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Analgesic activity of ZC88, a novel N-type voltage-dependent calcium channel blocker, and its modulation of morphine analgesia, tolerance and dependence

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Abstract

ZC88 is a novel non-peptide N-type voltage-sensitive calcium channel blocker synthesized by our institute. In the present study, the oral analgesic activity of ZC88 in animal models of acute and neuropathic pain, and functional interactions between ZC88 and morphine in terms of analgesia, tolerance and dependence were investigated. In mice acetic acid writhing tests, ZC88 (10–80 mg/kg) administered by oral route showed significant antinociceptive effects in a dose-dependent manner. The ED_{50} values of ZC88 were 14.5 and 14.3 mg/kg in male and female mice, respectively. In sciatic nerve chronic constriction injury rats, mechanical allodynia was ameliorated by oral administration of ZC88 at doses of 14, 28 and 56 mg/kg, suggesting ZC88 relieved allodynic response of neuropathic pain. When concurrently administered with morphine, ZC88 (20–80 mg/kg) dose-dependently potentiated morphine analgesia and attenuated morphine analgesic tolerance in hot-plate tests. ZC88 also prevented chronic exposure to morphine-induced physical dependence and withdrawal, but not morphine-induced psychological dependence in conditioned place preference model. These results suggested that ZC88, a new non-peptide N-type calcium channel blocker, had notable oral analgesia and anti-allodynia for acute and neuropathic pain. ZC88 might be used in pain relief by either application alone or in combination with opioids because it enhanced morphine analgesia while prevented morphine-induced tolerance and physical dependence.

Keywords: N-type voltage-sensitive calcium channel blocker; Analgesia; Anti-allodynia; Morphine; Tolerance; Dependence

1. Introduction

Opioids are widely used in pain relief, but their clinical use is greatly limited by the tolerance and dependence produced by prolonged administration. To avoid addiction, researchers have focused on developing novel analgesics acting on non-opioid receptors. Voltage-sensitive calcium channels regulate intracellular calcium concentration, which affects various important cellular functions such as cellular excitability, neurotransmitter release, hormone secretion, intracellular metabolism, and gene expression (Hess, 1990; Olivera et al., 1994; Miljanich and Ramachandran, 1995). Based on their electrophysiological and pharmacological properties, the voltage-sensitive calcium channels are classified into six subtypes (T, L, N, P, Q and R). N-type calcium channels encoded by $Ca_v2.2$ gene are mainly located on presynaptic terminals in central and peripheral neurons and directly mediate spinal transmission of pain signals from the peripheral to the central nervous system (Miljanich and Ramachandran, 1995). Thus, N-type calcium channels are considered as a potential target for the treatment of pain.

Ziconotide (SNX-111, Prialt), a 25-amino acid residue polypeptide, is the synthetic form of ω-conotoxin MVIIA. Ziconotide selectively blocks N-type voltage-sensitive calcium channels with high potency, and shows antinociceptive activity in both preclinical and clinical trials when administered

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intrathecally (Miljanich, 2004; Wermeling, 2005; Prommer, 2006; Lynch et al., 2006). FDA has been filed ziconotide for its application in pain management. Despite its potent analgesia in clinic application, ziconotide has some obvious undesirable properties, including neuropsychiatric adverse effects and a risk of meningitis due to possible contamination of the microinfusion device. Therefore, current efforts have been focused on the development of non-peptide, small molecular inhibitors of N-type calcium channels. Some small molecular N-type calcium channel blockers have been reported (Hu et al., 2000; Seko et al., 2003; Teodori et al., 2004). We designed and synthesized a series of compounds with a 4-aminopiperidine scaffold and got a patent. Among these compounds, ZC88 inhibited calcium currents mediated by N-type calcium channel with high potency and high selectivity. It blocked recombinant human N-type voltage-sensitive calcium channels ($\alpha_{1B}/\beta_{1b}/\alpha_2\delta$) transiently expressed in HEK-293 cells with 0.45±0.09 μM of IC_{50} value (Zhang et al., in review). However, up to 100 μM ZC88 had no effect on the L-type ($\alpha_{1C}/\beta_{1b}/\alpha_2\delta$), R-type ($\alpha_{1E}/\beta_{1b}/\alpha_2\delta$) and P/Q-type ($\alpha_{1A}/\beta_{1b}/\alpha_2\delta$) voltage-sensitive calcium channel currents transiently expressed in *Xenopus* oocytes (unpublished data), as well as on the voltage-gated sodium and potassium channel currents in primary cultured hippocamal neurons of rat (Zhang et al., in review). Therefore, the present study investigated the oral analgesic activity of ZC88 in animal models of acute and chronic neuropathic pain. To learn more about the possible functional interactions between N-type calcium blockers and conventional opioid analgesics, the effects of ZC88 on morphine analgesia, tolerance and dependence were investigated as well.

2. Materials and methods

2.1. Animals

Kunming mice and Sprague-Dawley (SD) rats were obtained from Beijing Animal Center (Beijing, China) and housed at an ambient temperature of 22±1 °C and relative humidity of 50–60%. Animals were maintained on a 12 h light/dark cycle (lights on between 7:00 A.M. and 7:00 P.M.) and given ad libitum access to food and water, in strict compliance with the guidelines set for the use of experimental animals by the International Association for the Study of Pain (IASP) and the local Committee on Animal Care and Use.

2.2. Chemicals

ZC88 (*N*-[1-(2,3-dimethoxyphenyl)-4-methyl-3-pentene-1-yl]-1-(5-bromo-furfuryl)- 1-piperidyl-4-amine dihydrochloride) and ZC1 ((4-benzyloxy-phenyl)-[1-(4- dimethylamino-benzyl)-piperidin-4-yl]-(3-methyl-but-2-enyl)-amine) (Hu et al., 2000) were synthesized by Beijing Institute of Pharmacology and Toxicology. Morphine was purchased from Qinghai Pharmaceutical Factory (Xining, China). Naloxone was obtained from Sigma-Aldrich Sigma Chemical Company (St. Louis, MO, USA). All drugs were dissolved in 0.9% saline. Drug administrations were made in a volume of 10 ml/kg. [^{3}H] diprenorphine (50 Ci/mmol) was purchased from PerkinElmer Life Sciences (NEN™, Boston, MA, USA).

2.3. Antinociceptive tests

2.3.1. Acetic acid writhing tests

Male and female Kunming mice weighing 18–22 g were injected with 0.4 ml of 0.6% acetic acid (ip). ZC88 or ZC1 (10 to 80 mg/kg) was administrated by oral route 40 min prior to acetic acid injection. The number of writhing, characterized by a wave of contraction of the belly followed by extension of the hind limbs, was counted within 15 min after i.p. injection of acetic acid 5 min. ED_{50} values were calculated by the method of Bliss (probit regression analysis). This method maximizes the log-likelihood function to fit a parallel set of Gaussian sigmoid curves to the dose–response data, and provides ED_{50} values and 95% confidence intervals (Bliss, 1967). To study the role of opioid receptors in ZC88 analgesia, naloxone (1 mg/kg, sc) was injected 15 min before ZC88 administration.

2.3.2. Hot-plate tests

Female Kunming mice weighing 18–22 g were individually placed on a hot-plate maintained at 55±0.2 °C and the time of licking of the hind paws was recorded as the latency period. The cut-off time was 60 s to avoid tissue damage. Before drug administration, baseline latency was examined. Animals with baseline latency lower than 5 s or above 30 s were excluded. After ZC88 administration (po) 40 min, the paw withdrawal latency was tested again. The maximum possible effect (MPE) was calculated as: MPE%=(latency after drug administration −baseline latency)/(60−baseline latency)×100.

To observe the effect of ZC 88 on morphine analgesia, vehicle (saline, 10 ml/kg, po) or ZC 88 (80 mg/kg, po) was administrated approximately 15–20 min before morphine injection (2.5 mg/kg, sc). 30 min after morphine injection, the latency was tested.

2.3.3. Mechanical allodynia tests in chronic constriction injury of the sciatic nerve in rats

The sciatic nerve chronic constriction injury model was made according to the method described by Bennett and Xie (1988). Briefly, male SD rats (180–220 g) were anesthetized with sodium pentobarbital (60 mg/kg, i.p.), and the left common sciatic nerve was exposed at the level of the middle of the thigh, and four ligatures were loosely tied around the nerve by using 4-0 chromic gut sutures with 1 mm space. The surgical incision site was sutured and disinfected. Sham operation was performed in the same manner except for sciatic nerve ligation. On the post-operative day 14, mechanical allodynia was evaluated by application of von Frey hairs (0.4–26 g, North Coast Medial Inc., San Jose, CA, USA) according to previous report (Yang et al., 2004). Each von Frey hair was applied to the plantar surface of the left hind paw ten times. The cut-off force was 26 g. The 50% paw withdrawal threshold (g) was taken as the lowest force that caused 4–6 withdrawals out of the ten consecutive stimuli. Before and after vehicle or ZC88

administration of 30–40 min, the 50% paw withdrawal threshold was tested respectively. The MPE was calculated as: MPE%=(50% paw withdrawal threshold after drug administration−50% paw withdrawal threshold before drug administration)/(cut-off point (26 g)−50% paw withdrawal threshold before drug administration)×100.

2.4. Chronic morphine tolerance in mice

The antinociceptive effects of morphine were determined under acute conditions by hot-plate test. After the mice received morphine (10 mg/kg, sc) and tested, a chronic administration regimen was initiated that consisted of morphine (30 mg/kg, sc) in combination with vehicle (0.9% saline, 10 ml/kg, po) or ZC88 (20, 40, 80 mg/kg, po). Vehicle or ZC 88 was administered 30 min prior to morphine injection. Mice received these treatments three times daily for 3 consecutive days. 12 h after the last administration, the antinociceptive effects of morphine (10 mg/kg, sc) were re-determined.

2.5. Morphine dependence test in mice

2.5.1. Morphine-induced physical dependence and withdrawal

Male Kunming mice weighing 18–22 g received ascending doses of morphine injections three times daily for 5 consecutive days (day 1: 30 mg/kg, day 2: 40 mg/kg, day 3: 50 mg/kg, day 4: 60 mg/kg, day 5: 70 mg/kg, sc). 6 h after the last morphine administration, naloxone (5 mg/kg, ip) was injected and the number of jumping was counted within 30 min. To observe the effect of ZC 88 on the development of morphine dependence, vehicle or ZC 88 (20, 40, 80 mg/kg, po) was administrated 30 min prior to morphine injection. In order to assess the effect of ZC88 on the expression of morphine dependence, mice received vehicle or ZC88 (20, 40, 80 mg/kg, po) every 2 h for three times and the first vehicle or ZC88 administration was given 2 h after the last morphine treatment. 30 min after the last vehicle or ZC88 administration, naloxone (5 mg/kg, ip) was injected.

2.5.2. Morphine-induced conditioned place preference

Male Kunming mice weighing 18–22 g were used for the experiment. The place preference apparatus consisted of two equal-size compartments (30×30×52.5 cm) connected by a communicating tunnel (30×12×52.5 cm). The compartments had walls of different colors (black *vs* white) and also distinct floor textures (wide grid in the black compartment and fine mesh in the white compartment). Access to the tunnel could be blocked by a removable partition.

Conditioned place preference procedure consisted of three phases: preconditioning (day 1–day 3), conditioning (day 4–day 11), and postconditioning (day 12). During the preconditioning phase (day 1–day 3), mice were given free access to all compartments of the apparatus for 15 min each day. On day 3, the time spent by animals in each compartment was recorded. Those few which showed a strong unconditioned preference (more than 600 s) for any compartment were discarded, as animals used in this experiment did not typically show an unconditioned preference for either of the compartments (395.0±74.5 s in white side *vs* 384.0±86.1 s in black side). Therefore, the unbiased method was used in this study. During the conditioning phase of 8 consecutive days (day 4–day 11), the animals were conditioned by vehicles and drugs. For conditioning, mice were treated with drugs (morphine or ZC88) and confined to one of the two compartments. Following vehicle (saline) treatment, they were confined to the other compartment. Conditioning sessions (eight drug and eight saline sessions), each 45 min in duration, were conducted over 8 days. Mice received alternate two trials daily with at least 6 h interval between the drug and saline training sessions. During the testing (postconditioning) phase (day 12), the mice were placed in the apparatus for 15 min with free access to both compartments and the time spent in each compartment was recorded. Meanwhile, the locomotor activity was measured.

To find out whether ZC88 alone could induce conditioned place preference, mice were treated with vehicle or ZC88 (10–100 mg/kg, po) 30 min before conditioning training. To evaluate the effect of ZC88 on morphine-induced conditioned place preference, vehicle or ZC88 (10–100 mg/kg, po) was administrated 30 min prior to morphine (10 mg/kg, sc) injection. After morphine administration of 5 min, conditioning training was carried out.

2.6. Measurement of locomotor activity in open field

Male and female Kunming mice weighing 18–22 g were used for the experiment and the experiment was performed between 8:00 A.M. and 12:00 A.M. The locomotor activity in open field was carried out in the SuperState v3.0 system (AniLab Software & Instruments Co., Ningbo, China). The activity chambers (27×30×26 cm, width×length×height) were placed in soundproof boxes with dim illumination and a fan. Horizontal locomotor activity was recorded by the breaking of infrared beams. To assess the effect of ZC88 on locomotor activity, mice received ZC88 (10, 40, 80 mg/kg, po) administration 30 min and then placed in the activity chambers individually. After 5-min acclimatization, the locomotor activity was recorded for 45 min.

2.7. Membrane preparation and [^{3}H]diprenorphine binding assays

Male SD rats weighing 200–250 g were scarified and their brain was quickly excised into ice-cold normal saline. The cerebellum was removed, and remainder of the brain was homogenized in the ice-cold homogenization buffer (320 mM sucrose, 50 mM Tris–HCl, pH 7.6) containing 0.1 mM phenylmethyl sulfonylfluoride (w/v, 1/10). The homogenate was centrifugated at 1000 g, 4 °C for 10 min. The supernatant was centrifugated at 40,000 g, 4 °C for 20 min. The pellets were resuspended in the ice-cold homogenization buffer to homogenize, and centrifugated again (4 °C, 40,000 g, 20 min). The pellets were resuspended in ice-cold 50 mM Tris–HCl buffer (pH 7.5) and the protein content was determined by Bradford method (Bradford, 1976).

[^{3}H]diprenorphine binding assay was carried out in a total volume of 0.5 ml, which contained 0.2 mg membrane preparation and 50 mM Tris–HCl buffer (pH 7.5). The final concentration of [^{3}H]diprenorphine was 1 nM and naloxone (10 μM) was used to define specific binding to opioid receptors. Competitor was 10 μM ZC88. After 30 min incubation at 37 °C, bound and free [^{3}H]diprenorphine were separated by filtration with GF/C filters under reduced pressure, and the filters were washed rapidly three times with 50 mM Tris–HCl buffer (pH 7.5). Radioactivity in filters was determined by liquid scintillation counting.

2.8. Statistical analyses

Data were expressed as mean±S.E.M. Statistical analyses were done with Student *t*-test or one-way ANOVA with Dunnett's *t*-test for two groups or multiple comparisons, respectively.

3. Results

3.1. Analgesia of ZC88 in acetic acid writhing tests

In mice acetic acid writhing tests, both ZC1, an N-type Ca^{2+} channel blocker previously reported, and ZC88 administered by oral route showed significant antinociceptive effects in a dose-dependent manner (Fig. 1A). In male mice, the ED_{50} value of ZC88 for analgesia was 14.5 mg/kg ([10.0, 18.6], 95% confidence intervals), which was lower than that of ZC1 with 27.2 mg/kg ([23.5, 31.1], 95% confidence intervals) (*U*-test, $U=3.618$, $P<0.01$). In female mice, the ED_{50} value of ZC88 was 14.3 mg/kg ([9.3, 18.7], 95% confidence intervals), which was similar to that of ZC1 with 18.4 mg/kg ([15.0, 21.7], 95% confidence intervals) (*U*-test, $U=1.263$, $P>0.05$). Our previous study showed that ZC88 (0.01–10 μM) blocked N-type calcium channel currents in a concentration-dependent manner and the IC_{50} value of ZC88 (0.45±0.09 μM) was similar to that of ZC1 (0.34±0.04 μM) (Zhang et al., in review). This result suggested that ZC88, a novel N-type calcium channel blocker, had oral activity for analgesia.

Though ZC88 has been proved to block N-type calcium channels, whether it binds to opioid receptors or not is unclear. In [^{3}H]diprenorphine, an opioid receptor antagonist, binding assay, no binding of ZC88 (up to 10 μM) to opioid receptors was detected. Additionally, opioid receptor antagonist naloxone failed to block the analgesic activity of ZC88 (80 mg/kg, po) in acetic acid writhing tests in both male and female mice, while it completely abolished the analgesia of morphine (Fig. 1B). These indicated that the analgesic

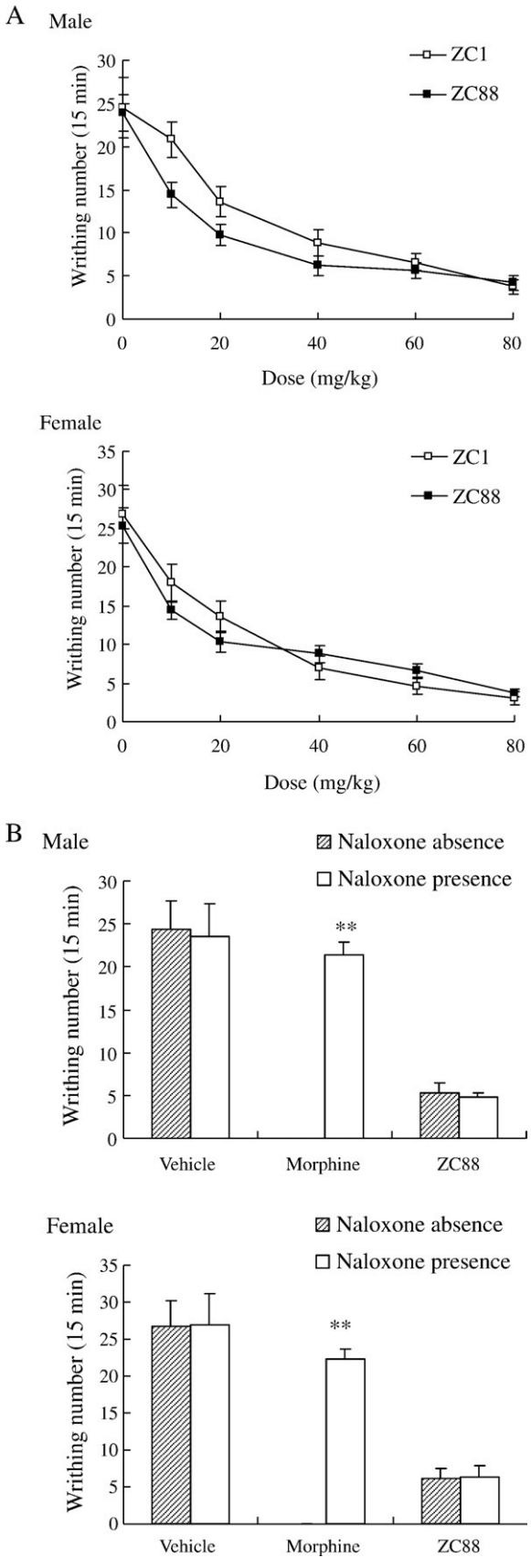

Fig. 1. Analgesia of ZC88 in mice acetic acid writhing tests. (A) Analgesia of ZC88 and ZC1 in male and female mice. ZC88 or ZC1 orally administered 40 min prior to acetic acid injection. Data were expressed as mean±S.E.M. $n=10$. (B) Effect of naloxone on analgesia of ZC88 in male and female mice. Naloxone (1 mg/kg) was injected (sc) 15 min before ZC88 (80 mg/kg, po) administration. Data were expressed as mean±S.E.M. $n=10$. $^{**}P<0.01$, *vs* naloxone absence, Student *t*-test.

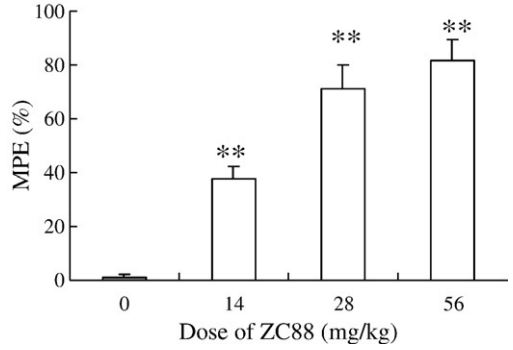

Fig. 2. Effect of ZC88 on mechanical allodynia in the rats with chronic constriction injury of the sciatic nerve. Data were expressed as mean±S.E.M. $n=11$. $^{**}P<0.01$, *vs* saline, one-way ANOVA with Dunnett's *t*-test.

activity of ZC88 was not mediated by activation of opioid receptors.

3.2. The amelioration of allodynia by ZC88 in chronic constriction injury of the sciatic nerve in rats

On the post-operative day 14, rats with chronic constriction injury of the sciatic nerve displayed mechanical allodynia as shown by the reduction in 50% paw withdrawal threshold to 2 g or lower, compared with that of 15–26 g in sham operated rats. When rats were orally administered with ZC88 at doses of 14, 28 and 56 mg/kg, mechanical allodynia induced by chronic constriction injury of the sciatic nerve was ameliorated in a dose-dependent manner (Fig. 2). The 50% paw withdrawal threshold increased from 2 g to 11.09, 19.09 and 21.55 g at ZC88 of 14, 28, and 56 mg/kg, respectively. And the 50% paw withdrawal threshold of the contralateral paws was 26 g which wasn't altered by ZC88 (14–56 mg/kg, po) treatment. No behavioral abnormalities were observed at any dose of ZC88 throughout the observation period.

3.3. The potentiation of ZC88 to morphine analgesia in hot-plate tests

In mice 55 °C hot-plate tests, a heat-stimulated acute pain model, morphine (2.5 mg/kg, sc) produced an obvious analgesic effect. ZC88 (20–80 mg/kg, po) given alone had no significant analgesia (Fig. 3A), while it enhanced morphine analgesia in a dose-dependent manner (Fig. 3B). When ZC88 (40 and 80 mg/kg) was concurrently administrated with morphine, the MPE of morphine (2.5 mg/kg, sc) increased from 17.9% to 41.8% and 59.1%, respectively.

3.4. The attenuation of ZC88 to morphine tolerance

Before chronic morphine administration, morphine (10 mg/kg, sc) produced absolute analgesia in mice hot-plate assay. Repeated administration of morphine at the dose of 30 mg/kg (sc, tid) for 3 days induced tolerance to analgesia, in which the MPE of morphine (10 mg/kg, sc) decreased from 100% to 26.2%. When ZC88 co-pretreated with morphine, ZC88 (20–80 mg/kg) attenuated morphine tolerance in a dose-dependent

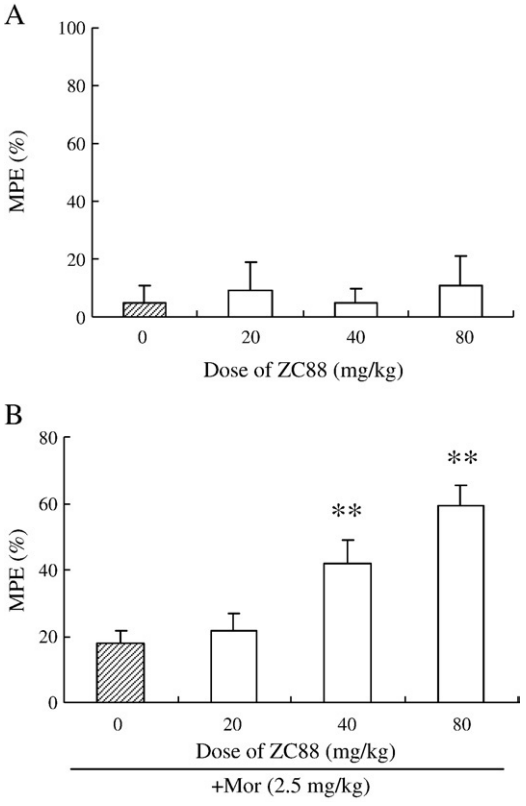

Fig. 3. The potentiation of ZC88 to morphine analgesia in 55 °C hot-plate tests. (A) Effect of ZC88 alone on analgesia. ZC88 orally administered 40 min prior to test. Data were expressed as mean±S.E.M. $n=15–18$. (B) The potentiation of ZC88 to morphine analgesia. ZC88 administered approximately 15–20 min (po) prior to morphine injection (2.5 mg/kg, sc). After morphine injection 30 min, the latency was tested. Data were expressed as mean±S.E.M. $n=20$. $^{**}P<0.01$, *vs* morphine alone (ZC88 0 mg/kg), one-way ANOVA with Dunnett's *t*-test.

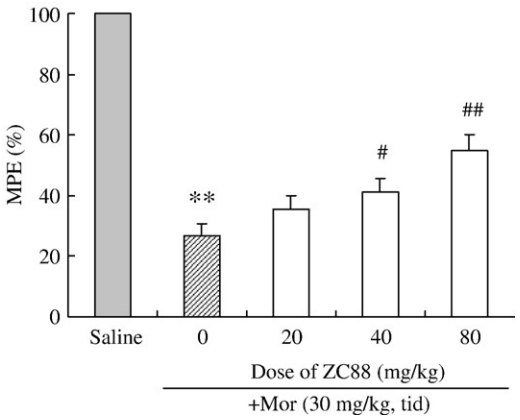

Fig. 4. The attenuation of ZC88 to morphine tolerance in 55 °C hot-plate tests. Mice received morphine (30 mg/kg, sc) in combination with vehicle (0.9% saline, 10 ml/kg, po) or ZC88 (20, 40, 80 mg/kg, po) three times daily for 3 consecutive days. Saline group: mice received saline (10 ml/kg, sc, tid) for 3 days and then received a challenge dose of morphine (10 mg/kg, sc) to determine the morphine analgesia. Data were expressed as mean±S.E.M. $n=35–36$. $^{**}P<0.01$, *vs* saline-pretreated group (saline), Student *t*-test. $^{\#}P<0.05$ and $^{\#\#}P<0.01$, *vs* morphine alone-pretreated group (ZC88 0 mg/kg), one-way ANOVA with Dunnett's *t*-test.

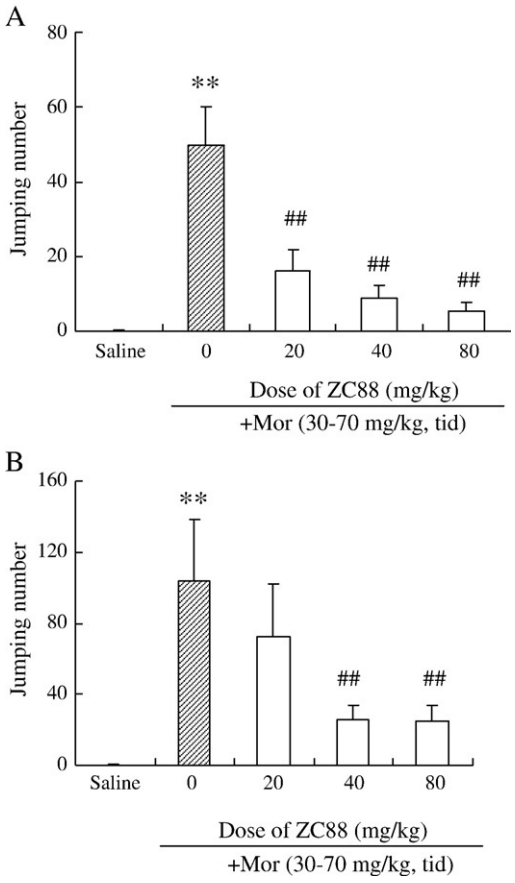

Fig. 5. The attenuation of ZC88 to morphine-induced physical dependence. (A) The prevention of ZC88 to the development of morphine-induced physical dependence. Mice received ZC88 (20–80 mg/kg, po) in combination with ascending doses of morphine (30–70 mg/kg on day 1 to day 5, sc) injections three times daily for 5 consecutive days. 6 h after the last morphine administration, naloxone (5 mg/kg, ip) was injected and the number of jumping was counted within 30 min. Data were expressed as mean±S.E.M. $n=10$. $^{**}P<0.01$, *vs* saline-pretreated group (saline), Student *t*-test. $^{\#\#}P<0.01$, *vs* morphine alone-pretreated group (ZC88 0 mg/kg), one-way ANOVA with Dunnett's *t*-test. (B) The attenuation of ZC88 to the expression of morphine-induced physical dependence. Mice received ascending doses of morphine (30–70 mg/kg on day 1 to day 5, sc) injections three times daily for 5 consecutive days. After the last morphine administration, mice received vehicle or ZC88 (20, 40, 80 mg/kg, po) every 2 h for three times and the first vehicle or ZC88 administration was given 2 h after the last morphine treatment. 30 min after the last vehicle or ZC88 administration, naloxone (5 mg/kg, ip) was injected. Data were expressed as mean±S.E.M. $n=10$. $^{**}P<0.01$, *vs* saline-pretreated group (saline), Student *t*-test. $^{\#\#}P<0.01$, *vs* morphine alone-pretreated group (ZC88 0 mg/kg), one-way ANOVA with Dunnett's *t*-test.

manner (Fig. 4), in which the analgesia of morphine (10 mg/kg, sc) increased to 41.4% and 55.0% at ZC88 40 mg/kg and 80 mg/kg, respectively.

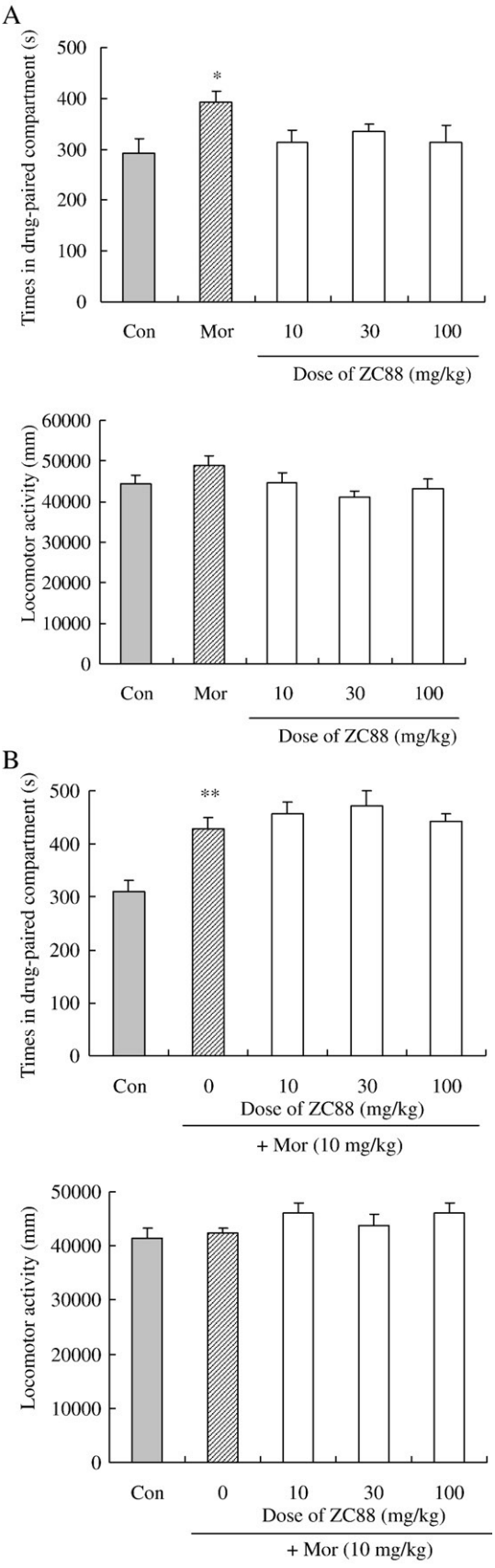

Fig. 6. Effect of ZC88 on morphine-induced conditioned place preference. (A) Effect of ZC88 alone on place conditioning. Mice were treated with vehicle or ZC88 (10–100 mg/kg, po) 30 min before conditioning training. Data were expressed as mean±S.E.M. $n=11$–12. $^{*}P<0.05$, *vs* control, Student *t*-test. (B) Effect of ZC88 on morphine-induced conditioned place preference. Vehicle or ZC88 (10–100 mg/kg, po) was administrated 30 min prior to morphine (10 mg/kg, sc) injection in conditioning sessions. After morphine administration 5 min, conditioning training was carried out. Data were expressed as mean±S.E.M. $n=12$–13. $^{**}P<0.01$, *vs* control, Student *t*-test.

3.5. The attenuation of ZC88 to morphine-induced physical dependence

Chronic administration of morphine in an ascending dose manner resulted in physical dependence, in which naloxone precipitation induced significant abstinent jumping. ZC88 (20, 40 and 80 mg/kg, po) concurrent administration with morphine significantly inhibited naloxone-precipitated abstinent jumping in a dose-dependent manner (Fig. 5A), indicating ZC88 could reverse the development of morphine-induced physical dependence.

To investigate the effect of ZC88 on naloxone-precipitated withdrawal, morphine-dependent mice received ZC88 administration for three times before naloxone precipitation. ZC88 (20–80 mg/kg, po) significantly attenuated naloxone-precipitated jumping, and the abstinent jumping number decreased from 105.8 to 25.0 at the dose of 80 mg/kg (Fig. 5B). These results suggested that ZC88 not only prevented the development of morphine-induced physical dependence, but also attenuated the expression.

3.6. The effect of ZC88 on morphine-induced conditioned place preference

Mice treated chronically with morphine (10 mg/kg, sc) for 8 days demonstrated preference to the drug-paired side ($t=4.50$, $P<0.01$, Student t-test). ZC88 (10, 30 and 100 mg/kg, po), however, failed to induce conditioned place preference or conditioned place aversion ($F(3, 43)=0.43$, $P>0.05$, one-way ANOVA) (Fig. 6A). Paring ZC88 (10, 30 and 100 mg/kg, po) with morphine (10 mg/kg, sc) during the conditioning sessions had no influence on the morphine-induced conditioned place preference ($F(3, 47)=0.83$, $P>0.05$, one-way ANOVA) (Fig. 6B). Additionally, the locomotion in the test session wasn't changed when ZC88 treated either alone or with morphine in conditioning training (Fig. 6A and B), so the observed effects could not be attributed to any changes in locomotion. These results inferred that ZC88 had no tendency of psychological dependence, and did not affect morphine-induced psychological dependence as well.

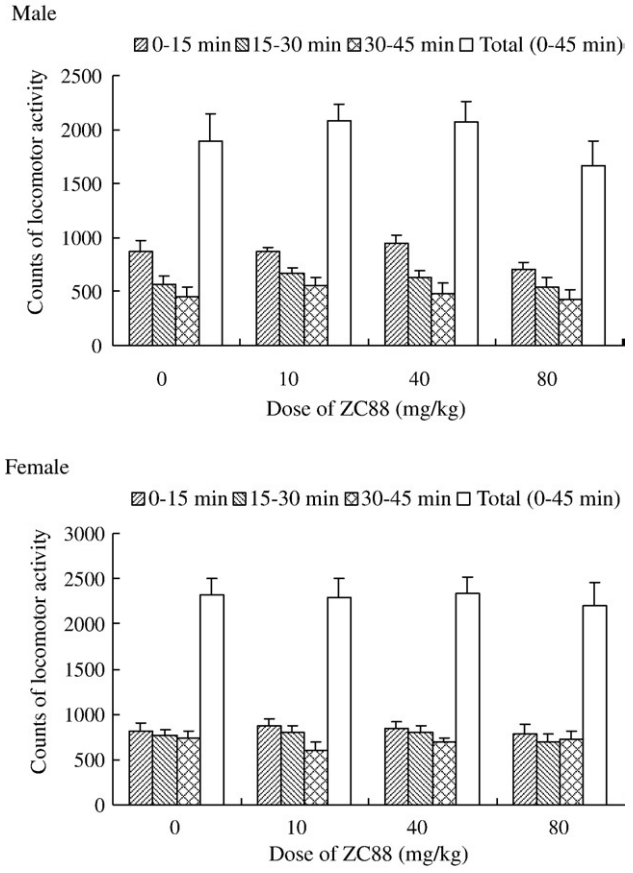

Fig. 7. Effect of ZC88 on the locomotor activity. Mice received vehicle or ZC88 (10, 40, 80 mg/kg, po) administration 30 min and then placed in the activity chambers individually. After 5-min acclimatization, the locomotor activity was recorded for 45 min. Data were expressed as mean±S.E.M. $n=10$.

3.7. The effect of ZC88 on locomotor activity

To rule out the false positive results in analgesia, the effect of ZC88 on locomotor activity was observed. In both male and female mice, ZC88 at doses of 10, 40 and 80 mg/kg did not affect locomotor activity during the 45-min observation period (males: $F(3, 36)=0.874$, $P>0.05$; females: $F(3, 36)=0.088$, $P>0.05$; one-way ANOVA) (Fig. 7).

4. Discussion

In the present study, we found that ZC88, a new N-type voltage-sensitive calcium channel blocker synthesized by ourselves, significantly inhibited acute and neuropathic pain by oral route. Moreover, ZC88 enhanced morphine analgesia, and attenuated morphine-induced tolerance and physical dependence, but not psychological dependence.

Based on the clinical observations reported on ziconitide, it is suggested that selective and orally active N-type calcium blockers could be useful for the treatment of pain. We designed and synthesized a series of 4-aminopiperidine derivatives including ZC88 as novel candidates for N-type calcium channel blockers. Our previous study proved that ZC88 is a selective N-type calcium channel blocker. In HEK293 cells transiently expressing the N-type calcium channel ($\alpha_{1B}/\beta_{1b}/\alpha_2\delta$), ZC88 reversibly inhibited calcium currents mediated by the N-type calcium channel with 0.45 μM of IC_{50} value. While up to 100 μM, ZC88 affected neither calcium currents mediated by L, P/Q, or R-type calcium channels, nor voltage-gated sodium and potassium currents. As a novel N-type calcium channel blocker with high affinity and selectivity, could ZC88 produce analgesia by the oral route? The present study showed that ZC88 not only attenuated acute pain, but also ameliorated allodynia of neuropathic pain. In acetic acid writhing tests in mice, ZC88 administered by oral route showed significant antinociceptive effects in a dose-dependent manner, in which ED_{50} values were lower than or similar to that of positive control ZC1, a 4-piperidinylaniline analog previously reported by Hu et al. (2000), in male and female mice. Since some narcotic analgesics such as fentanyl have the structure of 4-anilidopiperidine that is similar to our designed compounds, the possibility that the analgesia of ZC88 is due to binding to opioid receptors

should be considered. Naloxone, an opioid receptors antagonist, failed to antagonize the analgesic activity of ZC88 in the acetic acid writhing model. Furthermore, ZC88 did not bind to opioid receptors in ^{3}H-diprenorphine binding assays. These results suggested that the antinociception of ZC88 was mainly associated with blocking the N-type calcium channel, rather than activating opioid receptors.

Although other calcium channels are expressed in peripheral and central nervous systems, N-type calcium channels somehow dominate perception of chronic pain states (McGivern and McDonough, 2004). There are several reports on the increases in N-type calcium channel currents or expression in chronic pain states, especially neuropathic pain (Matthews and Dickenson, 2001; Cizvoka et al., 2002). Moreover, in the spinal nerve ligation model of neuropathic pain, homozygous $Ca_v2.2$ knockout mice displayed markedly reduced thermal and mechanical allodynia (Kim and Chung, 1992). Thus, N-type calcium channels might be an ideal target for treatment of neuropathic pain. In fact, ziconotide has been proved to provide pain relief to patients suffering from intractable neuropathic pain. In our present study, ZC88 (14–56 mg/kg) dose-dependently attenuated mechanical allodynia in the sciatic nerve chronic constriction injury model of neuropathic pain when administered orally. Besides ZC88, other N-type calcium channel blockers synthesized by us or reported by E. Teodori et al. also displayed noticeable anti-allodynic activity on neuropathic pain (Teodori et al., 2004). These indicate that the development of small molecular N-type calcium channel blockers for clinical use is valuable to treatment of neuropathic pain.

Opioids are conventional narcotic analgesics, but some severe side effects, especially addiction, limit their clinical use. One approach is the concomitant application of opioids and another kind of analgesics, which produces additive effect on analgesia but not on addiction. The combination of N-type calcium channel blockers and morphine might achieve this purpose. Previous studies showed that ziconotide or ω-conotoxin GVIA in combination with morphine produced additive or synergistic analgesia (Wang et al., 2000; Fukuizumi et al., 2003; Omote et al., 1996). In our present study, we found ZC88, a small molecular N-type calcium blocker, not only potentiated the analgesia of morphine, but also reduced tolerance and dependence induced by chronic exposure to morphine. Activation of μ opioid receptor by morphine and other opioids leads to inhibition of N-type calcium channels via G-protein βγ subunit by changing channel gating and altering ion permeation. Therefore, the inhibition of N-type calcium currents may be one of the mechanisms by which morphine produces analgesia. Since both ZC88 and morphine directly and indirectly inhibited N-type calcium channel activity, it can be reasonably presumed that they may act synergistically to produce analgesia. Indeed, our results showed ZC88 enhanced morphine analgesia in hot-plate tests, but whether it was a synergistic or additive effect was unclear.

Repeated administration of morphine results in tolerance to analgesia and dependence, but the mechanisms are not well understood. It has been reported that N-type voltage-sensitive calcium channels increased in mice brains after chronic morphine treatment (Suematsu et al., 1993). The inhibition of ZC88 to morphine-induced tolerance and physical dependence was observed in the present study. Since ZC88 selectively inhibited the function of N-type calcium channels, we presumed that the preventive effects of ZC88 on morphine tolerance and dependence might be due to its decreasing the up-regulation of N-type calcium channel functions during chronic morphine treatment. However, it was reported that ziconotide by intrathecal administration failed to prevent spinal morphine tolerance to analgesia (Wang et al., 2000). This contradiction between ZC88 and ziconotide might result from the differences in the administration route and characteristics of blocking N-type calcium channels. Surprisingly, ZC88 did not affect morphine-induced psychological dependence in the conditioned place preference model, though it noticeably inhibited both development and expression of morphine physical dependence. Why this happens remains unknown, and further studies are needed.

In conclusion, ZC88, a novel small molecular N-type voltage-sensitive calcium channel blocker we synthesized, produced oral activity for analgesia and anti-allodynia in acute and neuropathic pain. When it was concurrently administered with morphine, ZC88 enhanced morphine analgesia but inhibited tolerance and physical dependence. These suggested that ZC88 might be used in pain management by either application alone or in combination with opioids.

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