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Antinociceptive effects of the 6-O-sulfate ester of morphine in normal and diabetic rats: Comparative role of mu- and delta-opioid receptors

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Abstract

This study determined the antinociceptive effects of morphine and morphine-6-O-sulphate (M6S) in both normal and diabetic rats, and evaluated the comparative role of mu-opioid receptors (mu-ORs) and delta-opioid receptors (delta-ORs) in the antinociceptive action of these opioids. *In vitro* characterization of mu-OR and delta-OR-mediated signaling by M6S and morphine in stably transfected Chinese hamster ovary (CHO-K1) cells showed that M6S exhibited a 6-fold higher affinity for delta-ORs and modulated G-protein and adenylyl cyclase activity via delta-ORs more potently than morphine. Interestingly, while morphine acted as a full agonist at delta-ORs in both functional assays examined, M6S exhibited either partial or full agonist activity for modulation of G-protein or adenylyl cyclase activity, respectively. Molecular docking studies indicated that M6S but not morphine binds equally well at the ligand binding site of both mu- and delta-ORs. *In vivo* analgesic effects of M6S and morphine in both normal and streptozotocin-induced diabetic Sprague-Dawley rats utilizing the hot water tail flick latency test showed that M6S produced more

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Conflict of interest

The University of Kentucky holds a patent on the compound M6S described in the current work. A potential royalty stream to PAC may occur consistent with University of Kentucky policy.

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potent antinociception than morphine in both normal rats and diabetic rats. This difference in potency was abrogated following antagonism of delta- but not mu- or kappa (kappa-ORs) opioid receptors. During 9 days of chronic treatment, tolerance developed to morphine-treated but not to M6S-treated rats. Rats that developed tolerance to morphine still remained responsive to M6S. Collectively, this study demonstrates that M6S is a potent and efficacious mu/delta opioid analgesic with a delayed tolerance profile when compared to morphine in both normal and diabetic rats.

Perspective—This study demonstrates that M6S acts at both mu- and delta-ORs, and adds to the growing evidence that the use of mixed mu/delta opioid agonists in pain treatment may have clinical benefit.

Graphical Abstract

In vivo analysis of morphine- and M6S-induced (i.p.) antinociception was examined by hot water tail flick latency test in naïve and diabetic rats. *In vitro* experiments to determine opioid receptor affinity and activation of post-receptor events utilized Chinese hamster ovary (CHO) cells transfected with human mu- or delta-ORs. M6S produced more potent antinociception than morphine in both naïve and diabetic rats. The data imply that the improved analgesic and tolerance profile of M6S over morphine may be due to the ability of M6S to act at both mu- and delta-ORs.

Keywords

Morphine-6-O-sulfate; morphine; streptozotocin-induced diabetes; antinociception; opioid receptors

1. Introduction

Treatment of chronic pain remains a significant problem. Existing non-opioid treatments are frequently of inadequate efficacy, while the use of otherwise powerful mu-opioid receptor (mu-OR) agonist-based therapies for chronic pain are limited by development of tolerance and side-effects, resulting in dose escalation and subsequent adverse effects [1]. In search of safer opioids, polyvalent mu-/delta-OR agonists have recently attracted attention [2, 3], with morphine-6-O-sulfate (M6S), a simple structural analog of the mu-OR-selective opioid, morphine, potentially also falling into this class of drugs [4, 5].

All animal studies to date unequivocally confirm the antinociceptive superiority of M6S over morphine with regard to the treatment of acute burning pain [6–9]. Furthermore, M6S is superior to morphine for relief of inflammatory, as well as superficial and deep neuropathic

pain [4]. Analgesic effects of M6S are observed at doses that are 10 times lower than those producing gastrointestinal transit problems or sedation [4]. The superior analgesic and side effect profile of M6S when compared to morphine is similar to other known mixed mu-/delta-OR agonists [10, 11] and is in agreement with reports that simultaneous activation of mu- and delta-ORs produces synergistic antinociception with reduced side effects [2, 3].

However, unlike the plethora of evidence for morphine acting as a mu-OR-selective opioid, existing evidence indicating that M6S acts as a mixed mu/delta-OR agonist is mostly indirect, and no definitive studies investigating this suggestion have been reported. Mechanistically, this suggestion has never been supported by either *in vitro* or computational receptor docking studies, and no animal studies utilizing selective OR antagonists have yet been conducted with M6S to directly determine the relative contribution of different ORs to M6S-mediated antinociception. The finding that the antinociceptive activity of M6S is mediated via interaction at both mu- and delta-ORs could have clinical benefits, since it implicates M6S as a potential opioid-rotation drug in patients who fail to respond to, or who develop tolerance to traditional mu-OR therapy, or who have not responded to the mu-OR drug from the onset of treatment. However, to confirm such beneficial actions, studies examining the development of cross-tolerance between morphine and M6S are needed. Finally, although the delta-OR pain control circuitry appears to be upregulated in diabetes [12–15], studies examining the antinociceptive efficacy of M6S, or other existing mu/delta OR agonists in animal models of diabetes have not yet been reported [16].

Considering the above information, in the current work we conducted *in vitro* studies to determine receptor affinity, G-protein activation and adenylyl cyclase inhibition of morphine and M6S in human Chinese Hamster Ovary (CHO-K1) cells transfected with human mu- and delta-ORs, as well as molecular docking studies of morphine and M6S with mu- and delta-opioid receptors. *In vivo* studies utilizing the hot water tail flick latency test were conducted to compare the acute and chronic effects of morphine and M6S, and potential cross-tolerance between morphine and M6S was assessed in streptozotocin (STZ)-induced diabetic rats, a condition in which the antinociceptive efficacy of morphine is frequently reported to diminish [17, 18]. Finally, studies utilizing OR-selective antagonists were conducted to define the comparative roles of mu-, delta- and kappa-ORs in the antinociceptive action of M6S.

2. Methods

2.1. Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Arkansas for Medical Sciences and were performed in compliance with the guidelines of the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats (250–350g); (Charles River Laboratories, Wilmington, MS) were housed three per cage with *ad libitum* access to food and water in the Institutional Division of Laboratory Animal Resources. Rats were randomly assigned to control or diabetic groups and then to specific drug treatment or vehicle control groups (4–12 rats per group).

To induce diabetes, overnight fasted animals were injected with STZ (Sigma Aldrich, St. Louis, MO). STZ was dissolved in 10mM citrate buffer (pH 4.0) immediately before injection and given intraperitoneal (i.p.) at a dose of 65 mg/kg. Control animals received the citrate buffer injection only. All STZ-injected animals in this study developed diabetes (randomly tested blood glucose values > 11 mM), which was confirmed by glucose measurements of blood samples obtained by the tail-prick technique on the 6th – 7th day after STZ injection (colorimetric Contour Next blood glucose monitoring system; Bayer HealthCare, Mishawaka, IN, USA). Diabetes was defined following the guidelines on Standards of Medical Care in Diabetes as the state of casual/random (measured at any time of day without regard to time since last food intake) blood plasma glucose equal or exceeding 11.1 mM levels [19]. As in humans, in rats, this criterion can be used as an alternative to fasting glucose or 2 hr-glucose in oral glucose tolerance test measurements to reliably distinguish normal, prediabetic and overt diabetic states [16, 20, 21].

2.2. Drugs

Morphine hydrochloride was purchased from Merck & Co., Inc. (Rahway, NJ). Morphine-6-*O*-sulfate (sodium salt) was prepared as described previously [22]. The delta-OR selective antagonist naltrindole hydrochloride (NLD) was purchased from Tocris Biosciences (Minneapolis, MN). The kappa-OR antagonist Norbinaltorphimine hydrochloride (nor-BNI) was a generous gift from Dr. David Wessinger (UAMS, Little Rock, AR). Naltrexone hydrochloride (NTX) was purchased from Sigma Aldrich (St. Louis, MO). All drugs were prepared in physiological saline obtained from Fischer Scientific (St Louis, MO) immediately prior to use. The volume of intraperitoneal (i.p.) injections for all animals was 2 mL/kg of rat weight. Animals in the vehicle-control group received an equivalent volume of the vehicle only.

To determine the role of different ORs in the antinociception produced by M6S in the hot water tail withdrawal latency (TWL) test, normal and diabetic animals were pre-treated with the nonselective OR antagonist NTX (2 mg/kg) [23, 24], the kappa-OR-selective antagonist nor-BNI (5 mg/kg) [25, 26], or the delta-OR-selective antagonist, NLD (1 mg/kg) [27, 28]. All drugs were injected i.p. Nor-BNI, NTX and NLD were injected 18 hr [29], 30 min [23, 24] and 30 min [27, 28], respectively, prior to the measurement of peak antinociception produced by M6S or morphine (30 minutes for morphine in diabetic rats, and 60 minutes in all other cases).

2.3. Behavioral testing

All experiments were conducted starting on the 2nd week and completed before the end of the third week (except for selected tolerance studies) after STZ/citrate buffer injection. Behavioral tests were carried out between 10 AM and 5 PM. Animals were allowed to acclimate to the room environment for about 1 hr before beginning of testing. In all experiments, TWL was measured at baseline and then at 15, 30, 45 and 60 min following drug or vehicle injection. For time-course studies and calculation of area-under-the-curve (AUC, integral antinociceptive effect), additional TWL readings were taken at 2, 3, 4 and 5 hr post-treatment. At least one training TWL measurement session was conducted to familiarize rats with the test procedures. In the dose-response studies, the same animals were

used to test the effect of low-to-moderate concentration (0.1 – 3 mg/kg) of a given drug. Morphine or M6S were administered in the sequence from low to higher doses with a 2 day washout period allowed between each increment. Animals treated with the 3 mg/kg dose were then continued with the same daily dose in the drug tolerance studies. Effects of higher 5.6 mg/kg and 10 mg/kg drug concentrations were studied using independent groups of 4 to 12 rats. In the tolerance studies, a 3 mg/kg dose of morphine or M6S was administered to rats daily (i.p.). Baseline and 45 minute post drug TWL readings were collected on days 1, 3, 7 and 9 of the experiment, and then at about 5 day intervals in a subset of experiments designed to monitor progression of tolerance to M6S.

During the test, a hot water bath (Fisher Scientific IsoTemp 102S) was maintained at a constant temperature level of 50 ± 0.5 °C. Three tail withdrawal latency (TWL) measurements (trials) per animal per test were recorded with a 1–2 min interval between consecutive measurements and individual values were averaged. During each trial, the distal 2 cm of the tail was immersed in the hot water. The latency time for an animal to flick its tail in response to the noxious stimulus (withdrawal reflex) was recorded by a second observer using a stopwatch and expressed in seconds. To avoid injury to the tail, a cutoff time of 12 sec was employed. The percentage of maximum possible effect (%MPE) was calculated by the following formula: $100\% \times [(drug\ response\ time - basal\ response\ time)/(12\ sec - basal\ response\ time)]$.

3. Cell Culture

Chinese hamster ovary (CHO-K1) cells were stably transfected (see following section) with either the human delta-OR (CHO-hDOR) or mu-OR (CHO-hMOR) containing an *N*-terminal FLAG or cMyc epitope tag, respectively. All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Mediatech Inc., Manassas, VA) containing 10% fetal calf serum (Gemini Bio products, Sacramento, CA), 0.05 IU/mL penicillin and 50 µg/mL streptomycin (Invitrogen, Carlsbad, CA). To maintain selection, CHO-hDOR and CHO-hMOR transfected cells were cultured with an additional 200 µg/mL of Hygromycin B (Life Technologies, GrandIsland, NY) or 250 µg/mL of Geneticin or (G418; Sigma-Aldrich, St. Louis, MO), respectively. Cells were maintained in a humidified chamber at 37°C with 5% CO₂, harvested weekly and only cells from passages 5–15 were used in the experiments.

CHO-hMOR transfected cells were created as reported previously [30]. To produce stably transfected CHO-hDOR cells, the cationic-lipid lipofectin (Life Technologies, Grand Island, NY) was used in a manner similar to that reported previously to create CHO-hMOR transfected cells. Briefly, CHO-K1 cells were cultured to 80% confluence (3×10^6 cells in 100-mm dishes) and incubated for 6 hr with 5 µg of pcDNA3.1 plasmids containing the cDNA encoding for the human delta-OR containing an *N*-terminal FLAG epitope tag, and 15 µg of lipofectin reagent in serum-free Opti-MEM. An antibiotic (1 mg/ml hygromycin) was added to the cell culture medium 48 hr after transfection, and surviving colonies were isolated 14 days after selection. To confirm delta-OR expression, competition binding studies using whole cells obtained from each colony were performed by employing [³H]-diprenorphine (1.0 nM) displaced by nonradioactive naloxone (10 µM), as described below. The clone expressing the highest level of specific delta-OR binding, was selected for use in

all the subsequent studies, and was maintained in DMEM containing 200 µg/ml hygromycin throughout the study.

3.1. Membrane Preparation

CHO-hMOR cells and CHO-hDOR cells were harvested and homogenized with 10 complete strokes of a 7 ml Ounce glass homogenizer in ice-cold buffer containing 50 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.4, 3 mM MgCl₂, and 1 mM ethylenediaminetetraacetic acid (EDTA), as described previously [31]. The homogenized samples were centrifuged at $40,000 \times g$ for 10 min at 4°C. Supernatants were discarded, and the pellets re-suspended in buffer, homogenized again, and centrifuged similarly twice more. After the final centrifugation step, supernatants were discarded and pellets were re-suspended in ice-cold 50 mM HEPES, pH 7.4 to achieve an approximate protein concentration of 10 mg/ml. Membrane homogenates were divided into aliquots and stored at 80°C for future use. A small aliquot of each membrane preparation was removed prior to freezing and the protein concentration was determined using the BCA™ Protein Assay (Thermo Fisher Scientific, Waltham, MA).

3.2. Competition Receptor Binding

Competition binding studies were conducted as described previously [30]. Each reaction mixture contained 100 µg of membrane homogenate, 1 nM of the high-affinity non-selective opioid antagonist [³H]-diprenorphine, 5 mM MgCl₂, and increasing concentrations of the non-radioactive competitive ligands morphine, M6S, [D-Pen^{2,5}]enkephalin (DPDPE) or [D-Ala²,N-MePhe⁴,Gly-ol]enkephalin (DAMGO) in a 50 mM Tris-HCl buffer (pH 7.4) containing 0.1% bovine serum albumin. The total volume of the reaction mixture was 1 ml. Reaction mixtures were vortexed and then allowed to reach equilibrium binding during incubation at room temperature for 90 min. Non-specific binding was defined as the amount of radioligand binding remaining in the presence of a single 10 µM concentration of non-radioactive naloxone. Binding was terminated by rapid vacuum filtration through glass fiber filters (Brandel, Gaithersburg, MD), followed by four 5 ml washes of ice-cold 50 mM Tris-HCl (pH 7.4) buffer containing 0.1% bovine serum albumin. Four ml of Scintiverse© scintillation fluid (Fisher Scientific, Waltham, MA) was added to the filters and the amount of radioactivity was quantified 24 hr later utilizing liquid scintillation spectrophotometry.

3.3. [³⁵S]GTPγS Binding

The GTPγS binding assay to measure G-protein activation was performed as previously described [32]. Briefly, in a total volume of 1 ml, 10 µg of membrane homogenate was added to a reaction mixture containing 0.1 nM [³⁵S]GTPγS, 20 mM HEPES, 10 mM MgCl₂, 100 mM NaCl, 10 µM GDP, 0.1% bovine serum albumin and the indicated concentrations of morphine, M6S, DPDPE or DAMGO. After mixing, reaction mixtures were incubated at 30°C for 30 min. (a time interval shown to produce optimal agonist-induced [³⁵S]-GTPγS binding levels, data not shown). Nonspecific binding was defined by the amount of radioactivity remaining in the presence of 10 µM non-radiolabeled GTPγS. Reactions were terminated by rapid vacuum filtration through glass fiber filters followed by four washes with ice cold 50 mM HEPES (pH 7.4) containing 0.1% bovine serum albumin. Four ml of Scintiverse© scintillation fluid (Fisher Scientific, Waltham, MA) was added to the filters and

the amount of radioactivity was quantified 24 hr later utilizing liquid scintillation spectrophotometry.

3.4. Measurement of Intracellular cAMP

CHO-hDOR or CHO-hMOR transfected cells were plated separately into 24-well culture plates at a density of 6×10^6 cells/plate and incubated overnight at 37°C in 5% CO₂. As previously described [32], the day after seeding, culture media was removed from each well and replaced with 500 µl of pre-incubation mixture containing DMEM with 0.9% NaCl, 500 µM 3-isobutyl-1-methylxanthine (IBMX), and 2 µCi/well [³H]-adenine. Cells were incubated for 5 hr at 37°C in 5% CO₂. The pre-incubation mix was removed and morphine, M6S, DPDPE or DAMGO was added to individual wells at the indicated concentrations for 15 min at 37°C in a Krebs-Ringer-HEPES solution (10 mM HEPES, 110mM NaCl, 25mM Glucose, 55mM Sucrose, 5mM KCl, 1mM MgCl₂, 1.8 mM CaCl₂, pH 7.4), containing 500 µM IBMX and 10µM forskolin. Reactions were terminated by adding 50 µl of 2.2N HCl and [³H]-cAMP was isolated by employing alumina column chromatography. Radioactivity contained in 4 ml of the final column elute was counted in a Packard-Tri-carb 2100/TR liquid scintillation counter after adding 10 ml of Scintiverse liquid scintillation cocktail.

3.5. Statistical analyses

The results of behavioral testing were analyzed using repeated measures two-way ANOVA followed by *post hoc* Bonferroni or one-way ANOVA followed by *post hoc* Tukey as appropriate (Graphpad Prism® software version 5.0). When only two groups were compared, a Student's *t*-test was employed. Regression analysis and non-linear curve-fitting procedures were performed using a Levenberg-Marquart algorithm of X² minimization [33] and Origin 10 software package (MicroCal, Northampton, MA). For *in vitro* studies, non-linear regression was used to determine IC₅₀ values for competition receptor binding, ED₅₀ and E_{max} values for G-protein studies, and IC₅₀ and I_{max} values for measurement of cAMP levels. The Cheng-Prusoff equation [34] was used to convert experimental IC₅₀ values obtained from competition receptor binding experiments to K_i values. All dissociation constants and measurements of potency were converted to pK_i, pEC₅₀, or pIC₅₀ values by taking the negative log of each value so that parametric tests could be used for statistical comparison. All *in vitro* values in this study were derived from a minimum of 3 replicates. Differences were considered as statistically significant at *p* < 0.05. Data are reported as the mean ± standard error of the mean (SEM).

4. Computational molecular docking

Atomic coordinates for the crystal structures of the target delta-OR (PDB 4N6H [35]) and mu-OR (PDB 4DKL [36]) were downloaded from the RCSB Protein Data Bank (<http://www.rcsb.org/pdb/>). Atomic coordinates for the small molecules morphine, M6S and naltrexone were generated using Marvin Sketch (ChemAxon). All input coordinate files for both the proteins and the small molecules were prepared for docking using the Dock Prep module in the UCSF Chimera software. Docking was performed using SwissDock (<http://www.swissdock.ch/>), which employs the EADock DSS algorithm [37]. Docking poses from multiple docking runs were ranked based on the resulting docking scores, and only the top-

scoring poses were considered further. Validity of the docking protocol was established by performing pilot docking runs, in which the antagonists found in the crystal structures of delta-ORs (naltrindole) and mu-ORs (β -funaltrexamine) were docked using the same docking protocol, and docking results were compared with the binding poses of these ligands from the original crystal structures. Use of the most exhaustive and unbiased option in SwissDock enabled the sampling of a maximum number of binding modes for each molecule.

5. Results

5.1. In vitro assays to compare opioid receptor affinity and intrinsic activity of M6S and morphine

5.1.1. Competition Receptor Binding—Competition receptor binding studies employed the high affinity non-selective opioid antagonist [^3H]-diprenorphine to characterize the affinity of opioids for delta-ORs or mu-ORs stably expressed in stably transfected CHO-hDOR or CHO-hMOR cells, respectively (Fig 1, Table 1). K_i values were converted to $\text{p}K_i$ values ($\text{p}K_i = -\text{Log}[K_i]$) so that parametric tests could be used for statistical comparison. In these experiments we found that morphine binds with high affinity ($K_i = 0.31 \text{ nM}$) to mu-ORs, and with much lower affinity for delta-ORs ($K_i = 66.7 \text{ nM}$). M6S exhibits high affinity for mu-ORs ($K_i = 0.98 \text{ nM}$); however, M6S also binds to delta-ORs with a K_i value in the low nanomolar range (11.1 nM). As anticipated, the selective delta-OR agonist DPDPE binds with high affinity ($K_i = 0.56 \text{ nM}$) to delta-ORs and the selective mu-OR agonist DAMGO exhibits a K_i value of 0.52 nM for mu-ORs. Most importantly, M6S exhibits a delta-/mu-OR affinity ratio of only 11, which is in marked contrast to the delta-/mu-OR affinity ratio of 215 for morphine.

5.1.2. [^{35}S]GTP γS Binding—Studies were next conducted to compare the intrinsic activity of M6S and morphine at delta- and mu-ORs. Initial studies examined the ability of these opioids to modulate G-protein activity via ORs (Fig. 2, Table 2). EC_{50} values were converted to pEC_{50} values ($\text{pEC}_{50} = -\text{Log}[\text{EC}_{50}]$) so that parametric tests could be used for statistical comparison. As shown in Fig 2, M6S and morphine activate G-proteins via mu-ORs expressed in CHO-hMOR cells with similar potencies ($\text{M6S-ED}_{50} = 35.0 \text{ nM}$; $\text{morphine-ED}_{50} = 17.9 \text{ nM}$) and efficacies ($\text{M6S-}E_{\text{max}} = 13.7\%$; $\text{morphine-}E_{\text{max}} = 12\%$). When compared to DAMGO ($E_{\text{max}} = 25\%$), both M6S and morphine act as partial agonists, producing less maximal activation of G-proteins. In contrast to observations with mu-ORs, M6S activates G-proteins via delta-ORs much more potently than morphine ($\text{M6S-ED}_{50} = 86.3 \text{ nM}$; $\text{morphine-ED}_{50} = 762 \text{ nM}$). However, similar to efficacy at mu-ORs, M6S is also a partial agonist at delta-ORs when compared to activation produced by the full agonist DPDPE ($\text{M6S-}E_{\text{max}} = 14.7\%$; $\text{DPDPE-}E_{\text{max}} = 24\%$). Although morphine acts as a partial agonist at mu-ORs, it activates G-proteins with similar efficacy ($\text{morphine-}E_{\text{max}} = 24.7\%$) to the full delta-OR agonist DPDPE in CHO-hDOR transfected cells.

5.2. Measurement of Intracellular cAMP Levels

To compare a second measure of intrinsic efficacy, the ability of M6S and morphine to modulate activity of the downstream intracellular effector adenylyl cyclase via delta- and

mu-ORs expressed in intact CHO-hDOR and CHO-hMOR cells was measured (Fig. 3, Table 3). IC_{50} values were converted to pIC_{50} values ($pIC_{50} = -\log[pIC_{50}]$) so that parametric tests could be used for statistical comparison. As expected for Gi/Go-coupled receptors, both morphine and M6S produce a concentration-dependent reduction in intracellular cAMP concentrations to about 20% of baseline values in intact CHO-hMOR transfected cells. Similar to modulation of G-protein activity, M6S and morphine modulate adenylyl cyclase activity with similar potencies (M6S- $IC_{50} = 109$ nM vs. morphine- $IC_{50} = 67.1$ nM) and efficacies (M6S- $I_{max} = 52.6\%$ vs. morphine- $I_{max} = 66.8\%$). Also, similar to G-protein regulation, M6S (but not morphine) acts as a partial agonist for regulation of adenylyl cyclase activity when compared to the full mu-OR agonist DAMGO ($I_{max} = 80.3\%$). Most importantly, G-protein activation studies demonstrate that M6S modulates adenylyl cyclase activity via delta-ORs in intact CHO-hDOR cells much more potently than morphine (M6S- $IC_{50} = 69.5$ nM; morphine- $IC_{50} = 372$ nM). All three agonists examined in CHO-hDOR transfected cells were full agonists, maximally reducing intracellular cAMP to similar levels.

6. Computational docking studies

Theoretical docking studies were conducted to evaluate if there were any significant differences in the interaction of morphine and M6S with mu- and delta-OR binding pockets. The predicted binding poses were ranked based on their FullFitness (FF) scores, and the top-scoring docked poses for morphine and M6S were compared. Of the top 10 docked poses of morphine that bound to the delta-OR, only one pose (FF score -1090 kcal/mol) was observed to bind at the same site as that occupied by the agonist naltrindole (Fig. 4A left panel; *magenta*; the naltrindole site is shown as a dashed circle). We refer to this site as the 'ligand-binding site' (LBS). The rest of the nine docked poses of morphine, including the pose with the highest FF score (-1101 kcal/mol) bound to a cleft that is located outside of the cavity that constitutes the LBS of naltrindole on the delta-OR ('allosteric site'). This distribution of the docked morphine poses between the two sites suggests that for morphine, binding to the allosteric site might be sterically and energetically more favorable, than binding to the LBS. In contrast, eight of the top ten docked poses (including the top-scoring pose; FF score -1118 kcal/mol) for M6S bound at the LBS on the delta-OR (Fig. 4A middle panel; *cyan*) while only two poses docked at the allosteric site. Again, this can be interpreted as suggesting a more favorable binding of M6S to the LBS of the delta-OR.

We also performed docking with the non-selective antagonist naltrexone to the delta-OR. In stark contrast to both morphine and M6S, the top 10 poses of naltrexone were distributed almost equally at both the LBS and the allosteric site (Fig. 4A right panel; *yellow*).

The single docked pose of morphine at the LBS makes atomic contacts with the same subset of delta-OR residues as that with the antagonist naltrindole (from the co-crystal structure). As shown in Fig. 4B, both naltrindole and morphine interact through polar contacts with side-chains of residues Asp128 and Tyr129 of the delta-OR. Naltrindole interacts with a few additional residues of the delta-OR through van der Waals contacts, as compared to morphine, presumably due to the larger size of the naltrindole molecule. Overall, the docking results suggest that morphine may bind preferably to the allosteric site as compared to the LBS on the delta-OR, which in turn, may lead to an ineffective activation of the

receptor, as compared to delta-OR-selective agonists. Further, docking results with M6S also suggest that M6S, by virtue of binding preferentially to the LBS of delta-OR, produces a much more effective receptor-activation than morphine.

Results of docking studies for morphine and M6S with the mu-OR provided quite different results. The top hits for both morphine and M6S were observed to bind to the β -funaltrexamine LBS on the mu-OR (Fig. 5A; the five poses with highest FF scores are shown). This indicates that unlike for the delta-OR, both these ligands could potentially bind favorably at the LBS of the mu-OR. Notably, the orientations of morphine and M6S at the mu-OR binding site are 'flipped' with respect to each other. As shown in Fig. 5B, many residues of the mu-OR that are involved in van der Waals' interactions with morphine and M6S are the same (i.e. residues Gln124, Asp147, Ile144, Lys233, Ile296, Val300, Trp318, and Tyr326); some are unique to morphine (residues Thr218 and Leu219), while some are unique to M6S (residues Tyr148 and Ile322). M6S shows some additional electrostatic interactions that are missing in morphine's interaction with the delta-OR. This is also reflected in the higher FF score of the top pose of M6S (−1934 kcal/mol) as compared to that of morphine (−1912 kcal/mol). The sulfate group of M6S is involved in ionic interactions with the side-chains of residues Tyr148 and Lys233, while the −OH group of M6S interacts with the *N*-atom of the indole ring of Trp318. The *N*-atom of M6S, which is 'flipped' with respect to its orientation in morphine, is involved in an interaction with the side chain of Asp147. This interaction is not possible in the case of morphine, because of the flip.

7. In Vivo Studies

7.1. In vivo comparison of the analgesic profiles for morphine and M6S in both normal and diabetic rats

7.1.1. Baseline characteristics of control and STZ-treated rats—For comparison of the antinociceptive effects of M6S and morphine *in vivo* in normal and diabetic rats, the hot water tail-flick latency (TWL) test was selected due to its simplicity and sensitivity for evaluation of antinociception in response to thermal pain [38].

Control animals weighed an average of 250 ± 4.5 g and during an average experimental period of 2–3 weeks, gained weight at a mean rate of 7.9 ± 0.3 g/day ($n=88$). To ensure development of diabetes all STZ-treated animals were injected with the toxin after overnight fasting. As a result, all STZ-injected animals developed hyperglycemia (random blood glucose concentrations of 30 ± 2.9 mM; compared to normal rat random blood glucose concentrations of 6.2 ± 0.1 mM; $n = 20$), polyuria and polydipsia. Water consumption and urination by diabetic rats exceeded those by control rats by 18–20 times (as measured in metabolic cage experiments; $n = 6$ rats per group). Furthermore, STZ-treated rats also gained weight at a rate about 2 times lower than control animals (3.4 ± 0.2 g/day; $n=87$; unpaired *t*-test, $t(173) = 12.45$; $p<0.001$; vs. control). The effect of morphine and M6S on tail flick pain modality was studied in 43 control and 40 STZ-treated rats of above. The remaining rats were used in other experiments, including other behavioral tests and/or other treatments; the results of these studies are not reported here. In the tail flick test, control rats ($n=43$) exhibited baseline latencies that were normally distributed around a mean of 4.21 ± 0.08 sec.

compared to STZ treated rats (n=40) mean latency of 3.98 ± 0.09 sec. the TWL of STZ-treated rats were lower than those of normal rats, suggesting development of heat hyperalgesia in diabetic rats. However this difference did not reach statistical significance (unpaired t-test, $t(81) = 1.750$, $p = 0.084$) (Fig. 6A). Random blood glucose concentrations of 43 diabetic animals used in TWL experiments were 30.1 ± 0.6 mM (95% CI: 28.9 – 31.2 mM), and not different from glucose concentrations measured in a larger population of STZ-treated rats (n = 87, see above), but statistically significantly higher than the maximum glucose concentration measured in control rats (7.45 mM; one-sample t-test).

7.1.2. Antinociception time course—The time course of antinociceptive action for morphine and M6S in the TWL test did not differ in control animals. With both drugs, TWL increased to a peak within 45–60 min post-injection, and then returned to baseline within 2–3 hours (Fig. 6B). When compared to control, in diabetic rats, the antinociceptive action of both drugs was accelerated; the peak effect occurred 30–45 min post-injection, with recovery to baseline within the next hour (one-way ANOVA both drugs $F(4, 25) = 12.29$; at 120 min $p > 0.05$ for (diabetic rats have prefix D) D-M6S and D-morphine vs. D-Vehicle; $p < 0.001$ for (control rats have prefix C) C-M6S and C-morphine vs C-Vehicle). Shortening of the time course of the antinociceptive effect in diabetic rats was more pronounced for morphine than for M6S (effect of time RM-ANOVA; $F(5, 175) = 75.39$; post-hoc Bonferroni test, at 60 min vs. 45 min $p < 0.001$ for D-morphine; $p > 0.05$ for all other treatment groups, Fig. 6B). Control treatment (vehicle injections) did not affect TWL in either naïve or diabetic rats (data not shown, RM-ANOVA, $F(6, 175) = 56.76$; $p > 0.05$).

7.2. Antinociceptive dose-response relationships

To determine dose-response relationships of morphine and M6S, effects of increasing doses of drugs from 0.03–10 mg/kg on TWL were examined. In control rats, M6S exhibited a %MPE₅₀ at a dose of 0.82 mg/kg (95%CI: 0.58–1.36) that was statistically significantly lower than that for morphine (3.41 mg/kg (95%CI: 2.76–4.77)). In diabetic rats, this difference between M6S and morphine was even more pronounced: %MPE₅₀ for M6S was 0.35mg/kg (95%CI: 0.30–0.46), while that for morphine was 6.47mg/kg (95%CI: 4.67–6.92). Thus, compared to normal rats, the antinociceptive potency of M6S in STZ-diabetic rats was enhanced, while no statistically significant differences in antinociceptive potency were observed for morphine in normal and diabetic rats. Nonetheless, due to changes in time course, the total antinociceptive score (AUC) of M6S decreased by 25% in diabetic rats compared to that in non-diabetic rats (Fig. 6D; one way ANOVA, $F(3, 20) = 61.07$ (M6S) $F(3, 20) = 13.35$ (morphine); $p < 0.001$, post hoc Tukey test). In marked contrast, the AUC for morphine in diabetic rats was reduced to a much greater extent (approximately 3-fold) when compared to normal animals (one way ANOVA; $F(3, 20) = 13.35$; $P < 0.001$, Fig. 6D).

7.3. Role of opioid receptors in M6S-mediated antinociception

To determine the role of different ORs in the antinociception produced by M6S in the TWL test, normal and diabetic animals were pre-treated with the nonselective OR antagonist NTX (2 mg/kg), the kappa-OR-selective antagonist nor-BNI (5 mg/kg), or the delta-OR-selective antagonist, NLD (1 mg/kg). All drugs were injected *via* the i.p. route of administration. Nor-BNI, NTX and NLD were injected 18 hr, 30 min and 30 min, respectively, prior to the

measurement of peak antinociception produced by M6S or morphine (30 minutes for morphine in diabetic rats, and 60 minutes in all other cases). Dose selection and timing of drug administration for antagonists were based on reports from the literature [23–27], as described in the Methods section.

Experiments with the non-selective opioid antagonist NTX confirmed that M6S produces antinociception in the TWL test in normal rats via activation of ORs (Fig 7A). NTX blocked $97.1 \pm 2.9\%$ of the antinociception produced by M6S. By comparison, pre-treatment with the kappa-OR antagonist nor-BNI did not affect M6S-induced antinociception in normal rats, while the delta-opioid receptor antagonist NLD decreased the antinociceptive effect of M6S by approximately 55% (Fig. 7A). Based on these observations, further experiments were focused on studies of the effects of NLD only (Fig. 7B and C). In these experiments, NLD did not affect peak antinociception of morphine in either normal or diabetic animals. In contrast, NLD reduced M6S-mediated antinociception in diabetic animals by 55%. Notably, in both control and diabetic rats, NLD reduced the analgesic action of M6S to levels that were indistinguishable from antinociception produced by morphine (Fig. 7B, C; morphine vs. M6S+NLD; $F(1, 12) = 2.33$, $p = 0.827$ by one-way ANOVA).

7.4. Tolerance and cross-tolerance studies

Repeated treatment with morphine (3 mg/kg, daily) resulted in a reduction of antinociceptive effect (tolerance) in both control and diabetic rats (Fig. 7D). Complete loss of antinociception was observed after 9 days of chronic treatment. In contrast, the decline in the antinociceptive action of M6S (3 mg/kg) during repeated daily treatment was much slower, with only approximately 10% reduction in effect observed by the end of the 9-day treatment period. Diabetes had no effect on the time course of tolerance progression to either morphine or M6S (Fig. 7D). Remarkably, three weeks of daily treatment with M6S were required to achieve the same level of tolerance that was observed following 9 days of repeated treatment with morphine (data not shown). Furthermore, while the antinociceptive action of M6S was preserved in morphine-tolerant rats (no cross-tolerance development), morphine failed to produce antinociception in M6S-tolerant rats (Fig. 7E).

8. Discussion

M6S is a sulfate ester derivative of morphine that in some preclinical studies appears to have superior analgesic and tolerance profile over morphine. The reasons for this superior profile are not clearly understood. In the current studies, by utilizing a set of *in vitro* and *in vivo* studies we have provided supporting evidence that the improved analgesic and tolerance profile of M6S over the selective mu-OR agonist morphine is likely due to the ability of M6S to act as a mixed mu/delta-OR agonist. Furthermore, our data suggest that M6S may constitute a powerful alternative to mu-OR agonists for treatment of pain in subjects with diabetes, as well as having the potential to serve as an opioid rotation drug in subjects who fail to respond to traditional opioids, or who become tolerant to chronic treatment with opioids.

In vitro receptor binding data demonstrate that morphine and M6S exhibit a 215- and 11-fold greater affinity for mu-ORs over delta-ORs, respectively. Consistent with the activity

expected for a mu-OR-selective agonist, morphine modulates both G-protein and adenylyl cyclase activity 43- and 6-fold more potently via mu-, relative to delta-ORs, respectively. In contrast, the much less selective mu/delta-OR agonist M6S regulates G-protein and adenylyl cyclase activity via mu- and delta-ORs with relatively similar potency. Not only do M6S and morphine differ in mu-/delta-OR selectivity, but our study also found that M6S and morphine also exhibit distinct intrinsic activity at mu- and delta-ORs. For example, both M6S and morphine act as partial mu-OR agonists to regulate both intracellular effectors examined. In contrast, when considering delta-ORs, morphine instead acts as a full agonist in both functional assays, while M6S exhibits either partial or full agonist activity to modulate G-protein or adenylyl cyclase activity, respectively. The apparent dichotomy between the partial versus full agonist modulation of G-protein and adenylyl cyclase activity by M6S via delta-ORs might be explained, in part, by the knowledge that regulation of adenylyl cyclase occurs downstream from activation of G-proteins. Therefore, production of cAMP (the endpoint of adenylyl cyclase regulation) is an amplified response relative to G-protein activation. In any case, if similar to reports for mu-OR partial agonists such as buprenorphine [39], the partial agonist activity of M6S at mu- and/or delta-ORs may provide several distinct advantages to treatment of chronic pain when compared to opioids exhibiting full agonism.

Molecular docking results support the *in vitro* data, indicating that M6S binds more favorably than morphine to the LBS on the delta-OR, whereas both ligands bind with equal efficiency at the LBS on the mu-OR. Specifically, M6S affords a higher docking score at the LBS of both the delta- and the mu-OR as compared to morphine (–1118 kcal/mol and –1090 kcal/mol, respectively, for M6S and morphine at the delta-OR; and –1934 kcal/mol and –1912 kcal/mol, respectively, for M6S and morphine, at the mu-OR). These observations could indicate a more favorable binding of M6S at the LBS of both receptors as compared to morphine.

As reported previously in the radiant heat tail flick test [4], in our hot water tail flick assay in normal rats, M6S displays a greater acute antinociceptive potency than morphine and maintains its efficacy, even during chronic treatment (Fig. 6 and 7).

In our present work, pretreatment with the kappa-OR specific antagonist nor-BNI did not affect M6S-evoked antinociception in the TWL test. In contrast, while the delta-OR specific antagonist NLD had no effect on morphine-induced antinociception, it suppressed the activity of M6S by about 50% in both normal and diabetic animals (Fig 6). These findings are consistent with our *in vitro* and computational results, and also compare well with our *in vivo* observations. In addition, co-activation of kappa-ORs has been demonstrated to decrease antinociception produced by morphine in the TWL test in rats [40]. Thus, if M6S were to act via kappa-ORs it would be expected to produce less, and not greater (Fig. 6) antinociception than morphine, which again supports the non-involvement of kappa-ORs in analgesic actions of M6S. Furthermore, although evoked heat pain is relayed by nociceptive fibers expressing both mu- and delta-ORs [41], the antinociceptive effects of morphine in the HPT and TWL tests in mice [42] and in rats [43], are mediated by activation of only mu-ORs, in contrast to M6S, which activates both receptors.

Selective mu-OR agonists often fail to afford adequate pain relief in diabetic patients [44] in line with several preclinical reports [17, 18, 45]. Our study assesses the antinociceptive potency and efficacy of M6S as a potential mu/delta-OR agonist alternative in diabetic animals in comparison to the selective mu-OR agonist, morphine.

We used a well-established rat model of STZ-induced diabetes. The effect of diabetes on thermal nociception in this model remains uncertain. In this respect, literature reports are often contradictory with some researchers claiming an increase in, while others citing either a decrease in, or no change in withdrawal threshold/latency to heat. Animal strain, severity and duration of diabetes, and specific test/protocol-related differences may contribute to these discrepancies (for a review see [16]). Our findings agree with reports of no changes in sensitivity to heat stimuli in STZ-treated rats [46]. It is important to note that our studies were conducted in rats with early-stage and relatively mild diabetes (rats continued to gain weight throughout the study).

We also found no effect of diabetes on the peak potency of morphine analgesia in either the acute or chronic treatment studies (Fig. 6C, 7D). This finding is in contrast to several previous studies of mu-OR agonists in STZ-induced diabetic rats [17, 18]. As discussed earlier, the duration or severity of diabetes may potentially contribute to differences between studies, but further research is required to resolve these issues. The diabetic condition in the current study greatly accelerated the time course of morphine- and M6S-induced antinociception compared to the normal condition, resulting in about a 45–60% decline in morphine's total analgesic score (AUC) in the TWL test (Fig 6). This finding is consistent with the reported enhanced renal function and 3-fold faster clearance of morphine in STZ-induced diabetic rats compared to normal rats (see [17] and references therein). Judging from the acceleration of the time course, the same may also be applicable to M6S (Fig. 7B); however, the effect of diabetes on AUC was substantially weaker (20%–30% decrease) than that for morphine, which might be explained by the increase in M6S potency observed in diabetic animals (Fig. 7C). In this respect there are contradictory findings in the literature regarding the reasons for the decrease in morphine's analgesic score in diabetic rats [17], [47], [48]. While some researchers have attributed the decrease to increased clearance of morphine in diabetic rats compared to normal rats, others have argued that it may be due to changes in pharmacological function/receptor number for mu opioid receptors in diabetic rats compared to normal rats. One can speculate that accelerated clearance by enhanced renal function in diabetic rats could potentially occur with both morphine and M6S. However, comparative pharmacokinetic studies on morphine and M6S in normal and diabetic rats are needed to directly address this issue. In this respect we have observed that there is almost a 20-fold difference in the clearance of M6S (~23ml/kg/mg) when compared to morphine (~413ml/kg/mg) after i.p. administration of these two drugs in normal Sprague Dawley rats [49]. Further studies are needed to fully determine whether increased clearance is responsible for the observed reduction in the duration of analgesia exhibited by both morphine and M6S in diabetic rats compared to normal rats.

Compared to morphine, M6S was found to be more potent in the TWL test during both acute and chronic treatments in normal rats. Furthermore, in diabetic rats the antinociceptive efficacy of M6S was found to be greater, and tolerance development to repeated M6S

treatment occurred slower even than in normal rats (Fig. 6C and 7E, respectively). Collectively, our data suggest that the differences in the antinociceptive actions of M6S and morphine may be attributed to the ability of M6S to act as a mixed mu/delta-OR agonist, in contrast to morphine, which acts primarily via activation of mu-ORs at therapeutically-relevant doses. While our data do not provide a direct evidence in support to this idea, they are in general agreement with findings from previous researchers, where other pharmacological agents with mixed mu/delta-OR activity (i.e. glycopeptide MMP-2200 [2]; fentanyl/enkephalin structure-based RV-Jim-C3 [50]; octapeptide biphalin derivatives [51]; and the 7-hydroxymitragynine derivative MGM-16 [52]) have consistently demonstrated potent analgesia and delayed tolerance development when compared to morphine. Furthermore, our observation of a leftward shift in the M6S antinociceptive dose-response curve in diabetic rats (Fig. 6C) together with our *in vitro* and *in vivo* data suggesting mixed mu/delta-OR agonism, also is in agreement with reports of an increase in the density/availability of delta-ORs in diabetic rodents [12–15]. Finally, involvement of delta-ORs may also be a key factor contributing to the lack of M6S cross-tolerance to morphine although this needs to be objectively confirmed in the future studies employing receptor knock out studies *in vivo*. (Fig. 7) [2].

9. Conclusions

Taken collectively, the data presented herein demonstrate that M6S is a potent and efficacious mu/delta opioid analgesic with delayed tolerance profile over morphine in both normal and diabetic rats. We have provided direct and indirect evidence suggesting the ability of M6S to activate mu-OR and most importantly also delta-OR pain control pathways as a chief determinant underlying the superiority of this drug over morphine. This notion is also supported by the results presented in both *in vitro* and *in vivo* assays. For example, *in vitro* receptor binding and functional studies demonstrate that while morphine acts as a relatively selective mu-OR agonist, M6S instead modulates mu-OR and delta-OR signaling in a relatively balanced manner. Such unbiased regulation of mu-OR and delta-OR signaling by M6S may be an underlying mechanism contributing to the previously reported superior analgesic and side effect profiles of M6S and other mixed mu-/delta-OR agonists when compared to morphine [10, 11]. Furthermore, the observed balanced activity of M6S at mu-/delta-ORs is consistent with observations that simultaneous activation of mu- and delta-ORs produces synergistic antinociception with reduced side effects [2, 3].

The molecular docking results also support the observations from our *in vitro* binding and functional studies that M6S binds more favorably than morphine to the LBS on the delta-OR, whereas both ligands bind with equal efficiency at the LBS on the mu-OR. These results would suggest that morphine may be effective in the activation of the mu-OR, while working only ineffectively at the delta-OR; M6S, on the other hand may be effective at both the receptors. Additionally, our docking results also provide a structural basis for the higher potency of M6S to elicit analgesia compared to morphine.

A final novel finding from our studies is that M6S, as would be expected for a mixed mu/delta-OR agonist, lacks cross-tolerance in morphine-tolerant diabetic animals. This

important observation indicates that M6S may serve as a useful alternative (rotational opioid) to morphine and other opioids for pain alleviation in diabetic patients.

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Abbreviations

M6S	morphine-6-sulphate
delta-OR	delta-opioid receptor
mu-OR	mu-opioid receptor
kappa-OR	kappa opioid receptor
CHO-K1	Chinese hamster ovary
STZ	streptozotocin
NLD	naltrindole hydrochloride
NTX	naltrexone hydrochloride
TWL	tail withdrawal latency
nor-BNI	norbinaltorphimine hydrochloride
MPE	maximum possible effect (MPE)
CHO-hDOR	Chinese hamster ovary human delta-opioid receptor
CHO-hMOR	Chinese hamster ovary mu-opioid receptor
DMEM	Dulbecco's Modified Eagle's Medium
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
EDTA	ethylenediaminetetraacetic acid
DPDPE	[D-Pen ^{2,5}]enkephalin
DAMGO	[D-Ala ² ,N-MePhe ⁴ ,Gly-ol]enkephalin
IBMX	3-isobutyl-1-methylxanthine
LBS	ligand-binding site
FF	FullFitness

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Fig. 1.

Determination of the binding affinity of test compounds for hMORs (A) and hDORs (B) by competition receptor binding performed with membrane homogenates.

Competition receptor binding studies utilized the high affinity non-selective opioid antagonist $[^3H]$ -diprenorphine to determine the affinity of ligands for delta-ORs or mu-ORs stably expressed in CHO-hDOR or CHO-hMOR transfected cells, respectively.

Fig. 2.

Modulation of basal G-protein activity by test compounds utilizing $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding in CHO-hMOR and CHO-hDOR membrane homogenates (A) and (B), respectively. CHO-hMOR and CHO-hDOR cell membranes (10 μg) were incubated in the presence of 0.1 nM $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ with increasing concentrations of morphine, M6S, DAMGO or DPDPE. Data are expressed as percent specific $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding to membranes incubated with vehicle and reported as individual ED_{50} and E_{max} values.

Fig. 3.

Modulation of adenylyl cyclase activity by test compounds in CHO-hMOR (A) and CHO-hDOR (B) transfected cells.

After loading with [^3H]-adenine, intact CHO-hMOR and CHO-hDOR transfected cells were incubated in the presence 10 μM forskolin and increasing concentrations of morphine, M6S, DAMGO or DPDPE. Data are expressed as percent of intracellular cAMP in cells incubated with vehicle and reported as individual IC_{50} and I_{max} values.

Fig. 4.

Two distinct binding sites are observed for the ligands morphine and M6S at the delta-opioid receptor (delta-OR).

(A) - The delta-OR protein (PDB 4N6H) is shown as a *wheat* cartoon. The dashed circle indicates the location of the ligand binding site (LBS) on the delta-OR occupied by naltrindole in the crystal structure. Distribution of the top 10 docked poses of morphine (*magenta*), M6S (*cyan*) and the non-selective antagonist naltrexone (*yellow*) between the LBS and the allosteric site (see text) are shown. As can be clearly seen, a majority of the docked poses of morphine bind at the allosteric site, while only one pose binds at the LBS. In contrast, for M6S, a majority of poses (8 out of 10) including the top-scoring pose, are bound at the LBS on the receptor. The top 10 docked poses for naltrexone are distributed equally between the two sites. (B) - Atomic contacts of naltrindole, morphine and M6S at the LBS of the delta-OR. The antagonist naltrindole (*yellow*) is bound at the LBS in the co-crystal structure with the delta-OR, and makes polar contacts with two delta-OR residues, Asp128 and Tyr129. These contacts and many of the van der Waals' contacts of other delta-OR residues are common between naltrindole, and morphine (*magenta*). The lone docked pose of morphine (FF score -1090 kcal/mol) shown here makes fewer van der Waals' contacts with delta-OR residues as compared to naltrindole at the LBS. In contrast, the top-scoring docked pose of M6S (*cyan*) shown here (FF score -1118 kcal/mol) makes an ionic interaction through its sulfate group to the side-chain of Lys214 of the delta-OR, as well as van der Waals' contacts with other residues common with morphine.

Fig. 5.

Top docked poses of both morphine and M6S bind at the LBS of the μ -OR.

(A) - The top 5 docked poses (based on FF scores) of morphine (*magenta*), and M6S (*cyan*) are shown bound to the LBS on the μ -OR, which is shown as a *wheat* cartoon. (B) - The μ -OR residues at the LBS within 4 Å of the respective ligands: β -funaltrexamine (*yellow*), present in the crystal structure, and the top-scoring docked poses of morphine (*magenta*) and M6S (*cyan*) are shown as *wheat* sticks and are labeled. The antagonist β -funaltrexamine, is bound at the LBS in the co-crystal structure with the μ -OR, and makes polar contacts with the sidechains of Lys303 and Asp147. Morphine is involved in polar interactions with the sidechains of Lys233 and Trp318. M6S shows a ‘flipped’ orientation as compared to morphine, and is involved in a greater number of ionic interactions; the sulfate group of M6S interacts with Lys233 and Tyr148, while the *N*-atom of M6S makes a polar contact with Asp147.

Fig. 6.

Comparison of the analgesic actions of morphine and M6S.

(A) - Distribution of baseline TWL in control and diabetic rats (open and grey bars, respectively). Smooth solid (control) and dashed (diabetes) lines represent the results of best fit of Gaussian function to the data. Best fit parameters, for control rat data: center = 4.21 ± 0.08 s, width (2 SD) = 0.68 ± 0.05 s, Adj. $R^2=0.961$ and for diabetic rat data: center = 3.98 ± 0.09 s, width = 0.77 ± 0.11 s, Adj. $R^2=0.867$. (B) - Time course of analgesic effects of morphine (morphine, triangles and dashed lines) and M6S (circles and solid lines) in the TWL test in control (C, solid lines; 3 mg/kg of morphine and 1 mg/kg of M6S) and diabetic (D, dashed lines; 3 mg/kg of morphine and 0.3 mg/kg of M6S) rats, respectively. Equianalgesic (producing a similar peak analgesic effect) doses of morphine and M6S in both diabetic and normal rats are represented for comparison (peak TWL increase was 3.6 ± 0.5 s and 4.3 ± 0.3 s, respectively, for morphine and M6S in normal rats, and 3.3 ± 0.3 s and 3.4 ± 0.5 s, respectively, for morphine and M6S in diabetic rats; $n=6$ rats per group). Nonetheless, to facilitate the time course comparison, in each set of experiments, data were normalized to respective maximum effect. (C) - Dose-response relationships for the analgesic effects of M6S and morphine in control and diabetic rats. Straight lines are results of best fit of linear function to the data (note logarithmic dose scale; adjusted $R^2 > 0.99$ for all 4 lines fitted). Horizontal dashed line is 50% of maximum possible drug effect (%MPE). An unpaired t-test was used for comparison of ED₅₀ values between C-morphine Vs C-M6S and for D-morphine vs D-M6S. The t-test showed significant difference between ED₅₀

values for C-morphine Vs C-M6S (unpaired t test; $t(7) = 4.0952$; $p = 0.0046$ and also between ED50 values for D-morphine vs D-M6S (unpaired t test; $t(8) = 3.3987$; $p = 0.0094$. **(D)** - Total antinociceptive score (magnitude x duration of antinociceptive effect; area-under-the-curve, AUC) for morphine and M6S at doses of 1 and 3 mg/kg i.p., in control and diabetic rats (n=6 rats per group). Text labels indicate p values for adjacent data set comparisons (one-way ANOVA, *post hoc* Tukey test).

Fig. 7.

Comparison of tolerance and cross-tolerance profiles for M6S and morphine and the effect of the delta-specific antagonist naltrindole on both drugs.

(A) - The effect of M6S (3 mg/kg) on TWL in the presence of opioid receptor antagonists (non-subtype-specific, NTX; kappa-OR-specific, Nor-BNI; and delta-OR-specific, NLD) relative to that without OR blockade. Asterisks – difference from other two groups at $p < 0.05$. Double asterisks – difference from nor BNI and NTX treated group. A one-way ANOVA followed by post hoc Tukey test was used to compare the differences between NTX, NBNI and NLD treated M6S rats. The one-way ANOVA revealed an overall significant difference between treatment groups, $F(2,17) = 30.10$; $P < 0.0001$. Furthermore, a post hoc Tukey test revealed that M6S + NLD treatment group (denoted by **) is significantly different from M6S + NTX and M6S + Nor BNI treated rats ($p < 0.05$). In addition, post hoc Tukey also revealed that M6S + Nor BNI treated rats are significantly different (denoted by *) from both M6S + NLD and M6S + NTX treated rats ($p < 0.05$). (B) - The effect of the delta-OR-specific antagonist naltrindole (1 mg/kg, i.p., 30 min prior peak effect) on tail withdrawal latencies observed in morphine (MOR)- (light grey) and M6S- (dark grey) treated control animals (3 mg/kg, i.p. for both drugs); Asterisk – different from all other groups at $p < 0.001$ by one way ANOVA. (C) - The effect of the delta-OR-specific antagonist naltrindole on morphine (MOR) and M6S antinociception in diabetic animals. All other conditions and labeling as in (B). (D) - Maximum analgesic effects of morphine (morphine and M6S) in the TWL test in control rats (open and closed circles) and diabetic rats (open and closed triangles) during daily drug administration. In each set of experiments data are normalized to respective maximum effect observed on day one. A two way repeated

measures ANOVA followed by post hoc Bonferroni test was used to determine the development of tolerance for C-MOR, C-M6S, D-MOR and D-M6S treatment groups. There was an overall significant effect of treatment: $F(3, 60) = 195.0$; $p < 0.001$; and an overall significant Effect of time: $F(3, 60) = 36.56$; $p < 0.001$. However, a post hoc Bonferroni test revealed that on Day 5 of treatment, C-M6S, C-MOR and D-MOR are significantly different from their day 1 values ($p < 0.01$ for C-M6S, $p < 0.001$ for C-MOR and D-MOR but not D-M6S ($p > 0.05$). Therefore, development of tolerance was delayed in diabetic M6S treatment compared to C-M6S treatment group. (E) - Cross-tolerance profile for morphine (MOR) and M6S in diabetic rats. The morphine/M6S group is morphine-tolerant rats (9 days of morphine injections) tested on the 10th day with M6S (equal dose of 3 mg/kg; i.p.) and the M6S/morphine group is M6S tolerant rats (27 days of M6S treatment) tested with morphine on the 28th day (equal dose of 3 mg/kg, i.p.); $F(3, 20) = 13.35$; $p < 0.001$ by one way ANOVA, *post-hoc* Tukey test for M6S/morphine. $n=6$ rats per group were used in all experiments presented in this figure.

Table 1

Competition binding employing CHO-cMyc-hMOR and CHO-Flag-hDOR membranes

³ H]CP-55,940 Binding						
CHO-cMyc-hMOR			CHO-Flag-hDOR			
Drug	K _i (nM)	pK _i	N	Drug	K _i (nM)	pK _i
Morphine	0.31	9.515 ± 0.224 ^a	4	Morphine	66.7	7.176 ± 0.045 ^{a, **}
M6S	0.98	9.011 ± 0.163 ^b	4	M6S	11.1	7.956 ± 0.135 ^{b, **}
DAMGO	0.52	9.288 ± 0.098 ^{a, b}	3	DPDPE	0.56	9.253 ± 0.040 ^c

^{a, b, c} pK_i values designated by different letters are significantly different from values within the same column; P<0.05, one-way ANOVA, Tukey *Post-hoc* test.^{**} pK_i values for hDOR receptors are significantly different from hMOR; P<0.01, student's *t*-test.

Table 2
Modulation of GTP- γ S binding employing CHO-cMyc-hMOR and CHO-Flag-hDOR membranes

GTP- γ S binding									
CHO-cMyc-hMOR					CHO-Flag-hDOR				
Drug	EC ₅₀ (nM)	pEC ₅₀	E _{MAX} (%)	N	Drug	EC ₅₀ (nM)	pEC ₅₀	E _{MAX} (%)	N
Morphine	17.9	7.746 $\pm$ 0.174 ^a	12.0 $\pm$ 2.1 ^a	3	Morphine	762	6.118 $\pm$ 0.074 ^{a, **}	24.7 $\pm$ 0.67 ^{a, **}	3
M6S	35.0	7.456 $\pm$ 0.217 ^a	13.7 $\pm$ 1.9 ^a	3	M6S	86.3	7.064 $\pm$ 0.175 ^b	14.7 $\pm$ 1.9 ^b	3
DAMGO	22.4	7.650 $\pm$ 0.243 ^a	25.0 $\pm$ 2.3 ^b	3	DPDPE	7.10	8.147 $\pm$ 0.320 ^c	24.0 $\pm$ 2.5 ^a	3

^{a, b, c}

pEC₅₀ or E_{MAX} values designated by different letters are significantly different from values within the same column; P<0.05, one-way ANOVA, Tukey *Post-hoc* test.

^{**} pEC₅₀ or E_{MAX} values for hDOR are significantly different from hMOR; P<0.01, student's *t*-test.

Table 3
Modulation of Intracellular cAMP Levels in CHO-cMyc-hMOR and CHO-Flag-hDOR Cells

Intracellular cAMP Levels									
<i>CHO-cMyc-hMOR</i>					<i>CHO-Flag-hDOR</i>				
Drug	IC ₅₀ (nM)	pIC ₅₀	I _{MAX} (%)	N	Drug	IC ₅₀ (nM)	pIC ₅₀	I _{MAX} (%)	N
Morphine	67.1	7.173 ± 0.067 ^a	66.8 ± 5.0 ^a	6	Morphine	372	6.430 ± 0.014 ^{a, **}	43.3 ± 9.7 ^{a, **}	3
M6S	109	6.962 ± 0.070 ^b	52.6 ± 5.8 ^b	5	M6S	69.5	7.158 ± 0.078 ^{b, **}	38.0 ± 7.2 ^{a, **}	4
DG	16.6	7.780 ± 0.039 ^c	80.3 ± 3.7 ^c	3	DP	0.39	9.413 ± 0.205 ^c	31.0 ± 1.5 ^a	3

^{a, b, c} pIC₅₀ or I_{MAX} values designated by different letters are significantly different from values within the same column; P<0.05, one-way ANOVA, Tukey *Post-hoc* test.

^{**} pIC₅₀ or I_{MAX} values for hDOR are significantly different from hMOR; P<0.01, student's *t* test.