In Vivo Characterization of MMP-2200, a Mixed δ/μ Opioid Agonist, in Mice

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ABSTRACT

We have previously reported the chemistry and antinociceptive properties of a series of glycosylated enkephalin analogs (glycopeptides) exhibiting approximately equal affinity and efficacy at δ opioid receptors (DORs) and μ opioid receptors (MORs). More detailed pharmacology of the lead glycopeptide MMP-2200 [$\text{H}_2\text{N-Tyr-D-Thr-Gly-Phe-Leu-Ser-(O-}\beta\text{-D-lactose)-CONH}_2$] is presented. MMP-2200 produced dose-related antinociception in the 55°C tail-flick assay after various routes of administration. The antinociceptive effects of MMP-2200 were blocked by pretreatment with the general opioid antagonist naloxone and partially blocked by the MOR-selective antagonist β -funaltrexamine and the DOR-selective antagonist naltrindole. The κ opioid receptor antagonist nor-binaltorphimine and the peripherally active opioid antagonist naloxone-methiodide were ineffective in blocking the antinociceptive effects of MMP-2200. At equi-antinociceptive doses, MMP-2200 produced significantly less stimulation of loco-

motor activity compared with morphine. Repeated administration of equivalent doses of morphine and MMP-2200 (twice daily for 3 days) produced antinociceptive tolerance ($\sim$ 13- and 5-fold rightward shifts, respectively). In acute and chronic physical dependence assays, naloxone precipitated a more severe withdrawal in mice receiving morphine compared with equivalent doses of the glycopeptide. Both morphine and MMP-2200 inhibited respiration and gastrointestinal transit. In summary, MMP-2200 acts as a mixed DOR/MOR agonist in vivo, which may in part account for its high antinociceptive potency after systemic administration, as well as its decreased propensity to produce locomotor stimulation, tolerance, and physical dependence in mice, compared with the MOR-selective agonist morphine. For other measures (e.g., gastrointestinal transit and respiration), the significant MOR component may not allow differentiation from morphine.

Introduction

Chronic pain continues to be a major health, social, and economic problem throughout the world. In the United States, it is estimated that up to 56 million adults suffer from chronic pain annually (Brennan et al., 2007). When un-

treated, chronic pain can interfere with sleep, limit activities of daily living, and decrease overall productivity. Costs associated with medical care and lost worker productivity caused by chronic pain have been estimated at more than \$50 billion annually (Burgoyne, 2007).

Opioid agonists are currently the most widely used analgesic class of drugs for the treatment of moderate to severe pain. They exert their effects primarily through the μ opioid receptor (MOR), which produces not only analgesic effects, but also a number of side effects (Gutstein and Akil, 2005). These side effects include the development of tolerance and physical dependence, addiction liability, urinary retention, constipation, and respiratory depression, all of which can limit their clinical application (McNicol et al., 2003). Because

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ABBREVIATIONS: MOR, μ opioid receptor; DOR, δ opioid receptor; KOR, κ opioid receptor; MMP-2200, $\text{H}_2\text{N-Tyr-D-Thr-Gly-Phe-Leu-Ser-(O-}\beta\text{-D-lactose)-CONH}_2$; CNS, central nervous system; PNS, peripheral nervous system; GI, gastrointestinal; BBB, blood-brain barrier; CHO, Chinese hamster ovary; C.I., confidence interval; β -FNA, β -funaltrexamine; nor-BNI, nor-binaltorphimine; DPDPE, [D-Pen^2 , D-Pen^5]enkephalin; DAMGO, (2S)-2-[[[(2R)-2-amino-3-(4-hydroxyphenyl)propanoyl]amino]propanoyl]amino]acetyl]-methylamino]-N-(2-hydroxyethyl)-3-phenylpropanamide; U69,593, (+)-(5 α ,7 α ,8 β)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]benzeneacetamide; U50,488, *trans*-3,4-dichloro-N-methyl-N[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide methanesulfonate; SNC-80, (+)-4-[(α R)- α -((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-N,N-diethylbenzamide.

many of the detrimental side effects of opioid treatment are mediated through the MOR, there is a renewed interest in targeting the δ opioid receptor (DOR) in an effort to produce adequate analgesia with potentially decreased side effects (Porreca et al., 1984; Bilsky et al., 2000).

DOR agonists produce broad-spectrum antinociceptive effects in preclinical rodent models. Several investigators have reported complex changes in DOR expression levels and function during acute and subacute inflammatory pain states (Scherrer et al., 2006; Cahill et al., 2007), as well as potent and efficacious antinociceptive effects (Petrillo et al., 2003). Similar profiles have been observed in the neuropathic pain states, as well as in animal models of bone cancer pain (Brainin-Mattos et al., 2006; Holdridge and Cahill, 2007). There is accumulating preclinical evidence for fewer classic opioid side effects with DOR agonists, including less respiratory depression, less inhibition of gastrointestinal transport, and reduced tolerance and physical dependence (Cowan et al., 1988; Sheldon et al., 1990; Su et al., 1998; Bilsky et al., 2000; Codd et al., 2009).

In addition to the promising antinociceptive activity of selective DOR agonists, there is a growing body of evidence suggesting that DOR and MOR interact with one another and that DOR/MOR interactions, either agonist/agonist (DOR + MOR) or agonist/antagonist (MOR – DOR) may produce synergism in terms of increased antinociception, while at the same time reducing undesirable side effects. For example, there are broad colocalizations of MOR and DOR on overlapping populations of neurons in pain-modulating regions of the CNS (Fields et al., 1980; Gray et al., 2006). Coadministration of DOR agonists apparently increases the potency and efficacy of MOR agonists (Sutters et al., 1990). Furthermore, studies have evaluated combinations of MOR and DOR agonists. Combinations of (+)-4-[(α R)- α -(2*S*,5*R*)-4-allyl-2,5-dimethyl-1-piperazinyl]-3-methoxybenzyl]-*N,N*-diethylbenzamide (SNC-80) and a MOR agonist produced leftward shifts in the dose-effect curves. The SNC-80 and MOR agonist mixture produced superadditive effects in the thermal antinociceptive assay, and subadditive or additive effects on behavioral measures associated with side effects (Stevenson et al., 2003). Although functionally distinct MOR-DOR heterodimers or hetro-oligomers have been proposed (Gomes et al., 2004), evidence suggests that MOR and DOR exist on separate populations of neurons (Scherrer et al., 2006). Taken together, the literature supports a functional interaction between MOR and DOR and a possible regulatory role for DOR agonists. Compounds possessing dual actions at DOR and MOR should produce broad-spectrum analgesic efficacy with less intense side effects compared with MOR-selective agonists (Schiller, 2010).

Our group previously reported that the glycosylation of enkephalin-based peptides increases stability and blood-brain barrier (BBB) penetration, thereby increasing CNS bioavailability (Bilsky et al., 2000; Elmagbari et al., 2004; Eggleton et al., 2005; Polt et al., 2005; Lowery et al., 2007). In this study, we report a more detailed *in vivo* characterization of a lead glycopeptide [MMP-2200; H₂N-Tyr-D-Thr-Gly-Phe-Leu-Ser-(O- β -D-lactose)-CONH₂] that has high affinity and efficacy for DOR and MOR. It was proposed that the antinociceptive potency of MMP-2200 was caused by its synergistic activity at these two receptors, and that this would produce a more favorable side effect profile compared with a MOR-

selective agonist such as morphine. The current studies were designed to assess the *in vitro* and *in vivo* activity of MMP-2200 and further define the optimal DOR/MOR receptor selectivity in an effort to enhance antinociception and limit detrimental side effects.

Materials and Methods

Drugs and Injections. Good manufacturing practice-compliant MMP-2200 was synthesized by PolyPeptide Labs of Torrance, CA, using previously published methods (Elmagbari et al., 2004). Morphine sulfate, naloxone, β -funaltrexamine (β -FNA), naltrindole, and norbinaltorphimine (nor-BNI) were obtained through the National Institute on Drug Abuse (Bethesda, MD) drug supply program. Naloxone-methiodide, [D-Pen²,D-Pen⁵]enkephalin (DPDPE), (2*S*)-2-[[2-[(2*R*)-2-[(2*S*)-2-amino-3-(4-hydroxyphenyl)propanoyl]amino]propanoyl]amino]acetyl]-methylamino]-*N*-(2-hydroxyethyl)-3-phenylpropanamide (DAMGO), and (+)-(5 α ,7 α ,8 β)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]benzeneacetamide (U69,593) were purchased from Sigma-Aldrich (St. Louis, MO).

All drugs were dissolved in distilled water for intracerebroventricular injections and in physiological saline (0.9% NaCl) for systemic injections. The intracerebroventricular injections were performed as described previously (Porreca et al., 1984). In brief, mice were lightly anesthetized with ether, and a 5-mm incision was made along the midline of the scalp. An injection was made using a 25- μ l Hamilton syringe (Hamilton Co., Reno, NV) at a point 2 mm caudal and 2 mm lateral from bregma. All systemic injections were given in a volume based on the weight of the animal (0.1 ml/10 g bodyweight). For intravenous injections, mice were restrained in a Plexiglas holder, and the distal portion of the tail was dipped into 40°C warm water for approximately 10 s to dilate the tail vein. The injection was made into the tail vein using a 30-gauge needle and a 1-ml syringe. For intraperitoneal injections, mice were restrained by grasping the nape of the neck and tail. Their abdominal regions were exposed by slightly bending their backs, and the injection was made with a 1-ml syringe and a 30-gauge needle inserted into the peritoneal cavity. Subcutaneous injections were performed by injecting the drug directly underneath the skin between the abdominal region and the anterior portion of the hip. For oral injections, an 18-gauge, 1 1/2-inch curved ball needle attached to a 1-ml syringe was used to prevent damage to the esophagus and from passing through the glottal opening into the trachea. The needle was passed gently through the mouth and pharynx into the esophagus, and substance was administered. Intrathecal injections were made by using a constant 5- μ l volume at the L5/6 level using a modification of the method used by Hylden and Wilcox (1980). Injections were made to lightly anesthetized mice using a Hamilton microliter syringe fitted with a 30-gauge needle.

Opioid Binding to Human MOR, DOR, or KOR. Chinese hamster ovary (CHO) cells stably transfected with the human δ opioid receptor (hDOR-CHO) or κ opioid receptor (hKOR-CHO) were obtained from Dr. Larry Toll (SRI International, Palo Alto, CA). CHO cells stably expressing the human μ opioid receptor (hMOR-CHO) were obtained from Dr. George Uhl (National Institute on Drug Abuse Intramural Program, Bethesda, MD). The cells were grown in 100-mm dishes in Dulbecco's modified Eagle's media supplemented with 10% fetal bovine serum and penicillin-streptomycin (10,000 units/ml) at 37°C in a 5% CO₂ atmosphere. At confluence, the CHO cells were scraped from the 100-mm dishes, and they were centrifuged at 1200g. The cell pellets were resuspended in 50 mM Tris-HCl, pH 7.5, by use of a Polytron. The membranes were centrifuged at 39,000g for 30 min at 4°C. The membranes were resuspended in 50 mM Tris-HCl, pH 7.5, at a protein concentration of approximately 5 mg/ml. The membranes were aliquoted into polypropylene tubes and frozen at –80°C until use. The protein concentration was determined using previously published methods (Bradford, 1976). Mem-

branes were incubated with 12 different concentrations of either morphine or MMP-2200 and the radiolabeled ligand in 50 mM Tris-HCl, pH 7.5, at a final volume of 1 ml. Nonspecific binding was measured by the inclusion of 10 μ M naloxone. Data were taken as the mean K_i values $\pm$ S.E.M. from three separate experiments each performed in triplicate. Incubation times of 60 min were used for the MOR-selective peptide [3 H]DAMGO and the KOR-selective ligand [3 H]U69,593. A 3-h incubation was used with the DOR-selective antagonist [3 H]naltrindole. The concentrations of [3 H]DAMGO, [3 H]naltrindole, and [3 H]U69,593 were 0.25, 0.2, and 1 nM, respectively. Binding was terminated by filtering the samples through Whatman (Clifton, NJ) no. 32 glass fiber filters using a Brandel Inc. (Gaithersburg, MD) 48-well cell harvester. Filters were soaked for at least 60 min in 0.25% polyethylenimine for [3 H]naltrindole and [3 H]U69,593 binding experiments. After filtration, filters were washed three times with 3 ml of cold 50 mM Tris-HCl, pH 7.5, and counted in 2 ml of Fisher Scintisafe 30% scintillation fluid (Thermo Fisher Scientific, Waltham, MA). The K_i values of unlabeled compounds were calculated using the equation $K_i = IC_{50}/(1 + S)$, where S = (concentration of radioligand)/(K_d of radioligand) (Cheng and Prusoff, 1973).

[35 S]GTP γ S Binding Studies. To prepare membranes for [35 S]GTP γ S binding, CHO cells stably expressing the human MOR, DOR, or KOR were scraped from tissue culture plates and centrifuged at 200g for 10 min at 4°C. The cells were resuspended in phosphate-buffered saline, pH 7.4, containing 0.04% EDTA. After centrifugation at 200g for 10 min at 4°C, the pellet was resuspended in membrane buffer, consisting of 50 mM Tris-HCl, 3 mM MgCl₂, and 1 mM EGTA, pH 7.4. The membranes were homogenized with a Dounce homogenizer, followed by centrifugation at 39,000g for 20 min at 4°C. The membrane pellet was resuspended in membrane buffer, and the centrifugation step was repeated. The membranes were resuspended in assay buffer, which consisted of 50 mM Tris-HCl, 3 mM MgCl₂, 100 mM NaCl, and 0.2 mM EGTA, pH 7.4. The hMOR-CHO (10 μ g of protein/tube), hDOR-CHO (10 mg of protein/tube), or hKOR-CHO (15 μ g of protein/tube) cells were incubated with 12 different concentrations of the agonist in assay buffer for 60 min at 30°C in a final volume of 0.5 ml. The reaction mixture contained 3 μ M GDP and 0.080 nM [35 S]GTP γ S. Basal activity was determined in the presence of 3 μ M GDP and in the absence of agonist, and nonspecific binding was determined in the presence of 10 μ M unlabeled GTP γ S. After the 60-min incubation, the membranes were filtered onto Whatman no. 32 glass fiber filters by vacuum filtration, followed by three washes with 3 ml of ice-cold 50 mM Tris-HCl, pH 7.5. Samples were counted in 2 ml of Fisher Scintisafe 30% scintillation fluid. Data are presented as the percentage of agonist stimulation of [35 S]GTP γ S binding normalized against DAMGO (MOR), SNC-80 (DOR), or *trans*-3,4-dichloro-*N*-methyl-*N*[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide methanesulfonate (U50,488) (KOR). All experiments were repeated three times and performed in triplicate.

Animals. Male ICR mice (25–35 g; Harlan, Indianapolis, IN) were used for all studies with the exception of the respiration studies. Male BALB/c mice (25–35 g; The Jackson Laboratory, Bar Harbor, ME) were used for respiration studies, because this strain of mice does not display robust hyperlocomotion to morphine when given at antinociceptive or supra-antinociceptive doses. A total of 680 mice (8–10 mice per group) were used for the experiments. All mice were housed in groups of five in Plexiglas chambers with food and water available *ad libitum*. All animals were maintained on a 12-h light/dark cycle (lights on at 7:00 AM) in a temperature- and humidity-controlled animal colony. All animal experiments were performed under an approved protocol in accordance with institutional guidelines and the *Guide for the Care and Use of Laboratory Animals* as adopted and promulgated by the National Institutes of Health.

Tests for Antinociception. Antinociception was assessed using the 55°C warm-water tail-flick test. The latency to the first sign of a rapid tail flick was taken as the behavioral endpoint (Janssen et al.,

1963). Each mouse was first tested for baseline latency by immersing its tail in the water and recording the time to response. Mice not responding within 5 s were excluded from further testing. Mice were then administered the test compound and tested for antinociception at various time points afterward. Antinociception was calculated using the following formula: percentage of antinociception = $100 \times (\text{test latency} - \text{control latency}) / (10 - \text{control latency})$. To avoid tissue damage, a maximum score was assigned (100%) to animals that failed to respond within 10 s.

Antagonist Studies. To determine the opioid receptors involved with MMP-2200-mediated antinociception, mice were pretreated with various opioid-selective antagonists: the nonselective opioid antagonist naloxone (1 mg/kg i.p.; $t = -30$ min), the peripherally selective antagonist naloxone-methiodide (3.2 mg/kg i.p.; $t = -30$ min), the DOR antagonist naltrindole (10 mg/kg s.c.; $t = -10$ min), the KOR antagonist nor-BNI (30 mg/kg i.p.; $t = -24$ h), and the MOR-selective antagonist β -FNA (19 nmol i.c.v.; $t = -24$ h). These doses have previously been shown to be selective for the intended opioid receptor type, and times chosen for pretreatment corresponded to times of peak antagonist effect in this assay (Heyman et al., 1986; Jiang et al., 1989; Horan et al., 1992;). Antinociceptive testing took place at the time of agonist peak effect.

Locomotor Studies. Locomotor activity was measured using an activity monitoring system (Coulbourn Instruments, Allentown, PA) and TruScan software (Coulbourn Instruments). Each of the eight chambers consisted of 10-in wide $\times$ 10-in long $\times$ 16-in high arena surrounded by Plexiglas walls. The floor consisted of a removable plastic drop pan that was cleaned between sessions. A sensor ring surrounded the arena on the bottom outside edges of the four sides of the chamber and contained 16 infrared beams that transected both the length and the width of the chambers on all four sides (beam spacing = 0.6 inches, resolution of 32×32 squares). Measurement of the animal position was determined every 100 ms, and the software calculated a number of parameters related to aspects of locomotor activity. The total distance traveled by each mouse was used as the primary measure of activity, with the data summed into 5-min intervals (bins). Mice were habituated to the chamber for 30 min and then removed from the chambers and administered saline, morphine, or MMP-2200 subcutaneously. Mice were immediately placed back into the chambers and monitored for an additional 90 min. Sessions were divided into 24 5-min bins with the distance traveled calculated for each mouse.

Tolerance Assay. Mice were administered twice-daily (8 AM and 5 PM) subcutaneous injections of morphine or MMP-2200 at respective A_{90} doses for 3 days. On day 4, a baseline latency was taken for each mouse using the 55°C tail-flick test. Mice were then injected subcutaneously with drug and tested for antinociception at the time of peak effect.

Acute Physical Dependence. Mice were injected subcutaneously with saline or doses of test drugs that were 16.8 times their respective A_{50} doses. This calculation was based on our previous studies, which demonstrated a robust level of dependence/withdrawal in morphine-treated animals at 100 mg/kg s.c. The modification evaluates all compounds on an equivalent micromolar/kilogram basis. Four hours after injection, withdrawal was precipitated with a single intraperitoneal injection of naloxone (10 mg/kg). Immediately after the injection, mice were placed into a clear Plexiglas cylinder with a filter paper bottom. Mice were videotaped for 20 min, and the number of vertical jumps was recorded by trained observers blinded to the experimental protocol.

Chronic Physical Dependence. Mice were administered twice-daily (8 AM and 5 PM) subcutaneous injections of saline, morphine (30 mg/kg), or MMP-2200 (30 mg/kg) for 4 days before withdrawal precipitation. Withdrawal was precipitated with a single intraperitoneal injection of naloxone (30 mg/kg) approximately 4 h after the last injection. Mice were videotaped for a 20-min session after their antagonist injection, and the numbers of vertical jumps, feces, urine

output, and bodyweight were recorded by trained observers blinded to the experimental protocol.

Gastrointestinal Transit Studies. Mice were deprived of food for 18 h before the experiment. On test day, saline, morphine, or MMP-2200 was administered intravenously at $t = -10$ min. At $t = 0$ min, charcoal was delivered orally at a constant volume of 250 μ l. The suspension was prepared the day of use as 10% charcoal (Sigma-Aldrich) with 2.5% arabic acid (Sigma-Aldrich) in water and mixed thoroughly and repeatedly to minimize needle obstruction and ensure a homogenous suspension. Mice were sacrificed at $t = 30$ min via cervical dislocation, and the small intestine (duodenum to cecum) was then dissected out and carefully uncoiled. The distance traveled by the charcoal was measured and compared with the total length of the small intestine for each mouse and expressed as percentage of GI transit (i.e., distance covered by the charcoal/total intestinal length $\times$ 100).

Respiration Studies. Respiratory function was measured in conscious, freely moving male BALB/c mice using whole body plethysmography technology (Buxco Electronics, Inc., Troy, NY). Chambers were maintained at room temperature (23–24°C) and mass flow controllers set the flow and composition of the gas. Saline, morphine, or MMP-2200 was administered intraperitoneally at $t = 0$ min. Mice were then immediately placed into the chambers for a 30-min habituation period. At $t = 30$ min, four different concentrations of carbon dioxide (0, 3, 5, and 8%) were administered every 7 min, at a constant O_2 saturation of 25%. Minute ventilation and respiratory rate were recorded for each CO_2 concentration.

Statistical Analysis. For antinociceptive tests, dose-response lines were constructed at times of agonist peak effect and analyzed using linear regression. Agonist A_{50} values [95% confidence intervals (C.I.)] were calculated from the linear portion of the dose-response curve (FlashCalc software; Dr. Michael Ossipov, University of Arizona, Tucson, AZ). A t test and analysis of variance followed by appropriate post hoc analysis were used for statistical analysis of the remaining behavioral data. In all cases, significance was established at $p < 0.05$.

Results

Figure 1 depicts the chemical structures of morphine and MMP-2200 and summarizes the binding affinities and functional activity at human MOR, DOR, and KOR. Morphine

displayed preferential binding to MOR over KOR and DOR, consistent with values reported in the literature (Smith et al., 2007). In contrast, MMP-2200 exhibited high affinity for both DOR and MOR, with approximately 8-fold lower affinity for KOR. The GTP γ S assay further characterized the functional activity of MMP-2200. The compound produced near-maximal stimulation (compared with the MOR agonist DAMGO and the DOR agonist SNC-80), although it is noteworthy that the EC_{50} for DOR was almost 16-fold lower (more potent) compared with MOR.

The dose- and time-related antinociceptive effects of morphine and MMP-2200 have been reported previously after intracerebroventricular or intravenous administration in the 55°C tail-flick test (Elmagbari et al., 2004). We extend these results to the subcutaneous route of administration, because it is commonly used for repeated tolerance and physical dependence injection regimens. As expected, morphine and MMP-2200 both produced dose- and time-related antinociception in this assay (Fig. 2). On a micromolar/kilogram basis, MMP-2200 was approximately 2.7-fold more potent than morphine with calculated A_{50} values (and 95% C.I.) of 8.9 (8.1–10.0) μ mol/kg for MMP-2200 and 23.8 (21.3–26.6) μ mol/kg for morphine. Figure 2 also shows the calculated area under the curves for the antinociceptive dose and time response curves. Both compounds exhibited similar area under the curves at equivalent antinociceptive doses ($F_{1,54} = 1.26$; $p > 0.05$). The two compounds were also tested for antinociceptive effects after intrathecal administration. MMP-2200 was approximately 19-fold more potent than morphine after intrathecal administration, with calculated A_{50} values (and 95% C.I.) of 0.049 (0.035–0.068) nmol for MMP-2200 and 0.929 (0.627–1.312) nmol for morphine (data not shown).

In an effort to complement our in vitro studies, a number of in vivo assays related to antinociceptive efficacy and side effects were performed (summarized in Table 1 and detailed below). The antinociceptive effects of MMP-2200 were mea-

Ligand	$[^3H]$ DAMGO K_i (nM)	$[^3H]$ Naltrindole K_i (nM)	$[^3H]$ U69,593 K_i (nM)	MOR:DOR:KOR	$[^{35}S]$ GTP γ S EC_{50} (nM)/ E_{max} (%)		
					MOR	DOR	KOR
Morphine	0.38 ± 0.029	270 ± 36	11 ± 0.39	1:710:29	44 ± 1.7	N.D.	30 ± 6.7
					95 ± 6.6	N.D.	97 ± 9.2
MMP-2200	5.0 ± 0.65	9.2 ± 1.7	42 ± 5.0	1:2:8	220 ± 28	14 ± 2.4	N.D.
					85 ± 2.7	92 ± 2.3	N.D.

Fig. 1. Structures and in vitro activity profiles of morphine and MMP-2200. Binding affinity and functional activity for morphine and MMP-2200 are summarized from studies in CHO cell membranes expressing human MOR, DOR, or KOR. Data are the mean K_i values $\pm$ S.E.M. from three experiments performed in triplicate. Mean EC_{50} and E_{max} values $\pm$ S.E.M. in $[^{35}S]$ GTP γ S assay are from three to four experiments performed in triplicate. The E_{max} values at the MOR are normalized to the stimulation observed with the MOR-selective peptide DAMGO. The E_{max} values obtained for $[^{35}S]$ GTP γ S binding mediated by the DOR and KOR were normalized to the DOR agonist SNC-80 and the KOR-selective agonist U50,488, respectively.

Fig. 2. A and B, antinociceptive dose- and time-response curves for subcutaneous morphine (A) and MMP-2200 (B) in the mouse 55°C tail-flick test. C and D, the calculated area under the curve graphs are depicted. Bars represent the S.E.M. for each dose and time point after injection.

TABLE 1

Summary of the in vivo data for morphine and MMP-2200

Ligand	Antinociceptive Potency Subcutaneously	Stimulation of Locomotor Activity	Antinociceptive Tolerance Shift	Physical Dependence	GI Transit Inhibition	Respiratory Depression
	$\mu\text{mol/kg}$					
Morphine	23.8 (21.3–26.6)	+++	12.8-fold	+++	+++	+++
MMP-2200	8.90 (8.10–10.0)	+	4.8-fold	+	+++	+++

+, mild; ++, moderate; +++, severe.

sured after various antagonist pretreatments. Figure 3A shows that the antinociceptive effects of an A_{90} dose of MMP-2200 (20 mg/kg s.c.) were blocked by pretreatment with the general opioid antagonist naloxone (1 mg/kg i.p.; $t = -30$ min), but not by the peripherally selective antagonist naloxone-methiodide (3.2 mg/kg i.p.; $t = -30$ min; $F_{2,18} = 160.8$; $p < 0.001$). The antinociceptive effects of MMP-2200 were partially blocked by pretreatment with the MOR-selective antagonist β -FNA (19 nmol i.c.v.; $t = -24$ h) (Fig. 3B). Under similar conditions, β -FNA fully blocked the antinociceptive effects of the MOR-selective agonist DAMGO (0.1 nmol i.c.v.; $F_{3,34} = 79.5$; $p < 0.001$). The antinociceptive effects of MMP-2200 (20 mg/kg s.c.) were also partially blocked by pretreatment with the DOR antagonist naltrindole (10 mg/kg s.c.; $t = -10$ min)

(Fig. 3C). Under similar conditions, naltrindole fully blocked the antinociceptive effects of the DOR agonist DPDPE (30 nmol i.c.v.; $F_{3,29} = 49.4$; $p < 0.001$). The antinociceptive effects of MMP-2200 (20 mg/kg s.c.) were not blocked by pretreatment with the KOR antagonist nor-BNI (30 mg/kg i.p.; $t = -24$ h). In contrast, the KOR agonist U69,593 (60 nmol i.c.v.) was fully sensitive to nor-BNI blockade ($F_{3,25} = 883.5$; $p < 0.001$) (Fig. 3D). A separate experiment assessed the effects of pretreatment with a combination of β -FNA (19 nmol i.c.v.; $t = -24$ h) and naltrindole (10 mg/kg s.c.; $t = -10$ min). This combination completely blocked the antinociceptive effects of MMP-2200 (data not shown).

MOR agonists strongly stimulate stereotypical patterns of movement and produce muscular rigidity and Straub tail in

Fig. 3. Antagonism of MMP-2200 antinociception using various opioid antagonists. Mice were pretreated with general (A) or subselective (B–D) antagonists and then injected with agonist and tested for antinociception in the mouse 55°C tail-flick assay at time of peak agonist effect. DH20, distilled water. ***, $p < 0.001$; †††, $p < 0.001$.

ICR mice. The locomotor effects (distanced traveled) produced by subcutaneous injection of morphine or MMP-2200 are quantified in Fig. 4. The first 30 min of the session habituated animals to the testing chambers with a general decrease in activity occurring in all groups. Saline control mice continued to habituate to the chambers over the next 90 min. Morphine injections produced an initial and transient (0–10 min) decrease in activity followed by robust and dose-related stimulation of forward locomotion (Fig. 4A). In contrast, at approximate equi-antinociceptive doses MMP-2200 produced a stronger initial decrease in locomotor activity that was followed by a weak stimulation of locomotor activity (Fig. 4B). Informal observations of the morphine- and MMP-2200-treated animals indicated less muscular rigidity and Straub tail with MMP-2200. Separate groups of mice were injected with doses of morphine or MMP-2200, and the degree of Straub tail was scored as described previously (Zarindast et al., 2002). A double Y plot of dose of compound versus antinociceptive or Straub tail effect is depicted in Fig.

4C. The dose-response curves for morphine on these two measures are virtually overlapping with only a 1.5-fold separation between doses that produce half-maximal effects. In contrast, the dose-response curves for MMP-2200 were separated by a 5-fold difference in potency (e.g., it took almost five times higher doses of MMP-2200 to produce a half-maximal effect on the Straub tail measure compared with doses that produced a 50% maximal possible effect in the tail-flick assay).

As mentioned above, both compounds initially depressed locomotor activity immediately after injection. MMP-2200 produced a significantly greater depressing effect on locomotor activity from $t = 30$ to 60 min based on the calculated area under the curve ($F_{1,114} = 38.6$; $p < 0.01$). To further examine these effects, a modified locomotor activity experiment was performed to evaluate this initial depression of activity observed with MMP-2200. The assay was modified to collect 1-min bins of data (increased temporal resolution), and mice were injected with drug immediately before they were placed

Fig. 4. Comparison of locomotor/muscular effects of morphine and MMP-2200. A and B, dose- and time-response curves for subcutaneous morphine and MMP-2200 in an open-field activity monitoring system. C, a dual plot of subcutaneous antinociception and degree of Straub tail for both drugs.

into the chambers to keep activity levels high to be sensitive to detecting the potential initial decrease in activity. As before, an A_{90} dose of MMP-2200 (20 mg/kg s.c.) decreased activity compared with vehicle-treated animals (Fig. 5). It was hypothesized that the observed decrease reflected the drug's actions on peripheral opioid receptors and/or activity at CNS KORs. Pretreatment with the KOR antagonist nor-BNI (30 mg/kg i.p.; $t = -24$ h) or the peripherally selective antagonist naloxone-methiodide (3.2 mg/kg i.p.; $t = -30$ min) partially reversed the locomotor-depressing effects of MMP-2200. A combination of nor-BNI (30 mg/kg i.p.; $t = -24$ h) and naloxone-methiodide (3.2 mg/kg i.p.; $t = -30$ min) fully reversed the MMP-2200-induced depression of locomotor activity to control levels (Fig. 5B). Neither antagonist treatment by itself altered activity levels compared with saline controls (Fig. 5B). An overall analysis of variance yielded an $F_{6,72} = 19.0$ and $p < 0.001$.

The development of antinociceptive tolerance and physical dependence were assessed using previously published

methods (Raehal et al., 2005). Twice-daily subcutaneous injections of equi-antinociceptive doses of morphine or MMP-2200 for 3 days resulted in approximate 12.8- and 4.8-fold rightward shifts in the dose-response curves, with calculated A_{50} values (and 95% C.I.) of 305.8 (255.3–366.4) μ mol/kg for morphine and 42.9 (37.5–49.0) μ mol/kg for MMP-2200 (Fig. 6). Neither compound produced any signs of overt behavioral toxicity or bodyweight loss compared with saline control mice (data not shown). In the acute physical dependence assay, naloxone (10 mg/kg i.p.) did not precipitate any vertical jumping in mice pretreated with saline (data not shown). In contrast, morphine-treated animals displayed significant naloxone-precipitated vertical jumping behavior compared with controls [$t(19) = 3.84$; $p < 0.001$]. In MMP-2200-pretreated animals, naloxone produced less vertical jumping behavior compared with equivalent treatments with morphine [$t(23) = 3.15$; $p < 0.01$], and this group was not significantly different from saline controls [$t(9) = 1.51$; $p > 0.05$].

Fig. 5. Modified locomotor assay evaluating the initial depression of activity observed with MMP-2200. For blockade studies, mice were pretreated with either the KOR antagonist nor-BNI or the peripherally selective antagonist naloxone-methiodide (NLX-MI) followed by subcutaneous MMP-2200. A, data summarized in 1-min bins. B, data summed over the entire 30-min session. ***, $p < 0.001$.

Fig. 6. Antinociceptive tolerance studies. Comparisons between repeated equivalent antinociceptive doses of morphine (A) and MMP-2200 (B) after subcutaneous administration in the 55°C tail-flick assay.

A longer treatment regimen (twice daily for 4 days) of morphine or MMP-2200 was used to further assess development of physical dependence. Chronic saline-treated animals displayed no vertical jumping after naloxone injection (Fig. 7A). As expected, naloxone precipitated significant withdrawal jumping in morphine-pretreated mice compared with mice pretreated with saline [$t(9) = 2.16$; $p < 0.05$]. Animals receiving chronic MMP-2200 displayed less naloxone-precipitated jumping behavior compared with the morphine controls but was not significant [$t(11) = 1.69$; $p = 0.12$]. The MMP-2200 group was no different from saline controls [$t(9) = 1.27$; $p > 0.05$]. Additional indices of withdrawal included bodyweight loss, fecal boli, and urine output (Figs. 7, B–D, respectively). All groups lost bodyweight after injection of naloxone

with the saline controls losing approximately 0.3 g in the 20-min observation session. Morphine-treated animals lost significantly more bodyweight compared with saline controls [$t(17) = 3.29$; $p < 0.01$]. The MMP-2200 group displayed intermediate bodyweight loss [$t(18) = 1.41$; $p > 0.05$] compared with the morphine group. The MMP-2200 group was no different from vehicle controls [$t(17) = 1.77$; $p > 0.05$]. Similar patterns of withdrawal severity were observed on the fecal boli and urine output measures (Fig. 7, C and D). Morphine-treated animals produced significantly more fecal boli compared with saline controls [$t(17) = 7.9$; $p < 0.001$]. The MMP-2200 group produced significantly less fecal boli compared with morphine-treated animals [$t(18) = 7.7$; $p < 0.001$]. Morphine-treated animals produced significantly

Fig. 7. Naloxone-precipitated withdrawal in mice treated chronically with equivalent doses of morphine or MMP-2200. MMP-2200-pretreated mice exhibited significantly less naloxone-precipitated withdrawal behaviors compared with morphine including vertical jumping (A), bodyweight loss (B), number of feces (C), and urine output (D). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ††, $p < 0.01$; †††, $p < 0.001$.

more urine output compared with saline controls [$t(17) = 2.81$; $p < 0.05$]. The MMP-2200 group produced significantly less urine output compared with morphine-treated animals [$t(18) = 3.09$; $p < 0.01$] and was no different from saline controls [$t(17) = 0.19$; $p > 0.05$].

The effects of morphine or MMP-2200 doses on gastrointestinal transit and respiration are depicted in Figs. 8 and 9, respectively. Morphine and MMP-2200 both dose-dependently inhibited gastrointestinal transit compared with saline controls: $F_{4,41} = 45.6$, $p < 0.001$ and $F_{4,41} = 44.9$, $p < 0.001$, respectively. On a micromolar per kilogram basis, morphine and MMP-2200 had calculated ID_{50} values (and 95% C.I.) of 4.7 (3.7–6.0) $\mu\text{mol/kg}$ and 0.51 (0.35–0.75) $\mu\text{mol/kg}$, respectively. In the respiration assay, whole-body plethysmography was used to quantify the effects of varying CO_2 levels on respiratory rate, tidal volume, and minute ventilation. The minute ventilation data are used as the primary endpoint (Fig. 9). Minute ventilation was stimulated in a concentration-dependent manner as the level of CO_2 increased. Morphine and MMP-2200 both decreased minute ventilation under normal air (0% CO_2) conditions: $F_{4,39} = 41.0$, $p < 0.001$ and $F_{4,38} = 26.9$, $p < 0.001$, respectively. The slopes of the CO_2 response curves were also significantly decreased at higher doses of both morphine and MMP-2200: $F_{4,39} = 4.45$, $p < 0.01$ and $F_{4,38} = 4.80$, $p < 0.01$, respectively.

Discussion

Targeted glycosylation of enkephalin-based peptides can increase peptide stability, BBB penetration, and antinociceptive potency (Bilsky et al., 2000; Elmagbari et al., 2004; Dhanasekaran and Polt, 2005; Eggleton et al., 2005; Lowery et al., 2007). MMP-2200, a lead glycopeptide, exhibits low nanomolar affinity and high efficacy at DOR and MOR and potency/time course profiles similar to morphine in an acute thermal nociceptive assay (Elmagbari et al., 2004). Synthesis of MMP-2200 (>98% purity) is efficient and scalable, allowing us to pursue advanced in vivo testing in rodents and nonhuman primates (Eggleton et al., 2005; Do Carmo et al., 2008).

The in vitro assays indicated that MMP-2200 exhibited affinity and efficacy for both DOR and MOR. This profile was further substantiated by the in vivo antagonist studies. Pretreatment with naloxone and naloxone-methiodide verified that CNS, but not PNS, opioid receptors were critical for producing the antinociceptive effects after systemic administration. DOR- and MOR-selective (but not KOR-selective) antagonists reduced MMP-2200 antinociception, supporting the in vitro profile of the compound and the hypothesis that the antinociceptive potency of MMP-2200 is caused by mixed DOR/MOR activity. Additional studies need to be performed

Fig. 8. The effects of morphine and MMP-2200 on gastrointestinal transit. Morphine (A) and MMP-2200 (B) both dose-dependently inhibited gastrointestinal transit. **, $p < 0.01$; ***, $p < 0.001$. C, the dose-response curves for inhibition of GI transit is summarized.

Fig. 9. The effects of morphine and MMP-2200 on respiration using whole-body plethysmography. Morphine (A) and MMP-2200 (B) produced similar effects on the slopes of CO₂ and minute ventilation plots.

to determine whether these interactions are additive/synergistic (Stevenson et al., 2003).

Two of the hallmarks of MOR agonist activity in ICR mice are stereotypic circling behavior and Straub tail/muscular rigidity. These behaviors are frequently observed at doses of MOR agonists producing full antinociception. We initially

observed much less circling behavior and Straub tail and muscular rigidity in mice injected with MMP-2200 and quantified forward locomotion. Morphine produced robust time- and dose-related stimulation of forward locomotion and stereotypic circling behavior. In contrast, equivalent doses of MMP-2200 produced only mild stimulation of locomotion and

stereotypic circling behavior. Closer inspection of the results in Fig. 4 indicated that both morphine and MMP-2200 initially produced a decrease in locomotor activity compared with saline. The effects were transient, although more robust with all of the doses of MMP-2200 tested. Figure 4C plots the antinociceptive and Straub tail data for both compounds. The morphine dose-response curves for each effect overlap, whereas there is significant separation between the two effects with MMP-2200 (i.e., the mixed DOR/MOR compound is more potent at producing antinociception versus muscular rigidity/Straub tail).

The decrease in activity observed almost immediately after injection with MMP-2200 (versus saline) could be related to 1) strong activation of peripheral opioid receptors, 2) residual agonist activity at CNS KORs, or 3) some off-target effect of the glycopeptide that directly or indirectly affects motor systems (e.g., histamine release/pruritus). To address these possibilities, we used a modified locomotor assay and pretreated different groups of mice with naloxone-methiodide or norn-BNI. These treatments both partially reversed the initial effects of MMP-2200, and when combined brought the level of activity back to saline controls. A separate set of experiments compared intracerebroventricular injections of morphine and MMP-2200 in the locomotor assay to eliminate peripheral drug exposure. Approximate A_{90} doses of intracerebroventricular morphine produced robust hyperlocomotion and stereotypic circling, whereas equivalent doses of MMP-2200 produced only modest increases (data not shown). It is noteworthy that we did not see a decrease in initial activity with either drug administered intracerebroventricularly, indicating the role of peripheral opioid receptors in this effect.

In a model of antinociceptive tolerance, repeated administration of equivalent doses of morphine and MMP-2200 (twice daily for 3 days) resulted in approximate 13- and 5-fold shifts in the dose-response curves, respectively. In acute and chronic physical dependence assays, naloxone consistently precipitated more severe withdrawal in mice receiving morphine versus equivalent MMP-2200 dosing. The results may reflect differences in receptor activity profiles and suggest that a mixed DOR/MOR profile may offer a decreased propensity to produce tolerance/physical dependence compared with MOR-selective agonists. This is in line with previous studies demonstrating lower tolerance/dependence liabilities associated with DOR versus MOR agonists (Dondio et al., 2001; Codd et al., 2009). It may also reflect potential synergy of DOR/MOR effects for antinociception versus additivity/subadditivity with respect to mechanisms that drive development of tolerance/dependence (e.g., less occupancy of DOR and MOR populations needed to produce full antinociceptive effects) (Rozenfeld et al., 2007). Additional experiments need to be performed to test this hypothesis, and there are limitations to interpreting the current results. We attempted to match the dosing and time courses of the two compounds as closely as possible to provide equivalent CNS exposure, but we have not yet measured actual pharmacokinetic and distribution patterns for the compounds. Current efforts have developed analytical methods to quantify MMP-2200 levels in blood and brain tissue. We are in the process of running these more formal pharmacokinetic studies.

A number of additional side effects associated with MOR agonists have complicated the use of these agents for the treatment of acute and chronic pain. Based on the preclinical

literature, we predicted that a DOR/MOR agonist would produce less severe inhibition of GI transit and respiratory depression compared with morphine. In the GI transit assay, both drugs produced potent, dose-related inhibition of GI transit. For morphine, the effects were seen at doses that were very close to those needed to produce antinociception in the tail-flick test. In contrast, subantinociceptive doses for MMP-2200 produced significant inhibition. The potency of MMP-2200 in inhibiting GI transit may be caused in part by the activity at peripheral MORs and the relative levels of the compound at peripheral versus central sites. Although glycosylation dramatically improves CNS penetration, the ratio of CNS/PNS levels of MMP-2200 is still probably lower than that for the small-molecule morphine. This presumably results in a stronger stimulation of peripheral MORs to get an equivalent level of CNS DOR/MOR activation and antinociception. Our analytical efforts will help determine the CNS/PNS ratios, and we may be able to further optimize BBB transport to bring the CNS/PNS ratio closer to that of morphine.

The respiration data indicate that MMP-2200 may have a MOR agonist component too strong to differentiate it from morphine. Under the 0% CO_2 conditions, morphine produced dose-related inhibition of minute ventilation, decreasing this measure by more than 50%. MMP-2200 produced similar levels of inhibition of minute ventilation under normal air conditions. Analysis of the slopes of the CO_2 /minute ventilation curves indicated that both compounds reduced the respiratory drive associated with increasing levels of CO_2 and a reduction in blood pH. Our limited understanding of opioid pharmacology as it relates to respiratory physiology adds to the difficulty of drawing more definitive conclusions. For example, the relative contributions of peripheral versus central MORs, and the role that DORs play in modulating respiration, remain unclear and controversial (Pattinson, 2008). There is evidence that DOR selective agonists stimulate (or at least produce less inhibition of) respiration, and DOR agonists can reverse MOR-induced respiratory depression (Su et al., 1998; Dondio et al., 2001). Changes in motor activity in response to opioid administration can affect respiratory measures, including minute ventilation. We tried to address this confound by using a strain of mouse (BALB/c) in the respiration studies that has similar antinociceptive responses but does not hyperlocomote in response to MOR stimulation. Our current studies are characterizing more DOR-selective glycopeptides, and preliminary data indicate that the compounds produces significantly less respiratory depression compared with morphine and MMP-2200.

In summary, the current studies extend the pharmacological characterization of a lead enkephalin-based glycopeptide that exhibits approximately equal DOR/MOR agonist activity. This profile, along with enhanced stability and BBB penetration, may account for the compound's potent antinociceptive effects after systemic administration. We have profiled the compound in several subacute and chronic inflammatory and neuropathic pain models where it produces full reversal of the abnormal pain states (manuscript in preparation). The broad spectrum of activity and less intense side effects observed with MMP-2200 may be related to the simultaneous activation of DOR and MOR. Our working hypothesis is that there is synergy with respect to antinociception and only additivity/subadditivity with respect to tolerance and dependence generation, locomotor stimulation, muscular rigidity, etc. The optimal ratio of DOR/MOR activation is

unknown with respect to maximizing antinociception and minimizing classic opioid-mediated side effects. In the case of GI and respiratory parameters, it is likely that a higher degree of DOR selectivity is needed to reduce these side effects (Codd et al., 2009). We are currently characterizing next-generation glycopeptides that have >100-fold selectivity for DOR over MOR, and we are also working to eliminate the KOR agonist component of MMP-2200. Additional optimization of the glycosyl is ongoing to further enhance CNS delivery and reduce the PNS/CNS ratio of drug levels. These efforts have the goal of developing a viable drug candidate that can be advanced into clinical trials to determine whether a DOR-selective agonist will be an effective broad-spectrum analgesic with reduced side effects compared with MOR analgesics and whether the glycosylation strategy is a viable approach for delivering small peptides to the CNS after intravenous or oral administration.

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Authorship Contributions

Participated in research design: Lowery, Raymond, Giuvelis, Bidlack, Polt, and Bilsky.

Conducted experiments: Lowery, Raymond, and Giuvelis.

Contributed new reagents or analytic tools: Polt.

Performed data analysis: Lowery, Raymond, Giuvelis, Bidlack, Polt, and Bilsky.

Wrote or contributed to the writing of the manuscript: Lowery, Raymond, Giuvelis, Bidlack, Polt, and Bilsky.

Other: Bilsky and Polt acquired funding for the research.

References

- Bilsky EJ, Eggleston RD, Mitchell SA, Palian MM, Davis P, Huber JD, Jones H, Yamamura HI, Janders J, Davis TP, et al. (2000) Enkephalin glycopeptide analogues produce analgesia with reduced dependence liability. *J Med Chem* **43**: 2586–2590.
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**:248–254.
- Brainin-Mattos J, Smith ND, Malkmus S, Rew Y, Goodman M, Taulane J, and Yaksh TL (2006) Cancer-related bone pain is attenuated by a systemically available delta-opioid receptor agonist. *Pain* **122**:174–181.
- Brennan F, Carr DB, and Cousins M (2007) Pain management: a fundamental human right. *Anesth Analg* **105**:205–221.
- Burgoyne DS (2007) Prevalence and economic implications of chronic pain. *Manag Care* **16**(Suppl 3):2–4.
- Cahill CM, Holdridge SV, and Morinville A (2007) Trafficking of delta-opioid receptors and other G-protein-coupled receptors: implications for pain and analgesia. *Trends Pharmacol Sci* **28**:23–31.
- Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (I₅₀) of an enzymatic reaction. *Biochem Pharmacol* **22**:3099–3108.
- Codd EE, Carson JR, Colburn RW, Stone DJ, Van Besien CR, Zhang SP, Wade PR, Gallantini EL, Meert TF, Molino L, et al. (2009) JNJ-20788560 [9-(8-azabicyclo[3.2.1]oct-3-ylidene)-9H-xanthene-3-carboxylic acid diethylamide], a selective delta opioid receptor agonist, is a potent and efficacious antihyperalgesic agent that does not produce respiratory depression, pharmacologic tolerance, or physical dependence. *J Pharmacol Exp Ther* **329**:241–251.
- Cowan A, Zhu XZ, Mosberg HI, Omnaas JR, and Porreca F (1988) Direct dependence studies in rats with agents selective for different types of opioid receptor. *J Pharmacol Exp Ther* **246**:950–955.
- Dhanasekaran M and Polt R (2005) New prospects for glycopeptide based analgesia: glycoside-induced penetration of the blood-brain barrier. *Curr Drug Deliv* **2**:59–73.
- Do Carmo GP, Polt R, Bilsky EJ, Rice KC, and Negus SS (2008) Behavioral pharmacology of the mu/delta opioid glycopeptide MMP2200 in rhesus monkeys. *J Pharmacol Exp Ther* **326**:939–948.
- Dondio G, Ronzoni S, Farina C, Graziani D, Parini C, Petrillo P, and Giardina GA (2001) Selective delta opioid receptor agonists for inflammatory and neuropathic pain. *Pharmacol* **56**:117–119.
- Eggleston R, Bilsky E, Tollin G, Dhanasekaran M, Lowery J, Alves I, Davis P, Porreca F, Yamamura H, Yeomans L, et al. (2005) Biousian glycopeptides penetrate the blood–brain barrier. *Tetrahedron Asymmetry* **16**:65–75.
- Elmagbari NO, Eggleston RD, Palian MM, Lowery JJ, Schmid WR, Davis P, Navrati-lova E, Dhanasekaran M, Keyari CM, Yamamura HI, et al. (2004) Antinociceptive structure-activity studies with enkephalin-based opioid glycopeptides. *J Pharmacol Exp Ther* **311**:290–297.
- Fields HL, Emson PC, Leigh BK, Gilbert RF, and Iversen LL (1980) Multiple opiate receptor sites on primary afferent fibres. *Nature* **284**:351–353.
- Gomes I, Gupta A, Filipovska J, Szeto HH, Pintar JE, and Devi LA (2004) A role for heterodimerization of mu and delta opiate receptors in enhancing morphine analgesia. *Proc Natl Acad Sci USA* **101**:5135–5139.
- Gray AC, Coupar IM, and White PJ (2006) Comparison of opioid receptor distributions in the rat central nervous system. *Life Sci* **79**:674–685.
- Gutstein H and Akil H (2005) Opioid analgesics, in *Goodman & Gilman's The Pharmacological Basis of Therapeutics* (Brunton L, Lazo J, and Parker K eds) pp 547–590, McGraw-Hill, New York.
- Heyman JS, Koslo RJ, Mosberg HI, Tallarida RJ, and Porreca F (1986) Estimation of the affinity of naloxone at supraspinal and spinal opioid receptors in vivo: studies with receptor selective agonists. *Life Sci* **39**:1795–1803.
- Holdridge SV and Cahill CM (2007) Spinal administration of a delta opioid receptor agonist attenuates hyperalgesia and allodynia in a rat model of neuropathic pain. *Eur J Pain* **11**:685–693.
- Horan P, Taylor J, Yamamura HI, and Porreca F (1992) Extremely long-lasting antagonistic actions of nor-binaltorphimine (nor-BNI) in the mouse tail-flick test. *J Pharmacol Exp Ther* **260**:1237–1243.
- Hylden JL and Wilcox GL (1980) Intrathecal morphine in mice: a new technique. *Eur J Pharmacol* **67**:313–316.
- Janssen P, Niemegeers C, and Dorg J (1963) The inhibitory effects of fentanyl and other morphine-like analgesics on the warm water-induced tail withdrawal reflex in rats. *Arzneim-Forsch Drug Res* **13**:502–507.
- Jiang Q, Heyman JS, and Porreca F (1989) Mu antagonist and kappa agonist properties of β -funaltrexamine (β -FNA): long lasting spinal antinociception. *NIDA Res Monogr* **95**:199–205.
- Lowery JJ, Yeomans L, Keyari CM, Davis P, Porreca F, Knapp BI, Bidlack JM, Bilsky EJ, and Polt R (2007) Glycosylation improves the central effects of DAMGO. *Chem Biol Drug Des* **69**:41–47.
- McNicol E, Horowitz-Mehler N, Fisk RA, Bennett K, Gialeli-Goudas M, Chew PW, Lau J, Carr D, and American Pain Society (2003) Management of opioid side effects in cancer-related and chronic noncancer pain: a systematic review. *J Pain* **4**:231–256.
- Pattinson KT (2008) Opioids and the control of respiration. *Brit J Anaesth* **100**:747–758.
- Petrillo P, Angelici O, Bingham S, Ficalora G, Garnier M, Zaratini PF, Petrone G, Pozzi O, Sbaccia M, Stean TO, et al. (2003) Evidence for a selective role of the δ -opioid agonist [8R-(4bS*,8a α ,8a β ,12b β)]7,10-Dimethyl-1-methoxy-11-(2-methylpropyl)oxycarbonyl 5,6,7,8,12,12b-hexahydro-(9H)-4,8-methanobenzo-furo[3,2-e]pyrrolo[2,3-g]isoquinoline hydrochloride (SB-235863) in blocking hyperalgesia associated with inflammatory and neuropathic pain responses. *J Pharmacol Exp Ther* **307**:1079–1089.
- Polt R, Dhanasekaran M, and Keyari CM (2005) Glycosylated neuropeptides: a new vista for neuropsychopharmacology? *Med Res Rev* **25**:557–585.
- Porreca F, Mosberg HI, Hurst R, Hruby VJ, and Burks TF (1984) Roles of mu, delta, and kappa opioid receptors in spinal and supraspinal mediation of gastrointestinal transit effects and hot-plate analgesia in the mouse. *J Pharmacol Exp Ther* **230**:341–348.
- Raehal KM, Lowery JJ, Bhamidipati CM, Paolino RM, Blair JR, Wang D, Sadée W, and Bilsky EJ (2005) In vivo characterization of δ -naltrexol, an opioid ligand with less inverse agonist activity compared with naltrexone and naloxone in opioid-dependent mice. *J Pharmacol Exp Ther* **313**:1150–1162.
- Rozenfeld R, Abul-Husn NS, Gomez I, and Devi LA (2007) An emerging role for the delta opioid receptor in the regulation of mu opioid receptor function. *Sci World J* **7**:64–73.
- Scherrer G, Tryoen-Tóth P, Filliol D, Matifas A, Laustriat D, Cao YQ, Basbaum AI, Dierich A, Vonesh JL, Gavériaux-Ruff C, et al. (2006) Knockin mice expressing fluorescent delta-opioid receptors uncover G protein-coupled receptor dynamics in vivo. *Proc Natl Acad Sci USA* **103**:9691–9696.
- Schiller RW (2010) Bi- or multifunctional opioid peptide drugs. *Life Sci* **86**:598–603.
- Sheldon RJ, Qi JA, Porreca F, and Fisher LA (1990) Gastrointestinal motor effects of corticotrophin-releasing factor in mice. *Regul Pept* **28**:137–151.
- Smith PA, Selley DE, Sim-Selley LJ, and Welch SP (2007) Low dose combination of morphine and delta9-tetrahydrocannabinol circumvents antinociceptive tolerance and apparent desensitization of receptors. *Eur J Pharmacol* **571**:129–137.
- Stevenson GW, Folk JE, Linsenmayer DC, Rice KC, and Negus SS (2003) Opioid interactions in rhesus monkeys: effects of delta + mu and delta + kappa agonists on schedule-controlled responding and thermal nociception. *J Pharmacol Exp Ther* **307**:1054–1064.
- Su YF, McNutt RW, and Chang KJ (1998) Delta-opioid ligands reverse alfentanil-induced respiratory depression but not antinociception. *J Pharmacol Exp Ther* **287**:815–823.
- Sutters KA, Miaskowski C, Taiwo YO, and Levine JD (1990) Analgesic synergy and improved motor function produced by combination of mu-delta- and mu-kappa-opioids. *Brain Res* **530**:290–294.
- Zarrindast MR, Ramezani-Tehrani B, and Ghadimi M (2002) Effects of adrenoceptor agonists and antagonists on morphine-induced Straub tail in mice. *Pharmacol Biochem Behav* **72**:203–207.

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