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Antinociceptive effects of the novel spirocyclopiperazinium salt compound LXM-10 in mice

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Abstract

The compound LXM-10 (2,4-dimethyl-9-β-phenylethyl-3-oxo-6, 9-diazaspiro [5.5]undecane chloride) is a new spirocyclopiperazinium salt compound. This is the first article to evaluate its antinociceptive effect in the abdominal constriction test induced by acetic acid and the hot-plate test. In the abdominal constriction test, LXM-10 had a significant dose-response effect, and the maximal inhibition ratio was 79.2%. In the hot-plate test, LXM-10 had significant dose-response and time-response effects. The antinociceptive effect began at 1.0 h, peaked at 2.0 h, and persisted 3.0 h after s.c. administration. The hot-plate latency was increased by 126.8% at the dose of 12.0 mg/kg. The antinociceptive effect of LXM-10 was blocked by mecamylamine (a central and peripheral neuronal nicotinic acetylcholine receptor antagonist, 0.25, 0.5, 1.0 mg/kg, i.p.), hexamethonium (a peripheral neuronal nicotinic acetylcholine receptor antagonist, 0.2, 1.0, 5.0 mg/kg, i.p.), atropine (a central and peripheral muscarinic acetylcholine receptor antagonist, 0.2, 1.0, 5.0 mg/kg, i.p.), and atropine methylnitrate (a peripheral muscarinic acetylcholine receptor antagonist, 0.2, 1.0, 5.0 mg/kg, i.p.) in a dose-dependent fashion. In contrast, the effect was not blocked by naloxone (a non-selective opioid receptor antagonist, 2.0 mg/kg, i.p.) or yohimbine (a α_2-adrenergic receptor antagonist, 1.0, 2.5, 5.0 mg/kg, i.p.) in the hot-plate test. Therefore, the antinociceptive effects of LXM-10 involve the peripheral neuronal nicotinic and muscarinic acetylcholine receptors; they are not related to opioid receptors or α_2-adrenergic receptors. LXM-10 did not affect motor coordination, spontaneous activity, or body temperature. These findings with LXM-10 suggest that spirocyclopiperazinium derivatives could provide insight on new analgesics.

Keywords: LXM-10; Antinociception

1. Introduction

Pain is one of the most common symptoms that seriously affect patients' lives. Classifications of pain include acute, persistent, and neuropathic (Millan, 1999). Opioid analgesics, non-steroidal anti-inflammatory drugs (NSAIDs), certain anticonvulsants, and tricyclic antidepressants are commonly used to treat pain. Opioids are mostly used to treat severe pain, but they are less effective in treating neuropathic pain, and issues of tolerance and dependence limit their use (Przewlocki and Przewlocka, 2001). NSAIDs are effective in mild to moderate persistent pain, particularly inflammatory pain; however, they show little effect in severe pain and produce gastrointestinal side effects (Kingery, 1997). Anticonvulsants and tricyclic antidepressants are used to treat neuropathic pain, but these only achieve clinical pain relief in fewer than 50% of patients (McQuay et al., 1996).

Because of these limitations, the development of new powerful analgesics that lack substantial side effects is a priority. A great deal of emphasis has been placed on identifying novel molecular targets; muscarinic cholinergic agonists, neuronal nicotinic acetylcholine receptor agonists, neurokinin-1 receptor antagonists, sodium channel antagonists, *N*-methyl-D-aspartate receptor antagonists, and compounds that interact with purinergic neurotransmission are some of the agents under development, but no new breakthroughs have been made.

N,*N*-dimethyl-N_4-phenylpiperazinium iodide (DMPP) is a neuronal nicotinic acetylcholine receptor agonist that has

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antinociceptive activity by intracerebroventricular (i.c.v.) administration in the hot-plate test and tail-flick assay (Tadimeti et al., 1996; Dina et al., 1999). However, it has a quaternary nitrogen and does not readily cross the blood brain barrier, and systemic administration produces marked peripheral effects such as tachypnea, hypotension, ataxia, and vocalization (Tadimeti et al., 1996; Romanelli et al., 2001). Li and Sun et al. synthesized a series of novel spirocyclopiperazinium compounds with analgesic activity, which were based on structural modifications of DMPP (Gao et al., 2003; Wang et al., 2003, 2004). We screened these compounds for analgesic activity by hot-plate test and abdominal constriction test induced by acetic acid, and found that the compound LXM-10 (2,4-dimethyl-9-β-phenylethyl-3-oxo-6, 9-diazaspiro [5.5]undecane chloride, Fig. 1) had a stronger analgesic activity without obvious side effects. The purpose of the study was to explore potential antinociceptive effects and possible mechanisms of LXM-10.

2. Materials and methods

2.1. Animals

Both sexes of ICR mice, each weighing 20–22 g, were used (Department of Laboratory Animal Science of Peking University). Animals were housed in standard environmental conditions (22 °C±1 °C, humidity 60%±5% and a 12 h/12 h dark/light cycle—lights on at 7:00 a.m.), and water was constantly available. The research was conducted in accordance with the International Association for the Study of Pain ethical guidelines (Zimmermann, 1983) and approved by the Institutional Animal Care and Use Committee of Peking University. All behavioral tests were conducted in a blinded manner.

2.2. Drugs

The following drugs were used: LXM-10 (Department of Chemical Biology, Pharmaceutical School of Peking University, Beijing, P.R. China), acetic acid (Beijing Fine Chemical Co., Beijing, P.R. China), morphine hydrochloride (Neuroscience Research Institute of Peking University, Beijing, P.R. China), naloxone hydrochloride, atropine sulphate, atropine methylnitrate, mecamylamine hydrochloride, hexamethonium chloride, and yohimbine hydrochloride (Sigma Chemical Co., St. Louis, MO, USA). All drugs were dissolved in isotonic saline solution (NaCl 0.9%) immediately before use. Drug concentrations were prepared in such a way that the necessary dose could be administered in a volume of 10 ml/kg by subcutaneous injection (s.c.) or intraperitoneal injection (i.p.).

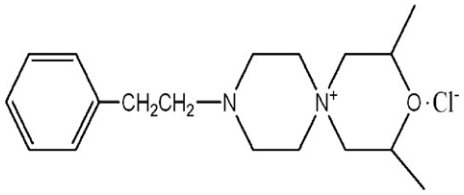

Fig. 1. The chemical structure of the compound LXM-10.

2.3. Antinociceptive tests

2.3.1. Abdominal constriction test

Both sexes of mice were administered LXM-10 (1.5, 3.0, or 6.0 mg/kg, s.c.), and control animals received saline; after 30 min, each animal was injected with 0.6% acetic acid (10 ml/kg, i.p.) and individually housed in a glass cylinder on a flat glass floor (Koster et al., 1959). The number of constriction movements was counted for 10 min, starting 5 min after acetic acid injection. The percentage of inhibition was determined for each experimental group by using the following formula:

$$\text{Inhibition } (\%) = [(\text{control} - \text{experiment})/\text{control}] \times 100\%.$$

2.3.2. The hot-plate test

The hot-plate test was used to measure response latencies according to the method described by Eddy and Leimbach (Eddy and Leimbach, 1953). Both sexes of mice were treated and placed individually on a hot plate (Model GJ-8401, P.R. China), maintained at 55.0 °C±0.5 °C, the time between placement of the animal on the hot plate and licking hind paws, shaking, or jumping off the surface was recorded as response latency. Basal latency was recorded as the mean value of three determinations before treatments. Mice with baseline latencies of less than 5 s or more than 10 s were eliminated from the study, and the cut-off latency time was set at 30 s to avoid tissue damage. Animals were selected 30 min before the assay. The hot-plate response latencies were measured at 1.0, 2.0, and 3.0 h after administering LXM-10 (3.0, 6.0, 12.0 mg/kg, s.c.), morphine (10.0 mg/kg, s.c.), or saline.

2.4. Investigation of the antinociceptive mechanisms of LXM-10

To address some of the antinociceptive mechanisms of LXM-10, both sexes of mice were pretreated with different receptor antagonists in the hot-plate test. The response latencies were measured before and at 1.0 and 2.0 h after the second injection. The doses of each receptor antagonist were selected on the basis of other experiments in the literature (Michael et al., 1998; Barocelli et al., 2004; Chaim et al., 2005) and our preliminary experiments.

2.4.1. Involvement of opioid system

Mice were pretreated with the non-selective opioid receptor antagonist naloxone (2.0 mg/kg, i.p.), and after 15 min the animals received the second injection of LXM-10 (6.0 mg/kg, s.c.), morphine (10.0 mg/kg, s.c.), or saline. Other animals were pretreated with saline as control, and after 15 min the animals received LXM-10 (6.0 mg/kg, s.c.), morphine (10.0 mg/kg, s.c.), or saline.

2.4.2. Involvement of neuronal nicotinic acetylcholine system

Mice were pretreated with mecamylamine (a central and peripheral neuronal nicotinic acetylcholine receptor antagonist, 0.25, 0.5, or 1.0 mg/kg, i.p.) or hexamethonium (a peripheral neuronal nicotinic acetylcholine receptor antagonist, 0.2, 1.0, or

5.0 mg/kg, i.p.); after 15 min the animals received the second injection of LXM-10 (6.0 mg/kg, s.c.) or saline. Other animals were pretreated with saline as control, and after 15 min the animals received the second injection of LXM-10 (6.0 mg/kg, s. c.) or saline.

2.4.3. Involvement of muscarinic acetylcholine system

Mice were pretreated with atropine (a central and peripheral muscarinic acetylcholine receptor antagonist, 0.2, 1.0, or 5.0 mg/kg, i.p.) or atropine methylnitrate (a peripheral muscarinic acetylcholine receptor antagonist, 0.2, 1.0, or 5.0 mg/kg, i.p.); after 15 min, animals received the second injection of LXM-10 (6.0 mg/kg, s.c.) or saline. Other animals were pretreated with saline as control, and after 15 min the animals received the second injection of LXM-10 (6.0 mg/kg, s. c.) or saline.

2.4.4. Involvement of α_2-adrenergic system

Mice were pretreated with yohimbine (a α_2-adrenergic receptor antagonist, 1.0, 2.5, or 5.0 mg/kg, i.p.), and after 15 min the animals received the second injection of LXM-10 (6.0 mg/kg, s.c.) or saline. Other animals were pretreated with saline as control, and after 15 min the animals received the second injection of LXM-10 (6.0 mg/kg, s.c.) or saline.

2.4.5. Assessment of motor performance, spontaneous activity, and body temperature

Motor coordination was assessed by the rota-rod test (Rosland et al., 1990). The apparatus (Model DXP-2, Institute of Materia Medica, Chinese Academy of Medical Sciences, P.R. China) consisted of a bar rod with a diameter of 2.5 cm, subdivided into four compartments. The bar rod rotated at a constant speed of 40 rpm, and animals were evaluated for the time until they fell from the rod. The animals were selected 24 h previously by eliminating those mice that did not remain on the bar for 60 s. The cut-off time used was 120 s. Spontaneous activity was assessed in the open-field test (Rodrigues et al., 2002). The apparatus consisted of a wooden box measuring 40 × 60 × 50 cm. The floor of the arena was divided into 12 equal squares, and the number of squares crossed with all paws crossing was counted in a 3-min session. Body temperature was assessed by using a probe (Digital Thermometer, Shanghai, P.R. China) that was inserted 3.0 cm into the rectum. Both sexes of mice were treated with LXM-10 (6.0, 12.0, or 24.0 mg/kg, s.c.), diazepam (2.0 mg/kg, i.p.), or saline (10 ml/kg, s.c.) in the three experiments. An individual animal was used only in a single experiment. Time to falling, spontaneous activity, and body temperature were measured before and 0.5, 2.0, and 3.5 h after treatment.

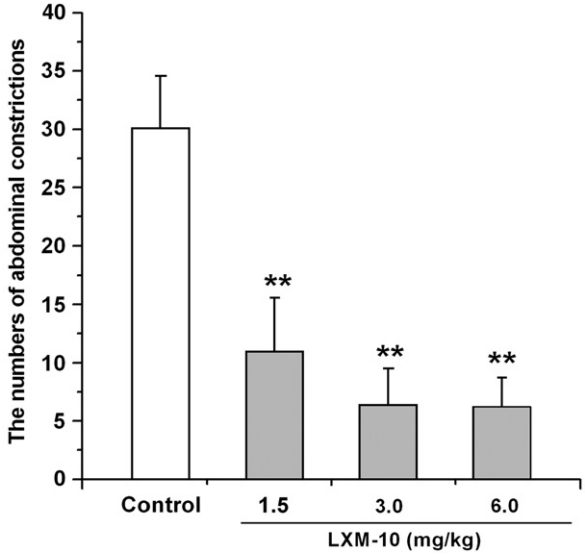

Fig. 2. Antinociceptive effect of LXM-10 in the abdominal constriction test in mice. The mice were administered LXM-10 (1.5, 3.0, or 6.0 mg/kg, s.c.), 30 min prior to injection of 0.6% acetic acid (10 ml/kg, i.p.). Each column represents the numbers of abdominal constrictions as mean ± S.E.M of 8 mice per group. One-way ANOVA followed by LSD was used to test the difference between groups. **P < 0.01 vs. control.

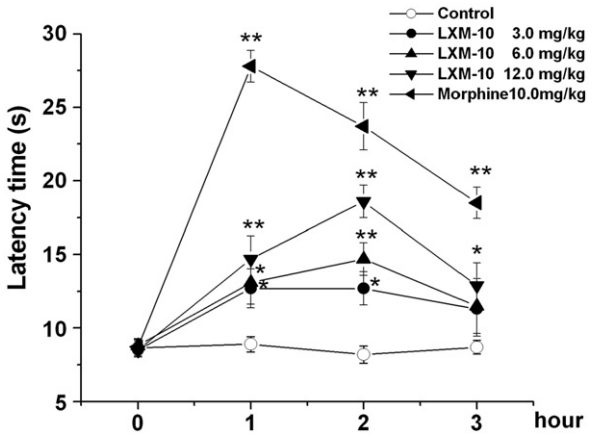

Fig. 3. Antinociceptive effect of LXM-10 in the hot-plate test in mice. The mice were administered LXM-10 (3.0, 6.0, or 12.0 mg/kg, s.c.) or morphine (10.0 mg/kg, s.c.). Each group represents the latency time as mean ± S.E.M of 10 mice per group. Repeated-measures ANOVA followed by LSD was used to test the difference between groups. *P < 0.05, **P < 0.01 vs. control group at the same time.

2.4.6. Statistical analysis

The results were presented as mean ± S.E.M. Data were analyzed by means of repeated-measures ANOVA or one-way ANOVA followed by least significant difference test by using SPSS 13.0 for windows. Statistical significance was indicated by P values < 0.05.

3. Results

3.1. Antinociceptive tests

3.1.1. Antinociceptive activity of LXM-10 in the abdominal constriction test

Fig. 2 shows that LXM-10 significantly reduced the number of abdominal constrictions as compared with control group at the doses of 1.5, 3.0, and 6.0 mg/kg; inhibition ratios were 63.4%, 78.7%, and 79.2%, respectively. One-way ANOVA relative to the number of abdominal constrictions showed a

significant effect between the groups [$F(3, 31)=8.999$, $P<0.001$].

3.1.2. Antinociceptive activity of LXM-10 in the hot-plate test

Fig. 3 shows that LXM-10 significantly delayed the hot-plate latency times as compared with the control groups. The antinociceptive effect of LXM-10 began at 1.0 h, peaked at 2.0 h, and persisted 3.0 h after s.c. administration. The hot-plate latency times were increased by 54.8%, 79.2%, and 126.8%, respectively, at 3.0, 6.0, and 12.0 mg/kg at peak time. Repeated-measures ANOVA showed significant differences for treatment groups [$F(4,45)=38.7$, $P<0.001$], times [$F(3,135)=44.7$, $P<0.001$], and interaction between treatment groups and times [$F(12,135)=9.06$, $P<0.001$].

3.2. The antinociceptive mechanisms of LXM-10

3.2.1. Involvement of opioid system

Fig. 4 shows that LXM-10 (6.0 mg/kg, s.c.) produced significant antinociception at both 1.0 and 2.0 h after administration, and the non-selective opioid antagonist naloxone (2.0 mg/kg, i.p.) did not block the effects, whereas it blocked the antinociception caused by morphine (10.0 mg/kg, s.c.) in the hot-plate test. Naloxone (2.0 mg/kg, i.p.) did not affect the latency time at any time point in this experiment. Repeated-measures ANOVA showed significant differences for treatment groups [$F(5,54)=16.1$, $P<0.001$], times [$F(2,108)=37.1$, $P<0.001$], and interaction between treatment groups and times [$F(10,108)=9.15$, $P<0.001$].

3.2.2. Involvement of neuronal nicotinic acetylcholine system

Fig. 5 shows that LXM-10 (6.0 mg/kg, s.c.) produced significant antinociception at both 1.0 and 2.0 h after administration; mecamylamine, a central and peripheral neuronal nicotinic acetylcholine receptor antagonist (0.25, 0.5, and 1.0 mg/kg, i.p.) blocked the effect in the hot-plate test in a dose-dependent manner. Repeated-measures ANOVA showed significant differences for treatment groups [$F(5,54)=16.1$, $P<0.001$], times[$F(2,108)=32.8$, $P<0.001$], and interaction between treatment groups and times [$F(10,108)=5.22$, $P<0.001$]. At the same time, the peripheral neuronal nicotinic acetylcholine receptor antagonist hexamethonium (0.2, 1.0, and

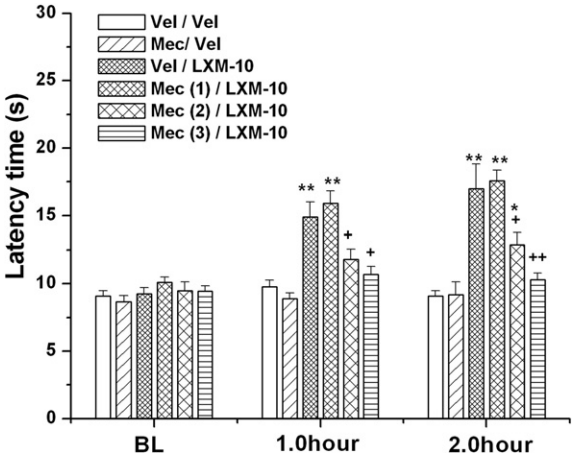

Fig. 5. Effect of mecamylamine on the LXM-10-induced antinociception in the hot-plate test. The mice were pretreated with mecamylamine [Mec (1), (2), and (3) represent the doses of 0.25, 0.50, and 1.0 mg/kg, i.p., respectively] or saline (Vel), 15 min before administration of LXM-10 (6.0 mg/kg, s.c.) or saline. Mice were tested by basal latency (BL) and 1.0 and 2.0 h response latency after the second injection. Each column represents the latency time as mean±S.E.M of 10 mice per group. Repeated-measures ANOVA followed by LSD was used to test the difference between groups. *$P<0.05$, **$P<0.01$ vs. Vel/Vel at the same time; $^{+}P<0.05$, $^{++}P<0.01$ vs. Vel/LXM-10 at the same time.

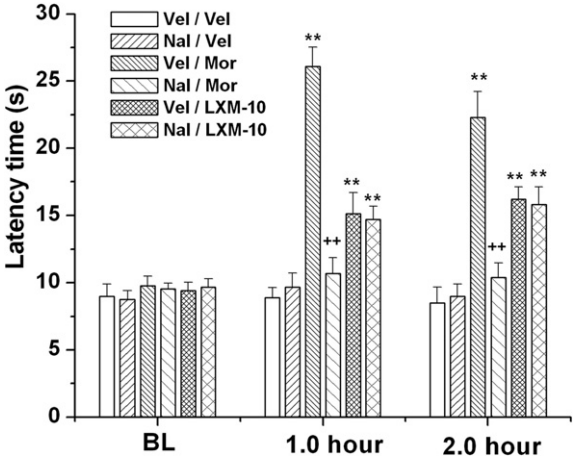

Fig. 4. Effect of naloxone on the LXM-10-induced antinociception in the hot-plate test. The mice were pretreated with naloxone (Nal, 2.0 mg/kg, i.p.) or saline (Vel), 15 min before administration with LXM-10 (6.0 mg/kg, s.c.), morphine (Mor, 10.0 mg/kg, s.c.) or saline. Mice were tested by basal latency (BL) and 1.0 and 2.0 h response latency after the second injection. Each column represents the latency time as mean±S.E.M of 10 mice per group. Repeated-measures ANOVA followed by LSD was used to test the difference between groups. **$P<0.01$ vs. Vel/Vel at the same time; $^{++}P<0.01$ vs. Vel/Mor at the same time.

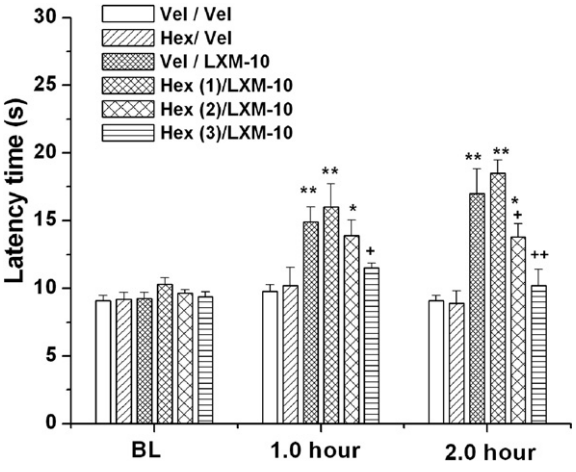

Fig. 6. Effect of hexamethonium on the LXM-10-induced antinociception in the hot-plate test. The mice were pretreated with hexamethonium [Hex (1), (2), and (3) represent the doses of 0.2, 1.0, and 5.0 mg/kg, i.p., respectively] or saline (Vel), 15 min before administration of LXM-10 (6.0 mg/kg, s.c.) or saline. Mice were tested by basal latency (BL) and 1.0 and 2.0 h response latency after the second injection. Each column represents the latency time as mean±S.E.M of 10 mice per group. Repeated-measures ANOVA followed by LSD was used to test the difference between groups. *$P<0.05$, **$P<0.01$ vs. Vel/Vel at the same time; $^{+}P<0.05$, $^{++}P<0.01$ vs. Vel/LXM-10 at the same time.

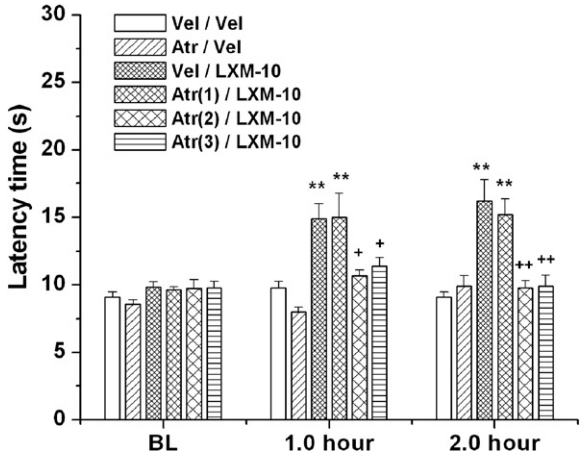

Fig. 7. Effect of atropine on the LXM-10-induced antinociception in the hot-plate test. The mice were pretreated with atropine [Atr (1), (2), and (3) represent the doses of 0.2, 1.0, and 5.0 mg/kg, i.p., respectively] or saline (Vel), 15 min before administration of LXM-10 (6.0 mg/kg, s.c.) or saline. Mice were tested by basal latency (BL) and 1.0 and 2.0 h response latency after the second injection. Each column represents the latency time as mean±S.E.M of 10 mice per group. Repeated-measures ANOVA followed by LSD was used to test the difference between groups. $^{**}P<0.01$ vs. Vel/Vel at the same time; $^{+}P<0.05$, $^{++}P<0.01$ vs. Vel/LXM-10 at the same time.

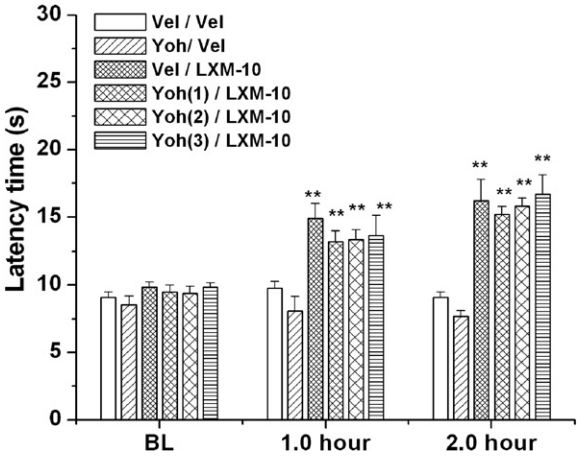

Fig. 9. Effect of yohimbine on the LXM-10-induced antinociception in the hot-plate test. The mice were pretreated with yohimbine [Yoh (1), (2), and (3) represent the doses of 1.0, 2.5, and 5.0 mg/kg, i.p., respectively] or saline (Vel), 15 min before administration of LXM-10 (6.0 mg/kg, s.c.) or saline. Mice were tested by basal latency (BL) and 1.0 and 2.0 h response latency after the second injection. Each column represents the latency time as mean±S.E.M of 10 mice per group. Repeated-measures ANOVA followed by LSD was used to test the difference between groups. $^{**}P<0.01$ vs. Vel/Vel at the same time.

5.0 mg/kg, i.p.) also blocked the effect in a dose-dependent manner. Repeated-measures ANOVA showed significant differences for treatment groups [$F(5,54)=15.0$, $P<0.001$], times [$F(2,108)=40.6$, $P<0.001$], and interaction between treatment groups and times [$F(10,108)=5.19$, $P<0.001$] (Fig. 6). Mecamylamine and hexamethonium did not affect the latency time at any time point in the experiment.

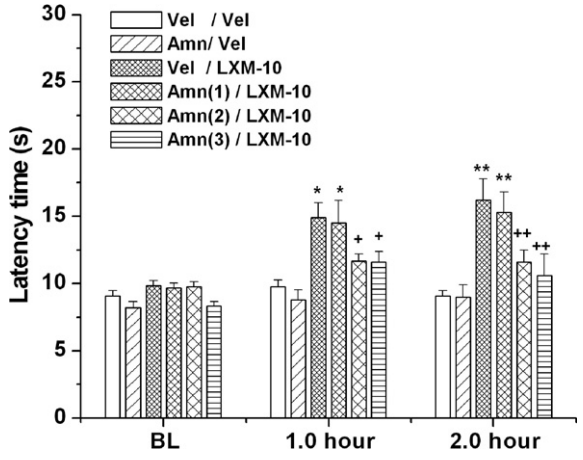

Fig. 8. Effect of atropine methylnitrate on the LXM-10-induced antinociception in the hot-plate test. The mice were pretreated with atropine methylnitrate [Amn (1), (2), and (3) represent the doses of 0.2, 1.0, and 5.0 mg/kg, i.p., respectively] or saline (Vel), 15 min before administration of LXM-10 (6.0 mg/kg, s.c.) or saline. Mice were tested by basal latency (BL) and 1.0 and 2.0 h response latency after the second injection. Each column represents the latency time as mean±S.E.M of 10 mice per group. Repeated-measures ANOVA followed by LSD was used to test the difference between groups. $^{*}P<0.05$, $^{**}P<0.01$ vs. Vel/Vel at the same time. $^{+}P<0.05$, $^{++}P<0.01$ vs. Vel/LXM-10 at the same time.

3.2.3. Involvement of muscarinic acetylcholine system

Fig. 7 shows that LXM-10 (6.0 mg/kg, s.c.) produced significant antinociception at both 1.0 and 2.0 h after administration. Atropine, a central and peripheral muscarinic acetylcholine receptor antagonist, did not block the effect at the lower dose (0.2 mg/kg, i.p.), but it blocked the effect completely at the higher doses (1.0 and 5.0 mg/kg, i.p.) in the hot-plate test. Repeated-measures ANOVA showed significant differences for treatment groups [$F(5,54)=15.2$, $P<0.001$], times [$F(2,108)=11.1$, $P<0.001$], and interaction between treatment groups and times [$F(10,108)=3.84$, $P<0.001$]. At the same time, atropine methylnitrate, a peripheral muscarinic acetylcholine receptor antagonist, did not block the effect at the lower dose (0.2 mg/kg, i.p.), but it blocked the effect at the higher doses (1.0 and 5.0 mg/kg, i.p.) in the hot-plate test. Repeated-measures ANOVA showed significant differences for treatment groups

Table 1
Effect of LXM-10 on the motor performance (rota-rod test) in mice

Group	Before treatment	After treatment		
		0.5 h	2.0 h	3.5 h
Saline	95.4±8.48	118.1±1.87	119.5±0.38	119.3±0.62
Diazepam (2.0 mg/kg i.p.)	102.5±6.48	48.8±14.08**	73.3±14.11**	111.7±6.70
LXM-10 (6.0 mg/kg s.c.)	96.7±9.17	106.5±8.88	110.0±5.17	116.2±3.75
LXM-10 (12.0 mg/kg s.c.)	96.6±8.93	109.6±10.37	109.3±8.15	119.6±0.38
LXM-10 (24.0 mg/kg s.c.)	101.2±9.15	112.7±5.18	117.5±2.50	119.0±1.00

The mice were treated with LXM-10 (6.0, 12.0, or 24.0 mg/kg, s.c.), diazepam (2.0 mg/kg, i.p.), or saline.
The results are given as the mean±S.E.M of 8 mice per group. Repeated-measures ANOVA followed by LSD was used to test the difference between groups. $^{**}P<0.01$ vs. saline group at the same time.

Table 2
Effect of LXM-10 on spontaneous activities (open-field test) in mice

Group	Before treatment	After treatment		
		0.5 h	2.0 h	3.5 h
Saline	45.3±2.53	40.2±2.51	41.6±2.42	41.5±4.00
Diazepam (2.0 mg/kg i.p.)	51.5±5.15	5.37±1.64**	10.8±3.10**	18.2±3.61*
LXM-10 (6.0 mg/kg s.c.)	46.8±1.96	46.6±2.63	42.3±3.38	48.0±5.00
LXM-10 (12.0 mg/kg s.c.)	47.7±2.55	48.0±6.21	48.1±6.14	48.0±7.85
LXM-10 (24.0 mg/kg s.c.)	50.2±2.31	43.5±4.21	45.5±6.05	46.6±3.69

The mice were treated with LXM-10 (6.0, 12.0, or 24.0 mg/kg, s.c.), diazepam (2.0 mg/kg, i.p.), or saline.
The results are given as the mean±S.E.M of 8 mice per group. Repeated-measures ANOVA followed by LSD was used to test the difference between groups. *$P<0.05$, **$P<0.01$ vs. saline group at the same time.

[$F(5,54)=7.05$, $P<0.001$], times [$F(2,108)=15.4$, $P<0.001$], and interaction between treatment groups and times [$F(10,108)=2.28$, $P<0.001$] (Fig. 8). Atropine and atropine methylnitrate did not affect the latency time at any time point in the experiment.

3.2.4. Involvement of α_2-adrenoceptor system

Fig. 9 shows that LXM-10 (6.0 mg/kg, s.c.) produced significant antinociception at both 1.0 and 2.0 h after administration and the α_2-adrenoceptor antagonist yohimbine (1.0, 2.5, and 5.0 mg/kg, i.p.) did not block the effect in the hot-plate test. Repeated-measures ANOVA showed significant differences for treatment groups [$F(5,54)=19.6$, $P<0.001$], times [$F(2,108)=41.1$, $P<0.001$], and interaction between treatment groups and times [$F(10,108)=5.06$, $P<0.001$].

3.3. Assessment of motor performance, spontaneous activity, and body temperature

The compound LXM-10 (6.0, 12.0, and 24.0 mg/kg, s.c.) had no significant effect on motor performance in the rota-rod test or spontaneous activity in the open-field test; nor did it decrease body temperature at 0.5, 2.0, or 3.5 h after administration in mice. However, diazepam (2.0 mg/kg, i.p.) significantly reduced performance and activity and decreased body temperature in mice (Tables 1, 2, and 3). The outcome of repeated-measures ANOVA relative to the falling time, the number of crossings, and body temperature are presented in Table 4.

Table 3
Effect of LXM-10 on the rectal temperature in mice

Group	Before treatment	After treatment		
		0.5 h	2.0 h	3.5 h
Saline	37.1±0.18	37.4±0.25	37.1±0.25	37.1±0.21
Diazepam (2.0 mg/kg i.p.)	37.6±0.17	35.1±0.26**	35.9±0.35**	36.5±0.31
LXM-10 (6.0 mg/kg s.c.)	37.7±0.34	37.3±0.35	37.3±0.28	37.5±0.29
LXM-10 (12.0 mg/kg s.c.)	37.1±0.09	37.0±0.26	37.1±0.24	37.1±0.20
LXM-10 (24.0 mg/kg s.c.)	37.0±0.18	37.5±0.21	37.0±0.19	37.3±0.16

The mice were treated with LXM-10 (6.0, 12.0, or 24.0 mg/kg, s.c.), diazepam (2.0 mg/kg, i.p.), or saline.
The results are given as the mean±S.E.M of 8 mice per group. Repeated-measures ANOVA followed by LSD was used to test the difference between groups. **$P<0.01$ vs. saline group at the same time.

Table 4
Outcomes of repeated-measures of ANOVAs in the rota-rod test, the open-field test, and body temperature

Experiment	Group	Time	Group * time
Rota-rod test	$F(4,35)=5.66$, $P=0.001$	$F(3,105)=7.65$, $P<0.001$	$F(12,105)=4.74$, $P<0.001$
Open-field test	$F(4,35)=14.2$, $P<0.001$	$F(3,105)=11.4$, $P<0.001$	$F(12,105)=6.33$, $P<0.001$
Body temperature	$F(4,35)=8.26$, $P<0.001$	$F(3,105)=3.85$, $P=0.012$	$F(12,105)=4.81$, $P<0.001$

4. Discussion

We assessed the antinociceptive effect of the new compound LXM-10 by using the acetic acid-induced abdominal constriction test and the hot-plate test. According to our findings, LXM-10 produced a dose-related antinociceptive effect with maximal inhibition of 79.2% by s.c. administration in the acetic acid-induced abdominal constriction test. The compound shows similar efficacy at 3.0 and 6.0 mg/kg in the assay, which suggests that the effect is close to its maximal efficacy. In the hot-plate test, LXM-10 increased the latency time in a dose-dependent and time-dependent manner. The antinociceptive effect began at 1.0 h, peaked at 2.0 h, and persisted 3.0 h after s.c. administration and maximally increased the latency time by 126.8%. The dose of LXM-10 in the hot-plate test was higher than that used in the abdominal constriction assay because of the different sensitivity of the two pain models to the compound.

In order to explore the antinociceptive mechanism of LXM-10, we undertook the naloxone antagonism test. In this experiment, the non-selective opioid receptor antagonist naloxone (2.0 mg/kg, i.p.) completely blocked the antinociceptive effect of morphine but did not block the effect of LXM-10. The result indicated that the opioid system was not involved in the antinociception of LXM-10, which means that LXM-10 may lack the problems associated with opioid analgesia.

The antinociceptive effects produced by neuronal nicotinic or muscarinic acetylcholine receptor agonists have been widely studied (James, 1999; Jürgen et al., 2003; Davis et al., 1932; Michael et al., 2004). The results indicated that the antinociceptive effect of LXM-10 was related to peripheral neuronal nicotinic acetylcholine receptors since the antinociception of LXM-10 was blocked by mecamylamine and hexamethonium. Although neuronal nicotinic agonist-induced antinociception on the hot-plate test is thought to be predominantly central, the pain pathway extends from the primary receptive fields in the periphery to the higher centers of the brain, and the pain signal transmission along

this pathway can be modulated at many levels, both peripheral and central. Neuronal nicotinic acetylcholine receptors are present in the central and peripheral nervous systems and non-neuronal systems such as muscle cells, macrophages, and skin cells (Le Novere and Changeux, 1995; Lindstrom, 2000; Khan et al. 2003; Gotti and Clementi, 2004). The antinociception of neuronal nicotinic acetylcholine receptors is mainly attributed to subtype $\alpha_4\beta_2$, and α_7, α_3 (Marubio et al., 1999; Bannon et al., 1998). So it is possible that LXM-10 acts on the peripheral neuronal nicotinic acetylcholine receptors.

The analgesic effects of muscarinic receptor agonists depend not only on central systems but also on peripheral systems, in which M_2 and M_4 subtypes play an important role (Hartvig et al., 1989; Iwamoto and Marion, 1993; Naguib and Yaksh, 1997; Swedberg et al., 1997; Bernardini et al., 2001a,b; Jürgen, et al., 2003). Our results show that the antinociceptive effect of LXM-10 is blocked by atropine and atropine methylnitrate, which suggests that the antinociceptive effect of LXM-10 may be related to peripheral muscarinic acetylcholine receptors. Besides, activation of α_2-adrenoceptors can elicit antinociception (Millan, 2002), but α_2-adrenoceptors are not believed to be involved in the antinociception of LXM-10 because the selective α_2-adrenoceptor antagonist yohimbine failed to affect the antinociception of LXM-10 in the study.

In addition to analgesic effects, nicotinic and muscarinic agonists are associated with side effects. For example, (−)-nicotine, (+)-epibatidine, and DMPP reduced motor activity and decreased body temperature; oxotremorine and arecoline produced tremor, incoordination, and polysialia (Bannon et al., 1995; Decker et al., 1994; Elisabetta et al., 2001). Consequently, we assessed the effect of LXM-10 on motor coordination in the rota-rod test, spontaneous activity in the open-field test, and body temperature. The results show that LXM-10 did not affect these behaviors and body temperature, nor did we observe significant changes in animals' gross behavior or polysialia at the minimal lethal dose (445.0 mg/kg, s.c.) in acute toxicity tests, which suggests that LXM-10 does not produce the typical side effects of muscarinic or nicotinic agonists. However, these results show that the antinociceptive effects of LXM-10 are not artifacts related to motor effects or hypothermia.

Activation of either muscarinic or nicotinic receptors alone has been reported to be sufficient to produce antinociception (Michael et al., 2004; Jürgen et al., 2003). We found that antagonizing either the muscarinic or nicotinic receptors was fully effective in blocking the antinociception of LXM-10, which implies that activation of both receptors were required. We assume that a peripheral pain modulating point F is activated by both muscarinic and nicotinic receptor agonists. There are reports that nicotinic receptor agonists epibatidine and ABT-594 produced antinociceptive activity by predominantly activating the $\alpha_4\beta_2$ subtype (Bannon et al., 1998), whereas the nicotinic receptor agonists GTS-21 and ABT-089 did not produce antinociception (Sullivan et al., 1997), though they were also activating the $\alpha_4\beta_2$ subtype, which suggests that $\alpha_4\beta_2$, may not be the only ultimate pain modulation site. Therefore, LXM-10 may activate point F by acting on muscarinic and nicotinic receptors, but we cannot say which subtype plays the most important role, and we will explore this question further in future study. In addition, LXM-10 did not produce side effects, which suggests that the analgesic effect induced by point F may avoid typical side effects of muscarinic or nicotinic agonists.

In conclusion, the compound LXM-10 produced antinociception in chemical and thermal models of nociception in mice without significant side effects. In addition, the antinociceptive effect of LXM-10 was achieved by activating peripheral neuronal nicotinic acetylcholine and muscarinic acetylcholine receptors, but the effect did not relate to opioid receptors or α_2-adreneceptors. Our findings with LXM-10 suggest that the study of spirocyclopiperazinium derivatives may provide insight on new analgesics.

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