Hydroxyamide analogues of propofol exhibit state-dependent block of sodium channels in hippocampal neurons: implications for anticonvulsant activity.

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Propofol Analogues Exhibit State-Dependent Block

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Abbreviations: ACSF, artificial cerebrospinal fluid; AED, antiepileptic drug; RSE, refractory status epilepticus; AP, action potential.
Abstract

Although propofol is most commonly known for its general anesthetic properties, at sub-anesthetic doses propofol has been effectively used to suppress seizures during refractory status epilepticus (RSE), a mechanism, in part, attributed to the inhibition of neuronal sodium (Na) channels. In this study, we have designed and synthesized two novel analogues of propofol, HS245 and HS357, and determined their effects on Na currents recorded from cultured hippocampal neurons. HS357 had greater affinity for the inactivated state of the Na channel than propofol and HS245 (0.22 versus 0.74 and 1.2 \( \mu \text{M} \), respectively) and exhibited the greatest ratio of affinity for the resting over the inactivated state. HS357 also demonstrated greater use-dependent block and delayed recovery from inactivation in comparison to propofol and HS245. Under current clamp conditions, action potentials from hippocampal CA1 neurons in slices were evoked by current injection, or following perfusion with a zero Mg\(^{2+}/7\) mM K\(^+\) artificial cerebrospinal fluid solution. Propofol and HS357 reduced the number of current-induced action potentials; however, HS357 caused a greater reduction in the number of spontaneous action potentials. Consistent with these electrophysiology studies, propofol and HS357 protected mice against acute seizures in the 6 Hz (22 mA) partial psychomotor model. Efficacious doses of propofol were associated with an impairment of motor coordination as assessed in the rotorod toxicity assay. In contrast, HS357 demonstrated a two-fold greater protective index than propofol. Thus, propofol analogues represent an important structural class from which not only effective, but also safer anticonvulsants may be developed.
Epilepsy is a neurological disorder that affects approximately 1-2% of the population. Treatment of epilepsy focuses on the suppression of seizures via the use of antiepileptic drugs (AEDs) (Browne and Holmes, 2001). First generation AEDs, such as phenytoin, carbamazepine, phenobarbital, and sodium valproate, along with second generation AEDs, such as topiramate, gabapentin, lamotrigine and zonisamide, offer a wide selection of possible therapeutic treatments for suppression of seizures. Although the number of AEDs has increased, the percentage of patients that fail to be treated successfully remains constant at an alarming 30% (Loscher and Schmidt, 2002). Substantial problems exist with toxicity, resistance, and idiosyncratic reactions in a number of the currently used AEDs (Brodie, 2001), all of which likely contribute to their failure rate. In view of this failure rate, there remains a need for a better understanding of the mechanisms involved in suppression of epileptic seizures with minimal side effects.

Voltage-gated sodium (Na) channels play an important role in determining neuronal excitability and constitute a proven target for the suppression of epileptic seizures. Sodium channels consist of a pore forming α subunit and auxiliary β subunits, which modulate the gating kinetics of the channel (Catterall, 2000). To date, nine α subunit isoforms and four β subunits have been cloned (Goldin, 2001). At hyperpolarized membrane potentials, Na channels exist in the closed state and transition through the open and into the inactivated state upon membrane depolarization (Goldin, 2003). Clinically used Na channel blockers are state-dependent; that is, they bind with higher affinity to the inactivated state of the Na channel than the resting state (Hille, 1977). This state-dependence is not only considered important for seizure suppression, but also is essential for a favorable side effect profile, since it allows AEDs to inhibit action...
potential (AP) bursts that occur during seizures without affecting normal neuronal activity (Rogawski and Loscher, 2004;Hille, 1977).

Propofol (2,6-diisopropylphenol), is most commonly known for its use as an intravenous general anesthetic; however, at sub-anesthetic doses, propofol is used clinically during refractory status epilepticus (RSE), a critical medical emergency with mortality rates as high as 76% in elderly patients (Claassen et al., 2002;Rossetti et al., 2004;Prasad et al., 2001;Marik and Varon, 2004). Propofol has been shown to inhibit Na currents in mammalian cell lines expressing rat Na1.2 (Rehberg and Duch, 1999), activate γ-aminobutyric acid (GABA) currents in hippocampal neurons (Orser et al., 1994), and to inhibit L-type calcium currents in cortical neurons (Martella et al., 2005). However, its modulation of Na currents (Martella et al., 2005) has been suggested to be important for its AED activity in both veratridine-induced seizures and (Otoom and Hasan, 2004) amygdala-kindled rats (Borowicz and Czuczwar, 2003). Although the mechanisms of action for propofol remains unclear, it continues to be an effective alternative to barbiturates and first-line AEDs when treating RSE (Rossetti et al., 2004).

In this study, we have used propofol as a scaffold to design novel analogues. The first propofol analogue, 2-(3-Ethyl-4-hydroxy-5-isopropyl-phenyl)-3,3,3-trifluoro-2-hydroxy-propionamide (HS245), incorporates an α-hydroxyamide moiety we have previously shown to be important for Na channel inhibition (Brown et al., 1999;Lenkowski et al., 2004). In a previous study, we have also shown that the replacement of one phenyl ring in the structure of phenytoin with a seven-carbon alkyl chain resulted in a potent and state-dependent Na channel blocker (Lenkowski et al., 2004). To further determine the optimum distance between the phenyl and α-
hydroxyamide moieties we designed 2-Hydroxy-8-(4-hydroxy-3,5-diisopropyl-phenyl)-2-
trifluromethyl-octanoic acid amide (HS357), which incorporates a six-carbon alkyl linker
(Fig 1).

The effects of propofol, HS245, and HS357 were characterized on Na channel
currents recorded from cultured hippocampal neurons and APs evoked in CA1 neurons
from rat hippocampal slices. In an effort to optimize in vitro conditions for burst firing
and epileptiform events, we employed a hyper-excitation model obtained by perfusing
brain slices with a zero Mg^{2+}/7 mM K^{+} artificial cerebrospinal fluid (ACSF) solution that
allows a higher magnitude depolarization and a stronger excitatory response (Mangan
and Kapur, 2004; Arias and Bowby, 2005). In agreement with our in vitro studies,
systemic administration of either propofol or HS357 protected mice from acute seizures
observed in the 6Hz (22mA) partial seizure model; however, in contrast to propofol, the
protection afforded by HS357 was not accompanied by motor impairment in the rotorod
toxicity assay.
Materials and Methods

Materials. Propofol was obtained from Sigma-Aldrich (St. Louis, MO). 2-(3-Ethyl-4-hydroxy-5-isopropyl-phenyl)-3,3,3-trifluoro-2-hydroxy-propionamide (HS245) and 2-Hydroxy-8-(4-hydroxy-3,5-diisopropyl-phenyl)-2-trifluromethyl-octanoic acid amide (HS357) were synthesized as described in the supplemental data (Supplemental Data HS245 Synthesis and Supplemental Data HS357 Synthesis).

Hippocampal Culture. All experimental protocols were approved by the Institutional Animal Care and Use Committee affiliated with the University of Virginia and the University of Utah. All efforts were made to minimize animal stress and discomfort. The preparation of hippocampal cultures has been previously described (Mangan and Kapur, 2004). Briefly, hippocampi were dissected from 18-day-old rat embryos, dissociated by trypsin, and triturated with a Pasteur pipette. The neurons were plated on coverslips coated with poly-L-lysine in minimal essential medium (MEM), with 10% horse serum at an approximate density of 25,000/cm². Once the neurons had attached to the substrate, they were transferred to a dish containing a glial monolayer and maintained for up to 4 weeks in serum-free MEM with N2 supplement. Voltage clamp recordings were made from 1-2 week-old cultured hippocampal neurons.

Brain Slice Preparation. Transverse hippocampal slices (250-300 µm) were prepared from Sprague-Dawley rats (150-200 g). Animals were euthanized with halothane, decapitated, and brains rapidly removed and placed in chilled (4°C) artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 125, KCl 2.5, NaH₂PO₄ 1.25, CaCl₂ 2, MgCl₂ 1, L-Ascorbic acid 0.5, glucose 10, NaHCO₃ 25, and Pyruvate 2 (oxygenated with 95% O₂ and 5% CO₂). Slices were prepared using a Vibratome.
(Vibratome 1000 Plus, St. Louis, MO, U.S.A.) and transferred to a chamber containing oxygenated ACSF, incubated at 37°C for 20-35 minutes, and then stored at room temperature. For recording, slices were held in a small chamber perfused with heated (32°C) oxygenated ACSF at 2 ml/min. CA1 hippocampal neurons were visually identified using a Zeiss Axioscope microscope (Zeiss, Oberkochen, Germany).

**Electrophysiology.** Na currents were recorded from cultured hippocampal neurons using the whole-cell configuration of the patch clamp recording technique with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). All voltage protocols were applied using Clampfit software (v9, Axon Instruments, CA, USA) and a Digidata 1322A (Axon, USA). Currents were acquired at 33 kHz and low pass filtered at 2 kHz. Borosilicate glass pipettes were pulled using a Brown-Flaming puller (model P-87, Sutter Instruments Co, Novato, CA) and heat polished to produce electrode resistances of 1.5 - 2.0 MΩ when filled with pipette solution containing (in mM): CsF 140, MgCl₂ 2, EGTA 1, HEPES 10, Na₂ATP 4, NaGTP 0.3 (pH adjusted to 7.3 with CsOH). Cultured neurons were perfused with solution containing (in mM): NaCl 30, Choline Chloride 110, KCl 3, CaCl₂ 1, CdCl₂ 0.1, MgCl₂ 2, HEPES 10, TEA-Cl 30 (pH adjusted to 7.3 with CsOH). Calcium and potassium currents were blocked by cadmium and TEA-Cl, respectively. On establishing the whole-cell configuration, neurons were held at -90 mV for 5 minutes to permit time for the equilibration of gating shifts. Currents were elicited from a holding potential of -90 mV by a step to -10 mV for 20 ms. Capacitive and leak currents were corrected for using standard P/4 protocols except during steady-state inactivation and use-dependent block protocols. All voltage clamp experiments were performed at room temperature (20-22 °C). Current clamp recordings were performed at 32°C in an ACSF.
extracellular solution described previously and an intracellular solution containing (in mM): Kgluconate 120, NaCl 10, MgCl₂ 2, K₂EGTA 0.5, HEPES 10, NaATP 4, NaGTP 0.3 (pH adjusted to 7.2 with KOH).

To induce spontaneous firing in hippocampal CA1 pyramidal neurons, slices were perfused (20 min) with a nominally zero Mg²⁺/7 mM K⁺ ACSF solution containing (in mM): NaCl 125, KCl 7, NaH₂PO₄ 1.25, CaCl₂ 2, NaHCO₃ 25, glucose 10 (oxygenated with 95% O₂ and 5% CO₂) and the same intracellular solution used for action potential (AP) current clamp recordings with the removal of MgCl₂. Compounds were prepared as 100 mM stock solutions in dimethylsulfoxide (DMSO) and diluted to desired concentrations in the respective extracellular solution. The total concentration of DMSO was maintained at less than 0.2% of the total volume.

**Data Analysis.** IC₅₀ values were obtained by least square fitting of data to the Hill equation:

\[
\frac{I}{I_{control}} = \frac{1}{1 + (C/IC_{50})^{n_H}} \quad (1)
\]

where C is the concentration, IC₅₀ is the concentration that blocks 50% of the current, I is the current in presence of the drug, I_{control} is the current in the absence of drug, and \( n_H \) is the Hill slope. Steady-state inactivation data were fitted to a Boltzman equation of the form:

\[
\frac{I}{I_{max}} = \frac{1}{[1 + \exp((V-V_{1/2})/k)]} \quad (2)
\]

where \( I/I_{max} \) is the normalized current, \( I_{max} \) is the maximum current, \( V_{1/2} \) the voltage of half-maximal inactivation, and \( k \) is the slope factor. The difference between the \( V_{1/2} \) value in the presence and absence of compound is shown as \( \Delta V_{1/2} \) (mV).
The affinities for the inactivated states of the channel were calculated using the steady state inactivation curves in the presence of various concentrations of compound (Bean et al., 1983; Kuo and Bean, 1994), according to the following equation:

\[ \Delta V_{1/2} = k \log \left[ \frac{1 + (D/K_i)}{1 + (D/K_r)} \right] \]  

where \( K_i \) is the binding affinity for the inactivated state, \( K_r \) is the binding affinity for resting state (calculated from dose-response curves at -90 mV), \( D \) is the drug concentration, \( k \) is the slope of the steady state inactivation curve and \( \Delta V_{1/2} \) is the shift of the steady state inactivation curve in the presence of drug compared to control.

Time constants for recovery from inactivation were obtained using a double exponential function:

\[ Y = A_1 \left( 1 - \exp \left( -t/\tau_1 \right) \right) + A_2 \left( 1 - \exp \left( -t/\tau_2 \right) \right) \]  

where \( A_1 \) and \( A_2 \) are the amplitudes of the fast and slow exponentials, \( t \) the time, and \( \tau_1 \) and \( \tau_2 \) the fast and slow time constants. The percentage of the current represented by the fast time constant was calculated from the equation:

\[ \frac{A_1}{A_1 + A_2} \times 100\% \]  

Time constants for use-dependent block at 10 Hz were obtained using a single exponential function.

Data analysis was performed using Clampfit software (v9, Axon Instruments, CA, USA), Origin (v6, Microcal Software, MA, USA) and Excel. Statistical analyses were performed using the standard one way ANOVA followed by Tukey’s post hoc test, or the paired \( t \)-test (SigmaStat, Jandel). Averaged data are presented as mean ± standard error of the mean (S.E.M.). Statistical significance was set at \( p < 0.05 \).
6 Hz Corneal Stimulation Seizure Model. Adult male CF-1 mice (Charles River Laboratories, Wilmington, MA) weighing between 20 and 30 g were used in all experiments. Anticonvulsant activity was assessed using the 6 Hz partial ‘psychomotor’ seizure model described previously (Barton et al., 2001). Briefly, seizure activity was evoked via corneal stimulation (6 Hz, 0.2 ms rectangular pulse width, 3 s duration, 22 mA) using a Grass S48 stimulator. A drop of 0.5% tetracaine anesthetic was applied directly to the eyes of all animals at the time of test compound administration. At the time of peak effect (TPE) for each compound, a drop of 0.9% saline was placed on each eye prior to corneal electrode placement. Following stimulation, mice were observed for the presence or absence of behaviors consistent with partial seizure activity (immobility, forelimb clonus, twitching of the vibrissae, and Straub-tail). All compounds were administered intraperitoneally (i.p.) in a volume of 0.01 ml/g body weight. Drugs were dissolved in 0.5% (weight/volume) methylcellulose (MC) or 0.9% sodium chloride (SAL). If a test compound did not produce a suitable suspension in MC or SAL, then Tween 80 (not to exceed 1-2 drops) and/or dimethylsulfoxide (DMSO, <1% total volume) was used. Protection was defined as the absence of a seizure, while toxicity was defined as impairment of motor-coordination assessed in the rotorod assay. Impairment was defined as the inability of a mouse to maintain equilibrium in three consecutive trials during a one minute test on a rod rotating at 6 rpm. For each compound we evaluated the time of peak effect (TPE), the ED\textsubscript{50}, and the TD\textsubscript{50} values. The TPE was determined by assessing the protection by propofol (30 mg/kg) and HS357 (100 mg/kg) at 5, 10, 15, 30, 60 and 120 minutes following i.p. administration. The protective index (PI) value is defined as the ratio TD\textsubscript{50}/ED\textsubscript{50} and was calculated for each test compound. At the
conclusion of each experiment, animals were sacrificed in accordance with the guidelines established by the Institute of Laboratory Resources and the University of Utah’s policy on the humane care and use of laboratory animals.
Results

Propofol and analogues block Na currents in hippocampal neurons.

The chemical structures of propofol, HS245 and HS357 are shown in Figure 1. We have previously shown that the replacement of the methyl group in themisone, an anticonvulsant used in the 1940s, with a tri-fluoro group yielded a compound with potent anticonvulsant activity in the maximal electric shock (MES) animal model of epilepsy (Choudhury-Mukherjee et al., 2003) (Fig 1). In this study, we have continued to investigate the importance of both the tri-flouro and the α-hydroxyamide groups of this themisone analogue. While HS245 explores the functional consequences of overlapping the phenyl rings of propofol with our themisone analogue, HS357 explores the effects of separating the phenyl and the tri-flouro/α-hydroxyamide portions of HS245 with a six-carbon alkyl linker (Fig 1). Previous comparative molecular field analysis (CoMFA) modeling studies have suggested that compounds with longer alkyl chains have an increased affinity for the inactivated state of the cardiac Na channel (Lenkowski et al., 2004). Interestingly, the structural changes made to the propofol scaffold resulted in different Log P, or lipophilicity values for propofol, HS245, and HS357 (3.63, 3.43, and 5.74, respectively).

Propofol and derivates affinities for the inactivated state.

To determine the affinity for the resting state of the Na channel, concentration dependent curves were generated at a negative holding membrane potential of -90 mV (Fig 2). When data were fit to the Hill equation, the IC₅₀ at -90 mV (Kᵥ) values for propofol, HS245, and HS357 were 17.7 µM, 100 µM, and 24 µM with corresponding Hill slopes of 0.98, 1.84, and 1.50, respectively (Fig 2A). Interestingly, both HS245 and
HS357 had Hill slopes greater than 1, suggesting that the compounds could be interacting with more than one binding site. Example current traces are shown in Figure 2B.

Propofol has previously been suggested to have a greater affinity for the inactivated state of the Na channel (Rehberg and Duch, 1999). To test if HS245 and HS357 also demonstrated a similar affinity a two-pulse steady state inactivation protocol with a 1 s pre-pulse was used (Figure 3A-C). Under drug-free conditions, the voltage of half maximal inactivation ($V_{1/2}$) was $-56.4 \pm 1.1$ mV with a slope value ($k$) of $5.6 \pm 0.2$ mV, ($n=28$). In a similar manner to propofol, HS245 caused a concentration dependent hyperpolarizing shift in $V_{1/2}$. HS357 at the same concentrations caused a much greater shift in the $V_{1/2}$ (Table 1). Slope factors ($k$) remained unchanged from those recorded in the absence of drug.

The affinity for the inactivated state of the channel for each compound was calculated from these concentration dependent shifts in steady-state inactivation and tonic block data using the equation described previously in the data analysis section (Fig 3 D; Table 1). HS357 demonstrated a greater binding affinity for the inactivated state ($K_i = 0.2 \ \mu$M) compared to propofol ($K_i = 0.7 \ \mu$M) while HS245 had a lower binding affinity ($K_i = 1.2 \ \mu$M). Comparing the binding affinity to the resting state versus the inactivated state ($K_r/K_i$) both HS245 and HS357 exhibited approximately a four to five-fold greater $K_r/K_i$ ratio than the parent compound, propofol (Table 1).

**Propofol and analogues exhibit use-dependent block.**

Use-dependent blocking characteristics are thought to be important since they allow further block of Na channels during sustained high-frequency depolarizations known to occur during seizure activity. To this end, use-dependent block of propofol and
our compounds were examined using 10 Hz trains of depolarizing pulses to -10 mV from a holding potential of -90 mV (Fig 4). Peak current amplitude was normalized to the first pulse in each experiment. Under control conditions, accumulation of inactivated channels resulted in a reduction in peak current amplitude by 30.4 ± 4.6% (n=16). Propofol and HS357 were both tested at 30 µM, a concentration close to their IC_{50}'s. At these concentrations propofol and HS357 further reduced the current amplitude by 60.8 ± 3.2% (n=5), and 79.5 ± 6.6% (n=6), respectively (p < 0.01) after 6 seconds. Interestingly, when tested at a concentration lower than its IC_{50} (30 µM), HS245 exhibited significant (p < 0.01) use-dependent block reducing the current amplitude to 50.7 ± 8.8% (n=5). To assess the rate of development of use-dependent block, data were fitted with a single exponential function. Time constants for propofol, HS245, and HS357 were 2 ± 0.2 s, 2.9 ± 0.7 s, and 1.6 ± 0.1 s, respectively.

**Delay of recovery from inactivation.**

Use-dependent block characteristics can arise from not only affinity for inactivated channels, but also from a delay in recovery from inactivation kinetics. Therefore, we determined the effects of propofol, HS245, and HS357 on this parameter. Recovery from inactivation was assessed using a two pulse protocol (Fig 5). From a holding potential of -90 mV, a 1 s test pulse to 0 mV was applied, followed by a recovery step to -90 mV ranging from 1 ms to 100 s; currents were subsequently elicited by a second test pulse to -10 mV to assess recovery. In these experiments, compounds were assessed at a concentration close to their IC_{50} values (i.e. 30 µM for propofol and HS357 and 100 µM for HS245). Data were normalized to the current amplitude during the first test pulse and fitted using a double exponential function. Table 2 summarizes the
recovery parameters for propofol and its analogues. Under control conditions recovery from inactivation was best fit by a double exponential function representing the recovery of both fast and slow inactivated channels (Chen et al., 2006). In the presence of all test compounds, both fast and slow time constants were increased indicating a slowing of recovery kinetics. This was coupled with a significant decrease in the proportion of channels recovering with a fast time constant. The greatest modulation of recovery kinetics was observed in the presence of HS357.

**Propofol and HS357 modulate membrane excitability and epileptiform activity.**

In comparison to HS245, HS357’s actions on Na channel gating kinetics were more pronounced. In view of this, HS357 was selected to determine its effects on membrane excitability. The effects of propofol and HS357 on AP discharge and key parameters of the AP were determined. Under current clamp conditions, depolarizing current injections (0.02 - 0.16 nA, 300 ms) were used to elicit AP discharges in CA1 neurons in hippocampal slices. Typical resting membrane potential (RMP) values for CA1 neurons under these recording conditions were -64.3 ± 2.4 mV (n =11) and remained unaltered in the presence of both propofol and HS357. The effects of propofol and HS357 on APs elicited after a 300 ms current injection of 0.14 nA are shown in Figure 6. Both propofol and HS357 caused a modest reduction in the number of APs evoked. At a concentration of 30 µM, APs were reduced from 6.8 ± 1.2 to 4.3 ± 0.9 (n = 4, p < 0.05) for propofol and from 6.9 ± 1.6 to 4.9 ± 1.6 (n = 7, p < 0.05) for HS357. These effects remained unchanged in the presence of picrotoxin (100 µM), a selective GABA<sub>A</sub> antagonist. Under these conditions, HS357 (30 µM) reduced the number of APs from 6.7 ± 0.4 in control to 5.0 ± 0.72 (p < 0.05, n=3) in the presence of drug. Neither
propofol nor HS357 altered input resistance, AP amplitude, AP duration, or afterhyperpolarization amplitude. In contrast to propofol, HS357 increased the rheobase by 20 pA ($p < 0.05$, $n = 7$, data not shown). All effects were fully reversible upon washout.

To test the effects of propofol and HS357 on sustained spontaneous ictal-like AP discharges, CA1 pyramidal neurons in hippocampal slices were perfused with ACSF containing zero Mg$^{2+}$/7 mM K$^+$. The resulting enhanced neuronal excitability is thought to be comparable to epileptiform activity and is partially attributed to increased NMDA receptor activity as a result of reduced magnesium block (Mangan and Kapur, 2004).

The perfusion of hippocampal slices ($n = 9$) with our modified ACSF solution (zero Mg$^{2+}$/7 mM K$^+$) resulted in sustained spontaneous discharges in CA1 pyramidal neurons (Fig. 7). The number of APs spontaneously generated during a 100 s period prior to perfusion of the test compound was considered as control and was compared with the number of APs elicited in the presence of test compounds and following washout. Under control conditions, the average number of ictal discharges was $42.2 \pm 6.5$ ($n = 9$) and was reduced to $13.2 \pm 2.7$ in the presence of propofol ($30 \mu$M, $n = 5$, $p < 0.01$). After a 20 min period, only partial washout was observed for propofol ($24.5 \pm 5.0$; Fig 7A). In comparison, HS357 ($30 \mu$M, $n = 4$, $p < 0.01$) caused a further reduction in the number of epileptiform events reducing it to $8.8 \pm 2.0$, and was completely reversible after a 20 min washout ($40.0 \pm 15.6$). Once again, the effects of HS357 were unaffected by the presence of picrotoxin (100 $\mu$M). Specifically, the number of APs counted in a 100 s interval prior to drug application was $118 \pm 40$ and was reduced to $19 \pm 11$ in the presence of both HS357 ($30 \mu$M) and picrotoxin (100 $\mu$M; $p < 0.05$, $n = 3$). Furthermore this effect, a 15.4
± 5.9 % reduction in the number of AP discharges in the presence of picrotoxin and HS357 was not significantly different (p = 0.79) from those observed in the presence of only HS357 (17.9 ± 8.0 % reduction).

**Anticonvulsant activity and toxicity of propofol and HS357**

To determine if the modulation of Na channel gating and decreased neuronal excitability observed *in vitro* would translate into anticonvulsant activity *in vivo*, test compounds were administered systemically to male CF-1 mice and tested in the 6 Hz (22 mA stimulation) partial psychomotor seizure model. Behavioral signs of toxicity were assessed using the mouse rotorod assay (Table 3). A time of peak effect (TPE) of 1.0 hour for HS357 and approximately 5 minutes for propofol were determined following i.p. administration of the compounds. At their respective TPE’s, both propofol and HS357 prevented partial seizure activity in the 6 Hz test. The ED₅₀ observed following i.p. administration of propofol (15.8 mg/kg) was 2.8-fold lower than that observed for HS357 (56.3 mg/kg). However, the protection afforded by propofol was associated with significant sedation and motor impairment, which resulted in a TD₅₀ of 18.0 mg/kg, consistent with propofol’s anesthetic properties. In contrast, HS357 was observed to be profoundly less toxic, as indicated by its 9-fold higher TD₅₀ (155.7 mg/kg) than propofol. The observed ED₅₀ and TD₅₀ values for propofol and HS357 resulted in estimated protective indices (PI) of 1.1 and 2.7, respectively. Taken together, these results demonstrate that HS357 not only exhibits anticonvulsant activity in the 6 Hz (22 mA) psychomotor seizure model, but also possesses fewer toxic side effects than propofol.
Discussion

Propofol at sub-anesthetic concentrations exhibits anticonvulsant properties in refractory status epilepticus patients (Brown and Levin, 1998; Prasad et al., 2001; Rossetti et al., 2004). However, several studies have cautioned against the use of propofol as an anticonvulsant due to serious side effects (Claassen et al., 2002; Marik, 2004). Although propofol is known to enhance GABA currents and inhibit Ca\(^{2+}\) channels, its modulation of neuronal Na channels is thought to be a major determinant for its AED activity (Martella et al., 2005; Rehberg and Duch, 1999; Orser et al., 1994). In this study, we have synthesized two novel analogues of propofol and characterized their effects on Na channel gating. One of the propofol derivatives, HS357, inhibited spontaneously generated APs and protected against seizures generated in 6 Hz (22 mA) psychomotor model with less behavioral toxicity than propofol.

Biophysical properties of propofol analogues parallel physicochemical and structural properties.

Propofol caused tonic inhibition of Na channel currents recorded from cultured hippocampal neurons with an IC\(_{50}\) value of 17 \(\mu\)M, consistent with values reported for Na\(_{\alpha}1.2\) \(\alpha\)-subunits expressed in CHO cells (Rehberg and Duch, 1999). In contrast, addition of an \(\alpha\)-hydroxyamide to the phenyl ring of propofol resulted in HS245 and a 5-fold lower affinity for the resting state but only a 1.6-fold difference in affinity for the inactivated state. Interestingly, HS357, which incorporates an alkyl linker between the amide and phenyl groups, exhibited a comparable resting state inhibition to that of propofol, but a 3.6-fold greater affinity for the inactivated state compared to propofol. These distinct biophysical properties observed for propofol and its derivatives may
parallel differences in their physicochemical and structural properties. First, it is possible that direct addition of the \(\alpha\)-hydroxyamide moiety to the phenyl ring of propofol destabilizes the molecular interactions of HS245 with the closed state of the channel, while having little effect on interactions formed with the inactivated state (Table 1). The addition of the \(\alpha\)-hydroxyamide moiety to HS245 would increase the steric bulk around the phenyl group, and this could provide steric hindrance for HS245, allowing a less favored interaction with the resting state binding site. Conversely, addition of the aliphatic chain in HS357 affords a greater distance between the bulky \(\alpha\)-hydroxyamide moiety and the phenyl ring, which could explain HS357’s similar resting state affinity to propofol. In support of this hypothesis, mutagenesis studies have shown that replacing the large hydrophobic residues L1465, N1466, and I1469 in D3-S6 with the smaller amino acid, alanine, caused a 2-fold increase in the resting state block by lamotrigine (Yarov-Yarovoy et al., 2001). This increase in resting state block by lamotrigine was attributed to additional space made available for access to the resting state binding site.

The second distinct structural feature of HS357 is its greater spatial volume compared with propofol and HS245, which could be important for HS357’s increased affinity for the inactivated state. The addition of the alkyl chain adds approximately 10.3 Å between the phenyl and \(\alpha\)-hydroxyamide moieties of HS357. It has been proposed that the local anesthetic/AED binding site is approximately 10 Å in length (Lipkind and Fozzard, 2005) and becomes more accessible to larger molecules upon proposed conformational changes of S6 segments during activation (Yarov-Yarovoy et al., 2001; Yarov-Yarovoy et al., 2002). Given that HS357 is at least 10.3 Å in length, it would nearly occupy the entire binding site and thereby allow for stronger and more
confined \( \pi-\pi \) stacking interactions between the phenyl and aromatic residues within the receptor site. Potential interactions sites are likely to be the aromatic residues F1764 and Y1771 on D4-S6, which are critical for binding of AEDs, local anesthetics, and other therapeutic agents (Nau and Wang, 2004; Ragsdale et al., 1996; Ragsdale and Avoli, 1998a; Yarov-Yarovoy et al., 2001). In addition to these sites, it is also possible that the alkyl side chain of HS357 could form hydrophobic interactions sites with more distant residues such as L1465 and I1469 in D3-S6. These amino acids have also been suggested to form part of the AED and local anesthetic binding site (Yarov-Yarovoy et al., 2001).

In fact, mutation of these residues to alanine decreased the affinity for the inactivated state for lamotrigine, its derivatives, and etidocaine (Yarov-Yarovoy et al., 2001; Ragsdale et al., 1994; Ragsdale et al., 1996; Yarov-Yarovoy et al., 2002).

Finally, HS357 possesses a greater lipophilicity value (Log P), than propofol and HS245. An increase in lipophilicity values may contribute to HS357’s ability to readily form hydrophobic interaction within the AED binding site, consequently allowing stronger binding constants. Several studies have correlated increased Log P values with increased Na channel inhibition; indicating that hydrophobic interactions may be formed by Na channel blockers (Brown et al., 1997; De Luca et al., 2003; Yarov-Yarovoy et al., 2001).

**Greater inhibition of epileptiform discharges by propofol analogue.**

Under current clamp conditions, we determined the effects of propofol and HS357 on both evoked APs by current injection and spontaneously occurring APs induced by the perfusion of a zero Mg\(^{2+}\)/7 mM K\(^+\) ACSF solution. Spontaneous APs generated by perfusion of zero Mg\(^{2+}\) are thought to be a useful model of seizure activity
in epileptic patients (Derchansky et al., 2004; Rafiq et al., 1995). Furthermore, clinically used AEDs such as valproate, gabapentin, and carbamazepine have demonstrated efficacy in this in vitro hyperexcitability model (Arias and Bowby, 2005). Both propofol and HS357 exhibited more pronounced block of epileptiform events than current-evoked APs. These results are consistent with previous studies showing selective inhibition by propofol of low Mg\(^{2+}\)-induced epileptiform activity over current-evoked APs in cortical neurons (Martella et al., 2005). In these studies, bicuculline abolished the effects of propofol on current-evoked APs, but not on low Mg\(^{2+}\)-induced APs. In contrast, HS357’s effects on both current-evoked APs and low Mg\(^{2+}\)-induced APs were unchanged in the presence of picrotoxin, a selective GABA\(_A\) antagonist, suggesting that HS357’s actions were not mediated by the potentiation of GABA currents. However, effects on other ion channel targets cannot be ruled out.

During spontaneous epileptic events, Na channels rapidly cycle through the closed, activated, and inactivated states. According to the modulated receptor hypothesis (Hille, 1977) Na channels accumulate in high affinity drug binding conformations (i.e. open and/or inactivated states) and therefore the inhibitory effects of propofol and HS357 on epileptiform events may be explained by their greater affinities for the inactivated state. HS357’s more pronounced inhibition of epileptic events compared to propofol could be attributed to its 4-fold greater K\(_d\)/K\(_i\) ratio than propofol, resulting in greater use-dependent block and further delay of recovery from inactivation. These mechanisms would reduce Na channel availability during high-frequency repetitive firing (Ragsdale and Avoli, 1998b; Rogawski and Loscher, 2004).
**In vivo studies and therapeutic implication for a novel propofol analogue.** Our behavioral studies in the 6 Hz partial seizure model support our hypothesis that structural changes in the propofol scaffold can result in novel compounds with anticonvulsant activity. At efficacious doses (30 mg/kg), propofol caused marked sedation and ataxia, while HS357 (100 mg/kg) displayed little or no side effects at higher doses. The approximate 2-fold difference in protective index (PI) values for propofol and HS357 may again be attributed to HS357’s far greater selectivity for the inactivated over resting state of the Na channel (i.e. K_r/K_i ratios). In other words, increased K_r/K_i ratios for Na channel blockers may indicate greater *in vivo* efficacy with fewer side effects by favoring block of sustained high-frequency firing, which is known to occur during epileptic activity, without interfering with normal neuronal firing.

The greater protective index of HS357 is a desirable characteristic for a potential AED and warranted in the therapeutic treatment of seizure suppression (Brodie, 2001). Equally important to improved tolerability is the identification of new AEDs with potential efficacy in therapy-resistance patients (Brodie, 2001). Previous studies have implicated the use of the 6 Hz model (22-44 mA) for identifying potentially useful AEDs and differentiating those which may be effective for patients with therapy-resistant epilepsies (Barton *et al.*, 2001). For example, levetiracetam, an AED used to treat refractory human partial seizures, was effective in the 6 Hz model (22, 32, and 44 mA) but inactive in the maximal electroshock (MES) and subcutaneous metrazole (ScMET) seizure models (Barton *et al.*, 2001). Other anticonvulsant agents, including phenytoin, lamotrigine, and ethosuximide, were effective at blocking seizures in the 6 Hz model at 22 mA, but were unable to maintain this protection at 32 mA (phenytoin, lamotrigine) or
at 44 mA (ethosuximide). Here, we show that HS357 is not only effective in the 6 Hz model at 22 mA, but also better tolerated than its parent compound, propofol. Given the activity identified at the 22 mA current intensity, it will be important to determine the potential utility of HS357 and/or propofol in the 6 Hz partial seizure model at higher current intensities in order to determine if these compounds would offer any potential benefit for refractory seizure activity.

In summary, clinical and experimental results have established Na channel blockers as effective treatments of epilepsy. Here, we describe the effects of propofol and two derivatives, HS245 and HS357, on Na currents recorded from hippocampal neurons. HS357 demonstrated a greater affinity for the inactivated state of the Na channel and protected mice against seizures in the 6 Hz seizure model. Furthermore, HS357 displayed a greater safety margin than propofol with regards to impairment of motor coordination, suggesting that propofol analogues could be an important structural class in the development of new AEDs.
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Footnotes

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Figure Legends

**Fig. 1.** Compound structures and design. Propofol, HS245, and HS357 are examined in this study. HS245 includes a hydroxyamide moiety while HS357 incorporates a 6-carbon linker between the phenyl and hydroxyamide moieties.

**Fig 2.** Tonic block of Na currents by propofol and analogues in cultured hippocampal neurons. (A) Concentration-dependent inhibition of Na currents by propofol, HS245, and HS357. Currents were elicited by a step depolarization to -10 mV for 20 ms from a holding potential of -90 mV. Data represent the mean ± S.E.M. Smooth lines represent the least squares fit when data were fitted with the Hill equation. The values in parentheses represent the sample size for each data point. (B) Representative current traces for application of propofol and each analogue.

**Fig 3.** Effects of propofol and analogues on steady-state inactivation curves in cultured hippocampal neurons. (A-C) From a holding potential of -90 mV, pre-pulse potentials ranging from -105 to -30 mV were applied for 1s followed by a depolarization step to -10 mV for 20 ms to elicit a current. Currents were normalized to the peak current elicited from the -105 mV test potential within each experiment. Smooth lines correspond to the average of the least squares fits when data were fitted by the Boltzmann equation as described in the data analysis section. *Propofol* (10 µM) $V_{1/2} = -68.3 ± 3.4$ mV, $k = 5.3 ± 0.3$ mV, $n = 4$; *Propofol* (30 µM) $V_{1/2} = -70.8 ± 1.5$, $k = 5.2 ± 0.3$, $n = 4$. *HS245* (10 µM) $V_{1/2} = -74.5 ± 1.8$ mV, $k = 5.1 ± 0.2$ mV, $n = 5$; *HS245* (30 µM) $V_{1/2} = -81.7 ± 0.9$, $k = 5.3 ± 0.3$, $n = 4$. *HS357* (3 µM) $V_{1/2} = -69.1 ± 2.7$ mV, $k = 5.4 ± 0.1$ mV, $n = 7$; *HS357* (30 µM) $V_{1/2} = -74.9 ± 2.0$, $k = 5.8 ± 0.3$, $n = 5$. Data points represent the mean ± S.E.M.
(D) Smooth lines correspond to the average of the least squares fits when data were fitted by the equation \[ \Delta V_{1/2} = k \log \left( \frac{1 + (D/K_i)}{1 + (D/K_r)} \right) \] to calculate the affinity for the inactivated state (K_i). Data points represent the mean ± S.E.M (n = 4 - 7).

**Fig 4.** Effects of propofol and derivatives on use-dependent block at 10 Hz in cultured hippocampal neurons. (A) Currents were elicited by a 20 ms pulse from -90 mV at a frequency of 10 Hz for 6 s. Each peak Na current was normalized to current amplitude at time equals 0.1 s. Values represent the mean ± S.E.M (n = 5-6). (B) Representative current traces for control and drug conditions. Vertical scale bars are equivalent to 200 pA and horizontal scale bars are equivalent to 2 ms.

**Fig 5.** Effects of propofol and analogues on the recovery from inactivation of Na currents. Recovery from inactivation was assessed using a two-pulse protocol. (A) A pre-pulse from -90 mV to 0 mV for 1 s was applied to inactivate channels. Subsequently, neurons were then held at -90 mV for a variable period of time (1 ms – 100 s) to allow channels to recovery and then the proportion of recovered channels were assessed with a 20 ms voltage step to -10 mV. Smooth lines correspond to the average of the least squares fits when data were fitted to a double exponential function as described in the data analysis section. (B) Representative current traces are shown for control and HS357. Data points represent the mean ± S.E.M. (n = 4-5).

**Fig 6.** Propofol and HS357 decrease AP discharges in hippocampal CA1 pyramidal neuron slices. Injection of depolarizing current pulses (-0.02 to 0.16 nA, 300 ms) induced reproducible AP discharges under control conditions in CA1 pyramidal neurons. Both propofol (30 µM: A) and HS357 (30 µM: B) caused a modest decrease in firing activity within 10 minutes of drug perfusion.
Fig 7. Propofol and HS357 inhibit epileptiform activity in CA1 pyramidal neurons. Perfusion of slices with zero Mg\textsuperscript{2+}/ 7 mM K\textsuperscript{+} ACSF (20 min) caused the appearance of epileptiform activity in hippocampal CA1 pyramidal neurons. (A) Representative trace shows perfusion of propofol (30 μM) caused a moderate decrease in the ictal-like discharges which was partially reversible upon wash (20 min). (B) Representative traces demonstrates perfusion of HS357 (30 μM) caused a marked reduction in ictal-like activity which was fully reversible upon wash (20 min). Expanded time scales of 100 s are shown in the inserts and (i) control (ii) drug and (iii) wash conditions are indicated for both propofol and HS357 experiments.
Table 1. Effects of propofol and novel analogues on steady-state inactivation parameters

<table>
<thead>
<tr>
<th></th>
<th>$\Delta V_{1/2}$ (mV)</th>
<th>$K_r$ (µM)</th>
<th>$K_i$ (µM)</th>
<th>$K_r/K_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 (µM)</td>
<td>10 (µM)</td>
<td>30 (µM)</td>
<td>100 (µM)</td>
</tr>
<tr>
<td>Propofol</td>
<td>-7.9 ± 1.0</td>
<td>-11.6 ± 1.3</td>
<td>-15.5 ± 1.4</td>
<td>n.d.</td>
</tr>
<tr>
<td>HS245</td>
<td>-4.3 ± 2.2</td>
<td>-11.6 ± 1.6</td>
<td>-16.9 ± 3.6</td>
<td>-19.9 ± 3.2</td>
</tr>
<tr>
<td>HS357</td>
<td>-14.4 ± 1.5**</td>
<td>-18.8 ± 1.5**</td>
<td>-23.2 ± 1.7**</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

**p < 0.01 versus propofol with same concentration by one way ANOVA (n=4-5); n.d. (not determined)
Table 2. The effects of novel propofol analogues on recovery from inactivation parameters

<table>
<thead>
<tr>
<th>Condition</th>
<th>$\tau_1$ (ms)</th>
<th>A1</th>
<th>$\tau_2$ (ms)</th>
<th>A2</th>
<th>Fast (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.7 ± 1.7</td>
<td>0.52 ± 0.08</td>
<td>1830 ± 620</td>
<td>0.51 ± 0.07</td>
<td>52.5 ± 7.4</td>
</tr>
<tr>
<td>Propofol 30 µM</td>
<td>71.0 ± 14.1**</td>
<td>0.28 ± 0.06</td>
<td>5710 ± 780**</td>
<td>0.73 ± 0.06</td>
<td>27.8 ± 6.1*</td>
</tr>
<tr>
<td>Control</td>
<td>6.8 ± 2.5</td>
<td>0.69 ± 0.14</td>
<td>1300 ± 400</td>
<td>0.45 ± 0.06</td>
<td>59.3 ± 7.9</td>
</tr>
<tr>
<td>HS245 100 µM</td>
<td>91.5 ± 8.4*</td>
<td>0.20 ± 0.05</td>
<td>11600 ± 2400**</td>
<td>0.81 ± 0.05</td>
<td>19.7 ± 4.8**</td>
</tr>
<tr>
<td>Control</td>
<td>5.6 ± 1.0</td>
<td>0.59 ± 0.07</td>
<td>1900 ± 360</td>
<td>0.53 ± 0.05</td>
<td>58.0 ± 11.8</td>
</tr>
<tr>
<td>HS357 30 µM</td>
<td>43.1 ± 15.2**</td>
<td>0.15 ± 0.05</td>
<td>15700 ± 1100**</td>
<td>0.85 ± 0.05</td>
<td>15.2 ± 4.7**</td>
</tr>
</tbody>
</table>

*p < 0.05; **p < 0.01 versus respective control by one way ANOVA (n = 4-5).
Table 3. Anticonvulsant effects of propofol and HS357 tested using the 6 Hz (22 mA) psychomotor seizure model and rotorod toxicity

<table>
<thead>
<tr>
<th>Compound</th>
<th>ED$_{50}$ (mg/kg)</th>
<th>TD$_{50}$ (mg/kg)</th>
<th>P.I.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propofol</td>
<td>15.8 (6.09 - 92.8)</td>
<td>18.0 (13.0-24.4)</td>
<td>1.1</td>
</tr>
<tr>
<td>HS357</td>
<td>56.3 (21.6-101.7)</td>
<td>155.7 (88.7 – 8295.1)</td>
<td>~2.8</td>
</tr>
</tbody>
</table>

( ) 95% confidence interval upper limit, P.I. = TD$_{50}$/ED$_{50}$

$^a$ Poor suspension obtained at concentrations >150 mg/kg precluded accurate determination of the P.I.
Figure 1

Propofol + Themisone Analogue → HS245 → HS357
Figure 6

A  Control  Propofol 30 μM

+68 mV

20 mV  200 ms

B  Control  HS357 30 μM

+61 mV

20 mV  200 ms
Figure 7

A

0 Mg$^{2+}$/ 7 mM K$^+$ ACSF

30 μM Propofol

-60 mV

(i) (ii) (iii)

20 mV

100 s