), 6.51 (d, *J* = 8.1 Hz, 1H), 6.45 (d, *J* = 8.1 Hz, 1H), 4.77 (s, 1 H), 4.45 (d, *J* = 8.1 Hz, 1 H), 3.52 (m, 1H), 2.90 (m, 2 H), 2.47 (m, 2 H), 2.21 (m, 2 H), 2.02 (m, 1 H), 1.87 (m, 1 H), 1.73 (m, 1 H), 1.48 (m, 1 H), 1.37 (m, 1 H), 1.21 (m, 1 H), 1.15 (m, 1 H), 0.73 (m, 1 H), 0.35 (m, 2 H), 0.01 (m, 2 H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ 161.54, 156.46, 153.90, 146.23, 146.18, 142.06, 140.46, 139.10, 138.86, 131.24, 131.05, 130.93, 123.52, 123.23, 118.47, 117.12, 90.39, 69.52, 61.75, 58.38, 51.90, 47.06, 43.66, 30.32, 29.97, 24.33, 22.19, 9.22, 3.65, 3.52. ¹⁹F NMR (75 MHz, DMSO-*d*₆) δ -192.29. IR (diamond, cm⁻¹) *V*_{max} 2942.19, 2829.33, 1655.64, 1529.02, 1487.78, 1454.25, 1416.41, 1322.93, 1238.29, 1204.29, 1186.88, 1152.58, 1131.78, 1100.14, 1034.82, 1017.27, 985.87, 942.11, 919.01, 898.28, 882.36, 799.14, 785.35, 763.19, 746.38, 667.00. Mass spectrum C₂₆H₂₈FN₃O₄ *m/z* calcd 465.2064, obsd 466.2159 [M + H]⁺. Mp 277.6–280.6 °C.

17-Cyclopropylmethyl-3,14β-dihydroxy-4,5α-epoxy-6β-[(3'-cyano-4'-pyridyl)acetamido]morphinan (Compound 2, NYP).—Compound 2 was synthesized as shown in the general procedure with a yield of 48%. ¹H NMR (400 MHz, DMSO-*d*₆) δ 9.15–9.13 (m, 2 H), 8.94 (s, 1 H), 7.92 (d, *J* = 3.6 Hz, 1 H), 7.44 (s, 1 H), 7.31 (s, 1 H), 7.18 (s, 1 H), 6.77 (d, *J* = 8.08 Hz, 1 H), 6.69 (d, *J* = 8.08 Hz, 1 H), 5.06 (d, *J* = 8.24 Hz, 1 H), 3.38–3.33 (m, 3 H), 3.11–3.06 (m, 3 H), 2.9 (m, 1 H), 1.93–1.90 (m, 1 H), 1.51–1.43 (m, 3 H), 1.10–1.09 (m, 1 H), 0.67 (m, 1 H), 0.60 (m, 1 H), 0.51 (m, 1 H), 0.42 (m, 1 H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ 167.45, 167.09, 156.63, 144.66, 142.37, 142.15, 139.45, 129.84, 125.95, 121.26, 120.30, 118.68, 117.50, 87.36, 70.14, 62.25, 57.32, 52.30, 47.03, 46.66, 30.59, 27.74, 23.65, 21.51, 6.32, 5.70, 3.24. IR (diamond, cm⁻¹) *V*_{max} 3111.05, 2087.54, 1721.23, 1626.30, 1501.34, 1467.64, 1371.61, 1322.77, 1079.34, 1031.03, 858.34, 731.40. Mass spectrum C₂₇H₂₈N₄O₄ *m/z* calcd 472.2111, obsd 474.2049 [M + 2H]⁺. Mp 222.8–224.5 °C.

17-Cyclopropylmethyl-3,14β-dihydroxy-4,5α-epoxy-6β-[(3'-nitro-4'-pyridyl)acetamido]morphinan (Compound 3).—Compound 3 was synthesized as shown in the general procedure with a yield of 52%. ¹H NMR (400 MHz, DMSO-*d*₆) δ 9.16 (s, 1 H), 8.94 (m, 1 H), 8.87 (m, 1 H), 7.53 (m, 1 H), 6.50 (d, *J* = 8.1 Hz, 1H), 6.44(d, *J* = 8.1 Hz, 1H), 4.35(d, *J* = 7.8 Hz, 1H), 3.42 (m, 1H), 2.90 (m, 2 H), 2.49 (m, 2 H), 2.22 (m, 2 H), 2.01 (m, 1 H), 1.88 (m, 1 H), 1.70 (m, 1 H), 1.57 (m, 1 H), 1.39 (m, 1 H), 1.19 (m, 2 H), 0.73 (m, 1 H), 0.35 (m, 2 H), 0.01 (m, 2 H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ 163.07, 154.58, 145.17, 142.56, 142.01, 140.49, 139.18, 131.16, 123.46, 122.82, 118.53, 117.14, 90.25, 69.52, 61.69, 58.32, 54.85, 52.11, 47.04, 43.66, 30.28, 29.84, 23.57, 22.18, 9.11, 3.68, 3.48. IR (diamond, cm⁻¹) *V*_{max} 3072.08, 2952.94, 2820.03, 1681.02, 1639.45, 1615.13, 1567.61, 1550.22, 1531.35, 1451.63, 1393.77, 1357.98, 1348.34, 1329.88, 1277.14, 1262.58, 1253.30, 1241.33, 1227.99, 1189.63, 1147.14, 1121.29, 1035.17, 984.65, 945.33, 931.99,

922.82, 915.19, 896.65, 882.54, 858.32, 828.95, 806.17, 763.88, 740.60, 732.83, 721.00, 672.69. Mass spectrum $C_{26}H_{28}N_4O_4$ m/z calcd 492.2009, obsd 493.2087 $[M + H]^+$. Mp 266.46–269 °C.

17-Cyclopropylmethyl-3,14 β -dihydroxy-4,5 α -epoxy-6 β [(3'-trifluoromethyl-4'-pyridyl)acetamido]morphinan (Compound 4).—Compound 4 was synthesized as shown in the general procedure with a yield of 51%. 1H NMR (400 MHz, DMSO- d_6) δ 9.38 (s, 1 H), 9.04 (s, 1 H), 9.02 (m, 1 H), 8.98 (m, 1 H), 7.56 (m, 1 H), 6.75 (d, J = 8.1 Hz, 1H), 6.66 (d, J = 8.1 Hz, 1H), 4.62 (d, J = 8.1 Hz, 1H), 3.38 (m, 1H), 3.04 (m, 2 H), 2.83 (m, 2 H), 2.43 (m, 2 H), 2.16 (m, 1 H), 1.82 (m, 2H), 1.84 (m, 1 H), 1.62 (m, 1 H), 1.41 (m, 1 H), 0.86 (m, 1 H), 0.76 (m, 2 H), 0.53 (m, 2 H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 164.68, 154.10, 147.20, 143.25, 142.09, 141.39, 129.55, 123.01, 122.22, 121.83, 121.17, 119.33, 117.98, 89.34, 69.64, 61.60, 60.67, 51.54, 45.67, 32.18, 29.30, 27.69, 22.97, 21.96, 13.95, 5.71, 5.05. ^{19}F NMR (75 MHz, DMSO- d_6) δ -57.80. IR (diamond, cm^{-1}) V_{max} : 3216.30, 2935.48, 2831.91, 1676.72, 1607.94, 1557.26, 1502.39, 1471.37, 1452.47, 1422.76, 1377.05, 1328.16, 1281.32, 1194.14, 1132.27, 1081.31, 1054.16, 1038.57, 1027.12, 999.69, 982.16, 927.80, 890.56, 858.47, 821.56, 785.26, 720.13, 691.36, 675.16. Mass spectrum $C_{27}H_{28}F_3N_3O_4$ m/z calcd 515.2032, obsd 516.2089 $[M + H]^+$. Mp 224.9–227.5 °C.

17-Cyclopropylmethyl-3,14 β -dihydroxy-4,5 α -epoxy-6 β [(3'-carboxyl-4'-pyridyl)acetamido]morphinan (Compound 5).—Compound 5 was synthesized as shown in the general procedure with a yield of 23%. 1H NMR (400 MHz, DMSO- d_6) δ 9.14 (d, J = 0.84 Hz, 1 H), 9.12 (d, J = 4.76 Hz, 1 H), 8.99 (s, 1 H), 7.91 (d, J = 1.08 Hz, 1 H), 7.90 (d, J = 0.92 Hz, 1 H), 6.62–6.52 (m, 2 H), 4.99 (d, J = 8.08 Hz, 1 H), 4.92 (s, 1 H), 3.91–3.82 (m, 1 H), 3.16 (d, J = 5.24 Hz, 1 H), 3.03 (t, 2 H), 2.97 (s, 1 H), 2.63–2.60 (m, 1 H), 2.57–2.53 (m, 1 H), 2.40–2.30 (m, 2 H), 2.24–2.18 (m, 1 H), 2.02–1.95 (m, 1 H), 1.59–1.55 (m, 1 H), 1.18–1.44 (m, 1 H), 1.41–1.34 (m, 1 H), 1.26–1.23 (m, 1 H), 0.87–0.82 (m, 1 H), 0.48–0.46 (m, 2 H), 0.13–0.11 (m, 2 H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 167.65, 167.25, 156.53, 144.44, 141.89, 141.13, 139.43, 125.90, 119.61, 117.53, 88.10, 70.04, 62.06, 58.80, 52.80, 49.03, 48.08, 44.14, 31.23, 22.66, 4.26, 4.00. IR (diamond, cm^{-1}) V_{max} 3345.73, 3039.98, 2478.91, 2079.72, 1782.60, 1725.96, 1651.70, 1630.27, 1614.51, 1556.21, 1505.72, 1455.55, 1398.02, 1361.26, 1263.68, 728.87, 708.62. Mass spectrum $C_{27}H_{29}N_3O_6$ m/z calcd 491.2056, obsd 474.2033 $[M-CH_3]^+$. Mp 271.2–273.7 °C.

17-Cyclopropylmethyl-3,14 β -dihydroxy-4,5 α -epoxy-6 β [(3'-methylformate-4'-pyridyl)acetamido]morphinan (Compound 6).—Compound 6 was synthesized as shown in the general procedure with a yield of 32%. 1H NMR (400 MHz, DMSO- d_6) δ 9.16 (s, 1 H), 9.12 (s, 1 H), 9.08 (s, 1 H), 9.00 (d, J = 3.68 Hz, 1 H), 7.91 (d, J = 3.68 Hz, 1 H), 7.76 (d, J = 3.52 Hz, 1 H), 7.31 (s, 1 H), 7.26 (d, J = 8.24 Hz, 1 H), 7.00 (d, J = 8.24 Hz, 1 H), 5.16 (7.26 (d, J = 8.24 Hz, 1 H)), 3.83 (s, 3H), 3.55 (s, 1 H), 3.50 (s, 1H), 3.36–3.35 (m, 1 H), 3.27–3.24 (s, 1 H), 3.23 (s, 2 H), 3.16–3.12 (m, 1 H), 2.95 (m, 1 H), 2.66–2.63 (m, 1 H), 1.99–1.95 (m, 1 H), 1.57–1.44 (m, 3 H), 1.23–1.19 (m, 1 H), 1.13 (m, 1 H), 0.69 (m, 1 H), 0.62 (m, 1 H), 0.54 (m, 1 H), 0.45 (m, 1 H). ^{13}C NMR (100 MHz, DMSO- d_6) δ 167.04, 165.51, 163.83, 156.57, 154.31, 150.66, 146.23, 144.61, 139.14, 134.01, 131.22, 130.14, 123.75, 122.67, 120.93, 117.47, 89.86, 70.03, 61.97, 57.38, 53.57, 52.41, 49.14, 46.62,

30.53, 27.58, 24.12, 21.11, 6.29, 5.70, 3.29. IR (diamond, cm^{-1}) V_{max} 3554.62, 2953.39, 2088.05, 1716.47, 1633.27, 1557.57, 1489.97, 1446.28, 1367.76, 1290.11, 1263.40, 1234.61, 1193.08, 1152.84, 1128.11, 1101.15, 1076.56, 1032.71, 995.14, 960.71, 938.56, 914.77, 892.11, 855.03, 834.94, 797.66, 780.76, 732.58, 685.21, 662.07. Mass spectrum m/z calcd $\text{C}_{28}\text{H}_{31}\text{N}_3\text{O}_6$ 505.2213, obsd 506.2227 $[\text{M} + \text{H}]^+$. Mp 210.5–212.3 °C.

17-Cyclopropylmethyl-3,14 β -dihydroxy-4,5 α -epoxy-6 β [(3'-ethyl-4'-pyridyl)acetamido]morphinan (Compound 7).—Compound 7 was synthesized as shown in the general procedure with a yield of 61%. ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 9.00(s, 1 H), 8.50 (m, 2 H), 7.25 (m, 1 H), 6.70 (d, J = 8.1 Hz, 1H), 6.54(d, J = 8.1 Hz, 1H), 4.53(d, J = 7.8 Hz, 1H), 3.61 (m, 1H), 3.01 (m, 2 H), 2.74 (q, J = 7.6 Hz, 2H), 2.56 (m, 2 H), 2.29 (m, 2 H), 2.12 (m, 1H), 2.00 (m, 1H), 1.82 (m, 1 H), 1.62 (m, 1 H), 1.48 (m, 1 H), 1.34 (m, 1 H), 1.25 (m, 1 H), 1.16 (t, J = 7.6 Hz, 3H), 0.84 (m, 1 H), 0.47 (m, 2 H), 0.12 (m, 2 H). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$) δ 166.80, 150.49, 147.32, 143.38, 142.17, 140.54, 135.81, 131.33, 123.50, 120.89, 118.39, 117.10, 90.38, 69.58, 61.74, 58.37, 54.86, 51.53, 47.05, 43.68, 30.09, 24.32, 23.14, 22.19, 15.54, 9.22, 3.65, 3.51. IR (diamond, cm^{-1}) V_{max} 3307.45, 3084.08, 2946.81, 2931.57, 2822.35, 1659.73, 1592.75, 1530.90, 1503.18, 1471.36, 1454.36, 1431.17, 1404.97, 1368.65, 1322.47, 1260.16, 1239.39, 1227.39, 1190.27, 1154.54, 1128.18, 1114.19, 1096.93, 1082.03, 1059.66, 1036.40, 1028.24, 974.96, 932.47, 922.72, 915.97, 899.73, 877.40, 855.23, 818.18, 799.14, 784.30, 761.82, 744.37, 709.36, 675.07. Mass spectrum m/z calcd $\text{C}_{28}\text{H}_{33}\text{N}_3\text{O}_4$ 475.2471, obsd 476.2539 $[\text{M} + \text{H}]^+$. Mp 238.6–241.3 °C.

17-Cyclopropylmethyl-3,14 β -dihydroxy-4,5 α -epoxy-6 β [(3'-isobutyl-4'-pyridyl)acetamido]morphinan (Compound 8).—Compound 8 was synthesized as shown in the general procedure with a yield of 48%. ^1H NMR (400 MHz, CDCl_3) δ 8.61 (d, J = 5.04 Hz, 1 H), 8.58 (s, 1 H), 8.48–8.46 (m, 2 H), 7.86 (d, J = 5.04 Hz, 1H), 7.23 (d, J = 4.92 Hz, 1 H), 6.96 (d, J = 8.16 Hz, 1 H), 6.74 (d, J = 8.20, 1H), 4.56 (d, J = 4.80 Hz, 1 H), 4.27–4.21(m, 1H), 3.15 (s, 1 H), 3.11 (s, 1 H), 2.90–2.86 (m, 1 H), 2.68–2.66 (m, 2 H), 2.41–2.39 (m, 1 H), 2.27–2.25 (m, 1 H), 1.96–1.85 (m, 1 H), 1.65–1.63 (m, 1 H), 1.26 (s, 1 H), 0.93 (d, J = 2.20 Hz, 2 H), 0.91 (d, J = 2.16 Hz, 2 H), 0.84 (d, J = 7.96 Hz, 6 H), 0.57–0.55 (m, 2 H), 0.16–0.15 (m, 2H). ^{13}C NMR (100 MHz, CDCl_3) δ 167.37, 164.01, 153.50, 152.53, 148.14, 143.75, 137.28, 136.07, 134.23, 132.66, 132.14, 123.90, 122.76, 120.83, 119.34, 92.94, 69.91, 62.25, 59.63, 49.70, 47.07, 43.86, 40.21, 39.46, 30.39, 30.21, 28.70, 23.25, 22.55, 4.19, 3.94, 0.13. IR (diamond, cm^{-1}) V_{max} 3212.54, 3055.22, 2957.95, 2869.52, 2580.83, 2089.08, 1756.35, 1659.98, 1633.88, 1446.95, 1257.27, 1234.91, 1057.99, 851.95, 772.04, 666.71. Mass spectrum m/z calcd $\text{C}_{30}\text{H}_{37}\text{N}_3\text{O}_4$ 503.2784, obsd 504.2653 $[\text{M} + \text{H}]^+$. Mp 195.5–197.8 °C.

17-Cyclopropylmethyl-3,14 β -dihydroxy-4,5 α -epoxy-6 β [(3'-cyclopentanyl-4'-pyridyl)acetamido]morphinan (Compound 9).—Compound 9 was synthesized as shown in the general procedure with a yield of 58%. ^1H NMR (400 MHz, CDCl_3) δ 8.66 (s, 1 H), 8.43 (m, 1 H), 7.05 (m, 1 H), 7.03 (m, 1H), 6.74 (d, J = 8.1 Hz, 1H), 6.58 (2 d, J = 8.1 Hz, 1H), 4.45 (d, J = 7.8 Hz, 1H), 3.99 (m, 1H), 3.33 (m, 1 H), 3.06 (m, 2H), 2.63 (m, 2 H), 2.36 (m, 2 H), 2.18 (m, 2H), 2.08 (m, 2H), 1.83 (m, 3H), 1.65 (m, 16 H), 1.49 (m, 2 H), 0.82

(m, 1 H), 0.52 (m, 2 H), 0.12 (m, 2 H). ^{13}C NMR (100 MHz, MeOH- d_4) δ 170.58, 149.17, 147.62, 146.33, 143.88, 142.80, 140.26, 122.25, 120.57, 119.18, 92.20, 71.59, 64.10, 59.53, 53.32, 41.77, 35.59, 35.51, 31.29, 26.59, 25.02, 24.07, 5.35, 3.80. IR (diamond, cm^{-1}) V_{max} 3135.82, 3042.97, 2956.45, 2868.49, 1635.34, 1543.77, 1504.22, 1451.51, 1406.10, 1324.84, 1242.99, 1156.26, 1124.74, 1060.06, 1032.41, 1012.61, 986.31, 919.54, 855.36, 799.44, 781.28, 747.15, 677.37. Mass spectrum $\text{C}_{31}\text{H}_{37}\text{N}_3\text{O}_4$ m/z calcd 515.2784, obsd 516.2896 $[\text{M} + \text{H}]^+$. Mp 231.1–232.8 °C.

17-Cyclopropylmethyl-3,14 β -dihydroxy-4,5 α -epoxy-6 β [(3'-cyclohexanyl-4'-pyridyl)acetamido]morphinan (Compound 10).—Compound 10 was synthesized as shown in the general procedure with a yield of 51%. ^1H NMR (400 MHz, CDCl_3) δ 8.66(s, 1 H), 8.46 (m, 1 H), 7.11 (m, 1 H), 6.89 (m, 1H), 6.74 (d, J = 8.1 Hz, 1H), 6.58(d, J = 8.1 Hz, 1H), 4.43(d, J = 7.8 Hz, 1H), 4.06 (m, 1H), 3.19 (m, 1 H), 2.91(m, 2H), 2.63 (m, 2 H), 2.36 (m, 2 H), 2.18 (m, 2H), 1.85 (m, 5H), 1.74 (m, 1 H), 1.66 (m, 2 H), 1.45 (m, 5H), 1.26 (m, 3 H), 0.82 (m, 1 H), 0.52 (m, 2 H), 0.12 (m, 2 H). ^{13}C NMR (100 MHz, MeOH- d_4) δ 172.30, 149.20, 147.66, 146.05, 143.84, 142.16, 141.31, 132.55, 125.43, 122.40, 120.11, 118.63, 92.74, 71.79, 63.78, 60.27, 53.44, 45.34, 40.59, 35.16, 34.94, 31.83, 31.36, 27.84, 27.73, 27.05, 25.56, 23.59, 10.25, 4.48, 4.20. IR (diamond, cm^{-1}) V_{max} 3269.93, 2925.57, 2850.60, 1644.45, 1537.65, 1504.66, 1450.26, 1411.24, 1374.41, 1324.67, 1240.14, 1188.89, 1149.13, 1130.35, 1095.73, 1035.49, 984.59, 918.19, 881.92, 856.04, 828.64, 798.39, 785.52, 762.60, 745.35, 728.43, 702.73, 677.85. Mass spectrum $\text{C}_{32}\text{H}_{39}\text{N}_3\text{O}_4$ m/z calcd 529.2941, obsd 530.3025 $[\text{M} + \text{H}]^+$. Mp 248.6–249.7 °C.

17-Cyclopropylmethyl-3,14 β -dihydroxy-4,5 α -epoxy-6 β [(3'-cycloheptanyl-4'-pyridyl)acetamido]morphinan (Compound 11).—Compound 11 was synthesized as shown in the general procedure with a yield of 65%. ^1H NMR (400 MHz, CDCl_3) δ 8.58(s, 1 H), 8.43 (m, 1 H), 7.07 (m, 1 H), 6.89 (m, 1H), 6.74 (d, J = 8.1 Hz, 1H), 6.58(d, J = 8.1 Hz, 1H), 4.44 (d, J = 7.8 Hz, 1H), 4.04 (m, 1H), 3.06(m, 3H), 2.63 (m, 2 H), 2.36 (m, 2 H), 2.18 (m, 2H), 1.47–1.92 (m, 17H), 0.83 (m, 1 H), 0.52 (m, 2 H), 0.13 (m, 2 H). ^{13}C NMR (100 MHz, MeOH- d_4) δ 170.34, 149.43, 147.35, 145.13, 143.83, 143.39, 142.18, 132.54, 125.41, 122.28, 120.11, 118.62, 92.74, 71.79, 63.78, 60.27, 53.43, 45.35, 42.38, 37.53, 37.42, 31.82, 31.36, 28.74, 28.73, 28.63, 28.47, 25.64, 23.59, 10.23, 4.48, 4.19. IR (diamond, cm^{-1}) V_{max} 3269.95, 3074.45, 2923.19, 2853.17, 1644.22, 1536.48, 1504.37, 1453.20, 1411.13, 1374.26, 1324.43, 1239.86, 1186.87, 1154.29, 1129.40, 1095.41, 1035.39, 984.37, 918.44, 896.79, 882.17, 856.61, 829.74, 798.64, 785.78, 762.42, 745.58, 728.45, 703.20, 676.77. Mass spectrum $\text{C}_{33}\text{H}_{41}\text{N}_3\text{O}_4$ m/z calcd 543.3097, obsd 544.3253 $[\text{M} + \text{H}]^+$. Mp 240.1–242.5 °C.

17-Cyclopropylmethyl-3,14 β -dihydroxy-4,5 α -epoxy-6 β [(3'-phenyl-4'-pyridyl)acetamido]morphinan (Compound 12).—Compound 12 was synthesized as shown in the general procedure with a yield of 60%. ^1H NMR (400 MHz, DMSO- d_6) δ 9.06(s, 1 H), 8.66(s, 1 H), 8.65 (m, 2 H), 7.48 (m, 4 H), 7.37 (m, 2H), 6.60 (d, J = 8.1 Hz, 1H), 6.51(d, J = 8.1 Hz, 1H), 4.43(d, J = 7.8 Hz, 1H), 3.48 (m, 1H), 3.17 (m, 1 H), 2.96 (m, 2H), 2.56 (m, 2 H), 2.30 (m, 2 H), 2.07 (m, 1H), 1.96 (m, 1H), 1.82 (m, 1 H), 1.54 (m, 1 H), 1.39 (m, 1 H), 1.23 (m, 3 H), 0.83 (m, 1 H), 0.46 (m, 2 H), 0.11 (m, 2 H). ^{13}C NMR (100

MHz, DMSO-*d*₆) δ 166.73, 150.11, 148.48, 143.55, 142.12, 140.48, 136.30, 133.45, 131.25, 128.25, 128.49, 127.80, 123.45, 121.48, 118.56, 117.07, 90.18, 69.51, 61.69, 58.33, 51.59, 48.57, 46.95, 43.67, 29.87, 23.71, 22.16, 9.18, 3.66, 3.49. IR (diamond, cm⁻¹) $\nu_{\max}$ 3267.19, 2926.62, 1759.45, 1655.72, 1537.27, 1490.98, 1477.79, 1445.55, 1399.94, 1329.53, 1277.45, 1236.21, 1200.83, 1154.28, 1131.52, 1095.64, 1068.52, 1037.44, 1006.16, 984.61, 934.19, 914.70, 880.93, 852.37, 793.70, 758.91, 699.07, 671.71. Mass spectrum C₃₂H₃₃N₃O₄ m/z calcd 523.2471, obsd 524.2552 [M + H]⁺. Mp 232.4–235.2 °C.

Biological Evaluation.

Drugs and Chemicals.: Morphine (morphine sulfate pentahydrate salt) and naloxone were procured from the National Institute of Drug Abuse (NIDA), Bethesda, MD, and then made into a 10 μ M stock solution by dissolving in distilled water, which was further diluted to the desired concentrations. All other reagents were purchased from either Sigma-Aldrich or Thermo Fisher.

Animals.: Male Swiss Webster mice (25–30 g, 7–8 weeks, Harlan Laboratories, Indianapolis, IN) were raised in animal care quarters and maintained at room temperature on light–dark cycle. Food and water were available ad libitum. Protocols and procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Commonwealth University and complied with the recommendations of the IASP (International Association for the Study of Pain).

Competitive Radioligand Binding and [³⁵S]GTP γ S Functional Studies.: In the competition binding assay, 30 μ g of membrane protein was incubated with the corresponding radioligand in the presence of different concentrations of test compounds in TME buffer (50 mM Tris, 3 mM MgCl₂, and 0.2 mM EGTA, pH 7.4). The bound radioligand was separated by filtration using the Brandel harvester. Specific (i.e., opioid receptor-related) binding at the MOR, KOR, and DOR was determined as the difference in binding obtained in the absence and presence of 5 μ M naltrexone, U50,488, and SNC80, respectively. The IC₅₀ values were determined and converted to K_i values using the Cheng–Prusoff equation.

In the [³⁵S]GTP γ S functional assay, 10 μ g of MOR-CHO membrane protein was incubated with 10 μ M GDP, 0.1 nM [³⁵S]GTP γ S, assay buffer (TME + 100 mM NaCl), and varying concentrations of the compounds under investigation for 90 min in a 30 °C water bath. Nonspecific binding was determined with 20 μ M unlabeled GTP γ S. 3 μ M DAMGO was included in the assay as maximally effective concentration of a full agonist for the MOR. All assays were determined in duplicate and repeated at least 4 times. Percent DAMGO-stimulated [³⁵S]GTP γ S binding was defined as (net-stimulated binding by ligand/net-stimulated binding by 3 μ M DAMGO) $\times$ 100.

Data Analysis of [³⁵S]GTP γ S Binding Assays.: All samples were assayed in duplicate and repeated at least four times for a total of 4 independent determinations. Results were reported as mean values $\pm$ SEM. Concentration–effect curves were fit by nonlinear regression to a one-site binding model, using GraphPad Prism software, to determine EC₅₀

and $E_{\max}$ values. IC_{50} values were obtained from Hill plots, analyzed by linear regression using GraphPad Prism 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.

Tail Immersion Test.: Swiss Webster mice (5 male mice for each group, 7–8 weeks, ENVIGO) were used for this experiment. Water bath temperature was maintained at 56 ± 0.1 °C. The baseline latency (control) was determined before the test compound was injected subcutaneously (sc) into the mice. The average baseline latency obtained for this experiment was 3.0 ± 0.1 s, and only mice with a baseline latency of 2–4 s were used. For the agonism study, tail immersion was conducted 20 min (time that morphine's antinociceptive effect starts to peak) after the test compound was injected. To prevent tissue damage, a 10 s maximum cutoff time was imposed. Antinociception response was calculated as the percentage maximum possible effect (%MPE), where $\%MPE = [(test - control)/(10 - control)] \times 100$. For the antagonism study, the test compound was given 5 min before morphine. Tail immersion was then conducted 20 min after giving morphine. %MPE was calculated for each mouse using at least five mice per drug. AD_{50} values were calculated using the least-squares linear regression analysis followed by calculation of 95% confidence interval by Bliss method.

Withdrawal Assay.: Swiss Webster mice (5 male mice for each group, 7–8 weeks, ENVIGO) were used for the withdrawal study. A 75 mg morphine pellet was implanted into the base of the neck of the mice, and the mice were then given time to recover in their home cages. Before the test began, 30 min was given to the mice for habituation to an open-topped, square, clear Plexiglas observation chamber ($26 \times 26 \times 26$ cm³) with lines partitioning the bottom into quadrants. All drugs and test compounds were administered by subcutaneous injection (sc). The withdrawal was precipitated 72 h from pellet implantation with naloxone (1 mg/kg, sc) and the test compounds at different doses. Withdrawal commenced within 3 min after antagonist administration. Escape jumps, paw tremors, and wet dog shakes were quantified by counting their occurrences over 20 min for each mouse (five mice per group). The data is shown as the mean $\pm$ SEM.

Statistical Analysis.: One-way ANOVA followed by the post hoc Dunnett test was performed to assess significance using Prism 6.0 software (GraphPad software, San Diego, CA).

Docking Study.: The crystal structures of the MOR (PDB code 4DKL), KOR (PDB code 4DJH), and DOR (PDB code 4EJ4) were obtained from the Protein Data Bank at <http://www.rcsb.org>. Sybyl-X 2.0⁵⁸ was used to add hydrogen atoms to each receptor and to sketch NFP (Tripos Inc., St. Louis, MO). NFP was then assigned Gasteiger–Hückel charges and energy minimized to a gradient of 0.05 under the TAFF in Sybyl-X 2.0.⁵⁸

GOLD 5.4 with default settings was used to conduct the docking study. The atoms within 10 Å of the γ -carbon atom of D^{3.32} of the MOR, KOR, and DOR were defined as the binding site in the three receptors. Automated docking was conducted with a distance constraint of 4 Å between the nitrogen atom at the 17' position of the epoxymorphinan skeleton and the

conserved D^{3.32}. In addition, a hydrogen bond constraint was applied between NFP's dihydrofuran oxygen and the phenolic oxygen of the conserved Y^{3.33}. The highest scored solutions (CHEM-PLP) were selected and merged into the receptor to obtain the optimal binding poses of NFP in the MOR, KOR, and DOR. After docking, the binding poses were energy minimized in Sybyl-X 2.0.7 with 5000 iterations under TAFF to remove the clashes and strain energy between NFP and the three receptors.⁷⁰

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS USED

OIC	opioid-induced constipation
GPCR	G-protein-coupled receptor
MOR	μ opioid receptor
KOR	κ opioid receptor
DOR	δ opioid receptor
NOP	nociception opioid peptide
GIRK	inwardly rectifying K^+
VGCC	voltage-gated Ca^{2+}
BBB	blood–brain barrier
CHO	Chinese hamster ovary
NFP	compound 1
NYP	compound 2
NMR	nuclear magnetic resonance
sc	subcutaneous injection
NIDA	National Institute of Drug Abuse
IACUC	Institutional Animal Care and Use Committee
NLX	naloxone
CL	confidence level

%MPE percentage maximum possible effect

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Figure 1.
MOR selective antagonists.

Compounds	R	Compounds	R
1	F	7	C ₂ H ₅
2	CN	8	Isobutyl
3	NO ₂	9	Cyclopentyl
4	CF ₃	10	Cyclohexyl
5	COOH	11	Cycloheptyl
6	COOCH ₃	12	Phenyl

Figure 2.
Structures of newly designed the third generation NAP derivatives.

Figure 3.

Tail immersion assay results for the third generation NAP derivatives as agonist (A) and antagonist (B) in the presence of morphine in mice at a single dose of 10 mg/kg. Morphine (10 mg/kg) and saline were used as positive and negative controls ($n = 5$, *** indicates $P < 0.005$).

Figure 4.

Dose-response studies of NFP (A) and NYP (B) as antagonists in mice. Morphine (10 mg/kg) and saline were used as positive and negative controls ($n = 5$, *** indicates $P < 0.005$).

Figure 5.

In vivo withdrawal study of NFP in morphine-pelleted mice ($n = 5$). The first column in each figure represents the withdrawal effects of NFP in placebo-pelleted mice, while the second to the seventh show the withdrawal effects of naloxone (NLX, 1 mg/kg) and NFP in morphine-pelleted mice ($n = 5$, * means $P < 0.05$, ** means $P < 0.005$, *** indicates $P < 0.0005$).

Figure 6.

In vivo withdrawal study of NYP in morphine-pelleted mice ($n = 5$). The first column in each figure represents the withdrawal effects of NYP in placebo-pelleted mice, while the second to the seventh show the withdrawal effects of naloxone (NLX, 1 mg/kg) and NYP in morphine-pelleted mice ($n = 5$, * indicates $P < 0.05$, ** indicates $P < 0.005$, *** indicates $P < 0.0005$).

Figure 7. MOR partial agonism and antagonism by NFP in mMORCHO cells. Ligand concentration–effect for stimulation of [35 S]GTP γ S binding in membranes from mMOR-CHO cells was determined alone (A) or in the presence of 0.3 μ M DAMGO (B). Data are the mean $\pm$ SEM ($n = 4-5$) of the percentage of maximal stimulation (produced by a maximally effective DAMGO concentration of 0.3 μ M).

Figure 8.
Chemical structure of NFP with atom notations from the docking studies.

Figure 9.

Docking studies results of NFP in the MOR (a, PDB code 4DKL), KOR (b, PDB code 4DJH), and DOR (c, PDB code 4EJ4), respectively, with the highest CHEM-PLP scores: NFP atoms, carbon (magenta); key residue atoms in MOR, carbon (green); key residue atoms in KOR, carbon (orange); key residue atoms in DOR, carbon (light blue); oxygen (red); nitrogen (blue). The red dashed lines represent the electrostatic interactions. The yellow dashed lines represent the hydrogen bonds.

Scheme 1.

Synthesis of 3'-Substituted Pyridylcarboxylic Acids for the Newly Designed NAP Derivatives

Scheme 2.

Synthesis Route of the Third Generation NAP Derivatives (Compounds 1–12)

Radioligand Binding Results at the MOR, KOR, and DOR and [³⁵S]GTPγS Functional Assay at the MOR for Third Generation NAP Derivatives

Table 1.

Compounds R	K _i (nM)		DOR		KOR		Selectivity		MOR [³⁵ S]GTPγS Binding	
	MOR						δ/μ	κ/μ	EC ₅₀ (nM)	% Emax of DAMGO
NTX ^a	0.26 ± 0.02		117.1 ± 8.9		5.15 ± 0.26		450	20	ND	7.75 ± 0.20
NAP ^a	0.37 ± 0.07		277.5 ± 8.0		60.7 ± 5.6		747	163	1.14 ± 0.38	22.72 ± 0.84
1(NFP)	0.36 ± 0.02		156.93 ± 7.81		4.80 ± 0.26		435.8	13.3	1.20 ± 0.19	34.97 ± 3.07
2(NYP)	0.87 ± 0.09		46.29 ± 7.86		2.74 ± 0.47		53.2	3.1	1.28 ± 0.20	26.05 ± 0.74
3	0.17 ± 0.02		95.61 ± 5.90		4.46 ± 0.11		562.4	26.5	0.53 ± 0.01	39.10 ± 2.57

Compounds R	K _i (nM)	DOR	KOR	Selectivity		MOR [³⁵ S][GTP-γ-S Binding]	% Emax of DAMGO	
	MOR			δ/μ	κ/μ	EC ₅₀ (nM)
4	0.43 ± 0.01	61.79 ± 5.0	12.53 ± 0.89	143.7	29.1	1.38 ± 0.20	31.88 ± 3.39
5	0.76 ± 0.08	55.58 ± 6.02	2.43 ± 0.19	73.2	3.2	1.83 ± 0.18	29.27 ± 1.42
6	4.31 ± 0.29	137.31 ± 17.55	11.74 ± 1.15	31.9	2.7	13.40 ± 1.36	29.19 ± 0.84
NMP ^b	0.58 ± 0.25	273.6 ± 1.8	96.7 ± 12.2	470	166	1.52 ± 0.26	30.63 ± 0.55	

Compounds R	K _i (nM)	DOR	KOR	Selectivity		MOR [³⁵ S]GTPγS Binding	% Emax of DAMGO
	MOR			δ/μ	κ/μ	EC ₅₀ (nM)
7	0.32 ± 0.05	42.86 ± 8.27	9.18 ± 1.36	134.1	28.8	2.29 ± 0.28	41.65 ± 3.14
8	7.20 ± 0.81	148.75 ± 15.96	75.63 ± 3.87	20.7	10.5	19.70 ± 2.37	27.30 ± 0.25
9	0.60 ± 0.06	33.99 ± 3.99	8.16 ± 1.17	56.7	13.7	1.85 ± 0.16	56.67 ± 3.14

Compounds R	K _i (nM)	DOR	KOR	Selectivity		MOR [³⁵ S]GTP-γS Binding	% Emax of DAMGO
	MOR			δ/μ	κ/μ	EC ₅₀ (nM)
10	0.78 ± 0.09	11.24 ± 1.91	3.32 ± 0.54	14.4	4.2	2.46 ± 0.30	51.59 ± 3.73
11	0.93 ± 0.13	14.97 ± 2.44	5.16 ± 0.54	16.1	5.6	5.11 ± 0.16	48.32 ± 3.34
12	0.63 ± 0.05	44.83 ± 3.70	3.42 ± 0.38	71.1	5.4	1.46 ± 0.18	44.42 ± 3.12

^aReference 44a.

Reference 57a.

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