Antinociceptive effects and toxicity of morphine-6-O-sulfate sodium salt in rat models of pain

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Abstract

Mu- opioids (i.e. morphine, oxycodone, hydrocodone) are considered to be the primary drugs for treatment of moderate to severe acute, chronic and cancer pain. Despite their analgesic effectiveness they have several clinically significant side-effects (cognitive, motor, respiratory, cardiovascular, gastrointestinal). They also have a limited spectrum of action, being more effective for nociceptive than neuropathic pain. In an effort to identify other opioid analgesics with greater effectiveness in mixed pain states and with a better side-effect profile compared to the classical mu- opioid agonist, morphine, a relatively little-known morphine derivative, morphine-6-O-sulfate, was characterized using a range of well-established rodent pain models. The present data demonstrated that morphine-6-O-sulfate was efficacious after several routes of administration, including neuroaxial (intrathecal), parenteral (intraperitoneal) and oral in the rat. It showed potent, dose-related, analgesic activities against acute nociceptive pain (the tail flick test), neuropathic pain (chronic constriction nerve injury hyperalgesia and allodynia) and inflammatory pain (formalin test). It had a good separation based on dose (at least 10-fold) between side-effects (incoordination, hypolocomotion, inhibition of gastrointestinal motility) and analgesia in all models of pain tested. In addition, morphine-6-O-sulfate had a more favorable potency ratio for delay of gastrointestinal transit and analgesia when compared to morphine. These preclinical findings suggest that morphine-6-O-sulfate is a potential candidate for development as a novel opioid for management of nociceptive, neuropathic and mixed pain states.

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neuropathic and inflammatory pain (Petrillo et al., 2003). The above promising findings lead us to further characterize morphine-6-O-sulfate with regard to its analgesic effect, in particular in neuropathic pain, and its toxicity.

The purpose of the present study in the rat was three-fold: 1) to determine if morphine-6-O-sulfate had antinociceptive activity after acute neuroaxial, parenteral and oral routes of administration, as well as to determine if tolerance develops with repeated doses in the tail-flick test; 2) to determine if morphine-6-O-sulfate had effectiveness in chronic pain; and specifically, if the drug was able to attenuate enhanced pain sensitivity (hyperalgesia, allodynia) in a chronic constriction nerve injury model of neuropathy and to inhibit flinching behavior in a formalin model of inflammatory pain; 3) to determine the morphine-6-O-sulfate side-effect profile by observing gastrointestinal motility (charcoal transit), coordination (rotarod) and locomotor activity. Findings on morphine-6-O-sulfate were compared to results using morphine as a positive control in some experiments.

2. Materials and methods

2.1. Animals

Male Sprague Dawley rats, approximately 90 days old, weighing about 350 g (Harlan, Indianapolis, IN), were housed individually in transparent cages with a sawdust-covered floor in a humidity- and temperature-controlled AAALAC-accredited facility (a 12 h alternative light/dark cycle) with free access to standard laboratory chow and tap water. Rats were fasted overnight for experiments requiring oral dosing. Rats were allowed to habituate to the housing facility for at least one week. Next, they were familiarized with the experimental environment/apparatus (three times) before the experiments were begun. On each day, rats were habituated to the experimental room for at least 30 min prior to testing. All experiments were conducted during the light phase of the cycle. Body weights were determined on the day of experimentation. All surgical procedures were performed under sterile conditions and pentobarbital sodium (40 mg/kg intraperitoneal, i.p.) anesthesia. At the end of the experiment, rats were euthanized with a pentobarbital overdose (150 mg/kg i.p.). A cross-over paradigm was used within an experiment (if possible) to minimize the number of rats. All experiments were performed according with a protocol approved by the University of Kentucky Animal Care and Use Committee (IACUC) and carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Publication No 85-23, revised 1983).

2.2. Drugs

Morphine-6-O-sulfate sodium was provided by Dr. P. Crooks (University of Kentucky, College of Pharmacy Lexington, KY). Morphine sulfate was purchased from Mallincrodt (St. Louis, MO). Drugs were dissolved in 0.9% saline and administered by the neuroaxial (intrathecal, i.t., 10 μg/rat), parenteral (i.p., 1 ml/kg) and oral (p.o., 1 ml/kg) routes in the rat. Saline served as a control.

2.3. Acute nociception (the tail flick test)

The antinociceptive effect was determined by the responsiveness to radiant heat in the tail-flick test (D’Amour and Smith, 1941). Tail flick latency (TFL, s) was measured by recording the time from the onset of heat stimulus to the tail to withdrawal of the tail from the heat source using a standard tail-flick apparatus (IITTA Life Science, Woodland Hills, CA). Heat intensity was adjusted to yield average baseline TFL equal to 2–3 s (cut-off time, 10 s). Responsiveness (TFL) was measured prior to (twice, 15 min apart) and at several time points after administration (until no significant antinociception was observed).

Four groups of rats were used to characterize the antinociceptive properties of morphine-6-O-sulfate in the tail flick test. 1) Rats were treated with morphine-6-O-sulfate (0.25, 0.5, 1, 1.5 mg/kg) administered by the i.p. route (randomized doses, weekly intervals). 2) Rats were treated with morphine-6-O-sulfate (1, 5, 10, 15, 20 mg/kg) administered orally with help of gavage feeding needle after overnight fasting (randomized doses; weekly intervals). 3) Rats were subjected to chronic catheterization of the spinal subarachnoid space performed according to Yaksh and Rudy (1976) with modification. Briefly, a 21 cm long P-10 polyethylene tubing (10 μl) which extended 8.5 cm beyond an incision in the atlanto-occipital membrane was secured to the skull with acrylic cement. The catheter tip rested in the vicinity of T-12 at the rostral face of the lumbar cord enlargement. One week after surgery, rats were administered with morphine-6-O-sulfate (0.125, 0.25, 0.5 μg) by the i.t. route. 4) Rats were treated with repeated doses of morphine-6-O-sulfate (1.5 mg/kg) administered twice daily (approximately at 0900 and 1700) by the i.p. route for 25 days. Responsiveness was determined 30, 45 and 60 min after injection. Each rat was considered tolerant when the maximum possible effect, % MPE, was equal or lower than 5%, as assessed by TFL (Holzman et al., 2004). In addition, morphine was administered acutely by i.t. (3, 10, 30 μg), i.p. (2, 3, 5, 7 mg/kg) and p.o. (10, 15, 20 mg/kg) routes, as well as chronically (7 mg/kg i.p., twice a day for 12 days) for comparison (the tail flick test).

2.4. Neuropathic pain (chronic constriction nerve injury)

2.4.1. Surgery

A well-established chronic constriction nerve injury (CCI) rodent model of neuropathic pain (Bennett and Xie, 1988) was utilized to determine the antihyperalgesic and antiallodynic effects of morphine-6-O-sulfate. Briefly, rats were subjected to a sciatic nerve ligation (left paw) and sham surgery (right paw) as follows. Proximal to the sciatic trifurcation, the nerve (approximately 7 mm) was freed from adhering tissue and four loose ligatures were tied around the nerve (1 mm apart) using 4.0 chromic catgut, barely constricting the diameter of the nerve. In sham surgery, the nerve was exposed but was not ligated. The incision was closed in layers with silk thread 3.0. This procedure results in enhanced sensitivity to both noxious stimuli (hyperalgesia) and normally non-noxious stimuli (allodynia) that develops typically within 7 days and last approximately 28 days after surgery in a CCI paw. There are no significant changes in the sham-operated paw.

2.4.2. Mechanical hyperalgesia

The presence of mechanical hyperalgesia was determined by the paw pressure test (32 g/s; cut-off 300 g) (Randall and Selitto, 1957) utilizing the Basile Analgesimeter (UGO Basile, Italy). Vocalization was used as the end point (vocalization threshold, VT, g). Experiments were carried out prior to (pre-surgery baseline) and on post-surgery days 7, 9, 11 and 14 when abnormal pain behavior was at a stable level. Responsiveness was determined prior to and after CCI, respectively, in control rats. Each rat received four doses of morphine-6-O-sulfate (0.1, 0.5, 0.75, 1 mg/kg i.p.; randomized doses). Responsiveness to mechanical noxious stimuli (VT) was assessed prior to (baseline) and at several time points (15–180 min) after injection in both CCI and sham paws. In addition, morphine (3, 5, 7 mg/kg i.p.) was tested for comparison in CCI rats.

2.4.3. Tactile allodynia

Responsiveness to normally non-noxious stimuli (allodynia) was assessed using von Frey filaments (Stoelting, Wood Dale, IL) with incremental stiffness (4, 8, 15 g) (Flatters and Bennett, 2004). Briefly, each von Frey hair was applied (and held for 5 s) to the plantar surface of each hind paw (10 times/paw) prior to (day 0) and on post-surgery days 9, 11, 14 and 16 when enhanced pain sensitivity was at a stable
maximum. A positive response was regarded as the sharp withdrawal of the paw (paw withdrawal frequency, PWF, expressed as an overall percentage response). Typically, PWF values were equal to 20% versus 90% (4 g), 50% versus 90% (8 g) and 80% versus 100% (15 g) prior to and after CCI, respectively, in control (saline) rats. Thus, enhanced responsiveness to 4 g filament (a normally innocuous stimulus) can be described as tactile allodynia, responses to 15 g filament are probably best described as hyperalgesia (heightened pain response from normally noxious punctuate stimulus) and the responses to 8 g filament are intermediate. Rats were treated with morphine-6-O-sulfate (0.1, 0.5, 0.75, 1 mg/kg i.p.; randomized doses) and tested with von Frey filaments prior to (baseline) and 60 min after injection. Effect of morphine (1, 2, 4 mg/kg i.p.) also was assessed in this paradigm.

2.5. Inflammatory pain (the formalin test)

The ability of morphine-6-O-sulfate to block formalin-induced flinching behavior was assessed using the well-established formalin test (Wheeler-Aceto and Cowan, 1991). Briefly, 50 μl of formalin (5%) was injected subcutaneously (s.c.) into the dorsal surface of the left hind paw. This procedure typically produces a biphasic behavioral response consisting of flinching, lifting and licking in control rats. The first phase (0–10 min) is thought to result from direct stimulation of nociceptors (nociceptive pain) whereas the second phase (20–60 min) involves central sensitization. Herein, rats were injected with morphine-6-O-sulfate (0.01, 0.1, 0.5, 1 mg/kg i.p.) 15 min prior to formalin injection (s.c.). Incidences of formalin-induced flinching were counted continuously in 5 min intervals for 60 min. Each rat received only one treatment. Morphine (1, 2.5, 5 mg/kg i.p.) also was characterized in the formalin test.

2.6. Gastrointestinal motility (the charcoal test)

Inhibition of intestinal propulsion was tested using the charcoal meal test in the rat (Megens et al., 1998). After the overnight fasting, rats were given 2 ml of oral charcoal solution (5% charcoal and 10% gum Arabic) 30 min after i.p. administration of morphine-6-O-sulfate (0.5, 1.5, 3, 6, 15 mg/kg) or morphine (2.5, 5, 10 mg/kg). Thirty minutes later rats were euthanized (CO₂ inhalation), stomach and intestine were removed, and the distance traveled by the charcoal was recorded as a percentage of the total length of the small intestine. Control rats were treated with saline.

2.7. Motor coordination (the rotarod test)

The motor effect of morphine-6-O-sulfate was determined using the rotarod performance test (Watzman et al., 1964). Intact rats were trained to run on the Rat Rota Rod (Ugo Basile, Comeno, Italy) at a constant speed (10 rpm) for 180 s at two consequent days. Thereafter, they were treated with morphine-6-O-sulfate (1.5, 3, 6, 15 mg/kg i.p.; randomized doses; weekly intervals) and latency to fall was assessed prior to and at several time points (15, 30, 45, 60, 90 and 120 min) after injection (cut-off = 180 s).

2.8. Locomotor activity

Locomotor activity was determined using the Opto-Varimex infrared photocell-based activity monitor (Columbus Instrument, Columbus, OH). Horizontal (ambulation) and vertical (rearing) activities were recorded during 5 min sessions prior to and after (30, 60, 90 and 120 min) administration of morphine-6-O-sulfate (1.5, 3, 6, 15 mg/kg i.p.; randomized doses, weekly intervals) in intact rats.

2.9. Data and statistics

Data were normalized for baseline values for each rat at each time point. The areas under the curves (AUC₀₋₄₅₀) were calculated for baseline-normalized data (the trapezoidal rule). Percent maximum possible effect (%MPE) was calculated as follows: tail-flick test: [(TFL−baseline)/(10−baseline)]*100; CCI (paw pressure test): [(PWT−baseline_postCCI)/(baseline_preCCI−baseline_postCCI)]*100; CCI (von Frey test): (baseline−PWF/baseline)*100; formalin test: (flinches₅₅₀−flinches₅₅₀)₅₅₀*100; rotarod test: (latency to fall/180)*100; locomotor activity (AUC₅₅₀−AUC₅₅₀/AUC₅₅₀)*100. The percent of inhibition of gastrointestinal transit (%IGT) was calculated as (GT₅₅₀−GT₅₅₀/GT₅₅₀)*100, where GT₅₅₀ and GT₅₅₀ is the percent of gastrointestinal transit in the saline- and drug-treated rats, respectively. Dose–response curves were generated for %MPE (at peak time) or %IGT as a function of a log dose. The dose–response curves were assessed by regression analysis. A dose of drug causing 50% MPE (ED₅₀) and 95% confidence limits (95% CL) were calculated using the method of Tallarida and Murray (1987). The data were analyzed using one-way analysis of variance (ANOVA) and post-hoc Student Newman Keuls (SNK) test (locomotor, charcoal), Kruskal–Wallis ANOVA followed by Dunn’s test (rotarod) and two-way RM ANOVA (tolerance). Statistical analysis was performed using SigmaPlot for Windows version 11.0 (Systat Software, Inc.). The level of significance was P ≤ 0.05. All data are mean ± S.E.M. (n rats).

3. Results

3.1. Morphine-6-O-sulfate produces an antinociceptive effect after neuroaxial, parenteral and oral routes of administration in the tail flick test

The effects of morphine-6-O-sulfate were examined after the neuroaxial (i.t.), systemic (i.p.) and oral administration in a rodent model of acute nociception (the tail-flick test). The present data demonstrated (Fig. 1) that morphine-6-O-sulfate produced a dose-related antinociception after all routes of administration tested: AUC₅₅₀₋₄₅₀ min = 72.8 ± 42.6 – 2241.7 ± 219.3 (i.t.); AUC₀₋₄₅₀ min = 96.1 ± 38.3 – 713.6 ± 75.5 (i.p.); AUC₀₋₂₄₀ min = 3.6 ± 32.1 – 499.6 ± 166.6 (p.o.). The ED₅₀ (95% CL) values were equal to: 0.29 (0.16–0.54) μg i.t., 0.54 (0.52–0.55) mg/kg i.p. and 4.97 (3.8–6.52) mg/kg p.o. A maximum antinociception (%MPE = 100%) was maintained for approximately 3 h after i.t. administration of morphine-6-O-sulfate (0.5 μg). The peak effects were achieved approximately at 30–45 min and 60 min after i.p. and p.o. administration, respectively. Morphine-6-O-sulfate was approximately 10 times less potent after oral administration than after parenteral delivery. A ceiling effect (%MPE–70%) was observed with oral dosing (10–20 mg/kg).

3.2. Tolerance to morphine-6-O-sulfate antinociception

The antinociceptive effectiveness of morphine-6-O-sulfate was also measured across time of chronic dosing (1.5 mg/kg i.p.; twice a day) in the tail-flick test. The present data showed (Fig. 2) that repeated exposure to morphine-6-O-sulfate resulted in a time-related reduction in the antinociceptive effect (tolerance). Each rat was considered tolerant when %MPE was equal to 5%. Only two out of six rats met this criterion on day 25 of chronic treatment.

3.3. Morphine-6-O-sulfate alleviates mechanical hyperalgesia and tactile allodynia in a chronic constriction nerve injury rodent model of neuropathic pain

Chronic constriction nerve injury (CCI) of the sciatic nerve resulted in the development of enhanced responsiveness to mechanical noxious stimuli (hyperalgesia) evident by decrease in vocalization...
threshold on post-CCI days 7, 9, 11 and 14 (VT, g = 135 ± 4.7, 87.5 ± 7.9, 90.8 ± 7.7 and 110 ± 11.8, respectively) compared to pre-CCI baseline value (VT, g = 240.8 ± 3.3). Sham surgery did not enhance pain sensitivity (VT, g = 235 ± 5.5, 230.8 ± 3.8, 217.5 ± 3.9, 225.8 ± 6.4 and 224.2 ± 4.9, respectively on days 0, 7, 9, 11 and 14). The present data showed (Fig. 3) that morphine-6-O-sulfate in a dose-related fashion attenuated mechanical hyperalgesia in a CCI paw (the paw pressure test). The ED50 (95% CL) value was equal to 0.4 (0.32–0.49) mg/kg i.p. The maximum possible effect (VT post-CCI – VT pre-CCI) was achieved within 30 min and was maintained for about 90 min after administration of a high dose (1 mg/kg i.p.).

In addition, CCI resulted in enhanced responsiveness to previously non-noxious stimuli (tactile allodynia) evident by an increase in responsiveness to 4 g von Frey hair on post-CCI days 9, 11, 14 and 16 (paw withdrawal frequency, PWF, %= 70 ± 9.4, 90 ± 4.9, 90 ± 4.8 and 73.3 ± 15.7, respectively) compared to pre-CCI baseline response (PWF, %= 20 ± 8). Effect of sham surgery was less pronounced (PWF, %= 16.7 ± 6.7, 30 ± 7.5, 33.3 ± 4.6, 40 ± 8 and 16.7 ± 8.8 on days 0, 9, 11, 14 and 16, respectively). Morphine-6-O-sulfate reduced, in a dose-related fashion, responsiveness to a 4 g von Frey filament (normally an innocuous stimulus) in a CCI paw with an ED50 (95% CL) value equal to 0.19 (0.098–0.406) mg/kg i.p. (Fig. 4A). In addition, morphine-6-O-sulfate, in a dose-related manner, reduced responsiveness to a 15 g von Frey filament (a noxious stimulus) (Fig. 4B). In this regard, a high dose of morphine-6-O-sulfate (1 mg/kg i.p.) only partially reversed mechanical hyperalgesia (%MPE = 50 ± 15.7%) assessed as a paw withdrawal response to punctuate noxious stimuli (the von Frey test). Notably, the same dose completely abolished mechanical hyperalgesia (%MPE = 98.5 ± 1.1%) evoked by ascending pressure applied into the paw with vocalization taken as endpoint (Randall Selitto test) (see Fig. 3).

3.4. Morphine-6-O-sulfate inhibits flinching behavior in a rodent model of inflammatory pain (the formalin test)

Formalin injection into the paw caused a biphasic flinching behavior in control (saline) rats. Herein, the effects of morphine-6-O-sulfate (i.p.) on the first phase (0–10 min), which is thought to result from direct stimulation of nociceptors, and the second phase (20–60 min), which involves central sensitization, were investigated. The present data demonstrated (Fig. 5) that morphine-6-O-sulfate attenuated, in a dose-related fashion, flinching during both phases in
The ED$_{50}$ (95% CL) values were equal to 1.28 (0.435–3.75) and 0.094 (0.049–0.178) mg/kg i.p. for the phase 1 and the phase 2, respectively.

3.5. The side effect (toxicity) profile of morphine-6-O-sulfate

The acute effects of morphine-6-O-sulfate on gastrointestinal motility (the charcoal meal test), motor incoordination (the rotarod test) and locomotor activity were tested after i.p. administration in intact rats. Morphine-6-O-sulfate caused a dose-related inhibition of the gastrointestinal propulsive activity ($F_{4,16} = 4.1; P < 0.025$; one-way ANOVA) with the effect produced by a high dose (15 mg/kg i.p.) being significantly different from the effect of saline ($P < 0.05$; post-hoc SNK) (Fig. 6). The ED$_{50}$ (95% CL) value was equal to 5.79 (2.83–11.85) mg/kg i.p. Morphine-6-O-sulfate also produced a dose-related motor incoordination evident by impairment of rotarod performance ($H_{3,20} = 20.5; P < 0.001$; one-way ANOVA on rank) (Fig. 7). Once again, the effect produced by a high dose (15 mg/kg) was significantly different from saline ($P < 0.05$; post-hoc Dunn's test).
different from saline ($P<0.05$; post-hoc Dunn’s test). The ED$_{50}$ (95% CI) value was equal to 5.55 (4.25–7.22) mg/kg i.p. In addition, morphine-6-$O$-sulfate decreased, in a dose-related fashion, locomotor activity ($F_{4,31} = 11.3; P<0.001$; one-way ANOVA) (Fig. 8). In this case, the effects produced by both 6 and 15 mg/kg i.p. of morphine-6-$O$-sulfate were significantly different from the effect of saline ($P<0.05$; post-hoc SNK test). The ED$_{50}$ (95% CI) value was equal to 7.39 (5.45–9.98) mg/kg i.p. With regard to morphine-6-$O$-sulfate mediated toxicity, it is important to emphasize that the undesirable side-effects tested (i.e. delay of gastrointestinal transit, motor incoordination and hypolocomotion) occurred at doses significantly higher (at least 10-fold) than doses required to produce the desirable analgesic effects in all the pain models utilized in this study (Fig. 9).

3.6. Comparisons between the effects of morphine 6-$O$-sulfate and morphine

Morphine-6-$O$-sulfate was compared to morphine with regard to analgesic potency (the antinociceptive, antihyperalgesic and anti-allodynic effects) and one of the clinically important adverse effects, inhibition of gastrointestinal motility. Several differences between these opioids were noted. First, morphine-6-$O$-sulfate had a greater analgesic activity than morphine in all rodent models of pain tested (Table 1). Specifically, morphine-6-$O$-sulfate was approximately 10-fold more potent compared to morphine in producing antinociception after neuroaxial (i.t.) administration in the tail-flick test. Maximum effect (%MPE = 100%) was maintained for a longer period of time after morphine-6-$O$-sulfate (i.t.) administration (approximately 180 min) than after morphine (i.t.) administration (approximately 90 min, data not shown). Morphine-6-$O$-sulfate also had a greater potency than morphine after the parenteral (i.p.) and oral routes of administration, 5- and 2-fold, respectively; however, in these cases the time courses (onset and duration of action) were similar for both drugs. Morphine-6-$O$-sulfate was more potent than morphine in alleviation of mechanical hyperalgesia and tactile allodynia, 6- and 8-fold, respectively in a CCI model of peripheral neuropathy and had a nearly 3-fold greater potency than morphine in inhibition of formalin-induced flinching behavior in a rodent model of inflammatory pain (the formalin test). Second, morphine-6-$O$-sulfate (1.5 mg/kg i.p., twice a day) had a long time course (at least 25 days) for development of tolerance to the antinociceptive effect in the tail-flick test. Conversely, morphine (7 mg/kg i.p., twice a day) exhibited a more rapid onset of tolerance (by Day 10) in this paradigm. With regard to tolerance development to antinociception, significant differences between drugs ($F_{1,111} = 30.9, P<0.001$) and time ($F_{7,111} = 35.9, P<0.001$), as well as significant drug–time interaction ($F_{7,111} = 6.4, P<0.001$) were demonstrated (two-way RM ANOVA). Of note, both drugs produced

![Fig. 8. Effect of morphine-6-$O$-sulfate on locomotor activity, (A; time–action curve) and (B; dose–response curve), after intraperitoneal (i.p.) administration in the rat. Mean ± S.E.M. (n = 6 rats). *Significantly different from saline ($P<0.05$; post-hoc SNK).](image)

![Fig. 9. Morphine-6-$O$-sulfate produced analgesia and side-effects following intraperitoneal (i.p.) administration in the rat. Mean ± S.E.M. (numbers of rats are indicated on Figs. 1–8).](image)

<table>
<thead>
<tr>
<th>Model of pain</th>
<th>Route of administration</th>
<th>ED$_{50}$ (95% CI)</th>
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<tbody>
<tr>
<td>Tail flick</td>
<td>i.t. (µg)</td>
<td>0.29 (0.16–0.54)</td>
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<td></td>
<td>i.p. (mg/kg)</td>
<td>0.54 (0.52–0.55)</td>
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<td></td>
<td>p.o. (mg/kg)</td>
<td>4.97 (3.8–6.52)</td>
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<tr>
<td>CCI (hyperalgesia)</td>
<td>i.p. (mg/kg)</td>
<td>0.40 (0.32–0.49)</td>
</tr>
<tr>
<td>CCI (alldynia)</td>
<td>i.p. (mg/kg)</td>
<td>0.19 (0.098–0.406)</td>
</tr>
<tr>
<td>Formalin test (phase 2)</td>
<td>i.p. (mg/kg)</td>
<td>0.094 (0.049–0.178)</td>
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<td>0.258 (0.135–0.492)</td>
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(Table 1). Specifically, morphine-6-$O$-sulfate was approximately 10-fold more potent compared to morphine in producing antinociception after neuroaxial (i.t.) administration in the tail-flick test. Maximum effect (%MPE = 100%) was maintained for a longer period of time after morphine-6-$O$-sulfate (i.t.) administration (approximately 180 min) than after morphine (i.t.) administration (approximately 90 min, data not shown). Morphine-6-$O$-sulfate also had a greater potency than morphine after the parenteral (i.p.) and oral routes of administration, 5- and 2-fold, respectively; however, in these cases the time courses (onset and duration of action) were similar for both drugs. Morphine-6-$O$-sulfate was more potent than morphine in alleviation of mechanical hyperalgesia and tactile allodynia, 6- and 8-fold, respectively in a CCI model of peripheral neuropathy and had a nearly 3-fold greater potency than morphine in inhibition of formalin-induced flinching behavior in a rodent model of inflammatory pain (the formalin test). Second, morphine-6-$O$-sulfate (1.5 mg/kg i.p., twice a day) had a long time course (at least 25 days) for development of tolerance to the antinociceptive effect in the tail-flick test. Conversely, morphine (7 mg/kg i.p., twice a day) exhibited a more rapid onset of tolerance (by Day 10) in this paradigm. With regard to tolerance development to antinociception, significant differences between drugs ($F_{1,111} = 30.9, P<0.001$) and time ($F_{7,111} = 35.9, P<0.001$), as well as significant drug–time interaction ($F_{7,111} = 6.4, P<0.001$) were demonstrated (two-way RM ANOVA). Of note, both drugs produced
maximum antinociception (MPE% = 100%) at the doses used on the first day of chronic treatment (see Fig. 2). Third, morphine-6-O-sulfate and morphine had similar potencies in affecting gastrointestinal transit in the rat, as indicated by the ED50 (95% CI) values equal to 5.79 (2.83–11.85) and 4.71 (3.21–6.93) mg/kg i.p., respectively (see Fig. 6). However, in the case of morphine-6-O-sulfate, significant inhibition of gastrointestinal motility occurred at a dose (15 mg/kg i.p.; %IGT = 68.4 ± 8.1%) which was approximately 10- to 20-fold higher compared to doses required to produce analgesia across pain models used, including the tail flick test (1.5 mg/kg i.p.; %MPE = 91.3 ± 9.5%); the CCI paw pressure test (1 mg/kg i.p.; %MPE = 98.5 ± 1.1%); the CCI von Frey test (0.75 mg/kg i.p.; %MPE = 73.3 ± 9.2%) and the formalin test (1 mg/kg i.p.; %MPE = 76.9 ± 5.9%). In contrast, a dose of morphine that caused significant inhibition of gastrointestinal transit (10 mg/kg i.p., %IGT = 64.6 ± 2.8%) was similar to doses producing maximum effects against nociception (7 mg/kg i.p.; %MPE = 92.5 ± 5.7%), hyperalgesia (7 mg/kg i.p.; %MPE = 100 ± 0%), allodynia (4 mg/kg i.p.; 92.6 ± 0.2%) and formalin-evoked behaviors (5 mg/kg i.p.; %MPE = 99.7 ± 0.2%).

4. Discussion

In an effort to identify an opioid with a better therapeutic index compared to the classical mu-opioid agonist, morphine, a relatively little-known morphine derivative, morphine-6-O-sulfate, was characterized with regard to analgesia and side-effect profile using a range of well-established rodent models. The present data demonstrated the potent analgesic activities of morphine-6-O-sulfate against acute nociception, neuropathic pain (hyperalgesia, allodynia) and inflammatory pain; a good separation based on dose (~10-fold) between analgesia and side-effects tested (motor, gastrointestinal); and apparently a more favorable gastrointestinal effect/analogic effect ratio for morphine-6-O-sulfate compared to morphine.

The initial objective was to assess the analogic effectiveness of morphine-6-O-sulfate with regard to dose, route and mode of administration utilizing several rodent models of pain. Our data in rats after neuroaxial (i.t.), systemic (i.p.) and oral routes of administration confirmed earlier reports in mice after central injection (i.c.v.) (Brown et al., 1985; Houdi et al., 1996; Mori et al., 1972; Zuckerman et al., 1999) that morphine-6-O-sulfate produced a dose-related analgesic effect in the tail-flick test. A novel finding with the present study was that morphine-6-O-sulfate alleviated pain sensitization occurring as the result of an injury to nerve or tissue. Specifically, it attenuated, in a dose-related manner, an enhanced sensitivity to a noxious (hyperalgesia) and previously non-noxious (allodynia) stimulus in a CCI model of peripheral neuropathy, as well as inhibited both the early and the late nociceptive responses in a formalin model of inflammatory pain. Of note, the high analgesic potency of morphine-6-O-sulfate was observed irrespective of the rat model of pain used, i.e. nociception (the tail flick test) and neuropathy (CCI). However, the later appeared to be related to the modality of mechanical hyperalgesia tested (non-punctuate > punctuate). Opioids (e.g. morphine) seem differently depress responses triggered by different fibers (Le Bars et al., 2001).

The next objective was to characterize the side-effect profile of morphine-6-O-sulfate. This was done by assessing motor function (rotarod), gastrointestinal transit (charcoal) and locomotor activity in intact rats. A consistent finding with this study was that morphine-6-O-sulfate produced motor incoordination, hypolocomotion and inhibition of gastrointestinal motility at doses significantly higher (10- to 100-fold) than those responsible for analgesia in rodent models of thermal nociception, nerve injury-evoked peripheral neuropathy and inflammatory pain following formalin injection. Whether this is also the case with respiratory depression (severe side-effect of opioids) warrants further study. Previous data with other clinically used mu-opioids (morphine, codeine, oxycodone, hydrocodone) showed that the side-effects of such drugs (motor, gastrointestinal, respiratory) occurred at doses similar to the analgesic effects in rodents (Meert and Vermeirsh, 2005).

The final objective was to compare the effects of morphine-6-O-sulfate with those of morphine (a prototypical mu-opioid), specifically the analogic potency and a common adverse effect, opioid-induced bowel dysfunction. 1) Analgesic potency. The previous finding of a greater analgesic potency of morphine-6-O-sulfate compared to morphine at the supraspinal level (30-fold, i.c.v.) in mice (Zuckerman et al., 1999) was confirmed herein both at the spinal level (10-fold, i.t.) and in the periphery (5-fold, i.p.) in rats (tail flick test). As can be seen, the ratio of potencies (morphine-6-O-sulfate/morphine) was markedly greater after central delivery compared to delivery via the spinal and systemic routes, which may suggest that these compounds differ in their distribution within the CNS. The previous studies (microdialysis) showed that morphine and its 6-β-glucuronide conjugate accumulated respectively in the intra- and extra-cellular fluids, which in turn, likely defined their availability at, and interactions with, neuronal opioid receptors (Smith and South, 2001, review). This may be the case with morphine-6-O-sulfate. Importantly, the present data also showed that morphine-6-O-sulfate was more potent than morphine in alleviation of pain in rodent models of neuropathy (6- to 8-fold, CCI) and inflammatory pain (3-fold, formalin test) (Table 1). This is a potentially meaningful finding, as chronic pain conditions, in particular neuropathic pain, are typically regarded as less responsive to mu-opioids (Ballantine and Shin, 2008; Martin and Eisenach, 2001; Przewlocki and Przewlocka, 2001, reviews). The reason for these differences is not clear. For example, it has been proposed that morphine-6-O-sulfate (like morphine-6-β-glucuronide) mediates its pain-relieving effects through a different mu receptor subtype (sensitive to 3-methoxynaltrexone) than morphine does (Pasternak, 2001, review). Indeed, the obviously greater analogic potency of morphine-6-O-sulfate than morphine (in particular after central administration; 30-fold) was not supported by the traditional in vitro binding data showing that this morphine derivative had only slightly improved (~3-fold) affinity (Ki, nM) compared to a parent drug for mu- (0.9 ± 0.01 versus 2.5 ± 0.3), delta- (18 ± 0.4 versus 58 ± 3.8) and kappa-3- (6.3 ± 0.3 versus 13.9 ± 4.1) opioid receptors in guinea pig brain homogenates (Crooks et al., 2006). On another note, the earlier work demonstrated similar affinities (Ki, nM) for mu-(1.8 ± 0.4 versus 0.8 ± 0.1) but significantly different affinities for delta- (48.4 ± 0.4 versus 161 ± 10.2) receptors for morphine-6-O-sulfate compared to morphine in rat brain homogenates (morphine-6-β-glucuronide had the Ki values equal to 10.4 ± 2.4 and 88.5 ± 10.7 nM for these receptors) (Oguri et al., 1987). Use of different species may explain discrepancy in these findings. The binding profile (delta/mu) was more favorable for morphine-6-O-sulfate than morphine (Oguri et al., 1987); thus, it is plausible to speculate that delta-opioid receptors may also play a role in morphine-6-O-sulfate pharmacology; i.e. enhanced potency in neuropathic and inflammatory pain in rats. This possible mechanism of action should be further tested with selective delta-receptor antagonists. 2) Duration of analgesia. A prolonged duration of action, in particular after neuroaxial (intrathecal, epidural) administration, is an appreciated property of an analgesic drug in the clinical setting. Morphine-6-O-sulfate had a longer time-course of activity compared to morphine after direct administration to the CNS, either i.c.v. (Brown et al., 1985) or i.t. (present study). Morphine-6-O-sulfate (a polar molecule) was expected to have a delayed distribution into the brain and a consequently slower onset and duration of analgesia than morphine. Neither was observed after the parental or oral administration (similar onsets and durations of activity for both drugs). Overall oral efficacy was more favorable for morphine compared to the more charged molecules, morphine-6-O-sulfate (ceiling effect; present study) and morphine-6-β-glucuronide (van Dorp et al., 2006). Thus, the above findings can only be explained in part by the differences in
the physicochemical properties of these compounds. 3) Tolerance. Diminished analgesic responsiveness (tolerance) frequently occurs with a long-term exposure to mu-opioids, and this undesired phenomenon leads typically to dose escalation and accompanying adverse effects (cognitive, motor, gastrointestinal). The development of tolerance to antinociception was notably slower for morphine-6-O-sulphate than morphine (25 versus 10 days) when these drugs were administered repeatedly at equipotent doses (%MPE – 100%, Day 1) in the tail flick test. This finding appears to support a general belief that the degree of tolerance is inversely correlated to potency of mu-opioids (Frey and Latasch, 2003; Pawar et al., 2007). 4) Therapeutic index (toxicity/analgesia). Opioid-induced bowel dysfunction is a significant clinical problem, as it occurs typically at analgesic doses, and contrary to the other side-effects of opioids (e.g. respiratory depression), does not decline with repeated dosing (a lack of tolerance). Herein, morphine and its sulfate conjugate inhibited, with similar potencies, the propulsive activity (charcoal test) in the rat. However, morphine-6-O-sulphate had a significant separation based on dose (at least 10-fold) between the undesirable gastrointestinal effect and the desirable analgesic activities in all pain models tested. This separation was significantly less for morphine (only 2-fold) either in rats (present study) or mice (Paul et al., 1989). This is a promising finding for morphine-6-O-sulphate especially in view of the fact that morphine-6-β-glucuronide, which is recognized for its potential priority over morphine (Smith and South, 2001; van Dorp et al., 2006, reviews), delayed gastrointestinal motility at doses far less than those required to produce antinociception (ED50 = 0.065 and 2.7 mg/kg s.c. respectively) in mice (Paul et al., 1989). In addition, morphine-6-β-glucuronide inhibited gastrointestinal transit with greater potency (10-fold) than morphine (Paul et al., 1989). The complexity of mu-opioid pharmacology is well recognized. The analgesic (supraspinal) and gastrointestinal effects of mu-opioids (i.e. morphine) are thought to be mediated by different subsets of receptors, likely mu-1 and mu-2, respectively (Pasternak, 2001; review). Previous data showed that morphine-6-O-sulphate had a greater affinity (10-fold) for mu-1 than mu-2 receptor subtype (Kc, nM = 0.8 ± 0.01 versus 10.2 ± 0.9) in mouse brain homogenates (Zuckerman et al., 1999). On the other hand, both morphine and its major active metabolite, morphine-6-β-glucuronide, competed with similar IC50 (nM) values for mu-1 and mu-2 receptor subtypes (morphine: 3.4 ± 0.8 versus 4.2 ± 1.1; morphine-6-β-glucuronide: 13.3 ± 3.2 versus 14.5 ± 2.7) in the same binding assay (Paul et al., 1989).

In conclusion, the present study in rats demonstrated potential clinical advantages of morphine-6-O-sulphate compared to morphine, based on the following findings: a significantly greater (5- to 10-fold) analgesic potency when assessed using various routes of administration (i.t., i.p.) across a range of nociceptive assays (nociceptive, neuropathic, inflammatory); a slower onset of tolerance development to antinociception (3-fold) and reduced side-effect liability manifested by a considerably greater separation between inhibition of gastrointestinal transit (charcoal test) and analgesia. Together, these preclinical data suggest that morphine-6-O-sulphate may be an interesting potential drug for further study and use for management of nociceptive, neuropathic and mixed pain states.

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References