Intravenously Administered Ganaxolone Blocks Diazepam-Resistant Lithium-Pilocarpine-Induced Status Epilepticus in Rats: Comparison with Allopregnanolone^S

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ABSTRACT

Ganaxolone (GNX) is the 3 β -methylated synthetic analog of the naturally occurring neurosteroid, allopregnanolone (ALLO). GNX is effective in a broad range of epilepsy and behavioral animal models and is currently in clinical trials designed to assess its anticonvulsant and antidepressant activities. The current studies were designed to broaden the anticonvulsant profile of GNX by evaluating its potential anticonvulsant activities following i.v. administration in treatment-resistant models of status epilepticus (SE), to establish a pharmacokinetic (PK)/pharmacodynamic (PD) relationship, and to compare its PK and anticonvulsant activities to ALLO. In PK studies, GNX had higher exposure levels, a longer half-life, slower clearance, and higher brain penetrance than ALLO. Both GNX and ALLO produced a sedating response as characterized

by loss of righting reflex, but neither compound produced a full anesthetic response as animals still responded to painful stimuli. Consistent with their respective PK properties, the sedative effect of GNX was longer than that of ALLO. Unlike other nonanesthetizing anticonvulsant agents indicated for SE, both GNX and ALLO produced anticonvulsant activity in models of pharmacoresistant SE with administration delay times of up to 1 hour after seizure onset. Again, consistent with their respective PK properties, GNX produced a significantly longer anticonvulsant response. These studies show that GNX exhibited improved pharmacological characteristics versus other agents used as treatments for SE and position GNX as a uniquely acting treatment of this indication.

Introduction

Naturally occurring neurosteroids, such as allopregnanolone (ALLO), elicit a broad range of anticonvulsant and psychotherapeutic responses in experimental animal models and are currently being evaluated for these activities in human clinical trials (Kokate et al., 1994; Frye, 1995; Kanes et al., 2017; Rosenthal et al., 2017). Neurosteroids elicit their anticonvulsant activities through positive allosteric modulation of endogenous GABA_A receptors located in the central nervous system (Belelli and Lambert, 2005; Belelli et al., 2006). Both neurosteroids and benzodiazepines positively modulate synaptically located GABA_A receptors comprised of α and γ subunits (Campo-Soria et al., 2006). However, neurosteroids act via a distinct binding site on the GABA_A receptor and, unlike benzodiazepines, also modulate extrasynaptic GABA_A receptors that are comprised of α and δ subunits (Akk et al., 2004; Belelli and Lambert, 2005; Belelli et al., 2006; Campo-Soria et al., 2006; Sigel and Steinmann, 2012). This distinctive receptor selectivity confers a unique pharmacological profile to neurosteroids. However, the utility of naturally

occurring neurosteroids as therapeutics is limited by their pharmacokinetic (PK) liabilities, including lack of oral bioavailability and metabolic stability (Kokate et al., 1994; Frye, 1995; Carter et al., 1997; Martinez Botella et al., 2015).

Ganaxolone (GNX; CCD-1042; 3 β -methyl-3 α -ol-5 α -pregnan-20-one; 3 α -hydroxy-3 β -methyl-5 α -pregnan-20-one) differs from naturally occurring ALLO by addition of a methyl group at the 3-position (Carter et al., 1997). The 3 β -methylation prevents back conversion to the hormonally active 3-keto derivative, eliminates affinity to the nuclear hormone progesterone receptor, and confers metabolic stability and oral bioavailability in experimental animals and humans (Carter et al., 1997; Nohria and Giller, 2007). Moreover, this chemical modification does not meaningfully modify the potency, efficacy, or selectivity of GNX to GABA_A receptors (Carter et al., 1997; Nik et al., 2017).

GNX is effective in a broad range of animal models of epilepsy and behavioral disorders but exhibits important pharmacological differences from the benzodiazepine class of GABA_A receptor modulators (Carter et al., 1997; Reddy and Rogawski, 2000, 2010; Pinna and Rasmusson, 2014; Yum et al., 2014). Unlike benzodiazepines, repeated GNX administration does not induce tolerance to the anticonvulsant response, and there is greater separation between anticonvulsant

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ABBREVIATIONS: AED, antiepileptic drug; ALLO, allopregnanolone; ANOVA, analysis of variance; CSE, convulsive SE; EEG, electroencephalographic; FFT, Fast-Fourier transform; GNX, ganaxolone; JVC, jugular vein catheter; PK, pharmacokinetic; SE, status epilepticus.

and sedating doses (Gasior et al., 1997, 2000; Mares and Stehlikova, 2010). On the basis of these distinctive pharmacological properties and broad preclinical efficacy, GNX is currently being evaluated for behavioral effects and anticonvulsant activities in clinical studies (Younus and Reddy, 2018).

Status epilepticus (SE) is an especially severe and life-threatening condition that frequently occurs in patients with epilepsy, as well as individuals without a history of epilepsy. Patients in SE almost always require treatment with parenterally (typically i.v.) administered drugs (Glauser et al., 2016). In clinical settings, the first-line treatment of control of SE is i.v. administration of benzodiazepines (Glauser et al., 2016). In patients for whom benzodiazepine treatment fails, the guidelines call for sequential treatment with standard anticonvulsant drugs such as phenytoin, valproic acid, phenobarbital, and/or levetiracetam. If SE remains uncontrolled, treatment with general anesthetics such as pentobarbital or propofol is initiated (Glauser et al., 2016). Patients with SE become progressively refractory to treatment over time from onset, and up to 30% of patients with SE cannot be successfully treated and die within 30 days (Al-Mufti and Claassen, 2014; Trinka et al., 2015). Thus, there is a clear unmet medical need for additional therapeutics effective against treatment-resistant forms of SE.

The lithium-pilocarpine rodent model of SE is a clinically translatable model of SE (Jones et al., 2002; Curia et al., 2008). Both the rodent model and clinical SE exhibit convulsive and electroencephalographic (EEG) seizures, mortality, and, in subjects that survive, cognitive deficits and neuronal degeneration (Lehmkuhle et al., 2009; Tang et al., 2011; White et al., 2012). Moreover, animal subjects exhibit similar response profiles to treatments that are effective in clinical SE (Jones et al., 2002; Zheng et al., 2010; Pouliot et al., 2013). The current studies were conducted to both evaluate the anticonvulsant efficacy of i.v.-administered GNX with administration delays up to 1 hour after seizure onset, and to establish a PK/pharmacodynamics relationship that would differentiate GNX from existing treatments for SE. These studies were additionally designed to compare GNX with ALLO with respect to degree of efficacy and duration of action in this preclinical model of benzodiazepine-resistant SE.

Materials and Methods

Drugs and Chemicals

Captisol vehicle (sulfobutylether- β -cyclodextrin) was acquired from Ligand (San Diego, CA). GNX and ALLO were formulated in 30% captisol/sterile water at concentration of 2.5 mg/ml. The GNX concentration in this formulation was kept constant for all studies, and dose levels were modified by adjusting dosing volume. All other chemicals were provided by standard commercial chemical suppliers.

Animals

Studies measuring behavior and EEG seizure activity were conducted at Melior Discovery (Exton, PA). Behavioral convulsive SE (CSE) studies were conducted at Neuroadjuvants (University of Utah, Salt Lake City, UT). PK studies were conducted at Bayside Biosciences (Santa Clara, CA) and Melior Discovery. Bioanalysis of drug plasma and brain levels was conducted at Climax Laboratories (San Jose, CA). The experimental procedures were approved by and conducted in accordance with the guidelines in the *Guide for the*

Care and Use of Laboratory Animals from the National Research Council for the respective institutions. Male Sprague–Dawley rats were used for all studies and were provided by either Charles River Laboratories (Raleigh, NC) or Harlan Laboratories (Frederick, MD). Rats were approximately 300 g at time of studies, except for CSE studies, in which rats were 100–150 g (4–6 weeks old) at time of the study. Prior to and during the study, animals were given food and water ad libitum and were maintained on a 12/12-hour light/dark schedule.

PK Studies

For PK studies, rats were administered GNX or ALLO via i.v. tail vein injection with blood and brains collected 5 minutes and periodically up to 8 hours after administration. There were four rats per treatment group/time point for each study. Blood was collected twice per animal, after the second blood draw; rats were then anesthetized, perfused with cold saline solution, and euthanized, and brains were collected for analysis. Plasma was prepared from blood and analyzed for levels of GNX and ALLO. Brains were homogenized in acetonitrile and centrifuged, and the resulting supernatant was analyzed for GNX and ALLO levels. Plasma and brain levels of GNX and ALLO were measured by liquid chromatography with tandem mass spectrometry (LC/MS/MS) analysis. Levels were compared with a standard curve of each compound that was prepared in the appropriate biologic matrix.

Behavioral Impairment Studies

Adult male rats (four per treatment group) were evaluated for behavioral response after i.v. administration of test compounds and compared with vehicle-treated rats. GNX and ALLO were administered via tail vein bolus injection, and rats were monitored for behavioral sedating effects. Rats were scored as follows: 0 = awake, absence of sedation, no change in observed locomotion or behavior; 1 = light sedation, slowed movement, intact righting reflex; 2 = sedation, loss of righting reflex, responsive to toe-pinch reflex; 3 = anesthesia, loss of toe-pinch reflex.

Behavioral CSE Studies

Male, Sprague–Dawley rats were divided into 20 treatment groups, as follows: Treatment groups 1–5 (administration at time of SE onset): 1) vehicle, 2) ALLO, 3) GNX (6 mg/kg), 4) GNX (9 mg/kg), 5) GNX (12 mg/kg); treatment groups 6–10 (administration 15 minutes after SE onset): 1) vehicle, 2) ALLO, 3) GNX (6 mg/kg), 4) GNX (9 mg/kg), 5) GNX (12 mg/kg); treatment groups 11–15 (administration 30 minutes after SE onset): 1) vehicle, 2) ALLO, 3) GNX (6 mg/kg), 4) GNX (9 mg/kg), 5) GNX (12 mg/kg); and treatment groups 16–20 (administration 60 minutes after SE onset): 1) vehicle, 2) ALLO, 3) GNX (6 mg/kg), 4) GNX (9 mg/kg), 5) GNX (12 mg/kg). There were 8–12 rats/treatment group/time point. Twenty-four hours prior to pilocarpine treatment, rats were administered lithium chloride (127 mg/kg; i.p.). On the study day, the rats then received pilocarpine hydrochloride (50 mg/kg; i.p. in 0.9% saline) and were continuously monitored carefully for the presence or absence of convulsive seizure activity by an experienced experimenter blinded to treatment condition. Animals were scored for seizure activity, according to the Racine scale (Racine, 1972): stage 3—bilateral forelimb clonus, stage 4—bilateral forelimb clonus and rearing, and stage 5—bilateral forelimb clonus with rearing and falling. SE onset was defined as presentation of a Racine stage 3 or greater seizure onset of stage 3, or greater seizure was taken as the onset of convulsive SE. Administration of pilocarpine induced stage 3 or greater seizures within 5–20 minutes.

GNX, ALLO, or vehicle was administered via tail-vein injection over a period of 20 seconds or less. Dose levels were adjusted by altering the dose volume. Test agent was administered at 0, 15, 30, or 60 minutes after SE onset. All rats were continuously observed and scored for stage 3–5 seizure severity for 120 minutes postdrug administration, and an experienced experimenter blinded to treatment conditions noted any accompanying behavioral effects. Animals

were considered protected with cessation of CSE activity (stage 3–5 seizures) at any point during the 120-minute observation period. Animals were often noted to have cessation of seizure activity within 15 minutes of compound administration. Any sedation was also noted by an experienced investigator blinded to treatment condition. At the conclusion of the 120-minute observation period, each surviving animal was administered a 3 ml dose of lactated Ringer's solution to replace any SE-induced fluid loss.

All animals were retained for 24 hours following onset of CSE for assessment of weight change and survival. The number of rats that survived the treatment paradigm in each group was also recorded and compared between study groups and time points. All surviving rats were euthanized 24 hours after CSE onset.

EEG Studies

Surgery. EEG activity was recorded in rats using standard methodology described elsewhere (Gruner et al., 2009). EEG signals were recorded from stainless steel screw electrodes chronically implanted in the skull ($0.80 \times 1/4$; Plastics-One, Roanoke, VA). One electrode was located 3.0 mm anterior to bregma and 2 mm to the left of midline, and the second 4.0 mm posterior to bregma and 2.5 mm to the right of midline. A ground electrode was located just rostral to the posterior skull ridge. The animals were allowed to recover from EEG surgery for 1 week, after which they underwent surgery to implant a jugular vein catheter (JVC) using a modified method described previously (Foley et al., 2002). Catheters were maintained with daily administration of heparin solution to maintain patency until time of study. After JVC implantation, animals were allowed to recover for 1 week prior to drug testing.

EEG Recording. One day prior to the initiation of seizure induction, animals were placed into a recording container ($30 \times 30 \times 30$ cm) with ad libitum access to food and water. The recording container was located inside a sound attenuation cabinet (ENV-018V; Med Associates, St. Albans, VT) that contained a ventilation fan, a ceiling light (light on from 7 AM to 7 PM), and a video camera.

Cortical EEG signals were fed via a cable attached to a commutator (Plastics-One), then to an amplifier (model 1700; $1000\times$ gain; A-M Systems; Carlsborg, WA), band pass filtered (0.3–1000 Hz), and finally digitized at 512 samples per second using ICELUS acquisition/sleep scoring software (M. Opp, University of Michigan, Ann Arbor, MI) operating under National Instruments (Austin, TX) data acquisition software (Labview 5.1) and hardware (PCI-MIO-16E-4).

EEG Seizure Induction, Onset Identification, and Treatment. To induce seizures, rats were dosed with lithium chloride (127 mg/kg; i.p.) before placing them in the recording chamber approximately 20 hours prior to recording onset. On the day of seizure induction, baseline EEG activity was recorded for 2 hours, after which food was removed from the cage and scopolamine methyl-bromide (1 mg/kg; i.p.) was administered. Thirty minutes later, pilocarpine (50 mg/kg; i.p.) was administered to induce seizure.

The time of SE onset was initially indicated by the presence of large amplitude slow wave EEG activity. Animals were continuously observed by video for the onset of tonic fore- and hind limb seizures (stage 3 on the Racine scale) that typically lasted several seconds and was coincident with large-amplitude EEG activity (Racine, 1972). This behavior was taken as the time of SE onset.

Treatments were administered via i.v. bolus injection via a JVC at 15 or 60 minutes after SE onset, after which EEG recording continued for 5 hours. The health of the animals was monitored throughout the recording period. In animals lacking postural support, core body temperature was monitored by rectal probe and a heating pad was used as needed to maintain temperature around 37°C . Animals were assessed for loss of righting reflex and reflex pain response to toe pinch. Other atypical or abnormal behavior or health issues, including mortality, were noted.

EEG Seizure Analysis. EEG power ($\mu\text{V}^2/\text{Hz}$) was analyzed by Fourier analysis [Fast-Fourier transform (FFT)] in 1 Hz frequency

bins from 1 to 96 Hz using the ICELUS software. Epochs containing artifacts were determined by visual inspection of the EEG recordings and excluded from the analysis.

Customized frequency ranges were selected based on inspection of the full frequency range and chosen as follows: 0–10 (0.3 up to 10 Hz, etc.); 10–30; 10–30; 30–50; 50–96 Hz. Initial EEG power analysis consisted of determining the average power (in mV^2/Hz) over successive 5-minute time periods. To minimize variables such as electrode size, contact with the brain, spacing, etc., several procedures were applied to the data. First, data were log transformed to linearize EEG power versus frequency and to minimize biasing of the results by the relatively large amplitude of low-frequency EEG activity. Second, baseline power normalization was used to minimize across-animal variations in EEG power due to differences in electrode and tissue characteristics. The baseline EEG for 2 hours prior to scopolamine treatment was used to normalize EEG power across animals. Specifically, the average integrated log-FFT value for the 2-hour baseline period from 0 to 96 Hz was used to obtain a single normalization constant K_{norm} for each animal:

$$K_{\text{norm}} = \sum_{f=0}^{96} \sum_{t=-120}^{-10} \log(\text{FFT}); f = \text{frequency (Hz)}; t = \text{time (min)}$$

K_{norm} was subtracted from all FFT power values prior to further processing. This procedure adjusted the average of the total baseline EEG power for each animal to zero. Following this procedure, the mean normalized baseline EEG power curves for each group closely overlapped. Third, the normalized EEG power values were initially averaged into successive 5-minute bins and then consolidated into the following additional activity time periods: 1) baseline, 2) scopolamine, 3) pilocarpine, 4) SE (the time between SE onset and treatment), and 5) post-treatment intervals: 0–15, 15–30, 30–60 minutes, and 1–2, 2–3, 3–4, and 4–5 hours. Fourth, each frequency range was evaluated individually as a function of time. For this purpose, the baseline EEG power value at each frequency for each animal was subtracted from the values at subsequent time points, resulting in a baseline value of 0 for each frequency range.

Statistical Analysis

All data are expressed as the mean $\pm$ S.E.M. For PK studies, the differences between GNX and ALLO were determined by two-way analysis of variance (ANOVA), followed by Holms–Sidak test. Individual PK parameter differences were determined by t test. For behavioral tests, data were analyzed by a nonparametric t test. For the CSE studies, the probit method was used to calculate statistical differences between treatment groups (Finney, 1971). For EEG studies, EEG power (baseline subtracted values) was evaluated by two-way ANOVA (treatment and time period) at each frequency range using Prism GraphPad (version 6). A post hoc Bonferroni multiple comparison test was used to determine differences between treatments at any given time point. Significance was set at $P < 0.05$ for all comparisons.

Results

Pharmacokinetics. Two PK studies were conducted. In the first study (shown in Fig. 1), GNX was administered at dose levels of 6, 9, 12, and 15 mg/kg (i.v.; bolus), and plasma levels of GNX measured out to 4 hours. GNX administration (at all doses) produced a first-order elimination curve with a linear dose-proportional exposure ($R^2 = 0.96$) from 6 to 15 mg/kg.

In the second study (shown in Fig. 2), GNX was compared with ALLO (both at 15 mg/kg; i.v.; bolus), and plasma measured out to 4 hours and brain levels measured at 0.25, 1, and 3 hours after administration. Both compounds were

Fig. 1. GNX PK. GNX was administered i.v. to rats, and blood was collected at time points between 5 minutes and 4 hours after administration. Plasma was prepared and analyzed for levels of GNX by liquid chromatography with tandem mass spectrometry (LC-MS-MS). (A) PK curve. (B) Exposure levels as measured by area under the curve. Each data point represents the average $\pm$ S.E.M. from four animals.

formulated identically in 30% captisol solution. In this study, GNX- and ALLO-administered i.v. exhibited first-order elimination curves. GNX plasma levels were significantly higher than ALLO plasma levels at each individual time point after administration. Moreover, overall exposure levels (as measured by area under the curve) of GNX were significantly higher (>2 -fold) than those of ALLO at the same dose level. ALLO exhibited a higher clearance than GNX (4.98 vs. 2.21 $\text{mg} \times \text{h/l}$). The peak brain levels (C_{max} brain) of GNX and ALLO were at the earliest time point measured (15 minutes). GNX levels were 2- and 3-fold higher than those of ALLO levels at 15 minutes and 1 hour, respectively, consistent with a 30% greater half-life. Overall, brain exposure levels of GNX were also more than 2-fold those of ALLO. Table 1 shows combined PK parameters for GNX at all doses and ALLO at a dose of 15 mg/kg.

Behavior. Sedative responses produced by GNX and ALLO are shown in Fig. 3. When administered i.v., both compounds produced sedation characterized by a loss of righting reflex. GNX produced a dose-dependent increase in sedation duration at doses of 6–15 mg/kg. Using restoration of the righting reflex as measure of recovery, the 6 mg/kg dose

group was nearly fully recovered within 30 minutes. By 1 hour the 9 mg/kg group was fully recovered, the 12 mg/kg group was partially recovered, and the 15 mg/kg group remained maximally sedated. In contrast, ALLO-treated animals (15 mg/kg) were fully recovered by 1 hour after administration. Rats were never fully anesthetized with either compound; i.e., they still showed a toe-pinch reflex (score of 2 of 3). This result contrasts with anesthetic agents such as pentobarbital that induce loss of toe-pinch response at dose levels >30 mg/kg (score of 3; data not shown) and therefore are considered to be fully anesthetized (Pouliot et al., 2013).

CSE. The effects of GNX and ALLO in the lithium-pilocarpine CSE model are shown in Table 2. All vehicle-treated rats (39 in total) exhibited convulsive seizures within the study time period. Administration of vehicle at all four time points tested was without any effect on CSE.

GNX produced a significant dose-dependent protection from seizure at all delayed administration time points (0, 15, 30, and 60 minutes) (Table 2). There was a significant effect of GNX at each administration time point (0 minute, $P < 0.01$; 15 minutes, $P < 0.05$; 30 minutes, $P < 0.001$; 60 minutes, $P < 0.001$). ALLO at 15 mg/kg also produced a significant protection from seizure at all time points ($P < 0.0001$). There were no significant differences between ALLO and GNX at any given time point.

Survival 24 hours after SE onset is another useful metric for behavioral CSE studies (Table 2). In this study, 17 of 39 total vehicle-treated rats (independent of treatment time after SE onset) survived 24 hours after seizure induction. Administration of GNX significantly improved survival relative to vehicle-treated rats at all time points tested (0 minute, $P < 0.05$; 15 minutes, $P = 0.05$; 30 minutes, $P < 0.01$; 60 minutes, $P < 0.001$). Administration of ALLO increased survival at 15 mg/kg ($P < 0.001$) at all time points tested with no difference from GNX treatment (Table 2).

EEG Seizure Response. Pilocarpine administration produced significant abnormalities and long-term increases in EEG response that closely resembled SE. The SE-onset times for all animals were between 15 and 45 minutes (26.0 ± 1.5 minutes; average $\pm$ S.E.M.) after pilocarpine administration and were not different between the treatment groups (ANOVA, $P > 0.4$).

EEG seizure responses are shown in Figs. 4–7. Figure 4 shows EEG recordings of representative animals in the vehicle, ALLO, and GNX treatment groups. These recordings are from 10 minutes prior to SE onset (time “A”) and up to 5 hours after SE onset. Figure 5 shows the EEG data that have been transformed to total EEG power (0–96 Hz). Figures 6 and 7 show the EEG power data divided into four frequency ranges, 0–10, 10–30, 30–50, and 50–96 Hz, and provide more detailed representation of the seizure pattern.

Differences in seizure response were found depending on the frequency range that was examined. For example, EEG seizures in the 0–10 Hz range remained elevated until the end of the monitoring period (5 hours after SE onset), whereas between 10 and 70 Hz EEG power declined over time. At 50–96 Hz, EEG power was still above baseline for up to 3 hours after SE onset (Figs. 6 and 7). In Supplemental Material, we show representative EEG traces (each 8 seconds in duration) at various times up to 4 hours after treatment (Supplemental Fig. 1). These traces show that the seizure pattern in vehicle-treated animals changes over time with rhythmic spike-wave complexes at later time points.

Fig. 2. Comparison of GNX to ALLO PK and brain penetrance. GNX and ALLO were administered i.v., and blood and brains were collected at time points between 5 minutes and 3 hours after administration. (A) Plasma PK curves. (B) Plasma exposure levels as measured by area under the curve. (C) Brain PK curves. (D) Brain exposure levels as measured by area under the curve. Data expressed as average $\pm$ S.E.M. Statistical significance: * $P < 0.05$; ** $0 < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

Effects of ALLO and GNX Administered 15 Minutes Post-SE Onset. ALLO (15 mg/kg) administered 15 minutes postseizure onset reduced EEG power beginning almost immediately, with a maximal effect achieved for approximately 1 hour postdosing (Figs. 4 and 5). ALLO-mediated suppression of EEG seizure response was observed across all frequency ranges (Fig. 6). However, there were differences in duration of suppression at these frequency ranges. At the lower frequency ranges (0–10 Hz), a significant reduction was present until 2 hours (Table 3). At higher frequency ranges (10–96 Hz), a significant reduction was sustained for only 1 hour after administration (Table 3).

GNX exhibited a dose-dependent suppression of EEG seizure response (Figs. 4 and 5). The duration of EEG suppression was

longer than that of ALLO. At 6 mg/kg GNX was inactive; i.e., EEG activity in all animals in this group was not different from vehicle group. At dose levels of 12 and 15 mg/kg, GNX reduced EEG power below the vehicle level for up to 5 hours (Figs. 4 and 5). When divided into distinct frequency ranges, GNX showed differences in duration of seizure suppression. At 0–10 Hz, EEG power was suppressed until the end of the study. At 10–30 Hz, EEG suppression lasted for 2 hours, whereas, at frequency ranges of 30–50 and 50–96 Hz, EEG power was reduced to values at or below the baseline level until study end (Fig. 6; Table 3).

Data in a supplemental figure (Supplemental Fig. 1) show that in GNX-treated animals, EEG pattern returned to normal (preseizure; baseline) and showed little high-amplitude spiking

TABLE 1

Comparison of ALLO to GNX:PK parameters

Alloprenanolone and GNX were administered via i.v. tail vein injection. Blood was collected between 5 minutes and 6 hours after administration, and plasma was prepared. Brains were collected between 30 minutes and 3 hours after injection. Samples were analyzed as described in *Materials and Methods*. There were four animals per time point.

Parameter	GNX (6 mg/kg)	GNX (9 mg/kg)	GNX (12 mg/kg)	GNX (15 mg/kg)	ALLO (15 mg/kg)
Plasma exposure (ng $\times$ h/mL; 0–6 h)	1548 $\pm$ 564	2224 $\pm$ 67	3642 $\pm$ 346	6545 $\pm$ 324***	3010 $\pm$ 406
Half life (min)	22 $\pm$ 1	16 $\pm$ 1	17 $\pm$ 0.5	21 $\pm$ 1	16 $\pm$ 1
Clearance (l/h per kilogram)	NC	4.1 $\pm$ 0.1	3.4 $\pm$ 0.3	2.3 $\pm$ 0.1*	5.3 $\pm$ 0.7
Vd (l/kg)	NC	1.2 $\pm$ 0.1	0.7 $\pm$ 1.0	0.87 $\pm$ 0.03	1.32 $\pm$ 0.21
Brain exposure (ng $\times$ h/g; 0–3 h)				8167 $\pm$ 88***	3789 $\pm$ 113

* $P < 0.05$; *** $P < 0.001$.

Fig. 3. GNX sedation. GNX or ALLO was administered via tail vein bolus injection, and rats were monitored for behavioral sedating effects from 0 to 4 hours. (A) GNX dose response. (B) Comparison of GNX to ALLO (15 mg/kg). Rats were scored as follows: 0 = awake, absence of sedation, no change in observed locomotion or behavior; 1 = light sedation, slowed movement, intact righting reflex; 2 = sedation, loss of righting reflex, responsive to toe-pinch reflex; and 3 = anesthesia, loss of toe-pinch reflex. $N = 4$ per treatment. Data are expressed as the average $\pm$ S.E.M. Statistical significance: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$.

or rhythmic spike-wave complexes 4 hours after treatment. In contrast, ALLO-treated animals reverted to a seizure response.

Effect of ALLO and GNX Administered 1 Hour Post-SE Onset. ALLO administration produced a reduction in EEG power when administered 1 hour after SE onset (Figs. 4 and 5). The effect of ALLO, although significant at the early time points, was transient and was not detectable 1 hour after seizure onset. When data were examined over different frequency ranges, the effect of ALLO was apparent up to 30 Hz but not significant above the 0–10 Hz range (Fig. 7; Table 4).

GNX administered at 1 hour post-SE onset at 12 and 15 mg/kg again strongly reduced EEG power (Figs. 4 and 5). The effect of GNX was long lasting and similar in duration to that when administered 15 minutes after SE onset. There were some subtle differences between effects of GNX dosed at 1 hour versus 15 minutes postonset. When administered 1 hour after SE onset, the EEG suppressive response was slightly delayed. Also, the effect of GNX was most pronounced at the 0–10 Hz range (Fig. 7; Table 4). Data shown in Supplemental Fig. 1 demonstrate that in GNX-treated animals, EEG patterns returned to normal (preseizure; baseline) and showed much less high-amplitude spiking or rhythmic spike-wave complexes when compared with the vehicle group (note amplitude scales). In contrast, ALLO-treated animals reverted to a seizure response similar to the vehicle group.

Discussion

Intravenous administration of GNX produced a complete and durable anticonvulsant response in the pilocarpine model of SE with administration up to 1 hour after SE onset. GNX blocked convulsions, improved survival, and prevented EEG seizures. Moreover, GNX exhibited pharmacological characteristics that are improvements over the naturally occurring neurosteroid, ALLO, and other standard-of-care agents used as treatments in SE.

The pilocarpine SE model is a clinically translatable model of SE. Similar to the human condition, rats subjected to experimental SE exhibit EEG abnormalities, convulsions, and mortality. Surviving animals show cognitive impairment and neurodegenerative pathology (Curia et al., 2008; Tang et al., 2011). In the present studies, rats in the behavioral convulsion study showed slightly more responsiveness to the effects of GNX than those in the EEG seizure studies. This may be due to younger rats used in the behavioral studies, or due to a transient convulsion suppression scored as a positive response not detected by EEG measurements. Alternatively, the difference may be that GNX is suppressing behavioral seizures by more potently affecting a brain region not detected

TABLE 2

Seizure protection and survival of GNX and ALLO in a CSE model

CSE was initiated by administration of lithium, followed by pilocarpine. GNX (6, 9, 12 mg/kg) or ALLO (15 mg/kg) was administered at time of seizure onset (0 min), 15, 30, or 60 minutes after seizure onset. Data are expressed as the percentage of animals that were protected from seizure (seizure protection) or survived for 24 hours. GNX (all dose levels) produced a statistically significant effect on seizure protection (probit analysis; $P < 0.01$) and survival (probit analysis; $P < 0.001$) independent of time of administration after seizure onset. ALLO also produced statistically significant response on seizure protection ($P < 0.01$) and survival ($P < 0.001$). There were no differences between ALLO and GNX at any given time point.

Time of administration	Seizure Protection				Survival (24 h)			
	0 min (%)	15 min (%)	30 min (%)	60 min (%)	0 min (%)	15 min (%)	30 min (%)	60 min (%)
VEH	0	0	0	0	50	33	44	44
GNX (6 mg/kg)	70	20	30	50	100	90	90	100
GNX (9 mg/kg)	90	40	40	80	90	90	100	100
GNX (12 mg/kg)	90	89	80	100	90	78	100	100
ALLO (15 mg/kg)	92	78	56	78	83	89	78	100

Fig. 4. Effects of GNX and ALLO on EEG activity following onset of CSE. EEG records from selected rats in the ALLO and GNX groups treated 15 or 60 minutes after SE onset, as indicated above each tracing. Records beginning 10 minutes after pilocarpine injection and between ~4 and 5 hours postdosing. Specific times in each record indicated by arrows: (A) SE onset; (B) 10 minutes post-SE onset; (C) dosing; (D) 10 minutes postdosing; (E) 30 minutes postdosing; (F) 1 hour postdosing; (G) 2 hours postdosing; (H) 3 hours postdosing; (I) 4 hours postdosing. Voltage scales in millivolt indicated in each record; time scale at bottom left. Examples of 10-second periods of EEG at several time points shown in Supplemental Fig. 1.

by EEG measurements. The EEG measurements were obtained via electrodes that spanned the frontal cortex and the contralateral temporo-parietal cortex. These electrodes can detect cortical and subcortical activity, including hippocampal activity, but not deeper brain region activity (Gruner

et al., 2011). In one study, ALLO administration completely suppressed generalized kindled convulsions but did not affect epileptogenic EEG activity in the amygdala, indicating that neurosteroids may have region-specific effects (Lonsdale and Burnham, 2007). The disconnect between the effective dose

Fig. 5. Comparison of GNX to ALLO in rat SE as measured by EEG power (0–96 Hz). Rats were preimplanted with cortical electrodes. On day of measurements, baseline EEG was recorded and then rats were administered scopolamine and pilocarpine (P) at the indicated times. SE (S) onset was determined by first convulsive seizure. GNX or ALLO was administered (A) 15 minutes after SE onset or (B) 60 minutes after SE onset. EEG readings were measured for 5 hours after SE onset.

levels required to suppress behavioral seizures and cortical EEG seizures may be due to region-specific effects of neurosteroids. Further studies measuring deep brain EEG response would be required to deconvolute this finding. However, the data clearly show that the measurements of EEG activity in the cortical and subcortical regions were effective in differentiating effects of GNX and ALLO by magnitude, and duration of action, and detected other differences between the compounds, such as expression of rhythmic bursting.

Animals subjected to lithium-pilocarpine-induced seizures respond to antiepileptic drugs (AEDs) that are used in the

clinic and, similar to the clinical situation, develop progressive resistance with delayed administration of anticonvulsants (Jones et al., 2002; Zheng et al., 2010; Pouliot et al., 2013). Guidelines for first-line treatment of SE call for the administration of benzodiazepines soon after seizure onset. With increased delays, patients become resistant to the anticonvulsant effects of benzodiazepines. Similar to the clinical situation, benzodiazepines such as diazepam attenuate EEG seizure response in pilocarpine-treated rats when administered shortly after seizure onset (typically less than 15 minutes) but become resistant with delayed pharmacotherapeutic intervention. Indeed, with delays of over 15 minutes, animals become unresponsive to the anticonvulsant effects of benzodiazepines (Jones et al., 2002). In contrast to benzodiazepines, GNX efficacy was independent of treatment delay times and elicited a complete effect with delays of administration until 1 hour after seizure onset. This effect was apparent as a block of convulsions, increased survival, and reduction in EEG seizure activity. The differences between benzodiazepines and GNX can be attributed to slightly different mechanisms of action. Although both agents are positive allosteric modulators of GABA_A receptors, benzodiazepines only modulate α and γ subunit-containing GABA_A receptors, but do not modulate δ subunit-containing GABA_A receptors (Campo-Soria et al., 2006; Sigel and Steinmann, 2012). These GABA_A receptor subtypes differ by location, ion channel dynamics, and refractoriness (Belelli and Lambert, 2005). The γ subunit GABA_A receptors are located synaptically, modulate phasic Cl[−] currents, and internalize and become unresponsive with chronic modulation and seizure (Farrant and Nusser, 2005; Brickley and Mody, 2012; Carver and Reddy, 2013). The internalization of the GABA_A receptor most likely accounts for the progressive resistance and development of tolerance to benzodiazepines (Sperk, 2007; Naylor, 2010).

In contrast, GNX modulates both γ and δ subunit-containing GABA_A receptors (Belelli and Lambert, 2005). GABA_A receptors containing δ subunits are located extrasynaptically, modulate tonic Cl[−] currents, and neither internalize nor become unresponsive with prolonged seizure or chronic modulation (Brickley and Mody, 2012). The modulation of extrasynaptically located δ subunit-containing GABA_A receptors explains the effectiveness of GNX with delayed administration during the typical pharmacologically resistant time period (Naylor et al., 2005; Sperk, 2007; Naylor, 2010). The stability of the δ subunit-containing receptors with chronic exposure to modulators also accounts for the lack of tolerance to the anticonvulsant effect of GNX with repeated administration.

GNX is also differentiated from existing second-line therapies for SE, such as phenytoin (or fosphenytoin), valproic acid, levetiracetam, and phenobarbital. Unlike GNX, both phenytoin and valproic acid are inactive on EEG seizure response SE animal models (Bankstahl and Loscher, 2008). Phenobarbital inhibits EEG seizures, but only when administered close to seizure onset (Jones et al., 2002). With delayed administration (≥ 10 minutes after seizure onset), phenobarbital is inactive (Jones et al., 2002). Levetiracetam administration blocks convulsions, but not EEG seizures, with delayed administration (Zheng et al., 2010). In SE, EEG seizures can occur in the absence of behavioral convulsions, and nonconvulsive EEG seizures can continue after control of the convulsive SE (DeLorenzo et al., 1998; Jones et al., 2002). GNX differs from these second-line AEDs in that it controls both convulsive and

Fig. 6. Examples of EEG power for animals dosed 15 minutes after SE onset and treated as indicated in the graph legend: GNX at 6, 12, or 15 mg/kg, and ALLO at 15 mg/kg. Data points represent averaged time range of 0–15, 15–30, 30–60, 60–120, 120–180, 180–240, and 240–300 minutes. EEG power for frequency range of (A) 0–10 Hz, (B) 10–30 Hz, (C) 30–50 Hz, and (D) 50–96 Hz. Statistical analysis for these data is shown in Table 2.

EEG seizure responses in experimental SE. Well-controlled clinical studies showing benefit of these AEDs in SE are lacking. Despite the limited clinical data, these AEDs are the recommended second-line treatments for established SE.

In the present studies, GNX effects with delayed administration were comparable to that produced by anesthetic agents, such as propofol and pentobarbital (Pouliot et al., 2013). However, GNX did not produce an anesthetic response at doses that conferred anticonvulsant effects. That is, rats still responded to painful stimuli at doses that blocked SE. This retention of the pain reflex at anticonvulsant doses indicates that GNX does not induce deep anesthesia. Escalating doses beyond those required to produce an anticonvulsant

response produced an extended duration of the sedative response without producing full anesthesia. Thus, GNX may be considered a sedating, nonanesthetizing anticonvulsant agent. GNX is also differentiated from other last-line SE treatments that produce deep anesthesia. Pentobarbital induces respiratory depression, and patients need to be intubated during treatment (Claassen et al., 2002). Propofol can induce propofol infusion syndrome, characterized by lactic acidosis, rhabdomyolysis, and cardiovascular collapse, and can be lethal (Claassen et al., 2002).

GNX demonstrated meaningful differences from ALLO, most notably in duration of action. These effects on duration are not likely to be driven by effects on receptor dynamics as

Fig. 7. Examples of EEG power for animals dosed 60 minutes after SE onset and treated as indicated in the graph legend: GNX at 12 or 15 mg/kg, and ALLO at 15 mg/kg. Data points represent averaged time range of 0–15, 15–30, 30–60, 60–120, 120–180, 180–240, and 240–300 minutes. EEG power for frequency range of (A) 0–10 Hz, (B) 10–30 Hz, (C) 30–50 Hz, and (D) 50–96 Hz. Statistical analysis for these data is shown in Table 3.

both ALLO and GNX show similar affinity and efficacy for γ and δ subunit-containing GABA_A receptors (Carter et al., 1997; Nik et al., 2017). In the SE models, GNX and ALLO produced a full anticonvulsant response when administered 15 minutes after seizure onset. However, the duration of action of GNX was at least twice that of ALLO and, depending on the frequency range examined, could extend to four times that of ALLO, with significant reductions in seizure response observed out to 5 hours after seizure onset in the lower frequency ranges. With a 60-minute delay, GNX produced similar anticonvulsant activity as when administered at 15 minutes, whereas ALLO had only a transient effect at 60 minutes. The increased duration of action of GNX compared with ALLO can be attributed to PK differences. GNX exhibited higher exposure levels and longer half-life than ALLO when administered at the same dose level. The brain: plasma ratios of GNX and ALLO were similar; however, brain exposure levels of GNX were significantly higher than those

of ALLO (approximately 2-fold) and consistent with GNX's higher plasma exposure. GNX contains a 3β -methylation substitution conferring improved metabolic stability (Carter et al., 1997; Nohria and Giller, 2007). Improvements in PK characteristics were extended to improvements in duration of antiseizure activity, with a 2- to 4-fold longer duration of action than that of ALLO. Note that in a recent report ALLO was found to produce a faster onset of action and a better antiepileptic response than GNX in a mouse model of SE (Zolkowska et al., 2018). However, this study was conducted with i.m. administration that produced higher initial ALLO plasma and brain levels than GNX.

Another feature differentiating GNX from ALLO is its oral bioavailability (Nohria and Giller, 2007). In humans, plasma levels of GNX following oral administration produced plasma levels consistent with anticonvulsant levels (Nohria and Giller, 2007). Clinical studies with orally administered GNX in various epileptic conditions are ongoing (Younus and Reddy, 2018).

TABLE 3

Statistical analysis of GNX and ALLO in SE: treatment 15 minutes after SE onset

Data were analyzed by one-way ANOVA, followed by a post hoc Dunnett's test. "Time" is from time of administration. Data correspond to that in Fig. 6.

EEG Power Frequency Bin (Hz)	Time	GNX (6 mg/kg)	GNX (12 mg/kg)	GNX (15 mg/kg)	ALLO (15 mg/kg)
0–10	0 to 15 min	ns	**	**	****
	15 to 30 min	ns	****	****	****
	30 min to 1 h	ns	****	****	****
	1 to 2 h	ns	****	****	****
	2 to 3 h	ns	****	****	*
	3 to 4 h	ns	****	****	ns
	4 to 5 h	ns	****	****	ns
10–30	0 to 15 min	ns	*	*	****
	15 to 30 min	ns	****	****	****
	30 min to 1 h	ns	****	****	****
	1 to 2 h	ns	****	****	****
	2 to 3 h	ns	****	****	ns
	3 to 4 h	ns	ns	ns	ns
	4 to 5 h	ns	ns	ns	ns
30–50	0 to 15 min	ns	****	****	****
	15 to 30 min	ns	****	****	****
	30 min to 1 h	ns	****	****	****
	1 to 2 h	ns	****	****	****
	2 to 3 h	ns	****	****	ns
	3 to 4 h	ns	*	*	ns
	4 to 5 h	ns	ns	ns	ns
50–96	0 to 15 min	ns	****	****	****
	15 to 30 min	ns	****	****	****
	30 min to 1 h	ns	****	****	****
	1 to 2 h	ns	****	****	****
	2 to 3 h	ns	****	****	ns
	3 to 4 h	ns	****	****	ns
	4 to 5 h	ns	ns	ns	ns

ns, not significant.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.001$ compared with vehicle control.

In SE, GNX could be administered orally to patients that recover following i.v. GNX treatment to avoid precipitous withdrawal upon cessation of i.v. treatment.

In summary, data from these studies show that i.v. administration of GNX can dose and time dependently attenuate seizure severity in a preclinical model of SE, and that its

TABLE 4

Statistical analysis of GNX and ALLO in SE: treatment 60 minutes after SE onset

Data were analyzed by one-way ANOVA, followed by a post hoc Dunnett's test. "Time" is from time of administration. Data correspond to that in Fig. 7.

EEG Power Frequency Bin (Hz)	Time	GNX (12 mg/kg)	GNX (15 mg/kg)	ALLO (15 mg/kg)
0–10	0 to 15 min	**	ns	**
	15 to 30 min	****	****	*
	30 min to 1 h	****	****	ns
	1 to 2 h	****	****	ns
	2 to 3 h	****	****	ns
	3 to 4 h	**	*	ns
	4 to 5 h	Ns	ns	ns
10–30	0 to 15 min	Ns	ns	ns
	15 to 30 min	**	ns	ns
	30 min to 1 h	**	*	ns
	1 to 2 h	**	**	ns
	2 to 3 h	**	*	ns
	3 to 4 h	Ns	ns	ns
	4 to 5 h	Ns	ns	ns
30–50	0 to 15 min	Ns	ns	ns
	15 to 30 min	Ns	ns	ns
	30 min to 1 h	*	*	ns
	1 to 2 h	**	*	ns
	2 to 3 h	*	ns	ns
	3 to 4 h	ns	ns	ns
	4 to 5 h	ns	ns	ns
50–96	0 to 15 min	ns	ns	ns
	15 to 30 min	*	ns	ns
	30 min to 1 h	*	**	ns
	1 to 2 h	****	**	ns
	2 to 3 h	**	*	ns
	3 to 4 h	ns	ns	ns
	4 to 5 h	ns	ns	ns

ns, not significant.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.001$ compared with vehicle control.

pharmacological profile shows differences and improvements over ALLO and other existing therapeutics for SE. These data support the use of GNX in the treatment of drug-refractory SE.

Authorship Contributions

Participated in research design: Saporito, Gruner, Barker-Haliski, White.

Conducted experiments: Hinchliffe, Barker-Haliski, DiCamillo.

Performed data analysis: Saporito, Gruner, Barker-Haliski, Hinchliffe, DiCamillo.

Wrote or contributed to the writing of the manuscript: Saporito, Gruner, Barker-Haliski, White.

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