Antiepileptic geissoschizine methyl ether is an inhibitor of multiple neuronal channels

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Geissoschizine methyl ether (GM) is an indole alkaloid isolated from Uncaria rhynchophylla (UR) that has been used for the treatment of epilepsy in traditional Chinese medicine. An early study in a glutamate-induced mouse seizure model demonstrated that GM was one of the active ingredients of UR. In this study, electrophysiological technique was used to explore the mechanism underlying the antiepileptic activity of GM. We first showed that GM (1–30 μmol/L) dose-dependently suppressed the spontaneous firing and prolonged the action potential duration in cultured mouse and rat hippocampal neurons. Given the pivotal roles of ion channels in regulating neuronal excitability, we then examined the effects of GM on both voltage-gated and ligand-gated channels in rat hippocampal neurons. We found that GM is an inhibitor of multiple neuronal channels: GM potently inhibited the voltage-gated sodium (NaV), calcium (CaV), and delayed rectifier potassium (I\(\mathrm{K}_{\text{D}}\)) currents, and the ligand-gated nicotinic acetylcholine (nACh) currents with IC\(50\) values in the range of 1.3–13.3 μmol/L. In contrast, GM had little effect on the voltage-gated transient outward potassium currents (I\(\mathrm{K}_{\text{A}}\)) and four types of ligand-gated channels (γ-aminobutyric acid (GABA), N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methylisoxazole-4-propionamide/kainite (AMPA/KA receptors)). The in vivo antiepileptic activity of GM was validated in two electricity-induced seizure models. In the maximal electroshock (MES)-induced mouse seizure model, oral administration of GM (50–100 mg/kg) dose-dependently suppressed generalized tonic-clonic seizures. In 6-Hz-induced mouse seizure model, oral administration of GM (100 mg/kg) reduced treatment-resistant seizures. Thus, we conclude that GM is a promising antiepileptic candidate that inhibits multiple neuronal channels.

**Keywords:** geissoschizine methyl ether; Uncaria rhynchophylla; antiepileptic drug; hippocampal neurons; action potential; nicotinic acetylcholine receptors; voltage-gated ion channels; mouse seizure model

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INTRODUCTION

Uncaria rhynchophylla (UR) is a commonly used traditional Chinese medicine that has been used to treat epileptic seizures for centuries [1, 2]. It is also one of the dried medicinal herbs in yi-gan san (yokukansan), a traditional herbal preparation widely used in China and Japan [3, 4]. Yokukansan has been approved by the Japanese Ministry of Health, Labor, and Welfare as a prescription drug for neurosis, insomnia, and irritability in children [4]. Various chemical components, such as rhynchophylline, isorhynchophylline, and geissoschizine methyl ether (GM), have been isolated from UR [2, 5]. Among these components, GM is known to be an active component of UR and a major contributor to the psychotropic nature of yokukansan [2, 4].

In a preliminary study, GM was found to exhibit moderate antiepileptic effects in a glutamate-induced seizure mouse model [6]. However, the mechanism underlying its antiepileptic activity remains unclear. The neuroprotective effects of GM have been observed in several glutamate-induced neuronal death experiments [7–10], and these effects are probably due to the inhibition of Ca\(^{2+}\) influx, the reduction in mitochondrial respiration and the suppression of the generation of reactive oxygen species (ROS) [11, 12]. Consistent with its neuroprotective effects, GM has also been found to ameliorate myelin deficiency in mature oligodendrocyte formation and to be involved in remyelination in the medial prefrontal cortex [13]. Moreover, GM may also behave as a partial agonist of the 5-HT\(_{1A}\) receptor and an antagonist of the 5-HT\(_{2A}\), 5-HT\(_{2C}\) and 5-HT\(_{7}\) receptors, which was thought to be the mechanism underlying the effects of yokukansan in the treatment of aggression and reduced sociality in mice [14–17]. The inhibitory activity on acetylcholinesterase could also contribute to the neuroprotective effects of GM against damage in Alzheimer’s disease models [2, 18]. However, neither of these proposed activities of GM provides an appropriate explanation for its antiepileptic effects.

Epilepsy is commonly characterized by abnormal spontaneous recurrent electrical discharge in the central nervous system [19]. Damage to the hippocampus was found in more than 50% of patients with temporal lobe epilepsy; thus, hippocampal neurons have been widely used for mechanistic studies of antiepileptic drugs (AEDs) [19, 20]. Voltage-gated sodium (Na\(_{\text{V}}\)), potassium (K\(_{\text{D}}\)), and
and calcium (Ca2+) channels play pivotal roles in neuron firing, and mutations in these channels may cause genetic epilepsy [21]. Impaired GABAergic inhibition and increased glutamatergic excitation are correlated with the generation of seizures [22, 23]. In addition, abnormalities in neuronal nicotinic acetylcholine (nACh) receptors and cholinergic neurotransmission are also involved in the pathophysiology of epilepsy, such as autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) and juvenile myoclonic epilepsy (JME) [24–26]. All of these voltage-gated and ligand-gated channels have been demonstrated to be relevant in the therapeutic activities of many AEDs, such as topiramate, phenytoin, and carbamazepine [27, 28]. However, AEDs may encompass different antiepileptic treatment spectrums in animal models of epilepsy [19]. In the present study, we explored the molecular mechanism underlying the antiepileptic activity of GM using a custom electrophysiology setup in hippocampal neurons and evaluated its antiepileptic treatment spectrum in the maximal electroshock (MES) and 6-Hz seizure models.

MATERIALS AND METHODS

Materials

GM was purchased from Standard Technology Co., Ltd (Shanghai, China). The purity of the synthetic GM was ≥98%, as assessed by HPLC. The compound was dissolved and stored in dimethyl sulfoxide (DMSO) to produce 20 mmol/L stock solutions that were then diluted in bath solution to obtain the final concentrations. DMSO at the final concentrations (≤0.5%) was well tolerated with no observable toxic effects to the neurons. To conduct research on animals, GM was dissolved in a mixture of 5% DMSO and 95% Tween-80. TTX was purchased from Aquaculture Technical Developing Company, Qinhuangdao, China. Topiramate (TPM), levetiracetam (LEV), γ-aminobutyric acid (GABA), N-methyl-D-aspartic acid (NMDA), α-αmino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA), kainic acid (KA) and acetylcholine chloride (ACH-Cl) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animals

Sprague-Dawley rats at postnatal days 1–7, 18-day-old C57BL/6 mouse embryos and male KM mice weighing 20 ± 2 g were purchased from the Shanghai SLAC Laboratory Animal Co., Ltd (Shanghai, China). All animal procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals under protocols strictly followed and approved by the guidelines of the IACUC (Institutional Animal Care and Use Committees). The IACUC checked all protocols and approved this study.

Primary cultured hippocampal neuron preparations

Hippocampal neurons were isolated from 18-day-old C57BL/6 mouse embryos. Briefly, the dissected hippocampal tissues were enzymatically dissociated with 3 mg/mL proteinase (Sigma-Aldrich) and incubated at 32°C for 8 min. One mg/mL of bovine serum albumin (Sangon Biotech, Shanghai, China) and trypsin inhibitor (Sigma-Aldrich) was then added to the solution to stop digestion. The tissues were then triturated into single cells using a fire-polished glass Pasteur pipette. The neurons were collected by centrifugation, resuspended in DMEM/F12 ( Gibco, Grand Island, USA) with 10% FBS (Gibco), and, finally, plated at a density of 150,000 cells/mL on poly D-lysine-coated glass coverslips in 24-well plates and cultured at 37°C in 5% CO2. The medium was replaced after 6 h with the same volume of serum-free Neurobasal-A (Gibco) containing 2% B-27 ( Gibco), 0.5 mmol/L GlutaMAX (Gibco), and 1% penicillin and streptomycin (Gibco). Thereafter, half of the old medium was removed and replaced with the same volume of fresh medium every 3 days. Neurons were used in electrophysiological experiments after 12 days of culture.

Acutely isolated hippocampal neuron preparations

Hippocampal neurons were obtained from Sprague-Dawley rats at postnatal days 1–7. Similar to the mouse neurons, the hippocampal tissues were triturated into single cells with a dissociation solution after digestion with proteinase. The acutely isolated hippocampal neurons were used in the following 6 h. As calcium-free dissecting solution has been reported to be beneficial for dissociating the hippocampal tissues and reducing the activity of intrinsic proteases, calcium was omitted in the solution [29]. The dissecting solution contained (in mmol/L): 82 Na2SO4, 30 K2SO4, 5 MgCl2, 1 NaPy, 10 HEPES, and 20 glucose at pH 7.4, adjusted with NaOH.

Electrophysiological recordings

Pipettes were pulled from borosilicate glass capillaries, and the resistances were 3–5 MΩ when filled with the intracellular solution. Current-clamp mode was used to record mouse and rat neuronal firing properties. The intracellular solution contained (in mmol/L) 140 KCl, 1 MgCl2, 10 EGTA, and 10 HEPES, with the pH adjusted to 7.2 using KOH; the bath or extracellular solution contained (in mmol/L) 140 NaCl, 5 KCl, 1 CaCl2, 1.25 MgCl2, 10 glucose, and 10 HEPES (pH 7.4 adjusted with NaOH). During recording, the cells were held at a potential of −60 mV, and the bath solution was continuously perfused using a bath perfusion system. Ligand-gated ion channel currents (GABA, AMPA, NMDA, KA, and nACh) were activated by the corresponding ligands in rat hippocampal neurons kept at the holding potential of −60 mV. The preapplication of GM for 60 s followed by the coapplication of GM and each ligand for 2 s (GABA, AMPA, NMDA, and KA) or 5 s (ACH-Cl) resulted in a change in the amplitude of the peak. To elicit sodium, calcium, potassium currents in rat hippocampal neurons, the standard whole-cell voltage-clamp technique was used. Sodium currents were recorded in the intracellular solution that contained (in mmol/L) 120 CsCl, 10 NaCl, 10 TEA-Cl, 1 CaCl2, 1 MgCl2, 10 EGTA and 10 HEPES (pH 7.2 adjusted with CsOH); the extracellular solution contained (in mmol/L) 120 NaCl, 5 KCl, 1 CaCl2, 1.25 MgCl2, 20 TEA-Cl, 10 glucose, and 10 HEPES (pH 7.4 adjusted with NaOH). The holding potential was −90 mV and was depolarized to −20 mV for a duration of 50 ms. Calcium currents were recorded in an intracellular solution containing (in mmol/L) 130 CsCl, 10 TEA-Cl, 5 MgATP, 10 EGTA, and 10 HEPES, pH 7.2 adjusted with CsOH; the extracellular solution contained (in mmol/L) 130 NaCl, 5 CaCl2, 20 TEA-Cl, 10 glucose, and 10 HEPES, with pH adjusted to 7.4 using NaOH. The calcium currents were evoked by steps from the holding potential of −90 to 0 mV. Potassium currents and Kir currents were recorded in an intracellular solution containing (in mmol/L) 140 KCl, 1 CaCl2, 1 MgCl2, 10 EGTA, and 10 HEPES (pH 7.2 adjusted with KOH); the extracellular solution contained (in mmol/L) 140 NaCl, 5 KCl, 1 CaCl2, 1.25 MgCl2, 10 glucose, and 10 HEPES, with pH adjusted to 7.4 using NaOH. The holding potential was −50 mV and was hyperpolarized to −110 mV for a duration of 500 ms. Then, whole-cell potassium currents were evoked by steps from −110 to 40 mV. To elicit delayed rectifier potassium currents (IK1), the potential was depolarized to −50 mV for a duration of 50 ms to deactivate the fast transient K+ currents (IK). The peak amplitude of IK1 was measured. The amplitude of the IK1 was measured with 300 ms latency. To record Kir2.1 currents, human embryonic kidney 293 (HEK-293) cells stably expressing hKir2.1 channels were clamped at a holding potential of −70 mV and a subsequent 500 ms test pulse to −140 mV. Data acquisition was achieved using a Axopatch 200B amplifier (Axon Instruments, Burlingame, CA, USA), and the signals were filtered at 2 kHz and digitized at 10 kHz with a Digitida 1440 A interface (Axon Instruments, Burlingame, CA, USA). Current and voltage recordings were made at room temperature.

MES-induced seizure assays

Electroconvulsions were generated by an alternating current (5.4 s stimulus duration, fixed current intensity of 4 mA, maximum stimulation voltage of 160 V) delivered via ear-clip electrodes through a physiological and pharmacological electronic stimulator.
(Ji-nan, China). The criterion for the onset of seizures was the presence of tonic-clonic generalized seizures that result in tonic hind-limb extension. The day before the experiment, mice who had experienced generalized tonic-clonic seizures induced by MES were selected to test the anticonvulsant effects of the compounds. Mice were separately administered GM (50, 80, or 100 mg/kg) or vehicle orally 1 h before electrical stimulation. Animals were thought to be protected from MES-induced seizures in the absence of the hind-limb tonic extension component of the seizure.

6-Hz corneal kindling epilepsy test
The acute 6-Hz seizure model was conducted as explained previously [30–32]. Corneal stimulation (0.2 ms rectangular pulses at 6 Hz for 3 s) was delivered by a constant-current device (CCU1) connected to an S48 Square Pulse Stimulator (Grass Technologies, West Warwick, RI, USA). A fixed current intensity of 32 mA was used to allow this data to be directly compared with data obtained in the aforementioned studies. Animals were randomly divided into three groups with ten animals in each group. Before stimulation, a drop of saline was applied to each eye to ensure optimal current conductivity. Seizures were characterized by a stunned or fixed posture often accompanied by rearing, forelimb clonus, and twitching. The duration of seizure activity was measured as the criterion for the protective effect of the compound. The animal was considered to be protected if it resumed its normal posture after drug administration or if the duration of the seizure was longer than 7 s.

Data analysis
All patch clamp data were processed using Clampfit 10.4 (Molecular Device, Sunnyvale, CA, USA) and then analyzed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). Voltage-dependent activation curves were fitted to the Boltzmann equation: $G = G_{\text{min}} + (G_{\text{max}} - G_{\text{min}}) / (1 + \exp(V - V_{1/2})/S)$, where $G_{\text{max}}$ is the maximum conductance, $G_{\text{min}}$ is the minimum conductance, $V_{1/2}$ is the voltage to reach 50% of the maximum conductance, and $S$ is the slope factor. Steady-state inactivation curves were constructed by plotting the normalized peak currents during the test pulses as a function of the prepulse potential. The data were fitted to the Boltzmann equation: $I/I_{\max} = 1/(1 + \exp(V - V_{\text{mid}})/K_I)$, where $I$ is the amplitude of peak current at each voltage; $I_{\max}$ is the maximal peak current value, $V$ and $V_{\text{mid}}$ are the prepulse potential and the half-maximal potential for inactivation, respectively, and $K_I$ is the inactivation slope factor. Dose-response curves were fitted to a 3-parameter Hill equation: $Y = \text{Bottom} + (\text{Top-Bottom})/\left[1 + 10^{(X-\text{LogIC}_{50})}\right]$, where Bottom and Top are the minimum and maximum inhibition, respectively; $X$ is the log of the concentration; $Y$ is the value of $I_{\text{drug}}/I_{\text{Control}}$ and $\text{IC}_{50}$ is the drug concentration producing a half-maximum response. The data are presented as the means ± SEM, and the significance was estimated using paired two-tailed Student’s $t$ tests unless otherwise stated.

RESULTS
Inhibitory effects of GM on action potential firing in cultured hippocampal neurons
The effects of GM on action potential firing were individually examined in cultured mouse and rat hippocampal neurons using whole-cell-current-clamp technique. For mouse neurons, under increasing doses of GM (1, 3, 10 and 30 μmol/L), the firing frequency decreased, with inhibition ratios of 45.3% ± 3.7%, 49.6% ± 5.1%, 82.3% ± 1.1% and 97.8% ± 0.5% (Fig. 1a, e, $n = 6$), respectively. GM also decreased the amplitude of single action potentials recorded from cultured mouse a and rat b hippocampal neurons without or with GM at indicated concentrations. Representative traces showing the effects of 10 μmol/L GM on the half-width of action potential from cultured mouse c and rat d hippocampal neurons ($n = 6$). e Summarized data showing the inhibitory effects of GM on the firing frequency ($n = 6$). f Amplitudes of action potentials before and after the application of GM ($n = 6$). g Summarized data showing the effects of GM on the half-width of action potential ($n = 6$). Paired Student’s $t$ test, $P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. control.

Fig. 1 GM inhibited the neuronal excitability of rat and mouse hippocampal neurons. Spontaneous firing and representative traces of action potentials recorded from cultured mouse a and rat b hippocampal neurons without or with GM at indicated concentrations. Representative traces showing the effects of 10 μmol/L GM on the half-width of action potential from cultured mouse c and rat d hippocampal neurons ($n = 6$). e Summarized data showing the inhibitory effects of GM on the firing frequency ($n = 6$). f Amplitudes of action potentials before and after the application of GM ($n = 6$). g Summarized data showing the effects of GM on the half-width of action potential ($n = 6$). Paired Student’s $t$ test, $P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. control.
potentials in a dose-dependent manner. The amplitude was reduced by 9.5% ± 1.8%, 20.9% ± 3.2% and 100.0% in the presence of 3, 10 and 30 µmol/L of GM, respectively (Fig. 1a, f, n = 6). Notably, in addition to a decrease in the amplitude, the duration of the action potential was also affected. In the presence of 10 µmol/L GM, the half-width of the action potential was prolonged to 2.7 ± 0.4 ms from 2.0 ± 0.2 ms in the controls (Fig. 1g, n = 6). Notably, in addition to a decrease in the amplitude, the duration of the action potential was also affected. In the presence of 10 µmol/L GM, the half-width of the action potential was prolonged to 2.7 ± 0.4 ms from 2.0 ± 0.2 ms in the controls (Fig. 1g, n = 6). Intriguingly, the resting membrane potential of the action potential did not change after GM perfusion (Fig. 1a). GM caused effects on action potential firing in rat neurons similar to those caused in mouse neurons, including a reduction in the firing frequency and the inhibition of the amplitude and prolongation of the duration, which supports the idea that there are no species differences in the activity of GM in mouse and rat neurons (Fig. 1d–g). Rat neurons were used in the following electrophysiological experiments unless otherwise stated.

Effects of GM on epilepsy-causing voltage-gated ion channels in hippocampal neurons
NaV, CaV, and Kv channels form a superfamily of ion channels that allow hippocampal neurons to generate and propagate action potentials [33]. Mutations in the genes encoding these channels have been linked to various genetic epilepsy disorders, such as Dravet syndrome, benign familial neonatal-infantile epilepsy and episodic ataxia [34]. These channels have been proposed to be the targets of some AEDs, such as phenytoin, carbamazepine and LEV [19]. To reveal the potential mechanisms underlying the antiepileptic activity of GM, the effects of GM on NaV, CaV, and Kv channels were individually examined in acutely isolated rat hippocampal neurons.

GM inhibits NaV currents. To elicit sodium currents, a 50 ms test pulse was applied to a holding potential of −90 mV, depolarizing it to −20 mV. The peak currents decreased as the concentration of GM increased in the perfusion solution (Fig. 2a). As shown in Fig. 2c, GM inhibited NaV currents in a dose-dependent manner with an IC50 of 10.2 (9.3–11.2) µmol/L (n = 7). Thus, a concentration of 10 µmol/L was used in the following experiments. To understand how GM inhibits NaV channels, the effects of GM on NaV channel kinetics were further characterized. To plot the activation curves, NaV currents were elicited by applying step pulses to a holding potential of −90 mV in 10 mV increments, ranging from −80 to +30 mV, for 50 ms. Representative current traces before and after the bath perfusion of 10 µmol/L GM are shown in Fig. 2d. Activation curves showed that no significant changes occurred in the half-activation voltage after the perfusion of GM (Fig. 2e). The values in the absence and presence of 10 µmol/L GM were −45.1 ± 1.0 mV and −42.7 ± 0.8 mV, respectively. Next, the influence of GM on steady-state inactivation was evaluated using a 500 ms conditioning pulse ramping from −120 to −20 mV in 10 mV increments, followed by a 20 ms test pulse at −20 mV (Fig. 2f). In contrast to its lack of effect on the activation curves, GM caused a hyperpolarizing shift in steady-state inactivation. The value of V1/2 was shifted from −54.4 ± 0.5 mV...
in the control condition to $-61.4 \pm 0.6$ mV in the presence of $10 \mu$mol/L GM (Fig. 2g). These data showed that GM is an inhibitor of Na$_V$ channels distributed in hippocampal neurons and preferentially affects channel inactivation.

**GM inhibits Ca$_V$ currents.** Ca$_V$ currents were elicited by a 300 ms test pulse from a holding potential of $-90$ mV to 0 mV. The peak currents decreased in a dose-dependent manner in the presence of GM, with an IC$_{50}$ of $13.3 \ (11.4-15.5) \ \mu$mol/L (Fig. 3a, c, n = 6). The Ca$_V$ currents in the isolated rat hippocampal neurons could be separated into two categories: high-voltage-activated (HVA) and low-voltage-activated (LVA) calcium currents [35]. With two protocols, HVA and LVA calcium currents could be evoked in different proportions, allowing a separation of currents based on their biophysical characteristics [35]. From a holding potential of $-90$ mV, both LVA and HVA components were evoked by depolarizing stimulations, whereas the majority of HVA currents were evoked when using a holding potential of $-50$ mV. Following the subtraction of the HVA currents, LVA currents were isolated. After perfusion with $10 \mu$mol/L GM, the evoked HVA and LVA currents were dramatically reduced by 28.5% $\pm$ 10.9% and 48.4% $\pm$ 6.9%, respectively (Fig. 3d, n = 7). These data demonstrated that GM preferentially inhibits LVA currents. Kinetic analysis showed that the activation curves of both HVA and LVA currents did not exhibit a remarkable change after $10 \mu$mol/L GM treatment (Fig. 3f, i). The effects of GM on the inactivation kinetics of HVA and LVA currents were then examined individually. To induce the inactivation of HVA currents, neurons were clamped at $-50$ mV, and then, a 300 ms prepulse ramping from $-80$ to $+10$ mV in $10$ mV increments was applied, followed by a 300 ms test pulse to 0 mV. No significant shift in the inactivation curves of HVA currents was observed, despite the peak currents being significantly smaller after the application of $10 \mu$mol/L GM (Fig. 3e, g, n = 9). The inactivation of LVA currents was examined using a range of depolarization pre pulses (from $-80$ to $10$ mV in $10$ mV increments) from a holding potential of $-90$ mV, followed by a 300 ms test pulse to $-30$ mV. The lack of an effect on LVA current inactivation was similar to that seen for HVA inactivation (Fig. 3h, j, n = 5). Taken together, GM dose-dependently inhibits Ca$_V$ currents in hippocampal neurons without observable effects on channel kinetics.

**GM inhibits K$_V$ currents.** Potassium currents in hippocampal neurons are composed of a rapidly inactivating component and a noninactivating, or only slowly inactivating, component, which are named the transient outward potassium current ($I_{to}$) and delayed rectifier potassium current ($I_{kr}$), respectively. These two distinct components could be distinguished by applying voltage steps from a holding potential of $-50$ mV, at which $I_{to}$ was almost completely inactivated and $I_{kr}$ remained. $I_{to}$ could be isolated by subtracting $I_{kr}$ from the total outward current. According to these electrophysiological properties, the effects of GM on K$_V$ currents were examined (Fig. 4a), revealing IC$_{50}$ values of 1.6 (1.3-2.0) $\mu$mol/L and 50.0 (30.3-82.3) $\mu$mol/L for $I_{to}$ and $I_{kr}$, respectively (Fig. 4b, n = 5). The effects of GM on inwardly rectifying potassium (Kir) channels that lack voltage-sensing domains (VSD) were then investigated. In contrast to the potent inhibition of K$_V$ currents, we found that $10 \mu$mol/L GM did not inhibit human Kir2.1 channels heterologously expressed in HEK-293 cells (Fig. 4c, d). These data showed that GM is an inhibitor of K$_V$ channels.

Effects of GM on ligand-gated ion channels associated with epilepsy in hippocampal neurons
In addition to voltage-gated ion channels, some ligand-gated ion channels, such as ionotropic nACh, GABAergic, and glutamatergic...
respectively. Paired Student’s t test, *P < 0.05, ***P < 0.001 vs. control.

Antiepileptic activity of GM in MES and 6-Hz seizure models

The potent inhibitory effects of GM on channels involved in epilepsy and neuronal firing in hippocampal neurons propelled us to evaluate its antiepileptic activities in other epilepsy models. First, a primary antiepileptic assay was performed in MES-induced seizure model mice. The incidence of hind-limb extension was recorded. If extension did not occur during the assay, the animal was thought to be protected from MES-induced seizure [36]. In contrast to that in the vehicle group, tonic hind-limb extension decreased in mice orally administered GM. Protection against MES in mice was achieved at a rate of 0%, 10%, 50% and 80% when vehicle or 50, 80, and 100 mg/kg of GM was administered, respectively (Table 1, n = 10). Dose-dependent relationship analysis revealed that the ED50 of GM was 78.9 (60.3–103.3) mg/kg.

Differing from the MES model, which represents generalized tonic-clonic seizures, the 6-Hz test is commonly regarded as a model for treatment-resistant seizures. To explore whether GM has broad-spectrum antiepileptic activity, the antiepileptic activity of GM was then evaluated in the 6-Hz psychomotor seizure model. LEV, an inhibitor of SV2A and CαT channels, was used as a positive control in the experiment [37]. After the oral administration of 100 mg/kg GM 60 min prior to stimulation, 70% of the tested mice were protected from 6-Hz-induced seizures. The vehicle group exhibited seizures, whereas no seizures were observed in the mice administered 100 mg/kg LEV (Table 2). The protective effects of GM in the 6-Hz model were comparable to those seen in the MES model. GM not only dose-dependently relieves generalized tonic-clonic seizures in the MES model but also reduces treatment-resistant seizures in the 6-Hz model, suggesting that GM is a promising antiepileptic drug candidate.

DISCUSSION

The antiepileptic activity of GM has been previously observed in a glutamate-induced seizure mouse model [6]. Consistent with its antiepileptic activity, GM also exhibited neuroprotective effects against glutamate-induced cell death in multiple types of neurons [6–10]. Most recently, GM was found to reduce ROS generation in mitochondria and enhance neuron glycolysis in a glutamate-induced neuron toxicity model, suggesting an indirect neuroprotective mechanism [11]. In the present study, we found that GM inhibited voltage-gated channels (NaV, CaV, and K) and a ligand-gated channel (nACh) expressed in neurons, suggesting that the...
antiepileptic activity of GM may arise through its modulation of multiple neuronal channels.

GM dose-dependently inhibited NaV currents with an IC50 of 10.2 (9.3–11.2) µmol/L (Fig. 2c). During action potential firing, NaV channels are thought to mediate the explosive and regenerative inward current during the rising phase [33]. Consistent with its inhibition of NaV channels, the amplitudes of action potentials were indeed reduced after the administration of 10 µmol/L GM (Fig. 1f). A kinetics study showed that 10 µmol/L GM did not affect the voltage-dependent activation curve. In contrast, under identical experimental conditions, GM shifted the inactivation curve in the negative direction (the half-maximal inactivation changed from –54.4 mV in the control group to –61.4 mV after 10 µmol/L GM administration) (Fig. 2g), which is in line with the notion that increasing the proportion of inactivated NaV channels would reduce neuronal excitability [38]. Indeed, action potential firing in hippocampal neurons decreased in a dose-dependent manner after the administration of GM (Fig. 1a, b, g). Our study supported the idea that the inhibition of NaV channels in hippocampal neurons promotes GM-produced antiepileptic activity.

GM dose-dependently inhibited calcium currents with an IC50 of 13.3 (11.4–15.5) µmol/L (Fig. 3c). Consistent with our finding in neurons, 10 µmol/L GM has been shown to be able to relax the calcium channel agonist Bay K8644- or Ca2+–induced contractions in isolated rat aorta strips [39], suggesting an inhibitory effect of GM on CaV channels expressed in vascular tissues. The inhibitory effect of GM on the LVA calcium current was stronger than that on the HVA current. After the administration of 10 µmol/L GM, the reduction in LVA currents was 48.4% ± 6.9%, whereas the reduction in HVA was only 28.5% ± 10.9% (Fig. 3d). Considering the key roles of LVA during the generation of neuronal burst firing, a much more potent inhibition of LVA currents might be beneficial to ameliorate hyperexcitability disorders, including epilepsy [40]. Therefore, it is reasonable to speculate that the inhibitory effects of GM on CaV currents, particularly on LVA currents, contribute to the inhibition of firing activity in hippocampal neurons and the antiepileptic activity of GM in vivo.

In addition to inhibiting NaV and CaV currents, GM was found to inhibit neuronal Kv currents \( (I_K, I_A) \). The IC50 values for \( I_K \) and \( I_A \) channels are thought to mediate the explosive and regenerative inward current during the rising phase [33]. Consistent with its inhibition of NaV channels, the amplitudes of action potentials were indeed reduced after the administration of 10 µmol/L GM (Fig. 1f). A kinetics study showed that 10 µmol/L GM did not affect the voltage-dependent activation curve. In contrast, under identical experimental conditions, GM shifted the inactivation curve in the negative direction (the half-maximal inactivation changed from –54.4 mV in the control group to –61.4 mV after 10 µmol/L GM administration) (Fig. 2g), which is in line with the notion that increasing the proportion of inactivated NaV channels would reduce neuronal excitability [38]. Indeed, action potential firing in hippocampal neurons decreased in a dose-dependent manner after the administration of GM (Fig. 1a, b, g). Our study supported the idea that the inhibition of NaV channels in hippocampal neurons promotes GM-produced antiepileptic activity.

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**Table 1.** Antiepileptic activities of GM in the MES-induced epilepsy mouse model (n = 10).

| Dosage (mg/kg) | Mortality | Tonic hind-limb extension | Protection rate (%) |
|---------------|-----------|---------------------------|---------------------|
| Vehicle –     | 0/10      | 10/10                     | 0                   |
| GM 50         | 0/10      | 9/10                      | 10                  |
| 80            | 0/10      | 5/10                      | 50                  |
| 100           | 0/10      | 2/10                      | 80                  |
| TPM 40        | 0/10      | 0/10                      | 100                 |

Vehicle was 5% DMSO + 95% Tween-80; TPM (Topiramate) was used as a positive control.

**Table 2.** Antiepileptic activity of GM in the 6-Hz corneal kindling epilepsy mouse model (n = 10).

| Dosage (mg/kg) | Mortality | Tonic hind-limb extension | Protection rate (%) |
|---------------|-----------|---------------------------|---------------------|
| Vehicle –     | 0/10      | 10/10                     | 0                   |
| GM 100        | 0/10      | 3/10                      | 70                  |
| LEV 100       | 0/10      | 0/10                      | 100                 |

Vehicle were 5% DMSO + 95% Tween-80; LEV (Levetiracetam) was used as a positive control.

Fig. 5 Effects of GM on ligand-gated receptors associated with epilepsy in rat hippocampal neurons. a Representative acetylcholine-evoked current traces recorded from cultured rat hippocampal neurons without or with GM at indicated concentrations. b Dose-response curve of GM on nACh receptors. The IC50 value was 1.3 (1.1–1.6) µmol/L (n = 4). c–f Representative traces of GABA (c), AMPA (d), NMDA (e) and KA (f) were shown. GM did not affect glutamate and GABA receptors in hippocampal neurons. The rat hippocampal neurons were clamped at –60 mV and the currents were induced by 2 s exposure to specific ligands at the indicated concentrations (n = 4). Paired Student’s t test, **P < 0.01, ***P < 0.001 vs. control.
were 1.6 (1.3–2.0) μmol/L and 50.0 (30.3–82.3) μmol/L, respectively (Fig. 4b). During action potential firing, the outward potassium currents mediated by Kv channels terminate the action potential by repolarizing the membrane potential [33]. Therefore, the inhibition of Kv channels may prolong the duration of an action potential. Consistently, the duration of action potentials recorded in hippocampal neurons was indeed significantly longer after perfusion with 10 μmol/L GM (Fig. 1c, d, g). In addition, the inhibition of Kv initially increases neuron firing frequency [41, 42]. However, we did not observe an increase in the firing frequency after GM administration. We already know that in addition to inhibiting Kv, GM shows strong inhibitory activity on NaV and CaV channels (Figs. 2 and 3), resulting in the suppression of action potential firing. Mutations in human Kv genes, such as Kv7.2 and Kv7.3 mutations, result in a reduction in or the loss of channel activity cause human epilepsy [34]. Activators capable of augmenting these Kv functions have been indicated to be effective in the treatment of human epilepsy [36, 43]. However, the inhibition of Kv channels, particularly neuronal Is, has also been thought to contribute to the antiepileptic effects of AEDs. For example, LEV, a first-line AED, reduced the Is but not the Is in hippocampal neurons of rats and guinea pigs, resulting in a prolonged single action potential duration and the reduced generation of the repeated action potentials, which is thought to be an important mechanism underlying the antiepileptic effects of LEV [44]. Interestingly, similar to LEV, GM also preferentially inhibited Is rather than Is. However, whether and how the GM inhibition of Kv channels indeed contributes to its antiepileptic effects requires further investigation. It is worth noting that GM did not affect Kir2.1 channels (Fig. 4c, d), another major type of potassium channel expressed in neurons. Unlike Is and Is channels, both of which contain four VSD surrounding a central pore domain, Kir2.1 channels lack a VSD [45], highlighting the importance of the VSD in the effects of GM on Kv channels.

In terms of ligand-gated ion channels associated with epilepsy, GM potently inhibited ACh-induced currents, with an IC50 of 1.3 (1.1–1.6) μmol/L, but did not affect GABA, AMPA, NMDA, or KA-induced currents (Fig. 5b–f), which distinguishes GM from AEDs targeting either GABA or glutamate receptors, such as diazepam, phenobarbital, and perampanel [19]. nACh receptors, which are broadly expressed in the neuron soma, dendrites, preterminal axon regions, and axon terminals, play an important role in modulating neuron excitability and neurotransmission [25, 46]. It has been reported that nACh receptors can modulate the release of neurotransmitters such as glutamate, GABA, dopamine, and serotonin [46–48]. The reduction in neuron excitability and neurotransmission through the inhibition of nACh receptors is thought to be beneficial in the treatment of epilepsy [19]. Numerous preclinical studies have revealed that antagonists of nACh receptors can relieve seizures in various models [28]. At relatively low concentrations, some AEDs, such as carbamazepine, oxcarbazepine and lamotrigine, inhibit nACh receptors [28]. Among these AEDs, carbamazepine has been used to treat human ADNFLE, a genetic form of epilepsy caused by mutations in nACh genes (Tables 1 and 2). Rhynchophylline, another major component isolated from UR, also exhibited antiepileptic activity. However, in contrast to GM, rhynchophylline showed inhibitory effects on NMDA-induced currents [49]. Depressed locomotor activity is a potential side effect of UR extractions, such as isorhynchophylline and perhaps rhynchophylline [50]. However, no significant decrease in locomotor activity was observed after the administration of GM at concentrations as high as 60 mg/kg, suggesting the high tolerance of GM. Finally, it is interesting to note that of the two active antiepileptic components of UR, the blood–brain barrier permeability rate of GM is nearly five fold higher than that of rhynchophylline [51]. Therefore, further structure-activity relationship studies of GM-based derivatives might be considered as an appropriate approach for novel antiepileptic drug development.

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