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Synthesis and Biological Evaluation of an Orally Active Glycosylated Endomorphin-1

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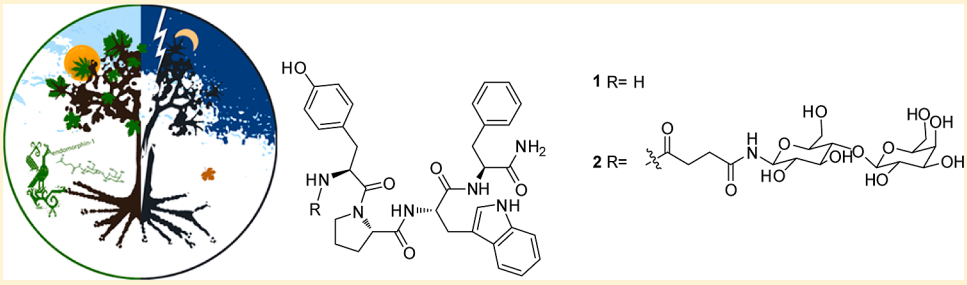
Supporting Information



ABSTRACT: The endogenous opioid peptide endomorphin-1 (**1**) was modified by attachment of lactose to the N-terminus via a succinamic acid spacer to produce compound **2**. The carbohydrate modification significantly improved the metabolic stability and membrane permeability of **2** while retaining μ-opioid receptor binding affinity and agonist activity. Analogue **2** produced dose-dependent antinociceptive activity following intravenous administration in a chronic constriction injury (CCI) rat model of neuropathic pain with an ED_{50} of 8.3 (±0.8) μmol/kg. The corresponding ED_{50} for morphine was 2.6 (±1.4) μmol/kg. Importantly, compound **2** produced dose-dependent pain relief after oral administration in CCI rats (ED_{50} = 19.6 (±1.2) μmol/kg), which was comparable with that of morphine (ED_{50} = 20.7 (±3.6) μmol/kg). Antineuropathic effects of analogue **2** were significantly attenuated by pretreatment of animals with the opioid antagonist naloxone, confirming opioid receptor-mediated analgesia. In contrast to morphine, no significant constipation was produced by compound **2** after oral administration.

INTRODUCTION

Neuropathic pain is a complex, chronic pain state that may develop secondary to damage to, or dysfunction of, the peripheral or central nervous system.[1] Pharmacological management of neuropathic pain is challenging for clinicians due to its heterogeneous nature. Currently available drug treatments, including anticonvulsants (e.g., gabapentin), antidepressants (e.g., amitriptyline), and antiarrhythmics, only cause pain relief in up to 50% of patients[2] and often produce dose-limiting side effects.[2,3] Additionally, morphine, the prototypic strong opioid analgesic, has reduced potency in neuropathic pain and produces a range of adverse effects including nausea, vomiting, constipation, sedation, analgesic tolerance, and cardiorespiratory depression.[3] Hence, considerable research attention has been directed toward investigation of endogenous opioid peptides as possible novel analgesic agents for producing potent analgesia with minimal opioid-related adverse effects. Two important members of this group are endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2, **1**) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2). They exhibit high selectivity and affinity for μ-opioid receptors (MOR),[4] the main opioid receptor type that mediates analgesia. Centrally administered endomorphins produce potent antinociception in rodent models of acute[5] and neuropathic pain[6] with less cardiorespiratory depression than opioid alkaloids.[7] In spite of the relatively high stability of endomorphins when compared with other opioid peptides, the rapid degradation of endomorphins by membrane-bound exo- and endopeptidases in the blood limits their usefulness as analgesics.[8,9] Furthermore, the blood−brain barrier (BBB) contains several enzymes that can degrade peptides and inhibit their penetration into the brain.[10] Because of their hydrophilicity, endomorphins, like all small peptides, are not able to cross the BBB by transmembrane diffusion.[11] Previous studies demonstrated that endomorphins do not elicit antinociceptive activity following peripheral administration in rodents.[12] Even after delivering the peptides directly into the central nervous system in animal models, endomorphins had a short duration of action (<30 min).[13,14] As a result, modification of the structure of endomorphins presents a simple and viable method to enhance their bioavailability to the brain.

Oral administration is the most convenient and favorable route for delivering drugs; nevertheless, it has proven to be

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extremely challenging for peptides and proteins.[15] In an attempt to overcome the shortcomings associated with oral bioavailability of peptides, a diverse array of chemical modifications have been explored including esterification,[16] amidation[17] (dermorphin analogues), and cyclization (α-conotoxin Vc1.1).[18] A well-studied approach to produce systemically active peptide analogues is conjugation to saccharide units.[19] Peptide glycosylation can promote biological stability as well as increase permeability across biological barriers via active or facilitated transport.[20,21] Previously, we showed that incorporation of sugars into the structure of short peptides can improve their oral bioavailability.[22] In the present study, we applied this approach to stabilize the structure of parent peptide **1**, reduce its vulnerability to proteolysis, and confer oral activity for the relief of neuropathic pain.

Previous work by our group showed that attachment of glucose succinamic acid to the N-terminus of peptide **1**, reduced MOR binding affinity considerably.[23] However, lactose modification has been reported to promote the uptake and activity of CNS peptides.[24,25] To investigate this, we modified the N-terminus of peptide **1** using lactose with a succinamic acid spacer. The resulting compound **2** was studied for its in vitro biological activity including plasma stability, membrane permeability, MOR, δ-opioid receptor (DOR), and κ-opioid receptor (KOR) binding affinity as well as MOR agonist activity. Additionally, the in vivo analgesic activity of derivative **2** was assessed in a rat model of neuropathic pain. The nonselective opioid receptor antagonist, naloxone hydrochloride was used to confirm the role of opioid receptors in mediating pain relief. In vivo pharmacological characterization of analogue **2** was also extended to evaluate its potency relative to morphine to induce constipation in rats.

RESULTS

Compounds **1** and **2** (Figure 1) were synthesized via solid phase peptide synthesis (see Supporting Information, Scheme 1) using the Fmoc in situ neutralization protocol[26] and purified to a single peak (>95% purity) by analytical reverse phase high performance liquid chromatography (RP-HPLC) and characterized using electrospray ionization mass spectrometry (ESI-MS). Purified yields of the compounds varied from 84% for peptide **1** to 24% for analogue **2**. In vitro assays were used to demonstrate the stability, membrane permeability, and functional activity of peptide **2**. Incubation of compound **2** with human plasma showed an increase in the stability. The half-life of the parent peptide **1** was 2.7 min, while the half-life of its analogue **2** was 57.3 min. The apparent permeability (P_{app}) values were evaluated using Caco-2 cell monolayers. Propranolol was used as a positive control, with P_{app} of 1.5 (±0.03) × 10^{-4} cm/s, because it is recognized as being completely absorbed from the gastrointestinal tract (GI).[27] Peptide **1** displayed low permeability through Caco-2 cell monolayers with P_{app} 1.1 (±0.5) × 10^{-7} cm/s, whereas compound **2** exhibited significant permeability with P_{app} 7.6 (±0.2) × 10^{-5} cm/s, which was a remarkable 700-fold increase from that of the parent peptide ($p < 0.001$).

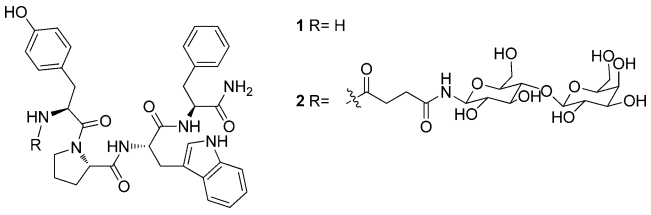

Figure 1. Structure of compounds **1** and **2**.

The possible role of a lactose receptor and lactose selective transporter[28,29] was examined by assessing the Caco-2 cell permeability of analogue **2** in the presence of 20 mM lactose, glucose, or galactose. The addition of lactose inhibited the absorption of analogue **2**, suggesting receptor-mediated absorption. Galactose, the terminal monosaccharide of lactose, caused less inhibition of the uptake of compound **2**. The presence of glucose had no effect on the permeability of compound **2**, suggesting that the absorption did not utilize any of the known glucose transporters (see Supporting Information, Figure 1).

Receptor Binding and Functional Activity. Receptor-binding scintillation proximity assay (SPA) was performed to investigate the affinity of compounds **1** and **2** at MORs, DORs, and KORs using membranes derived from stably transfected CHO-K1 cells which express the corresponding receptors. Confirmation of the agonist behavior of the peptide derivatives at MORs was attained by measuring the accumulation of cyclic adenosine monophosphate (cAMP) upon stimulation of SH-SY5Y cells with forskolin. SH-SY5Y is a human neuroblastoma cell line which endogenously expresses MORs and DORs. Treatment with MOR agonists has an inhibitory effect on the cAMP pathway, resulting in a reduction of intracellular cAMP formation.[30]

Compound **2** exhibited a decrease in binding affinity for the MORs compared with the parent peptide **1** at $K_{i\mu}$ 14.9 (±0.65) and 0.29 (±0.019) nM, respectively (Figure 2, Table 1). Like parent peptide **1**, compound **2** exhibited weak receptor binding affinities at DOR and KOR with $K_{i\delta}$ and $K_{i\kappa}$ values above 1000 nM (Table 1). The binding affinity of morphine was also tested at MOR, DOR, and KOR for comparison with $K_{i\mu}$, $K_{i\delta}$, and $K_{i\kappa}$ values 0.14 (±0.032), 295 (±19.8), and 13.3 (±1.7) nM, respectively.

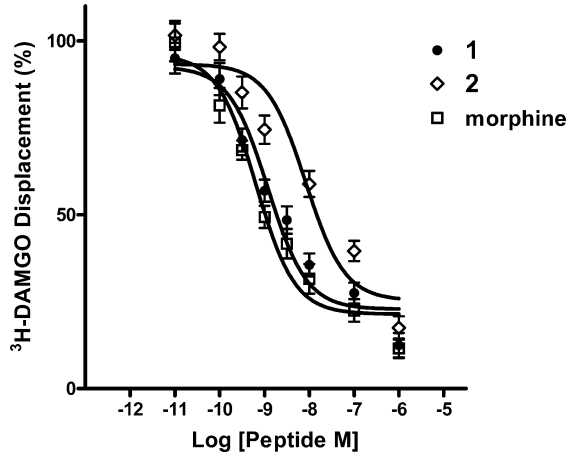

Figure 2. Binding affinities for compounds **1**, **2**, and morphine determined by competitive displacement of [^{3}H]DAMGO. Parent peptide **1** and morphine exhibited a higher binding affinity at MOR compared with compound **2**. Data presented are mean ± SEM from three separate experiments, each performed in triplicate.

Compounds **1** and **2** inhibited forskolin-stimulated cAMP formation in a concentration-dependent manner, with the carbohydrate derivative displaying a 9-fold lower potency than

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Table 1. Summary of in Vitro Results

compd	plasma stability,$t_{1/2}$ (min)	P_{app} (× 10^{-7}, cm/s)[a]	$K_{i\mu}$ (nM)[b]	$K_{i\delta}$ (nM)[c]	$K_{i\kappa}$ (nM)[d]	$K_{i\delta/\mu}$	$K_{i\kappa/\mu}$	cAMP, IC_{50} (nM)[e]
1	2.70 ± 1.9	1.1 ± 0.52	0.29 ± 0.019	2765 ± 176	2048 ± 424	9534	7062	65.4 ± 1.13
2	57.3 ± 6.6*	760.0 ± 19.9***	14.9 ± 0.65	4751 ± 354	1566 ± 165	319	105	590.2 ± 11.9
morphine	ND	ND	0.14 ± 0.032	295 ± 19.8	13.3 ± 1.7	2107	95	ND

[a]The apparent permeability (P_{app}, cm/s) through Caco-2 cell monolayers. The data were quantified by LC/MS and each point is expressed as mean ± SD (n = 4). [b]$K_{i\mu}$ versus [^{3}H]DAMGO. [c]$K_{i\delta}$ versus [^{3}H]DPDPE. [d]$K_{i\kappa}$ versus [^{3}H]diprenorphine. Binding affinity values were determined using eight concentrations of each compound in the range 10 pM to 1 μM and performed in three independent experiments, each in triplicate. Data are expressed as mean ± SEM. [e]The IC_{50} values (nM) were estimated from concentration−response curves using nonlinear regression for inhibition of forskolin-stimulated cAMP formation. *p < 0.05, ***p < 0.001 compared with compound **1** in the same experiment.

the parent peptide at MORs. IC_{50} values increased from 65.4 (±1.13) nM for compound **1** to 590.2 (±11.9) nM for compound **2** (Figure 3, Table 1).

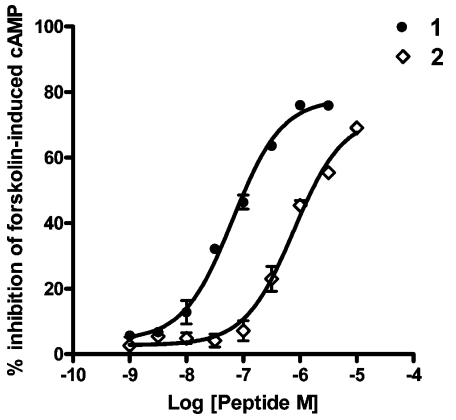

Figure 3. Concentration−response curves for the inhibitory effects of compounds **1** and **2** on forskolin-stimulated cAMP formation in SH-SY5Y cells. Data presented are mean ± SEM from three separate experiments, each performed in triplicate. Nonlinear regression as implemented in GraphPad Prism was used to estimate IC_{50} values.

Oral Absorption. To examine whether the high in vitro permeability of compound **2** translated into oral absorption of the peptide, 10 μmol/kg of compound **2** was administered to rats via oral gavage and the plasma concentrations of the peptide were measured at 30, 60, and 90 min postdosing. The peak plasma concentration of 22 (±5.1) nmol/mL was observed at 60 min postdosing for compound **2** (Figure 4). In contrast, the parent peptide **1** was not detected in plasma at any time following oral administration of the same dose.

Antinociceptive Activity. To investigate the impact of lactose conjugation on the pain-relieving effects of parent peptide **1** following systemic administration, the CCI rat model of neuropathic pain was used. Single doses of compounds **1** and **2** from 0.16 to 16 μmol/kg were administered intravenously (iv) and orally (po) to CCI rats. Morphine was used at 2 (iv) and 8 (po) μmol/kg p.o. Compound **2** produced significant analgesic activity at 5 and 16 μmol/kg, with a rapid onset of action at 15 min postdosing and a duration of action of 1.5 h following iv bolus administration (Figure 5a,b). The ED_{50} (±SEM) values for compound **2** and morphine were determined at 8.3 (±0.8) and 2.6 (±1.4) μmol/kg, respectively, after iv administration. Compound **1** did not produce significant analgesia after iv administration even at the highest dose tested (Figure 5a). The apparent membrane permeability coefficient for analogue **2** (Table 1), as well as its presence in plasma following oral administration (Figure 4), suggested its possible oral absorption. On the basis of these data, the efficacy

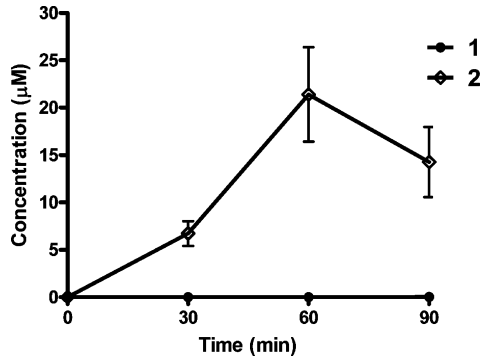

Figure 4. Plasma concentration versus time curves for compounds **1** and **2** following oral administration of single doses at 10 μmol/kg. The plasma concentrations were quantified by LC/MS, and each concentration is expressed as mean ± SEM (n = 4).

of compound **2** was evaluated in CCI rats after oral administration at the doses that produced pain relief following iv administration. Oral doses of compound **2** produced dose-dependent relief of mechanical allodynia in the ipsilateral (injured side) hindpaw of CCI rats. Compound **2** produced significant pain relief at 8 and 16 μmol/kg with the peak effect at 0.75−1 h postdosing and was active for up to 2 h (Figure 6a). Compound **2** at 8 μmol/kg was equi-effective, with morphine at the same dose after oral administration (Figure 6b) with ED_{50} (±SEM) values of 19.6 (±1.2) and 20.7 (±3.6) μmol/kg respectively.

Bolus iv and oral doses of compound **2** at 5 and 8 μmol/kg, respectively, produced insignificant antinociception in the contralateral (uninjured) hindpaw in contrast to morphine which produced significant antinociception in the contralateral hindpaw at corresponding doses (see Supporting Information, Figure 2, p < 0.001).

Naloxone Hydrochloride Pretreatment. Pretreatment of CCI rats with a single bolus subcutaneous (sc) dose of naloxone at 10 mg/kg abolished the analgesic effects of compound **2** at 5 μmol/kg iv and morphine at 2 μmol/kg iv (Figure 7, p < 0.001).

Constipation-Inducing Properties. The constipating potency of compound **2** relative to morphine was investigated using the castor oil-induced diarrhea assay and the charcoal test in order to assess the effect on stool hydration and GI motility, respectively. Single oral doses of compound **2** up to 16 μmol/kg (p > 0.05) did not significantly inhibit castor oil-induced diarrhea or GI transit of a charcoal meal. The mean (±SEM) GI propulsion (%) was 58.5 (±2.2) % for rats treated with compound **2**, which was not significantly different (p > 0.05) from that observed for vehicle-treated animals (66.5 ± 3.8%). By contrast, single oral doses of morphine at 8 μmol/kg significantly inhibited castor oil-induced diarrhea (p < 0.001)

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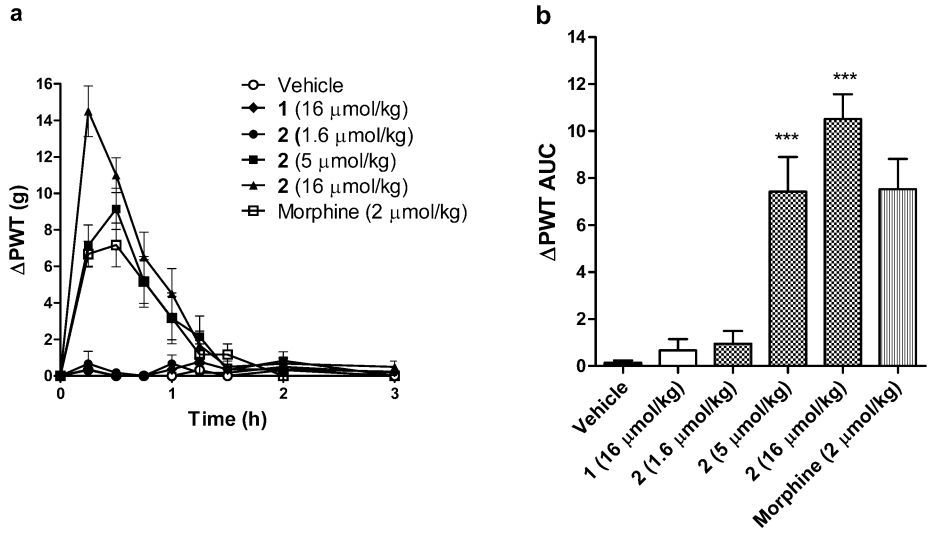

Figure 5. Intravenous dosing of compound **2** produced significant pain relief in the ipsilateral hindpaw of the CCI rat model of neuropathic pain. (a) Normalized (Δ) paw withdrawal thresholds (PWT) versus time curves for single iv bolus doses of compounds **1** and **2**, morphine, and vehicle in CCI rats. (b) Extent and duration of analgesia (areas under the curve (AUC) versus time, ΔPWT AUC values) shows that single iv bolus doses of compound **2** evoked significant analgesia at 5 and 16 μmol/kg, relative to vehicle. The ΔPWT AUC at 5 μmol/kg is comparable with that for morphine at 2 μmol/kg. Each value represents mean ± SEM (n = 6). ***p < 0.001 compounds **1** and **2** vs vehicle treated CCI rats.

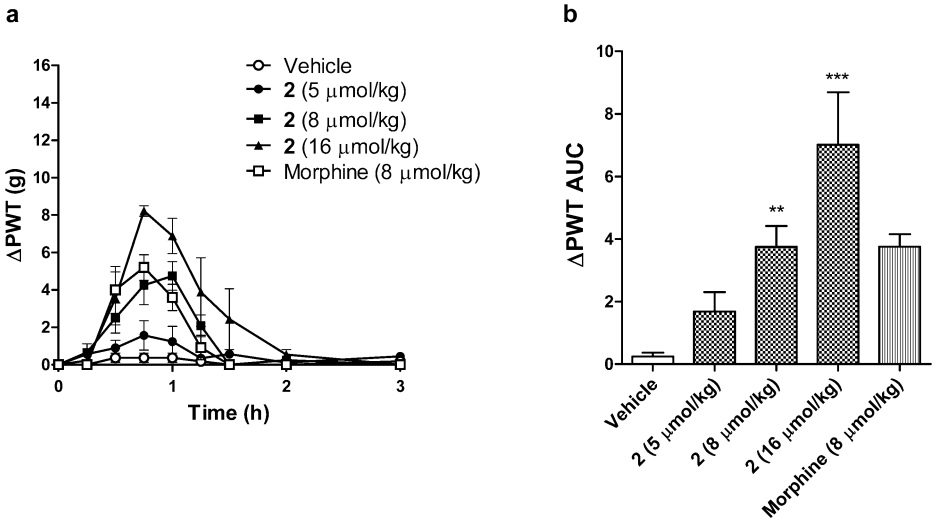

Figure 6. Dose-dependent analgesic activity was produced by single oral doses of compound **2** in the ipsilateral hindpaw of the CCI rat model of neuropathic pain. (a) The time course for the antinociceptive effects of compound **2**, morphine, and vehicle in CCI rats after oral gavage. (b) ΔPWT AUC values showing significant relief of mechanical allodynia produced by oral gavage of compound **2** at 8 and 16 μmol/kg relative to the vehicle. The effect at a dose of 8 μmol/kg is comparable with the effect of morphine at the equivalent dose. Each value represents mean ± SEM (n = 6–8). **p < 0.01, ***p < 0.001 compounds **2** vs vehicle treated CCI rats.

(Figure 8a) and markedly decreased GI transit of a charcoal meal (36.7 ± 2.9%, p < 0.001) (Figure 8b).

DISCUSSION

We showed that modification of peptide **1** with a lactose residue in position 1 (**2**) resulted in significant pain relief in CCI rats after both iv and oral administration.

The in vitro data demonstrated very low stability for peptide **1**, which is consistent with literature reports.[31,32] Conversely, compound **2** was significantly more stable in human plasma than the parent peptide **1**. There are a group of enzymes in plasma that can specifically hydrolyze peptide bonds. Dipeptidyl peptidase IV (DPP IV, EC.3.4.14.5) is a membrane-bound serine protease, which is the main enzyme degrading endomorphins.[33] Endomorphins are also the substrates of the proline-specific aminopeptidases, such as aminopeptidase P (APP; EC 3.4.11.9) and aminopeptidase M (APM, EC 3.4.11.2).[34] Carboxypeptidase Y (CPY, EC 3.4.16.1) can hydrolyze peptide amides such as endomorphins[35] and unlike many carboxypeptidases does not require a free C-terminal carboxyl group.[36]

The MOR, DOR, and KOR binding affinities of the synthesized peptides were determined by competitive displacement assay using [^{3}H]DAMGO, [^{3}H]DPDPE, and [^{3}H]-diprenorphine to label MOR, DOR, and KOR, respectively. In line with previous reports,[4] peptide **1** showed a high affinity and selectivity for MORs. While the lactose modification in compound **2** led to a drop in receptor binding affinity and agonist activity at MORs, it was still in the nanomolar range.

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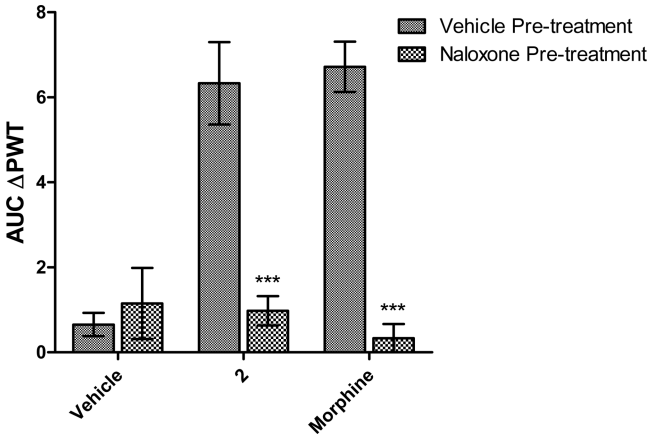

Figure 7. Pretreatment with the opioid receptor antagonist, naloxone hydrochloride (10 mg/kg sc), abolished the antinociception produced by a single iv bolus dose of compound **2** in the ipsilateral hindpaws of opioid-naive CCI rats (5 μmol/kg, $n = 6$) in a manner similar to morphine (2 μmol/kg, $n = 6$) ***$p < 0.001$, vs vehicle pretreated rats.

No increase in DOR and KOR binding affinities was observed for analogue **2**, demonstrating that it maintained selectivity for MORs. Morphine produced profound affinity for MORs, however, it showed a lack of selectivity for these receptors, which was consistent with previous studies.[30,37]

Despite the decrease in binding affinity and agonist activity of analogue **2** at the MORs, an unprecedented 700-fold increase in membrane permeability relative to the native peptide was observed. Lactose conjugation not only increased the stability and permeability of the peptide in vitro but also enhanced absorption across the GI mucosa in vivo. Hence, significant systemic exposure of compound **2** was observed after oral dosing in rats in contrast to the parent peptide **1**. Lactose significantly inhibited the membrane permeability of compound **2**, while galactose and glucose showed low or no inhibitory effect in the Caco-2 cell monolayer assay. These findings suggested receptor-mediated or lactose-selective transporter-mediated absorption of compound **2** as previously reported.[28,29] Furthermore, glycopeptides are believed to interact reversibly with the membrane and subsequently promote transport through brain capillaries by transcytosis.[32] According to the Biopharmaceutics Classification System, there is a high correlation between Caco-2 cell permeability coefficients (P_{app} values) and fractional absorption values (Fa) in humans. In this comparison, it was shown that for compounds with high Fa (%), the corresponding P_{app} values are in the range, 10^{-5}–10^{-6} cm/s.[38] On the basis of this classification, compound **2** is within the range of highly permeable compounds across barriers like the BBB. The Caco-2 cell model has been shown to be a reliable model when studying CNS targeted compounds, for either passive or active transport across the BBB.[39,40]

Previous studies demonstrated that glycosylation improved the brain delivery and intestinal absorption of some other peptides such as somatostatin,[41] met-enkephalin,[42] dermorphin, and deltorphin analogues.[16,43] These studies postulated that the improved bioavailability of the glycosylated peptide analogues stemmed from improved diffusion from peripheral sites into the bloodstream due to increased water solubility and active transport via glucose transporter 1 (GLUT-1), resulting in a higher rate of influx of the compounds from the blood to the brain.[44]

Following oral administration to CCI rats, compound **2** evoked significant analgesia. N-terminal conjugation of peptide **1** with lactose succinamic acid produced a derivative with significantly improved oral activity and comparable potency with morphine, for which various oral dosage forms are currently in clinical use. The pain-relieving activity of compound **2** in CCI rats was naloxone-sensitive, which is consistent with previous reports on the key role of opioid receptors in mediating the analgesic activity of peptide **1**.[14] Structure–activity studies of enkephalin-based opioid glycopeptides revealed that disaccharide derivatives were significantly more potent than any of the monosaccharides after peripheral administration.[24] In line with these findings, conjugation of peptide **1** with glucose residues resulted in over a 100-fold decrease in MOR binding.[23] The exact mechanism by which glycosylation improves BBB or GI transport has yet to be elucidated.[42] However, it is believed that glycopeptides promote negative membrane curvature on the surface of endothelial cells, which may increase endocytosis and result in enhanced transport through membranes via transcytosis.[32,45,46] Conjugation of peptide **1** with a lactose residue caused alterations in the excretion profile of the peptide and subsequently led to a significant systemic exposure of compound **2** after oral dosing in rats. Opioid peptides are predominantly cleared from the body via fecal–oral routes.[47,48] It has previously been shown that shifting from hepatic to renal

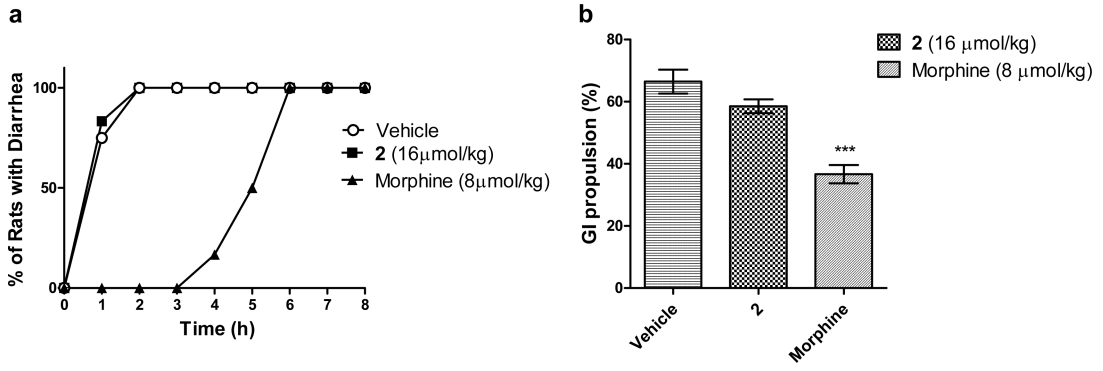

Figure 8. Assessment of the constipation-inducing activity of compound **2** relative to morphine in drug-naive rats following oral administration. (a) Lack of inhibitory effect of compound **2** in doses up to 16 μmol/kg on induction of diarrhea following oral administration of castor oil in contrast to significant inhibitory effects of morphine at 8 μmol/kg. (b) Lack of effect of compound **2** (16 μmol/kg) and vehicle on the gastrointestinal transit of a charcoal meal in contrast to the inhibitory effect of morphine (8 μmol/kg) in rats. Data are the mean ± SEM ($n = 8$). ***$p < 0.001$, vs vehicle.

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clearance can increase plasma stability and antinociceptive effects of opioid peptides.[48] Because hydrophilicity of the glycopeptides is increased compared with the parent peptides, it is plausible that glycosylation shifts elimination to the renal pathway and this may contribute to the higher stability of these conjugates.

The pain-relieving effects of compound **2** were selective to the injured hindpaw with insignificant effects produced in the contralateral hindpaw. In contrast, morphine produces pain relief affecting both the injured and uninjured hindpaws of CCI rats. It is plausible that derivative **2** produces an antineuropathic effect by a different mechanism to that of morphine. These observations are consistent with a previous report which showed selective antihyperalgesic responses in the inflamed hindpaw following continuous central (intrathecal) administration of peptide **1** in carrageenan-induced inflammatory pain in rats.[49]

For new opioid analgesics to be considered for clinical applications, it is essential that they have less opioid-related adverse effects than morphine. A common side effect produced by MOR agonists is constipation, comprising a reduction in stool hydration as well as inhibition of GI motility, resulting in delayed GI transit.[50] Two methods were applied to evaluate the constipation-inducing propensity of derivative **2**. These included assessment of inhibition of castor oil-induced diarrhea and reduced gastrointestinal transit of a charcoal meal. No significant constipation was observed in either assay following oral administration of compound **2** at doses higher than those required to fully reverse allodynia. In contrast, morphine induced significant constipation in both tests at a dose equal to the effective analgesic dose in CCI rats.

■ CONCLUSION

We have successfully designed and developed an orally active sugar-modified derivative of native peptide **1** with high potential for the treatment of neuropathic pain. Analogue **2** is the first reported orally active derivative of this opioid peptide. Its modification with a lactose moiety facilitates GI uptake possibly by providing a substrate to enhance transporter-mediated uptake associated with oral delivery. Remarkably, this compound exhibited strong antineuropathic activity without producing constipation, a major side effect of existing opioid analgesics. This is a highly novel finding in the opioid analgesic field and will be further investigated to reveal the underlying mechanisms. Our present findings suggest that analogue **2** can be developed as a novel opioid analgesic agent to treat neuropathic pain.

■ EXPERIMENTAL SECTION

General. Peptide synthesis grade dimethylformamide (DMF), trifluoroacetic acid (TFA), triisopropylsilane (TIPS), and piperidine were purchased from Merck Biosciences (Kilsyth, VIC, Australia). HPLC-grade acetonitrile was purchased from RCI Labscan Ltd. (Bangkok, Thailand). Fmoc-protected amino acids and Rink amide MBHA resin (100−200 mesh, 0.4−0.8 mmol/g loading) were obtained from Novabiochem (Melbourne, VIC, Australia) or Mimotopes (Clayton, VIC, Australia). Frozen membranes providing human MOR, KOR, and DOR derived from stably transfected CHO-K1 cells (1 × 400 units/μL), wheatgerm agglutinin (WGA) coated poly vinyl toluene SPA beads, [^{3}H]DAMGO (49.6 Ci/mmol, [Tyrosyl-3,5-^{3}H(N)]−), [^{3}H]DPDPE (25.2 Ci/mmol, [Tyrosyl-2,6-^{3}H(N)]−), [^{3}H]diprenorphine (50 Ci/mmol, [15,16-^{3}H]−), 384-well OptiPlates and ProxiPlates, and clear adhesive Topseal-A were purchased from Perkin-Elmer (Massachusetts, USA). All other chemicals were purchased from Sigma Aldrich (VIC, Australia). All cell culture reagents were purchased either from Sigma Aldrich or Gibco (VIC, Australia).

Hydrogenation was performed using a H-Cube continuous-flow hydrogenation reactor from ThalesNano Nanotechnology Inc., Hungary. Absorbance measurements were performed on a Varian Cary 50 Bio UV/vis spectrophotometer (λ = 570 nm). Electrospray ionization mass spectrometry (ESI-MS) was carried out on a PE Sciex API3000 triple quadrupole mass spectrometer using a mixture of solvent A (0.1% formic acid in water) and B (0.1% formic acid in 9:1 acetonitrile/water) at 0.05 mL/min. LC-MS was carried out using solvents A and B at a flow rate of 0.3 mL/min, and a splitter after the Phenomenex Luna C18 column (5 μm, 50 mm × 2.0 mm) was used to achieve a flow rate of 0.05 mL/min into the ion source of the mass spectrometer. Preparative HPLC was carried out on a Waters 600 controller and pump with a 490E programmable multiwavelength UV/visible detector, and analytical HPLC was performed on an Agilent 1100 system fitted with a binary pump, autosampler, and a UV detector set to a wavelength of 214 nm.

Chemistry. *2,3,6-Tetra-O-acetyl-4-O-(2′,3′,4′,6′-tetra-O-acetyl-β-D-galactopyranosyl)-β-D-glucopyranosylbromide (**3**).* A mixture of β-D-lactose (15 mmol, 5.1 g), lithium perchlorate (15 mmol, 1.6 g), and acetic anhydride (150 mmol, 14 mL) was stirred at 40 °C under an argon atmosphere for 16 h. Then the reaction mixture was cooled on an ice bath to 4 °C, and 32% hydrogen bromide in acetic acid (80 mmol, 14.3 mL) was added dropwise. Once the addition was complete, the solution was stirred for 5 h and subsequently diluted with 50 mL of dry dichloromethane (DCM). This was washed with saturated bicarbonate (5 × 30 mL) and brine (1 × 30 mL), dried over magnesium sulfate, and then filtered. Upon evaporation of the solvent, the product (6.7 g, 64%) spontaneously crystallized and was used without further purification for the next step.

*2,3,6-Tetra-O-acetyl-4-O-(2′,3′,4′,6′-tetra-O-acetyl-β-D-galacto-pyranosyl)-β-D-glucopyranosylazide (**4**).* Compound **3** (9.6 mmol, 6.7 g) was dissolved in 40 mL of acetone and a solution of sodium azide (11.5 mmol, 0.75 g) in 10 mL of water was added carefully. After the mixture had been stirred at room temperature for 17 h, it was concentrated under reduced pressure and the resulting white crystals were washed with ice−water and dried under vacuum (6.2 g, 98%).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.35 (dd, 1H, H-4′), 5.10 (m, 2H, H-2′, H-3), 4.95 (m, 2H, H-3′, H-2), 4.64 (d, 1H, H-1), 4.48 (d, 1H, H-1′), 4.11 (m, 3H, H-6$_a$′, H-6$_b$′, H-6$_a$), 3.87 (m, 1H, H-5′), 3.80 (m, 2H, H-4, H-6$_b$), 3.71 (m, 1H, H-5), 2.15 (s, 3H, OAc), 2.10 (s, 3H, OAc), 2.08 (s, 3H, OAc), 2.05 (s, 3H, OAc), 2.04 (s, 3H, OAc), 2.03 (s, 3H, OAc), 2.01 (s, 3H, OAc). Melting point: 83−85 °C.

*4-Oxo-4-[(2,3,6-tetra-O-acetyl-4-O-{2′,3′,4′,6′-tetra-O-acetyl-β-D-galactopyranosyl}-β-D-glucopyranosyl)amino]butanoic Acid (**5**).* Compound **4** (0.4 mmol, 260 mg) was dissolved in 20 mL of dry DCM and then pumped through an H-Cube continuous-flow hydrogenation reactor, which was operating with the following settings: T 35 °C, PH2 40 bar, flow rate 2 mL/min. The reaction was complete after 1 h, and the solvent was evaporated and replaced with fresh dry DCM for the next reaction step. Pyridine (1.5 mL) and 4-dimethylaminopyridine (0.04 mmol, 5 mg) were added, and the solution was cooled in an ice bath. Succinic anhydride (0.8 mmol, 80 mg) was added in small portions, and the mixture was stirred for 24 h, letting it warm to room temperature slowly. The solution was diluted with DCM (20 mL) and washed with 5% hydrochloric acid (3 × 30 mL). The organic phase was dried over magnesium sulfate and filtered, and the solvent was removed under reduced pressure. The residue was purified using gradient flash column chromatography (2:1 ethyl acetate/hexane to 100% ethyl acetate, 0.5% acetic acid) to yield a white powder (165 mg, 56%).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.44 (d, 1H, $J_{NH,1}$ = 9.3 Hz, NH), 5.35 (dd, 1H, $J_{4',5'}$ = 1.0 Hz, $J_{3',4'}$ = 3.5 Hz, H-4′), 5.29 (dd, 1H, $J_{3,4}$ = 8.9 Hz, $J_{2,3}$ = 9.6 Hz, H-3), 5.22 (dd, 1H, $J_{NH,1}$ = 9.3 Hz, $J_{1,2}$ = 9.3 Hz, H-1), 5.10 (dd, 1H, $J_{1',2'}$ = 7.9 Hz, $J_{2',3'}$ = 10.4 Hz, H-2′), 4.95 (dd, 1H, $J_{3',4'}$ = 3.5 Hz, $J_{2',3'}$ = 10.4 Hz, H-3′), 4.84 (dd, 1H, $J_{1,2}$ = 9.6 Hz, $J_{2,3}$ = 9.6 Hz, H-2), 4.47 (d, 1H, $J_{1',2'}$ = 7.9 Hz, H-1′), 4.43 (m, 1H, H-6$_a$), 4.14 (m, 2H, H-6$_b$,H-6$_a$′), 4.07 (m, 1H, H-6$_b$′), 3.87 (m, 1H, H-5′),

3.76 (dd, 1H, $J_{3,4}$ = 8.8 Hz, $J_{4,5}$ = 8.8 Hz, H-4), 3.73 (m, 1H, H-5), 2.72 (m, 1H, CH_2CO), 2.63 (m, 1H, CH_2CO), 2.47 (m, 2H, CH_2CO), 2.15 (s, 3H, OAc), 2.11 (s, 3H, OAc), 2.07 (s, 3H, OAc), 2.05 (s, 3H, OAc), 2.05 (s, 3H, OAc), 2.04 (s, 3H, OAc), 1.96 (s, 3H, OAc). Melting point: 97−98 °C.

Solid-Phase Peptide Synthesis. The peptide was assembled on Rink amide MBHA resin using HBTU (4 equiv)/DIPEA (5 equiv) activation and the in situ neutralization protocol for Fmoc chemistry.[51] The following protected amino acids were used and double coupled: Fmoc-Phe, Fmoc-Trp(Boc), Fmoc-Pro, and Fmoc-Tyr(tBu). Compound **5** (1.3 equiv) was activated with HBTU (1.25 equiv)/DIPEA (2 equiv) and coupled overnight. The resin was then washed with DMF, DCM, and MeOH and treated with 75% hydrazine hydrate in MeOH twice for 30 min. After washing with MeOH, the resin was dried under vacuum overnight. Cleavage of the peptide was achieved by treatment of the resin with TFA/triisopropylsilane/water (95:2.5:2.5) for 2 h. The solvent was removed under a stream of air, the crude peptide precipitated by addition of cold diethyl ether and then dissolved in acetonitrile/water/TFA (50:50:0.1) and lyophilized.

Purification. The crude peptide was purified by preparative RP-HPLC using a Vydac C18 column (22 mm × 250 mm) with a gradient of 20% to 60% solvent B (solvent A, 0.1% TFA in water; solvent B, 90% acetonitrile and 0.1% TFA in water) over 70 min at a flow rate of 10 mL/min. The purity of peptides in the collected fractions was confirmed by ESI-MS and analytical RP-HPLC using a Vydac C18 column (5 μm, 4.6 mm × 250 mm) and a gradient of 100% A to 100% B over 30 min at a flow rate of 1 mL/min. Fractions containing pure peptides were combined and lyophilized.

Compound **1** Yield: 108 mg, 84%. Exact mass calculated, 611.2976; found, 611.2987 [M + H^+].

Compound **2** Yield: 452 mg, 24%. Exact mass calculated, 1056.4173, found, 1056.4152 [M + Na^+].

In Vitro Experiments. *Cell Culture.* The Caco-2 human epithelial colorectal adenocarcinoma cell line was obtained from the American Type Culture Collection (Rockville, USA). Caco-2 cells were maintained in T-75 flasks (Nunc, Australia) with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% nonessential amino acids at 95% humidity at 37 °C in an atmosphere of 5% CO_2. The culture medium was changed every second day. When the cells reached 80% confluence, they were subcloned using 0.25% trypsin−EDTA. Passage numbers 55−75 were used in the assays. SH-SY5Y cells were maintained in DMEM:Hams F12 medium supplemented with 10% FBS, 1% glutamine, 1% nonessential amino acids, and 1000 U/mL streptomycin/penicillin. Cells were subcloned in the same manner as Caco-2 cells. Passage numbers 10−20 were used in the assays.

In Vivo Experiments. *Animals.* Male specific pathogen free (SPF) Sprague−Dawley (SD) rats weighing between 160 and 200 g were purchased from The University of Queensland Biological Resources breeding facility and kept in groups of three for at least a 3 day acclimatization period prior to initiation of experimental procedures. Rats were housed in an artificially lit room on a 12 h light/12 h dark cycle at controlled temperature (22.2 ± 0.2 °C; mean ± SEM) and humidity (51.6−65.2%) with food and water available ad libitum. The animal experimentation protocols were approved by The University of Queensland Animal Ethics Committee (AEC no. CIPDD/366/09/NHMRC).

Surgery Procedure. Chronic constriction injury (CCI) of the sciatic nerve was performed according to the method of Bennett and Xie[52] in adult male SD rats. Briefly, while rats were deeply anaesthetized with 3% isoflurane delivered in oxygen, the left common sciatic nerve was exposed at midthigh level by blunt dissection through the biceps femoris. Proximal to the trifurcation, ≈10 mm of nerve was freed of adhering tissue, four loose ligatures (3.0 silk) were tied around the sciatic nerve (≈1 mm apart), and the incision was closed in layers. After surgery, topical antibiotic powder (benzylpenicillin) was applied to the wound to prevent infection and rats were kept warm during surgical recovery. Rats were housed individually and inspected from the time of CCI surgery until experimentation on day 14 post-CCI surgery with regard to posture of the affected hindpaw, exploring behavior, body weight, and signs of autotomy.

Assessment of Von Frey Paw Withdrawal Thresholds. Paw withdrawal thresholds (PWTs) were assessed in the rat hindpaws using a set of calibrated Von Frey filaments. For each testing session, rats were acclimatized for at least 15 min in wire mesh metabolic cages. Von Frey filaments which delivered forces in the range 2−20 at 2 g increments were used to quantify the threshold for hindpaw withdrawal in response to application of a graded non-noxious stimulus for each hindpaw. Commencing with 6 g, the Von Frey filament was applied to the plantar surface of the footpad of the hindpaw so that the filament bent slightly for 3 s. Lifting the hindpaw or avoidance of the filament was considered a positive response. If the rat responded, the next filament in a descending sequence was tested. If no response was observed, the next filament in an ascending sequence was applied. Mechanical allodynia was fully developed in the ipsilateral (injured side) hindpaw by 14 days post-CCI surgery (PWTs ≤ 6 g). Rats not responding to any of the filaments were arbitrarily assigned a PWT value of 20 g.

Evaluation of Oral Absorption. Male Sprague−Dawley rats, weighing 280−310 g, were gavaged with 500 μL of a 20 mM solution of compound **1** or **2** in water. Blood samples were taken from the tail vein after 30, 60, and 90 min and collected in heparin-coated tubes. The blood was centrifuged to remove red blood cells, and 100 μL of plasma was added to 300 μL of acetonitrile to precipitate proteins. The suspension was centrifuged a second time, and the supernatant was analyzed by LC-MS as described in the analysis of the Caco-2 monolayer permeability assay analysis. Ethics approval was obtained from The University of Queensland Animal Ethics Committee for these experiments.

Naloxone-Sensitivity of Analgesia. Groups of CCI rats (n = 6) were pretreated with single subcutaneous doses of either vehicle or naloxone hydrochloride (10 mg/kg) at 5 min prior to iv administration of single bolus doses of compound **2** (5 μmol/kg), morphine (2 μmol/kg), or vehicle. PWTs were determined just prior to vehicle or naloxone administration and at the following times after test item administration: 0.25, 0.5, 0.75, 1, 1.25, 1.5, 2, and 3 h.

Assessment of Constipation Inducing Properties. *Castor Oil-Induced Diarrhea Test in Rats.* The constipating capacity of **2** was compared to that of morphine using the castor oil-induced diarrhea method. Drug-naive rats weighing 200−250 g were fasted for approximately 16 h before the start of experimentation but were permitted free access to water. Rats were then placed into individual wire-mesh cages without access to food or water for a 1 h acclimatization period prior to drug or vehicle administration.

At completion of the acclimatization period, each rat received compound **2** (16 μmol/kg), morphine (8 μmol/kg), or vehicle via oral administration. Five minutes after test item or vehicle administration, 1 mL of castor oil was administered by oral gavage. Rats were observed hourly up to 8 h after the administration of castor oil. At each observation time, the presence or absence of diarrhea was recorded. A scoring system was used based on the weight of feces boli and the feces consistency. Weight was scored from 0 (no feces) to 3 (>3 g of feces), and consistency was scored from 0 (normal-shape feces) to 3 (shapeless feces with large amounts of liquid). Diarrhea was considered to be present when both weight of feces and the feces consistency were scored at ≥2.

Charcoal Test. GI transit was assessed using the charcoal meal test in groups of drug-naive rats. Animals (200−250 g) were fasted for approximately 16 h before experimentation but had free access to water. Groups of rats (n = 8 per group) received oral doses of compound **2** at 16 μmol/kg, morphine at 8 μmol/kg, and vehicle just prior to oral gavage of an aqueous activated charcoal suspension (1 mL of 10% activated charcoal and 5% gum Arabic). One hour later, rats were euthanized with intraperitoneal (ip) sodium pentobarbital (65 mg per rat) followed by cervical dislocation. The intestine was carefully removed and separated from the omentum while avoiding stretching. The total length of the small intestine was measured from the pylorus to the ileocecal junction as well as the most distal point of migration of the charcoal meal. GI propulsion was calculated as the

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percentage of the distance traveled by the charcoal meal relative to the total length of the small intestine. GI propulsion <55% indicated the inhibition of bowel propulsion.

Data Analysis. For in vivo studies, the PWT versus time curve data were normalized by subtracting predosing baseline PWT values from the PWT of each time point for each individual rat to obtain ΔPWT values as follows:

$$\text{normalized } (\Delta)\text{ PWT value} = \text{postdosing PWT} - \text{predosing baseline PWT}$$

ΔPWT versus time curves were then constructed. The extent and duration of analgesia (area under the curves (AUC) versus time for ΔPWT; ΔPWT AUC values) was estimated using trapezoidal integration for individual CCI rats. Mean (±SEM) ΔPWT AUC versus log dose curves were constructed and the AUC values were normalized relative to the maximum possible ΔPWT AUC value to give % maximum possible effect (% MPE). Nonlinear regression as implemented in the GraphPad Prism (v5.0) software program (GraphPad Software) was used to estimate the ~ED_{50} values.

Statistical Analysis. During all animal experiments, the investigator was blinded to the type and dose of the administered compounds. All data are expressed as mean (±SEM). One-way ANOVA and Dunnett's post hoc test were used to compare stability and permeability results, normalized ΔPWT AUC between the ipsilateral and contralateral hindpaws, in addition to inhibition of GI propulsion and castor-oil induced diarrhea produced by compound **2** and morphine relative to the vehicle. All comparisons between two treatment groups, including comparisons between ΔPWT AUC values of naloxone and vehicle pretreated CCI rats and the ED_{50} value for compound **2**, were performed using the nonparametric Mann–Whitney test as implemented in the GraphPad Prism (v5.0) software package. The statistical significance criterion was $P < 0.05$.

■ ASSOCIATED CONTENT

Supporting Information

Scheme for synthesis of compounds **1** and **2** and additional figures. Full experimental details of plasma stability assay, Caco-2 cell permeability assay, receptor-binding scintillation proximity assay (SPA), inhibition of cAMP accumulation, and description of the test and control item administration for in vivo pharmacology experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

δ, chemical shift in parts per million downfield from tetramethylsilane; AUC, area under the curve; BBB, blood–brain barrier; Boc, *tert*-butoxycarbonyl; cAMP, 3',5'-cyclic adenosine monophosphate; DCM, dichloromethane; DMF, dimethylformamide; ED_{50}, dose effective in 50% of test subjects; EDTA, ethylenediaminetetraacetic acid; ESI, electrospray ionization; Fmoc, 9-fluorenylmethoxycarbonyl; GI, gastrointestinal; GLUT-1, glucose transporter 1; HPLC, high-performance liquid chromatography, high-pressure liquid chromatography; IC_{50}, half-maximum inhibitory concentration; iv, intravenous; K_i, , inhibition constant; LC-MS, liquid chromatography-mass spectrometry, ; m, multiplet (spectral), meter(s), milli, isotopic mass, magnetic quantum number (ESR and NMR spectroscopy); sc, subcutaneous; t-Bu, *tert*-butyl; $t_{1/2}$, half-time

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