Anesthetic Pharmacology of the Mint Extracts L-Carvone and Methyl Salicylate

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Keywords

Anesthesia · Euthanasia · γ -Amino butyric acid type · N-Methyl-D-aspartate · Voltage-gated sodium channel

Abstract

Introduction: Hydrocarbons with sufficient water solubility allosterically modulate anesthetic-sensitive ion channels. Mint extracts L-carvone and methyl salicylate water solubility exceeds modulation cutoff values for γ -amino butyric acid type A (GABA_A) receptors, N-methyl-D-aspartate (NMDA) receptors, and type-2 voltage-gated sodium (Na_v1.2) channels. We hypothesized that mint extracts modulate these channels at concentrations that anesthetize rats. **Methods:** Channels were expressed separately in frog oocytes and studied using 2-electrode voltage clamp techniques at drug concentrations up to 10 mM. Normalized current effects were fit to Hill equations. Mint compounds were formulated in a lipid emulsion and administered IV to rats. When unresponsive to the tail clamp, rats were exsanguinated, and plasma drug concentrations were measured. **Results:** Both mint compounds caused concentration-dependent inhibition of all channels except for methyl salicylate which inhibited GABA_A receptors at low concentrations and potentiated

at high concentrations. Plasma drug concentrations in anesthetized rats were 7.9 mM for L-carvone and 2.7 mM for methyl salicylate. This corresponded to $\geq 53\%$ NMDA receptor inhibition and $\geq 78\%$ Na_v1.2 channel inhibition by both compounds and 30% potentiation of GABA_A receptors by methyl salicylate. **Conclusion:** L-Carvone and methyl salicylate allosterically modulate cell receptor targets important to molecular actions of conventional anesthetics at concentrations that also induce general anesthesia in rats.

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Introduction

Common ion channel targets for anesthetics include γ -amino butyric acid type A (GABA_A) receptors, N-methyl-D-aspartate (NMDA) receptors, and type-2 voltage-gated sodium (Na_v1.2) channels. Many injectable anesthetics exhibit high-affinity for only one of these receptor targets. As examples, anesthetic effects of propofol are primarily due to GABA_A receptor potentiation, ketamine effects from NMDA receptor inhibition, and lidocaine effects from sodium channel blockade, including Na_v1.2 channels. In contrast, inhaled anesthetics, such as isoflu-

rane, sevoflurane, and desflurane, exhibit comparatively low receptor affinity but simultaneously modulate a large number of anesthetic-sensitive cell receptors and channels – including GABA_A, NMDA, and Na_v1.2 – at concentrations relevant to clinical anesthesia [1, 2].

Nonselective, low-affinity interactions with anesthetic-sensitive ion channels are not limited to conventional inhaled agents. Presumably by competing with water molecules occupying allosterically active amphipathic binding pockets in the protein, all hydrocarbons appear able to modulate anesthetic-sensitive ion channel currents, provided that the molar water solubility of the hydrocarbon exceeds that of the ion channel's measured solubility cutoff value [3–5]. The molar water solubility cutoff is defined as that minimum molar water solubility required of an amphipathic hydrocarbon to be able to allosterically modulate receptor function at sufficiently high concentrations (up to concentrations that equal the molar water solubility of the hydrocarbon). The average molar water solubility cutoff is 0.12 mM for GABA_A receptors, 1.1 mM for NMDA receptors, and 1.4 mM for Na_v1.2 channels [4, 5].

Terpenoids and salicylate esters are a large and diverse group of naturally occurring plant compounds, many of which have water solubility in excess of the cutoff values for common anesthetic-sensitive channels and receptors. Chemically, the “backbone” of terpenoid molecules is formed by linking 5-carbon units of isoprene (2-methylbuta-1,3-diene) that can subsequently undergo cyclization and cross-linking as per the isoprene rule and addition of oxygen-containing functional groups. Terpenoids are classified according to the number of isoprene units that form their linear backbone: hemiterpenoids (C₅H₈) contain 1 isoprene unit, monoterpenoids (C₁₀H₁₆) contain 2 units, and sesquiterpenoids (C₁₅H₂₄) contain 3 units [6]. Salicylate, 2-hydroxybenzoic acid (C₇H₆O₃), is formed in plants as a metabolite of cinnamic acid via the phenylpropanoid pathway. Condensation of the salicylate carboxylic acid group with an alcohol creates a salicylate ester [7].

L-Carvone is a monoterpene found as an essential oil component of spearmint (*Mentha spicata*). Methyl salicylate is found as an essential oil component of wintergreen (*Gaultheria* spp.). The Chemical Abstracts Service database of the American Chemical Society lists a calculated molar water solubility for L-carvone of 7.8 mM and for methyl salicylate of 21 mM at pH = 7. Because both of these compounds have molar water solubilities higher than the cutoff values for GABA_A and NMDA receptors and Na_v1.2 channels, both L-carvone and methyl salicy-

late should exhibit low-affinity interactions at sufficiently high concentrations and be able to modulate function of all of 3 anesthetic-sensitive ion channels.

We hypothesized that L-carvone and methyl salicylate each cause dose-dependent modulation of ion channel currents when GABA_A and NMDA receptors and Na_v1.2 channels are separately expressed in oocytes. If either compound is an anesthetic and not a convulsant, it should potentiate GABA_A receptors or inhibit NMDA receptors or inhibit Na_v1.2 channel currents. For compounds producing anesthetic-like modulation of ion channels, we predicted general anesthetic activity in vivo at plasma concentrations that correspond to their anesthetic-sensitive receptor effects in vitro.

Methods

In vitro Electrophysiology Studies

Oocyte Collection and Channel Expression

Ovaries removed from sexually mature *Xenopus laevis* frogs were bluntly dissected to remove surrounding connective tissue and then digested with 0.2% type I collagenase in an oocyte Ringer's solution. Once free from thecal cells and connective tissue, individual oocytes were rinsed, sorted, and stored in a modified Barth's solution [8–11].

Plasmids containing channel subunit genes were provided as a generous gift from Dr. Adron Harris. All genes were sequenced and compared to the National Center for Biotechnology Information nucleotide data to confirm gene identity. Heteromeric GABA_A receptors were expressed in oocytes by blind intranuclear injection of 1 ng DNA encoding the human α_1 (GABRA1) and rat β_2 (GABRB2) subunits in a 1:1 ratio. NMDA receptors were expressed by intracytoplasmic injection of 5 ng RNA encoding rat NR1 (GRIN1) and rat NR2a (GRIN2A) subunits in a 1:1 ratio. Na_v1.2 channels were expressed by injection of 5 ng RNA encoding the human SCN2A gene.

GABAA Receptor Experiments

Two-electrode voltage clamp studies were carried out in a 0.25-mL linear perfusion chamber. Perfusates were delivered by using a syringe pump at 1.5 mL/min using gastight glass syringes and polytetrafluoroethylene tubing.

Oocytes were impaled by two 3 M KCl-filled 0.2–1 M Ω borosilicate glass electrodes (KG-33; King Precision Glass, Claremont, CA, USA) connected to separate headstages (Axon Instruments HS2A; Molecular Devices, San Jose, CA, USA) through which voltage was clamped at –80 mV and current was passed via a computer-controlled amplifier (Axon Instruments GeneClamp 500B; Molecular Devices). Oocytes were perfused with frog Ringer's (FR) solution containing 115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl₂, and 10 mM HEPES prepared from ACS-grade chemicals and 18.2 M Ω H₂O, filtered and pH adjusted to 7.4. At 5-min intervals, the solution was switched to FR containing 50 μ M GABA; this concentration produces a chloride current equal to 35–40% of a maximum GABA agonist response. After 30 s, the perfusate was switched back to FR solution. This process was repeated until a stable base-

line response was achieved. Next, FR containing either L-carvone (99%; Sigma-Aldrich, St. Louis, MO, USA) or methyl salicylate ($\geq 99\%$; Sigma-Aldrich) at the test concentration was used to perfuse the oocyte for 1.5 min, and then the oocyte was perfused with FR + GABA solution containing this same drug and concentration for 30 s. Last, the solution was switched back to FR to wash out the drug for 5 min, and then FR + GABA was perfused for 30 s to confirm that the post-drug agonist response had returned to within 10% of the pre-drug response. Uninjected oocytes served as negative controls. Only one drug and concentration were studied per oocyte.

NMDA Receptor Experiments

Studies were carried out using equipment identical to that described for GABA_A receptors. Transmembrane potential was clamped at -80 mV, and oocytes were perfused with a barium-containing FR solution (Ba-FR) which substituted equimolar BaCl₂ for calcium salts and contained 0.1 mM EGTA as a calcium chelator. After 5 min, the perfusate was changed for 30 s to barium FR containing 0.1 mM glutamate plus 0.01 mM glycine (Ba-FREG) as receptor agonists which produce an NMDA receptor current $>99\%$ of the maximal response. Next, Ba-FR containing the study drug was perfused for 5 min and then switched to Ba-FREG containing the same test article for 30 s. The drug was then washed out with a 5-min perfusion of Ba-FR followed by a 30-s exposure to Ba-FREG to confirm post-drug currents had returned to within 10% of pre-drug values.

Na_v1.2 Channel Experiments

Using the same equipment as above, oocytes were perfused with FR and clamped at -80 mV transmembrane potential. Every 2 min, a 6-s step potential to 0 mV was applied to open the channel; this was repeated until baseline responses stabilized. Next, FR containing the test drug was perfused for 2 min followed by another 6-s step voltage change to 0 mV. Finally, FR was perfused for 5 min to wash out the drug, and another 6-s step clamp to 0 mV was applied to confirm that post-drug currents had returned to within 10% of pre-drug values.

Data Analysis

The change in whole cell peak current before and during agonist exposure (for GABA_A and NMDA receptors) or before and during the voltage step clamp (for Na_v1.2 channels) was measured in the tracings immediately before and during drug exposure. Percent change in current was calculated as follows:

$$\% \Delta = \frac{I_D - I_B}{I_B} \times 100,$$

where I_B is the baseline pre-drug current peak and I_D is the drug current peak.

For each drug and ion channel, data were fit to a Hill equation using nonlinear regression with sequential quadratic programming and bootstrap estimates of parameter standard errors (SPSS, v. 27; IBM, Armonk, NY, USA). In the model, maximum drug inhibition ($I_{\max}$) was constrained to not be $<-100\%$. Initial parameter estimates were seeded with values based on visual inspection of respective dose-response curves in order to facilitate model convergence.

In vivo Anesthesia Studies

Pilot Experiments

Solutions of 10% (v/v) L-carvone or methyl salicylate were prepared sterilely in 20% intralipid (Fresenius Kabi, Lake Zurich, IL, USA). Lateral tail vein catheters were placed in twenty-one 69-day-old, male, Sprague Dawley rats weighing 291 ± 17 (mean $\pm$ SD) grams. Each rat received a single intravenous bolus injection of L-carvone solution (0.2 or 0.4 mL), methyl salicylate solution (0.1, 0.15, 0.2, or 0.4 mL), or a 1:1 mixture of the L-carvone and methyl salicylate solutions over 15 s. Because L-carvone density is 0.9673 g/cm^3 at 25°C , 0.2 mL and 0.4 mL of solution contained 19 mg and 39 mg of L-carvone, respectively [12]. The density of methyl salicylate is 1.1798 g/cm^3 at 25°C ; hence, 0.2 mL and 0.4 mL of solution contained 24 mg and 47 mg of methyl salicylate, respectively [13].

Spontaneous motor activity and loss-of-righting reflex was assessed at 5- to 10-s intervals, and nociception response to an alligator clamp applied to the tail was tested if the righting reflex was absent. Animals administered the lower doses of L-carvone, methyl salicylate, and the drug mixture were recovered and returned to the vivarium. Animals were weighed 24 and 48 h after the study and then euthanized for gross pathology examination.

Plasma Drug Concentrations in Anesthetized Rats

Lateral tail veins were catheterized in eight, 70-day-old, male, Sprague Dawley rats weighing 309 ± 6 g. A 10% solution of L-carvone or methyl salicylate in 20% intralipid was each administered IV to 8 rats (4 animals per drug treatment) by using a syringe pump at a rate of 12 mL/h. When rats lost their righting reflex and were unresponsive to a tail clamp, a midline laparotomy was performed, the abdominal aorta catheterized, and the rat exsanguinated. Blood was collected in a heparin tube and centrifuged; plasma was separated and stored in cryogenic tubes at -80°C until analysis.

Measurement of Drug Concentrations

Serum calibrations were prepared by dilution of working standard solutions of methyl salicylate and L-carvone with drug-free rat serum (Innovative Research, Novi, MI, USA) to concentrations ranging from 0.05 to 500 $\mu\text{g/mL}$. Calibration curves and negative control samples were prepared fresh for each quantitative assay. In addition, quality control samples (drug-free rat serum fortified with analyte at 3–4 concentrations within the standard curve) were included as an additional check of accuracy.

Prior to analysis, 100 μL of each serum sample was diluted with 100 μL of acetonitrile (ACN):1 M acetic acid (9:1, v:v) and 50 μL of methanol to precipitate proteins. The samples were vortexed for 2 min to mix, refrigerated for 20 min, vortexed for an additional 1 min, centrifuged (4,300 rpm/3,102 g) for 10 min at 4°C , and 10 μL injected into the liquid chromatography tandem mass spectrometry system.

Quantitative analysis was performed on an LTQ XL Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA, USA) coupled with a Waters Acquity UPLC (Milford, MA, USA). The system was operated at a resolution of 15,000 ($M/\Delta M$, at full width at half maximum of the mass peaks) using positive electrospray ionization (ESI[+]). Detection and quantification were conducted using full-scan accurate mass from 145 to 160 (m/z). The responses were plotted using a 20-ppm data window for methyl salicylate (mass-to-charge ratio 153.05443 [m/z]) and L-carvone (mass-to-charge ratio 151.11160 [m/z]). The spray voltage was 3,500 V, and

Fig. 1. Sample electrophysiology tracings for GABA_A receptors (A), NMDA receptors (B), and Na_v1.2 channels (C) before, during, and after exposure to 1 mM L-carvone. Tracings for methyl salicylate exposures were qualitatively similar. GABA_A, γ-aminobutyric acid type A; NMDA, N-methyl-D-aspartate; Na_v1.2, type-2 voltage-gated sodium.

Fig. 2. Linear-log plot of the percent current change from baseline of GABA_A receptors exposed to L-carvone. Data are from 4–8 oocytes at each concentration. The solid line is the best fit to the Hill equation:

$$-100 \frac{C^{1.3}}{(7.2 \times 10^{-5}) + C^{1.3}},$$

where C is the L-carvone concentration. GABA_A, γ-amino butyric acid type A.

Table 1. Bootstrap parameter estimates for the nonlinear regression fit of the percent current change

	$I_{\max}$	IC_{50}	n_I	$E_{\max}$	EC_{50}	n_E
GABA _A						
L-Carvone	-100±0	6.5E-4±6.5E-5	1.3±0.1	–	–	–
Methyl salicylate	-73±13	1.1E-7±3.4E-6	2.5±2.8	223±34	5.3E-3±8.1E-3	0.21±0.06
NMDA						
L-Carvone	-100±0	2.4E-3±4.3E-4	0.8±0.1	–	–	–
Methyl salicylate	-100±0	2.3E-3±3.4E-4	0.8±0.1	–	–	–
Na _v 1.2						
L-Carvone	-100±0	7.5E-4±1.8E-4	0.9±0.5	–	–	–
Methyl salicylate	-100±0	1.5E-3±1.0E-4	2.2±0.3	–	–	–

Bootstrap parameter estimates for the nonlinear regression fit of the percent current change to the Hill equation:

$$I_{\max} \frac{C^{n_I}}{IC_{50}^{n_I} + C^{n_I}} + E_{\max} \frac{C^{n_E}}{EC_{50}^{n_E} + C^{n_E}},$$

where $I_{\max}$ and $E_{\max}$ are the respective maximum percent current inhibition or enhancement, IC_{50} and EC_{50} are the drug concentrations C producing respective median inhibitory or enhancement of current, and n_I and n_E are the respective Hill coefficients for the inhibitory and enhancing response curves

The methyl salicylate effect on GABA_A receptors was described by both inhibitory and enhancement Hill equations terms. All other drug-channel interactions were only inhibitory. GABA_A, γ-amino butyric acid type A; NMDA, N-methyl-D-aspartate; Na_v1.2, type-2 voltage-gated sodium.

the sheath and auxiliary gas was 45 and 20, respectively (arbitrary units). Chromatography employed an Accucore Vanquish C18 + 10 cm × 2.1 mm 1.5 μm column (Thermo Scientific) and a linear gradient of ACN in water with a constant 0.2% formic acid at a flow rate of 0.1 mL/min. The initial ACN concentration was held at 1%

for 0.4 min and ramped to 99% over 9 min before re-equilibrating for 14 min at initial conditions.

The precision and accuracy of the assay was determined by assaying quality control samples in replicates ($n = 6$). Accuracy was reported as percent nominal concentration and precision as per-

Fig. 3. Linear-log plot of the percent current change from baseline of GABA_A receptors exposed to methyl salicylate. Data are from 4–10 oocytes at each concentration. The solid line is the best fit to the Hill equation:

$$-73 \frac{C^{2.5}}{(4.0 \times 10^{-18}) + C^{2.5}} + 223 \frac{C^{0.21}}{(0.33) + C^{0.21}},$$

where C is the methyl salicylate concentration. GABA_A, γ-amino butyric acid type A.

cent relative standard deviation. For both methyl salicylate and L-carvone, all values were within 10% of the nominal concentration. The technique was optimized to provide limits of quantitation of 0.5 µg/mL and 0.05 µg/mL and limits of detection of approximately 0.1 µg/mL and 0.025 µg/mL for methyl salicylate and L-carvone, respectively.

Results

In vitro Electrophysiology Studies

Sample tracings for GABA_A receptor, NMDA receptor, and Na_v1.2 channel experiments are shown in Figure 1. L-Carvone caused dose-dependent inhibition of all 3 ion channels (Fig. 2, 4, 6) with near or complete loss of channel currents at the 10 mM drug dose. Potency of L-carvone, as assessed by the median inhibitory concentration (IC₅₀) for each channel (Table 1), was ranked as follows: GABA_A ≥ Na_v1.2 > NMDA.

Methyl salicylate also dose-dependently inhibited NMDA receptors and Na_v1.2 channels (Fig. 5, 7) with the drug exhibiting 50% greater potency for the latter (Table 1). However, GABA_A receptors were potently inhibited by methyl salicylate (Fig. 3) with an IC₅₀ of 0.11 µM indicative of a high-affinity receptor binding site (Table 1). At high concentrations, methyl salicylate binds a second site to potentiate GABA_A currents with a median effective concentration estimated at 5.3 mM, the weakest drug-receptor interaction observed in this study.

Hill coefficient estimates for the dose-response models were close to 1 for all ion channels with L-carvone and for NMDA receptors with methyl salicylate (Table 1). This is consistent with drug-receptor binding independent of agonist binding (in the case of GABA_A and NMDA receptors) and either a single drug-protein binding site or non-cooperative binding at multiple sites. The Na_v1.2 dose-response curve for methyl salicylate was significantly >1, suggesting cooperative binding that might occur from two or more drug molecules binding the channel at two or more interactive sites. In contrast, the Hill coefficient <1 for low-affinity methyl salicylate binding at the GABA_A receptor potentiation site indicates negative cooperative binding, possibly from interactions with methyl salicylate binding at the high-affinity GABA_A receptor inhibitory site.

In vivo Anesthesia Studies

In the pilot study IV bolus injections, 0.2 mL of the L-carvone emulsion ($N = 4$ rats) resulted in transient excitement followed by loss-of-righting reflex in 40 ± 6 s and loss of tail clamp response in 54 ± 11 s. During this time, the respiratory rate was transiently decreased for approximately 10 s. Half of the rats exhibited whole-body muscle hypertonia for 15 s, after which muscles were relaxed. Recovery was rapid with righting reflex returning in 58 ± 14 s and the rats walking and active in 60 ± 13 s. Body weight over the next 2 days was within 1.9 ± 1.4 percent of base-

Fig. 4. Linear-log plot of the percent current change from baseline of NMDA receptors exposed to L-carvone. Data are from 5–7 oocytes at each concentration. The solid line is the best fit to the Hill equation:

$$-100 \frac{C^{0.8}}{(8.0 \times 10^{-3}) + C^{0.8}},$$

where C is the L-carvone concentration. NMDA, N-methyl-D-aspartate.

line, and no gross pathology findings were noted on examination of the heart, lung, liver, or kidney. Similar intravenous injection of 0.4 mL of L-carvone ($N = 4$ rats) induced unconsciousness and flaccid muscle tone in 20 ± 4 s and cardiopulmonary arrest in 35 ± 18 s.

A 0.1 mL IV bolus of the methyl salicylate emulsion in a single rat caused loss- and return-of-righting reflex in 33 and 60 s, respectively, but never resulted in general anesthesia, as defined by immobility in response to a tail clamp. Increasing the methyl salicylate injection dose to 0.15 mL ($N = 4$ rats) caused loss-of-righting in 35 ± 5 s and anesthesia in 39 ± 2 s, and both returned in 53 ± 3 and 64 ± 8 s after drug injection with normal ambulation without ataxia at 76 ± 10 s. Following a 0.2 mL methyl salicylate emulsion injection ($N = 3$ rats), loss-of-righting and general anesthesia occurred in 35 ± 10 and 37 ± 13 s, respectively. However, 1 rat exhibited cardiopulmonary arrest 35 s following this dose of methyl salicylate. The other 2 rats regained tail clamp and righting responses an average of 48 s and 58 s, respectively, and were ambulating normally 110 s following drug administration. For those rats receiving sublethal methyl salicylate doses, body weight was within 97–98% of baseline values for the next two days, and no gross abnormalities were found on necropsy 48 h after drug administration. Increasing the methyl salicylate emulsion dose to 0.4 mL ($N = 3$ rats) resulted in unconsciousness in 20 ± 5 s and cardiopulmonary arrest in all animals $31 \pm$ sec after beginning injection.

Co-administration of 0.1 L-carvone emulsion plus 0.1 mL methyl salicylate emulsion ($N = 2$ rats) produced almost identical signs over a similar time scale as the anesthetic doses of described for either L-carvone emulsion or methyl salicylate emulsion administered alone. Body weights for both animals over the next 2 days ranged between 98 and 101% of baseline, and necropsy examinations were unremarkable. As there was no obvious benefit to this combination over administration of only the single agent, the pharmacodynamics of L-carvone/methyl salicylate mixtures were not further evaluated.

Continuous 0.2 mL/min IV infusions of L-carvone emulsions produced muscle fasciculations and loss-of-righting reflex followed by tonic muscle contractions suggestive of seizure over 2–4 min after the start of injection. After this time, there was no response to toe pinch and tail clamp, and the animals were euthanized by exsanguination. Similar injection with methyl salicylate emulsions in rats caused transient excitement in half of the animals 30 s after injection. Loss-of-righting and absent tail clamp responses occurred within 3–4 min after start of injection, after which rats were euthanized by exsanguination. No tonic or clonic muscle activity was observed in any rat administered methyl salicylate infusions.

The mean plasma concentration ($\pm$ SEM) of L-carvone during IV infusion in rats ($N = 4$) was 7.9 ± 3.0 mM ($N = 4$). This concentration of L-carvone corresponded to 96% inhibition of GABA_A receptors (Fig. 2), 71% inhibition of

Fig. 5. Linear-log plot of the percent current change from baseline of NMDA receptors exposed to methyl salicylate. Data are from 5–7 oocytes at each concentration. The solid line is the best fit to the Hill equation:

$$-100 \frac{C^{0.8}}{(7.7 \times 10^{-3}) + C^{0.8}},$$

where C is the methyl salicylate concentration. NMDA, N-methyl-D-aspartate.

NMDA receptors (Fig. 4), and 90% inhibition of $\text{Na}_v1.2$ channels (Fig. 6) for in vitro electrophysiology studies. Methyl salicylate concentration during IV infusions in rats ($N = 4$) was 2.7 ± 0.4 mM. This methyl salicylate concentration was associated with 30% potentiation of GABA_A receptors (Fig. 3), 53% inhibition of NMDA receptors (Fig. 5), and 78% inhibition of $\text{Na}_v1.2$ channels (Fig. 7) in vitro.

Discussion

Amphipathic compounds with sufficient molar water solubility can modulate anesthetic-sensitive ion channels such as GABA_A receptors, NMDA receptors, and $\text{Na}_v1.2$ channels via low-affinity interactions, often at hundreds of micromolar concentrations or higher. The mint compounds L-carvone and methyl salicylate are more soluble in water than the cutoff values for modulation of these 3 anesthetic-sensitive ion channels [4, 5]. We have shown that both mint compounds do indeed modulate GABA_A receptor, NMDA receptor, and $\text{Na}_v1.2$ channel currents at concentrations associated with general anesthesia in rats.

L-Carvone application to the sciatic nerve axon of frogs decreases compound action potential amplitude with an IC_{50} of 1.4 mM [14]. This is similar to the IC_{50} for human $\text{Na}_v1.2$ channels in the present study (Table 1). Given the central role of nodal $\text{Na}_v1.2$ channels for action

potential propagation along nerve axons, results support a local anesthetic mechanism of drug action. L-Carvone also decreases compound action potentials in rat sciatic nerves, but at an IC_{50} of 8.7 mM [15]. This potency variability suggests significant species differences in drug-receptor binding affinity.

A previous 2-electrode voltage clamp study of human benzodiazepine-sensitive $\alpha_1\beta_2\gamma_2\delta$ GABA_A receptors expressed in frog oocytes showed minimal response to L-carvone concentrations up to 0.12 mM [16]. In rat cerebral cortex cell cultures, 0.75 mM L-carvone exerted a negative allosteric effect with GABA_A -induced benzodiazepine binding reduced by 36% [17]. However, even greater GABA_A receptor modulation occurs at higher L-carvone concentrations. GABA_A receptor currents were almost entirely inhibited by 10 mM L-carvone, the highest concentration studied here.

Mint oils have been used as immersion anesthetics in fish. Baths containing either 1.9–4.5 mM L-carvone or 0.8–2.3 mM methyl salicylate caused anesthesia in carp with the highest doses associated with faster onset and longer recovery times [18]. Co-administration of the 2 mint extracts as an emulsion reduced the concentration required for each drug by 75% suggesting possible drug synergy action, at least in fish [19, 20]. The rat pilot experiments reported here with L-carvone, methyl salicylate, and the 1:1 administration of both compounds did not show as obvious a difference in dose requirement, onset of action, or recovery time. These similar anesthetic effects reflect

Fig. 6. Linear-log plot of the percent current change from baseline of type-2 voltage-gated sodium channels exposed to L-carvone. Data are from 3–7 oocytes at each concentration. The solid line is the best fit to the Hill equation:

$$-100 \frac{C^{0.9}}{(1.5 \times 10^{-3}) + C^{0.9}},$$

where C is the L-carvone concentration.

Fig. 7. Linear-log plot of the percent current change from baseline of type-2 voltage-gated sodium channels exposed to methyl salicylate. Data are from 6–9 oocytes at each concentration. The solid line is the best fit to the Hill equation:

$$-100 \frac{C^{2.2}}{(6.1 \times 10^{-7}) + C^{2.2}},$$

where C is the methyl salicylate concentration.

similar receptor drug actions and potency at NMDA receptors and Na_v1.2 channels.

The one remarkable difference in the mechanism of action between mint extracts occurred at the GABA_A receptor. However, L-carvone caused dose-dependent inhibition, and methyl salicylate exhibited a biphasic effect on GABA_A receptor currents (Fig. 2, 3). This implies the presence of at least 2 separate salicylate ester allosteric binding sites on the GABA_A receptor. Negative allosteric modulation occurred at a high-affinity binding site with an IC₅₀ of approximately 0.1 μM methyl salicylate. This

site may be the same GABA_A binding site as for the high-affinity anesthetic ligands propofol and propanidid, which share structural similarities to L-carvone and methyl salicylate [6]. At about 50,000 times higher concentration, methyl salicylate binds a second, noncompetitive positive allosteric site that, at a saturated aqueous phase concentration, produced a predicted GABA_A current potentiation to equal to half of the maximum observable enhancement possible with the agonist concentration used in this study. This binding site may be similar to the amphipathic, water-filled, allosterically active

pockets predicted for low-affinity interactions with inhaled anesthetics and other simple hydrocarbons [4, 5].

GABA_A receptor potentiation typically causes central nervous system depression; GABA_A receptor inhibition causes convulsions [21]. During anesthesia maintenance, L-carvone caused tonic muscle contractions suggestive of seizure activity at concentrations corresponding to near total inhibition of GABA_A receptor currents. Although methyl salicylate also inhibits these same currents at low concentrations, a rapid rise in plasma drug concentration to agonist levels during IV administration probably circumvented seizures in these rats. Additionally, inhibition of glutamate receptors and sodium channel currents decreases neuronal excitability and may have stopped seizure activity during L-carvone anesthesia and prevented visible seizures following high-dose bolus euthanasia in pilot rat experiments [22].

Differences between mint extract effects on GABA_A receptors may also explain differences in drug potency. GABA_A receptor inhibition increases neuronal excitability and increases anesthetic median effective concentrations [23]. Presumably, this is because increased basal excitability must be overcome through more depressant effects at other anesthetic-sensitive receptor targets, such as from NMDA receptor inhibition [24]. At the time general anesthesia was achieved and the aorta catheterized, plasma concentration of L-carvone was 3 times that for methyl salicylate and corresponded to GABA_A receptor inhibition for L-carvone but GABA_A receptor potentiation for methyl salicylate. GABA_A receptors would therefore contribute to methyl salicylate anesthesia but antagonize L-carvone anesthesia. These higher L-carvone plasma concentrations during anesthesia equate to 15–30% greater inhibition of NMDA receptors and Na_v1.2 channels than was present at the methyl salicylate plasma concentrations (Fig. 4–7). Increased action at these and perhaps other receptor targets allows L-carvone to induce an anesthesia effect despite contrary activity at GABA_A receptors. Indeed, a similar phenomenon has been described for conventional anesthetics, such as the inhaled agents isoflurane and sevoflurane that exhibit low-affinity interactions with structurally diverse anesthetic-sensitive ion channels and receptors. Antagonism of spinal GABA_A receptors in rats increases the isoflurane EC₅₀ for immobilization by up to 40% [23]. However, isoflurane requirement is not greater with further GABA_A receptor inhibition, presumably because increased isoflurane concentrations produce increased effects at other molecular targets – such as NMDA receptors [25], voltage-gated sodium channels [26], 2-pore domain potassium channels

[27], and glycine receptors [28] – that combined are sufficient to depress CNS function and cause immobility. Similarly, antagonism of spinal glycine receptors in rats increases the sevoflurane EC₅₀, at which general anesthesia is mediated by greater inhibition of NMDA receptors produced by this greater sevoflurane concentration [29].

The present study focused on an anesthetic endpoint, but these same receptor systems exert other important neurophysiologic effects. Sodium channel blockers and glutamate receptor antagonists act at sites within both the peripheral and central nervous system where they decrease pain [22]. In addition, both mint extracts can modulate other anesthetic-sensitive ion channels important for analgesia, such as the transient receptor potential cation channel TRPV1 [30, 31]. It is possible that antinociceptive effects may be achieved at subanesthetic drug concentrations.

Like L-carvone and methyl salicylate, conventional volatile anesthetics modulate anesthetic-sensitive ion channels through low-affinity interactions. However, conventional volatile anesthetics are not natural compounds, so why should these or other amphipathic compounds modulate anesthetic-sensitive ion channels at all? It has been proposed that pervasive environmental exposure to amphipathic chemicals from the beginnings of life to the present creates selective pressures for ion channels to decrease cation currents or increase anion currents in order to prevent uncontrolled and deleterious cell depolarization [32]. Indeed, animals are awash in potential anesthetic-acting compounds such as ammonia [33], amino acids [34], ketones [35], carboxylic acids [36], carbon dioxide [37, 38], and even the nitrogen in air itself [39, 40]. Among plants, L-carvone and methyl salicylate are just two of at least several dozen terpenoids and salicylate esters with molar water solubility values sufficient to predict activity on anesthetic-sensitive ion channels and receptors. However, GABA_A receptor inhibition by plant terpenoids and salicylate esters can increase cell excitability and suggests there may not be selective pressure in favor of any particular directional channel response to anesthetic-like compounds. Rather, the concentration-response data reported here for mint compounds, as well as previously for other metabolites and gases, indicate there may be selective pressure for anesthetic-sensitive ion channel insensitivity to amphipathic compounds at concentrations commonly present in the environment or internal milieu. It is only because channels are unresponsive to these low concentrations that, in the words of Shakespeare, we can “shake off this downy sleep.”

Conclusions

As predicted by their molar water solubility values, the mint extracts L-carvone and methyl salicylate modulate GABA_A receptor, NMDA receptor, and Na_v1.2 channel currents at millimolar concentrations indicative of low-affinity drug-ligand binding. Furthermore, drug concentrations associated with anesthetic-sensitive ion channel effects also induce anesthesia in rats. It is possible that these or similar edible terpenoids and salicylate esters may offer utility as injectable anesthetic, analgesic, or euthanasia agents in animals, particularly in livestock where drug residues may be of concern.

Statement of Ethics

All animal work was conducted with approval by the Institutional Animal Care and Use Committee at the University of California, Davis (Protocol No. 21535 and 21559).

Conflict of Interest Statement

Dr. Brosnan has assigned a provisional patent to the University of California related to the use of food-derived terpenoids and salicylate esters as injectable anesthetic, analgesic, and euthanasia agents. Other authors report no conflicts of interest.

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Author Contributions

Brosnan conceived the project, designed studies, participated in data acquisition, analyzed and interpreted data, and wrote the manuscript draft. Ramos participated in data acquisition, assisted with data analysis, and reviewed the manuscript. Aguiar participated in data acquisition, assisted with data analysis, and reviewed the manuscript. Cenani participated in data acquisition, assisted with data analysis, and reviewed the manuscript. Knych participated in data acquisition, assisted with data analysis, and reviewed the manuscript.

Data Availability Statement

Computer files containing experimental measurements used for the data summaries and analyses of this study are available at Dryad (doi:10.25338/B8463X).

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