Characterization of the antinociceptive effects of the individual isomers of methadone after acute and chronic administrations
Richard W. Morgan and Katherine L. Nicholson

Methadone is a long-acting opioid used in the treatment of various pain states and substitution therapy in heroin addiction. Extensive behavioral characterization has been carried out using the racemate, but limited investigation has been performed with the individual isomers. The L-isomer is a potent opioid agonist, whereas the D-isomer has weak μ-opioid activity and has also been shown to possess N-methyl-D-aspartate antagonist properties. Increasing dose ratios of D-methadone to L-methadone were administered chronically to determine the ability of the D-isomer to modulate antinociceptive tolerance to the L-isomer. Acutely, both L-methadone (0.1–5.6 mg/kg, subcutaneously) and D-methadone (3.0–56.0 mg/kg, subcutaneously) produced antinociception, although the efficacy of the D-isomer was limited at 55°C. These effects were dose dependently blocked by naltrexone (0.01–1.0 mg/kg, subcutaneously). Administered chronically, D-methadone (1.7–10 mg/kg, subcutaneously) dose dependently blocked tolerance development to the L-isomer (1.7 mg/kg, subcutaneously). These findings support the antinociceptive effects of the isomers being opioid receptor mediated with the L-isomer functioning as a full-efficacy agonist, whereas the D-isomer seems to have lower efficacy. The ability of nonracemic doses of the D-isomer to prevent tolerance development to the L-isomer may be attributed to partial μ-opioid activity; however, N-methyl-D-aspartate antagonist activity cannot be discounted. 

Introduction
Methadone (6-dimethylamino-4,4-diphenyl-3-heptanone) is a synthetic opioid of the diphenylmethanone class originally characterized as an analgesic (Scott and Chen, 1946). Dole and Nyswander (1965) first proposed primary clinical use of methadone as a substitution therapy in heroin addiction in 1965. This was the only approved use of methadone in the United States until 1976 when it was also approved for use as an analgesic (Schmidt, 1976). Since this time, methadone has continued to be used in opioid-dependence therapy and for the treatment of chronic pain conditions such as those associated with neuralgia and cancer (for reviews, see Layson-Wolf et al., 2002 and Nicholson, 2007). Reasons for its continued clinical success include high oral availability and a long half-life, both of which make fewer daily doses possible (Gourlay et al., 1986).

In clinical use, methadone is available as the racemic mixture of the D-isomer and L-isomer. The analgesic effects of methadone were ascribed to activity at opioid receptors as evidenced by high affinity for the μ subtype (Kᵢ = 5.6 nmol/l in human and 19 nmol/l in rat μ receptor expressing COS-7 cells, Raynor et al., 1995). Furthermore, these effects were attributed primarily to the L-isomer, which has 25-fold greater affinity for the μ receptor than the D-isomer (Terenius, 1974). In addition, both isomers have affinity for δ opioid (IC₅₀ = 9.532 and 0.371 μmol/l for D-isomer and L-isomer, respectively), α-3, β-4 nicotinic acetylcholine (IC₅₀ = 2.5 and 2.0 μmol/l), and N-methyl-D-aspartate (NMDA) glutamate (Kᵢ = 7.4 and 3.4 μmol/l) receptors (Kristensen et al., 1995; Gorman et al., 1997; Xiao et al., 2001). Many studies have demonstrated the ability of antagonists of the NMDA receptor to alter the effects of opioids including: potentiation of opioid analgesic activity; attenuation, prevention, and reversal of opioid tolerance; and attenuation of opioid physical dependence (Trujillo and Akil, 1991; Popik et al., 2000; Kozela et al., 2001; Zhu and Barr, 2001; Kozela et al., 2003). In-vitro data show that both isomers of methadone bind with low affinity within the NMDA channel and functionally inhibit channel currents (Ebert et al., 1995; Gorman et al., 1997; Callahan et al., 2004). Consistent with the actions of other NMDA antagonists, in-vivo studies have shown that the D-isomer is able to block morphine antinociceptive tolerance in the tail-flick test and NMDA-induced hyperalgesia as assessed by thermal paw-withdrawal latency (Davis and Inturrisi, 1999).
Thus, it is possible that the $\delta$-isomer of methadone may modify the opioid receptor-mediated effects of the L-isomer when clinically used as the racemate. Indeed, it has been proposed that this characteristic of the methadone isomers is responsible for production of analgesia by methadone in patients no longer responsive to other opioids (Morley, 1998) and the slower development of tolerance to methadone relative to morphine (Inturrisi et al., 1990).

Other studies, however, suggest that the effects produced by $\delta$-methadone are due to its weak opioid activity. When applied to dorsal horn neurons, $\delta$-methadone was able to inhibit NMDA-induced activity, but this was prevented by pretreatment with the opioid antagonist naloxone (Chizh et al., 2000). C-fibre activity, which is predominantly mediated by $\mu$-opioid and not by NMDA receptor, was inhibited by $\delta$-methadone, an effect which was reversed by naloxone (Carpenter et al., 2000). In behavioral experiments, $\delta$-methadone is able to produce antinociception that is naloxone reversible in the Randall–Selitto model of inflammation (Chizh et al., 2000) and in acetic acid-induced stretching (Smits and Myers, 1974).

Given the conflicting reports regarding the primary mechanism of action of $\delta$-methadone, we have conducted a series of experiments to further investigate the behavioral effects produced by the individual isomers. In the first set of experiments, the acute analgesic effects of the individual isomers were determined using the warm-water, tail-withdrawal (WWTW) measure of antinociception in rats. The ability of naltrexone to antagonize antinociceptive effects produced by the isomers was evaluated in the same subjects. Subsequently, the development of tolerance to the antinociceptive effects of the L-isomer after chronic administration of different dose ratios of the $\delta$ and L-isomers was evaluated using the WWTW procedure in separate groups of rats.

**Methods**

All studies were conducted at Virginia Commonwealth University (VCU). VCU is accredited by the American Association for the Accreditation of Laboratory Animal Care. All laboratory practices and animal care were consistent with the current National Institutes of Health guidelines and all experimental protocols were approved by the VCU Institutional Animal Care and Use Committee.

**Subjects**

A total of 58 male Sprague-Dawley rats weighing 250 g upon arrival were obtained from Charles River (Raleigh, North Carolina, USA). Subjects were housed individually in the University Vivarium and maintained on a 12:12 h light–dark cycle and allowed free access to food and water.

**Apparatus**

Experiments were conducted in flat-bottomed restraint tubes (model FB-Large) obtained from Braintree Scientific (Braintree, Massachusetts, USA). The clear plastic semicylindrical tubes measured 8.6 cm × 21.6 cm with a guillotine-style closure in the caudal end, which had a central opening at the bottom allowing protrusion of the tail.

**Procedure**

The experimental design was based on procedures used by Cook et al. (2000). In brief, subjects were habituated to restraint by placing them in the restraint tubes with their tails freely extending out of the guillotine closure. During habituation and all testing, an opaque cloth cover was extended above the restraint tubes. This served not only to enhance habituation due to the restricted light exposure, but also to minimize the impact of any ambient drafts on the tail withdrawal. Time spent in the restraint tubes was gradually increased from 20 to 90 min over the 5-day period before testing. A mark was placed on each rat's tail, 5 cm from the distal end. On test days, rats were brought to the laboratory and left undisturbed for 10 min before being placed into the restraint tubes for an additional 10 min. Subsequently, rats underwent a qualifying test to identify any subject that would exhibit tail withdrawal under non-noxious conditions and thus were ineligible for testing. In the qualifying tests, the distal 5 cm of each rat's tail was immersed in water at 40°C contained in an insulated thermos and the latency to tail withdrawal was manually recorded. To qualify for continued testing during the same session, latency to tail withdrawal had to equal the designated cutoff time (15 s) for at least two of three trials separated by 2 min. The cutoff time of 15 s was used to prevent tissue damage.

**Acute drug administration**

Immediately after qualifying tests, all rats meeting criteria were given an injection of vehicle (saline). Fifteen minutes postinjection, tail-withdrawal latencies were recorded at water temperatures of 50 and 55°C. Testing with the two temperatures was separated by 2 min and the order of presentation was randomized. During the 2-min separation time, in both qualifying and testing trials, the rats' tails were dried with Delicate Task Kimwipes (Kimberly-Clark, Roswell, Georgia, USA). After determination of the saline control-withdrawal times, the rats were administered cumulative doses of L-methadone (0.1–5.6 mg/kg, $n = 8$) or $\delta$-methadone (3.0–56.0 mg/kg, $n = 8$) every 15 min and tested for withdrawal latencies using the same procedure. Incremental dosing and testing ended when the average latency for tail withdrawal reached 80% maximum possible effect (MPE) for tail withdrawal from the 55°C stimulus. All latencies were measured manually. The testing procedure was repeated at most once per week to avoid the development of any tolerance to or physical dependence on the isomers of methadone. In subsequent testing, various doses of naltrexone (0.01–1.0 mg/kg) were given in lieu of saline to evaluate the impact of opioid ($\mu$) receptor blockade on...
the antinociceptive effects of the isomers. On the final test day, the highest dose of naltrexone necessary to completely block antinociception was administered followed by four injections of saline to control for any effect of naltrexone administration.

**Chronic administration**

Similar procedures were used in individual groups of rats (n = 5–7 per group) to evaluate the development of tolerance to the antinociceptive effects of L-methadone after chronic treatment with various ratios (0:1, 1:1, 3:1, 6:1, 10:1, or 6:0) of D-methadone to L-methadone doses. These ratios corresponded to the administration of the following doses of D-methadone with 1.7 mg/kg of L-methadone: 0 mg/kg (saline); 1.7; 5.1, 10; and 17 mg/kg. In addition, a control group was administered, 5.1 mg/kg of D-methadone with 0 mg/kg of L-methadone (saline) repeated providing our 6:0 dose ratio group. On the first day (day 0), an acute L-methadone dose–effect curve was generated using the WWTW procedure as described above. Rats were then assigned to one of six chronic treatment groups and administered twice daily injections on days 1–7. Each group received an injection of saline or D-methadone followed, 30 min later, by an injection of 1.7 mg/kg of L-methadone or saline. This combination of injections was repeated every 12 h. On day 8, an L-methadone cumulative dose–effect curve was regenerated in the morning. Twelve hours later, the rats received a final set of D-methadone and L-methadone injections. On the morning of day 9, a morphine cumulative dose–effect curve was generated. The morphine data were compared with results from a separate group of rats that had completed an acute morphine dose–effect curve only.

**Drugs**

D-methadone and L-methadone HCl were obtained through the National Institute on Drug Abuse Drug Supply Program (Bethesda, Maryland, USA). Morphine sulfate and naltrexone HCl were obtained from Mallinckrodt Pharmaceuticals (St. Louis, Missouri, USA). All drugs were dissolved in physiological saline (0.9% NaCl) to achieve the desired concentration for the individual doses. D-methadone and L-methadone and morphine were administered subcutaneously and naltrexone was administered intraperitoneally in a volume of 1 ml/kg.

**Data analysis**

Percent MPE was calculated for each subject at each test dose using the following formula: [{(Test latency – Baseline latency) / (Cutoff time – Baseline latency)}] × 100. Each data point represents the mean % MPE for all subjects tested in each condition. For both the acute and chronic experiments, full antinociception was defined as more than or equal to 80% MPE. ED\textsubscript{50} values for antinociception were calculated using regression analysis of the linear portions of the dose–effect curves after a log\textsubscript{10} transformation of the dose (Tallarida and Murray, 1987). The ED\textsubscript{50} potency ratios and 95% confidence limits were calculated for each treatment condition comparison. If in each of these comparisons the upper and lower potency ratio 95% confidence limits did not encompass a value of 1, the ED\textsubscript{50} values between the dose–effect curves compared were considered significantly different (Bliss, 1967).

**Results**

Figure 1 shows the results for testing antinociception after administration of acute L-methadone and D-methadone alone and in combination with varying doses of naltrexone at two stimulus intensities. At both water temperatures, L-methadone produced full antinociception (100 and 96% MPE). Naltrexone pretreatment dose dependently blocked the antinociception produced by L-methadone at both stimulus intensities. For the 50°C stimulus, D-methadone dose dependently produced antinociception with the highest dose (56 mg/kg) producing full antinociception (82% MPE). Naltrexone pretreatment dose dependently attenuated the antinociceptive effects produced by D-methadone. At the 55°C stimulus, D-methadone produced only partial antinociception (maximum of 57% MPE) when a dose up to 56 mg/kg was administered. When tested in combination with increasing doses of naltrexone (0.01, 0.1, and 0.3 mg/kg) the antinociceptive effects of D-methadone were dose dependently blocked at both the 50° and 55°C stimuli. When administered alone, naltrexone did not produce any antinociceptive or hypernociceptive effects in either group (data not shown). For each isomer, the ED\textsubscript{50} values for the production of antinociception and the ED\textsubscript{50} potency ratios between stimulus intensities and between isomers are presented in Table 1. When comparing the ED\textsubscript{50} values of the individual isomers at the two different water temperatures, L-methadone was approximately 3-fold and D-methadone was approximately 6-fold more potent at producing antinociceptive effects at the 50°C stimulus than the 55°C stimulus. Comparing ED\textsubscript{50} values between the isomers, L-methadone was approximately 30-fold and 70-fold more potent than D-methadone at the 50 and 55°C water temperatures, respectively. ED\textsubscript{50} values could not be calculated for D-methadone after pretreatment with the 0.1 and 0.3-mg/kg doses of naltrexone nor for L-methadone pretreated with 1-mg/kg naltrexone because these pretreatments blocked antinociception resulting in % MPE values well below 50%.

The antinociceptive effects of L-methadone before and after chronic administration of increasing ratios of D: L-methadone dosage combinations are shown in Fig. 2. Before chronic dosing, L-methadone dose dependently produced full antinociception (> 80% MPE) at both stimulus intensities in all subjects. After 7 days of twice-daily dosing with 1.7 mg/kg of L-methadone pretreated with saline, L-methadone produced full antinociception...
at both the 50 and 55°C water temperatures (100 and 85% MPE, respectively), however, there was a rightward shift in the dose–effect curves. As shown in Tables 2 and 3, the ED$_{50}$ values for antinociception at 50 and 55°C (0.22 and 0.79 mg/kg, respectively) after chronic dosing were significantly ($P < 0.05$) greater than the initial ED$_{50}$ values (2.7-fold increase at 50°C and 3.4-fold increase at 55°C).

For each of the different dose ratio combinations of the isomers administered chronically (1 : 1, 3 : 1, 6 : 1, and 10 : 1 D- to L-methadone), L-methadone was able to produce full antinociception in the WWTW procedure before and after chronic dosing as shown in Fig. 2. As the ratio of D-methadone was increased, the rightward shifts in the dose–effect curves determined before and after chronic dosing became less pronounced. When comparing ED$_{50}$ values (Tables 2 and 3), it can be seen that the potency ratios diminished when increasing doses of D-methadone were administered chronically in combination with 1.7 mg/kg of L-methadone indicating a dose-dependent blockade of tolerance development to L-methadone.

### Table 1  ED$_{50}$ values and their potency ratios for individual methadone isomers in rats tested in the warm-water tail-withdrawal procedure at 50 and 55°C water temperatures

<table>
<thead>
<tr>
<th>Dosing condition</th>
<th>ED$_{50}$ mg/kg (95% CL) at 50°C</th>
<th>ED$_{50}$ mg/kg (95% CL) at 55°C</th>
<th>ED$_{50}$ potency ratio (55/50°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-methadone</td>
<td>0.27 (0.2–0.4)</td>
<td>0.73 (0.6–0.9)</td>
<td>2.70</td>
</tr>
<tr>
<td>D-methadone</td>
<td>8.64 (3.4–21.7)</td>
<td>51.32 (32.4–81.2)</td>
<td>5.94</td>
</tr>
<tr>
<td>ED$_{50}$ potency ratio (D/L-isomer)</td>
<td>32.00</td>
<td>70.30</td>
<td>–</td>
</tr>
</tbody>
</table>

CL, confidence limits.
When evaluated at the 50°C stimulus intensity, the 3:1 ratio of isomers (5 mg/kg of D-methadone:1.7 mg/kg of L-methadone) prevented any significant potency change for L-methadone (potency ratio of 1.04). At the 55°C stimulus intensity, the development of tolerance was no longer seen after chronic administration of the 6:1 ratio.

Antinociceptive effects of L-methadone as assessed by warm-water tail withdrawal before and after chronic treatment with increasing dose ratios of D:L-methadone at both 50°C (left column) and 55°C (right column) water temperatures. (a) 0:1 D-isomer to L-isomer, n=5, (b) 1:1, n=6, (c) 3:1, n=7, (d) 6:1, n=5, and (e) 10:1, n=6. MPE, maximum possible effect; SEM, standard error of the mean.
isomer combination (potency ratio 1.35). The effects of chronic D-methadone administration only (10 mg/kg of D-methadone combined with saline) on tolerance development to L-methadone are shown in Fig. 3. Chronic exposure to D-methadone did not alter the antinociceptive effects of acute L-methadone at either stimulus intensity as evidenced by no significant difference between prechronic and postchronic ED50 values (Tables 2 and 3).

Table 2  Relative antinociceptive potency of L-methadone and ED50 potency ratios before and after chronic administration of various dose ratios of D/L-methadone in the warm-water tail-withdrawal procedure at 50°C

<table>
<thead>
<tr>
<th>Dosing condition</th>
<th>Prechronic ED50 mg/kg (95% CL)</th>
<th>Postchronic ED50 mg/kg (95% CL)</th>
<th>ED50 potency ratio (95% CL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0:1</td>
<td>0.22 (0.14–0.34)</td>
<td>0.79* (0.65–0.96)</td>
<td>2.70 (1.22–6.80)</td>
</tr>
<tr>
<td>1:1</td>
<td>0.22 (0.13–0.37)</td>
<td>0.83* (0.64–1.09)</td>
<td>3.50 (1.94–6.46)</td>
</tr>
<tr>
<td>3:1</td>
<td>0.52 (0.46–0.58)</td>
<td>0.37 (0.05–3.01)</td>
<td>1.04 (0.53–1.88)</td>
</tr>
<tr>
<td>6:1</td>
<td>0.44 (0.28–0.68)</td>
<td>0.33 (0.18–0.62)</td>
<td>0.80 (0.32–1.89)</td>
</tr>
<tr>
<td>10:1</td>
<td>0.35 (0.29–0.42)</td>
<td>0.33 (0.29–0.36)</td>
<td>0.94 (0.80–1.10)</td>
</tr>
<tr>
<td>6:0</td>
<td>0.32 (0.26–0.39)</td>
<td>0.31 (0.26–0.38)</td>
<td>0.98 (0.77–1.24)</td>
</tr>
</tbody>
</table>

CL, confidence limits.

*aPostchronic ED50 significantly different from prechronic at \( P < 0.05 \).

Table 3  Relative antinociceptive potency of L-methadone and ED50 potency ratios before and after chronic administration of various dose ratios of D/L-methadone in the warm-water tail-withdrawal procedure at 55°C

<table>
<thead>
<tr>
<th>Dosing condition</th>
<th>Prechronic ED50 mg/kg (95% CL)</th>
<th>Postchronic ED50 mg/kg (95% CL)</th>
<th>ED50 potency ratio (95% CL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0:1</td>
<td>0.54 (0.39–0.75)</td>
<td>1.78* (1.48–2.15)</td>
<td>3.41 (2.04–5.76)</td>
</tr>
<tr>
<td>1:1</td>
<td>0.69 (0.58–0.83)</td>
<td>1.06* (0.88–1.28)</td>
<td>1.49 (1.15–2.02)</td>
</tr>
<tr>
<td>3:1</td>
<td>0.78 (0.66–0.92)</td>
<td>1.09* (0.88–1.34)</td>
<td>1.40 (1.03–1.95)</td>
</tr>
<tr>
<td>6:1</td>
<td>0.76 (0.64–1.91)</td>
<td>1.04 (0.75–1.44)</td>
<td>1.35 (0.89–2.13)</td>
</tr>
<tr>
<td>10:1</td>
<td>0.94 (0.80–1.12)</td>
<td>0.94 (0.85–1.03)</td>
<td>0.99 (0.88–1.12)</td>
</tr>
<tr>
<td>6:0</td>
<td>0.63 (0.55–0.71)</td>
<td>0.68 (0.57–0.81)</td>
<td>1.08 (0.94–1.23)</td>
</tr>
</tbody>
</table>

CL, confidence limits.

*aPostchronic ED50 significantly different from prechronic at \( P < 0.05 \).

Fig. 3

Antinociceptive effects of L-methadone as assessed by warm-water tail withdrawal before and after chronic dosing with 10 mg/kg of D-methadone at both 50°C (left column) and 55°C (right column) water temperatures, \( n = 6 \). MPE, maximum possible effect; SEM, standard error of the mean.

(10 mg/kg of D-methadone:1.7 mg/kg of L-methadone) isomer combination (potency ratio 1.35). The effects of chronic D-methadone administration only (10 mg/kg of D-methadone combined with saline) on tolerance development to L-methadone are shown in Fig. 3. Chronic exposure to D-methadone did not alter the antinociceptive effects of acute L-methadone at either stimulus intensity as evidenced by no significant difference between prechronic and postchronic ED50 values (Tables 2 and 3).

On day 9, a morphine dose–effect curve was generated for each group dosed chronically with different dose ratios of D-methadone to L-methadone to assess the development of opioid cross-tolerance (Fig. 4). Data from these curves were compared with a control group of animals that were tested acutely with morphine. In the latter group, the ED50 values for morphine antinociception at 50 and 55°C were 1.25 and 5.13 mg/kg, respectively. Repeated administration of 1.7 mg/kg of L-methadone with saline resulted in a rightward shift in the morphine dose–effect curve at both stimulus intensities. Addition of D-methadone during chronic dosing showed a dose-dependent attenuation of the rightward shift (Fig. 4). The ED50 potency of morphine was significantly different between the morphine-only group and the 0:1 and 1:1 D-methadone to L-methadone treatment groups \( (P < 0.05) \) at both temperatures suggesting the development of cross-tolerance (Table 4). In the groups receiving higher chronic
doses of D-methadone and L-methadone (3:1, 6:1, and 10:1) dose combinations and D-methadone alone, ED50 potencies for the antinociceptive effects of morphine were not significantly different from the control group that had received morphine only at either temperature, indicating a lack of cross-tolerance (Table 4).

### Discussion

The behavioral effects produced by L-methadone and D-methadone in this study suggest that the primary site of action for the acute effects of both isomers is at the μ-opioid receptors. In the WWTW procedure, as expected, L-methadone produced dose-dependent antinociception that was antagonized by naltrexone at both stimulus intensities (Fig. 1). Similarly, D-methadone produced full antinociception at the 50°C water temperature reaching 82% MPE at the 56-mg/kg dose (Fig. 1). The D-isomer also produced a dose-dependent antinociception at the 55°C stimulus, however, it only produced a maximum of 57% MPE at the highest dose tested. Greater doses of D-methadone to determine its ability to produce full antinociception at the higher stimulus intensity were not tested due to toxic side-effects. The antinociceptive effects of the D-isomer were blocked by pretreatment with naltrexone lending further evidence that D-methadone prevents noxious stimulus transmission primarily by agonist actions at the μ-opioid receptor. Our results are comparable with those seen when testing morphine, meperidine, etorphine, or buprenorphine with naloxone or naltrexone in similar models of acute or physiological pain (Leander, 1980; Walker et al., 1994). The inability of the D-isomer to produce full antinociception at the higher stimulus intensity suggests it functions as a partial μ agonist. When tested at varying water temperatures, partial agonists such as butorphanol, buprenorphine, and pentazocine show full antinociceptive efficacy at low-stimulus, but not high-stimulus, intensities (Morgan et al., 1999a, 1999b; Terner et al., 2003).

**Table 4** Relative antinociceptive potency of morphine and ED50 potency ratios for a morphine control group (no chronic treatment) compared with groups after chronic administration of various dose ratios of α: methadone as assessed in the warm-water tail-withdrawal procedure

<table>
<thead>
<tr>
<th>Dosing condition</th>
<th>Morphine ED50 mg/kg (95% CL) at 50°C</th>
<th>ED50 potency ratio (95% CL)</th>
<th>Morphine ED50 mg/kg (95% CL) at 55°C</th>
<th>ED50 potency ratio (95% CL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Morphine control</td>
<td>1.25 (0.66–2.37)</td>
<td>−</td>
<td>5.13 (4.05–6.51)</td>
<td>−</td>
</tr>
<tr>
<td>0:1</td>
<td>6.87* (5.13–9.19)</td>
<td>4.44 (1.90–10.28)</td>
<td>15.18* (13.02–17.69)</td>
<td>2.16 (1.06–4.37)</td>
</tr>
<tr>
<td>1:1</td>
<td>3.27 (1.97–5.41)</td>
<td>2.45 (1.04–5.63)</td>
<td>10.03* (7.98–12.67)</td>
<td>1.94 (1.31–2.84)</td>
</tr>
<tr>
<td>3:1</td>
<td>1.31 (0.67–2.56)</td>
<td>1.18 (0.44–3.01)</td>
<td>9.39 (3.65–24.19)</td>
<td>2.05 (0.64–3.88)</td>
</tr>
<tr>
<td>6:1</td>
<td>2.00 (1.02–3.93)</td>
<td>1.59 (0.64–4.50)</td>
<td>3.98 (2.55–6.20)</td>
<td>0.83 (0.45–1.52)</td>
</tr>
<tr>
<td>9:1</td>
<td>2.12 (1.80–2.49)</td>
<td>1.68 (0.99–3.02)</td>
<td>5.21 (4.43–6.12)</td>
<td>1.04 (0.79–1.39)</td>
</tr>
<tr>
<td>6:0</td>
<td>1.09 (0.92–1.29)</td>
<td>0.87 (0.48–1.71)</td>
<td>3.98 (3.43–4.61)</td>
<td>0.81 (0.61–1.08)</td>
</tr>
</tbody>
</table>

*CL, confidence limits.

*Significantly different from morphine control at P < 0.05.
Potency differences between the isomers are highlighted by the L-methadone ED$_{50}$ value being 32-fold and 70-fold more potent than that for D-methadone at 50 and 55°C, respectively (Table 1). The ED$_{50}$ potency difference for the isomers is consistent with their relative in-vitro measurements of μ opioid receptor binding. The D-isomer was 25-fold less potent than the L-isomer in displacement of [H]dihydromorphone (IC$_{50} = 1 \times 10^{-7}$ vs. $4 \times 10^{-9}$ mol/l, respectively; Terenius, 1974). Similarly, Horng et al. (1976) investigated [H]dihydromorphone and [3H]-naloxone displacement by the isomers of methadone and showed D-methadone to have approximately 30-fold lower affinity for μ opioid receptors than L-methadone. Our results are consistent and remarkably similar to these in-vitro findings (Table 1). The greater than predicted potency difference based on binding affinity at the higher stimulus intensity could reflect the D-isomer acting as a lower efficacy agonist relative to the L-isomer. This is consistent with whole cell current-clamp and voltage-clamp recordings, which showed a greater intrinsic activity for L-methadone relative to D-methadone (Matsui and Williams, 2010). However, further testing with combinations of the two isomers will be required to demonstrate definitively that, consistent with these in-vitro assays, D-methadone functions as a partial efficacy μ agonist in vivo. An additional consideration is the two D-methadone metabolites, α-L-methadol and α-L-dinormethadol, which have also been shown to have opioid receptor affinity similar to or greater than the parent compound (Horng et al., 1976). Previous studies have found that metabolites of D-methadone produce antinociception (Sullivan et al., 1972; Terenius, 1974; Sullivan et al., 1975), which was attributed primarily to formation of the α-L-normethadol metabolite. Alpha L-methadol has been found in rat lung tissue 1 h after administration of D-methadone (Sullivan et al., 1975). Therefore, it is very possible that the metabolites also contribute to antinociceptive effects of D-methadone in this study.

The majority of previous studies evaluating D-methadone have found evidence of μ opioid receptor-mediated antinociceptive effects. In contrast, Shimoyama et al. (1997) reported that intrathecal D-methadone produced no antinociception in a radiant-heat tail-flick test and was ineffective in phase one but effective, and naloxone irreversible, in phase 2 of the formalin test. The latter results suggest that the primary action of D-methadone is through a nonopioid mechanism and are consistent with actions as an NMDA antagonist. In addition, Leander and McCleary (1982) found that decreases in keypecking behavior elicited by D-methadone in pigeons was unaffected by concurrent naloxone treatment. The present results are more similar to those of Smits and Myers 1974 and Chizh et al. 2000 who found that D-methadone produced naloxone-reversible antinociception in acetic acid-induced stretching (ED$_{50}$ = 6.1 mg/kg) and the Randall–Selitto model of inflammatory pain (ED$_{50}$ = 6.6 mg/kg), respectively. Furthermore, Chizh et al. (2000) also found that naloxone reversed D-methadone inhibition of noxious stimulation of single motor units. Similarly, the ability of D-methadone to inhibit C-fibre evoked activity can be prevented by naloxone (Carpenter et al., 2000). An explanation for discrepancies between the behavioral results in the different experiments could be the doses of drugs used, the pain model used, animal species used, and/or the route of administration. Shimoyama et al. (1997) reported testing only one dose of D-methadone and naloxone was administered intrathecally. It is possible that the dose of naloxone (30 μg) given was insufficient to displace D-methadone (250 μg) from a sufficient number of opioid receptors to reverse any opioid-mediated effects. Alternatively, the contribution of NMDA antagonism versus μ-opioid receptor activation may vary dependent on the pain model used and the route of administration (i.e. intrathecal versus systemic).

The relevance of the pain model used is supported by the current testing system evaluating antinociception in a spinally mediated model of acute pain, both the D and L-isomers seem to produce antinociception by opioid receptor-mediated mechanisms.

The ability of D-methadone to block the development of opioid antinociceptive tolerance to a fixed dose of L-methadone (1.7 mg/kg) given for 7 days was demonstrated. Combinations of 5.1, 10.0, and 17.0 mg/kg of D-methadone with 1.7 mg/kg of L-methadone blocked tolerance at 50°C, whereas only 10.0 and 17.0 mg/kg of D-methadone doses combined with 1.7 mg/kg of L-methadone blocked tolerance development when evaluated at 55°C (Fig. 2, Tables 2 and 3). After chronic administration, it was also demonstrated that D-methadone (5.1, 10.0, and 17.0 mg/kg, Table 4) combination treatment with L-methadone was also able to block cross-tolerance to morphine-induced antinociception. In addition, another group was repeatedly administered with only the D-isomer (10 mg/kg) for 7 days to determine its effects on acute administration of L-methadone and morphine. This moderate dose of D-methadone was chosen because it was able to block tolerance when combined with the L-isomer. This treatment did not result in any change in the antinociceptive potency of L-methadone or morphine indicating that D-methadone itself does not induce tolerance to opioids nor result in a hyperalgesic state at this dose. It also demonstrated that chronic administration of the D-isomer does not cause an altered response to acute administration of the potent...
opioid agonists that might enhance their effects and thus appear as a blockade of tolerance (Fig. 2, Tables 2–4). It is possible that higher doses of D-methadone could produce tolerance due to a greater stimulation of opioid receptors, as has been seen with chronic administration of partial agonists (Walker and Young, 2001; Grecksch et al., 2006).

Similar blockade of opioid tolerance development has been found using an acute pain model with D-methadone given in combination with morphine for 5 days (Davis and Inturrisi, 1999). On the basis of the current chronic administration study and previous studies, it is possible that blockade of opioid tolerance by coadministration of D-methadone was due to action of the isomer as an antagonist of NMDA receptors. However, it is also possible that the current results were due to the D-isomer acting as a lower affinity, lower efficacy agonist at μ-opioid receptors. In rats administered with morphine chronically, pretreatment with low doses of an opioid antagonist (naloxone and naltrexone) or with a low-efficacy opioid agonist (nalbuphine) was able to block the development of antinociceptive tolerance (Lee et al., 1997; Powell et al., 2002; Jang et al., 2006). This suggests that concurrent opioid receptor blockade or occupancy by a low-efficacy agonist during repeated exposure to a high-efficacy agonist can prevent the development of tolerance.

The relative binding potency of the isomers to μ-opioid receptors versus NMDA channel sites in vitro provides further support for opioid-mediated antinociception and tolerance-attenuating behavioral effects for the D-isomer (Ebert et al., 1995; Gorman et al., 1997; Callahan et al., 2004). D-methadone is approximately 5-fold more potent at activating μ-opioid than inhibiting NMDA receptors (ED50 = 0.6 μmol/l vs. IC50 = approximately 3.5 μmol/l, respectively; Matsui and Williams, 2010). This comparison seems particularly relevant, as the doses-attenuating tolerance development were similar to or lower than those showing naloxone-antagonized antinociceptive effects. Clinically, plasma levels achieved in methadone-maintained patients approximates the levels associated with opioid receptor activation (Kreek, 1973), whereas doses associated with plasma levels in the NMDA receptor IC50 range have been associated with overt toxicity (Perret et al., 2000). Thus, with the much greater affinity for μ-opioid than NMDA receptors, the role of NMDA antagonism in the antinociceptive and tolerance-blocking actions of D-methadone could be questioned. It must be noted that the level of NMDA receptor blockade (i.e. percent of receptors blocked) necessary to block tolerance development is unknown; therefore, it cannot be ruled out that a very low level of NMDA channel blockade did contribute to the tolerance attenuation. In addition, binding studies show the density of NMDA receptors is much greater than that of μ-opioid receptors in brain tissue thus providing more targets for the D-isomer to exert its glutamatergic effects (Goodman and Pasternak, 1985; Monaghan and Cotman, 1985). Interestingly, L-methadone has slightly higher affinity for binding within NMDA channels than D-methadone (Gorman et al., 1997). However, L-methadone is approximately 600-fold more potent (IC50 = 5.0 nM vs. 3.4 μM, respectively) in binding to μ-opioid receptors relative to NMDA channel binding. Therefore, the very potent effects of the L-isomer at μ-opioid receptors prevent in-vivo administration of doses, which would produce central nervous system concentrations sufficient to block NMDA receptor channels.

In conclusion, both D-methadone and L-methadone isomers produce naloxone-reversible antinociception after acute administration, although the D-isomer was less efficacious at higher levels of noxious stimuli, and the L-isomer was significantly more potent under all conditions. The D-isomer was also shown to block antinociceptive tolerance development after chronic L-methadone administration as well as cross-tolerance to morphine without having any effects on tolerance when administered alone. The acute antinociceptive effects seem to be the result of D-methadone acting as a partial μ-opioid agonist. Although the current results after chronic administration cannot conclusively be attributed solely to μ-opioid receptor activity, the role of NMDA antagonism to account for these findings can be questioned. Further studies to investigate the potential utility of varying combinations of D-methadone and L-methadone could be informative for the clinical use of methadone as an analgesic with less susceptibility for the development of tolerance. Methadone is currently available in the USA as a 1:1 ratio of the D and L-isomers. This ratio of the isomers would not be expected to modify the actions of the L-isomer based on the current results. However, it is possible that the use of higher ratios of D:L methadone clinically could delay or prevent the development of analgesic tolerance.

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Conflicts of interest
There are no conflicts of interest.

References
Characterization of the antinoceptive effects

Morgan and Nicholson 557


