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Evaluation of analgesia, tolerance and the mechanism of action of morphine-6-*O*-sulfate across multiple pain modalities in Sprague-Dawley rats

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Abstract

Background—Morphine-6-*O*-sulfate (M6S) is a mixed μ -/ δ -opioid receptor (OR) agonist and potential alternative to morphine for treatment of chronic multimodal pain.

Method—To provide more support for this hypothesis, the antinociceptive effects of M6S and morphine were compared in tests that access a range of pain modalities, including hot plate threshold (HPT), pinprick sensitivity threshold (PST) and paw pressure threshold (PPT) tests.

Results—Acutely, M6S was 2–3-fold more potent than morphine in HPT and PST tests. Specifically, derived from best-fit analysis of dose-response relationships morphine / M6S ED₅₀ ratios (lower, upper 95%CI) were 2.8 (2.0, 5.8) in HPT and 2.2 (2.1, 2.4) in PST tests. No differences in analgesic drug potencies were detected in the PPT test (morphine / M6S ED₅₀ ratio 1.2 (95%CI: 0.8, 1.4). After 7–9 days of chronic treatment, tolerance developed to the antinociceptive effects of morphine, but not to M6S, in all three pain tests. Morphine-tolerant rats

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were not cross-tolerant to M6S. The antinociceptive effects of M6S were not sensitive to κ -OR antagonists. However, the δ -OR antagonist, naltrindole, blocked M6S-induced antinociception by $55 \pm 4\%$ (95%CI: 39, 75) in the HPT test, $94 \pm 4\%$ (95%CI: 84, 105) in the PST test, and $5 \pm 17\%$ (95%CI: -47, 59) or $51 \pm 14\%$ (95%CI: 14, 84; 6 rats per each group) in the PPT test when examined acutely or after 7 days of chronic treatment, respectively.

Conclusions—Activity via δ -ORs thus appears to be an important determinant of M6S action. M6S also exhibited favorable antinociceptive and tolerance profiles compared to morphine in three different antinociceptive assays, indicating that M6S may serve as a useful alternative for rotation in morphine-tolerant subjects.

1. Introduction

1.1. Background

Treatment of chronic pain remains a vexing problem worldwide ¹. Mu-opioid receptor (μ -OR) agonists such as morphine and its analogs are considered the most effective therapeutic option for chronic pain alleviation ². However, multiple adverse effects undermine the use of this class of drugs in most chronic pain cases ³. Furthermore, as with other classes of drugs, not all pain modalities are equally responsive to a particular opioid ^{4,5}. Therefore, further efforts toward the discovery of safe and effective opioid therapeutics for multimodal chronic pain treatment are urgently needed. Recent advances suggest that opioids with mixed μ /delta-opioid receptor (μ -/ δ -OR) activity demonstrate a potential therapeutic alternative to traditional μ -OR-selective agonists ⁶. The present study is part of our continuing efforts aimed at providing complete preclinical characterization of one such opioid, morphine-6-*O*-sulfate (M6S).

1.2. Rationale

M6S was initially synthesized as a part of a study on morphine 3- and 6-*O*-glucuronides, ethereal sulfates and phosphate conjugates ⁷. It was quickly established and confirmed *in vivo* that M6S was more potent than morphine in the radiant-heat tail flick test (TFL; superficial burning pain, ⁸⁻¹¹). Further, it was reported ¹¹ that compared to morphine, M6S: 1) is more potent in the alleviation of deep pressure or superficial mechanical pain in the chronic constriction nerve injury (CCI) rat model of mononeuropathy, and in alleviating spontaneous pain in the rat model of formalin-induced inflammation; 2) demonstrates delayed development of tolerance during chronic treatment (TFL); and 3) when compared to morphine has a superior therapeutic window, as determined by motor coordination and gastric transit tests in normal rats. In efforts to explain the above observations ¹¹, previous studies have suggested that unlike the pure μ -OR agonist morphine, M6S may act through both μ - and δ -ORs ^{8,12-14}. Although mixed μ / δ -agonists other than M6S also have demonstrated potent antinociceptive action and an improved tolerance profile when compared to morphine ¹⁵⁻¹⁸, M6S holds unique promise for future clinical applications because it is the easiest of the known mixed μ / δ -agonists to prepare from the readily available opium alkaloid, morphine ¹³. However, despite this promise, multiple preclinical studies with M6S remain to be conducted. In this respect, considering the current status of preclinical development of M6S, most studies conducted to date have only compared the

ability of morphine and M6S to relieve burning pain in the TFL test ^{8–11,14}; and the evidence that M6S might be also useful for treating multimodal pain comes from only a single study ¹¹. Furthermore, little or nothing is known concerning the progression of tolerance or cross-tolerance to M6S action, or the potential for development of opioid-induced hyperalgesia (OIH) in different pain assays following chronic administration. Finally, while an important role for δ -ORs in the mechanism of action of M6S for amelioration of superficial burning pain appears to be firmly established ¹⁴, potential involvement of δ -ORs in pain control by M6S involving other pain pathways has not yet been determined.

1.3. Objectives

The current study was designed to investigate the role of different OR subtypes in mediating the antinociceptive actions of M6S in several pain modalities. Three evoked pain modalities (evoked superficial burning, pricking, and deep tissue pressure pain; most frequently exaggerated evoked pain modalities in human subjects with diagnosed neuropathic pain ¹⁹) were assessed in rats following acute and chronic M6S treatment protocols.

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 (UAMS) and 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, MA) were housed three per cage with free access to food and water in the animal facility maintained by the UAMS Division of Laboratory Animal Medicine. Rats were randomly assigned to specific drug or vehicle treatment groups (6–12 rats per group).

2.2. Drugs

Drugs used in this study were: morphine hydrochloride (Merck & Co., Inc., Rahway, NJ); morphine-6-*O*-sulfate (sodium salt, prepared in our laboratory as described previously¹³); the δ -OR selective antagonist naltrindole hydrochloride (NLD; Tocris Biosciences, Minneapolis, MN); the κ -OR selective antagonist norbinaltorphimine hydrochloride (nor-BNI; a generous gift from Dr. Philip Portoghesi, University of Minnesota, Minneapolis, MN); and non-selective opioid antagonist naltrexone hydrochloride (NTX; Sigma Aldrich, St. Louis, MO). All drugs were prepared in physiological saline immediately prior to use. The volume of intraperitoneal (i.p.) injections for all animals was 2 mL/kg of rat body weight. The control group of animals received an equivalent volume of the vehicle only. Treatment of animals with OR antagonists was conducted as described previously. Drugs were injected i.p. 30 min (NTX 2 mg/kg) ^{14,20} (NLD 1 mg/kg) ^{14,21} or 18 hr (nor-BNI 5 mg/kg) ^{14,22} prior the time of peak antinociceptive effect of M6S or morphine.

2.3. Antinociceptive Assays

Antinociceptive testing was conducted between 10 AM and 5 PM. Animals were allowed to acclimate to the laboratory environment for at least 1 hr before the onset of testing. At least one training session was conducted to familiarize rats with the test procedures.

In all the experiments, the pain thresholds were recorded one hour before treatment (baseline) and then at 15- to 60-min intervals after drug injection. In dose-response studies, the same animals were used to test the effect of low-to-moderate doses (0.1–1.0 mg/kg) of morphine or M6S. Drugs were administered in an escalating sequence from low to higher doses with a 2-day washout period between treatments. A 2 day washout period has been shown to be sufficient for complete recovery from the effects of previously administered opioids in oral doses as high as 64 mg/kg ²³. Effects of higher doses (1.5–10 mg/kg) were studied using independent groups of 6 to 12 rats. Studies with animals treated with the 1.5 mg/kg dose were then continued utilizing the same daily dose in chronic, daily treatment studies.

2.3.1 Hot plate threshold assay—Hot plate threshold (HPT) was measured using an IITC Life Sciences incremental hot-plate apparatus (Model PE-34, Woodland Hills, CA) according to a previously described standard protocol ²⁴. During the test, the plate temperature was increased at a linear rate of 5 °C/min from 35 °C baseline to a cut-off temperature of 55 °C. The HPT was defined as the temperature at which the animal abruptly withdrew either hind paw from the plate and licked it. Test sessions continued at 10-min intervals until three determinations were made for each animal. The average of these three determinations was used for statistical analyses.

2.3.2. Pinprick sensitivity threshold assay—Pinprick sensitivity threshold (PST) was measured using a sewing needle (150 µm tip with a 30 degree angle) attached to a hand-held force transducer (FORT1000, World Precision Instruments, Inc. Sarasota, FL, USA). Force transducer-generated signals (pinprick force waveforms) were recorded using a differential amplifier (ISO-80, World Precision Instruments), digitized at 50 Hz sampling rate using a Micro 1401 analog-to-digital converter. Threshold recordings were analyzed using Signal-4 software (Cambridge Electronic Design, Cambridge, UK). Rats were placed in an elevated test cage with a wire mesh bottom and allowed to acclimate for 15 min. During a test session, six pinprick stimuli (trials) were administered to the central ventral aspect of each hind paw with care taken not to apply the probe to locations within the tested area more than once. The inter-trial rate was 2 to 6s. The PST force was defined as peak force in grams (g) that produced withdrawal of the hind paw, or a maximum 50 g cut-off value for trials in which animals failed to demonstrate a withdrawal response. PST readings (total of 12/rat/test) were filtered for outliers using the mean $\pm$ SD rule; remaining values were averaged and used for the analysis.

2.3.3. Paw pressure threshold assay—Paw pressure threshold (PPT) was measured with a Randall-Selitto analgesia meter as described previously ²⁴. Each test session consisted of three to five trials conducted at 5 minute intervals on both hind paws (total of 6 to 10 trials per rat). In each trial, pressure was applied to the center of the hind paw at a linearly increasing rate of 16 g/s until the animal struggled to withdraw, or succeeded in withdrawing its paw. The pressure cut-off was set at 250 g. The nociceptive pain thresholds (in mass units, g) were recorded and filtered for outliers using the mean $\pm$ SD rule; remaining values were averaged and used for the analysis.

2.4. Statistical analyses

All drug effects in this study are presented in the text and figures in absolute threshold change units, or as a percent of absolute peak of the antinociceptive effect (mean $\pm$ SEM). All data were checked for normality of distribution. Non-parametric statistical tests were used to statistically compare data that were not normally distributed. Normally distributed data were analyzed by using a two-way ANOVA followed by a *post hoc* Bonferroni test, or a one-way ANOVA followed by a *post hoc* Tukey test. The repeated measures ANOVA was used as appropriate (Graphpad Prism[®], 5.0). When a statistically significant between-factor interaction was detected by a two-way ANOVA the focus of the analysis was on results of post-hoc comparisons instead of main effects. In situations where no interactions were detected, no collapsing data over time or over drug concentration was made, because a priori we were interested in the analysis of the antinociceptive effect of each particular concentration of the drug and in each specific time point of treatment. When only two groups were compared, a Student's *t*-test was employed. Regression analysis and non-linear curve-fitting procedures were performed using the Origin 6.0 software package (MicroCal, Northampton, MA). Effects were considered as statistically significant at $p < 0.05$ corrected for the multiple comparisons using Tukey or Bonferroni correction (Graphpad Prism[®], 5.0).

Sample size justification and power analysis: The primary goal of this study was to establish whether M6S produces physiologically meaningful antinociception in a given pain test. We defined as physiologically meaningful an increase in pain threshold of 1.96SD (effect size = 1.96) from baseline: 1.2 °C, 44.8 g and 4.4 g in HPT, PPT and PST tests, respectively. Control population mean $\pm$ 1.96SD value is accepted as a cut off for the diagnosis of hyper- and hypoalgesia in human evoked pain studies¹⁹. Using G*Power 3.1 software²⁵ we estimated that with this effect size, six animals (number of animals per group in the most of experiments in our study) was sufficient to detect drug effects in any statistical test used in this study, at an α value of 0.05 with a power of 0.85 or greater.

3. Results

3.1. Hot-plate threshold (HPT) assay

Baseline HPT values averaged 46.7 ± 0.1 °C ($n = 91$). No statistically significant changes in HPT values were observed following vehicle treatment, indicating that repeated HPT testing was not associated with accommodation or sensitization (Fig. 1A, triangles). Treatment with either morphine or M6S resulted in statistically significant increases in HPT. However, the effect of M6S was greater (Fig. 1A) and lasted longer than that of morphine (Fig. 1B; asterisk).

3.1.1. HPT dose-response curves—For both morphine and M6S, over the dose range tested (0.1 to 5.6 mg/kg), dose-response relationships could be described by a single component sigmoidal curve. Comparison of these relationships shows that M6S was about 3 times more potent and 1.4 times more efficacious than morphine (Fig 1C). The 10 mg/kg dose of both M6S and morphine produced a much larger increase in HPT approaching the test cut-off value (3.5 °C), which was well above the best-fit maximum values determined by the sigmoidal curve fitting procedure (Fig 1C, at arrow). These observations suggest

existence of a second, low potency component of HPT analgesia produced by M6S and morphine. This component likely represents effects of sedation rather than activation of an additional antinociceptive pathway. Indeed, for both morphine and M6S the “sedative” component in the HPT test was observed at drug doses higher than 5.6 mg/kg, which are similar to ED₅₀ values for sedative effects reported for morphine on locomotor activity and rotarod tests in rats (5.8 to 7.4 mg/kg^{11,26,27}). Analysis of AUC values confirmed that M6S produces a greater total antinociceptive score than morphine in the HPT test (Fig. 1D).

3.1.2. Tolerance and cross-tolerance studies—In acute treatment experiments the peak effects of 1.5 mg/kg morphine or M6S on HPT did not differ (difference -0.36 ± 0.18 °C; 95%CI $-0.74, 0.01$ °C; 12 rats per group; $p = 0.0578$; unpaired t-test). Furthermore, at this concentration both drugs produced near maximal effects (84% for morphine and 90% for M6S of the respective maximum antinociceptive effects predicted by best-fit of dose-response relationships; Fig. 1C). Thus, these minimal, but efficacious, drug doses were used in the tolerance and cross-tolerance studies described below. Drugs were administered once daily, while HPT was measured before (baseline) and at 1–1.5 hr after injection (the approximate time of peak analgesia) on days 3, 5, 7 and 9 of treatment. Chronic treatment did not alter baseline HPT values in vehicle- and morphine-treated rats. Baseline HPT however, decreased by about 1 °C (~2.5%) in rats treated daily with M6S (Fig 2A, asterisks). In contrast to M6S, rats chronically treated with morphine developed antinociceptive tolerance in the HPT test (Fig 2B). Development of morphine/M6S cross-tolerance in the HPT test was not observed (Fig 2B, grey column).

3.1.3. Role of opioid receptors—Pre-treatment of rats with the relatively non-selective opioid receptor antagonist NTX completely antagonized the effects of morphine and M6S in the HPT test, demonstrating that both drugs acted *via* opioid receptors (data not shown). However, while pre-treatment with either the selective κ -opioid antagonist nor-BNI or the selective δ -opioid antagonist NLD did not diminish the antinociceptive effect of morphine in the HPT tests, pre-treatment with NLD, but not nor-BNI, reduced the antinociceptive effects of M6S by 45% (Fig. 2C; asterisk).

3.2. Paw pressure threshold (PPT) assay

The mean baseline value observed in the PPT test was 120 ± 9.6 g ($n = 122$). Time-profile studies revealed no significant differences between the antinociceptive effects of M6S and morphine (5.6 mg/kg) at any time point examined. For both drugs, antinociceptive effects peaked between 30 and 60 min, and then declined to below baseline values by 240 and 270 min after injection (Fig 3A). Analysis of vehicle-treated rats and vehicle-corrected M6S and morphine data suggested that this “hyperalgesia-like” effect occurring at the longer time points in the PPT test is likely due to potential “sensitization” or “learning” during the repeated test procedure (Fig. 3B).

3.2.1. PPT dose-response curves—Both morphine and M6S produced similar dose-related antinociception in the PPT test (Fig. 3C). As observed in the HPT assay, the highest dose of morphine and M6S tested (10 mg/kg) produced greater effects than the plateau observed for the 1.5 and 5.6 mg/kg doses of these compounds. As such, this dose was

excluded from the curve-fitting analysis (Fig 3C, arrow). As in the time-course and dose-response studies, morphine and M6S afforded similar total antinociceptive scores, as measured by AUC (Fig 3D).

3.2.2. Tolerance and cross-tolerance studies—For studies involving chronic treatment, baseline PPT values remained stable in vehicle-treated rats, but decreased by day 7 in rats chronically treated with either morphine or M6S (Fig. 4A; asterisks). Over the same time period, complete tolerance developed to the antinociceptive effects of morphine in the PPT test. In marked contrast, the antinociceptive effects of M6S did not differ between different days of the study (Fig 4B). In morphine/M6S cross-tolerance studies, less tolerance (50–60%; Fig. 4B, grey column) was observed with M6S in rats chronically treated with morphine, than to morphine in M6S-chronically treated animals.

3.2.3. Role of opioid receptors—Pre-treatment with the δ -selective opioid antagonist NLD (1 mg/kg) had no effect on M6S-induced antinociception in the PPT test when examined acutely, but decreased analgesia by $55 \pm 10\%$ in chronically treated animals (Fig 4C, asterisk). Pre-treatment with the κ -selective opioid antagonist nor-BNI (5 mg/kg, i.p.) was not tested in PPT experiments with M6S following acute administration. However, pre-treatment with nor-BNI had no effect on antinociception produced by M6S in the PPT test in chronic treatment studies (Fig. 4C).

3.3. Pinprick sensitivity threshold (PST) assay

The mean PST value measured at baseline was 10.2 ± 0.7 g ($n = 38$). Repeated testing in vehicle-treated rats did not change baseline PST (Fig. 5A, triangles). The effects of acute treatment with M6S and morphine (5.6 mg/kg) were markedly different. Morphine produced a weak and transient increase in PST (antinociception) followed by a distinct and prolonged phase of hypersensitivity/hyperalgesia (decrease in PST below baseline; Fig 5A, B, opened circles), whereas treatment with M6S resulted in antinociception only (Fig. 5A, B; closed circles).

3.3.1. PST dose-response curves—The analysis of dose-response relationships demonstrated that M6S was ~2-fold more potent and efficacious than morphine as an analgesic in the PST test (Fig 5C). Differences between the effect of M6S and morphine on PST were even more pronounced when total antinociceptive scores (AUC) were considered (Fig 5D). Furthermore, while the total antinociceptive effect of M6S as measured by the AUC could be fitted to a sigmoidal curve (Adjusted $R^2 = 0.998$), the antinociceptive and apparent pronociceptive effects of morphine were best fit by linear regression (Fig 5D). Furthermore, unlike what was observed in the HPT and PPT tests (see Figs. 2C and 4C; 10 mg/kg drug dose), no indication of a sedative component was observed in PST dose-relationship studies with either M6S or morphine (Fig. 5C).

3.3.2. Tolerance and cross-tolerance studies—For consistency with previously described chronic treatment studies, a 1.5 mg/kg dose of morphine and M6S was used for chronic drug administration in the PST studies. Baseline PST values did not change over the 7-day injection schedule in vehicle-treated animals, but decreased by approximately 20% in

both morphine- and M6S-treated rats (Fig 6A, asterisks). As observed acutely, morphine was ineffective in the PST test, while M6S maintained high efficacy throughout the entire 7- day chronic treatment period (Fig. 6B). Administration of M6S to morphine-tolerant rats (1.5mg/kg) resulted in an increase in PST that was not statistically different from that produced by the same dose of M6S in either the acute or the chronic M6S treatment studies (Fig 6B, column).

3.3.3. Role of opioid receptors—Pre-treatment with the selective δ -opioid antagonist NLD (1 mg/kg, i.p.), decreased the antinociceptive effect of M6S in the PST test by about 95% (Fig 6C asterisk). In contrast, pre-treatment with the selective κ -opioid antagonist, nor-BNI, did not alter antinociception produced by M6S in this pain assay (Fig 6C).

4. Discussion

This study is the first to directly compare antinociception produced by M6S and morphine in three distinct evoked pain modalities (superficial burning, pricking and deep pressure pain). Among others, these three evoked pain modalities are most frequently exaggerated in human subjects with neuropathy and therefore are of specific interest with regard for development of novel drug treatments¹⁹. With regard to end-points measured and neuronal circuitry involved in each test, PST and PPT are thresholds of simple withdrawal reactions, evoking a minimal supraspinal involvement. In this respect, PST and PPT most resemble thresholds to pinprick and blunt pressure pain in humans^{19,28}. In contrast, the HPT test uses paw withdrawal and licking as end-points, evokes much greater supraspinal involvement, and is more representative of heat pain tolerance than threshold detection (see²⁹).

A summary of results from this study is presented in Table 1. The effects of morphine and M6S were compared using six different categories of potential clinical relevance: (1) potency, (2) efficacy, (3) duration of action, (4) development of tolerance, (5) development of acute hyperalgesia, and (6) development of chronic opioid-induced hyperalgesia (OIH). Based on the data presented, an arbitrary “superiority score” was calculated for each pain modality, and is presented as a fraction of categories listed above in which M6S exhibited “more desirable” characteristics than morphine, compared to the total number of categories examined. “More desirable” was defined as traits that would be expected to confer clinical advantage, such as greater potency, longer duration of action and higher efficacy, and lower degree of OIH or tolerance (see Table 1, last column). Additionally, information concerning the efficacy of M6S as a potential rotation drug in morphine-tolerant subjects (cross-tolerance) and estimates of the involvement of δ -OR-mediated effects in M6S-produced analgesia are listed for each pain modality.

In support of the reports of pain pathway/modality-specific action of analgesic drugs^{4,30,31}, data from the current study demonstrate distinct antinociceptive effects for both M6S and morphine across the pain modalities tested. However, in each test, M6S scored better than morphine with the rank-order of analgesic superiority scores for M6S versus morphine being PST > HPT > PPT (80%, 50% and 17%, respectively; see Table 1). Strikingly, this rank-order correlated well with the involvement of the δ -OR in M6S action in these tests, i.e. PST > HPT > PPT (95%, 55% and 5%, respectively). Furthermore, the “recovery from tolerance”

in the PPT test in rats treated chronically with M6S (Fig. 4B) also occurred in apparent association with an increase in the involvement of δ -ORs in the antinociceptive effect of M6S (from 5% to about 55% on day 1 and day 7 of treatment). Nevertheless, we acknowledge that further research is needed to assess whether tolerance to the antinociceptive effect of M6S in the PPT tests develops during first days of chronic treatment. These correlations agree with observations that morphine acts primarily as a pure μ -OR agonist at clinically relevant concentrations³², while analgesia produced by M6S appears to involve activation of both μ - and δ -ORs^{8,9,13,14}. Indeed, co-activation of μ - and δ -ORs has been reported to enhance the analgesic/antinociceptive efficacy of opioids *in vivo*^{15,16,18,33}. Furthermore, recruitment of δ -ORs to the neuronal membrane surface has been reported to enhance analgesic efficacy of δ -OR agonists in animals chronically treated with μ -OR agonists^{34–36}. Finally, analgesic tolerance develops more slowly following chronic treatment with mixed μ/δ -agonists when compared to that following chronic morphine treatment^{15,16,18}.

In addition to the development of tolerance, acute and chronic OIH constitutes another important limitation for the chronic use of opioids³⁷. We observed the development of acute OIH in the PST test in morphine-, but not in M6S-treated rats (Fig. 5A,B). Chronic OIH (steady decline in baseline pain threshold), however, was a more frequently observed phenomenon in our experiments. Development of chronic OIH was observed in the HPT test during chronic treatment with M6S (Fig. 2A) and in the PST and PPT tests during chronic treatment with either morphine or M6S (Fig. 4A and 6A). The mechanisms responsible for the development of OIH remain unclear³⁷. Nonetheless, our data suggest that mixed μ/δ -OR agonists are unlikely to offer distinct advantages over μ -OR agonists with respect to development of chronic OIH. Another important observation reported in this current study is that for every pain modality examined, tolerance, or the lack thereof, was observed regardless of the presence or absence of either acute or chronic OIH. This dissociation is not in accord with the hypothesis that OIH and tolerance to opioids are inter-linked phenomena³⁸. Furthermore, it suggests that different mechanisms may underlie acute and chronic OIH.

Differences in antinociceptive potency and efficacy of opioids in controlling various pain modalities may be associated with the differential involvement of specific subtypes of ORs in modulating the activity of a given pain pathway^{4,30,31}.

In agreement with studies reported in κ -OR knockout mice³⁹, results from the current studies using the κ -OR antagonist nor-BNI suggest these receptors play no role in the control of somatic pain sensations (PST, PPT and HPT). In contrast, with regard to δ -ORs, our results suggest these receptors are critical for controlling superficial pricking, moderate for superficial burning, and minor for deep pressure evoked pain.

Our finding that the PST pathway in rats is almost under exclusive control by the δ -OR system is in agreement with reports that mouse skin mechanoreceptors express δ - but not μ -ORs, and that the mouse paw von Frey filament withdrawal threshold is sensitive to δ - but not μ -OR-selective agonists^{40,41}. This finding is also in agreement with observations that

pinprick hyperalgesia in humans examined in experimental skin inflammation and post-surgical pain models respond poorly to μ -OR agonists^{42–44}.

Similarly, the finding that the selective δ -opioid antagonist NLD blocks about 50% of M6S-mediated antinociception in the HPT test (Fig. 2C) agrees with previous studies demonstrating equivalent involvement of δ - and μ -ORs in the control of pain associated with radiant heat in the TFL test^{14,16,18,45}. However, reports that TFL in mice is sensitive to μ -, but not δ -OR-selective agonists should be noted^{40,41}. Thus, additional studies will be required to clearly determine the relative role of different OR systems in burning pain tests (see also⁴⁶).

Deep aching pain is associated with activation of C- or A δ (small non-myelinated and thinly myelinated fibers, respectively) muscle afferents. These afferents serve both as ergo- and nociceptors⁴⁷. Studies of the rat exercise pressor reflex (homeostatic control, ergo-receptor function) suggest that muscle C- and A δ -afferents express μ - and δ -ORs^{48,49}, respectively. The current study failed to detect substantial input of δ -ORs for control of pain in the PPT test in normal rats (Fig. 4C). The central role of μ -ORs in the control of deep muscle pain is also supported by studies on the association of human OR gene polymorphisms with PPT⁵⁰, and by observations of the involvement of μ -OR pathways in fibromyalgia⁵¹. However, our data also suggest that during chronic treatment with M6S, the involvement of δ -ORs in controlling pain measured in the PPT test may increase from 5% to 60% over time (Fig. 4B, C). These observations agree with reports that chronic activation of μ -ORs (by M6S in the current studies) leads to recruitment of δ -ORs to the neuronal membrane^{34–36}. Thus, it is possible that while in acute treatment experiments M6S acts in the PPT test via μ -ORs only, after a few days of chronic treatment in the same test, M6S may realize its potential as a mixed μ/δ -OR agonist. This could explain the observed “recovery” from tolerance in the current study of daily treatment with M6S in rats in the PPT test (Fig. 4B). Tolerance is delayed in response to mixed μ/δ -OR agonists when compared to μ -OR agonists^{15,16,18}.

Use of multiple comparisons and relatively small sample size in many of experiments in this study constitute a potential limitation for interpretation of the results obtained, and call for further evaluation of M6S and mechanisms involved in its antinociceptive effects. However, while perhaps providing additional important information, it is our belief that such future studies will not considerably alter the significance or the main conclusions derived from the current study.

5. Conclusions

This study is the first to demonstrate that M6S exhibits a number of properties which indicate that this morphine derivative is likely superior to morphine for treatment of pain. This conclusion is based on the increased antinociceptive potency, efficacy, and duration of action, and the improved tolerance profile of M6S when compared to morphine. Thus, M6S has potential as a safe and efficacious alternative to traditional μ -OR agonists such as morphine for treatment of both acute and chronic multimodal pain. Furthermore, M6S may be a good candidate for opioid rotation in morphine-tolerant subjects. The potential ability of M6S to act as a mixed μ/δ -OR agonist appears to be a likely key determinant of

differences in antinociception produced by M6S and morphine in the pain modalities examined in this current study.

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Fig. 1. Time profiles, dose response relationships and total antinociception scores of morphine and M6S in the HPT assay (acute treatment)

A – Time course of analgesic effects of morphine (open circles and dashed lines), M6S (closed circles and solid lines) in the HPT assay at a dose of 5.6 mg/kg. Repeated testing of vehicle-treated rats (solid triangles and solid line) did not effect HPT thresholds (One way RM ANOVA, $F(4,29) = 0.995$; $p=0.433$). A two-way RM ANOVA demonstrated statistically significant ($p < 0.01$) main effects of both drug treatment ($F(1,30) = 12.63$) and time ($F(3,30) = 93.1$, $n = 6$ rats per group). However, a significant interaction between factors was also detected. Nonetheless, the Bonferroni post hoc test confirmed that the antinociceptive effect of M6S was greater than that of morphine at 1, 3, and 5 hrs post-injection (asterisks, $p < 0.01$). Mean morphine/M6S effect differences were, in $^\circ\text{C}$, -0.57 (95%CI: 1.1, 2.1), -0.8 (95%CI: 1.3, 2.3), -0.75 (95%CI: 0.2, 1.2) and 0.2 (95%CI: -0.3 , 0.7) $^\circ\text{C}$ for 1, 3, 5 and 7 hr time points, respectively).

B - In each set of experiments data were corrected for HPT changes observed in vehicle-treated rats and normalized to respective maximum effect. A two-way ANOVA does not reveal a significant interaction or overall effect of treatment (morphine vs. M6S; $F(1,10) = 3.05$; $p = 0.111$); however at the 5 hr time point morphine and M6S mean effect data are different at $p < 0.05$ by Bonferroni post-test (asterisk; $n=6$ rats per group; drug effect differences, in %, were: 0.01 (95%CI: $-0.28, 0.31$), -0.17 (95%CI: $-0.47, 0.13$), -0.45 (95%CI: $-0.75, -0.15$) and 0.14 (95%CI: $-0.16, 0.44$) % for 1, 3, 5 and 7 hr time points, respectively).

C - Dose-response relationships for the analgesic effects of M6S (solid circles and lines) and morphine (open circles and dashed lines) in the HPT assay. Sigmoidal curves for both the drugs were fitted using best curve fit procedures and a Boltzman sigmoidal function (note: logarithmic dose scale). The best-fit parameters for M6S are $ED_{50} = 0.35 \pm 0.07$ mg/kg (95%CI: $0.16, 0.54$ mg/kg) and efficacy = 1.4 ± 0.1 °C (95%CI: $1.07, 1.70$ °C; fit adjusted $R^2 = 0.902$). The best-fit parameters for morphine are $ED_{50} = 0.99 \pm 0.03$ mg/kg (95%CI: $0.92, 1.08$ mg/kg) and efficacy = 0.98 ± 0.01 °C (95%CI: $0.94, 1.07$ °C; adjusted $R^2 = 0.998$). Horizontal dashed lines represent the baseline (set to zero across the tests) and maximum effect possible in the test. Arrow indicates effect of 10 mg/kg of morphine and M6S, data points excluded from the fit procedures above ($n = 6$ to 12 rats per group; the legend as in panel B; Note Y-axis break at 2.5 °C HPT value).

D - Total antinociceptive score (area under the curve, AUC) for morphine (light grey bars) and M6S (dark grey bars) across 0.5–5.6 mg/kg drug dose ranges in HPT pain assays; Asterisks indicate statistical significant difference of adjacent data sets (two-way RM ANOVA, main effect of treatment, $F(1, 30) = 33.4$; $p < 0.01$; main effect of dose, $F(3, 30) = 10.4$; $p < 0.01$; no significant interaction between the factors was observed; 6 rats per group; drug effect differences (CI of difference) were 3.64 (95%CI: $1.04, 6.25$), 3.04 (95%CI: $0.44, 5.64$), 3.01 (95%CI: $0.41, 5.61$) and 3.51 (95%CI: $0.90, 6.11$) AUC units for 0.5, 1.5, 3 and 5.6 mg/kg drug dose, respectively (Bonferroni test).

Fig. 2. Chronic treatment effects of morphine and M6S; and effects of δ - and κ -OR-specific antagonists on M6S antinociception in the HPT assay

A: Baseline thresholds are expressed as percentages of respective first day baseline HPT values in rats chronically treated with vehicle, morphine and M6S (open, light and dark grey bars, respectively). Two way RM ANOVA detected statistically significant ($p < 0.05$) main effects of treatment, time and interaction between main effects. However, Bonferroni post-hoc test detected a statistically significant difference between day 1 vs. day 3, 7 or 9 baseline HPT in M6S-treated rats only ($p < 0.01$; 6 rats per group). Compared to the effect of M6S produced on day 1, effects measured on day 3, 7 and 9 were weaker by -0.98 (95%CI: $-1.90, -0.07$), -1.05 (95%CI: $-1.96, -0.14$) and -1.27 (95%CI: $-2.18, -0.35$) °C of the HPT change, respectively. In morphine- or vehicle- treated rats differences of baseline HPT on different days of the study never exceeded 0.38 °C (95%CI $-1.49, 0.74$; day 1 vs. day 3 baseline HPT difference observed in vehicle-treated rats)

B: Relative (% of the 1st day) analgesic effects of M6S (solid circles and solid lines) and morphine (open circles and dotted lines) on HPT during chronic daily treatment protocol. Two way RM ANOVA detected statistically significant ($p < 0.05$) main effects of treatment, time and interaction between main effects. However, statistically significant ($p < 0.01$, asterisks) development of tolerance to the antinociceptive effect was observed in morphine- but not M6S- treated rats (Bonferroni post hoc test; mean differences of effects of morphine on day 1 vs day 3, 7 and 9 were (in %) -43.85 (95%CI: $-109.1, 21.4$), -85.1 (95%CI: $-150.3, -19.80$) and -82.2 (95%CI: $-147.5, -16.9$). For M6S, the largest difference in the drug effects on HPT was observed between days 1 and day 9 of treatment (17.3% with 95%CI: $-48.0, 82.5$). Treatment of morphine-tolerant rats with 1.5 mg/kg M6S (dark grey bar) resulted an antinociceptive effect of M6S that was indistinguishable from that observed in either acute or chronic M6S-HPT studies (one-way ANOVA, $F(2,17) = 0.0306$, $p = 0.969$; 6 rats per group). No differences in M6S effects on HPT (in °C of HPT change) were

detected when morphine/M6S-replacement and acute M6S treatment groups of animals were compared (95%CI: -0.61, 0.61) and this difference constituted just 0.05 °C (95%CI: -0.66, 0.56) for morphine/M6S-replacement and chronic M6S treatment groups (Tukey's multiple comparison post hoc test).

C. Percent maximum effect on HPT for morphine and M6S alone or after pre-treatment with δ - or κ -OR-specific antagonists naltrindole (NLD, 1mg/kg, i.p.) or norbinaltorphimine (nor-BNI, 5mg/kg, i.p.). Asterisks indicates statistically significant difference between M6S and M6S +NLD rat groups (one-way ANOVA, $F(3,23) = 9.353$; $p = 0.0005$) The mean difference of relative effects of M6S given alone or after pre-treatment with NLD was 57% (95%CI: 23.9, 91.0%; $p < 0.01$) the same value for morphine was 10% (95%CI: -24.0, 43.1%; $p > 0.05$; post hoc Tukey test; 6 rats per group).

Fig. 3. Time profiles, dose response relationships and total antinociception scores of morphine and M6S in the PPT assay (acute treatment)

A – Time course of analgesic effects of morphine, open circles and dashed lines), morphine-6-*O*-sulfate (M6S, closed circles and solid lines) in the PPT assay at a dose of 5.6 mg/kg. Repeated testing of vehicle-treated rats (solid triangles and solid line) resulted in a steady decline in PPT (RM ANOVA, $F(5,29) = 4.392$; $p=0.0027$) with PPT measured at 180 and 270 min time points being statistically lower than baseline PPT ($p<0.05$; Tukey test). A two-way RM ANOVA detected significant effect of time ($F(6,60) = 34.34$; $p<0.01$) but no effect of treatment or time x treatment interactions. The largest difference in the effects of morphine and M6S on PPT (22.3 g greater effect in M6S vs morphine-treated rats) was observed at the 2-hr time point, but was not statistically significantly different from zero (95%CI: -28.3, 73.0; Bonferroi post-test; 6 rats per group).

B – Comparison of M6S and morphine analgesic time-profiles after the data presented in panel A were corrected for PPT changes observed in vehicle-treated rats and normalized to respective maximum effect.

C - Dose-response relationships for the analgesic effects of M6S (solid circles and lines) and morphine (open circles and dashed lines) in the PPT assay. Best fit parameters of sigmoidal functions describing the dose-response data were similar for both drugs: for morphine $ED_{50} = 1.4 \pm 0.1$ mg/kg (95% CI 0.8, 2.1 mg/kg) and $E_{max} = 72 \pm 7$ g (95% CI 43, 102g; adjusted $R^2 = 0.963$ and for M6S $ED_{50} = 1.2 \pm 0.1$ mg/kg (95% CI 0.9, 1.5 mg/kg) and $E_{max} = 70 \pm 6$ g (95% CI 53, 88 g; adjusted $R^2 = 0.951$). Horizontal dashed lines represent the baseline (set to zero across the tests) and maximum effect possible on the test. An arrow indicates effect of 10 mg/kg of morphine and M6S, data points excluded from the fit procedures above ($n = 6-12$ rats per group).

D - Total antinociceptive score (area under the curve, AUC) for morphine (light grey bars) and M6S (dark grey bars) did not differ across various dose ranges in the PPT assay. A two-way ANOVA detected a significant main effect of drug dose, $F(3, 40) = 23.58$; $p < 0.01$, but not of treatment and no treatment $\times$ drug dose interactions (6–12 rats per group). The largest difference in effects of morphine and M6S on PPT was observed at the 1.5 mg/kg dose, but this was not statistically significantly different from zero (95% CI: -14830, 2333; Bonferroi post hoc post-test).

Fig. 4. Chronic treatment effects of morphine and M6S, and effect of δ - and κ -OR-specific antagonists on M6S antinociception in the PPT assay

A: Baseline thresholds expressed as percentages of respective first day baseline PPT values in rats chronically treated with vehicle, morphine or M6S (open, light and dark grey bars, respectively). Two-way RM ANOVA detected main effect of time; $F(3, 66) = 5.44$, $P = 0.002$, but no interaction between the factors or the significant effects of treatment were observed. Nonetheless the baseline PPT in rats chronically treated with morphine and M6S was statistically lower on day 7 than on day 1 (asterisks, Bonferroni post hoc test; $p < 0.01$; 6 rats per group). This difference constituted -15.5% (95%CI: $-32.7, 0.86$) in morphine-treated rats and -18.4% (95%CI: $-34.7, -2.1$) in M6S-treated rats.

B: Relative (% of the 1st day value) analgesic effects of M6S (solid circles and solid lines) and morphine (open circles and dotted lines) on PPT during chronic daily treatment protocol. A two-way RM ANOVA revealed a significant ($p < 0.05$) effect of time and time x treatment interaction. A Bonferroni post hoc test detected decreased antinociception on days 5 and 7 (compared to day 1 respective mean differences were -34.2% (95%CI: $-63, -5.2$) and -38.9% (95%CI: $-67.9, -9.9$), asterisks; $p < 0.05$) in rats treated with morphine, but not with M6S. In the latter group, the largest relative decline in the antinociceptive action of M6S was observed on day 3 of experiment, but did not reach statistical significance: -23.0% (95%CI $-52.0, 5.9$). Treatment of morphine-tolerant rats with 1.5 mg/kg M6S (dark grey bar) resulted in weakened (50–60% of observed in experiments with either acute or day 7 chronic treatment) antinociceptive effect of M6S. This decrease however was not statistically significant; Kruskal-Wallis test, followed by Dunn's multiple comparison post hoc test; 12 rats per group).

C. Percent maximum effect on PPT for M6S alone or after pre-treatment with δ - or κ -OR-specific antagonists: naltrindole (NLD, 1mg/kg, i.p.) or norbinaltorphimine (nor-BNI, 5mg/kg, i.p.). Asterisks indicates statistically significant difference between M6S and M6S + NLD rat groups (one-way ANOVA, $F(3,23) = 9.353$; $p = 0.0005$; post hoc Tukey; $p < 0.001$; 6 rats per group). Pre-treatment with NLD decreased antinociceptive effect of M6S in PPT test by 33.6 g (95%CI: 1.78, 65.4).

Fig. 5. Comparison of time profiles, dose response relationships and total antinociception scores of morphine and M6S in the PST assay (acute treatment)

A – Time course of analgesic effects of morphine (open circles and dashed lines), M6S (closed circles and solid lines) in the PST assay at a dose of 5.6 mg/kg. Repeated testing of vehicle-treated rats (solid triangles and solid lines) did not effect PST. The antinociceptive effect of M6S was significantly stronger than that of morphine (6 rats per each group).

Furthermore, an acute phase of hyperalgesia developed after morphine, but not M6S treatment. A two-way (treatment x time) RM ANOVA confirmed a statistical significance of time and treatment as well as time x treatment interaction. The Bonferroni post hoc test for multiple comparisons confirmed the effects of M6S treatment being significantly different from those of morphine treatment at 30, 45, 60 and 120 min of experimentation ($p < 0.05$; asterisks). Mean morphine/M6S effect differences at these time points were (in grams of PST change), respectively: -33.9 (95% CI: $-40.7, -27.0$), -36.5 (95% CI: $-43.3, -30.0$), -37.3 (95% CI: $-44.1, -30.5$) and -10.0 (95% CI: $-16.9, -3.2$). At other time points studied,

differences never exceeded -5.2 g PST change value (95%CI $-12.1, 1.6$; 15 min post-treatment).

B - In each set of experiments, data were corrected for PST changes observed in vehicle-treated rats and normalized to the respective maximum effect. The Bonferroni post hoc test of normalized profiles detected significant differences at all time points studied except for the 4- and 5-hr time points (asterisks, at 4- and 5-hr time points mean drug effect differences were -0.31 g (95%CI: $-0.77, 0.15$) and -0.14 g (95%CI: $-0.60, 0.32$))

C - Dose-response relationships for the analgesic effects of M6S (solid circles and lines) and morphine (open circles and dashed lines) in the PST assay. The best-fit parameters for M6S are $ED_{50} = 2.3 \pm 0.1$ mg/kg (95%CI: $1.8, 2.7$ mg/kg) and efficacy = 40 ± 1 g (95%CI: $36, 44$ g; fit adjusted $R^2 = 0.991$). The best-fit parameters for morphine were $ED_{50} = 5.1 \pm 0.4$ mg/kg (95%CI: $3.8, 6.4$ mg/kg) and efficacy = 24 ± 2 g (95%CI: $19, 30$ g; adjusted $R^2 = 0.982$). Horizontal dashed lines represent the baseline (set to zero across the tests) and maximum effect possible on the test ($n = 6$ rats per group).

D - Dose-response relationships for total antinociceptive score (area under the curve, AUC) for M6S (closed circles) and AUC for antinociceptive (morphine analgesia, opened circles) and pro-nociceptive phases (morphine hyperalgesia, open squares) of morphine's acute effects on PST. The ED_{50} and E_{max} of M6S action were 2.3 ± 0.1 mg/kg and 3336 ± 134 g*hr (best fit of sigmoidal function parameters; adjusted $R^2 = 0.998$). Best fit of dose-response relationships for antinociceptive and hyperalgesic effects of morphine predicted very similar slopes of fitted lines: 81 ± 11 g*hr (95%CI $47, 115$ g*hr; adjusted $R^2 = 0.933$) and 101 ± 10 g*hr (95%CI $69, 133$ g*hr; adjusted $R^2 = 0.961$). Intercept was fixed to zero in both cases.

Fig. 6. Chronic treatment effects of morphine, and M6S, and the effect of δ - and κ -OR- specific antagonist on M6S antinociception in the PST assay

A: Baseline thresholds expressed as percentages of respective first day baseline PST values in rats chronically treated with vehicle, morphine and M6S (open, light and dark grey bars, respectively). Asterisks indicate a statistically significant difference of day 1 vs. day 3 and 7 baseline PST in M6S- and morphine-treated rats (two way RM ANOVA, main effect of time; $F(2, 30) = 8.54$, $P = 0.001$); followed by Bonferroni test; $p < 0.05$; 6 rats per group). Day 3 vs day 1 drug baseline PST differences constituted -1.93 g (morphine; 95%CI: $-3.92, 0.06$) and -1.37 g (M6S; 95%CI: $-3.36, 0.56$) and day 7 vs day 1 differences were -1.90 g (morphine; 95%CI: $-3.89, 0.09$) and -2.25 g (M6S; 95%CI: $-4.24, -0.26$).

B: Absolute (g of PST change) analgesic effects of 1.5 mg/kg M6S (solid circles and solid lines) and morphine (open circles and dotted lines) on PST during chronic daily treatment protocol. Antinociceptive efficacy of M6S is not different between 1st, 3rd and 7th days of chronic treatment (one way RM ANOVA, $F(2,17) = 1.861$; $P = 0.2804$). By Tukey post hoc test day 1 vs 3 and day 1 vs 7 mean differences of the effect of M6S on PST were not different from zero: -4.2 g (95%CI: $-10.3, 1.8$) and -2.8 (95%CI: $-8.9, 3.3$), respectively. The antinociceptive effect of M6S administered to rats chronically treated with morphine was not statistically different from its effects produced on any of the days studied in chronic M6S treatment experiments (dark grey bar, compare to data presented by filled circles; one-way RM ANOVA; $F(3,23) = 2.539$; $P = 0.096$; mean effect differences never exceeded 4.3 g (95%CI: $-1.1, 9.8$), the value observed when action of M6S in morphine-tolerant rats was compared to its action on day 3 of chronic treatment).

C. Percent maximum effect on PST for morphine and M6S alone or after pre-treatment with δ - or κ -OR-specific antagonists: naltrindole (NLD, 1 mg/kg, i.p.) or norbinaltorphimine (nor-BNI, 5 mg/kg, i.p.). Asterisks indicates statistically significant difference between M6S and M6S +NLD rat groups (one way ANOVA $F(2,17) = 56.55$; $P < 0.01$; post hoc Tukey test $p < 0.01$; 6 rats per group). Mean effect differences from control were 95.8 % (95%CI: $70.4, 121.3$) for M6S+NLD and 12.5 % (95%CI: $-13.0, 38.0$) for M6S+NBNI group of rats.

Table 1
Comparison of analgesic and safety performance of morphine and M6S across different evoked pain domains

Test (pain modality)	Parameter	Morphine	M6S	Score	Total Score
HPT (superficial burning); δ -OR fraction, $55 \pm 4\%$	1. Potency (ED_{50} , mg/kg)	0.99 ± 0.03	0.35 ± 0.07	+1	$3/6 = 0.50$
	2. Duration of action [*] , min	250 ± 13	332 ± 9	+1	
	3. Efficacy (E_{max} , °C)	0.98 ± 0.01	1.4 ± 0.1	+1	
	4. Hyperalgesia, acute ^{**}	None	None	0	
	5. Hyperalgesia, chronic ^{***}	None	$-2.7 \pm 0.8\%$	-1	
	6. Development of tolerance ^{***}	$-86 \pm 16\%$	None	+1	
PST (superficial pricking); δ -OR fraction, $94 \pm 4\%$	Cross-tolerance with morphine ^{****}		None		$4/5 = 0.80$
	1. Potency (ED_{50} , mg/kg)	5.1 ± 0.4	2.3 ± 0.1	+1	
	2. Duration of action, min	41 ± 7	105 ± 4	+1	
	3. Efficacy (E_{max} , g)	24 ± 2	40 ± 1	+1	
	4. Hyperalgesia, acute	$-47 \pm 12\%$	None	+1	
	5. Hyperalgesia, chronic	$-17 \pm 4\%$	$-20 \pm 5\%$	0	
PPT (deep tissue pain); δ -OR fraction, $5 \pm 17\%$ / $51 \pm 14\%$ (acute / chronic treatment)	6. Development of tolerance	?	None	?	$1/6 = 0.17$
	Cross-tolerance with morphine		?		
	1. Potency (ED_{50} , mg/kg)	1.4 ± 0.1	1.2 ± 0.1	0	
	2. Duration of action, min	154 ± 24	225 ± 30	0	
	3. Efficacy (E_{max} , g)	72 ± 7	70 ± 6	0	
	4. Hyperalgesia, acute	None	None	0	
	5. Hyperalgesia, chronic	$-12 \pm 4\%$	$-13 \pm 6\%$	0	
	6. Development of tolerance	$-83 \pm 23\%$	None	+1	
	Cross-tolerance with morphine		$-36 \pm 18\%$		

^{*} defined as time interval during which the drug effect is equal or exceeds 25% of respective peak antinociceptive effect

^{**} defined as a decrease in pain threshold below baseline level. Peak values of acute OIH expressed as % of maximal antinociceptive effect are shown. If no acute OIH was observed, "None" is stated

^{***} Chronic OIH and tolerance are defined, respectively, as a decrease of baseline pain threshold or peak antinociceptive drug effect measured on day 7 of treatment and expressed relative to respective day 1 values. Only statistically significant percent change values are shown, otherwise "None" is stated.

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defined as a decrease in M6S antinociceptive action after chronic treatment with morphine (relative to M9S action in acute experiments).

? – tested at 1.5 mg/kg morphine dose, which does not produce a significant effect in the PST test; therefore development of tolerance to morphine and morphine/M6S cross-tolerance remain to be determined at higher morphine concentrations.