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Nanoconjugated NAP as a Potent and Periphery Selective Mu Opioid Receptor Modulator To Treat Opioid-Induced Constipation

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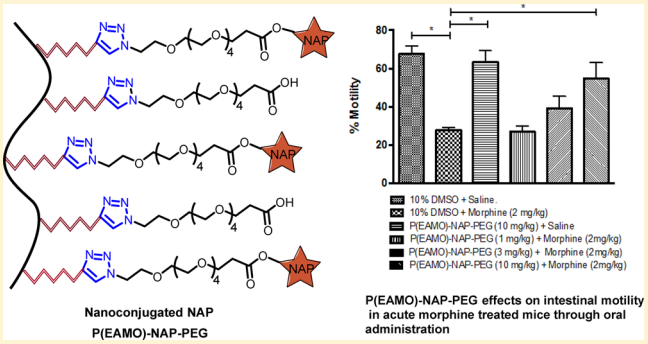
Supporting Information

ABSTRACT: Opioids are the mainstay for cancer and noncancer pain management. However, their use is often associated with multiple adverse effects. Among them, the most common and persistent one is probably opioid-induced constipation (OIC). Periphery selective opioid antagonists may alleviate the symptoms of OIC without compromising the analgesic effects of opioids. Recently our laboratories have identified one novel lead compound, 17-cyclopropylmethyl-3,14β-dihydroxy-4,5α-epoxy-6β-[(4′-pyridyl)acetamido]-morphinan (NAP), as a peripherally selective mu opioid receptor ligand carrying subnanomolar affinity to the mu opioid receptor and over 100-folds of selectivity over both the delta and kappa opioid receptors, with reasonable oral availability and half-life, and potential to treat OIC. Nanoparticle-based drug delivery systems are now widely considered due to their technological advantages such as good stability, high carrier capacity, low therapeutic side effects, etc. Herein we report nanoparticle supported NAP as a potential candidate for OIC treatment with improved peripheral selectivity over the original lead compound NAP.



KEYWORDS: *Opioid-induced constipation, Mu opioid receptor antagonist, Periphery selective, NAP, nanoconjugate*

Opioids are the mainstay for cancer and noncancer pain management. However, their use is associated with multiple adverse effects with the most common and distressing being constipation.[1] The high prevalence of opioid-induced constipation (OIC) among different populations and rare tolerance development to constipation significantly limit opioid usage.[2]

The traditional treatment of OIC by employing laxatives provides unsatisfactory clinical results.[3] Several other pharmacological interventions have been applied to address OIC, including opioid switch (such as switching from morphine to transdermal fentanyl,[4] transdermal buprenorphine,[5] methadone,[6] or tapentadol[7]), 5-HT$_4$ agonists (such as prucalopride[8]),), and type-2 chloride channel activators (such as lubiprostone[9]).

The pathophysiology of OIC is attributed primarily to the activation of peripheral mu opioid receptor (MOR) in the gastrointestinal (GI) tract,[10] though central effects cannot be totally ruled out.[11] Most of the aforementioned therapies have only limited effectiveness for OIC because they do not directly address this underlying pathophysiology of OIC. Therefore, selectively targeting the MOR in the GI tract should lead to a more effective treatment of OIC.

Naloxone (Figure 1), a relatively mu- and kappa-selective opioid antagonist (K_i ratios, $\delta/\mu \approx 96$, $\delta/\kappa \approx 69$),[12] has a low oral bioavailability (~2%) due to extensive hepatic first-pass metabolism.[13] Its role in OIC has been extensively studied for over a decade, yet reversal of desired analgesia and/or precipitation of withdrawal symptoms are frequently seen with only modestly improved laxation after immediate release of naloxone.[14] A fixed dose combination of extended-release naloxone and oxycodone overcame these drawbacks and

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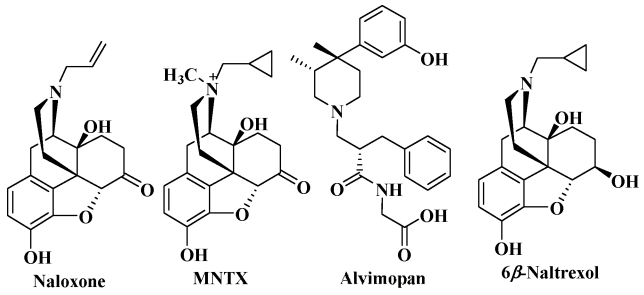

Figure 1. Mu opioid receptor selective antagonists targeting the peripheral nervous system.

significantly improved bowel function due to its improved pharmacokinetic property.[15] However, given its predetermined "recipe", this fixed-dose combination is not applicable to patients with chronic liver diseases or those using other opioid analgesics. It is therefore approved only in 13 European countries.[16]

Periphery-selective MOR antagonists will relieve OIC without compromising the systemic analgesic effects or inducing opioid withdrawal symptoms.[17] The first drug of this class, methylnaltrexone (MNTX, Figure 1),[17] was approved by the FDA in 2008 for palliative-care patients who suffer from OIC when laxatives are insufficient.[17] Since MNTX carries a permanently positive charge, it does not penetrate central nervous system (CNS) significantly. Another periphery-selective MOR antagonist, alvimopan (Figure 1), is efficacious in improving spontaneous bowel movements (SBM) compared to placebo[18] without any significant CNS effect due to its zwitterionic property. 6β-Naltrexol (Figure 1) has been reported to inhibit morphine-induced slowing of GI transit in healthy opioid-naive volunteers by acting as a peripheral opioid antagonist.[19] Naloxegol is another marketed drug for the treatment of opioid induced constipation in adult patients with chronic, noncancer pain. Naloxegol is a pegylated (polyethylene glycol-modified) derivative of naloxol. The pegylation of 5-alpha-hydroxyl side chain of naloxol improved the peripheral selectivity of naloxegol over the naloxone and methylnaltrexone.[20] Therefore, these clinical study results demonstrate that specifically blocking MOR in the GI tract indeed improves symptoms of OIC.

From our effort in identifying selective MOR antagonist, 17-cyclopropylmethyl-3,14β-dihydroxy-4,5α-epoxy-6β-[(4′-pyridyl)acetamido]morphinan (NAP) (Figure 2) was found to act as a periphery-selective MOR ligand based on *in vitro* and *in vivo* pharmacological and pharmacokinetic (PK) studies.[21,22] Not only did NAP display high binding affinity for the MOR (with more than 700-fold selectivity over the delta opioid receptor (DOR) and more than 150-fold selectivity over the kappa opioid receptor (KOR), but it also increased the intestinal motility in morphine-pelleted mice (PO ED_{50} = 0.009 mg/kg), nearly 300-fold more potent than MNTX, making it a novel lead to address the peripheral side effects of opioids.

Nanoparticle-based drug delivery systems have been widely considered due to their technological advantages, such as good stability, high carrier capacity, low therapeutic side effects, and feasibility of variable routes of administration. Nanoparticles have also been adopted as drug delivery system for opioid-type medications. For example, opioid peptide DPDPE was coupled to polyamidoamine (PAMAM) dendrimer G4.5 with polyethylene glycol (PEG) as the underlying carrier to construct

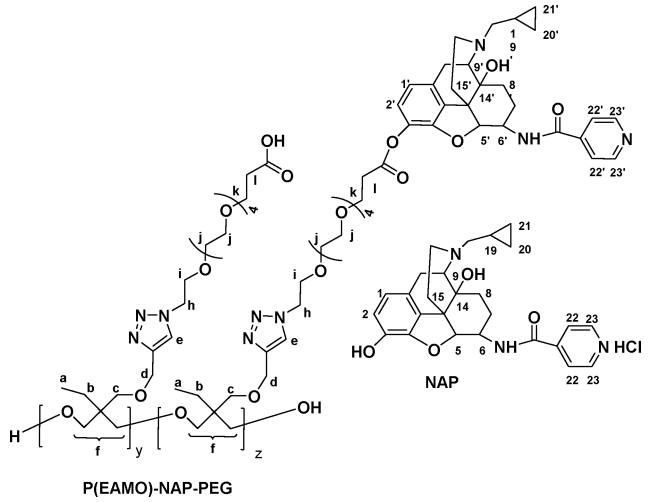

Figure 2. NAP and nanoconjugated NAP (P(EAMO)-NAP-PEG).

CNS therapeutic nanoparticles and was explored within the buccal mucosa as an alternative absorption site for administration of the dendritic nanoparticles.[23] Increased brain delivery of opioid peptide DAMGO by glutathione-PEGylated liposomes has also been reported.[24] More particularly, NTX-loaded micelles and nanogels showed increased therapeutic index and improved selectivity.[25] We recently synthesized water-soluble cytocompatible PEG-grafted polyoxetane brush polymers through ring-opening polymerization of acetylene-functionalized 3-ethyl-3-(hydroxymethyl)oxetane (EAMO) monomer to generate backbone of P(EAMO) followed by a click reaction with methoxypolyethylene glycol azide (mPEG-azide).[26] The uniformly distributed alkyne pendant groups make this new platform well suited for delivery of therapeutic and diagnostic agents.

In this work, we investigated the utility of this new carrier for NAP delivery in the GI tract. NAP was designed to link to P(EAMO) with PEG spacer, one end click coupled to the P(EAMO) polymer through triazole formation, the other end linking NAP through an ester bond (Figure 2). The formed NAP nanoparticles were then investigated for its therapeutic effectiveness through *in vitro* and *in vivo* experiments.

Similar to naloxegol, the nanoconjugated NAP is also a pegylated derivative. The phenol group of NAP is connected to PEG via an ester group. Structure-wise, the similar feature of our nanoconjugated NAP and naloxegol is the PEG component, while the most significant difference between them is that in nanoconjugated NAP the other end of PEG is connected to a polymer backbone via a triazole linkage and in naloxegol the other end of PEG is free. Ideally, nanoparticles would have advantages of high load ability and improved peripheral selectivity. The possible limitation of nanoconjugated NAP may include difficulty to control and optimize the surface coverage density and conformation.

NAP-PEG-azide was synthesized by coupling NAP to PEG-azide via the Steglich esterification (for details, see Supporting Information).[27] A click reaction between NAP-PEG-azide and P(EAMO) furnished the synthesis of NAP nanoconjugates. A singlet 7.94 ppm (Figure 3) was assigned to proton of the triazole ring, indicating the success of the click reaction. The ^{1}H NMR spectrum also indicated no free NAP present in the nanoconjugates (Figure 3). The FTIR spectrum of nanoconjugated NAP (see Supporting Information) clearly

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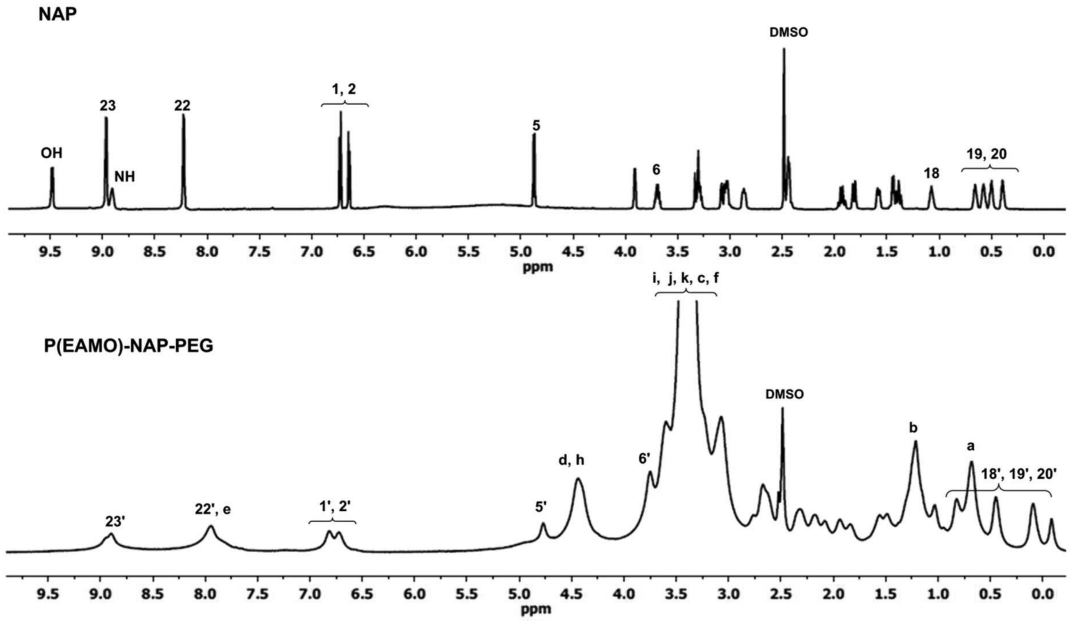

Figure 3. NMR spectra of (top) NAP and (bottom) P(EAMO)-NAP-PEG in d_6-DMSO.

presented absorption carried over from NAP and P(EAMO)-PEG, respectively. The vanishing of a weak alkyne absorption peak at 2108 cm^{-1} in nanoconjugated NAP further confirmed a 100% substitution. According to ^1H NMR spectroscopy analysis, 73% of NAP in the crude intermediate was conjugated to P(EAMO) through PEG. Combined analysis indicated that all alkynes were click coupled with PEG moieties, out of which 19 repeat units contained the drug. Conjugated NAP formula was then defined as P(EAMO)-$(PEG\text{-}acid)_{12}$-$(PEG\text{-}NAP)_{19}$.

The synthesized NAP nanoparticle was then evaluated for its critical micelle concentration (CMC).[28] Figure 4 presented the

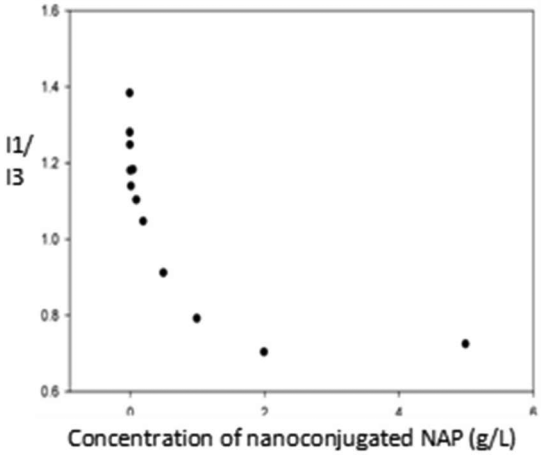

Figure 4. Critical micelle concentration evaluation. Plot of pyrene I1/I3 ratio versus concentration of nanoconjugated NAP.

pyrene I1/I3 ratio plot for nanoconjugated NAP in water. Using pyrene as a fluorescence probe, CMC of nanoconjugated NAP was determined to be 0.95 g/L, after which nanoconjugated NAP form stable micelles. Nanoconjugated NAP formed stable micelles with an average particle size of 541.5 ± 89.3 nm and zeta potential of 31.0 ± 13.7 mV at concentrations above CMC (see Supporting Information for detailed protocols).

The nanoconjugated NAP was then evaluated for its binding activities at opioid receptors.[21] Compared to naltrexone (NTX) and the parent lead compound NAP, nanoconjugated NAP retained high binding affinity at the MOR with a K_i of 1.76 nM (based on an average molecular weight of 23375 g/mol). The binding affinity of nanoconjugated NAP at the KOR was somehow higher than that of NAP itself. As a result, nanoconjugated NAP displayed a similar selectivity for MOR over KOR compared to NTX (Table 1). Nanoconjugated NAP only inhibited 18.27 ± 1.35% [^{3}H]naltrindole binding to DOR at 10 μg/mL, the highest concentration that can be used to maintain a minimum percentage of DMSO in the assay. Because of concentration limitation, the IC_{50} and K_i values of nanoconjugated NAP at DOR could not be determined under the tested conditions. Therefore, an arbitrary IC_{50} of >10 μg/mL was plugged into the Cheng−Prusoff equation to obtain a K_i value to estimate its selectivity. Apparently nanoconjugated NAP still maintained NAP's high selectivity over the DOR (see Supporting Information for detailed protocols). The MOR efficacy of nanoconjugated NAP was then evaluated using the MOR [^{35}S]GTPγS binding assay. Similar to NAP, nanoconjugated NAP acted as a low-efficacy MOR partial agonist (25.1% E_{max} of DAMGO) (Table 1). Interestingly, nanoconjugated NAP showed the lowest potency in activating MOR (EC_{50} = 6.44 ± 0.83 nM) compared to NTX and NAP. This is a desired characteristic for therapeutic purpose in order to reduce potential side effect related to activation of the MOR.

To investigate the peripheral selectivity of P(EAMO)-NAP-PEG, the warm water tail immersion test was performed in mice acutely treated with morphine (10 mg/kg) and P(EAMO)-NAP-PEG at increasing concentrations from 0.1 to 10 mg/kg (Figure 5)[29] (see Supporting Information for detailed protocols). P(EAMO)-NAP-PEG alone at the maximum dose of 10 mg/kg did not show any antinociceptive effects. Importantly P(EAMO)-NAP-PEG did not antagonize morphine antinociception at doses up to 10 mg/kg. As the formula of nanoconjugated NAP was defined as P(EAMO)-$(PEG\text{-}acid)_{12}$-$(PEG\text{-}NAP)_{19}$, the dose range of P(EAMO)-NAP-PEG (0.1 to 10 mg/kg) can be calculated as 0.037 to 3.7

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Table 1. Binding Affinity, Selectivity, and Efficacy of Nanoconjugated NAP[a]

compd	K_i (nM)			selectivity		MOR [^{35}S]GTPγS binding	
	μ	κ	δ	κ/μ	δ/μ	EC_{50} (nM)	$\%E_{max}$ of DAMGO
	0.39 ± 0.04	4.3 ± 1.1	127.8 ± 3.5	11	328	0.16 ± 0.04	5.4 ± 0.8
NAP[b]	0.37 ± 0.07	60.7 ± 5.6	277.5 ± 8.0	164	750	1.14 ± 0.38	22.7 ± 0.8
nanoconjugated NAP	1.76 ± 0.28	22.6 ± 2.6	[c]	13	>64[d]	6.44 ± 0.83	25.1 ± 1.0

[a]The values are the means ± SEM of at least three independent experiments. [^{3}H]naloxone, [^{3}H]diprenorphine, and [^{3}H]naltrindole were used to label MOR, KOR and DOR, respectively. The percentage stimulation to DAMGO is the E_{max} of the compound compared to that of DAMGO (normalized to 100%). NTX (naltrexone) was tested under the same conditions. [b]Data taken from our previously published results.[21] [c]Nanoconjugated NAP showed only 18.27 ± 1.35% inhibition of [^{3}H]naltrindole binding at 10 μg/mL (the highest concentration allowed to be used in the assay). [d]An arbitrary IC_{50} of >10 μg/mL was plugged into the Cheng−Prusoff equation to obtain a K_i value for selectivity calculation.

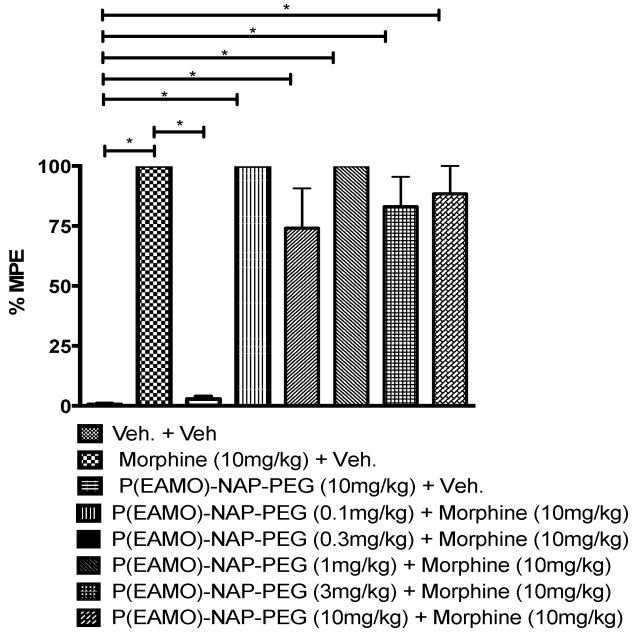

Figure 5. Antinociceptive effects of morphine in the presence of P(EAMO)-NAP-PEG as measured in the warm water tail immersion test. Data points are mean responses ± SEM, n = 4−5, *P < 0.05.

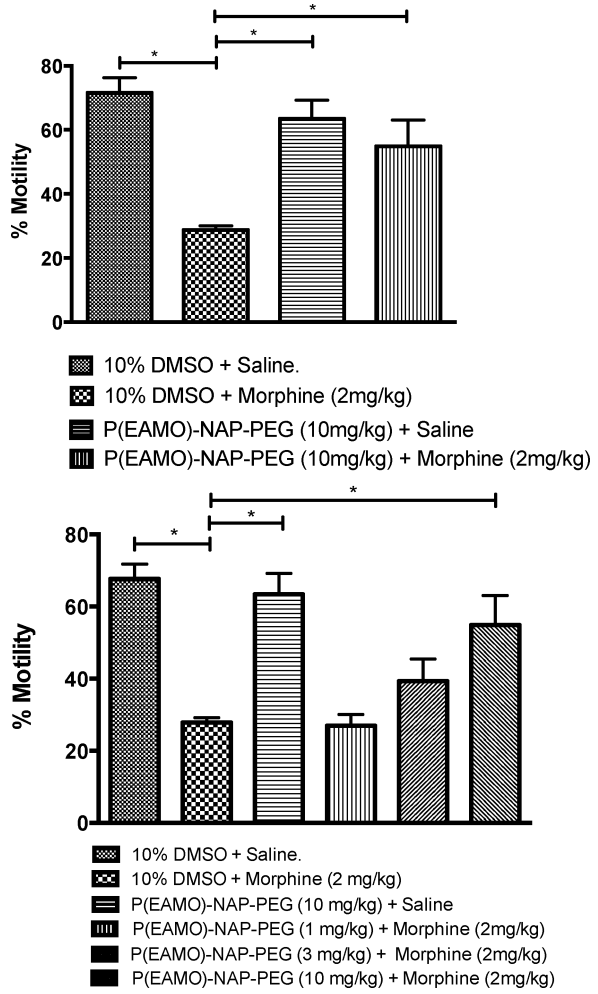

Figure 6. P(EAMO)-NAP-PEG effects on intestinal motility in acute morphine treated mice. (Top) s.c. injection of P(EAMO)-NAP-PEG. (Bottom) Oral administration of P(EAMO)-NAP-PEG. Data points are mean responses ± SEM, n = 4−9, *P < 0.05.

mg/kg regarding NAP. Due to the limited solubility of nanoconjugated NAP, a higher dose than 10 mg/kg was not tested. To be noticed, for NAP, the parent lead compound, though it did not carry any significant agonist activity, it did show certain degree of antagonist activity in the CNS and block the antinociceptive effect of morphine (AD_{50} at 4.98 mg/kg).[21] The diminished CNS activity of nanoconjugated NAP compared to NAP indicated that the nanoparticle skeleton (i.e., polymer carrier) further reinforced the peripheral selectivity of NAP, which made it to serve as a proof-of-concept for future drug development.

It is well-known that morphine significantly reduces GI motility. Given the diminished CNS activity of P(EAMO)-NAP-PEG as observed in the tail flick test, P(EAMO)-NAP-PEG was examined for its activity in the GI transit assay (see Supporting Information for detailed protocols). P(EAMO)-NAP-PEG alone showed no effect on GI motility when compared to vehicle treated groups. When administered via subcutaneous (s.c.) injection, P(EAMO)-NAP-PEG showed certain degree of reversal of morphine's effects on GI transit at the maximum dose used of 10 mg/kg (Figure 6A), but no dose response was observed (data not shown). However, when given via oral gavage, P(EAMO)-NAP-PEG did show a dose dependent reversal of morphine effects on GI motility and caused the highest reversal at 10 mg/kg (ED_{50} = 4.1 ± 2.9 mg/kg, Figure 6B).

In summary, nanoconjugated NAP was synthesized through the P(EAMO) carrier via the click reaction between the alkyne and azide and was characterized and structurally confirmed through ^{1}H NMR and FTIR. The NAP nanoparticles formed micelle over concentration of 0.95 g/L, with an average particle size of 541.5 ± 89.3 nm and zeta potential of 31.0 ± 13.7 mV at concentrations above CMC. In the *in vitro* and *in vivo* biological studies, the NAP nanoconjugates remained individual entities given that the concentration of nanoconjugated NAP was well

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below its CMC. Nanoconjugated NAP has proven to be a potent and selective MOR agent. Nanoconjugated NAP maintained the potency to treat opioid induced constipation with improved peripheral selectivity over NAP, the parent lead compound. These results further proved that nanotechnology application in drug delivery system IS very useful in medication development.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.6b00382.

Detailed synthetic procedures, nanoparticle characterization, critical micelle concentration and particle size determination protocols, and biological assay protocols (PDF)

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Author Contributions

Y.Z. and H.Y. conceived and oversaw the project. Y.Z., H.Y., H.I.A., D.E.S., and W.L.D. designed the experiments. G.G.X. conducted the characterization work and drafted the manuscript. O.Y.Z. conducted the chemical synthesis. D.A.W. conducted organ bath and animal studies. Y.Y. conducted radioligand binding assays under supervision of D.E.S. Y.Z. and H.Y. analyzed the data and discussed the results. Y.Z. and H.Y. finalized the manuscript. Y.Z. revised the manuscript.

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Notes

The authors declare no competing financial interest.

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