Lappaconitine, a C18-diterpenoid alkaloid, exhibits antihypersensitivity in chronic pain through stimulation of spinal dynorphin A expression

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Abbreviations: POMC, proopiomelanocortin; GNTI, 5’-guanidinonaltrindole; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; IL-1β, interleukin-1β; LPS, lipopolysaccharides; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GsPCR, Gs-protein-coupled receptor; MAPK, mitogen-activated protein kinase; ANOVA, analysis of variance; % MPE, % maximal possible effect; E_max, maximum effect; ED_{50} or EC_{50}, half-effective dose or half-effective concentration.
ABSTRACT

Lappaconitine is a representative C18-diterpenoid alkaloid extracted from *Aconitum Sinomontanum Nakai* and has been prescribed as a pain relief medicine in China for more than 30 years. This study evaluated its antihypersensitivity activity in the rat models of neuropathic and cancer pains and explored its underlying mechanisms. Subcutaneous injection of cumulative doses of lappaconitine produced dose-dependent mechanical antiallodynia and thermal antihyperalgesia in spinal nerve ligation-induced neuropathic rats. The cumulative dose-response analysis exhibited their $E_{max}$ values of 53.3% and 58.3% MPE, and $ED_{50}$ values of 1.1 and 1.6 mg/kg. Single intrathecal lappaconitine dose in neuropathy also dose and time dependently blocked mechanical allodynia, with an $E_{max}$ of 66.1% MPE and an $ED_{50}$ of 0.8 μg. Its multiple twice-daily intrathecal administration over 7 days did not induce mechanical antiallodynic tolerance. Subcutaneous cumulative doses of lappaconitine also produced dose-dependent blockade of mechanical allodynia in the rat bone cancer pain model induced by tibia implantation of cancer cells, with the $E_{max}$ of 57.9% MPE and $ED_{50}$ of 2.0 mg/kg. Furthermore, lappaconitine treatment stimulated spinal dynorphin A expression in neuropathic rats, and in primary cultures of microglia but not neurons or astrocytes. Intrathecal pretreatment with the specific microglia depletor liposome-encapsulated clodronate, dynorphin A antibody and κ-opioid receptor antagonist GNTI totally suppressed intrathecal and subcutaneous lappaconitine-induced mechanical antiallodynia. This study suggests that lappaconitine exhibits antinociception through directly stimulating spinal microglial dynorphin A expression.

**Keywords:** lappaconitine; neuropathic pain; bone cancer pain; dynorphin A; spinal microglia
1. Introduction

*Aconitum* plants are distributed in Asia, Europe and America and their active ingredients are alkaloids. To date, approximately 170 alkaloids have been identified from *Aconitum* plants and classified into four categories, i.e., C20-, C19-, C18-, and bis-diterpenoid alkaloids (Ameri, 1998; Wang et al., 2009a; Huang et al., 2016; Huang et al., 2017b). Lappaconitine is the main bioactive ingredient from the roots of *Aconitum Sinomontanum Nakai* and is classified as a representative C18-diterpenoid alkaloid (Ameri, 1998) and listed in Chinese Pharmacopoeia. The structures of the C18-diterpenoid alkaloid and lappaconitine are shown in Figure 1. Lappaconitine, formulated as pills, capsules, transdermal patches or injection forms, has been used clinically for more than 30 years in China to treat mild or moderate acute and chronic pains, including cancer-related pain, postoperative pain and neuropathic pain (Chen et al., 1996; Sun et al., 2009; Ou et al., 2011; Yuan and Wang, 2012; Gong and Li, 2015; Guo et al., 2015). In animal studies, systemic lappaconitine has been reported to have properties of analgesia, anti-inflammation, local anesthesia and hypothermia (Tang et al., 1983; Liu et al., 1987; Ono and Satoh, 1989; Wang et al., 2009b). Moreover, multiple daily intraperitoneal injection of lappaconitine blocked spontaneous pain in a mouse leukemia bone cancer pain model (Zhu et al., 2015).

The mechanisms underlying lappaconitine analgesia are complex and debatable. It was suggested that the analgesic effect of lappaconitine was associated with the blockage of neuronal voltage-dependent sodium channels (Ameri, 1998). It was also reported that activation of the central noradrenergic and serotonergic descending inhibitory pathways mediated lappaconitine antinociception (Guo and Tang, 1990a; Guo and Tang, 1990b; Ono and Satoh, 1992). Moreover, it was shown that lappaconitine exerted inhibitory effect on the nociceptive behaviors in neuropathy via decreasing the expression of the P2X3 receptors in the dorsal root ganglionic neurons (Ou et al., 2011). On the other hand, it was recently reported that the C19- and C20-aconitum alkaloids, including bulleyaconitine A (Li et al., 2016a), aconitine and benzolyaconine (Li et al., 2016b), and bullatine A (Huang et al., 2016; Huang et al.,
2017a), produced antinociception in a variety of rat models of chronic pain by stimulating spinal microglial dynorphin A release.

We, in this study, investigated the mechanical antiallodynia and thermal antihyperalgesia in the rat models of neuropathic pain and bone cancer pain and assessed whether lappaconitine induced antinociceptive tolerance. We then explored the mechanisms underlying lappaconitine antinociception, particularly the involvement of spinal microglial dynorphin A expression. Our study demonstrated that lappaconitine produced effective antihypersensitivity effects in neuropathic pain and bone cancer pain, apparently without induction of antinociceptive tolerance after long-term administration. In addition, the antinociceptive effects of lappaconitine is due to stimulation of spinal microglial expression of dynorphin A.

2. Materials and Methods

2.1. Drugs and reagents

Lappaconitine hydrobromide was obtained from the National Institute for the Food and Drug Control (Beijing, China) with purity not less than 95% determined by the manufacturer with high-performance liquid chromatography (HPLC). 5’-Guanidinonaltrindole (GNTI) and lipopolysaccharides (LPS) were obtained from Sigma-Aldrich (St. Louis, MO, USA), while Clophosome®-A (clodronate liposome) was purchased from FormuMax Scientific Inc. (Sunnyvale, CA, USA). The rabbit polyclonal antibody neutralizing dynorphin A, purchased from Phoenix Pharmaceuticals (Burlingame, CA, USA), was specific to dynorphin A, but not to dynorphin B, β-endorphin, α-neo-endorphin or leu-enkephalin, based on the manufacturer’s data. All the drugs and reagents were dissolved or diluted in 0.9% normal saline except metergoline and phentolamine mesilate that were dissolved in 1% ascorbic acid (pH adjusting to 7 by 1 M NaOH solution).

2.2. Experimental animals

Male and female adult (160-240 g body weight) and 1-day-old neonatal Wistar rats
were purchased from the Shanghai Experimental Animal Institute for Biological Sciences (Shanghai, China). The animal experiments were approved by the Animal Care and Welfare Committee of the Shanghai Jiao Tong University and carried out in accordance with the animal care guidelines of the National Institutes of Health. The adult animals were housed in plastic cages (4 per cage) and thick sawdust bedding in a temperature (22 ± 2°C)-controlled environment on a 12-hour light/dark cycle (7:00 a.m.-7:00 p.m.). All the animals received food and water ad libitum and were accustomed to the laboratory environment for at least 3 days before all the experiments. Experimental study groups (n=6 of each group) were randomly assigned and the researchers were blinded for the behavior tests.

2.3. Primary cell culture

The cortices and spinal cords of one-day-old neonatal rats (male or female) were collected and digested with 0.05% trypsin-ethylenediaminetetraacetic acid (Invitrogen, Grand Island, NY, USA) in the Dulbecco’s Modified Eagle’s Medium (DMEM) for 7 minutes and DMEM with 10% fetal calf serum (FBS) was used to prevent further digestion. The cell suspension was centrifuged (300 rpm) for 10 minutes and the cells were then suspended in the DMEM with 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL). For glial cell cultures, cell suspensions were plated in 75-cm² tissue culture flasks (1×10⁷ cells/flask) precoated with poly-L-lysine and maintained in a humidified atmosphere of 95% O₂ and 5% CO₂. To prepare microglia, the flasks after culture for 7 to 8 days were shaken at 260 rpm for 2 hours and the aliquots were then transferred to new plates and unattached cells were removed by washing with the serum-free DMEM. Harvested microglial cells exhibited a purity more than 95% as determined by Iba-1 immunoreactivity. To prepare astrocytes, the flasks after culture for 11 days were shaken for 2 hours followed by the incubation with 10 mL of 0.05% trypsin-ethylenediaminetetraacetic acid (Invitrogen) in a cell incubator for 15 minutes to separate the oligodendrocytes from the astrocytes. After trypsin neutralization with 10 mL of the completed DMEM media, the floating cell suspensions were discarded. A nearly intact monolayer of astrocytes was then trypsinized and
subcultured conventionally. Prepared astrocytes exhibited a purity >90%, as determined by glial fibrillary acidic protein (GFAP) immunoreactivity.

For neuronal cultures, cell suspensions were cultured for 4 hours in tissue plates precoated with poly-L-lysine (100 μg/mL) in the DMEM supplemented with 10% FBS. The medium was then changed to the Neurobasal (Invitrogen) containing B27 and 0.5 mM glutamine for further 7 to 8 day culture. Harvested neurons exhibited a purity >85%, as determined by neuronal nuclear antigen (NeuN) immunoreactivity (Gong et al., 2014b).

2.4. RNA isolation and quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

The rat spinal lumbar enlargements from neuropathic rats and primary neuron, astrocyte, and microglial cultures were collected for total RNA isolation using a TRIzol reagent (Invitrogen) (Zhang et al., 2013). A sample of 1 μg RNA was then reversely transcribed in to cDNA using a ReverTra Ace qPCR RT-Kit (Toyobo Co., Osaka, Japan). qPCR was carried out in a Mastercycler ep realplex (Eppendorf, Germany) using the Realmaster Mix (SYBR Green I) (Toyobo Co.). The fold change was calculated using the $2^{-\Delta\Delta Ct}$ method after normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primer sequences were GAPDH, 5'-CCAAGG TCA TCC ATG ACA AC-3' and 5'-TCC ACA GTC TTC TGA GTG GC-3' (Gong et al., 2014b); prodynorphin, 5'-CCT GTC CTT GTG TTC CCT GT-3' and 5'-AGA GGC AGT CAG GGT GAG AA-3' (Leitl et al., 2014; Huang et al., 2016); proopiomelanocortin (POMC), 5'-CCT ATC GGG TGG AGC ACT TC-3' and 5'-TGG CTC TTC TCG GAG GTC AT-3'; TNF-α, 5'-CCC CGA CTA TGT GCT CCT CAC-3' and 5'-AGG GCT CTT GAT GGC GGA-3'; IL-1β, 5'-GGA AGG CAG TGT CAC TCA TTG TG-3' and 5'-GTT CCT CAT CCT GGA AGC TCC-3'; IL-6, 5'-GGG ACT GAT GTT GAC AGC C-3' and 5'-CAT ATG TAA TTA AGC CTC CGA CTT GTG-3' (Fan et al., 2015).

2.5. Dynorphin A measurement

For spinal dorsal lumbar enlargements, rats were decapitated and the spinal dorsal
lumbar enlargements were collected and snap-frozen in liquid nitrogen until use. To measure dynorphin A level, the rat spinal dorsal lumbar enlargements were homogenized (4,000 rpm) for 15 seconds with a homogenizer (Fluko Equipment Co, Shanghai, China) in 10 mM Tris hydrochloric acid (pH 7.4) (1 g/5 mL) and then centrifuged (4,000 rpm) at 4°C for 15 minutes. For primary cell cultures, neurons, astrocytes and microglia were placed in 24-well plates (5×10^5 cells per well) and washed twice with 1 mL of warm DMEM containing 2 mg/mL bovine serum albumin and 15 mM N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid. All the cells were exposed to 100 μM lappaconitine for 2 hours and supernatant samples were collected and tested. Protein concentrations were determined by the bicinchoninic acid method using a commercially available kit (Beyotime Institute of Biotechnology, Shanghai, China).

The dynorphin A levels were assayed with an enzyme-linked fluorescent immunoassay kit (Phoenix Pharmaceuticals) that was validated by running linear standard curves with testing samples simultaneously to determine the peptide contents. According to the manufacturer, the cross-reactivity of the assay included dynorphin A (100%), but not dynorphin B (0%), β-endorphin (0%), or α-neo-endorphin (0%). The relative fluorescence units were measured using a Fluorescence Microplate Reader (ThermoLab systems, Vantaa, Finland), and the concentrations of the samples were determined by comparisons with a calibration curve running at the same time.

2.6. **Intrathecal catheterization and injection in rats**

An 18-cm polyethylene catheter (PE-10: 0.28-mm inner diameter and 0.61-mm outer diameter; Clay Adams, Parsippany, NJ, USA) with a volume of 13 μL was inserted into the rat lumbar level of the spinal cord under inhaled isoflurane anesthesia (4% for induction and 1% for maintenance) run by an anesthesia meter (Ugo Basile Gas Anesthesia System, Comerio, Italy). According to the length between the caudal ribs and the ventral iliac spines the tube was inserted inside the subarachnoid space and the rest tubing was placed subcutaneously and externally for the skin fixation and injection (Storkson et al., 1996). Penicillin powder was applied locally to avoid any wound
infections. Four days after animal recovery from anesthesia and surgery, the correct position of the catheter in the spinal cord was verified by administering 4% lidocaine (10 μL followed by 15 μL of saline for flushing) with a 50-μL microinjector (Shanghai Anting Microinjector Factory, Shanghai, China). Only rats that exhibited no motor impairment following insertion of the intrathecal catheter but developed immediate bilateral paralysis of the hindlimbs after intrathecal administration of lidocaine were selected and used for our experiments (Lu et al., 2012; Gong et al., 2014a).

2.7. Rat model of neuropathic pain

To induce neuropathic pain, adult male rats were subjected to spinal nerve ligation as described previously (Kim and Chung, 1992; Zhu et al., 2014; Wang et al., 2017). Briefly, under inhaled isoflurane anesthesia, the left L₅ and L₆ spinal nerves were isolated and tightly ligated with 6-0 silk thread. After ligation, the wounds were sutured and the rats were returned to their respective home cages to recover. Only those nerve-ligated rats with marked unilateral allodynia to mechanical stimulation, and no major impairment were selected and used for the study. When intrathecal injection was needed, the intrathecal catheterization was performed in rats at the same time just before spinal nerve ligation. Drug testing started on 2-4 weeks after the surgery of spinal nerve ligation.

2.8. Rat model of bone cancer pain

Adult female rats were anesthetized with intraperitoneal pentobarbital (50 mg/kg) as described previously (Huang et al., 2012; Zhang et al., 2013). In brief, incisions were made along the patellar ligament to expose the head of the tibia with minimal damage. A 23-gauge needle was inserted at the site of the intercondylar eminence and pierced 7 mm below the knee joint into the medullary cavity of the tibia. The needle was then removed and attached to a 10-μL microinjection syringe. Walker 256 breast carcinoma cells (4×10⁵) in 10 μL of the phosphate buffer solution were slowly injected into the left tibia cavity. While the syringe was removed the injection site was closed with aseptic bone wax quickly to prevent the carcinoma cells from leaking out. The wound was then
sutured and dusted with penicillin powder. Female rats were returned to their home cages for recovery for 2 weeks to allow developing unilateral mechanical allodynia in the ipsilateral paws.

2.9. Behavioral assessment of mechanical allodynia and heat hyperalgesia in rats

Before evaluation of mechanical allodynia, the animals were acclimatized for at least 30 minutes to the test environment, namely a plexiglass box on a metal grid. The bilateral hindpaw withdrawal threshold was measured by a 2391 CE Electronic von Frey (IITC Life Science Inc., Woodland Hill, CA, USA). The number 15 monofilament (ranging from 0.1 to 90 g) was applied perpendicularly to the surface of the hindpaws with increasing force until the rats suddenly withdrew their hindpaws. The lowest force to produce a withdrawal response was considered to the threshold and three repeated measurements with a 3-min interval were performed and the mean of the 3 threshold values for each hindpaw was calculated at each time point.

To assess heat hyperalgesia, rats were put in a plexiglass box on an elevated glass surface. After about 30 minutes of adaption, radiant heat (at a low density of 45 as indicated) was applied to the each hindpaw. The hindpaw withdrawal latency was measured by a 390G Plantar Test Analgesia Meter (IITC life Science Inc.). To prevent tissue damage, the latency cutoff was set at 30 seconds. The paw withdrawal latency was defined as the time from the onset of radiant heat application to the withdrawal of the hindpaws. Both hindpaws were tested independently three times with a 5-min interval between each experiment. The results of each test were calculated as the mean of the three repeated measurements.

2.10. Data evaluation and statistical analysis

The percentage of maximal possible effect (% MPE) was calculated using the following formula: \[ \frac{(\text{post-drug threshold in ipsilateral hindlimb} - \text{baseline threshold in ipsilateral hindlimb})}{(\text{baseline threshold in contralateral hindlimb} - \text{baseline threshold in ipsilateral hindlimb})} \times 100. \] The % MPE values near 100 indicate normal mechanical thresholds (i.e., near contralateral thresholds) and values near 0 indicate allodynia.
(Bowersox et al., 1996). For the dose–response curve analysis, the parameters, i.e., minimum effect ($E_{\text{min}}$), maximum effect ($E_{\text{max}}$), half-effective dose ($ED_{50}$) or half-effective concentration ($EC_{50}$), and Hill coefficient ($n$), were calculated by fitting non-linear least-square curves to the relation $Y = a + bx$, where $x = [D]^n / (ED_{50}^n + [D]^n)$ or $[C]^n / (EC_{50}^n + [C]^n)$. The values of $ED_{50}$ or $EC_{50}$ and $b$ ($E_{\text{max}}$) were projected by yielding a minimum residual sum of squares of deviations from the theoretical curve (Wang and Pang, 1993).

The data were expressed as means ± SEM or with 95% confidence limits, and there were no missing data in the study. The statistical significance was evaluated by unpaired and two-tailed Student’s t-test and one-way or repeated-measures two-way analysis of variance (ANOVA). The post-hoc Student–Newman–Keuls test was conducted when the effect of the drug (dose) (for the one-way ANOVA, the factor was drug [dose]; for the two-way ANOVA, the factors were drug [dose], time, and their interaction) was observed to be statistically significant. The probability values were two-tailed, and the statistical significance criterion P value was 0.05. The statistical evaluation was conducted using Prism (version 5.01, GraphPad Software, Inc., San Diego, CA, USA).

3. Results

3.1. Systemic lappaconitine produced mechanical antiallodynia and thermal antihyperalgesia in neuropathy

The antihypersensitivity activity of systemic lappaconitine was assessed in spinal nerve ligation-induced neuropathic rats that exhibited marked mechanical allodynia and thermal hyperalgesia in ipsilateral hindpaws. Two groups of neuropathic rats approximately 2 weeks after surgery received subcutaneous injection of the vehicle (0.9% normal saline, 1 mL/kg) or lappaconitine (0.3, 0.7, 2 and 7 mg/kg) sequentially in a 1-hour-interval to yield a cumulative dose of approximately 0.3, 1, 3 and 10 mg/kg. The mechanical and thermal paw withdrawal responses were measured before and 1 hour after each injection. As shown in Figure 2A, the mechanical paw withdrawal thresholds in both contralateral and ipsilateral paws of control rats remained unchanged during the
4-hour observation period after cumulative administration of saline. Cumulative subcutaneous injection of lappaconitine dose-dependently attenuated mechanical allodynia in ipsilateral hindpaws, without significantly affecting mechanical thresholds in contralateral hindpaws (Figure 2A). The dose-response analysis was projected from the % MPE values of each dose, resulting in a calculated ED$_{50}$ of 1.1 mg/kg (95% confidence limits: 0.6-2.1 mg/kg) and an $E_{\text{max}}$ of 53.3% MPE (Figure 2B). Similarly, cumulative subcutaneous lappaconitine also dose-dependently inhibited thermal hyperalgesia in ipsilateral hindpaws, without significantly affecting thermal latencies in contralateral hindpaws (Figure 2C). The projected ED$_{50}$ value was 1.6 mg/kg (95% confidence limits: 0.8-3.3 mg/kg) and $E_{\text{max}}$ value was 58.3% MPE (Figure 2D). There were no apparent sedation or motor side effects of lappaconitine observed during the study period of time.

3.2. Intrathecal lappaconitine attenuated mechanical allodynia without inducing tolerance

To investigate the spinal antiallodynic activity of lappaconitine, five groups of neuropathic rats received a single intrathecal injection of saline (10 μL) or lappaconitine at different doses (0.3, 1, 3 and 10 μg). Hindpaw withdrawal thresholds in both contralateral and ipsilateral paws to the mechanical stimulus were measured before and 0.5, 1, 2, and 4 hours after injection. Mechanical paw withdrawal thresholds in both contralateral and ipsilateral paws of the saline control rats remained unchanged during the 4-hour observation period. Intrathecal injection of lappaconitine attenuated mechanical allodynia in ipsilateral paws in a dose-dependent manner, but did not significantly alter withdrawal thresholds in contralateral paws. The antiallodynic effect was time-dependent, with a peak effect at 1 hour and lasted for 4 hours after injection (Figure 3A). The dose-response analysis showed the ED$_{50}$ value of 0.8 μg (95% confidence limits: 0.5-1.5 μg), and $E_{\text{max}}$ value of 66.1% MPE, as calculated from the 1 hour data after injection (Figure 3B). There were no apparent sedation or motor side effects of lappaconitine observed during the study period of time.

Two groups of neuropathic rats received multiple twice daily intrathecal injection
of saline (10 μL) or lappaconitine (10 μg) for 7 days. The paw withdrawal thresholds were measured 1 hour after each injection. Intrathecal lappaconitine produced a significant mechanical antiallodynic effect and importantly, the mechanical antiallodynia was not significantly changed over a period of 7 days during the treatments (P<0.05 using two-way ANOVA followed by post-hoc Student–Newman–Keuls test), indicating that long-term treatment with lappaconitine did not induce antiallodynic tolerance. Furthermore, when 10 μL of normal saline was intrathecally administered instead of 10 μg of lappaconitine on day 8, the paw withdrawal thresholds dropped and was not significantly different from those of the control animals that never received drug treatment. In addition, reapplication of multiple twice-daily intrathecal administration of lappaconitine still produced a comparable mechanical antiallodynic effect from day 9 to day 12, suggesting that its inability to induce antiallodynic tolerance was not due to its bona fide curative effect on neuropathic pain (Figure 3C).

3.3. Systemic lappaconitine produced mechanical antiallodynia in bone cancer pain

The mechanical antiallodynic effect of systemic lappaconitine was tested in a rat bone cancer pain model induced by tibia implantation of cancer cells. Two groups of rats with bone cancer pain produced approximately 2 weeks after tibia implantation of cancer cells received subcutaneously injection of saline (1 mL/kg) and 0.3, 0.7, 2 and 7 mg/kg of lappaconitine sequentially in a 1-hour-interval to yield a cumulative dose of approximately 0.3, 1, 3 and 10 mg/kg. As shown in Figure 4A, tibia implantation of cancer cells produced marked unilateral mechanical allodynia in ipsilateral paws. Withdrawal thresholds in contralateral and ipsilateral paws of control rats remained unchanged during the 4-hour observation period after cumulative saline treatment. Cumulative subcutaneous administration of lappaconitine did not significantly affect withdrawal thresholds in contralateral paws, but attenuated mechanical allodynia in ipsilateral paws in a dose-dependent manner, with an ED_{50} value of 2.0 mg/kg (95% confidence limits: 1.0–3.9 mg/kg) and an E_{max} value of 57.9% MPE (Figure 4B).
3.4. Lappaconitine stimulated spinal dynorphin A expression

To evaluate whether lappaconitine stimulated spinal dynorphin A expression, two groups of neuropathic rats received intrathecal injection of saline (10 μL) and lappaconitine (10 μg), respectively. We have previously demonstrated that the ipsilateral spinal levels of dynorphin A and prodynorphin gene measured 2-4 weeks after surgery (the same as our current experiment condition) in spinal nerve-ligated rats were the same as those in the contralateral side and in sham rats, and that the lappaconitine analogs bulleyaconitine A and bullatine A stimulated dynorphin A expression in both contralateral and ipsilateral spinal cords of neuropathic rats by the same degrees (Huang et al., 2016; Li et al., 2016a). Thus we in this study obtained the unseparated spinal dorsal lumbar enlargements 1 hour after injection of lappaconitine. Intrathecal injection of lappaconitine markedly increased spinal expression of the dynorphin A precursor prodynorphin mRNA for 2.0-fold, measured by qRT-PCR (P<0.05 using two-tailed and unpaired Student t-test), but did not significantly influence spinal expression of the β-endorphin precursor POMC mRNA (Figure 5A). Moreover, intrathecal lappaconitine significantly increased spinal level of dynorphin A by 89.3%, measured by an enzyme-linked fluorescent immunoassay (P<0.05 using two-tailed and unpaired Student t-test; Figure 5B).

In order to assess whether lappaconitine could directly stimulate dynorphin A expression in nervous cells, lappaconitine was incubated with primary cultures of microglia, astrocytes, and neurons originating from the cortex and spinal cord of neonatal rats. The intracellular expression of prodynorphin mRNA and the level of dynorphin A in the cultural medium were measured 2 hours after incubation. Although treatment with lappaconitine up to 300 μM did not affect the viability of microglia originating from the cortex, it did concentration-dependently (3, 10, 30, 100 and 300 μM) increase level of prodynorphin mRNA, with an EC₅₀ of 32.4 μM (95% confidence limits: 9.2–114.6 μM) (Figure 6A). As shown in Figure 6B, C, 100 μM of lappaconitine also markedly increased intracellular expression of prodynorphin mRNA and the level of dynorphin A in microglia originating from the spinal cord by 2- and 5-fold, respectively (P<0.05 using two-tailed and unpaired Student t-test). In contrast, it did
not significantly affect level of prodynorphin mRNA and dynorphin A release from either cultured neurons or astrocytes. On the other hand, treatment with lappaconitine did not significantly increased expression of POMC mRNA in microglia, neurons, or astrocytes originating from the spinal cord (Figure 6D).

We further assessed the ability of lappaconitine in intracellular expression of proinflammatory cytokines in microglial cells in the presence and absence of LPS. Cultured primary microglial cells originating from the cortex were treated with lappaconitine (100 μM) 1 hour prior to LPS (3 ng/mL) and were collected 2 hours after LPS treatment. As shown in Figure 7A-C, lappaconitine treatment did not stimulate or inhibit intracellular expression of TNF-α, IL-1β or IL-6 mRNA, whereas LPS treatment dramatically increased TNF-α, IL-1β and IL-6 mRNA expression by 9.0-, 15.9-, and 22.9-fold, respectively (P<0.05 using one-way ANOVA followed by post-hoc Student-Newman-Keuls tests). However, co-treatment with lappaconitine did not enhance or inhibit LPS-stimulated overexpression of proinflammatory cytokines.

3.5. Lappaconitine produced antiallodynia through spinal expression of dynorphin A

We first investigated whether the specific microglia depletor clodronate liposome could block lappaconitine-induced mechanical antiallodynia, given intrathecally and systemically. Two groups of neuropathic rats received an intrathecal injection of 10 μL normal or clodronate liposome (30 μg) (Van Rooijen et al., 1996; Dehghani et al., 2004), followed by an intrathecal injection of lappaconitine (10 μg) one day later. Hindpaw withdrawal thresholds to the mechanical stimulus were measured before and 0, 0.5, 1, 2, and 4 hours after the second administration. Intrathecal injection of lappaconitine produced a time-dependent mechanical antiallodynia in ipsilateral paws of saline-pretreated rats. Intrathecal injection of clodronate liposome did not alter baseline mechanical thresholds in contralateral and ipsilateral paws, but completely prevented lappaconitine-induced mechanical antiallodynia in ipsilateral paws (P<0.05 using repeated-measures two-way ANOVA followed by post-hoc Student-Newman-Keuls tests; Figure 8A). In addition, two groups of neuropathic rats received an intrathecal injection of saline (10 μL) or clodronate liposome (30 μg), followed by a subcutaneous
injection of lappaconitine (3 mg/kg) one day later. Pretreatment with intrathecal clodronate liposome completely suppressed subcutaneous lappaconitine-induced mechanical antiallodynia (P<0.05 using repeated-measures two-way ANOVA followed by post-hoc Student-Newman-Keuls tests; Figure 8B).

We further investigated the blockade effect of the dynorphin A antibody on lappaconitine-induced mechanical antiallodynia, given intrathecally and systemically. Two groups of neuropathic rats received an intrathecal injection of 10 μL rabbit serum (1:10 dilution) or the specific dynorphin A antibody (1:10 dilution) (Li et al., 2016a), followed by an intrathecal injection of lappaconitine (10 μg) 30 minutes later. Intrathecal injection of lappaconitine produced a time-dependent mechanical antiallodynia the rabbit serum-pretreated control rats. Intrathecal injection of the dynorphin A antibody did not alter baseline mechanical thresholds, but completely prevented lappaconitine-induced mechanical antiallodynia (P<0.05 using repeated-measures two-way ANOVA followed by post-hoc Student-Newman-Keuls tests; Figure 8C). In addition, two groups of neuropathic rats received an intrathecal injection of saline (10 μL) or the dynorphin A antibody (1:10 dilution, 10 μL), followed by an subcutaneous injection of lappaconitine (3 mg/kg) 30 minutes later. Pretreatment with intrathecal the dynorphin A antibody completely inhibited subcutaneous lappaconitine-induced mechanical antiallodynia in neuropathic rats (P<0.05 using repeated-measures two-way ANOVA followed by post-hoc Student-Newman-Keuls tests; Figure 8D).

We furthermore tested the blocking effect of the selective κ-opioid receptor antagonist GNTI on lappaconitine-induced mechanical antiallodynia. Two groups of neuropathic rats received an intrathecal injection of saline (10 μL) or GNTI (50 μg) (Huang et al., 2016), followed by an intrathecal injection of lappaconitine (10 μg) 30 minutes later. Intrathecal GNTI injection did not significantly alter baseline mechanical thresholds in ipsilateral paws, but completely blocked intrathecal lappaconitine-induced mechanical antiallodynia (P<0.05 using repeated-measures two-way ANOVA followed by post-hoc Student-Newman-Keuls tests; Figure 8E). In addition, two groups of neuropathic rats received an intrathecal injection of saline (10 μL) or GNTI (50 μg), followed by a subcutaneous injection of lappaconitine (3 mg/kg) 30 minutes later. As
shown in Figure 8F, pretreatment with intrathecal GNTI also completely inhibited subcutaneous lappaconitine-induced mechanical antiallodynia (P<0.05 using repeated-measures two-way ANOVA followed by post-hoc Student-Newman-Keuls tests).

4. Discussion

Our present study demonstrated that the C18-diterpenoid alkaloid lappaconitine given subcutaneously produced mechanical antiallodynia and thermal antihyperalgesia in spinal nerve ligation-induced neuropathic rats, although it was not effective to affect normal nociceptive thresholds. Its antinociceptive activities were similar in both mechanical alldyna and thermal hyperalgesia, with cumulative ED$_{50}$ values of 1.1 mg/kg (95% confidence intervals: 0.6-2.1 mg/kg) vs. 1.6 mg/kg (95% confidence intervals: 0.8-3.3 mg/kg) and E$_{\text{max}}$ values of 53% vs. 58% MPE, respectively. Moreover, subcutaneous injection of lappaconitine produced equivalent blockade of mechanical allodynia in the rat bone cancer pain model, with a cumulative E$_{\text{max}}$ value of 57.9% MPE and an ED$_{50}$ of 2.0 mg/kg (95% confidence limits: 1.0–3.9 mg/kg). Compared with the data on aconitine (Li et al., 2016b), bulleyaconitine A (Li et al., 2016a), bullatine A and guan-fu base A (Huang et al., 2016), we could rank the potencies of these diterpenoid alkaloids given intrathecally in mechanical antiallodynia of neuropathic pain as aconitine > bulleyaconitine A > lappaconitine = bullatine A > Guna-fu base A = no antinociception. In addition, we demonstrated that multiple twice daily administrations of lappaconitine over 7 days did not induce antinociceptive tolerance. Combined with data from previous studies (Ou et al., 2011; Zhu et al., 2015), our results provide a pharmacological base for long-term use of lappaconitine to treat clinical neuropathic pain and cancer pain and we speculate that diterpenoid alkaloids exhibit antinociception regardless of their basic C-18, C-19 and C-20 diterpenoid structure with C-20 diterpenoid alkaloids being less potent than C-19 ones.

Our data further demonstrated that lappaconitine antinociception is entirely through spinal microglial expression of dynorphin A. The notion is supported by our following findings in the current and previous studies. 1) Intrathecal injection of
Lappaconitine produced mechanical antiallodynia in neuropathic rats and increased spinal expression of dynorphin (but not β-endorphin). The effectiveness of intrathecal administration and the ED$_{50}$ ratio of approximately 340:1 for subcutaneous vs. intrathecal injection on mechanical antiallodynia suggest that the spinal cord is a principal site for lappaconitine to produce antinociception. 2) Pretreatment with intrathecal injection of the specific antibody neutralizing dynorphin A and κ-opioid receptor antagonist GNTI totally prevented intrathecal and systemic lappaconitine-induced mechanical antiallodynia in neuropathy. It is known that dynorphin A acts through activation of neuronal κ-opioid receptors to produce antinociception (Laughlin et al., 2001; Chavkin, 2013). Indeed, intrathecal injection of dynorphin A produced dose- and κ-opioid receptor-dependent antinociception in neuropathic rats (Huang et al., 2016). 3) Liposome-encapsulated clodronate has been extensively shown to specifically deplete and suppress peripheral macrophages and central microglia in vitro and in vivo via a “phagocytic suicide” approach, and is now frequently applied in studies aimed to unravel macrophage/microglia function (Van Rooijen et al., 1996; Kohl et al., 2003; Dehghani et al., 2004; Ellett et al., 2010; Drabek et al., 2012). Indeed, our recent data showed that intrathecal injection of liposomal clodronate specifically and reversibly depleted spinal microglia but not neurons or astrocytes, and its peak effect by more than 60% inhibition was obtained 1 day after injection (Wang et al., 2018). In this study, intrathecal injection of clodronate liposome one day after injection in the current study entirely attenuated the mechanical allodynic effect of lappaconitine, given intrathecally or subcutaneously, indicating that dynorphin A expressed in the spinal cord is originated from microglia. The conclusion is also supported by the facts that lappaconitine specifically expressed dynorphin A in cultured primary microglia but not astrocytes or neurons, and that intrathecal injection of the lappaconitine analog bullatine A stimulated dynorphin A expression directly from microglia but not astrocytes or neurons in the spinal cord, determined by a double immunofluorescence staining technique (Huang et al., 2016).

We have recently also performed similar experiments to conclude that the C-19 and C-20-diterpenoid alkaloids, including aconitine, bulleyaconitine A, benzolyaconine
and bullatine A, produced antinociception by direct stimulation of dynorphin A expression (Huang et al., 2016; Li et al., 2016a; Li et al., 2016b; Huang et al., 2017a; Li et al., 2017), rather than through interaction with the voltage-dependent sodium channels as originally accepted (Ameri, 1998; Wright, 2001). Furthermore, we demonstrated that bullatine A specifically stimulated dynorphin A expression only in microglia and not in neurons or astrocytes in primary cultures and the spinal cord of neuropathic rats, and that the microglial inhibitor minocycline completely blocked bullatine A and bulleyaconitine A-induced antinociception and spinal dynorphin A expression and release in neuropathic rats (Huang et al., 2016; Li et al., 2016a). In the current study, lappaconitine treatment specifically stimulated expression and secretion of dynorphin A (but not β-endorphin) from primary cultures of microglia, but not from neurons or astrocytes. Taken together, these results suggest that *aconitum* diterpenoid alkaloids produced antinociception through spinal microglial expression and secretion of dynorphin A, regardless of their skeletal structures of C-18, C-19 or C-20. The expanded notion is further supported by the correlation between the potencies of spinal mechanical antiallodynia and spinal expression of prodynorphin mRNA among the diterpenoid alkaloids given intrathecally in neuropathic rats (Figure 9). The ED50 values were derived from our current and previous studies (Huang et al., 2016; Li et al., 2016a; Li et al., 2016b).

The crucial role of spinal microglia has been recognized with regard to the initiation and development of chronic pain (Echeverry et al., 2008). Activated microglia have been implicated to produce proinflammatory cytokines, including TNF-α, IL-6 and IL-1β. These cytokines contribute to the central sensitization of neurons in the spinal dorsal horn and alter the excitatory or inhibitory synaptic transmission to contribute to pain facilitation (Inoue, 2006; Jha et al., 2012; Lisboa et al., 2016). However, we demonstrated that treatment with lappaconitine up to 100 μM did not suppress baseline and LPS-stimulated expression of proinflammatory cytokines in cultured microglial cells, which is in agreement with our recent finding that bullatine A did not inhibit LPS- or spinal never injury-induced overexpression of the proinflammatory cytokines (Huang et al., 2016). We have recently revealed that
bulleyaconitine A significantly induced cyclic AMP production and activation of cAMP-dependent protein kinase A (PKA). Subsequently, PKA specifically regulated the activity of p38 (but not ERK1/2 or JNK) and its translocation to the nucleus and then phosphorylated the transcription factor cyclic AMP-response element binding protein (CREB), leading to increase in dynorphin A expression (Li et al., 2017). Particularly, p38β but not p38α entirely accounted for bulleyaconitine A-induced dynorphin A expression (Li et al., 2017), whereas p38α and other mechanisms including activation of ERK but not p38β activation mediated LPS-induced expression of proinflammatory cytokines (Wu et al., 2017). The illustrated Gs-cAMP/PKA/p38β/CREB signal transduction pathway following activation of “aconitine receptors” supports the inability of aconitines to inhibit the expression of proinflammatory cytokines.

In conclusion, like C20- and C19-diterpenoid aconitum alkaloids, lappaconitine as a representative C18-diterpenoid alkaloid, given either systemically or intrathecally, attenuated mechanical allodynia and thermal hyperalgesia in neuropathic pain and bone cancer pain. Lappaconitine induced dynorphin A expression in cultured primary microglia and the spinal cord of neuropathic rats. Moreover, the antinociceptive effects of lappaconitine were entirely blocked by intrathecal injection of the specific dynorphin A antibody and κ-opioid receptor antagonist. Our results thus, suggest that lappaconitine, like other aconitum alkaloids, produces antinociception through stimulation of spinal microglial dynorphin A expression.

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Author contributions
Conceived and designed the experiments: YXW and MLS; performed the experiments: MLS, JPA, YRW, XYL, QH, and TFL; analyzed the data: YXW and MLS; and preparation of the paper: YXW and MLS.
Conflict of interest

The authors declare that there are no competing financial interests in this work.
References


Lisboa SF, Gomes FV, Guimaraes FS, Campos AC (2016), Microglial Cells as a Link between Cannabinoids and the Immune Hypothesis of Psychiatric Disorders.
Fronf Neurol 7:5.


Figure legends

Figure 1. Chemical structures of the C18-diterpenoid alkaloid and lappaconitine.

Figure 2. Mechanical antiallodynic (A, B) and thermal antihyperalgesic (C, D) effects of lappaconitine administered subcutaneously in neuropathic rats, induced by tight ligation of spinal L5/L6 nerves. Neuropathic rats received subcutaneous injection of the vehicle (0.9% normal saline, 1 mL/kg) or lappaconitine (0.3, 0.7, 2 and 7 mg/kg) sequentially in a 1-hour-interval to yield a cumulative dose of approximately 0.3, 1, 3 and 10 mg/kg. Cumulative dose-response analyses of lappaconitine on mechanical antiallodynia (B) and thermal antihyperalgesia (D) were best projected by the non-linear least-squares method using the data at 1 hour after each injection. The data are presented as means ± SEM (n=6 in each group).

Figure 3. Mechanical antiallodynic effects of single lappaconitine administrated intrathecally on mechanical allodynia in neuropathic rats induced by tight ligation of spinal L5/L6 nerves (A). B. Dose-response analysis of lappaconitine on mechanical antiallodynia was best projected by the non-linear least-squares method using the data at 1 hour after injection. C. Long-term mechanical antiallodynic effects of multiple twice-daily intrathecal injections of lappaconitine in neuropathic rats. The data are presented as means ± SEM (n=6 in each group). *denotes statistical significance compared with the respective saline groups (P<0.05 using repeated measures two-way ANOVA followed by post-hoc Student–Newman–Keuls tests).

Figure 4. Mechanical antiallodynic effects of lappaconitine administered subcutaneously in the rat bone cancer pain model induced by tibia implantation of cancer cells (A). Bone cancer pain rats produced approximately 2 weeks after surgery received subcutaneous injection of normal saline (1 mL/kg) or lappaconitine (0.3, 0.7, 2 and 7 mg/kg) sequentially in a 1-hour-interval to yield a cumulative dose of approximately 0.3, 1, 3 and 10 mg/kg. B. Cumulative dose-response analysis of
lappaconitine on mechanical antiallodynia was best projected by the non-linear least-squares method using the data at 1 hour after injection. The data are presented as means ± SEM (n=6 in each group).

**Figure 5.** Effects of intrathecal injection of lappaconitine on spinal expression of the dynorphin A precursor prodynorphin mRNA and β-endorphin precursor proopiomelanocortin (POMC) mRNA (A) and dynorphin A level (B) in neuropathic rats, induced by tight ligation of spinal L₅/L₆ nerves. Neuropathic rats received a single intrathecal injection of normal saline or lappaconitine and spinal lumbar enlargements were obtained 1 hour after injection. Expression of prodynorphin and POMC mRNA vs. GAPDH mRNA in cultured primary cells and dynorphin A level in the cultural medium were determined by using qRT-PCR and a commercial fluorescent immunoassay kit, respectively. The data are presented as means ± SEM (n=6 in each group). *denotes statistical significance compared with the saline group (P<0.05 using two-tailed and unpaired Student t-test).

**Figure 6.** Effects of lappaconitine on intracellular expression of the dynorphin A precursor prodynorphin mRNA, β-endorphin precursor proopiomelanocortin (POMC) mRNA and dynorphin A level in primary cultures of microglia, neurons, and astrocytes originating from the cortex (A) and spinal cord (B-D) of neonatal rats. Expression of the prodynorphin and POMC mRNA vs. GAPDH mRNA in cultured primary cells and dynorphin A level in the cultural medium were determined 2 hours after lappaconitine incubation using qRT-PCR and a commercial fluorescent immunoassay kit, respectively. The data are presented as means ± SEM (n=4 in each group with duplicate). *denotes statistical significance compared with the control (P<0.05 using two-tailed and unpaired Student t-test).

**Figure 7.** Effects of lappaconitine on expression of proinflammatory cytokines, tumor necrosis factor (TNF)-α (A), interleukin (IL)-1β (B) and IL-6 (C) in primary cultures of microglia originating from the cortex of neonatal in the presence and absence of
lipopolysaccharides (LPS). Expression of proinflammatory cytokine genes vs. GAPDH mRNA was determined 2 hours after lappaconitine and LPS incubation using qRT-PCR. The data are presented as means ± SEM (n=3 in each treatment in duplicate). *donates statistical significance compared to the control group (P<0.05 using one-way ANOVA followed by post-hoc Student–Newman–Keuls tests).

**Figure 8.** The blockade effects of intrathecal injection of the specific microglia depletor clodronate liposome (30 μg, A, B), dynorphin A antibody (1:10, C, D) and κ-opioid receptor antagonist 5'-guanidinonaltrindole (GNTI, 30 μg, E, F) on intrathecal and subcutaneous lappaconitine-induced mechanical antiallodynia in spinal L5/L6 nerve-ligated neuropathic rats. Neuropathic rats received an intrathecal injection of clodronate liposome, or the dynorphin A antibody or GNTI followed by an intrathecal or subcutaneous injection of lappaconitine one day or 30 minutes later. The data are presented as means ± SEM (n=6 in each group). *denotes statistical significance compared with the lappaconitine control group (P<0.05 using repeated-measures two-way ANOVA followed by post-hoc Student-Newman-Keuls tests).

**Figure 9.** Correlation between ED$_{50}$ values of mechanical antiallodynia in neuropathic rats and EC$_{50}$ values of microglial expression of the dynorphin A precursor prodynorphin mRNA among the C18, C19 and C20-diterpenoid alkaloids. The values of lappaconitine, bulleyaconitine A, aconitine, benzolyaconine, aconine, bullatine A and guan-fu base A were derived from the current study and our previous studies (Huang et al., 2016; Li et al., 2016a; Li et al., 2016b).
C\textsubscript{18}-diterpenoid alkaloids

Lappaconitine
Figure 3: Effects of intrathecal lappaconitine on paw withdrawal threshold.

A) Comparison of Paw Withdrawal Threshold (g) over time (hours) following intrathecal injection. Different doses of lappaconitine (0.3, 1, 3, 10 μg) compared to saline.

B) Dose-response curve showing the maximum possible effect (% MPE) of intrathecal lappaconitine (μg), with ED50 = 0.8 μg.

C) Changes in Paw Withdrawal Threshold (g) following bi-daily intrathecal injections over 12 days, comparing saline (10 μL, bid) and lappaconitine (10 μg, bid) treatments.
$R^2 = 0.90$
$P = 0.0012$

The graph shows a linear relationship between $ED_{50}$ in Spinal Antiallodynia (mol), Log and $EC_{50}$ in Microglial Expression of Prodynorphin (M), Log. The points correspond to different compounds labeled as Aconine, Guan-fu base A, Lappaconitine, Bullatine A, Benzolyaconine, Bulleyaconitine A, and Aconitine.