Lamiophlomis rotata, an Orally Available Tibetan Herbal Painkiller, Specifically Reduces Pain Hypersensitivity States through the Activation of Spinal Glucagon-like Peptide-1 Receptors

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ABSTRACT

Background: Lamiophlomis rotata is an orally available Tibetan herb prescribed for the management of pain, with shanzhiside methylester (SM) and 8-O-acetyl-SM as quality control ingredients. This study aimed to evaluate the antinociceptive properties of L. rotata, determine whether SM and 8-O-acetyl-SM are principle effective ingredients, and explore whether L. rotata produces antinociception through activation of spinal glucagon-like peptide-1 receptors (GLP-1Rs).

Methods: Formalin test, neuropathic pain, and bone cancer pain models were used, and the animal sample size was 5 to 6 in each group. Hydrogen peroxide–induced oxidative damage was also assayed.

Results: The L. rotata aqueous extract blocked formalin-induced tonic hyperalgesia and peripheral nerve injury– and bone cancer–induced mechanical allodynia by 50 to 80%, with half-effective doses of 130 to 250 mg/kg, close to the human dosage. The herb was not effective in alleviating acute nociceptive pain. A 7-day gavage with L. rotata aqueous extract did not lead to antiallodynic tolerance. Total iridoid glycosides, rather than total flavonoids, were identified by the activity-tracking method as effective ingredients for antihyperalgesia, whereas both SM and 8-O-acetyl-SM were principal components. Further demonstrations using the GLP-1R antagonist and gene silencer against GLP-1R at both the spinal and the cellular levels indicated that L. rotata inhibited pain hyperactivity by activation of spinal GLP-1Rs, and SM and 8-O-acetyl-SM appeared to be orthosteric, reversible, and fully intrinsic agonists of both rat and human GLP-1Rs.

Conclusions: Results support the notion that the activation of spinal GLP-1Rs leads to specific antinociception in pain hypersensitivity and further suggest that GLP-1R is a human-validated target molecule for the treatment of chronic pain. (Anesthesiology 2014; 121:835-51)

LAMIOPHLOMIS rotata (Benth.) Kudo (L. rotata), also called Tibetan Duyiwei (fig. 1), grows at an altitude of 3,000 m above sea level in Tibet and other southwestern areas in China. It has been traditionally used for the treatment of knife and gun wounds, and hemostasis for many centuries. After extensive clinical use and investigation, the orally available L. rotata aqueous extract was approved by the Food and Drug Administration of China (Beijing) in 1989 for the treatment of pain and is listed in Pharmacopoeia of China. The L. rotata aqueous extract, in formulations of pills and softgel capsules, is prescribed domestically for the management of moderate-to-severe cancer pain, postoperative and bone fracture pain, and neuropathic pain, although there appear no reports of clinical investigations outside China. Investigations of L. rotata in experimental pain models and its mechanism of action remain limited. Gavage with L. rotata extracts reduced antinociceptive pain responses in the mouse hot plate and acetic acid tests, whereas total iridoid glycosides blocked acetic acid-induced writhing response and formalin-induced tonic hyperalgesia but did not reduce formalin-induced acute flinching response or thermally evoked escape response.
Glucagon-like peptide-1 (GLP-1) induces insulin secretion and glucose regulation and shows therapeutic value in treating type 2 diabetes mellitus. These actions are mediated through GLP-1 receptors (GLP-1Rs). The single structurally identical GLP-1R expresses in pancreatic islets, the lungs, brain, dorsal root ganglia, and spinal cord. We recently discovered that GLP-1Rs are specifically expressed on spinal microglia. Spinal GLP-1R peptidic agonists exenatide and GLP-1 produce antihyperalgesia without affecting acute nociceptive pain. The antihyperalgesic effects of exenatide are prevented by pharmacologic blockade and genetic knockdown of GLP-1Rs. Exenatide antinociception is mediated via the action of β-endorphin released from spinal microglia. Our study provides the first evidence that activation of spinal GLP-1Rs leads to antinociception in pain hypersensitivity, notably refractory neuropathic pain, cancer pain, and painful diabetic neuropathy.

Flavonoids and iridoid glycosides represent major ingredients of L. rotata. Flavonoids include apigenin, luteolin, quercetin, and avicularin. Iridoid glycosides include shanzhiside methylester (SM), 8-O-acetyl-SM, 6-O-acetyl-SM, 8-dehydroxyshanzhiside, and loganin. SM and 8-O-acetyl-SM are major iridoid glycosides (approximately 1 to 3%) and the Chinese Pharmacopoeia lists the combination of these two compounds as a control index for the quality of L. rotata. Geniposide is one major iridoid glycoside of Gardenia jasminoides and shares a similar structure to SM and 8-O-acetyl-SM (fig. 1). Geniposide induced neuronal differentiation and protection against oxidative stress and regulated insulin secretion via GLP-1Rs.

The similarity in structure between geniposide, SM, and 8-O-acetyl-SM prompted us to explore whether L. rotata produces antinociception by activation of GLP-1Rs. We performed the following studies: (1) testing the antihyperalgesic effects in the formalin test, neuropathic, and bone cancer pain; (2) identifying effective ingredients; (3) determining primary sites for the antihyperalgesic effects; and (4) illustrating the potential role of GLP-1Rs in the antihyperalgesic effects. Our results demonstrate that L. rotata and its principle effective ingredients SM and 8-O-acetyl-SM specifically block pain hypersensitivity through direct activation of spinal GLP-1Rs, without induction of tolerance. SM and 8-O-acetyl-SM are orthosteric, reversible, and fully intrinsic agonists of both rat and human GLP-1R.

**Materials and Methods**

**Drugs and Reagents**

SM and 8-O-acetyl-SM were purchased from Chengdu Bio-purify Phytochemicals Co. (Chengdu, China) with a purity of more than 95% by high-performance liquid chromatography, and exendin(9–39) with a peptide content of 98% was purchased from Shanghai TASH Biotechnology Co. (Shanghai, China). Hydrogen peroxide and formaldehyde solution were purchased from Sinopharm Group Chemical Reagent Co. (Shanghai, China), and 5% formalin solution (v/v) was diluted from the original formaldehyde solution with saline. Obtained from Hainan Haishen Pharmaceutical Co. (Hainan, China) as a gift, the L. rotata aqueous extract was...
manufactured according to the protocol listed in the Chinese Pharmacopoeia, and its total SM and 8-O-acetyl-SM concentrations were 1.24 and 0.73%, respectively, as measured by high-performance liquid chromatography in house.

The total flavonoids extract and iridoid glycosides extract with different contents of iridoid glycosides represented by the combination of SM and 8-O-acetyl-SM were manufactured in house from L. rotata. The extract procedures were as follows. The powder of the aerial parts of L. rotata was decocted twice at 60°C with 10 volumes of distilled water for 1 h. After being cooled down and filtered, the aqueous extract was subjected to an XDA-1 macroporous adsorptive resin chromatographic column, which had been depurated with 95% ethanol at a flow rate of 2.0 ml/min. Ethanol at concentrations of 70% and 20 to 50% was used for total flavonoids and total iridoid glycosides, respectively, to elute the column after it had been eluted with distilled water to obtain a negative Molisch reaction. Total flavonoids and iridoid glycosides were obtained from the ethanol elution after they were vacuum-dried at 60°C. The total flavonoids sample contained approximately 90% total flavonoids, but no SM or 8-O-acetyl-SM, was present as determined by liquid chromatography-mass spectrometry. To refine iridoid glycosides, the powder of the total iridoid glycoside extract was dissolved with methanol in distilled water before being subjected to further chromatography on a silica gel column (200 to 300 mesh, 120 g). The 10 to 15 volumes of organic solvents (i.e., dichloromethane, ethyl acetate, or acetone) were then used to elute the column, resulting in subsequent total iridoid glycoside samples with differential contents. The contents of the SM and 8-O-acetyl-SM were determined by high-performance liquid chromatography analysis. Total iridoid contents were further calculated from peak areas of iridoids relative to SM and 8-O-acetyl-SM, respectively.

The small interfering RNA (siRNA) targeting the GLP-1R and its nonspecific oligonucleotide were synthesized by GenePharma Co. (Shanghai, China) according to the designation of Yin et al. The 19 nucleotide duplexes and 2 unpaired nucleotides overhanging the 3’ end were as follows: GLP-1R: 5’-GUA UCU CUA GGA CGA GUU-3’/5’-CUC GUC CUC GUA GAG AUA CUU-3’; negative control: 5’-UUC UCC UCC GAA CGU GUC ACG UUU-3’/5’-ACG UGA CAC GUU CGG AGA AUU-3’. Previous protocols were followed to formulate siRNAs. Linear polyethyleneimide (PEI) of 25 kDa (PolyScience, Niles, IL) was dissolved in 5% dextrose diethyl pyrocarbonate–treated water (pH 7.4), and 1 mg of RNA was dissolved in 1.5 mg PEI in a PEI:RNA ratio of 6 equivalents of PEI nitrogen per RNA phosphate to form RNA-polymer complexes at room temperature for 20 min.

**Experimental Animals**

All animal studies were performed under the protocols approved by the Laboratory Animal Use Committee of Shanghai Jiao Tong University School of Pharmacy (Shanghai, China). Adult male Swiss mice (20 to 28 g) and Wistar rats (160 to 240 g) were purchased from Shanghai Experimental Animal Institute for Biological Sciences (Shanghai, China). Animals were housed in plastic cages (2 to 3 per cage for rats and 4 to 5 per cage for mice) with soft bedding and free access to food and water under a 12/12-h reversed light–dark cycle (7:00 AM to 7:00 PM) at a constant temperature of 22°C ± 2°C. All animals were acclimated for 3 to 7 days before surgical and experimental procedures. Experimental study groups (n = 5 to 6 in each group) were assigned randomly, and the researcher was blinded to the behavior testing.

**Cell Culture and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide Assay**

Originally derived from a pheochromocytoma of the rat adrenal medulla, PC12 cells expressing GLP-1Rs were purchased from Shanghai Institute for Cell Biology Cell Bank (Shanghai, China) and were routinely cultured in high-glucose Dulbecco’s Modified Eagle’s Medium containing 5% fetal bovine serum, 10% horse serum, 100 U/ml penicillin, and 100 mg/l streptomycin. Human embryonic kidney 293 (HEK293) cells with stable expression of human GLP-1Rs (a generous gift from Bao-Hong Zhang, Ph.D., at BD Bioscience, Shanghai, China) and parent HEK293T just started as a chairman cells (purchased from Applied StemCell, Inc., Sunnyvale, CA) were cultured in high-glucose Dulbecco’s Modified Eagle’s Medium containing 10% fetal bovine serum, 2.0 mM L-glutamine, 100 μg/ml hygromycin B, 100 U/ml penicillin, and 100 mg/l streptomycin. Cells were cultured at 37°C in a humidified atmosphere of 95% and 5% CO2 and passed by trypsinization every 2 to 3 days. Afterward, cells were grown on 96-well plates at a density of 1.6×104 cells per well and attached after incubation of 24 h, and hydrogen peroxide was added for 15 min in PC12 cells (final concentration, 500 μM) or for 5 min in HEK293 cells (400 μM) or for 12 h in HEK293T cells (800 μM). After washing, PC12 and HEK293 cells were treated with SM or 8-O-acetyl-SM at different concentrations in the absence or presence of exendin(9–39) at three concentrations for 12 h. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was conducted to determine cell viability. Briefly, cells in 96-well plates were rinsed with phosphate-buffered saline, and 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added to each well. The microplates were incubated at 37°C for an additional 4 h. At the end of the incubation period, the medium with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was removed, and 200 μl dimethyl sulfoxide was added to each well. Plates were agitated on a plate shaker for 10 min, and the optical density was then read at 570 nm against a reference wavelength of 630 nm on a microplate reader (Multiskan MK3; Thermo Labsystems, Vantaa, Finland). Cell viability was expressed as a percentage of control.
Western Blotting

Rat spinal lumbar enlargements (L3 to L5) were quickly dissected after the rats were killed and homogenized in a radioimmunoprecipitation analysis buffer containing the protease inhibitor phenylmethylsulfonyl fluoride (Beyotime Institute of Biotechnology, Jiangsu, China). Protein concentrations of lysates were determined using a standard bicinchoninic acid protein assay (Beyotime Institute of Biotechnology). Lysate (20 μg of protein) was run on 12% sodium dodecyl sulfate polyacrylamide gel and transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA). The membrane was incubated with the GLP-1R antibody (ab39072, 1:2,000, rabbit polyclonal; Abcam, Cambridge, United Kingdom) and β-actin antibody (1:2,000, rabbit polyclonal; Proteintech Group, Wuhan, China). Protein bands were visualized using secondary antibody (IRDye 800 conjugated anti-rabbit immunoglobulin G; Rockland Immunochemicals, Gilbertsville, PA), with the Odyssey Infrared Imaging system from Li-Cor Biosciences (Lincoln, NE). The band intensity was quantitated using a computer-assisted image analysis program (Image J Software; National Institutes of Health, Bethesda, MD). To control sampling errors, the intensity ratio of the GLP-1R/β-actin band was calculated to quantify relative protein expression levels.

Immunofluorescence Staining

Immunofluorescence labeling of GLP-1Rs was performed on cultured PC12 cells, HeK293 cells, and HeK293T cells and observed on a confocal microscope (TCS SP8; Leica Microsystems, Buffalo Grove, IL). Cells were placed in six-well plates (5 x 10⁴ cells per well) with poly-L-lysine-coated glass coverslips at the bottom and were fixed in 4% paraformaldehyde and then incubated in 10% goat serum (vol/vol) and 0.5% Triton-X 100 (vol/vol) in phosphate-buffered saline for 1 h. Cells were incubated with the GLP-1R antibody at 4°C for 24 h. The antibody of GLP-1R was tested for optimal dilution, the absence of cross-reactivity, and nonspecific staining. The GLP-1R was visualized with the Alexa 555-conjugated goat anti-rabbit secondary antibody (1:200; Invitrogen, Shanghai, China). 4’,6-Diamidino-2-phenylindole (Sigma, Shanghai, China) staining was also used to determine cell nuclei.

Intrathecal Injection in Mice

Intrathecal injection in mice followed the previously described procedure.⁴⁹ In brief, a 10-μl microsyringe with a tube for delivering test drugs was inserted into the skin and through the L5 to L6 intervertebral space directly into the subarachnoid space. A tail flick provided a reliable indicator that the needle had penetrated the spinal arachnoid mater. Control and test articles in volumes of 5 μl, sufficient enough to spread consistently to the caudal thoracic vertebrae, which contained the lumbar enlargement of the spinal cord, were subsequently injected into the subarachnoid space. The injection success rate, confirmed by 2% lidocaine, was consistently over 95% in our laboratory.

Intrathecal Catheterization and Injection in Rats

The surgical procedure was performed under isoflurane (4% for induction and 1% for maintenance) in oxygen anesthesia according to protocols described earlier.⁴¹ An 18-cm polyethylene catheter (PE-10: 0.28-mm i.d./0.61-mm o.d.; Clay Adams, Parsippany, NJ) with volume of approximately 13 μl was inserted into the rat spinal cord at the lumbar level. Two days after recovery from anesthesia, correct intrathecal cannula placement was verified by administering 4% lidocaine. Only rats with no motor impairment following intrathecal catheter insertion were considered for the study, and only rats that developed immediate bilateral paralysis of their hind limbs following intrathecal administration of lidocaine were selected for the study. Our success rate for intrathecal catheterization was over 95%. The drugs were microinjected intrathecally with a 50-μl microsyringe (Shanghai Anting Micro-Injector Factory, Shanghai, China) in a volume of 10 μl followed by a saline flush in a volume of 20 μl.

Intracerebroventricular Catheterization and Injection in Rats

Methods for the intracerebroventricular catheterization and injection in rats have been described previously.⁴² Briefly, animals were anesthetized with intraperitoneal injection of pentobarbital sodium (40 mg/kg) and then placed on a stereotaxic instrument (Stoelting Co., Wood Dale, IL). After exposing the skull of rats, stainless steel screws were inserted into the somatosensory cortex (anterior/posterior: 0 mm, height: 2 mm) and into the somatosensory cortex (anterior/posterior: 3 mm, height: 1.5 mm relative to the bregma). A cannula for intraventricular injection was inserted at anterior/posterior: 0 mm (left side) and H: 4 mm relative to the bregma. After closure of the wound, the animals were returned to their home cages and allowed to recover for 2 to 3 days without using analgesics before entering the terminal experiments. Our preliminary study used 5 μl Indian ink dye to ensure accuracy and completeness of the surgical operation. Control and test articles were slowly delivered over 1 min through the injection needle (diameter: 0.3 mm) connected to a 10-μl microsyringe via a polyethylene tube, which was held in the same place for 5 min.

Mouse and Rat Formalin Test

The rat formalin test was performed as previously described.⁴¹ Animals were acclimated individually to the observation cage for 30 min before injecting 50 μl of 5% formalin in 0.9% saline subcutaneously on the dorsal side of the left hind paw. Nociceptive behavior in rats was manually quantified by counting the number of formalin-injected paw flinches in 1-min epochs, and measurements were taken at 10-min intervals beginning immediately after formalin injection and ending 90 min later. The mouse formalin test was performed as previously described.⁴³ In brief, 10 μl of 5% formalin in 0.9% saline was injected subcutaneously
on the dorsal side of the right hind paw, and the animal was immediately placed in a transparent observation box. The duration of nociceptive behaviors (mainly licking and some biting) was manually quantified in the pooled durations at 0 to 5 min and 20 to 40 min, which were considered the acute nociceptive response and tonic hyperalgesia, respectively.

**Rat Model of Peripheral Nerve Injury–induced Neuropathy**

The surgical procedure was performed under isoflurane (4% for induction and 1% for maintenance) in oxygen anesthesia via a facemask connected to a ventilator (Ugo Basile Gas Anesthesia System, Comerio, Italy). Neuropathy was created as described earlier. In brief, a partial excision of the left anatomic innervation area was performed. After ligation, the wound was inspected for hemostasis and then sutured, and the rats were allowed to recover. Rats showing a mechanical threshold of equal or less than 8.0 g and with no major motor impairment were defined as having appropriate neuropathy and selected for further studies.

**Rat Model of Bone Cancer–induced Mechanical Allodynia**

The bone cancer pain procedure followed the protocol as described in detail previously. In brief, female rats (commonly used for breast cancer cell inoculation) were anesthetized by intraperitoneal injection of sodium pentobarbital (40 mg/kg). A 1-cm-long rostra-caudal incision was made in the medial tibial skin. A hole was drilled with a 23-gauge needle 1 cm below the knee joint distal to the epiphyseal growth plate at the medial flat of the bone. Walker 256 rat mammary gland carcinoma cells (4 × 10^5 cells) in 10 μl of sterile phosphate-buffered saline in a 25-μl microsyringe (Shanghai Anting Micro-Injector Factory) were injected into the tibia, followed by immediate closure of the injection pore with aseptic bone wax.

**Behavioral Assessment of Mechanical Allodynia**

The hind limb withdrawal evoked by stimulation of the hind paw with a 2390 series electrical von Frey hair (ITTC Life Science Inc., Woodland Hills, CA) was determined while the mouse stood on a metal grid. The monofilaments, which produced forces ranging from 0.1 to 65 g, were applied to the footpad with increasing force until the mouse suddenly withdrew its hind limb. The lowest force producing a withdrawal response was considered the threshold, which was based on three repeated measurements, and the mean of three repeated threshold values for each hind paw at each time point was used.

**Rat Tail Immersion and Hot Plate Tests**

The rat tail immersion and hot plate tests were performed with the same animals according to the previous description with modifications. For the tail immersion test, a model SSY-H digital display thermostatic water bath (Shanghai Sanshen Medical Instrument Co., Shanghai, China) was used to maintain a constant water temperature of 50° ± 0.5°C. After the rats were placed in a tubular restrainer, their tails were immersed 3.5 cm into the water bath. The nociceptive threshold was defined as the time sustained to elicit a flick of the tail. The cutoff time was 30 s for tail-flick measurements to minimize tissue injury. For the hot plate test, the nociceptive reflex in response to thermal stimulus was measured using a YLS-6B Intelligence Hot Plate Analgesia Meter (Shandong Academy of Medical Sciences Device Station, Shandong, China). The surface of the hot plate was heated to a constant temperature of 55° ± 0.1°C, as measured by a built-in digital thermometer with an accuracy of 0.1°C and verified by a surface thermometer. Rats were placed on the hot plate, which was surrounded by a clear acrylic cage, and the start/stop button on the timer was activated. The latency to respond with a hind paw lick was measured to the nearest 0.1 s by deactivating the timer when the response was observed. To minimize tissue damage, trials were terminated if the animals did not respond within 50 s.

**Rat Rotarod Test and Locomotor Activity Test**

Motor coordination performance was assessed by a YLS-4C Rota Rod with automatic timers and falling sensors (Yiyan Scientific Ltd., Shandong, China). The rats were trained and tested by accelerating the rotarod speed from 5 to 25 rpm within 1 min followed by 25 rpm for 2 extra min. The accumulated time (seconds/3 min) for animals to spend on the rotarod was recorded during the 3-min observation period after the animals were trained once a day each for 9 min for 3 days. The accumulated time spent on the rod had to be at least 120 s to allow inclusion in the study. For the final testing, the accumulated time (seconds/5 min) spent on the rotarod was recorded during the 5-min observation period after receiving control or test articles. Locomotor activity was monitored by a YLS-1C electromagnetic activity monitor with a printer (Yiyan Scientific Ltd.) located in a quiet environment. Animals were each placed into an individual testing cage identical to their home cages with free access to food and water. Automatic counting of accumulated movements (counts/20 min) were continuously recorded and printed out at 20-min intervals for 4 h after test drug gavage.

**Data Analysis and Statistical Evaluation**

For dose–response curve analysis, the maximum effect (E_max), half-effective dose (ED_{50}), and Hill coefficient (n) of the parameters were calculated from individual dose–response curves. To determine the parameters of the dose–response curves, values of response (Y) were fitted by nonlinear least-squares curves to the relation \( Y = a + b \cdot x \), where \( x = [D]^{n} / (ED_{50} + [D])^{n} \), to give the value of half-effective dose (ED_{50}) and maximal inhibition (E_max) yielding a minimum residual sum of squares of deviations from the theoretical curve.
For Schild plots and pA2 analysis, the ratio of the dose of agonist ($A'$) to produce ED$_{50}$ in the presence of the antagonist ($B$) to the dose required in the absence of the agonist ($A$) is calculated following the equation: $[A]/([A] + K_D) = [A']/([A'] + K_{B} (1 + [B]/K_{B})$, where $K_D$ and $K_B$ are the equilibrium dissociation constants for $A$ and $B$, respectively.$^{51}$ pA$_2$ is practically determined by the application of at least three doses of antagonist, and then log (($A'/A) - 1$) versus the negative log B is plotted. If the regression of log (($A'/A) - 1$) on −log B is linear with a slope of −1, then this indicates that the antagonism is competitive, and by definition, the agonist and antagonist act at the same recognition sites. The x-intercept of the fitted regression line is an estimate of pA$_2$, which is the dose of the antagonist requiring a two-fold increase in agonist concentration.

Data are expressed as mean ± SEM, and no data were missing. Statistical significance was evaluated by an unpaired Student t test or one-way ANOVA with Prism (version 5.01; GraphPad Software Inc., San Diego, CA). This was followed by a post hoc Student–Newman–Keuls test when a statistically significant drug (dose) effect was observed. Probability values were two-tailed, and the statistical significance criterion P value was 0.05.

Results

L. rotata Exhibits Specific Antiallodynia and Antihyperalgesia in Pain Hypersensitivity States without Inducing Apparent Antiallodynic Tolerance

To determine differential blockade effects in acute nociceptive pain response and pain hypersensitivity states, the L. rotata aqueous extract was first tested in the formalin test, peripheral nerve injury– and bone cancer–induced mechanical allodynia, and thermally evoked acute nociceptive reflex responses in rats. Six groups of normal rats received gavage of saline or the L. rotata aqueous extract at different doses 30 min before formalin challenge. Subcutaneous injection of formalin in saline control rats provoked a biphasic response consisting of an initial and rapidly decaying acute phase followed by a slowly rising and long-lived tonic phase. Compared to the saline control, gavage with the L. rotata aqueous extract (30, 100, 300, 1,000, and 3,000 mg/kg) did not significantly inhibit the formalin-induced acute
flinching response \((P = 0.2284\) by one-way ANOVA) but produced dose-dependent blockade of the formalin-induced tonic hyperalgesia (fig. 2A). Further measured by the areas under the flinching response curve from 10 to 90 min in the tonic phase, dose–response analysis by best fit showed that the ED\(_{50}\) of the \(L.\) rotata aqueous extract was 214.6 mg/kg, and E\(_{\text{max}}\) was 73\% (fig. 2B).

Peripheral nerve injury causes clinically relevant painful peripheral neuropathy.\(^{52}\) Ligation of the L5 and L6 lumbar spinal nerves produces long-lasting mechanical allodynia on the ipsilateral hind paw. Six groups of neuropathic rats (at approximately 2 weeks after surgery) received gavage of saline and the \(L.\) rotata aqueous extract at different doses. Intragastric administration of \(L.\) rotata aqueous extract (30, 100, 300, 1,000, and 3,000 mg/kg) suppressed mechanical allodynia in ipsilateral paws in a dose-dependent manner but did not significantly alter withdrawal thresholds in contralateral paws (fig. 2C). The antiallodynic effect was time-dependent, with a peak effect at 1 h after gavage and a duration of more than 4 h. Dose–response analysis showed that the ED\(_{50}\) value was 236.5 mg/kg and the E\(_{\text{max}}\) value was 50\%, as calculated from 1-h values after gavage (fig. 2D).

Bone cancer developed from intratibial Walker 256 cells induces mechanical allodynia, reduction in weight bearing and increased incidence of spontaneous pain.\(^{53}\) Six groups of bone cancer pain rats, at approximately 20 days after cancer cell implantation, received gavage of saline and the \(L.\) rotata aqueous extract at different doses. Intragastric administration of the \(L.\) rotata aqueous extract (30, 100, 300, 1,000, and 3,000 mg/kg) reduced bone cancer–induced mechanical allodynia in ipsilateral paws in a dose-dependent manner but did not significantly alter withdrawal thresholds in contralateral paws (fig. 2E). The antiallodynic effect lasted for much longer than 4 h with a peak effect at 1 h after gavage. Dose–response analysis showed that the ED\(_{50}\) was

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Fig. 2. Effects of gavage with the \(Lamiophilomis rotata\) (\(L.\) rotata) aqueous extract on formalin-induced pain (A, B), peripheral nerve injury–induced mechanical allodynia (C, D), bone cancer–induced mechanical allodynia (E, F), thermally evoked nociceptive withdrawal responses (G, H), rotarod test (I), and locomotor activity test (J) in rats. Dose–response analysis of gavage with the \(L.\) rotata aqueous extract on formalin-induced tonic hyperalgesia measured by areas under the flinching response curve from 10 to 90 min (B), neuropathic pain (D) and bone cancer–induced mechanical allodynia (F), best fitted by the nonlinear least-squares method. (K) Effects of multiple daily gavage with \(L.\) rotata aqueous extract on mechanical allodynia in neuropathic rats. Data are presented as means ± SEM (\(n = 6\) in each group).
242.9 mg/kg and the $E_{\text{max}}$ was 54%, as calculated from 1-h values after injection (fig. 2F).

Conversely, gavage with the \textit{L. rotata} aqueous extract at a dose as high as 3,000 mg/kg did not reduce or potentiate either thermally evoked licking or flicking responses in the hot plate test (fig. 2G) or tail immersion test (fig. 2H), respectively, in rats during the 2-h observation period. No apparent sedation or motor side effects of the \textit{L. rotata} aqueous extract were observed during the study period. The rotarod and locomotor activity tests were conducted to further examine the possible motor side effects in rats. Gavage with the \textit{L. rotata} aqueous extract (3,000 mg/kg) did not significantly alter motor coordination (fig. 2I) or locomotor activity (fig. 2J) up to 4 h after observation.

Tolerance to analgesia is one of the major disadvantages of the clinical application of opiates, especially morphine. Given its wide clinical use in China for the treatment of chronic pain, we tested whether rats undergoing long-term treatment with \textit{L. rotata} developed tolerance to antiallodynia. Two groups of neuropathic rats (at approximately 2 weeks after surgery) received multiple daily gavage of saline and the \textit{L. rotata} aqueous extract (1,000 mg/kg) for 7 days. Both contralateral and ipsilateral paw withdrawal thresholds were measured 1 h after each gavage of saline or the \textit{L. rotata} aqueous extract over the 7-day period. Saline did not significantly affect withdrawal thresholds in either the contralateral or the ipsilateral paws, and its ineffectiveness remained the same throughout the 7-day gavage. In contrast, gavage with the \textit{L. rotata} aqueous extract produced marked antiallodynia by approximately 43% in the ipsilateral paws. The antiallodynic effect persisted throughout the 7-day gavage, suggesting that \textit{L. rotata} does not induce apparent antiallodynic tolerance (fig. 2K), in contrast to morphine administered with the same regimen.53

![Fig. 3. (Continued)](http://pubs.asahq.org/anesthesiology/article-pdf/121/4/835/266027/20141000_0-00027.pdf)
together, our results demonstrated that *L. rotata* specifically blocks pain hypersensitivity states but neither affects acute nociceptive pain response nor induces tolerance to antiallodynia.

**Iridoid Glycosides, Represented by SM and 8-O-acetyl-SM, Are Effective Ingredients for *L. rotata* Antihyperalgesia**

To identify effective ingredients for *L. rotata* antihyperalgesia, we used the activity-tracking method to investigate possible antihyperalgesic effects of the two major components, flavonoids and iridoid glycosides. Two groups of normal mice received gavage of saline and the total flavonoids extract, which contained approximately 90% flavonoids but without SM or 8-O-acetyl-SM, at a dose as high as 1,000 mg/kg, 30 min before formalin injection. Gavage with the total flavonoids extract from *L. rotata* did not significantly reduce either formalin-induced tonic hyperalgesia or acute flinching response compared to the saline control (fig. 3A).

We manufactured six iridoid glucose extracts from *L. rotata*. Total iridoid glycosides contained in these samples, as represented by the average of both SM and 8-O-acetyl-SM contents, were differentially distributed among 6% (the aqueous extract), 11% (the alcoholic extract), 22% (sample 3), 45% (sample 4), 57% (sample 5), and 62% (sample 6). We also prepared an “artificial extract sample” by mixing the pure SM with 8-O-acetyl-SM in a 1:1 ratio, resulting in a presumed content of 95% as an “artificial extract sample.” (I) Dose–response analysis of formalin-induced tonic pain, best fitted by the nonlinear least-squares method. Data are presented as means ± SEM (n = 5 in each group). *Statistical significance compared with the vehicle control group (P < 0.05 by one-way ANOVA followed by post hoc Student–Newman–Keuls test).

Fig. 3. Effects of gavage with *Lamiophlomis rotata*-derived total flavonoids (A) and total iridoid glycosides represented by shanzhiside methylester (SM) and 8-O-acetyl-SM (B–H), on formalin-induced flinching responses in mice. The total iridoid glycosides from extract samples were 6, 11, 22, 45, 57, and 62%, as represented by the combination of SM and 8-O-acetyl-SM. The last sample was made by mixing pure SM with 8-O-acetyl-SM in a 1:1 ratio, resulting in a presumed content of 95% as an “artificial extract sample.” (I) Dose–response analysis of formalin-induced tonic pain, best fitted by the nonlinear least-squares method.
Table 1. Total Iridoid Glycoside Contents of the Lamiophlomis rotata Extracts* and the ED50 Values of Their Antihyperalgesia in the Mouse Formalin Test

<table>
<thead>
<tr>
<th>Extract or Compound</th>
<th>Yield (%)</th>
<th>SM (%)</th>
<th>8-O-Acetyl-SM (%)</th>
<th>Total Iridoid* (%)</th>
<th>Average Total Iridoid (%)</th>
<th>ED50 (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous extract</td>
<td>36.6</td>
<td>1.2</td>
<td>0.7</td>
<td>7.1</td>
<td>5.2</td>
<td>6.2</td>
</tr>
<tr>
<td>Alcoholic extract</td>
<td>27.1</td>
<td>2.3</td>
<td>1.7</td>
<td>12.9</td>
<td>9.6</td>
<td>11.2</td>
</tr>
<tr>
<td>Sample 1</td>
<td>4.49</td>
<td>5.5</td>
<td>3.7</td>
<td>25.7</td>
<td>19.2</td>
<td>22.4</td>
</tr>
<tr>
<td>Sample 2</td>
<td>1.97</td>
<td>19.6</td>
<td>1.4</td>
<td>51.8</td>
<td>38.6</td>
<td>45.2</td>
</tr>
<tr>
<td>Sample 3</td>
<td>N.A.</td>
<td>13.8</td>
<td>4.2</td>
<td>53.4</td>
<td>60.3</td>
<td>56.9</td>
</tr>
<tr>
<td>Sample 4</td>
<td>N.A.</td>
<td>15.3</td>
<td>3.2</td>
<td>71.6</td>
<td>53.3</td>
<td>62.5</td>
</tr>
<tr>
<td>“Artificial extract sample”</td>
<td>N.A.</td>
<td>95</td>
<td>95</td>
<td>N.A.</td>
<td>N.A.</td>
<td>95</td>
</tr>
</tbody>
</table>

ED50 = half-effective dose; N.A. = not available; SM = shanzhiside methylester.

Iridoid glycoside contents are represented by the combination of SM and 8-O-acetyl-SM as determined by high-performance liquid chromatography.

* Total iridoid content relative to SM. † Total iridoid content relative to 8-O-acetyl-SM.

Fig. 4. Correlation between iridoid glycoside contents represented by the combination of shanzhiside methylester and 8-O-acetyl-shanzhiside methylester from the Lamiophlomis rotata extract samples (table 1) and their half-effective dose (ED50) values (in formalin-induced tonic hyperalgesia) in a nonlinear regression of one-phase exponential decay (A) and 1/ED50 values in a linear regression (B). The statistical significance was calculated for both correlations (P < 0.05 by the two-tailed Student t test).

nonlinear regression of one-phase exponential decay (R2 > 0.98, P < 0.05 by the two-tailed Student t test; fig. 4A) and to the 1/ED50 values in a linear regression (R2 > 0.98, P < 0.05 by the two-tailed Student t test; fig. 4B). Furthermore, the ED50 value for the combination of SM and 8-O-acetyl-SM was calculated from each extract sample and normalized by the percentage of its combination of both compounds. The result predicted that the theoretical ED50 would be 10.8 mg/kg, which was very close to our measured value of 9.6 mg/kg, suggesting that SM and 8-O-acetyl-SM are principle iridoid glycosides responsible for antihyperalgesia.

**SM and 8-O-acetyl-SM Produce Antihyperalgesia at the Spinal Cord Rather Than in the Brain or Peripheral Afferent Nociceptors**

In an effort to investigate the primary site responsible for *L. rotata* antihyperalgesia, we performed local direct injection of SM and 8-O-acetyl-SM. We first tested the antihyperalgesic effects of SM and 8-O-acetyl-SM via direct intrathecal injection into the spinal cord. Each of the six groups of rats (total 12 groups) chronically implanted with intrathecal cannulas received single intrathecal injections of saline (10 μl) or SM (10, 30, 100, and 300 μg) or 8-O-acetyl-SM (3, 10, 30, 100, and 300 μg) 30 min before formalin injection. Neither SM (fig. 5A) nor 8-O-acetyl-SM (fig. 5B) at concentrations of up to 300 μg significantly affected acute flinching response. Both compounds effectively blocked formalin-induced tonic hyperalgesia in a dose-dependent manner. The areas under the flinching response curve from 10 to 90 min were calculated, and dose–response analysis showed the Emx and ED50 values to be 76% and 24 μg for SM and 74% and 20.5 μg for 8-O-acetyl-SM, respectively (fig. 5C). No apparent sedation or motor side effects were observed with both compounds.

Furthermore, rats chronically implanted with intracerebroventricular cannulas received intraventricular injection of 5 μl saline or 300 μg SM or 8-O-acetyl-SM 30 min before formalin injection. The two compounds did not significantly reduce either the formalin-induced tonic or acute flinching response (fig. 5D). The same ineffectiveness was also demonstrated in paw subcutaneous coinjection with formalin, in which 300 μg SM or 8-O-acetyl-SM was not effective in reducing formalin flinching responses (fig. 5E). Taken together, all these results suggest that SM and 8-O-acetyl-SM produce antihyperalgesia primarily via the spinal cord rather than the brain or peripheral afferent nociceptors.

**L. rotata, SM, and 8-O-acetyl-SM Produce Antiallodynia and Antihyperalgesia by Activation of Apinal GLP-1Rs**

To test whether the antihyperalgesic and antiallodynic effects of *L. rotata*, SM, and 8-O-acetyl-SM occurred via activation of spinal GLP-1Rs, the specific GLP-1R antagonist...
exendin(9–39) was first applied intrathecally. Six groups of normal mice received intragastric administration of saline (10 ml/kg) or the L. rotata aqueous extract (800 mg/kg) and intrathecal injection of 5 μl saline or exendin(9–39) (0.1, 0.3, and 1 μg) 30 min before formalin injection. As shown in figure 6A, the intrathecal administration of 1 μg exendin(9–39) alone did not significantly affect either the formalin-induced tonic or the acute flinching response. Gavage with the L. rotata aqueous extract remarkably reduced the formalin-induced tonic hyperalgesia by 72%, which was dose-dependently and completely prevented by intrathecal exendin(9–39), with one-half reduction and complete prevention at doses of 0.4 and 1.0 μg, respectively (fig. 6B). In addition, intrathecal injection of 2 μg exendin(9–39) completely prevented the antiallodynic effect induced by gavage with the L. rotata aqueous extract (1,000 mg/kg) in spinal nerve ligation–induced neuropathic rats (fig. 6C).

Five groups of normal mice received intragastric administration of saline (10 ml/kg), SM (30 mg/kg), or 8-O-acetyl-SM (30 mg/kg) and intrathecal injection of 5 μl saline or 1 μg exendin(9–39) 30 min before formalin injection. Gavage with both SM and 8-O-acetyl-SM significantly suppressed formalin-induced tonic pain by 70 and 66%, respectively. Intrathecal injection of exendin(9–39) at 1 μg completely prevented antihyperalgesia produced by intragastric administration of SM or 8-O-acetyl-SM (fig. 6D).

The GLP-1R gene silencer (siRNA/GLP-1R) was applied to confirm the role of spinal GLP-1Rs in L. rotata antihyperalgesia. Four groups of intrathecally cannulated rats received multidaily intrathecal injections of PEI (control vehicle, 7.5 μg/day), nonspecific oligonucleotide (5 μg/day), or siRNA/GLP-1R (5 μg/day) for 7 days before gavage with saline (10 ml/kg) or the L. rotata aqueous extract (800 mg/kg) followed by formalin challenge. Intragastric L. rotata aqueous extract effectively blocked formalin-induced tonic hyperalgesia by 49 and 52% in PEI- and nonspecific oligonucleotide-treated control rats, respectively. Multidaily intrathecal injections of siRNA/GLP-1R completely blocked L. rotata aqueous extract-induced antihyperalgesia (fig. 6, E and F). The rats were then immediately killed after completion of the behavior test, and spinal homogenates for the Western blot test were obtained from L3–L5 lumbar enlargements of the spinal cord. Multidaily intrathecal injections of siRNA/GLP-1R significantly reduced GLP-1R protein expression (fig. 6G, inset). After scanning, the intensity ratio of the GLP-1R/β-actin band was calculated, and siRNA/GLP-1R reduced spinal GLP-1R protein expression by 84.3 and 79.5%, respectively, compared to the vehicle and nonspecific oligonucleotide control (fig. 6G). Multiple daily injections of siRNA/GLP-1R did not significantly reduce GLP-1R expression in the dorsal root ganglia. Our results further support the spinal cord as the primary site and suggest that activation of spinal GLP-1Rs is entirely responsible for the antihyperalgesic effects of L. rotata and its effective ingredients.

**SM and 8-O-acetyl-SM Activate Rat and Human GLP-1Rs without Apparent Species Difference, Presumably at an Exendin(9–39)-sensitive Site**

To examine whether SM and 8-O-acetyl-SM acted at exendin(9–39)-sensitive GLP-1Rs at the cellular level, we evaluated the protective effects of both compounds against hydrogen peroxide–induced oxidative damage in rat PC12 cells expressing GLP-1R. We first confirmed that specific GLP-1R immunostaining was present on PC12 cells (fig. 7A). Treatment with both SM and 8-O-acetyl-SM produced concentration-dependent inhibition of hydrogen peroxide–induced viability loss in PC12 cells, with EC50 values...
Fig. 6. Blockade effects of a single intrathecal injection of the specific glucagon-like peptide-1 receptors (GLP-1Rs) antagonist exendin(9–39) (A–D) and multiple daily injections of GLP-1R gene silencer small interfering RNA (siRNA)/GLP-1R (5 g/day) for 7 days (E–F) on the antihyperalgesic and antiallodynic effects of *Lamiophlomis rotata* (L. rotata) aqueous extract, shanzhiside methylester (SM), and 8-O-acetyl-SM in the mouse and rat formalin test and rat neuropathic pain test. (B) Dose–response analysis of the blockade effect of exendin(9–39) on antihyperalgesia produced by gavage with L. rotata in mice, best fitted by the nonlinear least-squares method. (F) Inhibitory effect of siRNA/GLP-1R on gavage L. rotata-induced antihyperalgesia of formalin-induced tonic flinching response measured by the area under the curve between 10 and 90 min (AUC10–90 min). (G) Inhibitory effect of intrathecal siRNA/GLP-1R for 7 days on GLP-1R protein expression in the spinal cord homogenates. The inset shows representative gels. The data are presented as means ± SEM (n = 6 in each group). *, #Statistical significance compared with the vehicle control group and L. rotata aqueous extract group, SM group, or 8-O-acetyl-SM group (P < 0.05 by one-way ANOVA followed by post hoc Student–Newman–Keuls test), respectively.
of 7.8 × 10⁻⁵ M and 1.4 × 10⁻⁴ M, respectively, and maximum protection of approximately 80% (fig. 7, B and C). Further treatment with exendin(9–39) at 1 × 10⁻⁷, 3 × 10⁻⁷, and 1 × 10⁻⁶ M shifted the above concentration response curves to the right without affecting the maximum protective values in a concentration-dependent manner. The Schild plots of exendin(9–39) against SM and 8-O-acetyl-SM were inserted in B and C, and E and F, respectively. Cells were grown on 96-well plates and attached after incubations of 24 h; hydrogen peroxide was added for 15 min in PC12 cells (final concentration, 500 μM), for 5 min in HEK293 cells (400 μM), and for 12 h in HEK293T cells (800 μM). PC12 and HEK293 cells after washing were treated with SM or 8-O-acetyl-SM in the absence or presence of exendin(9–39) for 12 h. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was conducted to determine cell viability. Data are presented as means ± SEM (n = 3 in each group). The study was repeated three times. Scale bars: 50 μm in A, D, and G. *Statistical significance compared with the vehicle control group (P < 0.05 by one-way ANOVA followed by post hoc Student-Newman-Keuls test).

Specific GLP-1R immunostaining staining was also confirmed on HEK293 cells with stable expression of human GLP-1Rs (fig. 7D). SM and 8-O-acetyl-SM produced concentration-dependent inhibition of hydrogen peroxide–induced viability loss in HEK293 cells, with EC₅₀ values of 9.5 × 10⁻⁵ M and 1.1 × 10⁻⁴ M, respectively, and maximum protection of approximately 100%. Treatment with exendin(9–39) at 1 × 10⁻⁷, 3 × 10⁻⁷, and 1 × 10⁻⁶ M also shifted the curves to the right in a concentration-dependent manner without affecting the maximum protective values. The Schild plots of exendin(9–39) in the insets of figure 7, E and F, show that the pA₂ values against SM and 8-O-acetyl-SM were 7.1 and 7.3, respectively, very similar to those of exendin(9–39) against exenatide in the same human GLP-1R assay.

To further confirm that SM and 8-O-acetyl-SM protected against hydrogen peroxide–induced oxidative damage via activation of GLP-1Rs, we also used parent HEK293T cells that do not express GLP-1Rs.⁵⁵,⁵⁶ Indeed, there was no specific GLP-1R immunostaining on HEK293T cells (fig. 7G). Treatment with SM and 8-O-acetyl-SM at concentrations as high as 1 mM did not protect against hydrogen peroxide–induced oxidative damage in these cells.
peroxide–induced oxidative damage (fig. 7H). In contrast, treatment with nicotinamide showed significant protection, in agreement with a previously reported finding.57 These results all indicated that SM and 8-O-acetyl-SM activate GLP-1Rs without apparent rat and human species difference and suggest that SM and 8-O-acetyl-SM act at the same recognition site as exendin(9–39) and exenatide.

Discussion
Our results systemically demonstrate that L. rotata is an antiallodynic and antihyperalgesic agent specifically effective in pain hypersensitivity states in experimental animal models. The results revealed that the L. rotata aqueous extract markedly and long- lastingly blocked both formalin-induced tonic hyperalgesia and peripheral nerve injury– and bone cancer–induced mechanical allodynia by 50 to 80%, with its similar half-effective doses ranging between 130 and 250 mg/kg. In contrast, L. rotata of up to 3,000 mg/kg was not effective in nociceptive pain responses, as evidenced by the ineffectiveness in the formalin-induced acute flinching response, mechanically stimulated paw withdrawal response and thermally evoked licking and flicking responses. In addition, chronic treatment with L. rotata did not lead to apparent antiallodynic tolerance, in contrast to morphine. Our results support the clinical application of L. rotata for long-term use. Indeed, a meta-analysis including 37 random control clinical trials showed that the long-term pain relief effects of an L. rotata capsule in a variety of pain states are superior to those of indomethacin tablets with fewer side effects.58

It is worth mentioning that although its in vitro potency is relatively low, the L. rotata aqueous extract was fairly potent in its effects on antiallodynia and antihyperalgesia in vivo: the half-effective doses ranged between 130 and 250 mg/kg for the aqueous extract and were approximately 10 mg/kg for its effective ingredients SM and 8-O-acetyl-SM. In addition, the efficacy of L. rotata against antiallodynia and antihyperalgesia ranged between 50 and 80%, and the duration for a single gavage was much longer than 4 h. The combination of fair potency, high efficacy, and long duration is of clinical significance when L. rotata is compared to gabapentin, a well-prescribed painkiller for chronic neuropathic pain and painful diabetic neuropathy. Gabapentin is dosed in a range of 100 to 300 mg/kg in rats and mice with a gavage duration usually of less than 4 h, whereas its dose ranges between 900 and 1,200 mg in patients. The doses of the L. rotata aqueous extract used in our animal studies are apparently parallel to its clinical treatment regimen in humans, with doses of 900 mg per dose three times a day resulting in a per-day dose of approximately 50 mg/kg. This appears to be true especially when the species factor is considered, in that humans generally (although not always) metabolize drugs more slowly than rodents by a percentage of 10 to 30% or even more.59 Nevertheless, a study should be conducted to compare the levels of the effective ingredients in human and rodent plasma after oral administration of L. rotata.

Flavonoids and iridoid glycosides represent major ingredients in L. rotata. Our study using an activity-tracking method identified total iridoid glycosides rather than total flavonoids, as the effective ingredients completely responsible for the antihyperalgesic property of L. rotata. Total iridoid glycosides comprise quite a few single isomers, such as SM, 8-O-acetyl-SM, 6-O-acetyl-SM, phlloyside II, 8-dehydroxyshanzhiside, loganin, penstemoside, 7,8-dehydropenstemoside, laminalorectine and iridoid diglycosides, and lamiophlomiol A and B.26–29 By measuring and adjusting the combination of SM and 8-O-acetyl-SM, we found the potency of the L. rotata extract antihyperalgesia to correlate well with that of the total iridoid glycoside contents. Our results also suggested that being nearly equally active, SM and 8-O-acetyl-SM are the major effective ingredients, although minor contributions of other iridoid glycosides are also recognized. This notion is supported by the following facts: (1) SM (1%) and 8-O-acetyl-SM (1 to 3%) represented major components, whereas other all iridoid glycosides were present in trace amounts56; (2) SM and 8-O-acetyl-SM, as well as loganin and other iridoid glycosides, had very similar efficacy and potency for GLP-1R activation in PC12 and HEK293 cells and for in vivo antihyperalgesia; (3) SM and 8-O-acetyl-SM were proved to be nearly entirely responsible for total iridoid glycosides antihyperalgesia by testing the “artificial extract sample.”

This study provides specific evidence to support the notion that L. rotata produces antiallodynia and antihyperalgesia entirely via direct activation of spinal GLP-1Rs. (1) Intrathecal injection of SM and 8-O-acetyl-SM produced dose-dependent antihyperalgesia, whereas their intravenous-tricular or subcutaneous injection at peripheral nociceptors was not effective. (2) The antihyperalgesic and antiallodynic effects of gavage with L. rotata aqueous extract, SM, and 8-O-acetyl-SM were completely prevented by intrathecal exendin(9–39) and genetic knockdown of spinal but not dorsal root ganglial19 GLP-1Rs. (3) SM and 8-O-acetyl-SM protected against hydrogen peroxide–induced oxidative damage in both rat PC12 cells expressing GLP-1Rs and HEK293 cells with stable expression of human GLP-1Rs but not in HEK293T cells not expressing GLP-1Rs. These results highlight a direct interaction between SM and 8-O-acetyl-SM and the spinal GLP-1Rs rather than a signal transduction pathway after the activation of the GLP-1Rs. (4) Exendin(9–39) competitively blocked the protective effects of SM and 8-O-acetyl-SM, with yielded pA2 values close to those of exendin(9–39) to compete against exenatide. These results suggest that SM and 8-O-acetyl-SM might act at the same binding site as exendin(9–39) and exenatide. GLP-1Rs belong to a class B1 family of G-protein-coupled receptors, which interact with peptidic ligands such as exenatide and GLP-1, probably with two binding domains.60–62 Peptidic exenatide and GLP-1 are not orally active and do not readily access the blood–brain barrier; thus, the search for orally active, nonpeptidic GLP-1R agonists has been a hot area of
research and development activities for antidiabetic drugs and possibly now for analgesics. However, because small molecules may not have sufficiently large mass and long diameter to bind to both binding domains, it is a challenge to design and synthesize small molecular GLP-1R agonists, and little success has been achieved so far.\(^6\)\(^3\)\(^6\)\(^4\) Orally available SM and 8-O-acetyl-SM derived from the *L. rotata* source in nature may represent one novel option for further discovery and development of small molecules for GLP-1R activation-related indications, particularly those related to chronic pain.

We recently revealed that GLP-1R is specifically expressed on seemingly all microglial cells rather than astrocytes or neurons in the spinal dorsal horn, and it is profoundly up-regulated accompanying microglial proliferation and hypertrophy following peripheral nerve injury. Activation of spinal GLP-1Rs by peptidic agonists exenatide and GLP-1 leads to specific antihyperalgesia and anti- allodynia, which are completely blocked by pharmacologic blockade and gene ablation of GLP1-Rs. The results suggest that spinal GLP-1Rs may be potential target molecules for the treatment of chronic pain.\(^1\)\(^9\) Our current findings, by taking advantage of the orally available small molecule GLP-1R agonists SM and 8-O-acetyl-SM and the *L. rotata* aqueous extract, support this notion at the animal level. Furthermore, *L. rotata* has been widely used for the clinical treatment of a variety of moderate-to-severe chronic pain states including cancer pain, postoperative and bone fracture pain, and neuropathic pain, with doses and regimens (900 mg per dose, three times per day, resulting in a per-day dose of approximately 50 mg/kg) apparently close to those of the animal studies. Our study further highlights the possibility that GLP-1R might be a human-validated drug target molecule for the management of chronic pain. This notion is further supported by the finding that SM and 8-O-acetyl-SM activate both rat and human GLP-1Rs without apparent species difference. However, further study is warranted to investigate GLP-1R-related side effects of *L. rotata* because GLP-1Rs are also widely expressed in peripheral organs and tissues, such as the pancreatic islets,\(^17\) lungs,\(^6\)\(^5\) and cardiovascular system.\(^6\)\(^6\)

**Competing Interests**

The authors declare no competing interests.

**Correspondence**

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**References**