Inhibition of Na\textsubscript{v}1.7 channels by methyl eugenol as a mechanism underlying its antinociceptive and anesthetic actions

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Aim: Methyl eugenol is a major active component extracted from the Chinese herb Asari Radix et Rhizoma, which has been used to treat toothache and other pain. Previous \textit{in vivo} studies have shown that methyl eugenol has anesthetic and antinociceptive effects. The aim of this study was to determine the possible mechanism underlying its effect on nervous system disorders.

Methods: The direct interaction of methyl eugenol with Na\textsuperscript{+} channels was explored and characterized using electrophysiological recordings from Na\textsubscript{v}1.7-transfected CHO cells.

Results: In whole-cell patch clamp mode, methyl eugenol tonically inhibited peripheral nerve Na\textsubscript{v}1.7 currents in a concentration- and voltage-dependent manner, with an IC\textsubscript{50} of 295 µmol/L at a -100 mV holding potential. Functionally, methyl eugenol preferentially bound to Na\textsubscript{v}1.7 channels in the inactivated and/or open state, with weaker binding to channels in the resting state. Thus, in the presence of methyl eugenol, Na\textsubscript{v}1.7 channels exhibited reduced availability for activation in a steady-state inactivation protocol, strong use-dependent inhibition, enhanced binding kinetics, and slow recovery from inactivation compared to untreated channels. An estimation of the affinity of methyl eugenol for the resting and inactivated states of the channel also demonstrated that methyl eugenol preferentially binds to inactivated channels, with a 6.4 times greater affinity compared to channels in the resting state. The failure of inactivated channels to completely recover to control levels at higher concentrations of methyl eugenol implies that the drug may drive more drug-bound, fast-inactivated channels into drug-bound, slow-inactivated channels.

Conclusion: Methyl eugenol is a potential candidate as an effective local anesthetic and analgesic. The antinociceptive and anesthetic effects of methyl eugenol result from the inhibitory action of methyl eugenol on peripheral Na\textsuperscript{+} channels.

Keywords: sodium channel blocker; methyl eugenol; peripheral Na\textsubscript{v}1.7 channels; local anesthetics; analgesic; asarum; Xixin

Introduction

Xixin (Asari Radix et Rhizoma) is a traditional herbal medicine that originates in the roots and rhizomes of \textit{Asarum heterotropoides} Fr Schmidt var \textit{manshuricum} (Maxim) Kitag, \textit{A sieboldii} Miq var \textit{seoulense} Nakai and \textit{A sieboldii} Miq\textsuperscript{[1]}. It has been widely used as a local anesthetic and a remedy for toothache, headache, and inflammatory diseases in China, Japan, and Korea\textsuperscript{[2]}. Xixin, applied by chewing its roots, is well known in China for its control of toothache.

Methyl eugenol (4-allyl-1,2-dimethoxybenzene) is a major active component isolated from Xixin and other plants\textsuperscript{[3, 4]}. Many biological actions of methyl eugenol have been reported. In addition to its actions as a fly attractant, it is also known for its anesthetic, antinociceptive, anti-epileptic properties, hypothermic and myorelaxant properties\textsuperscript{[5–9]}. The anesthetic property of methyl eugenol has been demonstrated by a loss of the righting reflex and the decreased sensitivity to a tail pinch in rats and mice, and a loss of the corneal reflex in rabbits\textsuperscript{[5, 7, 10]}. Methyl eugenol and methyl eugenol-enriched plant essential oil also display anti-epileptic action by inhibiting electroshock- or pentylenetetrazol-induced convulsions\textsuperscript{[6, 9, 11]}. More recently, antinociceptive effects of methyl eugenol have been shown through its relief of the secondary phase of formalin-induced pain in mice\textsuperscript{[12]}. Methyl eugenol displays antinociceptive and anesthetic effects similar to Xixin, implying that methyl eugenol is the main antinociceptive component of Xixin. Although
the antinociceptive and anesthetic effects of methyl eugenol and Xixin have been reported, the pharmacological basis underlying these actions has not been elucidated.

Na⁺ channels are a common target for anesthetics and analgesics[13]. Local anesthetics and analgesics prevent the transmission of nerve impulses via their binding to Na⁺ channels[14–16]. Anticonvulsants, such as carbamazepine (Tegretol, CBZ), are also used to treat neuropathic pain due to their interaction with Na⁺ channels[27]. Two main types of Na⁺ currents, termed tetrodotoxin (TTX)-sensitive and TTX-resistant, have been identified in the dorsal root ganglion (DRG)[18]. The TTX-sensitive Na⁺ channel isoform Na⁺,1.7 is highly expressed in the majority of small DRG neurons[19]. Studies have demonstrated a greater involvement of Na⁺,1.7 in inflammatory pain[20] and in mediating pain signaling[21]. TTX-resistant Na⁺ channels diminish following axotomy and are replaced by an abnormally high density of TTX-sensitive Na⁺ channels, illustrating that TTX-sensitive Na⁺ channels play a role in pathological pain development[22]. The involvement of Na⁺,1.7 in propagating pain information has been strongly indicated by the fact that a Na⁺,1.7 congenital channelopathy results in the inability to experience pain[23–25]. Recently, a study demonstrated an increase in Na⁺,1.7 expression in painful human dental pulp[26]. Based on the antinociceptive and anesthetic actions of methyl eugenol in vivo and of Xixin in dental clinics, the peripheral TTX-sensitive channel isoform, Na⁺,1.7, represents a good candidate for the channel involved in the effects of methyl eugenol.

Here, we characterize the inhibitory effect of methyl eugenol on Na⁺ channels using the whole-cell patch clamp technique in transfected cells that transiently express the human peripheral Na⁺,1.7 channel isoform.

**Materials and methods**

**Cell culture and transient transfections**

The cDNA clone of pNaEx8 plasmid encoding the Na⁺,1.7 α subunit[27] (gift of Dr Franz HOFMANN, Institut für Pharma-

Kologie und Toxikologie der Technischen Universität München, Germany) was transiently expressed in Chinese hamster ovary (CHO) cells. The cells were maintained in Ham’s F12 Media (Sigma, St Louis, MO, USA) fortified with 10% fetal bovine serum (Gibco, Grant Island, NY, USA), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco, Grant Island, NY, USA) and kept in a humidified incubator at 37°C and 5% CO₂.

Transient transfection was performed as follows: CHO cells were plated at 2.5×10⁵ cells/35-mm dish for 24 h prior to the transfection. The cells were exposed to 1 mL Opti-MEM (Gibco, Grant Island, NY, USA) media containing 0.8 µg of the α subunit plasmid DNA, 0.2 µg of a GFP indicator plasmid, and 6 µL of Lipofectamine reagent (Gibco, Grant Island, NY, USA) that was pre-incubated for 30 min. After incubation for 4 h, the cells were washed 3 times with control media and grown for 24 h under standard culturing conditions. The transfected cells were then plated on glass coverslips for the following day’s experiments. Only cells expressing GFP were selected for electrophysiological recordings.

**Whole-cell patch-clamp recordings**

Na⁺ currents were recorded using the whole-cell patch clamp recording technique. Cells were cultured on coverslips and then transferred to a handmade recording chamber and continuously perfused at room temperature with extracellular solution containing (in mmol/L): 130 NaCl, 4 KCl, 1.5 CaCl₂, 1.5 MgCl₂, 5 glucose, 5 HEPES, 20 sucrose, pH 7.4 adjusted with NaOH. The recording chamber volume was approximately 0.4 mL, and the flow rate was 0.6 mL/min. An MP-285 micromanipulator (Sutter Instrument Co, Novato, CA, USA) was used to place the electrode onto the cell. Patch pipettes were pulled from borosilicate glass capillaries (Drummond Scientific Co, Broomall, PA, USA) on an electrode puller (Model P-97, Sutter Instrument Co) and were filled with a 0.2 µmol/L filtered internal solution containing (in mmol/L): 90 CsF, 60 CsCl, 10 NaCl, 5 HEPES, pH 7.4 adjusted with NaOH. The pipettes had an input resistance of 0.8–1.4 MΩ. Recordings were performed at room temperature (22°C) using a patch clamp EPC 9 (HEKA Elektronik GmbH, Germany) and were filtered at 5 kHz. Leakage currents were subtracted using a P/4 or P/2 protocol. Pulse (HEKA elektronik GmbH, Germany) was used for the experimental control and basic data analysis. The methods of Na⁺ current recorded from Na⁺,1.7 expressing cells are similar to our previously published methods[28]. Cells with a series resistance of less than 2.5 MΩ were used for the drug test experiments, and only cells with a whole-cell maximal Na⁺ current of at least 1 nA were used in the analysis. To test for tonic inhibition evoked by the drug, the Na⁺ currents were recorded at 8 min after switching the perfusion solution to a solution containing methyl eugenol.

**Drug application**

Methyl eugenol was supplied by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Stock solutions of 500 mmol/L methyl eugenol were prepared in dimethyl sulfoxide (DMSO) and then diluted to the desired concentration for the experiments in the perfusion buffer. For all experiments, drugs were applied via perfusion. Control recordings showed that 0.2% DMSO had no detectable effect on the Na⁺ current in cells expressing Na⁺,1.7. The concentration of methyl eugenol used for most of the experiments was less than 625 μmol/L, which resulted in the perfusion buffer containing 0.125% DMSO.

**Statistical analysis**

The data were analyzed using a combination of PulseFit (HEKA Elektronik GmbH, Germany) and SigmaPlot 9.0 (Jandel Scientific, Corte Madera, CA, USA) software. All results are presented as the mean±SEM.

**Results**

In most culture preparations, approximately 30% of the GFP expressing cells displayed a fast, transient inward current following depolarization. The maximal peak inward current of Na⁺,1.7 expressing cells ranged from 0.8 to 7 nA; however, in a few cells, it was up to 12 nA. These currents were completely...
blocked by 0.5 µmol/L tetrodotoxin, confirming their identity as uncontaminated tetrodotoxin-sensitive Na⁺ currents under our recording conditions.

**Methyl eugenol tonically inhibits Naᵥ1.7 Na⁺ currents**
The application of methyl eugenol reversibly inhibited the peak amplitude of the Na⁺ current (Figure 1). The current traces of Naᵥ1.7 channels recorded at a variety of methyl eugenol concentrations are superimposed at a holding potential of -120 mV and -60 mV, respectively; the results indicate a tonic inhibition of Naᵥ1.7 channels by methyl eugenol in a concentration- and voltage-dependent manner (Figure 1A, IB). Most of the channels are in the resting state at a holding potential of -120 mV and are inactivated at a holding potential of -60 mV. These characteristics are observed by measuring the inactivation curve of Naᵥ1.7 (Figure 2A).

Figure 1C shows the current-voltage (I–V) relationship for Naᵥ1.7 in control cells and in those exposed to various concentrations of methyl eugenol. The shape of the I–V curve was unaffected by concentrations of methyl eugenol up to 625 µmol/L. This result suggests that at the concentrations tested, methyl eugenol had no effect on the voltage-dependence of Na⁺ channel activation. To further examine the tonic inhibition by methyl eugenol, we determined the concentration-response relationship at a holding potential of -100 mV, where most channels are in a resting state under control conditions.

Clinically, the control of toothache using the herbal medicine Asarum Xixin is usually mediated through chewing the herb or via topical administration, which allows for the usage of a higher concentration of the drug (in its alcohol extracted form). To link the above tested methyl eugenol concentrations with those showing therapeutic effects in the clinic, methyl eugenol concentrations up to 1.2 mmol/L were used to determine the concentration-response relationship. The average tonic inhibition by methyl eugenol at varying concentrations fit well to the Hill equation (Figure 1D). The fitted Hill coefficient (n) value was calculated to be 1.27, and it appeared that the stoichiometry of the drug and receptor interaction was 1:1. The IC₅₀ value was obtained by fitting the data to the Hill equation at -100 mV and estimated to be 295 µmol/L.

Figure 1. Tonic inhibition by methyl eugenol of Naᵥ1.7 channels. (A, B) Concentration-dependent tonic inhibition by methyl eugenol of Naᵥ1.7 channels in the resting state (holding potential at -120 mV) and inactivated state (holding potential at -60 mV). The current traces of Naᵥ1.7 channels recorded in control conditions and in the presence of varying concentrations of methyl eugenol are superimposed for the resting and inactivated states, respectively. The data are from the same representative cell. The currents were elicited using a 30-ms pulse to 0 mV from a holding potential of -120 mV or -60 mV. (C) Current-voltage relationships in the absence or presence of various concentrations of methyl eugenol were determined by stepping to various depolarized potentials (ranging from -80 to +100 mV in 10 mV increments) for 9 ms from a holding potential of -100 mV. (D) Concentration-response curve for the inhibition of Na⁺ current by methyl eugenol. The cells were held at -100 mV and stepped to 0 mV for 10 ms. The peak current in the presence of methyl eugenol were normalized to the control peak current, and then averaged. Each point was the mean±SEM of 4–8 cells. The lines represent the best fit for the data to the equation: \[ y = 1 - x^n / (K_d^n + x^n) \], where y is the fractional current, \( K_d \) is the apparent dissociation constant for methyl eugenol, and n is the Hill coefficient. \( K_d \) and n were estimated using a Marquadt nonlinear least-squares procedure.
Methyl eugenol shifts the steady-state inactivation curve

In previous studies using Na⁺ channel blockers such as phe

nytoin and carbamazepine, these drugs affected the voltage-
dependent availability of Na⁺ channels, as seen by a shift in the steady-state inactivation curve to more negative potentials.[29, 30] The larger tonic inhibition observed at a holding potential of -60 mV compared to -120 mV (Figure 1A, 1B) suggests that methyl eugenol preferentially interacts with channels in the inactivated state. Methyl eugenol shifted the inactivation curve toward more negative potentials (Figure 2A). The shift evoked by methyl eugenol was concentration-dependent. The current for each cell was normalized and averaged, then fit to a single Boltzmann relationship from which the mean $V_h$ and $k$ values were calculated. The slope factor ($k$) of the curve was not affected at the concentrations of methyl eugenol that we tested (12.5–625 µmol/L). Figure 2B shows the overall shift of the midpoint of the inactivation curve for Na,1.7 induced by different concentrations of methyl eugenol.

Estimation of methyl eugenol affinity for the resting versus inactivated state of Na⁺ channels

The above results indicate that methyl eugenol preferentially binds to Na,1.7 channels when they are in an inactivated state (Figure 2). We also observed a concentration-dependent tonic inhibition of methyl eugenol at negative holding potentials (Figure 1A, 1B), suggesting that methyl eugenol also interacts with channels in their resting state. To further analyze the effects of methyl eugenol on Na,1.7, we estimated the affinity of methyl eugenol for the resting and inactivated states of the channel using curve shift analysis ($\Delta V$) as described by Kuo and Bean.[31] The data in Figure 2B were transformed to $\text{Exp}(\Delta V/k)$, where $k$ is the Boltzmann inactivation curve slope factor obtained from the experiments on the shift of the inactivation curves, and then plotted against the methyl eugenol concentration (Figure 2C). The slope factor, $k$, of the curves was not affected by methyl eugenol under our experimental conditions and concentrations tested (Figure 2A, 2B). By fitting the data, the values of $K_R$ and $K_I$, where $K_R$ and $K_I$ are the equilibrium dissociation constants for the resting and inactivated states, respectively, were determined. The affinity of methyl eugenol for the resting and inactivated states is expressed as $1/K_R$ and $1/K_I$. The calculated values of $K_R$ and $K_I$ were 525 µmol/L and 82.0 µmol/L for the resting and inactivated states of Na,1.7 (Figure 2C), respectively. These values support our hypothesis that methyl eugenol binds preferentially to channels in the inactivated state.

Methyl eugenol evokes large use-dependent inhibition

Methyl eugenol has been shown to display anesthetic, anti-
epileptic and antinociceptive effects in vivo.[5, 7, 10, 12] It is well known that use-dependent inhibition plays an important role in anti-neuropathic pain and anti-convulsion.[28]

Different stimulus frequencies (2, 5, and 10 Hz with a 5-ms test duration) were used to test the use-dependence of methyl eugenol.

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**Figure 2.** The effects of methyl eugenol on the voltage-dependent inactivation of Na,1.7 channels and the estimation of methyl eugenol affinities. (A) The shift in the inactivation curve of Na,1.7 by 125 µmol/L methyl eugenol ($n=5$) is shown. The voltage dependence of steady-state inactivation ($h_s$) was examined by applying 500-ms prepulse potentials from -140 mV to -10 mV in 10-mV increments from a holding potential of -100 mV before stepping to the test potential (0 mV) for 35 ms. The peak current ($I$) for each cell was normalized with respect to the first value measured at the test potential (0 mV). The line through the data conforms to the equation: $y=1-1/[1-\text{exp}[(V-V_h)/k]]$, where $V$ is the membrane potential, $V_h$ is the prepulse potential where the current is half-maximal, and $k$ is the slope factor. (B) Concentration-dependent shift of the midpoint of inactivation ($\Delta V$) caused by 12.5, 62.5, 125, 250, and 625 µmol/L methyl eugenol for Na,1.7 ($n=4-9$). The line fits to a monoeponential equation. The mean $V_h$ at different concentrations of methyl eugenol was obtained by fitting a Boltzmann relationship as described above. (C) The data were fitted by: $\text{Exp}(\Delta V/k)=[1+(D/K)]/[1+(D/K_R)]$, where $\Delta V$ and $k$ are the shift in the midpoint of the inactivation curve and the slope factor, $D$ is the concentration of methyl eugenol, and $K_R$ and $K_I$ are the dissociation constants for the inactivated and resting states.
eugenol (Figure 3). At a lower pulse frequency (2 Hz), trains of stimulus pulses to 0 mV induced a modest use-dependent block in the presence of methyl eugenol that showed a gradual increase in the magnitude of the block (Figure 3A). At either 5 Hz or 10 Hz, methyl eugenol evoked a larger use-dependent inhibition with fast kinetics of the inhibition of Na⁺ currents (Figure 3B, 3C). The steady-state level of the use-dependent inhibition was reached after only a few test pulses (Figure 3B, 3C), suggesting that methyl eugenol may preferentially bind to channels in the open and/or inactivated states.

To more directly compare the potency of the methyl eugenol-mediated use-dependent inhibition at different stimulus frequencies, the difference between the normalized current in the absence and presence of methyl eugenol was calculated for each stimulus condition. Figure 3D shows the different potencies of the use-dependent inhibition and the kinetics of the block evoked by methyl eugenol (125 µmol/L and 500 µmol/L) on Naᵥ1.7 channels. Methyl eugenol displayed greater frequency-dependent inhibition at a higher stimulus frequency. At the 15th pulse at 10 Hz, 125 µmol/L methyl eugenol displayed two times more inhibition than at 5 Hz, and 5.4 times more than at 2 Hz. Similarly, 500 µmol/L methyl eugenol evoked 8.5 times greater inhibition at the 15th pulse at 10 Hz compared to 2 Hz. Methyl eugenol also changed the kinetic behavior of the use-dependent inhibition, as observed in Figure 3D. Methyl eugenol induced faster kinetics of inhibition at higher stimulus frequencies and with higher concentrations of the drug.

Binding kinetics of drug-channel interactions: methyl eugenol appears to bind preferentially to the fast-inactivated state of Naᵥ1.7

Figure 4 shows the effect of methyl eugenol binding on the development of the inactivation of Naᵥ1.7 channels. The holding potential was -100 mV. In the control condition, the development of channel inactivation was seen by a rapid decay of the peak current that was evoked by increasing the prepulse (0 mV) duration (Figure 4A). With 250 µmol/L methyl eugenol, Naᵥ1.7 currents decayed much faster and more prominently than in control conditions, presumably due to the binding of the drug to the inactivated channels (Figure 4A). The fraction of inactivated current, which developed as the duration of the prepulse increased, was fitted to a double exponential function. The Na⁺ channel inactivation evoked by a depolarization

![Figure 3](image3.png)

**Figure 3.** Use-dependent inhibition of Naᵥ1.7 by methyl eugenol. Cells were held at -100 mV and stimulated with a train of 5-ms pulses to 0 mV at a frequency of 2 Hz (A), 5 Hz (B), and 10 Hz (C) in the absence or presence of methyl eugenol. For each experiment, the current amplitudes were normalized with respect to the current evoked by the first pulse in the train \([I_{\text{pulse}}/I_{\text{pulse-1}}]\). For all 3 stimulus frequencies (2, 5, and 10 Hz), n=8 (control), 8 (125 µmol/L) and 4 (500 µmol/L). (D) The differences between the normalized currents in methyl eugenol and control at 2, 5, and 10 Hz were calculated. The data are from (A, B, and C).
of short duration (i.e., millisecond) is thought to represent classic “fast” inactivation..

Therefore, under the present experimental conditions, the enhanced decay of the Na+ current by methyl eugenol suggests that methyl eugenol may preferentially bind to a fast-inactivated state of the Na\textsubscript{v}1.7 channel. The contamination resulting from the normally inactivated channels under the experimental conditions was corrected by calculating the difference between the normalized current in the presence of methyl eugenol and the normalized current in the control conditions. Methyl eugenol evoked the largest current decay within a 10 ms prepulse duration, after which the decay tended to be relatively stable (Figure 4B). The decay of current due to methyl eugenol was concentration-dependent (see the insert in Figure 4B). The binding of methyl eugenol to Na\textsubscript{v}1.7 within the first 10 ms of the prepulse is consistent with the hypothesis that the binding of methyl eugenol exhibits rapid kinetics to induce use-dependent inhibition (Figure 3B, 3C).

**Effects of methyl eugenol on the recovery from inactivation**

The recovery of inactivated Na\textsubscript{v}1.7 channels in the presence of methyl eugenol was tested using a two-pulse protocol from a -100 mV holding potential. An inactivating, conditioning prepulse duration of 100-ms was utilized to allow for a complete fast inactivation without inducing slow inactivation and to allow for the complete binding of the drug to the inactivated channels. Figure 5A demonstrates that methyl eugenol slowed the rate of recovery from inactivation for Na\textsubscript{v}1.7 at increasing concentrations. The higher the concentration of methyl eugenol, the longer the recovery period to control levels. Within a 100-ms recovery period, the methyl eugenol effect was completely reversed at 25–125 µmol/L and was approximately 97.5% reversed at 250 µmol/L. However, at 500 µmol/L methyl eugenol, the effect was only reversed to 80.3%. Recovery curves were further tested using longer recovery periods up to 5 (Figure 5B). Interestingly, cells perfused with 500 µmol/L methyl eugenol failed to completely recover to control levels even after a 5 s recovery period (~86.7% recovery). To more directly compare the recovery rate of Na\textsubscript{v}1.7, the difference between the normalized currents in the presence and absence of methyl eugenol was calculated (Table 1). The maximal value of the methyl eugenol-evoked slowing of recovery involving fast-inactivated channels occurred when the interpulse interval was 15- to 30-ms (Figure 5C). The remainder of the methyl eugenol-bound inactivated channels did recover to control levels, albeit at a slower rate. The higher the concentration of methyl eugenol, the slower the second phase of the recovery period (Table 1).

**Discussion**

To clarify the mechanisms underlying the in vivo anesthetic and anti-nociceptive effects of methyl eugenol and the herbal
Methyl eugenol is a chemical derivative of eugenol. Cloves are used in Chinese medicine, and clove oil has been used for centuries in dentistry as an effective analgesic for dental emergencies\[^{33}\]. Eugenol and clove oil also have anesthetic and analgesic effects in rodents\[^{18,34}\]. Recently, the cellular mechanisms underlying eugenol’s analgesic action have been proposed, including the inhibition of Ca\(^{2+}\), Na\(^{+}\), and K\(^{+}\) currents and the potentiation of GABA\(_{A}\) receptors\[^{35–38}\]. Although methyl eugenol and eugenol are chemically similar compounds, they can exhibit different pharmacological effects, e.g., methyl eugenol has been shown to have hypothermic and muscle relaxant actions, while eugenol has not\[^{36}\]. However, both compounds have analgesic effects in vivo\[^{39}\]. By characterizing methyl eugenol’s action on the Na\(_{v}\)1.7 channel, we found that methyl eugenol displayed distinctly different characteristics compared to prior results obtained with eugenol. Cho et al.\[^{37}\] reported that eugenol reduced the maximal Na\(^{+}\) current with no frequency or use-dependent inhibition in rat dorsal root ganglia neurons. In contrast, methyl eugenol displayed greater use-dependent inhibition (Figure 3) as well as a slower rate of recovery for the inactivated channels (Figure 5). By comparing their effects on Na\(^{+}\) channels, we propose that methyl eugenol would be a good candidate to replace eugenol as an analgesic in dentistry.

Numerous studies have established that various drugs inhibit Na\(^{+}\) channels in different ways through the selective interaction with distinct channel structures or functional states\[^{39,39}\]. Our results indicate that methyl eugenol has a higher affinity for inactivated and/or open channels, with weaker binding to channels in the resting state. Through its rapid binding to open channels and stabilization of a non-conducting state, methyl eugenol can produce greater and faster use-dependent inhibition of Na\(^{+}\) current and slow the kinetics of recovery of channels in the inactivated state\[^{40}\].

Methyl eugenol rapidly reaches a steady-state level in a use-dependent manner (Figure 3B, 3C), similar to the use-dependency evoked by lidocaine on Nav1.4\[^{41,42}\], Nav1.7\[^{43,44}\],

medicine Xixin (Asari Radix et Rhizoma), we determined the effects of methyl eugenol on the Na\(^{+}\) channel isoform, Na\(_{v}\)1.7, using the technique of whole-cell patch clamp recording. Methyl eugenol reversibly inhibited Na\(_{v}\)1.7 current in a concentration- and voltage-dependent manner. Methyl eugenol shifted the steady state inactivation curve of Na\(_{v}\)1.7 to more negative potentials, and in the presence of methyl eugenol, a train of stimuli induced a greater use-dependent current reduction. The binding kinetics of methyl eugenol indicated that methyl eugenol preferentially binds to Na\(_{v}\)1.7 channels in the fast-inactivated state. Methyl eugenol also slowed the rate of recovery from inactivation of Na\(_{v}\)1.7 channels. An estimation of the affinity of methyl eugenol for the resting and inactivated states of Na\(_{v}\)1.7 also confirmed that methyl eugenol preferentially binds to channels in the inactivated state. These characteristics suggest that methyl eugenol may effectively limit the sustained firing or transient high-frequency bursts of firing that occur in peripheral nerves under conditions of neuropathic pain.

Figure 5. The effects of various concentrations of methyl eugenol on the recovery from inactivation of Na\(^{+}\) channels. The recovery from inactivation was measured with a two-pulse protocol that consisted of a 100-ms conditioning pulse to 0 mV from a -100 mV holding potential, followed by an interpulse interval of varying duration at a -100 mV holding potential, then a test pulse to 0 mV for 10 ms. The amplitude of the current elicited by the test pulses was normalized with respect to the current elicited by the conditioning pulses in each series and were plotted as a function of the recovery interval. (A) Methyl eugenol slowed the recovery rate from inactivation. An interpulse interval of 2.5–100 ms was used to show more clearly the initial slowing effect. The data fit to a double exponential function according to the equation \(y = 1 - A \exp(-t/\tau_1) - B \exp(-t/\tau_2)\), where \(y\) is the normalized current, \(A\) and \(B\) are the amplitudes of the corresponding components, \(t\) is the interpulse interval, and \(\tau_1\) and \(\tau_2\) are time constants for recovery. (B) Methyl eugenol slowed the rate of recovery from inactivation with an interpulse interval of varying duration (2.5–5000 ms), while a higher concentration of methyl eugenol (500 \(\mu\)mol/L) produced an incomplete recovery of inactivated Na\(_{v}\)1.7 channels. (C) The differences between the normalized currents in the presence of various concentrations of methyl eugenol and the corresponding normalized control currents are shown. Data are from (A).
Methyl eugenol induced a much slower recovery from channel inactivation, which implies that high concentrations of methyl eugenol may effectively limit or paralyze neuronal activity to exert a local anesthetic effect. In the dental clinic, the topical application or subcutaneous administration of methyl eugenol provides a means to produce a high concentration of the drug at a localized site for a certain period. Furthermore, a recent study has demonstrated an increased Na,1.7 expression in painful human dental pulp[29]. Therefore, given the inhibition of recovery by methyl eugenol, we propose that methyl eugenol could be a more effective analgesic for the treatment of toothache than clove oil. Additionally, understanding the differences in the patterns of Na⁺ channel inhibition by the chemical modification of eugenol to methyl eugenol should provide insights into the development of more potent compounds for clinical use.

In conclusion, the present results suggest that methyl eugenol inhibits peripheral nerve Na,1.7 channels with a higher affinity for inactivated and/or open channels and a weaker affinity to those in the resting state. Higher concentrations of the drug could drive more drug-bound, fast-inactivated channels into drug-bound, slow-inactivated channels that greatly delay the recovery to control levels of channel function. Our results provide a possible mechanism of the antinociceptive and anesthetic actions of methyl eugenol and the herbal medicine Xixin through the inhibition of peripheral Na⁺ channels.

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Author contribution
Ze-Jun WANG proposed the study concepts, designed and performed the experiments and data analysis; Ze-Jun WANG, Boris TABAKOFF and Thomas HEINBOCKEL prepared the manuscript; Simon R LEVINSON designed the experiments and contributed chemical reagents and recording equipment.

Abbreviations
Methyl eugenol, 4-allyl-1,2-dimethoxybenzene; DMSO, dimethylsulfoxide; CHO, Chinese hamster ovary; DRG, dorsal root ganglia; CNS, central nervous system; TTX, tetrodotoxin.

References
1 Chinese Pharmacopoeia Committee. Pharmacopoeia of the People’s Republic of China. Vol I. Beijing: Chemical Industry Press; 2005. p 159.
2 Li YL, Tian M, Yu J, Shang MY, Cai SQ. Studies on morphology and aristolochic acid analogue constituents of Asarum campaniflorum and a comparison with two official species of Asari Radix et Rhizoma. J Nat Med 2010; 64: 442–51.
3 Wang D, Wang X, Xia X. Analysis of season variation of methyleugenol and safrole in Asarum heterotropoides by gas chromatography. Se Pu 1997; 15: 85–6.
4 De Vincenzi M, Silano M, Stacchinetti P, Scazzochio B. Constituents of aromatic plants: I. Methyleneugenol. Fitoterapia 2000; 71: 216–21.
5 Cardini EA, Dallmeier K, Zeiger JL. Methyleneugenol as a surgical anesthetic in rodents. Experientia 1981; 37: 588–9.
6 Dallmeier K, Cardini EA. Anesthetic, hypothermic, myorelaxant and anticonvulsant effects of synthetic eugenol derivatives and natural analogues. Pharmacology 1981; 22: 113–27.
7 Sousa MB, Ximenes MF, Mota MT, Moreira LF, Menezes AA. Circadian variation of methyleugenol anesthesia in albino rats. Braz J Med Biol Res 1990; 23: 423–5.
8 Lima CC, Criddle DN, Coelho-de-Souza AN, Monte FJ, Jaffar M, Leal-Cardoso JH. Relaxant and antispasmodic actions of methyleugenol on guinea-pig isolated ileum. Planta Med 2000; 66: 408–11.

9 Sayyah M, Valizadeh J, Kamalinejad M. Anticonvulsant activity of the leaf essential oil of Laurus nobilis against pentylentetrazole- and maximal electroshock-induced seizures. Phytomedicine 2002; 9: 212–6.

10 Sell AB, Carlini EA. Anesthetic action of methyleugenol and other eugenol derivatives. Pharmacology 1976; 14: 367–77.

11 Dallmeier Zelger KR, Zelger JL, Carlini EA. New anticonvulsants derived from 4-allyl-2-methoxyphenol (Eugenol): comparison with common antiepileptics in mice. Pharmacology 1983; 27: 40–9.

12 Yano S, Suzuki Y, Yuzurihara M, Kase Y, Takeda S, Watanabe S, et al. Antinoceptive effect of methyleugenol on formalin-induced hyperalgesia in mice. Eur J Pharmacol 2006; 553: 99–103.

13 Waxman SG, Dib-Hajj S, Cummins TR, Black JA. Sodium channels and pain. Proc Natl Acad Sci U S A 1999; 96: 7635–9.

14 Nuss HB, Kambouris NG, Marbán E, Tomaselli GF, Balser JR. Isoform-specific lidocaine block of sodium channels explained by differences in gating. Biophys J 2000; 78: 200–10.

15 Baker MD. Selective block of late Na+ current by local anesthetics in rat large sensory neurons. Br J Pharmacol 2000; 129: 1617–26.

16 Chevrier P, Vijayaragavan K, Chahine M. Differential modulation of Nav1.7 and Nav1.8 peripheral nerve sodium channels by the local anesthetic lidocaine. Br J Pharmacol 2004; 142: 576–84.

17 Zakrzewska JM, Patsalos PN. Drugs used in the management of trigeminal neuralgia. Oral Surg Oral Med Oral Pathol 1992; 74: 439–50.

18 Elliott AA, Elliott JR. Characterization of TTX-sensitive and TTX-resistant sodium currents in small cells from adult rat dorsal root ganglia. J Physiol 1993; 463: 39–56.

19 Toledo-Aral JJ, Moss BL, He ZJ, Koszewski TJ, Levinson SR, et al. Identification of PN1, a predominant voltage-dependent sodium channel expressed principally in peripheral neurons. Proc Nat Acad Sci U S A 1997; 94: 1527–32.

20 Black JA, Liu S, Tanak M, Cummins TR, Waxman SG. Changes in the expression of tetrodotoxin-sensitive sodium channels within dorsal root ganglia neurons in inflammatory pain. Pain 2004; 108: 237–47.

21 Nassar MA, Stirling LC, Forani G, Baker MD, Matthews EA, Dickenson AH, et al. Nociceptor-specific gene deletion reveals a major role for Nav1.7 (PN1) in acute and inflammatory pain. Proc Natl Acad Sci U S A 2004; 101: 12706–11.

22 Rizzo MA, Kocsis JD, Waxman SG. Selective loss of slow and enhancement of fast Na+ currents in cutaneous afferent dorsal root ganglion neurons following axotomy. Neurobiol Dis 1995; 2: 87–96.

23 Cox JJ, Reimann F, Nicholas AK, Thornton G, Roberts E, Springell K, et al. An SCN9A channelopathy causes congenital inability to experience pain. Nature 2006; 444: 894–8.

24 Ahmad S, Dahlund L, Eriksson AB, Helgren D, Karlsson U, Lund PE, et al. A stop codon mutation in SCN9A causes lack of pain sensation. Hum Mol Genet 2007; 16: 2114–21.

25 Goldberg YP, MacFarlane J, MacDonald ML, Thompson J, Dubé MP, Mattice M, et al. Loss-of-function mutations in the Nav1.7 gene underlie congenital indifference to pain in multiple human populations. Clin Genet 2007; 71: 311–9.

26 Luo S, Perry GM, Levinson SR, Henry MA. Nav1.7 expression is increased in painful human dental pulp. Mol Pain 2008; 4: 16.

27 Klugbauer N, Lacinova L, Flockerzi V, Hofmann F. Structure and functional expression of a new member of the tetrodotoxin-sensitive voltage-activated sodium channel family from human neuroendocrine cells. EMBO J 1995; 14: 1084–90.

28 Wang ZJ, Snell LD, Tabakoff B, Levinson SR. Inhibition of neuronal Na+ channels by the novel antiepileptic compound DCUKA: identification of the diphenyureido moiety as an inactivation modifier. Exp Neurol 2002; 178: 129–38.

29 Ragsdale DS, Scheuer T, Catterall WA. Frequency and voltage-dependent inhibition of type IIA Na+ channels, expressed in a mammalian cell line, by local anesthetic, antiaarrhythmic, and anticonvulsant drugs. Mol Pharmacol 1991; 40: 756–65.

30 Schwartz JR, Grigat G. Phenytoin and carbamazepine: potential- and frequency-dependent block of Na currents in mammalian myelinated nerve fibers. Epilepsia 1989; 30: 286–94.

31 Kuo CC, Bean BP. Slow binding of phenytoin to inactivated Na+ channels in rat hippocampal neurons. Mol Pharmacol 1994; 46: 716–25.

32 Vedantham V, Cannon SC. Slow inactivation does not affect movement of the fast inactivation gate in voltage-gated Na+ channels. J Gen Physiol 1998; 111: 83–93.

33 Ohkubo T, Shibata M. The selective capsaicin antagonist capsazepine abolishes the antinoceptive action of eugenol and guaiacol. J Dent Res 1997; 76: 848–51.

34 Park CK, Kim K, Jung SJ, Kim MJ, Ahn DK, Hong SD, et al. Molecular mechanism for local anesthetic action of eugenol in the rat trigeminal system. Pain 2009; 144: 84–94.

35 Park CK, Li HY, Yeon KY, Jung SJ, Choi SY, Lee SJ, et al. Eugenol inhibits sodium currents in dental afferent neurons. J Dent Res 2006; 85: 900–4.

36 Lee MH, Yeon KY, Park CK, Li HY, Fang Z, Kim MS, et al. Eugenol inhibits calcium currents in dental afferent neurons. J Dent Res 2005; 84: 848–51.

37 Cho JS, Kim TH, Lim JM, Song JH. Effects of eugenol on Na+ currents in rat dorsal root ganglion neurons. Brain Res 2008; 1234: 53–62.

38 Sensch O, Vierling W, Brandt W, Reiter M. Effects of inhibition of calcium and potassium currents in guinea-pig cardiac contraction: comparison of beta-carophyllene oxide, eugenol, and nifedipine. Br J Pharmacol 2000; 131: 1089–96.

39 Ragsdale DS, McPhee JC, Scheuer T, Catterall WA. Common molecular determinants of local anesthetic, antiaarrhythmic, and anticonvulsant block of voltage-gated Na+ channels. Proc Natl Acad Sci U S A 1996; 93: 9270–5.

40 Wang GK, Strichartz GR. State-dependent inhibition of sodium channels by local anesthetics: a 40-year evolution. Biochem (Mosc) Suppl Ser A Membr Cell Biol 2012; 6: 120–7.

41 von Stein RT, Soderlund DM. Role of the local anesthetic receptor in the state-dependent inhibition of voltage-gated sodium channels by the insecticide metaflumizone. Mol Pharmacol 2012; 81: 366–74.

42 Arcisio-Miranda M, Muroi Y, Chowdhury S, Chanda B. Molecular mechanism of allostERIC modification of voltage-dependent sodium channels by local anesthetics. J Gen Physiol 2010; 136: 541–54.

43 Leffler A, Reiprich A, Mohapatra DP, Nau C. Use-dependent block by lidocaine but not amitriptyline is more pronounced in tetrodotoxin (TTX)-resistant Nav1.8 than in TTX-sensitive Na+ channels. J Pharmacol Exp Ther 2007; 320: 354–64.

44 Leffler A, Reckzeh J, Nau C. Block of sensory neuronal Na+ channels by the secreolytic ambroxol is associated with an interaction with local anesthetic binding sites. Eur J Pharmacol 2010; 630: 19–28.

45 Wang DW, Mistry AM, Kahlig KM, Kearney JA, Xiang J, George AL Jr. Propranolol blocks cardiac and neuronal voltage-gated sodium channels. Front Pharmacol 2010; 1: 1–12.

46 Hondegem LM, Katzung BG. Time- and voltage-dependent interactions of antiarrhythmic drugs with cardiac sodium channels. Biochim Biophys Acta 1977; 472: 373–98.