Licochalcone Mediates the Pain Relief by Targeting the Voltage-Gated Sodium Channel

Qianru Zhao††, Xu Zhang††, Siru Long††, Shaobing Wang†, Hui Yu†, Yongsheng Zhou†, Yi Li‡, Lu Xue‡, Yan Hu†, Shijin Yin†∗

†Department of Chemical Biology, School of Pharmaceutical Sciences, South-Central Minzu University, Wuhan, 430074, PR China
‡Institute for Medical Biology and Hubei Provincial Key Laboratory for Protection and Application of Special Plants in Wuling Area of China, College of Life Sciences, South-Central Minzu University, Wuhan, 430074, PR China

††These authors contributed equally to this work.
Running title: Analgesic Effects and Mechanisms of Licochalcones

*Corresponding author: Shijin Yin, Department of Chemical Biology, School of Pharmaceutical Sciences, South-Central Minzu University, No.182 Minzu Road, Hongshan District, Wuhan, Hubei Province, 430074, PR China.
Tel: +086 67841196. Fax: +086 67841196. E-mail address: yinshijinyf@163.com.

Number of text pages: 25
Number of tables: 0
Number of figures: 8
Number of references: 32
Number of words in the Abstract: 238
Number of words in the Introduction: 635
Number of words in the Discussion: 751

Abbreviations used:
APP, action potential peak; APT, action potential threshold; DMEM, Dulbecco’s modified eagle medium; DMSO, dimethyl sulphoxide; DRG, dorsal root ganglion; FBS, fetal bovine serum; gNa, membrane conductance of Na⁺; HBSS, Hank’s balanced salt solution; HPLC, high performance liquid chromatography; INa, sodium currents; LCA, Licochalcone A; LCB, Licochalcone B; LCC, Licochalcone C; LrB, Loureirin B; NaV, channel, voltage-gated sodium channel; opti-MEM, opti-minimal essential medium; RP, resting membrane potential; TTX, Tetrodotoxin.
Abstract
Licorice is a traditional Chinese medicine and recorded to have pain relief effects in
national Phamacopoeia, while the mechanisms behind these effects have not been
fully explored. Among the hundred compounds in licorice, Licochalcone A (LCA)
and Licochalcone B (LCB) are two important components belonging to the chalcone
family. In this study, we compared the analgesic effects of these two licochalcones
and the molecular mechanisms. LCA and LCB were applied in cultured dorsal root
ganglion (DRG) neurons, and the voltage-gated sodium (Nav) currents and action
potentials were recorded. The electrophysiological experiments showed that LCA can
inhibit Nav current and dampen excitability of DRG neuron, while LCB did not show
inhibition effect on Nav current. Because the Nav1.7 channel can modulate
Subthreshold membrane potential oscillations in DRG neuron which can palliate
neuropathic pain, HEK293T cells were transfected with Nav1.7 channel and recorded
with whole-cell patch clamp. LCA can also inhibit Nav1.7 channels exogenously
expressed in HEK293T cells. We further explored the analgesic effects of LCA and
LCB on formalin-induced pain animal models. The animal behavior tests revealed
that LCA can inhibit the pain responses during phase1 and phase2 of formalin test and
LCB can inhibit the pain responses during phase2. The differences of the effects on
Nav currents between LCA and LCB provide us with the basis for developing Nav
channel inhibitors. And the novel findings of analgesic effects indicate that
licochalcones can be developed into effective analgesic medicines.

Significance Statement
In this study, we found LCA can inhibit Nav currents and dampen excitabilities of
DRG neurons and inhibit the Nav1.7 channels exogenously expressed in HEK293T
cells. Animal behaviour tests showed that LCA can inhibit the pain responses during
phase1 and phase2 of formalin test while LCB can inhibit the pain responses during
These findings indicate that licochalcones could be the leading compounds for developing NaV channel inhibitors and effective analgesic medicines.

Key words
Licochalcone A, Licochalcone B, voltage-gated sodium channel subunit NaV1.7, analgesia
Introduction

Licorice is a commonly used traditional Chinese medicine consisting of *Glycyrrhiza uralensis* Fisch., *Glycyrrhiza inflata* Batalin and *Glycyrrhiza glabra* L. according to Chinese national Phamacopoeia (Zhonghua Renmin Gongheguo wei sheng bu yao dian wei yuan hui., 1995). The efficacy of licorice decoction includes stomach and spleen protection, pain relief, cough alleviation and phlegm elimination recorded in ancient Chinese medicine archives (Yang et al., 2015). More beneficial effects of licorice on multiple diseases were found with broader investigations in recent years. It is reported that licorice or its extracts have antiviral and antimicrobial activities (Adianti et al., 2014; Ahn et al., 2012). Recent studies also indicate that LCA could relieve the neuropathic pain in chronic constriction injury model and the inflammatory responses induced by lipopolysaccharide (Chu et al., 2012; Li et al., 2021).

The general effects of licorice could be explained by its complex constituents. For now, at least 20 triterpenoids and 300 flavonoids were found in licorice and each component has specific activities (Yang et al., 2015). This may explain why the effects of licorice are various while the mechanisms of these effects are little known. With the development of extraction and identification techniques, single molecules in licorice have been isolated, synthesized and tested under physiological situations. The molecular mechanisms behind the effects could be unveiled gradually. Among the hundred compounds in licorice, Licochalcone A (LCA) is the one that attracted the most attention. The main reports about LCA are related to its antitumor effects (Hong et al., 2019; Yang et al., 2015). We compared the backbone of LCA and another kind of chalcone - Loureirin B (LrB) and found they have high similarities with each other. Since LrB was reported to be a voltage-gated sodium (Na\textsubscript{V}) channel blocker and have analgesic effects (Chen et al., 2018), we applied LCA in cultured DRG neuron and found it could inhibit Na\textsubscript{V} current and dampen excitability of DRG neuron. Then we compared the effects of Licochalcone B (LCB) which is also component of licorice.
and shares the same basic structure with LCA (Yang et al., 2015). We found that LCB did not significantly inhibit the NaV current in DRG neuron. These differences of the effects on NaV currents between LCA and LCB may provide us with the basis for developing specific NaV channel inhibitors.

NaV channels are reported to consist of 10 different pore-forming α-subunits (NaV1.1-9 and NaVX) (Dib-Hajj and Waxman, 2019). Among them NaV1.7 is mainly expressed in the nociceptors and peripheral neurons such as DRG neurons thus becomes a potential drug target for pain (Ho and O’Leary, 2011; Li et al., 2018). NaV1.7 channel could modulate Subthreshold membrane potential oscillations in DRG neuron which can palliate neuropathic pain (Li et al., 2018; McDermott et al., 2019). Gain-of-function mutation in NaV1.7 expressing gene SCN9A was related to inherited erythromelalgia (Fertleman et al., 2006; Yang et al., 2004). While loss-of-function mutation in SCN9A was linked to channelopathy-associated congenital insensitivity (indifference) to pain (CIP) and hereditary sensory and autonomic neuropathy (HSAN) type IID (Gingras et al., 2014; Yuan et al., 2013). Inhibition of NaV1.7 might alleviate pain with few side effects because of its exclusive expression in nociceptors, while the number of effective and specific NaV1.7 inhibitors is still rare (Kingwell, 2019). In this research, we found that LCA could block the NaV currents in both the DRG neurons and the NaV1.7 overexpression HEK293T cells. Further investigations in animal behavior tests showed that LCA could inhibit the pain responses during phase 1 and phase 2 of the formalin test. These are novel findings that revealed the analgesic effects of licochalcones and the molecular mechanisms related to inhibition on voltage-gated sodium channel. This study indicates that licochalcones have the potentials to be developed into effective analgesic medicines.

Materials and methods

Chemicals

Poly-D-lysine (Cat. E607014) and HBSS (Cat. A003210) were ordered from Sangon
Co., Ltd (Shanghai, China). opti-MEM (Cat. 31985070), DMEM (Cat. 11965092), Neurobasal-A (Cat. 10888022) and B-27 supplement (Cat. 17504044) solutions were bought from Gibco (NY, United States), FBS was ordered from YEASON (Suzhou, China). LCA (Cat. B20409) and ATP-Mg (Cat. T31046) were bought from Yuanye Bio-Technology Co., Ltd (Shanghai, China). The purities of LCA and LCB were HPLC ≥ 98.0% and the accuracy of LCA and LCB structures were confirmed by Nuclear Magnetic Resonance technology. LCA and LCB were dissolved in DMSO and diluted with the external solution for electrophysiological experiment and the same volume of DMSO was added as the vehicle group. Penicillin-Streptomycin (Cat. C0230) was bought from Nobleryder (Peking, China), Papain (Cat. BS-190) from Biosharp (Hefei, China), Collagenase (Cat. 11179179001) and Dispase (Cat. 04942078001) from Roche (Basel, Switzerland). Borosilicate glass (Cat. B15013F) was bought from VitalSense Scientific Instruments Co., Ltd (Wuhan, China) and ExFect2000 Transfection Reagent (Cat. T202-01) from Vazyme (Nanjing, China). Mexiletine (Cat. 5370-01-4) was bought from Aladdin (Shanghai, China) and DMSO (Cat. D2650) from Sigma-Aldrich (St. Louis, MO, United States). Chlortal hydrate (Cat. 30037516) and 37% formalin solution (Cat. 33314) was ordered from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

**Plant materials**

The names of the licorice used in our research have been check on http://www.theplantlist.org website. *Glycyrrhiza inflata* Batalin was bought from Yarkand County Fuyuan Licorice Co., Ltd. (Yarkand, Xinjiang, China). Its flavonoids were extracted according to the methods previously reported (Miyazaki et al., 2020). Roots of *Glycyrrhiza inflata* Batalin were ground into powder and ethyl acetate was added. The mixture was refluxed at 75°C for 2 h and the drug residues were removed. The solvent was dried with nitrogen and methanol was added for HPLC analysis. Agilent 1100 liquid chromatograph with Agilent SDB C18 chromatographic column (Santa Clara, CA, United States) was used for HPLC analysis. Mobile phase A is
0.5% phosphoric acid aqueous solution and mobile phase B is acetonitrile. The gradient elution program is as follows: 0→5min, B from 30%→40%; 5→7min, B rose to 50%; 7→30min, B rose to 55%. The detection wavelength was 372 nm, the flow rate was 1.0 mL/min. The quantities of the licochalcones were measured according to the peak area of the HPLC chromatography. The content of LCA is 2.22% in *Glycyrrhiza inflata* Batalin and 22.20% in its extract. The *Glycyrrhiza inflata* Batalin extract was applied with 0.046 mg/mL mass-volume ratio in electrophysiological experiment to guarantee the molar concentration of LCA was 30 μM. *Glycyrrhiza uralensis* Fisch. flavonoids were bought from FEIYUBIO Co., Ltd (Cat. FY1246, Nantong, Jiangsu, China). The same mass-volume ratio of *Glycyrrhiza uralensis* Fisch. flavonoids were applied as negative control because it does not contain LCA or LCB according to HPLC analysis. The *Glycyrrhiza inflata* Batalin extract and *Glycyrrhiza uralensis* Fisch. Flavonoids were dissolved in DMSO and diluted with the external solution for electrophysiological experiment. The same volume of DMSO was added as the vehicle group. The inhibition rate of Nav current in DRG neuron was calculated as follows,

\[
\text{Inhibition rate} = \frac{(I_{\text{vehicle}} - I_{\text{drug}})}{I_{\text{vehicle}}}
\]

Where \(I_{\text{drug}}\) represents the amplitude of Nav current after the drug treatment, \(I_{\text{vehicle}}\) represents the amplitude of Nav current after the vehicle treatment.

**Preparation of dorsal root ganglion neurons**

Experiment animals were bought from Wuhan Center for Disease Control and Prevention. All animal experiments were conducted according to the rules of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and strictly following guidelines of the Institutional Animal Care and Use Committees (IACUC). The IACUC checked all protocols and the animal ethical approval number is 2020-scuec-028. 4-week female Kunming mice were anaesthetized with isoflurane and sacrificed. The whole spine was dissected out and put in ice-cold HBSS. Extra muscles and tissues were discarded. The spine was cut along the middle line into two
symmetrical parts with scissors. Dorsal root ganglions were picked under dissecting microscope. Fibers on DRG were trimmed and only transparent ganglions were left. The round ganglions were lysised with 2 mg/mL Papain in HBSS for 10 min under 37 °C. During this time, the Eppendorf (Ep) tubes containing ganglions were shaken frequently. Centrifuge the Ep tubes under 4200 rpm for 4 min. The supernatant was discarded, and the precipitates were resuspended with 3.75 mg/mL Collagenase and 3.75 mg/mL Dispase for 10 min under 37 °C. The Ep tubes were centrifuged under 4200 rpm for 4 min again. The supernatant was discarded and 1mL DMEM was added to stop enzyme digestions. DRG neurons were dissociated by trituration with a fire-polished glass Pasteur pipettes (Liu et al., 2009). Repeat centrifuging and suspending again and seeded the cells on coverslips preincubated with 10 µg/mL poly-D-lysine. Cells were cultured for 2 h and extra culture medium (Neurobasal-A with 1 × B-27 supplement) was added. After more than 16 h, DRG neurons were used for electrophysiology recordings.

**HEK293T cell culture and transfection**

HEK293T cell is a gift from Jing Yao lab (School of Life Sciences, Wuhan University, China). The cells were seeded in 24-well plate and cultured in DMEM with 10% FBS and 1% Penicillin-Streptomycin. When the cell density reached 60% - 80%, the cells were transfected with plasmids expressing the human Na\textsubscript{v}1.7 channel. The transfection procedure was briefly introduced as follows: 800 ng pcDNA3.1-SCN9A (NM_002977.3) plasmid which expresses Na\textsubscript{v}1.7 was co-transfected with pIRES2-EGFP plasmid expressing EGFP. Both pcDNA3.1 and pIRES2-EGFP plasmids are gifts from Wenxin Li lab (School of Life Sciences, Wuhan University, China). The full-length cDNAs for human Nav1.7 (SCN9A) was subcloned into pcDNA3.1 and sequenced. The HEK293T cells were transfected as the process reported previously (Shi et al., 2021). One Ep tube contained 40 µL opti-MEM mixed with pcDNA3.1-SCN9A and pIRES2-EGFP plasmids, another one contained 2 µL ExFect2000 in 40 µL opti-MEM. Make the two solutions stand still for 5 min without disturbance. After
that, they were mixed and left for 20 min again. The mixed solution was added gently in the culture medium of HEK293T cells and incubated for more than 36 h. When the expression of Na\textsubscript{v}1.7 and EGFP was confirmed with fluorescence microscope, cells were resuspended and seeded on coverslips. After the cells attached on the coverslips, they were picked for electrophysiology recordings.

**Electrophysiological study**

Whole-cell patch-clamp recordings were performed using an EPC9 amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany) at room temperature (22–24 °C). Pipettes pulled from borosilicate glass had resistances of 2-4 MΩ when filled with the internal solution. The internal pipette solution for recording Na\textsubscript{v} currents contained: CsCl 130 mM, NaCl 9 mM, MgCl\textsubscript{2} 1 mM, EGTA 10 mM, HEPES 10 mM (pH 7.3 with CsOH) (Chang et al., 2018). The external solution for recording Na\textsubscript{v} currents contained: NaCl 131 mM, TEACl 10 mM, CsCl 10 mM, 4-Aminopyridine 3 mM, MgCl\textsubscript{2} 2 mM, CaCl\textsubscript{2} 1 mM, CdCl\textsubscript{2} 0.3 mM, HEPES 10 mM, Glucose 10 mM (pH 7.4 with NaOH) (Chang et al., 2018). Electrophysiology data were analyzed using IGOR (WaveMetrics, Lake Oswego, OR, USA) software. Series resistance was compensated by 80%.

The holding potential was set at -120 mV. Sodium currents (I\textsubscript{Na}) were elicited from -80 mV to +15 mV in 5 mV step for 50 ms (Zheng et al., 2018). The interval between sweeps lasted for 5s. We evaluated steady-state I\textsubscript{Na} activation by measuring membrane conductance of Na\textsuperscript{+} (g\textsubscript{Na}) which was determined with the equation g\textsubscript{Na} = I\textsubscript{Na}/(V\textsubscript{m}-V\textsubscript{rev}), where V\textsubscript{m} is the membrane potential and V\textsubscript{rev} is the observed reversal potential for Na\textsuperscript{+} calculated through I-V curve. g\textsubscript{Na}/g\textsubscript{Na-max} was plotted against membrane potential and the putative curve was fitted with a sigmoidal function using the Boltzmann model: g\textsubscript{Na}/g\textsubscript{Na-max} = 1/{1 + exp [(V\textsubscript{m1/2} - V\textsubscript{m})/k] }. The half-activation voltage was got from the fitness curve. For fast inactivation curves, currents were elicited from -130 mV to 0 mV in 10 mV step for 500 ms, then were kept at 0 mV for 20 ms. For slow inactivation curves, currents were elicited from -140 mV to 20 mV in 10 mV step for
10 s, then were hyperpolarized to -120mV for 20 ms and depolarized to 0 mV for 20 ms. The currents elicited by depolarization after preconditional voltage steps were analyzed (Goldfarb et al., 2007; Zheng et al., 2018). The half-inactivation voltage was got by fitting \( \frac{I_{Na}}{I_{Na,max}} \) against membrane potential with a sigmoidal function using Boltzmann model: \( \frac{I_{Na}}{I_{Na,max}} = \frac{1}{1 + \exp \left[ (V_{m1/2} - V_m) / k \right]} \).

For the current clamp recording on DRG neurons, the internal pipette solution contained: KCl 140 mM, EGTA 0.5 mM, HEPES 5 mM, Mg-ATP 3 mM (pH 7.3 with KOH). The external solution for current clamp recording contained: NaCl 140 mM, KCl 3 mM, MgCl\(_2\) 2 mM, CaCl\(_2\) 2 mM, HEPES 10 mM (pH 7.3 with NaOH). Cells with rest membrane potential greater than -50 mV were discarded. Cells were clamped at the resting membrane potential and elicited with 0 to 1000 pA ramp current for 500 ms. After the cell state is stable, the action potentials of DRG neurons were recorded before and after drug administration. The number of action potentials, resting membrane potential (RP), neuronal action potential peak (APP), and action potential threshold (APT) were compared before and after the drug treatments. Action potential peak was determined by the difference between the action potential peak and the minimum voltage after hyperpolarization and action threshold was determined by the membrane potential which generated the action potential (Dustrude et al., 2016).

Tetrodotoxin (TTX)-sensitive Na\(_V\) currents were recorded in large-diameter (>30 μM) DRG neurons. After LCA treatment and the Na\(_V\) current washed back to the level before LCA treatment, 300 nM TTX diluted in external solution was applied and see whether TTX could inhibit the recorded currents. The Na\(_V\) current which could be completely inhibited by 300 nM TTX was putative TTX-sensitive Na\(_V\) current. And the effects of LCA on TTX-resistant Na\(_V\) currents were tested in small-diameter (<30 μM) DRG neurons. We applied 300 nM TTX in the recording bath and perfusion solution to block the TTX-sensitive Na\(_V\) currents. The residual Na\(_V\) currents was putative TTX-resistant Na\(_V\) currents.
Animal behavior test

Animals used for the behavior test were 4-week female Kunming mice (20 ± 2g), which were commonly used mice strain for formalin-induced pain model (Xu et al., 2019; Zhong et al., 2012). 5 - 6 mice per cage were housed and habituated to the environment for more than 1 week before the experiment. Behavior test was conducted during the daytime, starting at 9 a.m. in the morning. Mice were put in the observing box and habituated for 20 min. LCA or LCB were first diluted in DMSO (25 mg powder in 100 μL DMSO), then were diluted in saline to make the final concentration. Mice were injected subcutaneously with different doses of LCA or LCC including 25 mg/kg, 50 mg/kg, and 100 mg/kg 30 min before the formalin injection. The same volume of saline with diluted DMSO was injected subcutaneously as the Vehicle group. The Nav channel blocker (IC_{50} for Nav1.7 is 1.77 mM) and analgesic drug Mexilene was injected with 50 mg/kg dose in the mice as positive control (Blackburn-Munro et al., 2002; Wu et al., 2013). 30 min later, Licochalcone, Mexilene and Vehicle groups were injected with 10 μL 2% formalin solution in the plantar of right hind paws of the mice. 10 μL saline was injected in the plantar of right hind paw as the Saline group to screen out the effects of paw injection. Videos were recorded immediately after the paw injection for more than 30 min. The processes were separated into 2 phases. The first phase is 0-10 min and the second phase is 15-30 min (McNamara et al., 2007). The time of mice licking right hind paws was counted with stopwatch by two-blinded analyzers.

Statistics analysis

GraphPad Prism (San Diego, CA, United States) was used to analyze the data. All data were tested for the normality first. Statistical analysis of differences was carried out using Student’s t-test or Analysis of Variance (ANOVA) combined with Turkey post-hoc test if the data were normally distributed. Non-parametric statistical analysis was applied if the data did not distribute normally. P < 0.05 was considered
significantly different. Using IGOR (WaveMetrics, Lake Oswego, OR, USA) software, concentration–response curves of LCA were fitted according to the following modified Hill equation:

\[ \frac{I_{LCA}}{I_{\text{vehicle}}} = \frac{1}{1 + \left(\frac{[LCA]}{IC_{50}}\right)} \]

where \( I \) represent the amplitude of peak current; \([LCA]\) represent the concentration of LCA. The concentration of half-maximal inhibition (IC \(_{50}\)) was got by four-parametric non-linear regression analysis constraining bottom to 0 and top to 1.

**Results**

**LCA inhibits \( \text{Na}_V \) current in DRG neuron**

LCA is a kind of chalcone extracted from licorice and shares similar backbone with LrB (Fig. 1) which has been reported to significantly inhibit \( \text{Na}_V \) current in DRG neuron and has analgesic effects (Chen et al., 2018). We applied LCA to small-diameter (<30 μM) DRG neurons and recorded the \( \text{Na}_V \) currents to see whether LCA had similar effects with LrB on \( \text{Na}_V \) current. Our results showed that 30 μM LCA could significantly inhibit \( \text{Na}_V \) currents in DRG neurons (Fig. 2A). LCB is also a compound in licorice and shares similar backbone structures with LCA and LrB (Fig. 1). We tested the effects of LCB on small-diameter DRG neurons and recorded \( \text{Na}_V \) currents with patch-clamp technology. The voltage clamp results showed that LCB did not inhibit \( \text{Na}_V \) currents in DRG neurons (Fig. 2A).

We further recorded and analyzed gating properties of \( \text{Na}_V \) channel under LCA treatment. The maximum activation voltage was significantly higher in 30 μM LCA treatment group (-12.73 ± 4.28 mV) than in control (-15.45 ± 4.13 mV) and wash (-13.64 ± 4.72 mV) groups according to I-V curve (Fig. 2B, C and Fig. S1A). The activation and inactivation curves were fitted by single Boltzmann distributions (Griffith et al., 2019). The half-activation voltage was significantly higher in 30 μM LCA treatment group (-18.21 ± 2.49 mV) than in control (-25.41 ± 3.18 mV) and wash (-23.30 ± 1.01 mV) groups (Fig. 2D and Fig. S1A). And half-inactivation voltage
was significantly lower in LCA group (-51.45 ± 15.75 mV) than in control (-32.83 ± 7.57 mV) and wash (-43.14 ± 10.21 mV) groups (Fig. 2E, F and Fig.S1A).

**LCA dampens excitability of DRG neuron**

Na\textsubscript{V} channels play a key role in regulating action potentials in DRG neurons (Griffith et al., 2019; McDermott et al., 2019). We used the current clamp to record the changes of action potential before and after LCA treatment and see whether the inhibition of Na\textsubscript{V} channels by LCA could affect the DRG neuron excitability. We found that LCA significantly decreased the firing number (Control group: 8.00 ± 5.55; LCA treated group: 0.80 ± 1.16; Wash group: 3.80 ± 1.60) and action potential peak (APP) (Control group: 104.68 ± 6.31 mV; LCA treated group: 30.53 ± 20.35 mV; Wash group: 93.66 ± 10.62 mV) of the DRG neurons (Fig. 3A-C and Fig.S1B). LCA did not significantly change the action potential threshold (APT) (Control group: -42.75 ± 5.12 mV; LCA treated group: -43.57 ± 5.77 mV; Wash group: -43.21 ± 7.65 mV) or rest membrane potential (RP) (Control group: -59.44 ± 9.80 mV; LCA treated group: -62.80 ± 10.33 mV; Wash group: -63.64 ± 12.90 mV) of the DRG neurons (Fig. 3D, E and Fig.S1B). The results of the current clamp indicate that LCA could decrease the frequency and amplitude of the action potentials, thus dampening the excitabilities of DRG neurons.

**The effects of licorice extract on Na\textsubscript{V} current of DRG neuron**

It has been reported that LCA is a constituent exclusively produced by *Glycyrrhiza inflata* Batalin in Xinjiang (Miyazaki et al., 2020). We used ethyl acetate to extract the flavonoids from the roots of *Glycyrrhiza inflata* Batalin and detected LCA as the main constituents in this species with HPLC analysis (Fig. 4A-C). It is of interest to see whether the effects of *Glycyrrhiza inflata* Batalin extract are consistent with single compound LCA. We applied the *Glycyrrhiza inflata* Batalin extract on DRG neurons and recorded the Na\textsubscript{V} currents. The content of LCA in *Glycyrrhiza inflata* Batalin roots was 2.22% according to the peak area in HPLC chromatography. The
concentration of LCA in *Glycyrrhiza inflata* Batalin extract solution was 30 μM for electrophysiological recordings on DRG neurons. The flavonoids extracted from another species *Glycyrrhiza uralensis* Fisch. which does not contain LCA was used as negative control and applied with the same mass to volume ratio (0.046 mg/mL) (Fig. 4D). The *Glycyrrhiza inflata* Batalin extract could significantly inhibit NaV currents in DRG neurons and the inhibition percentage was 77.06 ± 5.92% while the *Glycyrrhiza uralensis* Fisch. extract did not show significant inhibition on NaV currents (Fig. 4E and F). These results indicate that the effects of *Glycyrrhiza inflata* Batalin extract on NaV currents in DRG neuron were similar with single compound LCA.

**LCA inhibits NaV1.7 channel exogenously expressed in HEK293T cell**

The NaV channels expressed in DRG neurons could be divided into TTX-sensitive and TTX-resistant types (Griffith et al., 2019; Ho and O’Leary, 2011; Li et al., 2018). TTX-sensitive NaV channels are expressed in both large- (>30 μm) and small-diameter (<30 μm) DRG neurons while TTX-resistant NaV channels are dominantly expressed in small-diameter DRG neurons (Griffith et al., 2019; Ho and O’Leary, 2011; Li et al., 2018). To identify which kind of NaV currents were inhibited by LCA, 300 nM TTX diluted with external solution was applied after drug treatment and wash steps in large-diameter DRG neurons and see whether TTX could inhibit the recorded currents. The Nav currents which could be completely inhibit by 300 nM TTX was putative TTX-sensitive and analyzed (Fig. 5A). The statistics analysis of I-V curves showed that the maximum activation voltage and the half-activation voltage were significantly higher in 30 μM LCA treatment group than in control and wash groups (Fig. 5B-D and Fig.S1C). Then we tested the effects of LCA on TTX-resistant NaV channels in small-diameter DRG neurons. 300 nM TTX was applied in the recording bath and drug perfusion solution. The residual NaV currents were putative TTX-resistant currents (Fig. 5E). The statistical analysis of I-V curves showed that the maximum activation voltage and the half-activation voltage were higher in 30 μM LCA treatment group than in the control and wash groups (Fig. 5F-H and Fig.S1D).
These results indicate that LCA could exert inhibition effects on both TTX-sensitive and TTX-resistant NaV channels.

According to the above results, the large-diameter DRG neurons exclusively expressing TTX-sensitive channels could be blocked by LCA. We expressed one subunit of TTX-sensitive Nav channel - NaV1.7 in HEK293T cells to observe the effects of LCA. Our results showed that LCA could both inhibit NaV1.7 currents and shift the I-V curve to depolarization direction (Fig. 6A-C). Analysis on voltage-gating properties indicated that LCA did not significantly increase the half-activation voltage of NaV1.7 channel (Fig. 6D and Fig.S1E). While LCA significantly decreased the fast and slow half-inactivation voltage of NaV1.7 channel (Fig. 6E, F and Fig.S1E). To get the IC50 value of LCA on NaV1.7, we treated the NaV1.7 expressing HEK293T cells with different concentrations of LCA for 1.5 min. The IC50 value (7.6 ± 3.5 μM, n = 5) was calculated by fitting concentration-response curve to Hill equation (Fig. 6G). We also applied LCB on HEK293T cells expressing Nav1.7 and analyzed the voltage-gating properties. The half-activation and inactivation voltage of Nav1.7 channel were not significantly changed by LCB (Fig. 7A-E).

The effects of LCA and LCB on formalin-induced pain responses

NaV1.7 has been reported to be predominantly expressed in nociceptors and closely related to pain in humans and animals (Kingwell, 2019; McDermott et al., 2019). Since LCA had significant inhibition effects on NaV currents in DRG neurons and NaV1.7 channels exogenously expressed in HEK293T cells, it might also have pain-relief effects. We used the classical behavior model - formalin test to investigate the effects of LCA on acute pain responses of animals (Uniyal et al., 2021). There are two phases in formalin tests: the first phase was mainly conducted by neuropathic pain while the second phase was related to the activation of complex downstream pathways (Fernandes et al., 2018; Uniyal et al., 2021). Our results showed that different concentrations (25 mg/kg, 50 mg/kg, 100mg/kg) of LCA could alleviate the
pain responses in both the first and the second phases of the formalin test (Fig. 8A and B). We applied 50 mg/kg Mexiletine which is a known Na\textsubscript{V} channel blocker and analgesic drug as the positive control during the tests (Blackburn-Munro et al., 2002). The results of Mexiletine showed that it could significantly decrease the licking time of mice after formalin injection in their right hind paws during both phases, which confirmed that our animal models are successful. The effects of LCA were consistent with their effects on Na\textsubscript{V} channels in DRG neurons and Na\textsubscript{V}1.7 expressed in HEK293T cells. LCB did not affect the responses during the first phase of formalin test but could inhibit the licking time during the second phase in a relatively high concentration (100mg/kg) (Fig. 8C and D). These results indicate that LCB behaves differently with LCA on Na\textsubscript{V} channels and neuropathic pain but may exert effects on molecular signaling pathways during the second phase of formalin test.

**Discussion**

As a traditional Chinese medicine, licorice is commonly applied to protect stomach and spleen, alleviate pain and improve the symptoms of cough and phlegm (Yang et al., 2015). Researchers recently discovered that LCA could relieve neuropathic pain and inflammatory responses (Chu et al., 2012; Li et al., 2021). Although licorice was proved to have effects on multiple diseases, the molecular mechanisms have rarely been disclosed. LCA and LCB are chalcones in licorice and share the same backbones with each other. The results of electrophysiological recording showed that LCA could inhibit Na\textsubscript{V} currents in DRG neurons. These results indicate that LCA has the potential to be developed into Na\textsubscript{V} inhibitors. Although LCA could significantly inhibit Nav currents in mouse DRG cells, LCB did not block the Nav currents. LCB also did not have effects on activation or inactivation of Nav1.7 channel. Structural analysis showed that the predicted physicochemical properties of LCA and LCB such as LogP and pKa are different (data not shown). This might be because LCA has olefin substituents consisted of five carbon atoms on the aromatic B ring, while LCB has no such side chain but one extra hydroxyl group, which suggests that the olefin
substituent on the aromatic B ring of licochalcone may play a key role in blocking the Nav channel. The discrepancy of effects between LCA and LCB may guide us to discover the key chemical groups to inhibit the specific channels and develop more effective NaV channel inhibitors.

NaV channels in DRG neurons could modulate the membrane potential oscillations and transduce sensories like pain and itch (Griffith et al., 2019; Li et al., 2018). We used current clamp to record the action potentials of DRG neurons to see whether inhibition of NaV channels by LCA could dampen the excitabilities of these neurons. The results showed that LCA could significantly decrease the firing frequencies and amplitudes of DRG neurons. We further conducted formalin test and observed that LCA had analgesic effects in these animal models. It has been confirmed that pain responses during phase1 of formalin test is neuropathic pain which is mediated by direct nociceptor neurotransmission while phase2 involves more complex signaling pathways such as immune cell infiltration and the activation of glia (Fernandes et al., 2018; Uniyal et al., 2021). Our results showed that LCA could decrease the licking time of mice during both phase1 and phase2 of formalin test. These results were consistent with changes of NaV currents in DRG neurons and inhibition of NaV1.7 exogenously expressed in HEK293T cells. Previous studies have reported that NaV1.7 is widely expressed in DRG neurons and is devoted both to neuropathic pain and inflammatory pain (Dib-Hajj et al., 2013; Nassar et al., 2004). Inhibition of NaV1.7 by LCA may lead to alleviation of pain responses during both phase1 and phase2 in formalin test. Although LCB did not show inhibitory effects on DRG NaV currents it could decrease the licking time during phase2 of formalin test. We suspect that LCB may be able to inhibit other channels or signaling molecules which were involved in the pain or inflammatory responses during phase2. It is of great interest to explore the mechanisms of this phenomenon.

DRG neurons could be divided into diverse groups according to their size, function
and molecular characteristics (Dib-Hajj et al., 2013; Ho and O’Leary, 2011). Small-size (< 30 μm) DRG neurons express both TTX-sensitive NaV channels such as NaV1.7 and TTX-resistant NaV channels such as NaV1.8 and NaV1.9 (Griffith et al., 2019; Ho and O’Leary, 2011). Large-size (> 30 μm) DRG neurons mainly express TTX-sensitive such as NaV1.1, NaV1.6 and NaV1.7 (Griffith et al., 2019; Ho and O’Leary, 2011). 300 nM TTX was applied after LCA administration and wash-back in large-size DRG neurons to confirm that the recorded NaV currents could be completely blocked. The results indicate that LCA could inhibit TTX-sensitive NaV channels in large-size DRG neurons. Although we observed the inhibition on Nav1.7 in HEK293T cells, the effects of LCA on other subunits such as Nav1.1 and Nav1.6 need to be explored. To separate the TTX-resistant NaV currents, 300 nM TTX was applied in the bath solution and the residual NaV currents in small-size DRG neurons were recorded. Similar to the results in large-size DRG neurons, LCA could inhibit TTX-resistant NaV currents in these cells. These results indicate that LCA may be able to inhibit TTX-resistant NaV channels like NaV1.8 and NaV1.9. We need further express other types of NaV channel subunits in HEK293T cells to observe the effects of LCA in future.

**Authorship contributions**

Qianru Zhao, Xu Zhang and Siru Long contributed equally to this work. Participated in research design: Shijin Yin. Conducted experiments: Qianru Zhao, Xu Zhang, Siru Long, Shaobing Wang, Yi Li, Hui Yu, Yongsheng Zhou, Lu Xue, Yan Hu. Performed data analysis: Shijin Yin, Qianru Zhao, Siru Long, Xu Zhang and Shaobing Wang. Wrote or contributed to the writing of the manuscript: Qianru Zhao, Shijin Yin. All authors read and approved the final manuscript.

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Beijing Shi.
Footnotes

$ This work is supported partly by grants from the National Natural Sciences Foundation of China
(81373379, 81641186) and the National Key R and D Program of China (2019YFC1712402) to SY, the National Natural Sciences Foundation of China (32000685) and Natural Sciences Foundation of Hubei Province (2020CFB348) to QZ, the Fundamental Research Funds for the Central Universities, South-Central Minzu University to SY(CZZ19005) and QZ (CZQ23026), and Knowledge Innovation Program of Wuhan-Shuguang Project to QZ (2022020801020412). All authors declare no interest conflicts.

This article has supplemental material available at molpharm.aspetjournals.org.

Figure Legends

Figure 1 Chemical structures of Licochalcone A (A), Licochalcone B (B) and Loureirin B (C).

Figure 2 Effects of LCA and LCB on NaV currents in DRG neurons. (A) Representative traces of NaV currents in DRG neurons under Vehicle, 30 µM LCA (top) or LCB (middle) and Wash treatments; Statistics of the Inhibition percentage of LCA (n = 7) and LCB (n = 5) compared to Vehicle (bottom); (B) Representative traces of NaV currents in DRG neurons with activation stimulus: cells were clamped at -80 mV and NaV currents were elicited from -60 mV to +10 mV in 10 mV step for 100 ms; (C) I-V curves of Vehicle, LCA treatment and Wash groups; (D) Activation curves of Vehicle, LCA and Wash treatments on single cell; (E) Representative traces of NaV currents in DRG neurons with inactivation stimulus: NaV currents were elicited from -60 mV to +10 mV in 10 mV step for 1000 ms, then were kept at -20 mV for 100 ms; (F) Inactivation curves of Control, LCA and Wash treatments on single cell. All data are presented as mean ± SEM for n independent observations. Statistical analysis of Inhibition percentage was carried out using one sample t-test and differences between groups was carried out using two-way ANOVA combined with Turkey post-hoc test, *, P < 0.05, **, P < 0.01, ***, P < 0.001.

Figure 3 Effects of LCA on action potentials of DRG neurons. (A) Representative action potential traces of DRG neurons under Vehicle (black), LCA treatment (red) and Wash (blue). Cells were clamped with rest membrane potentials and elicited with 0 to 1000 pA ramp current for 500 ms; (B-E) Statistics of the Number of Action Potentials (B), action potential peak (APP) (C), action potential threshold (APT) (D) and resting membrane potential (RP) (E) in DRG neurons with LCA treatment. All data are presented as mean ± SEM for n independent observations. Statistical analysis of differences between groups was carried out using one-way ANOVA combined with Turkey post-hoc test. *, P < 0.05; **, P < 0.01.

Figure 4 Effects of licorice extract on NaV channels of DRG neurons. (A) HPLC profiles of standard LCA samples, there is a single peak at 12.07 min; (A) HPLC profiles of standard LCB samples, there is a single peak at 3.72 min; (C) HPLC profiles of ethyl acetate Glycyrrhiza inflata Batalin extract, the peak at 11.99 min is putative to be LCA, the content of LCA in Glycyrrhiza inflata Batalin is 2.22%; (D) HPLC profiles of Glycyrrhiza uralensis Fisch. flavonoids; (E) Representative traces of NaV currents in DRG neurons under
Vehicle (black), *Glycyrrhiza inflata* Batalin extract (red), *Glycyrrhiza uralensis* Fisch. flavonoids (cyan) and Wash (blue) treatments, the mass-volume ratio of *Glycyrrhiza inflata* Batalin extract used was 0.046 mg/mL to guarantee 30μM concentration of LCA; (F) Inhibition percentage of *Glycyrrhiza uralensis* and *Glycyrrhiza inflata* Batalin extract on NaV currents of DRG neurons, n = 4, the data are presented as mean ± SEM. Statistical analysis of differences between the two groups was carried out using Student’s t-test. ***, P < 0.001.

Figure 5 Effects of LCA on TTX-sensitive and TTX-resistant NaV channels.
(A) Statistics of TTX-sensitive NaV currents recorded from large diameter DRG neurons; (B-D) I-V curve (B), activation curve (C) and inactivation curve (D) of TTX-sensitive NaV currents under Vehicle, 30 μM LCA and Wash treatments; (E) Statistics of TTX-resistant NaV currents recorded from small diameter DRG neurons; (F-H) I-V curve (F), activation curve (G) and inactivation curve (H) of TTX-resistant NaV currents under Vehicle, 30 μM LCA and Wash treatments. All data are presented as mean ± SEM for n independent observations. Statistical analysis of differences between groups was carried out using one-way ANOVA combined with Turkey post-hoc test. ***, P < 0.001.

Figure 6 Effects of LCA on NaV1.7 channels exogenously expressed on HEK293T cells.
(A) Representative traces of NaV1.7 currents in HEK293T cells before or after LCA treatment; (B) Representative traces of NaV currents in DRG neurons with activation stimulus: cells were clamped at -120 mV and NaV currents were elicited from -80 mV to 15 mV in 5 mV step for 50 ms; (C) I-V curves of NaV1.7 channel with LCA treatment, the data are presented as mean ± SEM for n independent observations, statistical analysis of differences between groups was carried out using two-way ANOVA combined with Turkey post-hoc test; (D-F) Activation curves (D), fast inactivation curves (E) and slow inactivation curves (F) with LCA treatment; (G) Concentration-response curve of NaV1.7 current with LCA treatment, y-axis represents the ratios of NaV current amplitudes (I_{LCA}/I_{vehicle}) and x-axis represents the concentration of LCA. The curve was fitted by Hill equation.

Figure 7 Effects of LCB on NaV1.7 channels exogenously expressed on HEK293T cells.
(A) Representative traces of NaV currents in HEK293T cells with activation stimulus: cells were clamped at -120 mV and NaV currents were elicited from -80 mV to 15 mV in 5 mV step for 50 ms; (B) I-V curves of NaV1.7 channel with LCA treatment, the data are presented as mean ± SEM for n independent observations, statistical analysis of differences between groups was carried out using two-way ANOVA combined with Turkey post-hoc test; (C-E) Activation curves (C), fast inactivation curves (D) and slow inactivation curves (E) with LCB treatment.

Figure 8 Effects of LCA and LCB on formalin-induced pain responses.
(A) Statistics of paw licking time during the first phase of formalin test under Vehicle (n = 9), LCA in different concentrations (n = 9 for 25 mg/kg, n = 10 for 50 mg/kg and n = 10 for 100mg/kg), Saline (n = 12) and Mexiletine (n = 7) treatments; (B) Statistics of paw licking time during the second phase of formalin test under Vehicle, LCA in different concentrations, Saline and Mexiletine treatments; (C) Statistics of paw licking time during the first phase of formalin test under Vehicle (n = 5), LCB in different concentrations (n = 5 for 25 mg/kg, n = 5 for 50 mg/kg and n = 5 for 100mg/kg), Saline (n = 12) and Mexiletine (n = 7) treatments; (D) Statistics of paw licking time during the second phase of formalin test under Vehicle, LCA in different concentrations, Saline and Mexiletine treatments. All data are presented as mean ± SEM for n independent observations. Statistical analysis of differences between groups was carried out using two-way ANOVA combined with Turkey post-hoc test. *, P < 0.05, **, P < 0.01**, ***, P < 0.001
compared with the Vehicle group.
Fig. 1

A

B

C
Licochalcone Mediates the Pain Relief by Targeting the Voltage-Gated Sodium Channel

Qianru Zhao††, Xu Zhang††, Siru Long††, Shaobing Wang†, Yi Li‡, Hui Yu†, Yongsheng Zhou†, Lu Xue‡, Yan Hu‡, Shijin Yin‖

†Department of Chemical Biology, School of Pharmaceutical Sciences, South-Central Minzu University, Wuhan, 430074, PR China

‡Institute for Medical Biology and Hubei Provincial Key Laboratory for Protection and Application of Special Plants in Wuling Area of China, College of Life Sciences, South-Central Minzu University, Wuhan, 430074, PR China

‖These authors contributed equally to this work.
Supplemental Figures

**Figure S1 Statistics for voltage-clamp and current-clamp in DRG neurons and HEK293T cells.**

A, Statistical analysis of gating properties of Na\(_V\) currents in DRG neurons before or after LCA treatment; B, Statistical results of Number, Action Potential Peak (APP), Action Potential Threshold (APT) and Resting Potential (RP) of DRG neuron action potential firings; C, Statistical analysis of gating properties of TTX-sensitive Na\(_V\) currents in DRG neurons before or after LCA treatment; D, Statistical analysis of gating properties of TTX-resistant Na\(_V\) currents in DRG neurons before or after LCA treatment; E, Statistical analysis of gating properties of Na\(_V\)1.7 channels expressed in HEK293T cells before or after LCA treatment. All data are presented as mean ± SEM for n independent observations. Statistical analysis of differences between groups was carried out using one-way ANOVA combined with Turkey post-hoc test. *, P < 0.05; **, P < 0.01; ns, not significant.