Title Page

Modulation of sodium channel inactivation gating by a novel lactam: Implications for seizure suppression in chronic limbic epilepsy.


Increased affinity for inactivation state by novel lactam

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Abbreviations: ACSF, artificial cerebrospinal fluid; AED, antiepileptic drug; Na, sodium; LTG, Lamotrigine; TLE, temporal lobe epilepsy; YW-I-92, (3-Hydroxy-3-(4-methoxyphenyl)-1-methyl-1,3-dihydro-indol-2-one).

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Abstract

Epilepsy remains a devastating neurological disorder associated with recurrent, unprovoked, spontaneous epileptic seizures. Current treatments involve seizure suppression using antiepileptic drugs (AEDs); however, many patients remain refractory to current treatments or suffer serious side effects. In view of this continued need for more effective and safer AEDs, we have designed a novel compound, YWI92, based on a lactam structural class and evaluated its modulation of hNav1.2 currents and hippocampal neuron action potential firing. Furthermore, we have tested its AED activity using a chronic and acute rat seizure model. In a similar manner to lamotrigine (LTG), a clinically used AED, YWI92 exhibited tonic block of hNav1.2 channels and caused a hyperpolarizing shift in the steady-state inactivation curve when using a 30 second inactivating prepulse. YWI92 also delayed the time constants of channel repriming after a 30 second inactivating prepulse and exhibited use-dependent block at 20 Hz stimulation frequency. In membrane excitability experiments, YWI92 inhibited burst firing in CA1 neurons of animals with temporal lobe epilepsy (TLE) at concentrations that had little effect on CA1 neurons from control animals. These actions on neuronal activity translated into AED activity in the maximal electroshock (MES) acute seizure model (ED$_{50}$ 22.96 mg/kg) and importantly, in a chronic temporal lobe epilepsy model, where the mean number of seizures were reduced. Importantly, YWI92 exhibited no sedative/ataxic side effects at concentrations up to 500 mg/kg. In summary, greater affinity for inactivated Na channels, particularly after long depolarizing prepulses, may be important for both anticonvulsant activity and drug tolerability.
Introduction

Epilepsy is a devastating neurological disorder, characterized by recurrent spontaneous seizures either in both brain hemispheres (general seizures) or localized in one or more parts of one or both hemispheres (partial seizures). Current treatment options for patients with epilepsy involve seizure suppression through the use of a myriad of currently available antiepileptic drugs (AEDs). Unfortunately, a substantial proportion of patients (~30%) continue to experience seizures even in the presence of optimal doses of AEDs and are considered pharmaco-resistant (Remy and Beck, 2006). In addition, many patients that achieve seizure control with medications suffer from medication induced neurotoxicity, sedation, and cognitive side effects (Brodie, 2001).

Since voltage-gated sodium (Na) channels play a critical role in the initiation and propagation of action potentials in excitable cells they remain a promising target for the development of new AEDs. The Na channel consists of a pore forming α subunit, which is sufficient to induce Na currents in heterologous expression systems, and a variable number of auxiliary β subunits that modulate the gating properties of the channel (Catterall, 2000). At resting membrane potentials Na channels are closed, but open upon depolarization, giving rise to a fast (transient) inward current, and in certain cells, a slowly inactivating (persistent) current (Goldin, 2003). The modulation of both the transient and persistent Na currents are believed to be a primary mechanism of action for many clinically used AEDs such as phenytoin, carbamazepine, and lamotrigine (Catterall, 2002). An underlying property of several AEDs is their state-dependent inhibition of the Na channel. In other words, these agents selectively bind to open and inactivated states visited at depolarized potentials. This feature is best described by the modulated receptor
hypothesis (Hille, 1977), and allows AEDs to preferentially inhibit action potential (AP) bursts that occur during seizures with minimal affects on normal neuronal activity (Rogawski and Loscher, 2004).

Inactivation of Na channels has at least two kinetic time courses; fast and slow. Fast channel inactivation and recovery from inactivation occurs within milliseconds and is best described by the ‘hinged lid’ mechanism. Slow inactivation develops and recovers over a much longer time course (i.e. seconds to minutes) and is a more complex process involving structural re-arrangement of the pore and other regions of the Na channel (Ulbricht, 2005). Disruptions to these inactivation processes have been associated with the incidence of generalized epilepsy with febrile seizures plus (GEFS+) and severe myoclonic epilepsy of infancy (SMEI) (Spampanato et al., 2001). Further evaluation of these disease causing mutations has revealed subtle changes in the steady-state inactivation properties, including entry into and recovery from slow inactivated states, which likely accounts for the increased persistent Na current observed in almost all of the mutations examined (Meisler and Kearney, 2005). Preferential targeting of slow inactivated states, or states present as a result of prolonged depolarization, may represent a new molecular target for AEDs in reducing Na channel activity and suppressing seizures, as has been recently proposed as a contributing mechanism of action for the novel AED lacosamide (Vimpat®)(Errington et al., 2008).

Previous studies have identified lactams as a structural class of effective AEDs, providing protection in either the acute maximal electric shock (MES) or the subcutaneous metrazole (ScMET) animal model of acute seizures (Grimm et al., 2003). Lactams can displace [3H]batrachotoxinin A 20-α-benzoate (BTX) binding from rat brain
cerebral cortex synaptosomes, suggesting that they share a similar binding site to many of the clinically used AEDs (Clare et al., 2000). In this study, we designed a novel benzolated lactam, 3-Hydroxy-3-(4-methoxy-phenyl)-1-methyl-1,3-dihydro-indol-2-one (YWI92) based on a simplified lactam motif (Scheme 1A). Synthesis was accomplished in high yield (Scheme 1B) and its effects determined on the human neuronal Na channel isoform Na,1.2, in comparison with Lamotrigine (LTG). YWI92’s ability to selectively suppress epileptiform AP discharges in CA1 neurons from chronic temporal lobe epilepsy (TLE) animals over control non-epileptic animals was also assessed. YWI92 exhibited a greater affinity for channels inactivated during long prepulses, shifting their steady-state inactivation curves to greater hyperpolarized potentials and accelerating the development, and slowing of their recovery. Furthermore, YWI92 was more effective at suppressing action potential firing in TLE CA1 neurons over control CA1 neurons. Consistent with these in vitro results, YWI92 inhibited seizures evoked in the MES acute seizure model with no sedative or ataxic side effects, and reduced the frequency of spontaneous seizures in the TLE rat model of chronic epilepsy. These studies suggest that the lactam moiety may represent an important structural feature for the development of state selective Na channel blockers with anticonvulsant activity.
Methods:

Synthesis of YWI92: $^1$H and $^{13}$C NMR spectra were measured at 300 MHz on a GE 300 MHz NMR spectrometer. Chemical shifts were reported relative to internal CDCl$_3$ (1H, 7.26 ppm and $^{13}$C, 77.0 ppm) and CD$_3$OD (1H, 3.30 ppm and $^{13}$C, 49.2 ppm). Flash column chromatography was performed on silica gel 60 (35-75 µm) and thin-layer chromatography on silica gel 60 F254 aluminum sheets. Melting points were determined on an Electrothermal melting apparatus. HRMS spectra were recorded using electrospray ionization (ESI) or MALDI techniques at the University of Illinois Urbana Champaign Mass Spectrometry Core Facility.

YW-I-92 (3-Hydroxy-3-(4-methoxyphenyl)-1-methyl-1,3-dihydro-indol-2-one). 4-methoxyphenylmagnesium bromide (6 ml, 2.5 mmol) was added dropwise to a solution of N-methylisatin (0.40 g, 2.5 mmol) in 10 ml of anhydrous tetrahydrofuran (THF) in a 100 ml two-neck round-bottom flask under N$_2$ atmosphere at 0 °C (Scheme 1B). The reaction mixture was warmed to room temperature, stirred for 24 h and quenched by addition of 10 ml 1 N HCl. The mixture was extracted with CH$_2$Cl$_2$ (20 ml x 3). The organic layers were combined, dried over anhydrous magnesium sulfate, filtered, and the solvent evaporated. The crude product was purified by column chromatography on silica gel eluting with EtoAc/ hexane to give a light yellow solid (0.58 g, 86 %), m.p. = 148-150 °C, $^1$H NMR (300 MHz, CDCl$_3$), d 7.38-7.24 (m, 4H), 7.13-7.02 (t, J=7.5 Hz, 1 H), 6.91-6.77 (m, 3 H), 3.80-3.71 (s, 3 H), 3.22-3.14 (s, 3 H). $^{13}$C NMR (300 MHz, CDCl$_3$) d 178.3, 160.0, 144.0, 132.7, 132.3, 130.2, 127.5, 125.4, 124.0, 114.4, 109.1, 78.1, 55.8, 27.0. HRMS: calculated for C$_{16}$H$_{15}$NO$_3$ 269.11, found 269.1019.
Lamotrigine (LTG) was obtained from Sigma (St. Louis, MO). Compounds were prepared as stock solutions in dimethyl sulfoxide (DMSO) and diluted to desired concentration in perfusion solution. The maximum DMSO concentration used was 0.1% and had no effect on Na current amplitude or action potential firing.

**Sodium Channel Electrophysiology**

Human embryonic kidney (HEK) cells stably expressing human Na$_v$1.2 were a kind gift from H.A. Hartmann (University of Maryland) and were grown in DMEM/F12 media (Invitrogen, Corp, CA, USA) supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 µg/ml) and G418 (500 µg/ml; Sigma, MO, USA). Cells were grown in a humidified atmosphere of 5% CO$_2$ and 95% air at 37 °C.

Na currents were recorded using the whole-cell configuration of the patch clamp recording technique with an Axopatch 200 amplifier (Axon Instruments, Foster City, CA). All voltage protocols were applied using pCLAMP 9 software (Axon, USA) and a Digidata 1322A (Axon, USA). Currents were amplified, low pass filtered (2 kHz), and sampled at 33 kHz. Borosilicate glass pipettes were pulled using a Brown-Flaming puller (model P97, Sutter Instruments Co, Novato, CA) and heat polished to produce electrode resistances of 0.5-1.5 MΩ when filled with the following electrode solution (in mM); CsCl 130, MgCl$_2$ 1, MgATP 5, BAPTA 10, HEPES 5 (pH adjusted to 7.4 with CsOH). Cells were plated on glass coverslips and superfused with solution containing the following composition; (in mM) NaCl 130, KCl 4, CaCl$_2$ 1, MgCl$_2$ 5, HEPES 5, and glucose 5 (pH adjusted to 7.4 with NaOH). All Na channel current experiments were performed at room temperature (20-22°C). After establishing whole-cell, a minimum series resistance compensation of 75% was applied and cells were held at –100 mV for 5
minutes to account for any equilibrium gating shifts. Capacitive and leak currents were corrected for using standard P/4 protocols except during steady-state inactivation and use-dependent block protocols. After control recordings, compound solutions were applied for five minutes to allow for bath equilibration. Tonic block was assessed by comparing peak sodium current in drug free conditions to peak current when drug was present. Dose response data were fitted using the Hill equation:

\[
\frac{I_{Na}}{I_{Na\text{ peak}}} = \frac{1}{1+(C / IC_{50})^H}
\]

Where C is the drug concentration, IC_{50} is the concentration that blocks 50% of the current and H is the hill coefficient.

Conductance as a function of voltage was derived from the current-voltage relationship using the equation \( g = \frac{I_{Na}}{V-E_{Na}} \), where V is the test potential and \( E_{Na} \) is the reversal potential. The voltage dependence of activation and steady state inactivation data were fitted by the equation:

\[
y = \frac{1}{1+\exp \left( \frac{V-V_{1/2}}{k} \right)}
\]

where y is the normalized conductance \( (g/g_{max}) \) or the normalized current for activation and inactivation respectively, \( V_{1/2} \) is voltage of half-maximal activation or 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). Time constants for recovery from inactivation and development of inactivation were obtained using either a single or a double exponential function:

\[
y = A_1 (1-\exp (-t / \tau_1)) - \text{single}
\]

where \( A_1 \) is the coefficients for the exponential, t is time (ms) and \( \tau_1 \) is the time constant. 

\[
y = A_1 (1-\exp (-t / \tau_1)) + A_2 (1-\exp (-t / \tau_2)) - \text{double}
\]
where $A_1$ and $A_2$ are the coefficients for the fast and slow exponentials, $t$ is time (ms) and $\tau_1$ and $\tau_2$ are the fast and slow time constants respectively. The percentage of the current represented by the fast time constant was calculated from the equation $100\% \times \frac{A_1}{A_1+A_2}$, where $A_1$ and $A_2$ are the amplitudes of the fast and slow gating modes respectively.

The apparent affinity ($K_i$) for the inactivated state of the channel was calculated using the steady-state inactivation and dose-response curves (Kuo and Bean, 1994). Data were fit using a Boltzmann function and the $V_{1/2}$ and $k$ values were calculated. This method employs the use of the following equation:

$$K_i = \frac{D}{((1+D/K_r)/e^{\Delta h/k})-1)}$$

where $\Delta h$ is the shift of the steady state curve, $k$ is the slope of the steady state inactivation curve, $D$ is the drug concentration tested and $K_r$ is the affinity for the resting state ($IC_{50}$) taken from the dose response curve (Bean et al., 1983).

**Brain Slice Preparation.** Transverse hippocampal slices (250-300 µm) were prepared from animals (300 - 450 grams; Sprague-Dawley rats) with temporal lobe epilepsy (TLE) and age matched controls. TLE animals used were documented as having four or more spontaneous seizures per day by EEG recordings, 2 to 3 months after the induction of status epilepticus. 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, NaH$_2$PO$_4$ 1.25, CaCl$_2$ 2, MgCl$_2$ 1, L-Ascorbic acid 0.5, glucose 10, NaHCO$_3$ 25, and Pyruvate 2 (oxygenated with 95% O$_2$ and 5% CO$_2$). 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 and perfused at 2 ml/min. CA1 hippocampal neurons were visually identified by infra-red video microscopy using a Zeiss Axioscope microscope (Zeiss, Oberkochen, Germany). Whole cell current clamp recordings were performed using borosilicate glass pipettes with resistances of 3.5-4.0 MΩ when filled with an intracellular recording solution containing (in mM): Kgluconate 120, NaCl 10, MgCl₂ 2, K₂EGTA 0.5, HEPES 10, Na₂ATP 4, NaGTP 0.3 (pH adjusted to 7.2 with KOH). Action potentials were evoked with a series of depolarizing current injection steps.

**In Vivo Experiments**

**Maximal electroshock (MES) acute seizure tests.**

Adult male Sprague Dawley rats (100-150 grams) were obtained from Charles River Laboratories (Raleigh, NC) and were fed, handled and housed in a matter consistent with the recommendations of the National Council’s “Guide for the Care and Use of Laboratory Animals”. No insecticides capable of altering hepatic drug metabolism were used. All animals had free access to food (Prolab RMH 3000) and water except during the times they were used in experimental procedures. At the conclusion of each experiment, animals were euthanized in accordance with the guidelines established by the National Institutes of Health guide for the care and use of laboratory animals, the Institute of Laboratory Resources and the University of Utah’s policy on the humane care and use of laboratory animals. For the MES acute seizure test, 60 Hz of alternating current was
delivered via corneal electrodes for a period of 0.2 seconds. A drop of anesthetic solution containing 0.5% tetracaine hydrochloride in normal saline (SAL) was placed on each eye of each animal prior to placement of the corneal electrodes for stimulation.

Supramaximal seizures were elicited at a current intensity of 150 mA, five times that necessary to evoke maximal threshold seizures. Immediately following the stimulation, rats were observed for elicited seizure activity characterized by ataxia manifested by evidence of abnormal or uncoordinated gait and stance. Since individual animals may have peculiarities in gait, equilibrium and placing response, all rats used for evaluating toxicity were examined before the test drug was administered for evidence of pre-existing impairment. Protection was defined as the absence of these behaviors. All drugs were either dissolved or suspended in 0.5% (weight/volume) methylcellulose (MC) or 0.9% sodium chloride (SAL). The time of peak effect (TPE) for YWI92 was determined at 0.25, 0.5, 1, 2, 4, 6 & 8 hours following oral administration. Toxicity was determined at TPE using the above noted criteria at time points ¼, ½, 1, 2, 4, 6, 8 and 24 hours. Rats were observed by trained and experienced technicians observing for motor impairment, ataxia, or other signs of behavioral toxicity.

For each compound, the time of peak effect (TPE) was determined and then used to calculate an ED$_{50}$ and TD$_{50}$.

**Spontaneous temporal lobe seizures (TLE) model.**

YWI92 was assessed in a rat model of spontaneous temporal lobe seizures, a model that has many features in common with human temporal lobe epilepsy. The following protocol was used.
Surgery: Adult male 250-300 gram Sprague-Dawley rats received bipolar twisted pair stainless steel electrodes to either hemisphere bilaterally in the posterior ventral hippocampus for stimulation and recording (coordinates from bregma AP -5.3 mm, ML ~4.9 mm, DV ~5.0 mm, bite at ~3.3 mm) (Paxinos and Watson G, 1996). Additional monopolar indifferent reference and ground electrodes were placed over the cerebellum. All electrodes, intracerebral and reference, were attached to Amphenol connectors and secured to the skull with jeweler's screws and dental acrylic.

Induction of Status Epilepticus: One week following surgery, rats were stimulated through the hippocampal electrode to induce limbic status epilepticus using a protocol previously described by ourselves (Lothman et al., 1989). In brief, animals were stimulated for 90 minutes with 10 second trains of 50Hz, 1 ms biphasic square wave with a maximum intensity of 400 μA peak to peak delivered every 11 seconds. After 90 minutes, stimulation was stopped and hippocampal activity was recorded for a minimum of 8 hours to ensure that a prolonged period of continuous EEG seizure activity was maintained. Animals that exhibited continuous electrographic seizure activity for at least 8 hours after stimulation were at uniform risk for development of limbic epilepsy. Animals (about 15%) that did not meet the EEG criteria of minimum continuous seizure activity were not maintained, as their chance of developing chronic epilepsy was extremely low.

Following the induction of and recovery from limbic status epilepticus, rats were placed in standard laboratory housing. Three months after the induction of status epilepticus, animals were evaluated for the presence and frequency of spontaneous temporal lobe seizures, as the seizure pattern and frequency will have plateaued by this
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time (Bertram and Cornett, 1994). During the monitoring phase, rats were placed in specially designed cages, which allowed full mobility of the animals, good visualization for video monitoring, and a stable recording environment. Animals had free access to food and water, as well as a standard 12 hour light-dark cycle. Seizures were recorded and documented using a commercial computerized EEG program (Harmonie, Stellate Systems). All data are reviewed at an offline reading station connected to the vivarium computers via a local area network. The time of occurrence, behavioral severity (Racine 5 point scale) and duration for all seizures were noted.

Seizure Determination: Electrographic seizures in the rats were characterized by the paroxysmal onset of high frequency (greater than 5 Hz) increased amplitude discharges that showed an evolutionary pattern of a gradual slowing of the discharge frequency and subsequent post-ictal suppression. Seizure duration was measured from the onset of the high frequency activity or initial spike to the cessation of the terminal regular electrographic clonic activity. Testing of YWI92 was carried out on animals exhibiting a regular pattern of a minimum of 4 seizures over a 6 hour period from 10AM to 4PM every day. This choice was made to be certain that any effect following the administration of YWI92 was a true drug effect and not the result of random variation in seizure frequency.

Drug Administration: To provide stable and consistent levels, YWI92 was administered intraperitoneally. Under halothane anesthesia, a silicon tube was placed in the abdomen through a small puncture in the midline. The tube was sewn into place, and then tunneled subcutaneously to the back, where it exited just behind the electrode headset. The tube was secured to the headset through a small tube that was built into the headset. The line
was used so that the animals would not be disturbed by an intraperitoneal injection of YWI92. All containers and materials for drug injection were sterilized. YWI92 was suspended in normal saline using a high speed shear mixer, and the injections were made within 30 minutes of mixing.

Data Analysis

Electrophysiology data analysis was performed using Clampfit software (v9, Axon Instruments, CA, USA) and Origin (v6, Microcal Software, MA, USA). Statistical analyses were performed using the standard one way ANOVA followed by Tukey’s or Dunn’s post hoc test (SigmaStat, Jandel). Averaged data are presented as means ± standard error of the mean (S.E.M). Statistical significance was set at $p < 0.05$. 
Results

YWI92 inhibits hNa1.2 channel currents in a voltage-dependent manner

To determine the affinity of LTG and YWI92 for the resting state of Na\textsubscript{v}1.2 channels stably expressed in HEK 293 cells, a single pulse voltage protocol was used. Depolarization of cells from a holding potential of -100 mV to +10 mV for a duration of 12 ms evoked transient inward Na currents that inactivated within a few milliseconds. Both LTG and YWI92 inhibited the recorded Na currents in a concentration-dependent manner, which was reversible on washout (Fig. 1, A and B). The calculated IC\textsubscript{50} values for LTG and YWI92 using the Hill equation were: 2.6 mM with a Hill slope of 0.9 and 1.1 mM with a Hill slope of 1.0, respectively. At a more depolarized holding potential (-60 mV), both LTG and YWI92 yielded lower IC\textsubscript{50} value’s of 172 \(\mu\)M and 57.1 \(\mu\)M. The Hill slope for YWI92 remained unchanged while the Hill slope for LTG was reduced to 0.7. These results suggest that both LTG and YWI92 have a higher affinity for Na channels in the inactivated state over those in the resting state. At a holding potential of -60 mV, 30 \(\mu\)M YWI92 and LTG exhibited similar tonic block (33.2 \(\pm\) 3.5 % and 27.8 \(\pm\) 3.7 % respectively). We compared the effects of both compounds on Na\textsubscript{v}1.2 channel gating at this concentration.

Modulation of Na channel activation

To study activation parameters, Na currents were elicited by applying a 25 ms voltage step ranging from -80 to +20 mV in 5 mV increments from a holding potential of -120 mV (Fig. 2). In the presence of YWI92 (30 \(\mu\)M), a small hyperpolarizing shift in half-activation voltage (\(V_{1/2}\)) by -6.2 mV was observed (from \(V_{1/2} = -19.0 \pm 1.2 \text{mV; n =}\) 16...
14 in control to -25.2 ± 2.1 mV; n = 6 in the presence of YWI92: p < 0.005). Slope factors (k) were unchanged (k = -5.1 ± 0.3 mV in control and -5.7 ± 0.5 mV in presence of YWI92). LTG (30 µM) had no effect on activation parameters.

YWI92 has greater affinity for channels inactivated during long depolarizing pre-pulses.

Since both LTG and YWI92 exhibited greater tonic block at depolarized potentials, their effects on inactivation equilibrium gating were determined. Inactivation was examined using either a short 10 ms prepulse, or a longer 30 s prepulse as previously described by others (Xie et al., 2001). Cells were initially held at -120 mV then subjected to a prepulse at voltages ranging from -135 mV to +20 mV. This was followed by a test pulse to +10 mV to establish the extent of channel inactivation (Fig. 3, A-B and Table 1). LTG (30 µM) had no effect on inactivation when using a short 10 ms inactivating prepulse. In contrast, YWI92 (30 µM) caused a small, but significant (P<0.05), hyperpolarizing shift in the inactivation curve by -10 mV and increased the slope value (k).

To evaluate the affinity of LTG and YWI92 for channels inactivated by a longer 30 s inactivating prepulse a similar protocol was used except that a 50 ms pulse at -100 mV was applied following the longer depolarizing prepulse to recover unblocked fast inactivated channels (Sandtner et al., 2004). In contrast to its effects on inactivation using a short 10 ms inactivating prepulse, LTG (30 µM) significantly shifted the inactivation curve resulting from the longer depolarizing prepulse by -10.8 mV. A similar effect was seen with YWI92 (30 µM) resulting in a shift of -15.4 mV. Slope factors remained unchanged (Fig. 3C and Table 1).
From the inactivation and dose-response curves, the apparent affinity for the inactivated state ($K_i$) was calculated using the following equation: 

$$K_i = \frac{D}{((1+D/K_R)/e^{\Delta \nu/k})-1}$$

first described by (Bean, Cohen, and Tsien, 1983). Using this equation, YWI92 was calculated to have an apparent IC$_{50}$ of 7.4 µM for the channels inactivated by a long 30 s prepulse ($K_i$). Given that the affinity for the resting state of the channel ($K_r$) was previously determined to be 1.2 mM (holding potential of -100 mV), a ratio of $K_r/K_i$ suggests that YWI92 has a 135-fold greater affinity for channels inactivated during long depolarizing prepulses than over the resting state of the channel. Using the same assumption, the apparent affinity for inactivated channels for LTG was calculated to be 15.9 µM, yielding a $K_r/K_i$ ratio value of 163.

**Delay of Recovery from Inactivation at -60 mV**

During epileptic seizures neuronal firing is thought to increase in frequency. Compounds that delay recovery from inactivation ultimately reduce the number of channels available to open and pass current during prolonged depolarizations. We examined the effects of LTG and YWI92 on recovery from both short and long inactivating prepulses (Fig. 4, Table 2). To evaluate the effects of each compound on recovery after short inactivating prepulses, cells were held at -120 mV for 1 s and inactivated with a depolarizing step to 0 mV for 10 ms. Na channels were subsequently recovered at -60 mV, a typical resting membrane potential for neurons, for a variable length of time (1 ms to 100 s) and then subjected to a test pulse of +10 mV to determine the extent of recovery. Data were normalized to the peak current amplitude under control, drug-free conditions and best fitted using a single exponential function. In the
presence of LTG (30 µM), but not YWI92 (30 µM), the fast time constant (τ₁) was significantly (P < 0.05) increased, suggesting a delay in the recovery kinetics of channels inactivated using a short 10 ms inactivating pre-pulse.

To determine whether LTG and YWI92 exhibited a preference for channels inactivated by short or long prepulses, we increased the duration of the depolarization step used to 30 s. This longer inactivating prepulse allows channels to occupy additional inactivated states that have been referred to as slow-inactivated states. Recovery from these states require longer time periods of several seconds. Data were normalized to the peak current amplitude recorded under control, drug-free conditions. Under control conditions, the rates of recovery were best fit with a double exponential function. (Fig. 4 C&D, Table 2). In the presence of YWI92 and LTG (both at 30 µM), channel recovery was best fit to a single exponential function. The most profound effect was a substantial decrease in the proportion of channels that had recovered after 100 s at -60 mV in the presence of both test compounds.

Development of Inactivation at 0 mV

To determine if LTG and YWI92 could modulate the rate at which Na channels entered the inactivated state, a development of inactivation protocol was developed (Fig. 5). From a holding potential of -120 mV an inactivating prepulse to 0 mV was applied ranging from 1 ms to 100 s, followed by a step to -100 mV for 50 ms and a final step to +10 mV for 12 ms to assess the extent of Na channel inactivation. Data were normalized to the peak current amplitude and fitted using a double exponential function. Under control conditions, the time constants for the development of inactivation were τ₁ = 2.38
YWI92 significantly reduced both time constants, accelerating the rate at which Na channels developed inactivation and significantly reduced the percentage of development represented by the fast time constant $\tau_1$ compared with both control and LTG, (30 $\mu$M YWI92; $\tau_1 = 0.41 \pm 0.02$ sec; $\tau_2 = 6.40 \pm 0.62$; $A_1 = 42 \pm 4\%$: n = 4; P < 0.05). Time constants were also reduced in the presence of LTG, but were not different from control (30 $\mu$M LTG; $\tau_1 = 1.58 \pm 0.24$ sec; $\tau_2 = 12.2 \pm 1.76$; $A_1 = 65 \pm 4\%$: n = 5).

**Block by YWI92 is use-dependent**

In addition to voltage-dependent block, AEDs often exhibit use-dependent block. This characteristic is considered important since it allows the enhanced block of high-frequency action potential discharges that occur during epileptic seizures (Catterall, 1999; Rogawski and Loscher, 2004). The effects of LTG and YWI92 on a train of depolarizing voltage steps from a holding potential of -120 mV to +10 mV for 12 ms at a frequency of 20 Hz pulse are shown in figure 6. Under control, drug free conditions, accumulation of inactivated channels as a result of the protocol resulted in an 8.8 $\pm$ 1.3 % (n = 11) reduction in peak current. In the presence of LTG and YWI92 (30 $\mu$M), current amplitudes were further reduced by 15.6 $\pm$ 4.0 % (P = 0.051, n = 4) and 21.2 $\pm$ 2.7 % (P < 0.001, n = 7), respectively.

**YWI92 inhibits burst firing in CA1 neurons of chronic limbic epilepsy animals.**

Since Na channels play a critical role in the generation of action potentials, the actions of YWI92 on Na channel currents should affect action potential generation in
neurons. In order to explore this idea we examined the actions of YWI92 on membrane excitability of CA1 neurons from both control brain slices and brain slices prepared from animals with chronic temporal lobe epilepsy (TLE; fig. 7). To standardize our tests the resting membrane potential was recorded under current clamp conditions and then maintained at -65 mV (indicated by arrow) by injection of a DC current prior to stimulation. Depolarizing current injections (range - 20 pA to 260 pA, for 300 ms) were used to elicit action potential (AP) discharges from control CA1 neurons (fig. 7A) and TLE CA1 neurons (fig. 7B). Resting membrane potentials between control (-64.4 ± 0.4 mV; n = 22) and TLE CA1 neurons (-63.7 ± 0.8 mV; n =10) were not significantly different. For comparison purposes, the number of AP elicited by a depolarizing current injection of 140 pA was used to determine the inhibitory actions of YWI92. Under these conditions, the most profound observation was the significantly greater number of AP’s that were evoked in TLE neurons compared with control neurons (6.0 ± 2.1; n = 6, for TLE compared to 1.3 ± 0.2; n= 16, for control: p<0.05). In control CA1 neurons, YWI92 at 10 μM (n=3) was without effect but did decrease the number of AP’s evoked by 16.5 ± 10.5 % at 30 μM (n=5) and 67.4 ± 12.3 % at 100 μM (n=7). In contrast, YWI92 caused a profound inhibition of AP firing in CA1 neurons from TLE animals reducing the number AP’s evoked by 76.6 ± 15.8 % (n=4) and 88.8 ± 6.7 % (n=5) for 10 and 30 μM respectively. At 100 μM, a complete inhibition of AP firing was observed (n=4; fig.7C). These effects of YWI92 on AP firing were fully reversible on washout.

_YWI92 exhibits anticonvulsant activity in an acute seizure model and a chronic limbic epilepsy model._
To determine if the inhibition of epileptiform burst firing in TLE CA1 neurons would translate into AED activity, YWI92 was tested in both the maximal electroshock (MES) acute seizure model and the TLE chronic seizure model. In the MES model orally administered YWI92 yielded an ED$_{50}$ of 22.96 mg/kg. In the rotorod toxicity test a TD$_{50}$ for YWI92 could not be determined since no signs of sedation or ataxia were observed at doses up to 500 mg/kg.

In the TLE model of chronic epilepsy intraperitoneal administration of 100 mg/kg YWI92 significantly reduced the frequency of spontaneous seizures to less than 25% of baseline frequency in the 6 hours following the drug’s administration (fig. 8, $p<0.01$; n=4). Although there were also reductions in seizure duration and behavioral severity, these changes did not achieve significance.
Discussion

Lactams represent an important structural class of compounds that exhibit anticonvulsant activity (Grimm et al., 2003). In this study, we have utilized the lactam structural motif and designed a novel compound, YWI92, incorporating a rigid analogue of the α-hydroxyamide moiety, a structural feature previously shown to be important for increased affinity for the inactivated state of the Na channel (Grimm et al., 2003; Jones et al., 2007).

Lactams have been shown to displace [3H]batrachotoxinin A 20-α-benzoate (BTX) binding from rat brain cerebral cortex synaptosomes, suggesting that they share the same binding site as many clinically used AEDs (Brouillette et al., 1988; Clare et al., 2000). Here we have compared the actions of YWI92 and lamotrigine (LTG), a clinically useful AED, for inhibition of the Na channel isoform, Na,1.2. This particular isoform represents a reasonable target for the evaluation of potential AEDs since it is abundantly expressed within the hippocampus, with specific expression along fine axonal fibers and varicosities, indicating close localization to presynaptic release sites (Jarnot and Corbett, 2006). Furthermore, mutations in SCN2A, the gene encoding Na,1.2, have been associated with epilepsy, supporting its role in controlling neuronal activity (Meisler and Kearney, 2005).

A common feature of many AEDs is their ability to bind with greater affinity to open and inactivated channels over channels at rest (Kuo and Bean, 1994; Kuo et al., 1997). This voltage- and frequency-dependent block is considered critical for the selective suppression of epileptiform activity prevalent during epileptic seizures, whilst sparing normal channel function (Rogawski and Loscher, 2004). This characteristic is
best described by the modulated receptor hypothesis which states that drug affinity for the channel receptor is dependent on the channel state, transitioning from a low to high affinity site during inactivation of the channel (Hondeghem and Katzung, 1977). In agreement with the modulated receptor hypothesis, block of Na\textsubscript{v}1.2 by both YWI92 and LTG was voltage dependent, with greater block at more depolarized holding potentials, suggesting a greater affinity for inactivated channels. However, Na channels can exist in multiple inactivated states, including fast, slow and even an ultra slow inactivated state (Goldin, 2003), and it is still unclear if selective affinity for a particular inactivated state is predictive of AED activity. We determined the effects of LTG and YWI92 on channels inactivated using a short 10 ms depolarizing prepulse and on channels inactivated using a 30 s depolarizing prepulse (Sandtner et al., 2004). Using short depolarizing prepulses, channels are thought to enter a fast inactivation state, involving the short intracellular loop between domains III and IV (West et al., 1992). This state is important for the voltage dependent block by the local anesthetic lidocaine, but not for its use-dependent block (Vedantham and Cannon, 1999). Fast inactivated channels are also thought to be targeted by the anticonvulsants LTG and phenytoin, although the rate of binding is considered to be extremely slow (Kuo and Bean, 1994; Kuo and Lu, 1997). For this to occur, the channel must continue to occupy the fast inactivated conformation even after prolonged depolarizations, leading others to conclude that it may preferentially bind to slow inactivated channels rather than fast (Xie et al., 1995). Slow inactivated channels are targets for mibefradil and lidocaine, (Chen et al., 2000; McNulty and Hanck, 2004), and more recently, for the AED lacosamide (Vimpat ®)(Errington et al., 2008). Slow inactivation is distinct from fast inactivation, and does not involve the fast inactivation
gate. Instead, mutational and sulfhydryl modification studies point towards a rearrangement of the channel pore, involving the P-segment and the S6 segments (Vilin and Ruben, 2001).

Our studies suggest that YWI92 has affinity for channels inactivated during both a short and long depolarizing prepulse. YWI92 caused a small hyperpolarizing shift in the inactivation curve when using a short 10 ms prepulse, and shifted the activation curve, although recovery from inactivation was not affected. When using a longer depolarizing prepulse, YWI92 caused a hyperpolarizing shift in inactivation and attenuated the proportion of channels recovered after a 30 s depolarizing prepulse. Furthermore, YWI92’s acceleration of the development of inactivation was only apparent following inactivating prepulses that were longer than 30 ms. Although the effects of YWI92 on inactivation and recovery from inactivation were more pronounced at longer prepulses, it is difficult to conclude that YWI92 preferential associates with slow inactivated channels over fast channels since slow association with fast inactivated channels cannot be ruled out.

In contrast to YWI92, LTG had no effect on inactivation parameters when using a short 10 ms prepulse, but did cause a hyperpolarizing shift in the inactivation curve when using a longer 30 s depolarizing prepulse. Again, these differences can be accounted for by either slow binding of LTG to fast inactivated channels (Kuo and Lu, 1997), or greater affinity for slow inactivated channels (Xie et al., 1995). Interestingly, LTG did delay recovery from fast inactivation suggesting that it can bind to channels inactivated during a short 10 ms prepulse, delaying their recovery.
Several studies have now highlighted the importance of specific Na channel inactivation states in regulating neuronal activity, synaptic integration and neuronal spiking. For example, in cortical pyramidal neurons, Na channel slow inactivation accounted for the reduction in spike rate during high frequency stimulation and the eventual failure to evoke a spike (Fleidervish et al., 1996). Reduction in Na channel activity by either PKC/PKA phosphorylation or activation of G-protein coupled receptors (GPCR) has been attributed to the increased development of slow inactivated channels (Carr et al., 2003). Development of slow inactivation during high frequency action potential discharges is also thought to play a major role in attenuating action potential back propagation into dendritic regions in CA1 hippocampal neurons, controlling synaptic integration and postsynaptic firing in the axon (Jung et al., 1997). In view of the importance of slow inactivation in modulating synaptic integration and activity, YWI92’s ability to bind to channels inactivated by prolonged a depolarization could be important for the suppression of action potential firing in CA1 hippocampal neurons. It is unclear why YWI92 was more effective at inhibiting epileptiform burst firing in chronically epileptic rats. However, Na channel isoform expression patterns and gating kinetics are distinct in epileptic neurons and could exist in a state that is preferentially targeted by AEDs. (Ketelaars et al., 2001).

Protection against both acute and spontaneous seizures were assessed using the maximal electroshock (MES) model of acute seizures and an electrical stimulation model of TLE with spontaneous seizures. The TLE model has similar pathology to that of humans with mesial temporal lobe epilepsy (MTLE) (Margerison and Corsellis, 1966) and has many common parallels with human drug resistant epilepsy (Stables et al., 2003).
In the MES model, orally administered YWI92 afforded profound protection against the acutely evoked generalized tonic-clonic seizures (ED$_{50}$ of 22.96 mg/kg) without any sedative or ataxic side effects (rotorod toxicity test, TD$_{50}$ > 500 mg/kg). These actions of YWI92 are encouraging considering that phenytoin, a clinically used AED, had a similar ED$_{50}$ value of 27.5 mg/kg, but a TD$_{50}$ of 35.6 mg/kg (Lenkowski et al., 2007). Importantly, YWI92 significantly decreased the number of seizures in the TLE model, providing protection in a chronic model of epilepsy with spontaneous seizures.

In summary, we propose that a greater affinity for the inactivated state of a Na channel is important for the inhibition of epileptiform discharges observed in neurons from animals with chronic TLE and also for anticonvulsant activity in animal models of TLE. This molecular state of Na channels may be an important target for the development of new anticonvulsant medication with tolerable side effect profiles. Furthermore, the lactam moiety itself may offer an important structural lead from which future development work can begin.
Acknowledgements

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Xie X, Dale TJ, John VH, Cater HL, Peakman TC, and Clare JJ (2001)
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Footnotes

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Legends for Figures.

YWI92 was designed using a progression strategy based on a lactam pharmacophore.
The synthesis was accomplished by addition of the methoxyphenyl through a
nucleophilic addition to commercially available methylisatin.

Fig 1. Concentration dependent curves for LTG and YWI92. Na currents were elicited
by a step depolarization to +10mV for 12 ms from a holding potential of either -60 mV or
-100 mV. Dose-response relationship for A) Lamotrigine (LTG) and B) YWI92 are
shown along with example current traces of drug block at 100 µM at a holding potential
of -100 mV or -60 mV. The IC50 values at a holding potential of -100 mV and -60 mV for
LTG were 2.6 mM and 172 µM, respectively and 1.2 mM and 57.1 µM respectively for
YWI92. The chemical structures of LTG and YWI92 are shown within the respective
dose response curves. Data represent Mean ± S.E.M. Smooth lines represent the least
squares fit when data were fitted with the Hill Equation \[ \frac{I_{Na}}{I_{Na\ peak}} = \frac{1}{1+(C/IC_{50})^H} \], Where C is the drug concentration, IC_{50} is the concentration that blocks 50% of the
current and H is the hill coefficient.

Fig 2. The voltage dependence of channel conductance was derived from the current-voltage relationship as described in the methods. Under drug free conditions the V_{1/2} and
k values were -19.0 ± 1.2 mV and -5.1 ± 0.3, n=14 respectively. LTG (30 µM) had no
effect on channel activation parameter values (V_{1/2} = -20.0 ± 1.6 mV, k = -5.4 ± 0.4, n =
8). In contrast, YWI92 (30 µM) caused a significant hyperpolarizing shift of the half-
activation voltage ($V_{1/2} = -25.2 \pm 2.1 \text{ mV}$, $k = -5.7 \pm 0.5$, $p < 0.005$, $n = 6$). Slope values were unchanged between control and drug conditions. Data represent Mean ± S.E.M. Smooth lines correspond to the average of the least squares fits when data were fitted with the Boltzmann equation.

**Fig 3.** Steady-state fast and slow inactivation. Steady-state inactivation was determined using either a short conditioning pulse of 10 ms (A: LTG) and (B: YWI92) or a longer 30 s pulse (C) from a holding potential of -120 mV. Data was recorded under drug free conditions or in the presence of LTG or YWI92 (30μM). Under control drug free conditions, $V_{1/2}$ for fast inactivation was $-41.7 \pm 1.8$ mV with a slope $k$ of $9.2 \pm 0.5$ mV, $n = 10$. LTG (A) had no effect on fast inactivation ($V_{1/2} = -43.8 \pm 2.9$ mV, $k = 8.2 \pm 0.2$ mV, $n = 5$). YWI92 (B) shifted fast inactivation in a hyperpolarized direction ($V_{1/2} = -51.7 \pm 4.2$ mV, $k = 11.7 \pm 0.8$ mV, $n = 5$, $P < 0.005$).

Both LTG and YWI92 shifted the slow inactivation curve in the hyperpolarized direction (C). Control: $V_{1/2} = -47.5 \pm 0.6$ mV, $k = 9.9 \pm 0.5$ mV, $n = 9$; LTG (30 μM): $V_{1/2} = -58.3 \pm 3.9$ mV, $k = 9.9 \pm 0.3$ mV, $n = 4$, $P < 0.05$; YWI92 (30 μM): $V_{1/2} = -62.9 \pm 1.7$, $k = 10.6 \pm 0.4$, $n = 5$, $P < 0.005$. In panel D, example traces for the effects of LTG and YWI92 at a 30 s prepulse voltage of -50 mV. Horizontal bar represents a duration of 0.5 ms. Data represent mean ± S.E.M. Smooth lines correspond to the average of the least squares fits when data were fitted with the Boltzmann.

**Fig 4.** Recovery from inactivation at -60 mV is delayed. Modulation of recovery from fast and slow inactivation was examined for LTG and YWI92 at a concentration of 30
μM. Recovery from fast and slow inactivation was assessed using a two-pulse protocol. From a holding potential of -120 mV, a step to 0 mV for 10 ms was used to assess recovery from fast inactivation (A: LTG; B: YWI92) while a longer step of 30 s was applied to assess recovery from slow inactivation (C). Recovery was determined at -60 mV using a variable period of 1 ms to 100 s. The proportion of recovered channels assessed with a final voltage step to +10 mV. In panel D, example traces for the effects of LTG and YWI92 on recovery from slow inactivation at 100 s. Horizontal bar represents a duration of 1 ms. Data points represent the mean ± S.E.M. Smooth lines correspond to the average of the least squares fits when data were fitted by either a single or a double exponential function.

Fig 5. Development of slow inactivation at 0 mV. Development of inactivation was assessed using a two pulse protocol. From a holding potential of -120 mV a prepulse to 0 mV was applied ranging from 1 ms to 100 s followed immediately by a step to -100 mV for 50 ms to recover fast inactivated channel. Availability of Na channels was assessed using a step to +10 mV for 12 ms. Data points represent the mean ± S.E.M. Smooth lines correspond to the average of the least squares fits when data were fitted by a double exponential function.

Fig 6. Use dependent block by YWI92 and LTG (30 μM) was assessed using a depolarizing pulse to +10 mV for 12 ms at a 20 Hz pulse frequency for 30 pulses. Peak current amplitude was normalized to the first pulse in each experiment in order to account for tonic block. Data points represent the mean ± S.E.M.
Fig 7. YWI92 selectively inhibits action potential discharges in TLE CA1 neurons. To standardize our tests the resting membrane potential was recorded and then maintained at -65 mV (indicated by arrow) by injection of a DC current prior to stimulation. Action potentials were evoked using a series of DC injection steps from -20 pA to 260 pA in 10 pA steps for 300 ms with a 5 s inter-pulse interval. In fig. 7A the effects of 100 µM YWI92 on control CA1 neurons is shown. In Fig 7B the effects of both 30 µM and 100 µM YWI92 on CA1 neurons from TLE animals is shown. In both cases only current steps of -20, 0, 30, 60 and 140 pA DC current injections are shown for clarity. Concentration dependent effects of YWI92 on both control (10 µM; n=3, 30 µM; n=5, 100 µM; n=7: stripped box) and TLE (10 µM; n=4, 30 µM; n=5, 100 µM; n=4: filled box) neurons is shown in Fig. 7C. Data points represent the mean ± S.E.M.

Fig. 8. YWI92 (100 mg/kg; n=4) reduces the mean number of seizures in animals with chronic TLE over the 6 hours following drug administration (vertical frequency scale is seizures/6 hours). Although there were reductions in mean seizure duration (seconds) and in behavioral severity (Racine 5 point scale; BSS) these changes did not achieve significance. Data points represent the mean ± S.E.M. * P <0.01.
Tables

**TABLE 1. Steady state inactivation parameters.**

<table>
<thead>
<tr>
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<th>Fast Inactivation</th>
<th>Slow Inactivation</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>(V_{1/2}) (mV)</td>
<td>(k) (mV)</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>-41.7 ± 1.8</td>
<td>9.2 ± 0.5</td>
</tr>
<tr>
<td><strong>YWI92 (30 µM)</strong></td>
<td>-51.7 ± 4.2^a^</td>
<td>11.7 ± 0.8^a,b^</td>
</tr>
<tr>
<td><strong>LTG (30 µM)</strong></td>
<td>-43.8 ± 2.9</td>
<td>8.2 ± 0.2</td>
</tr>
</tbody>
</table>

^a\ P<0.05 vs control ; ^b\ P<0.01 vs LTG ; ^c\ P<0.001 vs control.
**TABLE 2. Recovery from inactivation.**

<table>
<thead>
<tr>
<th></th>
<th>Recovery from Fast Inactivation</th>
<th>Recovery from Slow Inactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\tau_1$ (msec)</td>
<td>n</td>
</tr>
<tr>
<td>Control</td>
<td>17.5 ± 1.5</td>
<td>8</td>
</tr>
<tr>
<td>YWI92 (30μM)</td>
<td>19.6 ± 0.8</td>
<td>4</td>
</tr>
<tr>
<td>LTG (30 μM)</td>
<td>21.2 ± 1.8</td>
<td>4</td>
</tr>
</tbody>
</table>

*P<0.05 vs control; P<0.001 vs control; P<0.001 vs LTG.*
Scheme 1

A. Design strategy

Simplified lactam motif

B. Synthesis of YWI92

86% yield
Figure 1A

- $V_H = -60 \text{ mV}$
- $V_H = -100 \text{ mV}$

Fractional Block vs. Concentration LTG (μM)

- LTG 100 μM
- Control

$V_H = -60 \text{ mV}$

$V_H = -100 \text{ mV}$
Figure 1B

Concentration YWI92 (μM) vs. Fractional Block

- $V_H = -100$ mV
- $V_H = -60$ mV

100 μM YWI92 control

100 μM YWI92

200 pA 1 ms

fractional block
Figure 3

A

Normalized Na Current

Voltage (mV)

-120 -80 -40 0

0.0 0.5 1.0

Control

30 μM LTG

B

Normalized Na Current

Voltage (mV)

-120 -80 -40 0

0.0 0.5 1.0

Control

30 μM YWI92

C

Normalized Na Current

Voltage (mV)

-90 -60 -30

0.0 0.5 1.0

Control

30 μM LTG

30 μM YWI92

D

Normalized Na Current

Voltage (mV)

-120 -80 -40 0

0.0 0.5 1.0

Control

YWI92

LTG

control

control
Figure 4

A

Normalized Na Current

Time (ms)

Control
30 μM LTG

B

Normalized Na Current

Time (ms)

Control
30 μM YWI92

C

Normalized Na Current

Time (ms)

Control
30 μM LTG
30 μM YWI92

D

LTG
YWI92
control
control

0 mV / 30 s
-60 mV
+10 mV

-120 mV
T = 1 ms to 100 s
Figure 5

Normalized Na Current

Time (ms)

-120 mV to 100 mV

T = 1 ms to 100 s

Control

30 μM LTG

30 μM YWI92
Figure 6

Normalized Na Current

- 1.0
- 0.8
- 0.6
- 0.4
- 0.2
- 0.0

Pulse #

- 0
- 10
- 20
- 30

Control

30 µM LTG

30 µM YWI92

-120 mV
+10 mV
12 ms
x 30 (20 Hz)
Figure 7

A

control

100 μM YWI92

Washout

20 mV

100 ms

B

TLE

30 μM YWI92

100 μM YWI92

Washout

20 mV

50 ms

C

Number of Action Potentials (%)

<table>
<thead>
<tr>
<th>YWI92</th>
<th>10 μM</th>
<th>30 μM</th>
<th>100 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>TLE</td>
<td>10</td>
<td>30</td>
<td>100</td>
</tr>
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