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Chinese Journal of Natural Medicines 2019, **17**(6): 0413–0423
Chinese Journal of Natural Medicines

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3'-Methoxydaidzein exerts analgesic activity by inhibiting voltage-gated sodium channels

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Available online 20 June, 2019

[ABSTRACT] Isoflavones are widely consumed by people around the world in the form of soy products, dietary supplements and drugs. Many isoflavones or related crude extracts have been reported to exert pain-relief activities, but the mechanism remains unclear. Voltage-gated sodium channels (VGSCs) play important roles in excitability of pain sensing neurons and many of them are important nociceptors. Here, we report that several isoflavones including 3'-methoxydaidzein (3MOD), genistein (GEN) and daidzein (DAI) show abilities to block VGSCs and thus to attenuate chemicals and heat induced acute pain or chronic constriction injury (CCI) induced pain hypersensitivity in mice. Especially, 3MOD shows strong analgesic potential without inducing addiction through inhibiting subtypes $Na_V1.7$, $Na_V1.8$ and $Na_V1.3$ with the IC_{50} of 181 ± 14, 397 ± 26, and 505 ± 46 nmol·L^{-1}, respectively, providing a promising compound or parent structure for the treatment of pain pathologies. This study reveals a pain-alleviating mechanism of dietary isoflavones and may provide a convenient avenue to alleviate pain.

[KEY WORDS] 3'-Methoxydaidzein; Analgesic; Isoflavone; Chronic pain; Sodium channel

[CLC Number] R965 **[Document code]** A **[Article ID]** 2095-6975(2019)06-0413-11

Introduction

In general, chronic pain may result from either central or peripheral somatosensory system damage or from the constant nociceptor activation [1] and is recognized as a common health problem. Community-based surveys have found that 15%–25% of adults suffer from chronic pain at any given time, with the incidence increasing to 50% in those older than 65 years [2]. Chronic pain exists in different forms. It is easy to recur and difficult to cure permanently. However, many analgesics have limited efficacy and dose-limiting side-effects with long-term medication. Voltage-gated sodium channels (VGSCs) play critical roles in neuron nociceptive signal transduction and many of them are important nociceptors. Several different sodium channels have been proven to be potentially therapeutic targets for pain, such as $Na_V1.3$, $Na_V1.7$ and $Na_V1.8$. $Na_V1.3$ is upregulated in sensory neurons following chronic inflammation and nerve injury, and it has been connected to central neuropathic pain [3]. $Na_V1.7$ is highly expressed in nociceptors and humans who lack functional

[Received on] 24-Mar.-2019
[Research funding] This work was supported by Ministry of Science and Technology of China (Nos. 2013CB911304 and 2014ZX0800949B-002), the National Natural Science Foundation of China (Nos. U1302221, 31372208 and 31630075), Innovation Programe of Chinese Academy of Sciences (Nos. XDA12020340 and 2015CASEABRI002), Biological Resources Programme of Chinese Academy of Sciences (No. KFJ-BRP-008), the Natural Science Foundation of Yunnan Province (Nos. 2015BC005, 2016FA006 and 2015HA023) and the Natural Science Foundation of Jiangsu Province (Nos. Q0201600440, BE2016742).
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These authors have no conflict of interest to declare.

$Na_V1.7$ channels exhibit complete insensitivity to pain [4]. Specifically block of $Na_V1.7$ significantly reduces pain reactions in rodent pain models [5]. $Na_V1.8$ is also implicated in inflammatory and neuropathic pain mechanisms, and selective blockage of $Na_V1.8$ alleviates both of these types of pain [6].

Natural isoflavones mostly exist in Fabaceae plants, commonly known as the bean family, and they are particularly abundant in soybeans. Foods and drugs rich in isoflavones have been consumed all over the world for a long time especially in Asian countries. Studies have revealed many beneficial or protective effects of isoflavones related to different diseases such as pain [7, 8], obesity and diabetes [9], arteriosclerosis [10] and other cardiovascular disease [11], breast and prostate cancer [12], bone loss [13] and hot flash symptoms suffered by women during menopausal phase [14]. However, despite the widespread consumption, isoflavones have not been intensively studied to establish the underlying mechanisms corresponding to their beneficial functions. In this study, we investigate the effects of several dietary isoflavones on voltage-gated sodium channels and pain. 3MOD is found to efficiently inhibit $Na_V1.7$, $Na_V1.8$ and $Na_V1.3$ sodium channel subtypes, and thus to exert potentially analgesic abilities in rodent models.

Materials and Methods

Materials

3′-Methoxydaidzein (3MOD), genistein (GEN) and daidzein (DAI) were obtained from Yuanye Bio-Technology Company (Yuanye, Shanghai, China), and dissolved in DMSO (MP Biomedicals, USA). Tetrodotoxin was purchased from MedChem Express (MedChem Express, Shanghai, China). Dulbecco's modified eagle's medium (DMEM) was purchased from Corning Cellgro (Corning, USA). Enzymes and electrolytes were obtained from Sigma-Aldrich (Sigma-Aldrich, Shanghai, China) unless otherwise mentioned. Formalin and acetic acid were obtained from Xilong Scientific (Xilong, Guangdong, China).

Patch-clamp recording on rat DRG neurons

Rat dorsal root ganglion (DRG) neurons were acutely dissociated and maintained in short-term primary culture, as previously described [5]. Briefly, 4 weeks Sprague-Dawley rats of either sex were killed without anesthetization, the dorsal root ganglia were quickly removed from the spinal cord, and then they were transferred into DMEM containing collagenase (1.0 $g \cdot L^{-1}$, type IA), trypsin (0.5 $g \cdot L^{-1}$, type III) and DNase (0.1 $g \cdot L^{-1}$, type III) to digest at 37 °C with 100 $r \cdot min^{-1}$ for about 30 min. Trypsin inhibitor (1.5 $g \cdot L^{-1}$, type II-S) was used to terminate enzyme treatment. The DRG cells were transferred into 35-mm culture dishes containing 95% DMEM, 5% newborn calf serum, and penicillin-streptomycin. The dorsal root ganglion cells were incubated in a CO_2 incubator (5% CO_2, 95% air, 37°C) for 2–4 h before patch-clamping. Na^+, K^+, and Ca^{2+} currents were recorded using the whole-cell patch-clamp technique with an Axon Multiclamp 700B amplifier (Molecular Devices) as previously described [15]. Data were acquired and analyzed using Clampfit 10.0 (Molecular Devices, USA) and SigmaPlot 9.0 (Systat Software, USA). All the experimental protocols using animals in this work were approved by the Animal Care and Use Committee at Kunming Institute of Zoology, Chinese Academy of Sciences (SMKX2017024).

Patch-clamp recording of sodium channel subtypes

Sodium channel subtypes [human (h) or rat (r) sodium channel subtypes, $rNa_V1.3$, $rNa_V1.4$, $hNa_V1.5$, $hNa_V1.7$, $rNa_V1.8$], $\beta 1$ and eGFP were co-transiently transfected into HEK293T or ND7/23 cells according to the manufacturer's instructions (lipo2000, Invitrogen, Carlsbad, CA, USA). The bath solution contained the following components: 140 $mmol \cdot L^{-1}$ NaCl, 3 $mmol \cdot L^{-1}$ KCl, 1 $mmol \cdot L^{-1}$ $MgCl_2$, 1 $mmol \cdot L^{-1}$ $CaCl_2$ and 10 $mmol \cdot L^{-1}$ HEPES, pH 7.3. The internal solution contained: 10 $mmol \cdot L^{-1}$ NaCl, 3 $mmol \cdot L^{-1}$ KCl, 140 $mmol \cdot L^{-1}$ CsF, 10 $mmol \cdot L^{-1}$ $MgCl_2$ and 1 $mmol \cdot L^{-1}$ EGTA, pH 7.3. Experimental data were acquired and analyzed using Clampfit 10.0 (Molecular Devices, USA) and SigmaPlot 9.0 (Systat Software, USA).

Formalin-induced paw licking

Analgesic effects were evaluated in male C57BL/6J mice (18–22 g, n = 10). Formalin-induced paw licking was carried out by intraplantar injection of formalin in the right hind paws, and pain attenuation was compared in mice pre-injected with 3MOD or its analogues dissolved in the vehicle (5 μL DMSO in 200 μL saline) intraperitoneally. After 15 minutes' pretreatment, animals were injected with 10 μL 2% (*V*/*V*) formalin in saline at the plantar surface of the right hind paw. Mice were then placed individually into open polyvinyl cages (20 cm × 40 cm × 15 cm). The total licking time of the injected paw was recorded by digital video camera during phase I (0–5 min postinjection) and phase II (15–30 min postinjection).

Thermal pain test

The pain threshold of mice subjected to intense heat was measured by a photothermal pain detector (YLS-12A, Jinan, China). C57BL/6J mice (male, 18–22 g, n = 10) were injected intraperitoneally with 3MOD or its analogues dissolved in the vehicle (5 μL DMSO in 200 μL saline) 15 min before photothermal heating. Control group received the same volume of the vehicle. Mice were fixed and a constant intensity radiant heat source (with a power of 26 W) was aimed at the middle area of the tail. Tail withdrawal latency was measured as the time taken to withdraw the tail from the light beam.

Abdominal writhing induced by acetic acid

C57BL/6J mice (male, 18–22 g, n = 10) were injected intraperitoneally with 3MOD or its analogues dissolved in the vehicle (5 μL DMSO in 200 μL saline) 15 min before the administration of acetic acid. 200 μL 0.8% (*V*/*V*) acetic acid in saline was intraperitoneally injected to induce hind limb stretching and abdominal contractions. Mice were placed into open polyvinyl cages (20 cm × 40 cm × 15 cm) immediately after acid challenge, and abdominal constrictions were counted

over a period of 30 min.

Mice chronic constriction injury (CCI) induced pain hypersensitivity

The CCI model was originally described for rats [16] and was adapted for mice in this study [17]. Briefly, 18–22 g C57BL/6J male mice (n = 10) were anesthetised with intraperitoneal injection of 60 mg·kg^{-1} sodium pentobarbital (Merck, P11011), then the right common sciatic nerve was exposed and three ligatures were loosely tied around it at intervals of about 0.5 mm until they elicited a brief twitch in the related hind paw. Sham-operated mice (sciatic exposure without ligation) were used as control. The day after the surgery, 3MOD or its analogues suspended in the vehicle (5 μL DMSO in 200 μL saline) were orally administrated twice a day at a dosage of 50 mg·kg^{-1}. Responses to thermal and mechanical stimuli were measured before and 3, 6, 9 and 14 days (2 h after the last administration of testing samples) after the surgery.

Thermal hyperalgesia was tested using photothermal pain detector (YLS-12A, Jinan, China). Briefly, mice were placed in smaller clear plexiglass cubicles and allowed to acclimatize. A constant intensity radiant heat source (with a power of 23 W) was aimed at the midplantar area of the hind paw. The time from initial heat source activation until paw withdraw was recorded as withdrawal latency (s).

Mechanical allodynia was assessed using the Dynamic Plantar Aesthesiometer (Ugo Basile, Comerio, Italy). Animals were placed in a test cage with a wire mesh floor, and the rigid tip of a von Frey filament (punctate stimulus) was applied to the skin of the midplantar area of the hind paw. The filament exerted an increasing force ranging up to 5 g in 20 s, starting below the threshold of detection and increasing until the animal removed its paw. Withdrawal threshold was expressed in grams.

Plasma level of 3MOD after gavage

C57BL/6J male mice (18–22 g) were fasted for 12 h with free access to water and divided into 8 groups (9 in each group) according to the delivery time of 3MOD. Plasma samples were collected at 0, 0.5, 1, 2, 4, 8, 12 and 24 h after the gavage of 50 mg·kg^{-1} 3MOD. Concentration of 3MOD in plasma was measured according to the methods described previously [18]. In brief, 400 μL of mouse plasma was mixed with 400 units of β-glucuronidase (G0251, sigma) and 10 units of sulfatase (S9626, sigma), 400 μL of 0.1 mol·L^{-1} sodium acetate buffer (pH 5.0) containing 10 mmol·L^{-1} EDTA and 0.1% ascorbic acid. The mixture was incubated for 37 °C for 3 h to determine total 3MOD. Then samples were extracted for three times with 1 mL diethyl ether and centrifugation at 15 000 g at 4 °C for 10 min, the pooled extracts were dried in rotary evaporator (Heidolph, Gemany), dissolved in DMSO and subjected to reverse-phase high performance liquid chromatography (RP-HPLC) with Waters 1525 binary HPLC pump and C_{18} column (5 μm particle size, 4.6 mm × 250 mm), eluted by isocratic elution (50% acetonitrile and 50% water) at a flow rate of 1 mL·min^{-1} and monitored by Waters 2465 electrochemical detector.

Surface ECG measurements

C57BL/6J male mice (18–22 g) were anesthetized by intraperitoneal injection of pentobarbital sodium (60 mg·kg^{-1}). The animals were placed supinely, and three limbs (two front paws and right leg) were attached to gel-covered silver wire loops. A three-lead surface ECG was recorded before and after drug administration. The signals were displayed on a computer (ECG parameters gain 1 mV, high-pass filtering 0.1 s, low-pass filtering 100 Hz). ECG data were acquired and analyzed using BL-420F (Taimeng, Chengdu, China) preamp and TM wave MFC Application 1.0 software (Taimeng, Chengdu, China).

The conditioned place preference (CPP) conditioning procedure

The apparatus for CPP training was provided by Dr. WANG Jian-Hong from the Institute of Kunming Zoology at the Chinese Academy of Sciences. The procedure was followed as previously described [19]. Briefly, CPP conditioning procedure consisted of three days pre-conditioning, six days conditioning and one day post-conditioning phases. The pre-conditioning phase started around 09:00. During this phase, C57BL/6J mice (male, 18–22 g) were allowed to explore the entire apparatus freely for 15 minutes. The videos obtained on the third day were assessed and the time spent in the conditioning chambers was calculated as a pre-conditioning baseline. The conditioning phase lasted for six days: mice received morphine or 3MOD (20 mg·kg^{-1}) at 09:00 and vehicle (5 μL DMSO in 200 μL saline) at 21:00, intraperitoneally. After each injection, they were confined to the corresponding chambers (morphine and 3MOD with a non-preferred chamber that was referred to as the drug-paired chamber; and saline with a preferred chamber that was called the vehicle-paired chamber) for 40 minutes. The post-conditioning test was carried out 24 h after the last conditioning. Mice were placed in the intermediate room with the doors open and allowed free access to the conditioning chambers for 15 minutes, while the videos were filmed for further assessment. The CPP score represents the index of place preference of each mouse, calculated by the following formula: CPP score = time in drug paired chamber/ (time in drug-paired chamber + time in vehicle-paired chamber).

Statistical analysis

Statistical significance was assessed by Student's t-test. Analyses were performed with GraphPad Prism 6.1 software. Results were reported as mean ± SD with significance accepted at $P < 0.05$.

Results

Effects of isoflavones on voltage-gated Na^+, K^+ and Ca^{2+} currents in DRG neurons

Chemical structures of the isoflavones studied here are shown in figure. 1A. Effect of isoflavones on voltage-gated sodium channel (Na_V) currents was detected in rat DRG neurons. Isolated DRG neurons were held at –80 mV and equilibrated for at least 5 minutes, then current traces were evoked using a 50-ms step depolarization to –10 mV every second. Tetrodotoxin (TTX), a toxin contained in the ovaries and liver

of pufferfish, has proven to be a useful tool in the electrophysiological study of Na_V. TTX-resistant current is insensitive to micromolar concentrations of tetrodotoxin, with a low single-channel conductance, slow activation and inactivation kinetics and a more depolarized activation threshold than other channels [20]. TTX -resistant (TTX-r) and TTX -sensitive (TTX-s) sodium currents were separated by adding 200 nmol·L^{-1} TTX to the bathing solution [21]. $Na_V1.5$, $Na_V1.8$, and $Na_V1.9$ are TTX-r, whereas all other subtypes are TTX-s sodium channels. Both TTX-s (Fig. 1B) and TTX-r (Fig. 1C) currents were significantly inhibited by 1 μmol·L^{-1} 3MOD. Inhibition of TTX-s and TTX-r currents by 3MOD was dose-dependent with an IC_{50} of 0.71 ± 0.11 and 1.08 ± 0.95 μmol·L^{-1}, respectively (Fig. 1D). Comparison analysis revealed that 1 μmol·L^{-1} genistein (GEN) also inhibited TTX-s (Fig. 1E) and TTX-r (Fig. 1F) currents, and the IC_{50} for TTX-s and TTX-r current was 4.14 ± 0.23 and 9.49 ± 0.81 μmol·L^{-1}, respectively (Fig. 1G). However, daidzein (DAI) showed a weaker suppression effects on DRG TTX-s (Fig. 1H) and TTX-r (Fig. 1I) currents with an IC_{50} of 16.61 ± 0.82 and 21.89 ± 1.88 μmol·L^{-1}, respectively (Fig. 1J). 3MOD failed to block voltage-gated potassium (K_V) and calcium (Ca_V) channels in DRG neurons even at the concentration up to 50 μmol·L^{-1} (Fig. 1K, 1L). 3MOD at the concentration of 1 μmol·L^{-1} shifted the conductance-voltage relationship of TTX-r sodium channel currents to a more positive potentials (~4 mV), but it had little effect on the voltage-dependence of steady-state inactivation (Fig. 1M).

Selectivity of 3MOD on Na_V channel subtypes

3MOD was more effective than GEN or DAI in reducing both TTX-s and TTX-r sodium channel currents in DRG neurons (Fig. 1), we next evaluated the selectivity of 3MOD on Na_V channel subtypes using human (h) or rat (r) sodium channel subtypes ($rNa_V1.3$, $rNa_V1.4$, $hNa_V1.5$ and $hNa_V1.7$) expressed in HEK293T cells, and $rNa_V1.8$ expressed in ND7/23 cells [22]. Currents were elicited by a 20-ms depolarizing potential of –10 mV from a holding potential of –80 mV every 5s. At the concentration of 500 nmol·L^{-1}, 3MOD potently blocked $Na_V1.3$, $Na_V1.7$, and $Na_V1.8$ currents by ~50%, 70% and 60% (Fig. 2A, 2D and 2E), with the IC_{50} of 505 ± 46, 181 ± 14, and 397 ± 26 nmol·L^{-1}, respectively (Fig. 2F). Meanwhile, 500 nmol·L^{-1} 3MOD only depressed $Na_V1.4$ and $Na_V1.5$ currents by ~25% and 15% (Fig. 2B, 2C) with the IC_{50} of 1.98 ± 0.25 and 2.63 ± 0.46 μmol·L^{-1}, respectively (Fig. 2F). At the concentration of 200 nmol·L^{-1}, 3MOD did not change the conductance-voltage relationship (Fig. 3A) and steady-state inactivation of $Na_V1.7$ (Fig. 3B). In contrast, conductance-voltage relationship of $Na_V1.8$ positively shifted by approximately 4 mV (Fig. 3C), while a ~3 mV negative shift in steady-state inactivation of $Na_V1.8$ was detected in the presence of 400 nmol·L^{-1} 3MOD (Fig. 3D). As for $Na_V1.3$, 6 mV positive shift of conductance- voltage relationship (Fig. 3E) and 8 mV negative shift of steady-state inactivation (Fig. 3F) were detected in the presence of 500 nmol·L^{-1} 3MOD.

Effects of 3MOD against acute pain

Since $Na_V1.7$, $Na_V1.8$ and $Na_V1.3$ play key roles in nociception in humans [3-6], the analgesic effect of 3MOD and its analogues were tested in several rodent pain models in which pain was induced by noxious chemicals, acid, or heat. Two-phase pain response was evoked by intraplantar injection of formalin. An early nociceptive response (0–5 min), called phase I, caused by direct stimulation of TRPA1 in a subpopulation of C-fiber nociceptors was followed by phase II (15–30 min), a quiescent period that precedes the second phase of nociceptive behavior because of peripheral inflammation and central sensitization [23]. Intraperitoneal injection of 3MOD remarkably decreased both phase I and phase II responses compared with the vehicle (5 μL DMSO in 200 μL saline) group (Figs. 4A and 4B). 3MOD reduced the duration of paw licking during phase I by 6%, 18% and 41% at the dosage of 0.26, 1.3 and 6.5 mg·kg^{-1}, while that for GEN was 1%, 7% and 32% and for DAI was 0, 3% and 18%, respectively (Fig. 4A). 3MOD was highly effective at alleviating the second phase of nociceptive behavior following formalin injection. Paw licking duration was significantly reduced by 31%, 52% and 69% with the administration of 0.26, 1.3 and 6.5 mg·kg^{-1} 3MOD, while the reduced duration for GEN was 3, 31 and 50%, and for DAI was 1%, 2% and 14%, respectively (Fig. 4B). 3MOD was also more effective than GEN and DAI in reducing abdominal writhing movements induced by intraperitoneal injection of acetic acid. At the dosage of 0.26, 1.3 and 6.5 mg·kg^{-1}, 3MOD decreased the number of writhing movements by 21%, 53% and 68%, while GEN decreased 1.5%, 35% and 46%, and DAI decreased 0, 13% and 24%, respectively (Fig. 4C). In mice subjected to photothermal heat, tail withdrawal latency was increased from 4.8 s in the vector-treated control group to 5.1, 6.1 and 8.0 s in mice treated with 0.26, 1.3 and 6.5 mg·kg^{-1} 3MOD, while that for GEN was 4.8, 5.8, and 7.3 s, respectively. DAI did not alleviate photothermal induced tail twitch at any given dosage (Fig. 4D).

The effect of 3MOD on thermal hyperalgesia and mechanical allodynia

Previous study revealed that subcutaneous administrated genistein reversed sciatic nerve chronic constriction injury (CCI) induced pain hypersensitivity [17]. We evaluated the pain hypersensitivity alleviation activity of 3MOD and its analogues (50 mg·kg^{-1}, twice a day) on CCI mice in which testing samples were orally administrated the day after surgery. Thermal hyperalgesia and mechanical allodynia were all evaluated 2 h after the last sample administration. Three days after the surgery, mice developed a significant decrease in thermal withdrawal latency only at the paw ipsilateral to the injury, as compared to sham-operated animals (4.2 s *vs* 8.5 s, $P < 0.001$), and the latency gradually decreased to 3.9, 3.3 and 3.1 s on the 6, 9 and 14th day after the surgery (Fig. 4E). 3MOD significantly attenuated CCI induced thermal hyperalgesia to the latency period of 5.7, 7.0, 7.6 and 9.0 s over time, whereas GEN attenuated CCI induced thermal hyperalgesia to the latency period of 4.7, 5.2, 6.6 and 7.9 s on the 3, 6, 9 and 14th day, respectively. However, DAI only extended the latency period to 4.5 and 4.7s on the 9 and 14th day, respectively (Fig. 4E).

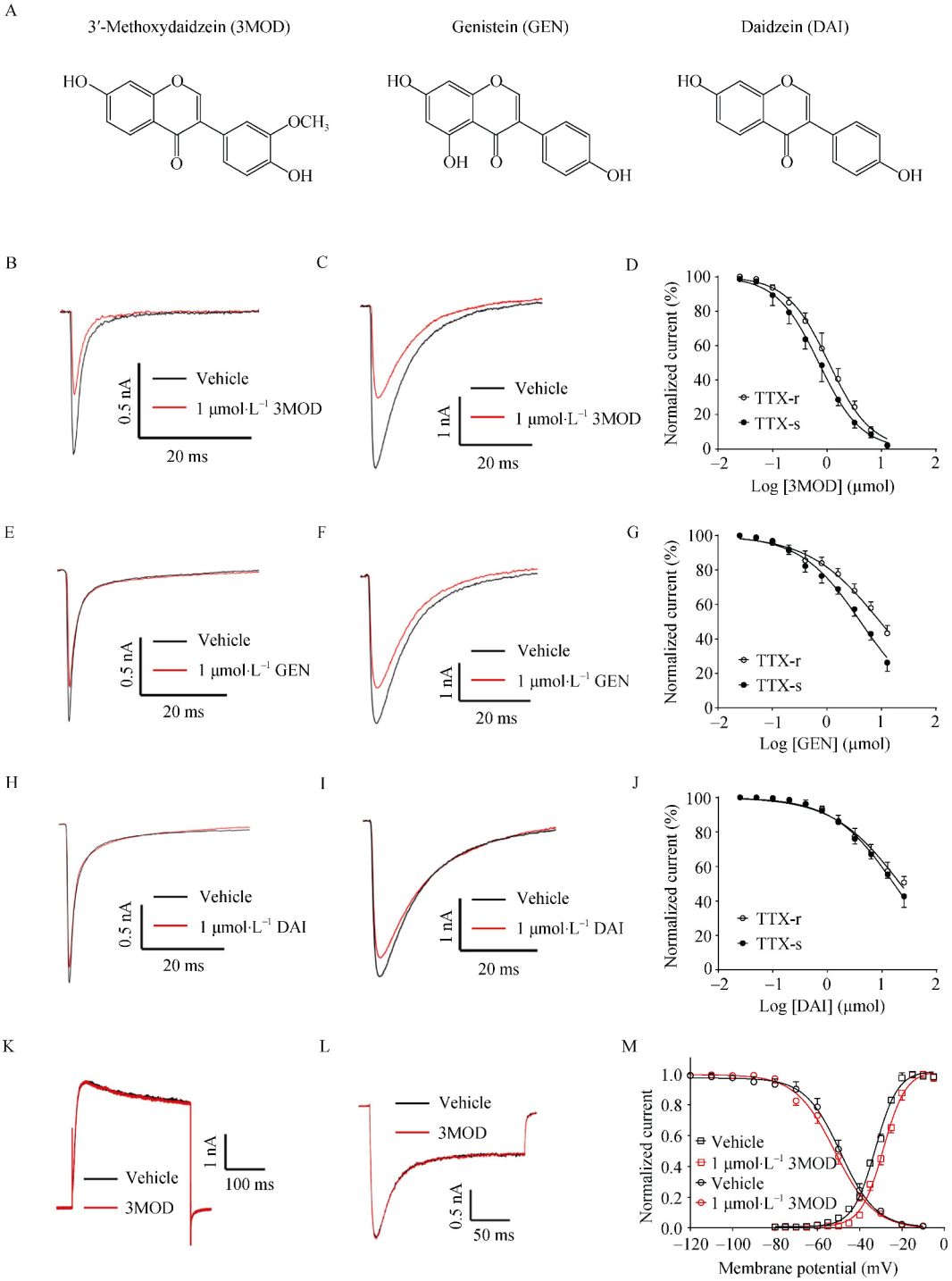

Fig. 1 Effect of 3'-methoxydaidzein and its analogues on voltage-gated sodium channel (Na_V) currents in rat DRG neurons. (A) Chemical structures of 3'-methoxydaidzein (3MOD), genistein (GEN) and daidzein (DAI). (B–J) Na_V currents' inhibitory effect of 3MOD and its analogues. Control currents are shown in black and current traces showing inhibition of DRG Na_V channels by 3MOD or its analogues are shown in red. Inhibition of TTX-s (B) and TTX-r (C) sodium channel currents by 1 μmol·L^{-1} 3MOD. (D) Concentration-response curve for inhibition of TTX-s and TTX-r sodium channel currents in DRG neurons by 3MOD (n = 5). Inhibition of TTX-s (E) and TTX-r (F) sodium channel currents by 1 μmol·L^{-1} GEN. (G) Concentration-response curve for inhibition of TTX-s and TTX-r sodium channel currents in DRG neurons by GEN (n = 5). Inhibition of TTX-s (H) and TTX-r (I) sodium channel currents by 1 μmol·L^{-1} DAI. (J) Concentration-response curve for inhibition of TTX-s and TTX-r sodium channel currents in DRG neurons by DAI (n = 5). 50 μmol·L^{-1} 3MOD has no effect on DRG voltage-gated potassium (K_V) (K) and calcium (Ca_V) (L) channel currents (n = 3). (M) Effect of 1 μmol·L^{-1} 3MOD on the conductance-voltage relationship (square) and voltage dependence steady-state inactivation (circle) on Na_V channel currents in rat DRG neurons. Data points are mean ± SD (n = 5).

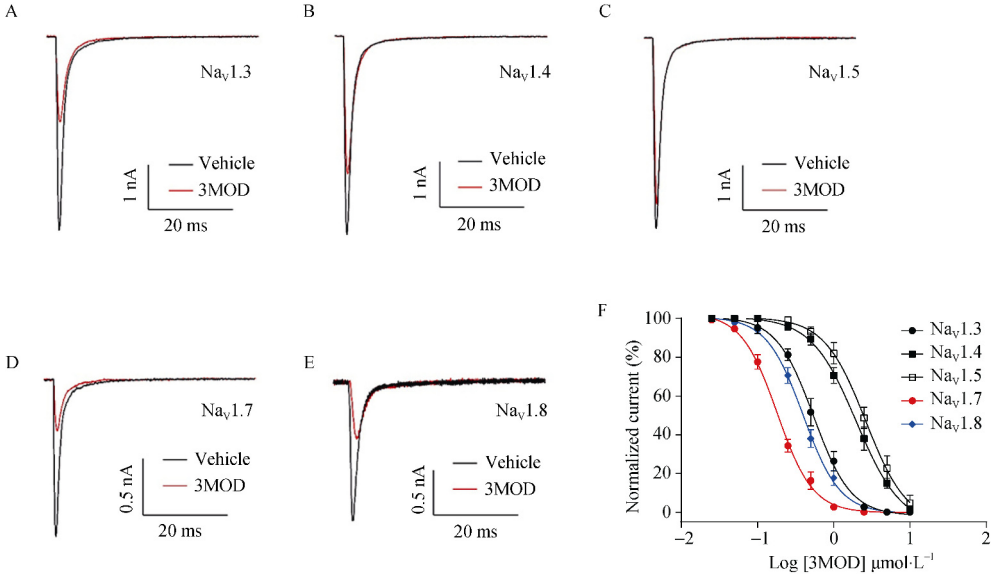

Fig. 2 Inhibitory effect of 3MOD on $rNa_V1.3$, $rNa_V1.4$, $hNa_V1.5$, $hNa_V1.7$ and $rNa_V1.8$. Current traces were evoked by a 50-ms step depolarization to –10 mV from a holding potential of –80 mV every 5 s. Control currents are shown in black and current traces showing inhibition of $rNa_V1.3$ (A), $rNa_V1.4$ (B), $hNa_V1.5$ (C), $hNa_V1.7$ (D) and $rNa_V1.8$ (E) by 500 nmol·L^{-1} 3MOD are shown in red. (F) Concentration-response curves for inhibition of $rNa_V1.3$, $rNa_V1.4$, $hNa_V1.5$, $hNa_V1.7$ and $rNa_V1.8$ by 3MOD. Data points are mean ± SD (n = 5).

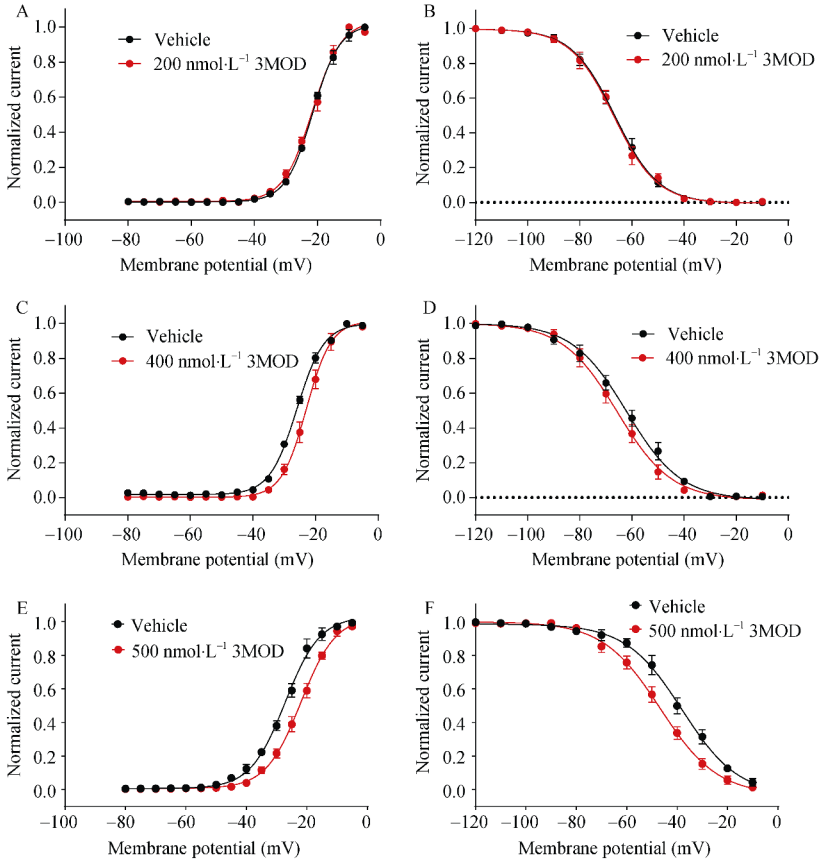

Fig. 3 Effect of 3MOD on current-voltage relationships of $hNa_V1.7$, $rNa_V1.8$ and $rNa_V1.3$. 200 nmol·L^{-1} 3MOD had no effect on I-V curves (A) and steady-state inactivation (B) of $hNa_V1.7$. 400 nmol·L^{-1} 3MOD induced a ~4 mV depolarizing shift in the I–V curves (C) and ~3 mV negative shift in steady-state inactivation (D) for activation of $rNa_V1.8$. 500 nmol·L^{-1} 3MOD induced a ~5 mV depolarizing shift in the I–V curves (E) and ~7 mV negative shift in steady-state inactivation (F) for activation of $rNa_V1.3$. Data points are mean ± SD (n = 5).

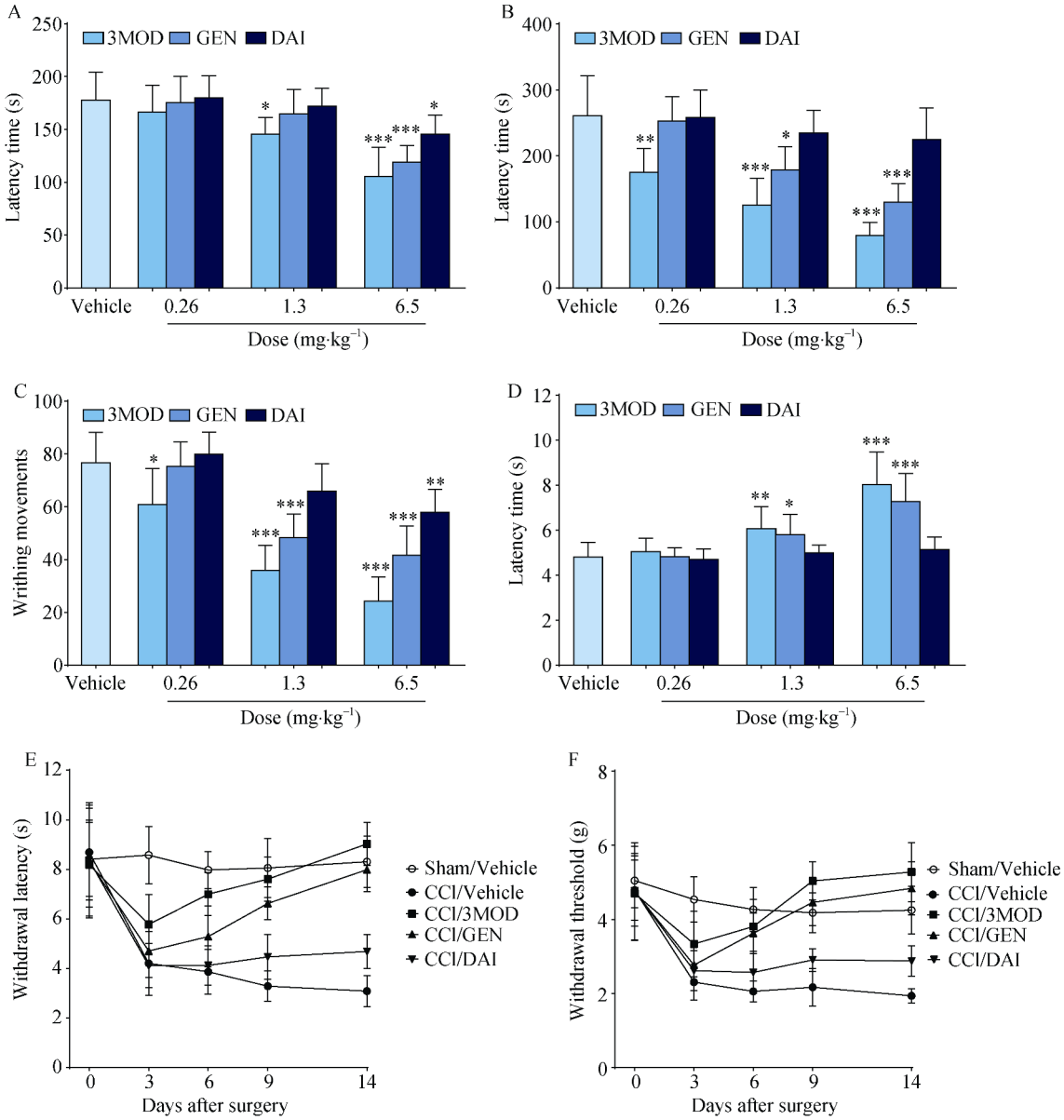

Fig. 4 Analgesic effects of 3MOD and its analogues on mice. Acute (A–D) and chronic (E, F) pain models were used in this experiment. (A) Effect of 3MOD and its analogues on phase I (0–5 min post-injection) (A) and phase II (15–30 min post-injection) (B) paw licking nociceptive behavior following intraplantar injection of formalin. (C) Effect of 3MOD and its analogues on abdominal writhing induced by intraperitoneal injection of acetic acid. (D) Effect of 3MOD and its analogues on the photothermal pain threshold in mice subjected to tail heating. Time-course of the effect on thermal hyperalgesia (E) and mechanical allodynia (F) of 3MOD or its analogues given daily to neuropathic (CCI) mice after injury for 14 days. Data points are mean ± SD (n = 10). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* the control group

Three days after the surgery, neuropathic mice also developed mechanical allodynia to normally innocuous stimulation with von Frey filament compared to sham-operated animals (2.3 *vs* 4.5 g, $P < 0.001$), and the withdrawal threshold remained 2.1, 2.2, and 1.9 g on the 6, 9 and 14th day after the surgery (Fig. 4F). 3MOD significantly attenuated CCI induced mechanical allodynia to the threshold of 3.3, 3.8, 5.0 and 5.2 g as time goes on, whereas GEN increased the threshold to 2.8, 3.6, 4.5 and 4.8 g on the 3, 6, 9 and 14th day, respectively. However, DAI only slightly attenuated allodynia threshold to 2.9 and 2.8 g on the 9 and 14th day after the surgery, respectively (Fig. 4F).

Plasma concentration and side effects assessment of 3MOD

After oral administration of 3MOD (50 mg·kg^{-1}) for 120 min, plasma 3MOD was reached to approximately 1.83 ± 0.25 μmol·L^{-1} (Fig. 5A), which was much higher than the IC_{50} for $Na_V1.3$, $Na_V1.7$ and $Na_V1.8$ currents (Fig. 2). $Na_V1.5$ is a cardiac sodium channel that plays an important role in the

excitability of atrial and ventricular cardiomyocytes and rapid impulse propagation. Some $Na_V1.5$ inhibitors may shorten the electrocardiograph (ECG) QT interval and increase QRS amplitude in rats [24]. Since 3MOD shows ability to inhibit sodium channel current in $hNa_V1.5$ overexpressed HEK293T cells (Fig. 2F), we compared the mice ECG signals 15 min after intraperitoneal injection of 6.5 mg·kg^{-1} 3MOD and vehicle (5 μL DMSO in 200 μL saline). There was no change in the ECGs after 3MOD administration (Fig. 5B, C). 20 mg·kg^{-1} daily intraperitoneal injection of 3MOD did not alter the two-week body weight changes compared with control and morphine (Fig. 5D). Conditioned place preference (CPP) assessment revealed that the daily injection of 20 mg·kg^{-1} 3MOD was not addictive to male C57BL/6J mice whereas mice were addicted to morphine at the same dose drastically (Fig. 5E). Intraperitoneal injection of 3MOD suspension (with 16 μL DMSO in 200 μL saline) at 800 mg·kg^{-1} was not lethal for C57BL/6J mice, but caused decreased motion within 15 min compared with the vehicle group. However, this effect was completely reversed in two or three hours (data not shown).

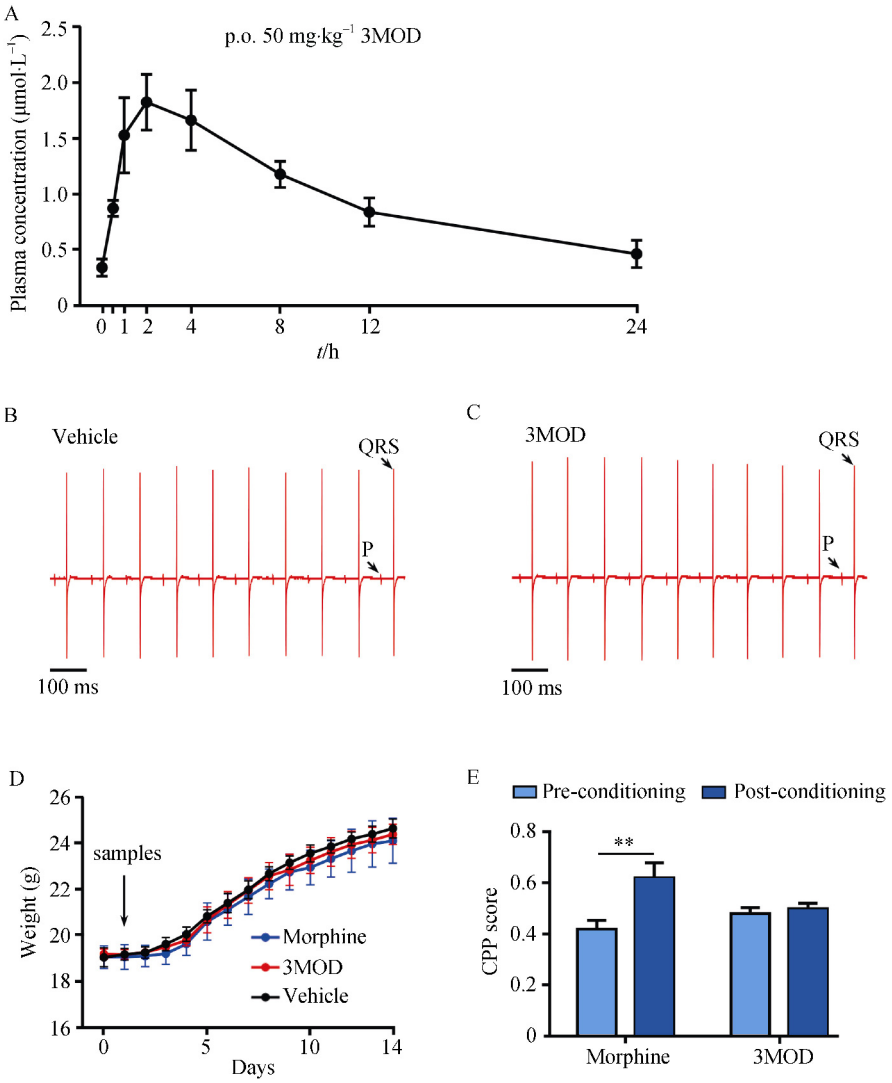

Fig. 5 Serum concentration and side effects assessment of 3MOD. (A) Blood concentrations of 3MOD after oral administration of 50 mg·kg^{-1} in C57BL/6J mice. Data points are mean ± SD (n = 3). C57BL/6J mice were pre-treated with vehicle (5 μL DMSO in 100 μL saline) (B) or 6.5 mg·kg^{-1} body weight 3MOD (C) *via* intraperitoneal injection and the electrocardiograms were monitored by BL-420F biological function experiment systems 15 min after the injection (n = 5). (D) Two-week body weight changes of mice daily injected with 20 mg·kg^{-1} 3MOD and morphine. Data points are mean ± SD (n = 8). (E) The establishment of conditioned place preference (CPP) by repeated pairing of morphine and 3MOD. The preference was determined by a comparison between the post-conditioning CPP score and the pre-conditioning baseline. CPP score in the drug-paired compartment was expressed as mean ± SD (n = 8). $^{**}P < 0.01$ *vs* the control group

Discussion

Some isoflavones were thought to suppress the development of neuropathic pain behavior in rats undergoing partial sciatic nerve ligation injury [25]. The average menstrual migraine frequency was also be decreased by more than 50% by the intake of isoflavones in a randomized, controlled trial with female adults [26], but the precise mechanisms remained unknown. Recent studies revealed that isoflavone genistein ameliorated painful neuropathy by anti-oxidant and anti-inflammatory effects in mouse sciatic nerve chronic constriction injury (CCI) [17] and diabetes mode [27]. Previous study also revealed that isoflavones genistein and daidzein block $^{22}Na^+$ influx through VGSCs in cultured rat brain neurons with a half-maximum effect of 60 and 192 μmol·L^{-1}, respectively [28]. The inhibition of VGSCs by genistein occurs in a direct manner without being mediated by its tyrosine kinase inhibition activity [28]. Controversially, in $Na_V1.7$ highly expressed rat superior cervical ganglia (SCG) neurons, genistein and daidzein inhibit Na^+ currents with an IC_{50} of 9.1 and 20.7 μmol·L^{-1}, respectively, and the inhibition of Na^+ currents is significantly reversed by protein tyrosine phosphatases antagonists. This suggests that genistein inhibits VGSCs in SCG neurons through protein tyrosine kinase-dependent and kinase-independent mechanisms [29].

3′-Methoxydaidzein (3MOD) is a dietary isoflavonoid which is widely exists in common Fabaceae plants, such as Pueraria [30], mung beans [31] and Dalbergia [32]. 3MOD is a derivative of daidzein. We find here that 3MOD effectively inhibits TTX-s and TTX-r VGSCs with the IC_{50} of 0.71 ± 0.11 and 1.08 ± 0.95 μmol·L^{-1} in rat DRG neurons, which was more potent than GEN or DAI on the same conditions (Fig. 1). DRG belongs to primary sensory neurons which constitute the first link in the chain of neurons that make up somatosensory pathways [33]. They become hyperexcitable following some injuries and give rise to unprovoked spontaneous action potential activity or pathological bursting, which contribute to chronic pain [34]. Subtype detection indicates that 3MOD inhibits $Na_V1.7$, $Na_V1.8$ and $Na_V1.3$ potently, with IC_{50} of 181 ± 14, 397 ± 26 and 505 ± 46 nmol·L^{-1}, respectively (Fig. 2F). Additionally, the isoflavones may selectively block VGSCs with a preference for $Na_V1.7$, $Na_V1.8$ and $Na_V1.3$, which are thought to be potential targets for pain therapeutics. 3MOD showed stronger analgesic activities compared to GEN or DAI both in acute and chronic rodent pain models (Fig. 4). The results suggest that the isoflavones have direct effects on sodium channel nociceptors and bring about analgesic functions apart from anti-oxidant and anti-inflammatory pain-alleviating pathways. Estrogen receptors are engaged in pain modulation. ERβ rather than ERα knockout female mice present less nociceptive behaviors than wild type females during the interphase and early phase II in formalin test. However, no difference in phase I, interphase and phase II was observed between ER knockout and wild type males groups [35-36]. Male mice were used in our pain tests, and our compounds showed pain alleviating activity during the whole phases (Figs. 4A–4B). Despite isoflavonoids are structurally similar to estrogen, it's not possible that pain reliving effects of 3MOD dependent on estrogen receptors. As shown in Fig. 5A, orally administrated 3MOD was absorbed quickly and maintained for a long time in plasma with a terminal half-life of approximate 10 h. After orally administration of 50 mg·kg^{-1} 3MOD for 120 min, plasma 3MOD was reached to approximate 1.83 μmol·L^{-1}, which is much higher than the IC_{50} for $Na_V1.3$, $Na_V1.7$ and $Na_V1.8$ currents (Fig. 2F). Orally administrated 3MOD (50 mg·kg^{-1}, twice a day) significantly relieved mouse CCI induced pain hypersensitivity (Figs. 4E–4F), suggesting that it is appropriate for alleviating human chronic pain with a calculated oral dose of 5.5 mg·kg^{-1} 3MOD twice a day approximately (according to the relative body surface area). In addition, 3MOD is found to be an efficient pain-relief molecule with little toxicity (Figs. 5B–5E). Despite that 3MOD depressed $Na_V1.5$ current with an IC_{50} of 2.63 ± 0.46 μmol·L^{-1} *in vitro* (Fig. 2), it did not change the ECGs of mice under the concentration in which robust analgesic effects were observed (Figs. 5B–5C). Long term administration of 3MOD was not addictive to mice as shown in CPP experiment (Fig. 5E), and had no undesirable effect on their diet and growth (Fig. 5D). Intraperitoneal injection of 3MOD was not lethal to mice at the concentration up to 800 mg·kg^{-1}.

Isoflavone-rich legumes and bean products are common food around the world, especially in Asian countries. The much larger estimated daily intake of isoflavones by Asians is an important factor accounting for the lower incidence and mortality from breast cancer in Asian women [37]. Food and Drug Administration (FDA) report revealed that, although varied by conditions, there is a lower prevalence of chronic pain in Asian populations [38]. Additionally, Asians have the lowest prevalence of back pain among other races in America [39]. Since the Asians consume a diet richer in isoflavones, we assume that high-isoflavone diet may have a protective effect against chronic pains. However, this assumption should be confirmed through statistical research among different populations, and randomized, controlled trials need to be carried out to give a reasonable recommendation on the prevention or treatment of chronic pains with isoflavones.

In conclusion, despite the possible effects of structural variations on the blockage of nociceptive neuron sodium currents, isoflavones exert more direct and powerful analgesic functions by VGSCs inhibition. Isoflavones, such as 3MOD, might be promising compounds to treat human chronic pain pathologies.

Acknowledgements

We thank Dr. WANG Jian-Hong for her instructions during CPP training and other lab members who provided great research assistance.

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Cite this article as: XU Run-Jia, FEI Shuo-Han, CHEN Lin-Yan, WANG Gan, LIU Ming, ZHANG Wen-Sheng, YAN Xiu-Wen, LAI Ren, SHEN Chuan-Bin. 3'-Methoxydaidzein exerts analgesic activity by inhibiting voltage-gated sodium channels [J]. *Chin J Nat Med*, 2019, **17**(6): 413-423.

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