Bioisosteres of ethyl 8-ethynyl-6-(pyridin-2-yl)-4H-benzo[f]imidazo [1,5-a][1,4]diazepine-3-carboxylate (HZ-166) as novel alpha 2,3 selective potentiators of GABA_A receptors: improved bioavailability enhances anticonvulsant efficacy

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Non-Standard Abbreviations

ADD: after discharge duration

ADT: after discharge threshold

AMPA: α -amino-3-hydroxy-5-methyl- 4-isoxazolepropionic acid

AP-4: 4-Aminopyridine

β -CCT: Beta-Carboline-3-carboxylate-t-butyl ester

CTZ: cyclothiazide

EACSF: excitable artificial cerebral spinal fluid

GYKI 53773 = LY300164: 7-acetyl-5-(4-aminophenyl)-8,9-dihydro-8-methyl-7H-1,3-dioxolo(4,5H)- 2,3-benzodiazepine

HZ-166: ethyl 8-ethynyl-6-(pyridin-2-yl)-4*H*-benzo[*f*]imidazo[1,5-*a*][1,4]diazepine-3-carboxylate

KRM-II-81: 5-(8-ethynyl-6-(pyridin-2-yl)-4*H*-benzo[*f*]imidazo[1,5-*a*][1,4]diazepin-3-yl)oxazole

KRM-II-82: 5-(8-ethynyl-6-phenyl-4*H*-benzo[*f*]imidazo[1,5-*a*][1,4]diazepin-3-yl)oxazole

LFP: local field potentials

MP-III-080: : 3-ethyl-5-(8-ethynyl-6-(pyridin-2-yl)-4*H*-benzo[*f*]imidazo[1,5-*a*][1,4]diazepin-3-yl)-1,2,4-oxadiazole

PAM: positive allosteric modulator

PI: protective index

Abstract

HZ-166 has previously been characterized as an $\alpha 2,3$ -selective GABA_A receptor modulator with anticonvulsant, anxiolytic, and anti-nociceptive properties but reduced motor effects. We discovered a series of ester bioisosteres with reduced metabolic liabilities, leading to improved efficacy as anxiolytic-like compounds in rats. In the present study, we evaluated the anticonvulsant effects KRM-II-81 across several rodent models. In some models we also evaluated key structural analogs. KRM-II-81 suppressed hyper-excitation in a network of cultured cortical neurons without affecting the basal neuronal activity. KRM-II-81 was active against electroshock-induced convulsions in mice, pentylenetetrazole (PTZ)-induced convulsions in rats, elevations in PTZ-seizure thresholds, and amygdala-kindled seizures in rats with efficacies greater than that of diazepam. KRM-II-81 was also active in the 6Hz seizure model in mice. Structural analogs of KRM-II-81 but not the ester, HZ-166, were active in all models in which they were evaluated. We further evaluated KRM-II-81 in human cortical epileptic tissue where it was found to significantly-attenuate picrotoxin- and AP-4-induced increases in firing rate across an electrode array. These molecules generally had a wider margin of separation in potencies to produce anticonvulsant effects vs. motor impairment on an inverted screen test than did diazepam. Ester bioisosteres of HZ-166 are thus presented as novel agents for the potential treatment of epilepsy acting via selective positive allosteric amplification of GABA_A signaling through $\alpha 2/\alpha 3$ -containing GABA receptors. The in vivo data from the present study can serve as a guide to dosing parameters that predict engagement of central GABA_A receptors.

Introduction

Epilepsy is not fully controlled despite a plethora of drugs with diverse mechanisms of action (Guerreiro, 2016; Shih et al., 2017). One well established and characterized mechanism involves the facilitation of inhibitory neuronal control of gamma-aminobutyric acid (GABA)ergic neurotransmission (c.f., Gasior et al., 1999). Although drugs used in the control of epilepsy acting through this mechanism have efficacy in some patients and are generally safe (Jain et al., 2016), global enhancement of GABAergic neurotransmission across the central nervous system (CNS) can produce undesirable effects of sedation, motor and memory impairment, dependence and abuse liability (Drummer, 2002; Canadian Agency for Drugs and Technologies in Health, 2014). The motor-impairing effects of GABA_A receptor agonists might, in addition, impede the achievement of maximally-efficacious drug levels due to motor impairment. Since GABA_A receptors are composed of multiple protein subunits and since different subunit configurations exist within native CNS structures (Sieghart and Ernst, 2005), selective positive allosteric modulation of only a subset of GABA receptors might imbue therapeutic advantage (Rowlett et al., 2005). Convergent evidence from transgenic animals and molecules with selectivity for the α proteins required for ligand-gating has suggested that while α_1 -comprised GABA receptors mediate sedative/ataxic effects of drugs, the α_2 - and α_3 -comprised GABA receptors mediate the anticonvulsant effects of drugs distinct from sedative/ataxic effects (Rivas et al., 2009; Ralvenius et al., 2015). Indeed, in humans, allosteric potentiators with selectivity for $\alpha_2/3$ over α_1 protein assemblies show reduced sedative effects compared to those without selective activity (Zuiker et al., 2016). Consequently, efforts to discover modulators of GABA_A receptors that positively amplify signaling selectively through $\alpha_2/3$ vs α_1 receptor configurations are currently ongoing.

One such $\alpha_2/3$ ligand, HZ-166 (**Fig. 1**), displays selectivity over α_1 -containing receptors. HZ-166 has demonstrated efficacy against convulsions in mice and rats at the same time showing a reduced liability for producing sedative/ataxic effects in rodents compared to non-selective agonists (Rivas et al., 2009). However, HZ-166 has pharmacokinetic liabilities that result in low exposures of plasma and brain after intraperitoneal (i.p.) or oral (p.o.) dosing in rats (Poe et al., 2016). The ester functionality on the imidazole ring is labile and results in the low bioavailability of HZ-166; replacement of the ester with bioisosteres (**Fig. 1**) produced marked improvements in bioavailability without negatively impacting either potency or selectivity for $\alpha_2/3$ vs α_1 (Poe et al., 2016; Lewter et al., 2017). For example, KRM-II-81 demonstrated μ M activity at the α_3 protein while at the same time retaining selectivity over α_1 (>20 fold). Furthermore, KRM-II-81 generated biologically-relevant plasma concentrations upon systemic dosing (Poe et al., 2016; Lewter et al., 2017). In vivo proof of concept demonstrated that, in contrast to HZ-166, KRM-II-81 prevented the suppression of responding of rats in a Vogel conflict test (Poe et al., 2016), a result extended to other molecules in this series (Witkin et al., 2017) and thus demonstrating anxiolytic-like activity in this rodent model. This in vivo translation has been also extended with these molecules into the pain domain (Lewter et al., 2017; Fischer et al., 2017).

The present series of studies was conducted to characterize the antiepileptic potential of KRM-II-81. We primarily focused attention upon KRM-II-81 in this work due to its broader characterization at the time of this study as a potential anxiolytic agent (Poe et al., 2016) and since we had sufficient material to conduct these experiments. However, we also studied some structural analogs (Poe et al., 2016) where supplies of material were available (KRM-II-82 and MP-III-080). The doses of these molecules to block or prevent seizures were compared to the doses producing motor-impairing effects. The results of these experiments demonstrated that when compared under the same conditions, the ester bioisosteres were equally efficacious or more efficacious than diazepam in multiple anticonvulsant models in rats whereas HZ-166 was not effective. There was generally no separation in the doses of diazepam inducing motor impairment and doses significantly attenuating convulsion measures. In contrast, the bioisosteres displayed efficacy at non-motor-impairing doses. Given the improved pharmacological profile of KRM-II-81, we further studied this molecule in human cortical epileptic tissue where it was found to significantly-attenuate picrotoxin- (GABA_A) or 4-aminopyridine (AP-4) (K^+)-induced increases in firing rate across an electrode array.

Methods

Compounds. KRM-II-81 and KRM-II-82 were synthesized by us (K.R. Methuku) as previously described (Poe et al., 2016) as was MP-III-080 (M. Poe) (Poe et al., 2016). HZ-166 and β -CCT were synthesized at the University of Wisconsin, Milwaukee (J.M. Cook laboratory). The other compounds (diazepam, valproic acid, and pentylenetetrazole) were obtained from Sigma-Aldrich, St. Louis, MO, USA. We primarily focused attention on KRM-II-81 as this was the best characterized molecule at the time of the current experiments (Poe et al., 2016) and the compound of which we had sufficient material to carry out studies in each of the assays.

Diazepam and β -CCT were dissolved in 1% hydroxyethylcellulose/0.25% Tween-80/0.05% Dow antifoam in water. KRM-II-81, KRM-II-82, MP-III-080 and HZ-166 were suspended in carboxymethylcellulose. The dose volume for diazepam and valproate was 1 ml/kg for rats and 10 ml/kg was used for mice. HZ-166 and its bioisosteres were dosed in volumes of 10 ml/kg in mice. HZ-166 was dosed in a volume of 5 ml/kg in rats and KRM-II-81 was dosed at 1 ml/kg in rats below doses of 30 mg/kg; 30 mg/kg (dosed at 3 ml/kg), 60 mg/kg (dosed at 6 ml/kg). All potential anticonvulsant compounds were dosed i.p., 30 min prior to convulsant administration with the exception of the 6Hz model where dosing was p.o., 2 hr prior to testing.

Doses and routes of administration of the GABA_A compounds were based upon prior in vivo studies with these molecules in vivo (Poe et al., 2016; Witkin et al., 2017). Doses tested are shown in the figures and tables. The compounds were generally dosed in $\frac{1}{2}$ log increments from 3 mg/kg to 30 mg/kg with the exception of diazepam that was given at doses beginning at 0.1 mg/kg (PTZ seizure threshold), 0.3 mg/kg (amygdala kindling), and 1 mg/kg (maximal electroshock). HZ-166 was dosed up to 60 mg/kg, and the doses in the 6Hz model were 10-50 mg/kg. Valproic acid was given at 300 mg/kg.

Pentylenetetrazole was given by s.c. injection as a seizure inducer at 35 mg/kg (producing ~97% of rats to convulse), and was given by i.v. infusion in the studies designed to assess drug effects on seizure thresholds.

Electrophysiological effects in neuronal cultures These experiments were conducted to determine if the compounds under investigation were effective in damping aspects of cortical neuronal network activity under basal and hyper-excited conditions.

Microelectrode array (MEA) preparation. Meastro 12 well plates (Axion Biosystems, Atlanta, GA) were treated with a solution of 0.1% polyethylenimine for 2-4 hours, rinsed with sterile H₂O and let dry overnight. Prior to plating of neurons the MEAs were treated with solution of 20 μ g/ml laminin for a minimum of 1 hour.

Primary neuronal culture. Cortices isolated from E18 rats (MEA) were obtained from BrainBits LLC, (Springfield, IL) and digested enzymatically with TrypLE Express (Gibco). After 15 minutes of digestion the tissue was mechanically dissociated with a series of sterile fire-polished glass pipettes of decreasing diameter. The dissociated neurons were plated directly onto substrate-integrated MEA plates and incubated in Nb Active1 (Brainbits LLC, Springfield, IL) supplemented with 5% dialyzed fetal bovine

serum, 0.25% Glutamax (Gibco). Cell cultures were maintained in tissue incubator (37°C, 6% CO₂) and fed twice a week by exchanging half of the medium. The experiments were performed on DIV 19-25 thus allowing partial maturation of the neurons. Prior to the experiment the cell culture media was replaced with external buffer containing (in mM): 129 NaCl, 5 KCl, 2 CaCl, 1 MgCl, 10 HEPES, 10 glucose. To achieve hyper-excitation the external buffer was modified by removal of magnesium or by addition of 1mM 4aminopyridine (4-AP).

MEA recording. The recordings were obtained at 37C using Meastro System with integrated AxIS 2.3 analysis software (Axion Biosystems, Atlanta, GA). Channels were sampled simultaneously with a gain of 1200× and a sampling rate of 12.5 kHz/channel. On-line spike detection was done with the AxIS adaptive spike detector. For recordings, a Butterworth band-pass filter (with a high-pass cutoff of 75 Hz and low-pass cutoff of 4000 Hz) was applied along with a variable threshold spike detector set at 7× standard deviation of the rms-noise on each channel. Only wells that did show spontaneous activity (more than 0.3 Hz) on the day planned for the experiment were treated with a compound. Burst was defined as a minimum of 5 spikes occurring with an interspike interval of less than 100ms. A minimum of one burst per minute was required for bursting analysis. Compounds were added by manual pipetting and the activity was sampled for six minutes prior and post compound addition.

Statistical analysis. The data were normalized to baseline activity and reported as mean ± standard error of the mean (SEM). For single treatment a single sample t-test was used. To compare between group effects, analysis of variance (ANOVA) with Dunnett's multiple comparison test were utilized; P<0.05 was considered significant.

Human Epileptic Cerebral Cortex Multi-Electrode Array Electrophysiology.

Patient data. Experiments were conducted with tissue from two patients undergoing cortical transection for pharmacologically-refractory epilepsy. The first patient was an 11-year-old female with a history of medically refractory epilepsy with increasing seizure frequency. She presented with localization related (focal), (partial) epilepsy and epileptic syndromes with complex partial seizures and intractable epilepsy. Medications and vagal stimulation failed to prove medically viable. Multiple diagnostics led to the decision to operate. A right frontotemporal parietal craniotomy for resection of her right frontal tumor and seizure focus, using intraoperative Stealth, stereotactic-guided electrocortical graphic localization of her seizure activity, and phase reversal mapping for localization of the central sulcus/precentral gyrus (motor cortex) as well as resection of the anterolateral aspect of the temporal lobe was performed. The anterior 4.5 cm of the middle temporal gyrus, inferior temporal gyrus, fusiform gyrus, and parahippocampal gyrus were resected along with the anterior 3 cm of the superior temporal gyrus on the right side. The hippocampus was left intact.

The second patient was a 12-yr-old boy with medically refractory epilepsy. He underwent a left frontotemporal craniotomy for resection of his anterolateral and mesial left temporal lobe seizure focus using intraoperative electrocorticography and Stealth stereotactic guidance. During surgery, there were abnormal epileptic spikes coming from the electrodes one through three on the left superior temporal gyrus, one through five on the left middle temporal gyrus, left inferior temporal gyrus, and left fusiform gyrus. The

anterior 3 cm of the superior temporal gyrus was resected and used for the electrophysiological recordings. The anterior 5 cm of the middle temporal gyrus, inferior temporal gyrus, fusiform gyrus, and parahippocampal gyrus was resected on the left as well. In addition, a left amygdalohippocampectomy was performed without incidence. These later tissue were not studied in the electrophysiological experiments.

Tissue preparation and recording. The tissue was prepared and treated as previously described (Zwart et al., 2014). Slices were maintained at 37° C and were perfused at 1.0 ml/min first with normal ACSF (NACSF) solution for one hour to see if the tissue was spontaneously active. If spontaneous activity did not develop after one hr, the tissue was bathed in excitable ACSF (EACSF) solution containing 5 mM K⁺ and 0 mM Mg²⁺ to induce robust local field potential (LFP) activity in cortical brain slices. Tissue was then recorded in EACSF with a concentration of 10 μM picrotoxin for 1 hr. Subsequently, tissue was recorded in EACSF with a concentration of 10 μM of picrotoxin with 30 μM KRM-II-81 and recorded for 1 hour. Following the KRM-II-81 addition, tissue was recorded in a washout solution of EACSF and 10 μM Picrotoxin for 1 hr. The concentration of KRM-II-81 was based upon data collected in human cortical slices with perampanel (Zwart et al., 2014) and upon the need to be conservative in concentration estimates to maximize the opportunity to see an effect given limited human tissue opportunities. A second experiment was conducted using AP-4 (50 μM) as an activator instead of picrotoxin.

Recordings were performed on microelectrode arrays of 60 electrodes as previously described (Zwart et al., 2014). LFPs with sharp negative peaks below a threshold set at 3 standard deviations of the signal were marked, and the time of the maximum excursion was recorded as the time of that LFP. Time points were binned at 4 ms. Two-way ANOVA was performed to ascertain whether KRM-II-81 was effective and whether the drug effect was dependent upon electrode location.

Rodent behavioral assays. All studies were performed in accordance with guidelines of the National Institutes of Health and by local animal care and use committees. The local animal care and use committee and veterinary staff provided direct oversight of the animals by inspections, protocol reviews, laboratory site visits, and animal health monitoring.

Animals were housed separately by species in a quiet, ventilated-, temperature- and humidity-controlled vivarium that met AALAAAC accreditation. Lighting was controlled with a 12 h light-dark cycle (lights on at 6 am). Food and water were available to the animals at all times when the animals were in their home cages. They were maintained in the colony room for at least 3 days before testing. Animals were moved to a quiet room 1 hour prior to the start of the test.

Experimental tests were conducted to evaluate the effects of the test compounds in a battery of tests that detect different aspects of anticonvulsant efficacy (Swinyard et al., 1989).

Mouse Studies.

Electroshock-Induced Convulsions. This assay detects effects of compounds that produced generalized seizures and those that dampen seizure spread. Male, CD1 mice (Taconic Farms) were studied at weights of 21-32g. Mice (n=10/dose) were used with Wahlquist Model H stimulator with 0.2 sec stimulation with corneal electrodes. Mice were observed for approximately 10 sec after administration of the electrical stimulus (10uA) and the types of convulsions were recorded (0 = no convulsion, 1 = clonus, 2 = tonic flexion, 3 = tonic extension). Mice were euthanized immediately following the test. Tonic extension was used as the primary endpoint. The percentage of animals exhibiting convulsions was analyzed by Fisher's Exact probability test.

6Hz Seizure Model. This seizure model is utilized to screen for drugs that might be detected by other screening approaches. For example, the highly used anticonvulsant leviteracetam was effective in this test but not in the pentylenetetrazole assay. Adult, male mice were subjected to 6Hz stimulation at 44 mA as originally described by Barton et al. (2001) and conducted per protocol of the NIH Anticonvulsant Screening Program (<https://panache.ninds.nih.gov/Model1A.aspx>). Briefly, a mouse was dosed with KRM-II-81, p.o., and 2 hr later given 6 Hz stimulation for 3 sec delivered through corneal electrodes at 44 mA and observed for the presence or absence of seizure activity. A separate group of mice was dosed with higher doses of KRM-II-81 and observed for potential deficits in motor performance and overt signs of toxicity at 4 hr post dosing.

Rat Studies.

Inverted Screen Test. This assay was conducted to evaluate potential motor impairing effects of drugs. Male, Sprague Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) were used and weighed 90-110 g when evaluated in experiments. Prior to dosing rats with PTZ, they were tested for potential motor impairment on an inverted screen. The apparatus consisted of four 13 x 16 cm squares of round hole, perforated stainless steel mesh (18 holes/square inch, 3/16 inch diameter, ¼ inch staggered centers, 50% open area) mounted 15 cm apart on a metal rod, 35 cm above the table top.

Rats were dosed with test compound and returned to their home cage. Twenty five min after dosing, animals were tested on the inverted screen and were scored after 60 seconds as follows: 0=climbed over to top of screen, 1= hanging on to screen, 2= fell off).

Pentylenetetrazole (PTZ)-Induced Seizures. This assay is utilized as one of the initial screens for efficacy of anticonvulsant drugs can detect drugs that dampen generalized seizures. After the inverted screen test, the rats were dosed with pentylenetetrazol (PTZ)(35 mg/kg, s.c.) in a volume of 1 ml/kg and placed in an observation cage (40.6x20.3x15.2 cm) with a floor containing 0.25 inches of wood chip bedding material. PTZ dose was determined to be ~ EC₉₇ for producing clonic convulsions. The rats were then observed for 30 min post PTZ for clonus (defined as clonic seizure of fore- and hindlimbs during which the rat demonstrated loss of righting) or for tonic seizures as exemplified by loss of righting accompanied with tonic hindlimb extension. The percentage of animals exhibiting convulsions was analyzed by Fisher's Exact probability test.

Pentylenetetrazole (PTZ)-Induced Seizure Threshold. This test is used to detect effects of drugs on dampening of seizure threshold. The threshold dose of PTZ to

produce seizures was measured in rats with and without drug pretreatment during i.v. PTZ infusion. Male F-344 Harlan rats (Indianapolis, IN) were randomly assigned to treatment groups and dosed with vehicle or test compound. Rats were placed in a restrainer and a winged infusion needle was inserted into the lateral tail vein. Intravenous infusion with 10 mg/ml PTZ at a rate of 0.5 ml/min was initiated until a clonic convulsion occurred, and the time to clonic convulsion was recorded in sec or a maximum of 4 min was recorded. Following infusion, rats are euthanized. The dose of PTZ required to elicit a clonic convulsion was calculated using the infusion rate, concentration of PTZ, time to clonic convulsion, and animal weight.

Basolateral Amygdala Kindling. Seizure kindling models evaluate effects of drugs on the sensitization of the nervous system to seizure induction. In this test, drugs were evaluated for their ability to impact seizure parameters in rats that were fully kindled by daily electrical stimulation of their amygdala. Valproic acid was used as a positive control. Experiments were conducted using the general procedures of Zwart et al. (2014) where details can be found. A bipolar electrode for electrical stimulation and EEG recording was stereotactically implanted into one hemisphere of the basolateral amygdala (AP -2.2, ML -4.8, DV -8.5 mm, relative to bregma) of male Wistar rats. After post-operative recovery, electrical kindling begins, where a subthreshold constant current (400 μ A, 1 ms, monophasic square-wave pulses, 60 Hz for 1 sec) is given once a day Monday-Friday for about 4-6 weeks until a rat is fully kindled. A fully kindled rat has experienced 10 consecutive stage 5 seizures or 10 of its last 12 were stage 5 according to the Racine Scale.

Twelve fully kindled rats were assigned to this study, and eight rats were selected and randomized to initial compound treatment groups from baseline after discharge threshold (ADT), seizure severity score, and after discharge duration (ADD). A pseudo within-subjects Latin Square design was used for subsequent testing, as replacement rats were used in the event that an assigned rat did not meet the pre-compound testing baseline criteria or a rat lost its head cap during a seizure. On test day, rats were dosed 30 min prior to beginning stimulation. After the pre-treatment, rats were stimulated using an ascending staircase sequence beginning at 10 μ A and increasing in log unit steps of 10, 16, 25, 40, 65, 100, 160, 250, and 400 μ A. Animals were stimulated until they were assigned a Seizure Severity Score for a visually observable seizure or they reached the 400 μ A threshold limit. ADD was determined following testing. ADT was the current that induced a seizure; measurements were scale-adjusted to capture the stimulation scale change required to observe a seizure from the previous baseline to the ADT scored on test day. When seizures were completely blocked, animals were assigned a scaled score of 0 for data analysis. The average scale adjusted ADT was approximately 0.63 μ A in vehicle treated rats. *Seizure Severity*: Racine score of behavioral response to stimulation: 0 = no behavioral response; 1 = immobility, staring and/or facial clonus; 2 = head nodding, jaw clonus, and/or tongue protrusion; 3 = unilateral forelimb clonus; 4 = bilateral forelimb clonus and/or rearing; 5 = bilateral forelimb clonus with rearing and loss of balance. *After-Discharge Duration (ADD)* is the duration of the first after-discharge.

ADT, ADD, and severity scores were analyzed by ANOVA (Norman, 2010). Seizure severity data was also analyzed by the nonparametric Skillings-Mack test, a generalization of the Friedman test for one-way repeated measures designs (Chatfield and

Mander, 2009). Posthoc tests when reported were either by Dunnett's method (seizure severity) or using Wilcoxon sign rank tests (ADT and ADD). Valproic acid, used as a positive control was not used in ANOVA calculations.

Data summarization. For the in vivo assays, we have calculated minimal effective doses (MED) in mg/kg. MED was defined as the lowest dose administered that produced statistically significant protection compared to vehicle control in the anticonvulsant assays. The MED (or minimal toxic dose (MTD) for motor impairment was the minimal dose required to produce statistically-significant impairment in motor performance. Doses tested are shown in the figures and tables and are generally dosed in $\frac{1}{2}$ log increments. The protective index (PI) was calculated at the MTD/MED. Thus, a PI of 1 indicates that a compound was equipotent in producing motor impairment and producing anticonvulsant effects. A PI > 1 indicates that anticonvulsant efficacy was achieved at doses lower than those producing motor impairment.

Plasma and brain exposures. Study samples were analyzed by LC-MS/MS using a Sciex API 4000 triple quadrupole mass spectrometer (Applied Biosystems/MDS; Foster City, CA) equipped with a TurboIonSpray interface, and operated in positive ion mode. The analyte was chromatographically separated using a Betasil C18 5 μ m 20x2.1mm Javelin (Thermo Electron Corp. Cat# 70105-022106 (pk of 4)). The pumps were Shimadzu LC-10AD units with a SCL-10A controller (Kyoto, Japan), and a Leap CTC liquid handler (LEAP Technologies, Carrboro, NC) was used as the autosampler. Water/1M NH₄HCO₃ (2000:10, v/v) (Mobile Phase A), and MeOH/1M NH₄HCO₃ (2000:10, v/v) (Mobile Phase B). The gradient profile changed from 10% B at 0 min, 47% B at 0.01 min to 0.20 min, 77% B at 0.30 to 0.40 min, and 98% B at 0.41 to 0.72 min where B is a solvent strength parameter for elution and separation purposes. The flow rate was 1.5 mL/min from 0 to 0.49 min, and increased to 2.5 mL/min from 0.51 to 0.72 min. Chromatography was performed at ambient temperature, with flow directed to the mass spectrometer between 0.25 and 0.50 min. The selected reaction monitoring (SRM) (M+H) transition m/z was 352.2 > 297.1. The TurboIonSpray temperature was maintained at 750C, with collision, curtain, nebulizing, and desolvation gas (nitrogen) settings of 40, 10, 70, and 50, respectively. The ion-spray voltage was set to 4500 V, while the respective declustering, entrance, and exit potentials were 80, 10, and 8.

Calibration standards were prepared by serial dilution of a 100 ug/mL analyte stock solution (95:5:1 Formamide:water:5N HCl) with methanol/water (1:1, v/v) w/0.1% Acetic Acid, which were then used to fortify control plasma and brain homogenates to yield analyte concentrations of 1, 5, 10, 20, 50, 100, 500, 1000, 2000, 4000, and 5000 ng/mL. A 25 uL aliquot of each study sample, appropriate calibration standard and control matrix were transferred to a 96-well plate. The study samples, calibration standards, and control matrix sample were then mixed with 25 uL of acetonitrile/methanol (1:1, v/v) w/0.1% Acetic Acid containing internal standard and 150 uL of ACN. After mixing, the samples were centrifuged to pellet the precipitated proteins, and 150 uL of the resulting supernatants were transferred to a clean 96-well plate and evaporated under nitrogen at 40C. Samples were then reconstituted with 50 uL of 0.5% HFBA in water. Ten microliter aliquots were analyzed by LC-MS/MS.

Results

Electrophysiological effects in neuronal cultures.

Cultured rat cortical neurons were used to assess the activity of KRM-II-81 on the electrical activity of neurons under basal conditions and under two different conditions of hyper-excitation (**Fig. 2A, C**). There was a reversible potentiation of spontaneous neuronal activity by removal of magnesium reflected in increased spiking and bursting frequency. Addition of 1mM 4-aminopyridine to the external solution primarily increased the frequency of spikes with smaller effect on frequency of bursts.

KRM-II-81 suppressed the hyper-excitation in the network of cortical neurons but not the spontaneous neuronal activity in normal magnesium containing external solution (**Fig. 2 B, D**). In the presence of a normal magnesium containing external solution (1mM Mg^{++}) the addition of 3 μ M KRM-II-81 produced no significant change in the frequency of spiking or bursting in neuronal network. When magnesium was omitted from the external solution, 3 μ M KRM-II-81 significantly depressed both the frequency of spiking and the frequency of bursting. A similar depression of neuronal activity was observed in the presence of 1mM 4AP with significant decreases in frequency of spiking and bursting. A smaller, nonsignificant depression of neuronal activity occurred under conditions of reduced magnesium (0.1mM Mg^{++}).

Rodent Studies.

Effects of HZ-166 and some ester bioisosteres were compared for potency and efficacy differences in various seizure provocation models in mice and rats. Diazepam was used as a benzodiazepine comparator standard.

Electroshock-Induced Seizures. Both diazepam, KRM-II-81 and KRM-II-82 fully prevented electroshock-induced seizures in mice with diazepam being 5x more potent than KRM-II-81 to induce full seizure protection. HZ-166 was not efficacious up to 30 mg/kg (**Fig 3, Table 1**).

Pentylenetetrazole (PTZ)-Induced Seizures. Diazepam and KRM-II-81 fully prevented PTZ-induced clonus in rats with diazepam being 10x more potent than KRM-II-81 to induce full seizure protection. HZ-166, while showing a tendency toward efficacy, did not significantly separate from vehicle up to 30 mg/kg (**Fig 4, Table 1**).

The motor effects of these two molecules were studied on the inverted screen where diazepam had a minimal effective dose of 10 mg/kg and KRM-II-81 had a minimal toxic dose (MTD) of 150 mg/kg (**Table 2**). Thus when evaluating the protective index (PI)($MED_{inverted\ screen}/MTD_{PTZ\ convulsions}$), the PI for diazepam was 1 and that for KRM-II-81 was 5 (**Table 3**).

Both KRM-II-82 and MP-III-080 also dose-dependently suppressed convulsions induced by PTZ with MP-II-080 being more potent (**Fig. 5**). Up to 30 mg/kg, both compounds were without significant effect on motor performance (**Fig. 5**). The MTD for producing motor impairment by KRM-II-82 was 150 mg/kg. In contrast, valproate (300 mg/kg), used as a second positive control in addition to diazepam (**Fig. 4**), produced full motor impairment (**Fig. 5**).

Effects of Diazepam. Diazepam (30 mg/kg, p.o.) markedly and significantly impaired motor performance on the inverted-screen test (2.0 ± 0 vs 0 ± 0 for vehicle, $p < 0.05$, $n = 5$ rats/group). In the presence of the $\alpha 1$ -preferring antagonist of GABA_A receptor, β -CCT (10 mg/kg, i.p.), the motor-impairing effects of diazepam were completely prevented (0 ± 0 , $p < 0.05$, $n = 5$ rats/group) but the anticonvulsant effects were retained (PTZ alone = 5/5 convulsions; diazepam + PTZ = 0/5 convulsions; β -CCT + diazepam + PTZ = 0/5 convulsions).

Pentylentetrazole (PTZ)-Induced Seizure Threshold. The dose of PTZ required to produce convulsions was 35.1 ± 1.2 mg/kg, which was the dose (35 mg/kg) used to produce clonic convulsions when given as a bolus in the PTZ-induced seizure experiments described above. Pretreatment of rats with diazepam, KRM-II-81, or KRM-II-82 (MP-III-080 was not studied) increased the dose of PTZ required to induce convulsions (**Fig. 6**). Diazepam was about 10x more potent than the other two molecules but was less efficacious at this dose range (both diazepam and KRM-II-81 were equally efficacious against electroshock and acute PTZ). HZ-166 was not active up to 60 mg/kg (**Fig 4, Table 1**). HZ-166: $F_{3,28} = 1.1$, $p = 0.36$; KRM-II-81: $F_{3,28} = 8.5$, $p < 0.001$; KRM-II-82: $F_{2,21} = 13.5$, $p < 0.001$ (vehicle control values used in ANOVA for this compound); Diazepam: $F_{2,21} = 1.7$, $p = 0.20$.

Amygdala Kindling. In amygdala kindled rats, diazepam, KRM-II-81, and KRM-II-82 (MP-II-080 was not studied) prevented multiple parameters of the seizure induced (Fig. 7). The adjusted after-discharge thresholds (ADT) were significantly increased by diazepam, KRMII-81 and KRM-II-82, but not by HZ-166. Potencies of diazepam and KRM-II-81 on this dependent measure were approximately equivalent with KRM-II-82 being somewhat less potent (**Fig 7, Table 1**). ADT data were scale adjusted to capture the stimulation scale change required to observe a seizure from the previous baseline to the ADT scored on test day. When seizures were completely blocked, animals were assigned a scaled score of 5 for data analysis. The average scale adjusted ADT was $0.75 \mu A$ in vehicle treated rats. Since complete block of seizures contributed to the ADT, the seizure free rates are provided. Seizure free scores (seizure severity = 0) were 0/8 for HZ-166, 1/8 for KRM-II-82, 2/8 for diazepam, and 7/8 for KRM-II-81.

In contrast, significant decreases in the after-discharge duration (ADD) were produced only by KRM-II-81 with a trend for a significant effect of diazepam and KRM-II-82 at the highest doses tested (**Fig 7, Table 1**). The seizure severity score was decreased significantly by diazepam and KRM-II-81 with a trend toward efficacy in the dose-effect curve for KRM-II-82 but not for HZ-166 itself. Of these molecules, KRM-II-81 was the most efficacious and potent on measures of amygdala kindling (**Fig 7, Table 1**).

Statistical analysis confirmed these result descriptions. After-discharge threshold- HZ-166: $F_{3,28} = 0.9$, $p = 0.92$; KRM-II-81: $F_{2,21} = 27$, $p < 0.0001$; KRM-II-82: $F_{2,21} = 3.7$, $p < 0.05$; diazepam: $F_{3,28} = 4.2$, $p < 0.015$. After-discharge duration – HZ-166: $F_{3,28} = 0.06$, $p = 0.98$; KRM-II-81: $F_{2,21} = 10.1$, $p < 0.001$; KRM-II-82: $F_{2,21} = 0.76$, $p = 0.48$; diazepam: $F_{3,28} = 1.4$, $p = 0.28$. Seizure severity score – HZ-166: $F_{4,27} = 1.29$, $p = 0.30$; KRM-II-81: $F_{3,20} = 29.21$, $p < 0.0001$; KRM-II-82: $F_{3,21} = 5.28$, $p < 0.01$; diazepam: $F_{4,27} = 10.46$, $p < 0.0001$.

We provided a second statistical analysis of the seizure severity data using the Skillings-Macks test. This analysis confirmed the statistical results presented above. Under analysis by the Skillings-Macks test, the results were as follows for the dose-

response analysis: KRM-II-81 ($p < 0.001$), KRM-II-82 ($p < 0.001$), and diazepam ($p < 0.001$), and HZ-166 ($p = 0.45$).

6 Hz-Induced Seizures in Mice. Mice ($n=8$) subjected to 6 Hz stimulation were protected by orally-administered KRM-II-81 when tested 2 hr post oral dosing (**Table 2**). Mild tremor was observed at 100 mg/kg and loss of righting was observed in one 1/8 mice at 150 mg/kg and 1/8 mice at 200 mg/kg, a dose at which more severe tremor was noted in 1/8 mice.

Protective Indices. When comparing potencies of diazepam and KRM-II 81 on a measure of motor deficit (inverted screen) to its anticonvulsant potencies (**Table 3**), a protective index can be calculated as $\text{Potency}_{\text{inverted screen}}/\text{Potency}_{\text{anticonvulsant}}$. PI values > 1 indicate a margin between efficacy and side-effect doses; PI values $\equiv 1$ indicate that side-effects and efficacy do not quantitatively separate and a PI value of < 1 indicates that the potency to produce efficacy is less than the potency to produce motor impairment. PI values for the various seizure models are shown in **Table 3**. These data reveal that the bioisosteres had better separation in the dose producing efficacy vs. the dose producing motor effects than that of diazepam.

In order to better estimate the separation between motor side-effects and anticonvulsant efficacy, we obtained plasma and brain levels of KRM-II-81 (at the ED50 for efficacy (17.3 mg/kg) and ED50 for motor impairment (121 mg/kg). (**Table 4**). Using these unbound drug concentrations, the PI based on drug levels in plasma was 3 and that based upon brain drug levels was 3.8.

Human Epileptic Cerebral Cortex Electrophysiology.

Slices of resected human cortical tissue are typically not active in NACSF alone (Hobbs et al. 2010) as was the case in the current study. By bathing the slice in 10 μM picrotoxin, elevated K^+ and reduced Mg^{2+} , however, we evoked field potentials on at least 30 channels of the array. We first recorded activity of the slice for 1 hr in 10 μM of picrotoxin to establish a baseline. The average firing rate (**Fig. 8**) was 0.05 ± 0.01 Hz. We then added KRM-II-81 (30 μM) and recorded activity for another hour again in the presence of 10 μM of picrotoxin. The average firing rate decreased to 0.01 ± 0.005 Hz (**Fig. 5A**). These decreases were statistically significant by two-way ANOVA ($F_{1,32} = 30.7$, $p < 0.0001$), whereas the electrode location was not a significant factor ($F_{32,32} = 1.1$, $p = 0.36$).

We also evaluated the effect of KRM-II-81 on the firing frequencies in slices from a second epileptic patient. In this second study, we stimulated firing with AP-4. In this slice preparation, activity was observed across all 60 channels of the micro-electrode array. When 30 μM KRM-II-81 was added to the bath, significant attenuation of firing was observed (0.08 ± 0.01 vs. 0.01 ± 0.005) ($F_{1,58} = 593$, $p < 0.0001$). The electrode location was not a significant factor in this drug effect ($F_{58,58} = 1.2$, $P = 0.22$). For both experiments with human tissue, we also evaluated the recovery of firing after suppression by KRM-II-81. In both cases, significant recovery was not observed as previously reported with other anticonvulsant mechanisms (Zwart et al., 2014).

Discussion

The clinical value of classical 1,4-benzodiazepine modulators of GABA is evidenced by the fact that some of them have been marketed for over 50 years without a suitable replacement (anxiety, epilepsy, sleep). However, for therapeutic use, benzodiazepines have some issues that impact prescribing practices and safety; these include abuse and dependence liabilities, as well as cognitive and motor impairing effects (Drummer, 2002; Canadian Agency for Drugs and Technologies in Health, 2014). Retaining the valuable therapeutic utility of benzodiazepine site modulation of GABAergic neurotransmission without the safety issues has thus been a goal of drug discovery efforts since 1979 (Klepner et al., 1979; Skolnick, 2012).

A compelling clinical opportunity exists in the development of $\alpha 1$ -sparing subtype-selective GABA_A ligands. These ligands are expected to result in superior treatments for seizures and anxiety without causing amnesia and ataxia, or the propensity for addiction/dependence (see Ator et al., 2010; Attack et al., 2011; Ralvenius et al., 2015; Zuiker et al., 2106). In particular, positive allosteric modulators (PAMs) of $\alpha 2/\alpha 3$ -comprised GABA_A receptors have been shown to be anxiolytic (Attack et al., 2006; Mirza et al., 2008; Fischer et al., 2010; Poe et al., 2016; Witkin et al., 2017), and analgesic (Ralvenius et al., 2015; Lewter et al. 2017) in animal models.

HZ-166 is an $\alpha 2/\alpha 3$ -selective benzodiazepine that has been reported to have anticonvulsant activity at non-motor-impairing doses in both mice (maximal electroshock, and PTZ) and rats (maximal electroshock, PTZ, and hippocampal kindling) (Rivas et al., 2009). However, HZ-166 was not active in any of the mouse or rat assays that detected efficacy with diazepam in the present study. The disparity of these negative findings with the anticonvulsant effects reported earlier is hypothesized to be due to differences in exposure of HZ-166 and/or differences in experimental parameters of the epilepsy models across studies. In general, doses were higher in the work of Rivas et al. (2009) than in the present study.

More extensive analysis of the metabolic fate of HZ-166 has determined that this molecule produces low systemic exposure that could limit its efficacy and underestimate its potential side-effect liabilities (Poe et al., 2016). Replacement of the ester function with an oxazole bioisostere in KRM-II-81 resulted in marked enhancements in systemic and central drug exposures and augmentation of anxiolytic-like effects in rats (Poe et al., 2016; Witkin et al., 2017) as well as analgesic efficacy (Lewter et al., 2017).

KRM-II-81 was active in all of the anticonvulsant assays tested and in multiple cases demonstrated broader and greater efficacy than diazepam. Furthermore, KRM-II-81 displayed a wider margin of efficacy to motor side effects than diazepam providing further support for the idea that GABA_A receptor PAMs with greater efficacy against containing $\alpha 2/3$ subunits than $\alpha 1$ subunits can be anticonvulsant with lower liability for motor impairment than PAMs with greater intrinsic efficacy at $\alpha 1$ subtypes. Evidence that agonist activity at $\alpha 1$ -containing GABA_A receptors can impede motor function was provided by Rudolph and colleagues (1999). They showed that genetic inactivation of $\alpha 1$ -containing GABA_A receptors blocked motor-impairing effects of diazepam but had reduced impact on its anticonvulsant effects. Our data with the $\alpha 1$ -selective antagonist β -CCT (Huang et al., 2000) is confirming of this classic data. β -CCT significantly attenuated the profound motor-impairing effects of diazepam without significantly

altering its anticonvulsant effects. These findings are consistent with, but not alone sufficient to prove, the idea that KRM-II-81 and its analogs produce anticonvulsant effects through positive allosteric amplification of $\alpha 2/3$ -containing GABA_A receptors. Comparable studies were conducted with diazepam in the antidepressant-detecting assay, the forced-swim test. KRM-II-81, KRM-II-82, and MP-III-080 were all antidepressant-like in this test; diazepam was not. However, blockade of the motor-impairing effects of diazepam by β -CCT revealed an underlying antidepressant phenotype in mice (Methuku et al, 2018). KRM-II-81 and the other analogs, where tested, were active with full or nearly full efficacy against multiple seizure types in both mice and rats. The compounds were also active against both acute and chronic seizure provocation models with both chemical and electrical stimuli. Molecules were effective against MES-induced seizures, a model of generalized seizures, and importantly against 6Hz seizures in mice. The 6Hz seizure model detects efficacious anticonvulsants used for partial seizures that might not be detected by other preclinical tests (Barton et al., 2001). PTZ-induced clonus is known to be sensitive to GABA_A PAMs and is used to detect effects of drugs on generalized seizures as is the PTZ seizure threshold model that additionally determines the magnitude by which a compound can prevent seizures while animals are being intravenously infused with PTZ. Seizure kindling of the basolateral amygdala is used to detect compounds that can depress the seizures generated in animals after they have been sensitized to the seizure inducing effects of electrical stimulation after many days of repeated exposure. In contrast to the other models, the seizures blocked by this model have been generated in a seizure sensitized state.

Compound KRM-II-82 was equally effective in the rat PTZ model as KRM-II-81 while MP-III-80 exhibited similar activity to diazepam (Figures 4 and 5). While KRM-II-082 is more potent than KRM-II-81 in vitro its equal efficacy in the PTZ assay is presumably due to lower metabolic stability and therefore lower exposure in vivo (Poe, et al. 2016).

Given the exceptional anticonvulsant profile of KRM-II-81, we had the opportunity to evaluate its potential efficacy in human epileptic brain. Under conditions that have been reported to show the anticonvulsant activity of the AMPA receptor antagonist perampanel (Fycompa), we showed here that KRM-II-81 also decreased picrotoxin-induced increases in cortical firing rates across an electrode array. Two weaknesses of these human data are first, the study of only one patient for each compound, and second, the use of picrotoxin as an amplifier of firing rates. The first issue will be remedied by future systematic replications of these findings when other patients present themselves. The use of picrotoxin is standard in our work as a neuroexcitant and is generally needed as human epileptic tissue is very rarely active without excitation (Hobbs et al., 2010). In cortical tissue from a second epileptic patient, we utilized a 4-aminopyridine or AP-4 as the excitant. KRM-II-81 was also efficacious in suppressing AP-4-enhanced firing (K^+ channel-driven) in the present study. Thus, the ability of KRM-II-81 to act as an anticonvulsant in human epileptic tissue occurs through general anticonvulsant mechanisms and not due to competition with picrotoxin at GABA_A receptors per se. In these experiments, recovery of firing rates post washout of KRM-II-81 was not observed. This is a typical state of affairs in this assay system (Zwart et al., 2014). Importantly, it has been observed from other experiments, that the lack of recovery is not due to run down in the slice, that is, electrical activity is enduring

in slices without drug addition (Hobbs et al., 2010). The fact that KRM-II-81 suppressed firing under both picrotoxin stimulation and under AP-4 stimulation increases the generality and strength of these data. Nonetheless, these experiments with $n=1$ per condition require systematic replication when human tissue again becomes available for investigation.

In conclusion, the present series of experiments has exemplified the qualitatively superior *in vivo* pharmacology of KRM-II-81 vs. its ester-substituted analog, HZ-166. Further, the data shown here document the superiority of KRM-II-81 over that of the known anticonvulsant diazepam in terms of broad anticonvulsant activity and maximal effect. Moreover, at equi-efficacious doses, KRM-II-81 also demonstrated a wider margin of effect compared to undesirable motor-impairing effects. Future studies will be needed to determine if KRM-II-81, as a bioavailable $\alpha 2/\alpha 3$ -selective ligand, is qualitatively distinct from non α -subtype selective GABA_A modulators in animal models of other neurological and psychiatric conditions. It will also be valuable to more fully evaluate any other potential strengths (e.g., reduced tolerance/dependence liabilities) or weaknesses (e.g., additional side-effects). In the meantime, KRM-II-81 is presented as a valuable tool for interrogating the biological significance and potential therapeutic value of selective allosteric amplification of GABA signaling through $\alpha 2/\alpha 3$ -containing GABA_A receptors. Moreover, while not studied as extensively as KRM-II-81, KRM-II-82 and MP-III-080 could also potentially be viewed as tool compounds but with different properties as KRM-II-81. The *in vivo* data from the present study can thus serve as a guide to dosing parameters for KRM-II-81 that predict engagement of central GABA_A receptors.

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References

Atack, J. R. GABAA receptor subtype-selective modulators. I. $\alpha 2/\alpha 3$ -selective agonists as non-sedating anxiolytics. *Curr. Top. Med. Chem.* 2011, 11, 1176 – 1202

Atack JR, Wafford KA, Tye SJ, Cook SM, Sohal B, Pike A, Sur C, Melillo D, Bristow L, Bromidge F, Ragan I, Kerby J, Street L, Carling R, Castro JL, Whiting P, Dawson GR, McKernan RM. TPA023 [7-(1,1-dimethylethyl)-6-(2-ethyl-2H-1,2,4-triazol-3-ylmethoxy)-3-(2-fluorophenyl)-1,2,4-triazolo[4,3-b]pyridazine], an agonist selective for $\alpha 2$ - and $\alpha 3$ -containing GABAA receptors, is a nonsedating anxiolytic in rodents and primates. *J Pharmacol Exp Ther.* 2006 316:410-422

Ator NA, Atack JR, Hargreaves RJ, Burns HD, Dawson GR. Reducing abuse liability of GABAA/benzodiazepine ligands via selective partial agonist efficacy at $\alpha 1$ and $\alpha 2/3$ subtypes. *J Pharmacol Exp Ther.* 2010 332:4-16.

Barton ME, Klein BD, Wolf HH, White HS. Pharmacological characterization of the 6 Hz psychomotor seizure model of partial epilepsy. *Epilepsy Res.* 2001;47:217-27

Chatfield M and Mander A (2009) The Skillings-Mack test (Friedman test when there are missing data). *The Stata Journal* 9:299-305.

Benzodiazepines - Effects on Human Performance and Behavior. **Drummer OH.**

Forensic Sci Rev. **2002** Feb;14(1-2):1-14.

Fischer BD, Licata SC, Edwankar RV, Wang ZJ, Huang S, He X, Yu J, Zhou H, Johnson EM Jr, Cook JM, Furtmüller R, Ramerstorfer J, Sieghart W, Roth BL, Majumder S, Rowlett JK. Anxiolytic-like effects of 8-acetylene imidazobenzodiazepines in a rhesus monkey conflict procedure. *Neuropharmacology.* 2010 59:612-618.

Fischer BD, Schlitt RJ, Hamade BZ, Rehman S, Ernst M, Poe MM, Li G, Kodali R, Arnold LA, Cook JM. Pharmacological and antihyperalgesic properties of the novel $\alpha 2/3$ preferring GABA_A receptor ligand MP-III-024. *Brain Res Bull.* 2017 131:62-69

Gasior, M., Carter, R. B. and Witkin, J. M. Neuroactive steroids: Potential therapeutic use in neurological and psychiatric disorders. *Trends in Pharmacological Sciences* 20: 107-112, 1999.

Guerreiro CA. Epilepsy: Is there hope? *Indian J Med Res.* 2016 Nov;144(5):657-660. doi: 10.4103/ijmr.IJMR_1051_16.

Aberrant neuronal avalanches in cortical tissue removed from juvenile epilepsy patients. Hobbs JP, Smith JL, **Beggs JM.** *J Clin Neurophysiol.* 2010 Dec;27(6):380-6. doi: 10.1097/WNP.0b013e3181fdf8d3.

Huang Q, He X, Ma C, Liu R, Yu S, Dayer CA, Wenger GR, McKernan R, Cook JM . Pharmacophore/receptor models for GABA_A/BzR subtypes ($\alpha 1\beta 3\gamma 2$, $\alpha 5\beta 3\gamma 2$, and $\alpha 6\beta 3\gamma 2$) via a comprehensive ligand-mapping approach. *J Med Chem* 2000 43:71–95.

Efficacy and safety of anti-epileptic drugs in patients with active convulsive seizures when no IV access is available: Systematic review and meta-analysis.

Jain P, Sharma S, Dua T, Barbui C, Das RR, Aneja S.
Epilepsy Res. **2016** May;122:47-55. doi: 10.1016/j.epilepsyres.2016.02.006

Resolution of two biochemically and pharmacologically distinct benzodiazepine receptors.
Klepner CA, Lippa AS, Benson DI, Sano MC, Beer B.
Pharmacol Biochem Behav. 1979 Oct;11(4):457-62

Lewter LA, Janet L. Fisher, Justin N. Siemian, Kashi Reddy Methuku³,
Poe MM, James M. Cook³, Jun-Xu Li. 2017 Antinociceptive effects of a novel $\alpha 2/\alpha 3$ -subtype selective GABA_A receptor positive allosteric modulator. *ACS Chemical Neuroscience*, 8(6):1305-1312. doi: 10.1021/acschemneuro.6b00447

Methuku K.R., X. Li, R. Cerne, S. D. Gleason, J. M. Schkeryantz, V. V. N. P. B. Tiruveedhula, L. K. Golani and G. Li, M.M. Poe, Md. T. Rahman, J. M. Cook, J. L. Fisher, and J. M. Witkin An Antidepressant-Related Pharmacological Signature for Positive Allosteric Modulators of $\alpha 2/3$ -Containing GABA_A Receptors, *Pharmacology, Biochemistry, and Behavior*, 2018 (in press).

Mirza, N. R.; Larsen, J. S.; Mathiasen, C.; Jacobsen, T. A.; Munro, G.; Erichsen, H. K.; Nielsen, A. N.; Troelsen, K. B.; Nielsen, E. O.; Ahring, P. K. NS11394 [3'-(5-(1-Hydroxy-1-methyl-ethyl)-benzoimidazol-1-yl)-biphenyl-2-carbonitrile], a unique subtype-selective GABA_A receptor positive allosteric modulator: In vitro actions, pharmacokinetic properties and in vivo anxiolytic Efficacy. *J. Pharmacol. Exp. Ther.* 2008, 327, 954-968.

Likert scales, levels of measurement and the "laws" of statistics.

Norman G.
Adv Health Sci Educ Theory Pract. **2010** Dec;15(5):625-32. doi: 10.1007/s10459-010-9222-y.

Poe MM., Kashi R. Methuku,^{1,‡} Guanguan Li,¹ Kelly A. Teske,^{1,#} Douglas C. Stafford,² Leggy A. Arnold,^{1,2} Jeffrey W. Cramer,³ Timothy M. Jones,³ Jeff S. McDermott,³ Jeffrey M. Witkin,³ James M. Cook,^{1,2} and Jeffrey M. Schkeryantz. Synthesis and characterization of a novel GABA_A receptor ligand, 5-(8-Ethynyl-6-(pyridin-2-yl)-4H-benzo[f]imidazo[1,5-a][1,4]diazepin-3-yl)oxazole (KRM-II-81), that combines exceptional drug stability, pharmacokinetics, and anxiolytic efficacy. *J. Med. Chem.*, in press, 2016. ^{UPDATE}

Ralvenius WT, Benke D, Acuña MA, Rudolph U, Zeilhofer HU. Analgesia and unwanted benzodiazepine effects in point-mutated mice expressing only one benzodiazepine-sensitive GABA_A receptor subtype. *Nat Commun.* 2015 Apr 13;6

Rivas FM, Stables JP, Murphree L, Edwankar RV, Edwankar CR, Huang S, Jain HD, Zhou H, Majumder S, Sankar S, Roth BL, Ramerstorfer J, Furtmüller R, Sieghart W, Cook JM.

Antiseizure activity of novel gamma-aminobutyric acid (A) receptor subtype-selective benzodiazepine analogues in mice and rat models. *J Med Chem.* 2009 52(7):1795-8.

Different GABAA receptor subtypes mediate the anxiolytic, abuse-related, and motor effects of benzodiazepine-like drugs in primates.

Rowlett JK, Platt DM, Lelas S, Atack JR, Dawson GR.

Proc Natl Acad Sci U S A. **2005** Jan 18;102(3):915-20

Rudolph U, Crestani F, Benke D, Brünig I, Benson JA, Fritschy JM, Martin JR, Bluethmann H, Möhler H. Benzodiazepine actions mediated by specific gamma-aminobutyric acid(A) receptor subtypes. *Nature.* 1999 401(6755):796-800. Erratum in: *Nature* 2000 404(6778):629.

Epilepsy treatment in adults and adolescents: Expert opinion, 2016.

Shih JJ, Whitlock JB, Chimato N, Vargas E, Karceski SC, Frank RD.

Epilepsy Behav. 2017 Apr;69:186-222. doi: 10.1016/j.yebeh.2016.11.018.

Sieghart, W.; Ernst, M. Heterogeneity of GABA_A receptors: revived interest in the development of subtype-selective drugs. *Curr. Med. Chem.: Cent. Nerv. Syst. Agents* 2005, 5, 217 - 242.

Skolnick P. Anxiolytic drugs: on a quest for the Holy Grail. *Trends Pharmacol Sci.* 2012 33(11):611-620.

Swinyard, E.A., Woodhead, J.H., White, H.S. & Franklin, M.R. (1989): General principles: experimental selection, quantification, and evaluation of anticonvulsants. In: *Antiepileptic Drugs*, Third Edition R.H. Levy, R.H. Mattson, B. Melrum, J.K. Penry and F.E. Dreifuss eds, pp. 85-102. New York: Raven Press

Witkin JM, Cerne R, Wakulchik M, S J, Gleason SD, Jones TM, Li G, Arnold LA, Li JX, Schkeryantz JM, Methuku KR, Cook JM, Poe MM. Further evaluation of the potential anxiolytic activity of imidazo[1,5-a][1,4]diazepin agents selective for $\alpha 2/3$ -containing GABA_A receptors. *Pharmacol Biochem Behav.* 2017 Jun;157:35-40

Zuiker, R. G. J. A.; Chen, X.; Osterberg, O.; Mirza, N. R.; Muglia, P.; de Kam, M.; Klaassen, E. S.; van Gerven, J. M. A. NS11821, a partial subtype-selective GABA_A agonist, elicits selective effects on the central nervous system in randomized controlled trial with healthy subjects. *J. Psychopharmacol.* 2016, 30, 253 - 262.

Zwart, R.; Sher, E. ; Ping, X. ; Jin, X.; Sims, J.R.; Chappell, A.S.; Gleason, S.D.; Hahn, P.J.; Gardinier, K.; Gernert, D.; Beggs, J.M.; Hobbs, J.; Smith, J.L.; Valli, S.N. ; Witkin, J.M. Perampanel (Fycompa®), the First FDA-Approved Antagonist of α -amino-3-hydroxy-5-methyl- 4-isoxazolepropionic acid (AMPA) Receptors for the Treatment of Epilepsy: Studies in Human Epileptic Brain, Non-Epileptic Brain, and in Rodent Models. *J. Pharmacol. Exp. Ther.*, 2014, 351:124-133

Legends for Figures

Figure 1. Structures of HZ-166, KRM-II-81, KRM-II-82, and MP-III-080.

Figure 2. KRM-II-81 suppresses the hyper-excitation in a network of cortical neurons.

A,C; Reversible potentiation of spontaneous neuronal activity (A-spiking, C-bursting) by removal of magnesium or by addition of 1mM 4-aminopyridine (4AP) to external solution. Removal of magnesium produced an increase in spiking frequency ($347 \pm 81\%$ of control, $p=0.04$, $n=8$) and an increase in bursting frequency ($443 \pm 128\%$ of control, $p=0.02$, $n=6$). Addition of 1mM 4-aminopyridine to the external solution primarily increased the frequency of spikes ($327 \pm 46\%$ of control, $p=0.005$, $n=12$) with smaller effect on frequency of bursts ($120 \pm 21\%$ of control, $p>0.05$, $n=10$) (Fig. 2A, C). **B;** KRM-II-81 ($3\mu\text{M}$) had no significant effect on spiking ($n=7$) in the network of cortical neurons bathed in normal magnesium containing external solution and produced small depression of spiking under conditions of reduced magnesium (0.1mM Mg^{++} , $n=4$, $p>0.05$). When the network was hyper-excited by removal of magnesium in external solution the addition of $3\mu\text{M}$ KRM-II-81 suppressed the frequency of spiking to $62.2 \pm 3.8\%$ of control ($p=0.03$, $n=7$). A similar effect of $3\mu\text{M}$ KRM-II-81 was observed in a neuronal network hyper-excited by addition of 1mM 4AP where the frequency of spiking was reduced to $50.5 \pm 7.5\%$ of control ($p=0.004$, $n=7$). **D;** KRM-II-81 ($3\mu\text{M}$) had no significant effect on bursting in normal magnesium ($n=7$) and in reduced magnesium containing external solution (0.1mM Mg^{++} , $n=4$, $p>0.05$). When the network was hyper-excited by removal of magnesium $3\mu\text{M}$ KRM-II-81 reduced the frequency of bursting to $54.8 \pm 7.1\%$ of control, ($p=0.06$, $n=5$). A similar effect of $3\mu\text{M}$ KRM-II-81 was observed in a neuronal network hyper-excited by addition of 1mM 4AP where the frequency of bursting was reduced to $50.9 \pm 8.8\%$ of control ($p=0.03$, $n=6$). The data were normalized to baseline activity and reported as mean $\pm$ standard error of the mean (SEM). One parameter t-test to determine statistical difference (A,C); analysis of variance (ANOVA) with Dunnett's multiple comparison test was utilized to compare between group effects (B, D); $P<0.05$ was considered significant (asterisk). MEA recordings from a culture of rat E18 cortical neurons (DIV 19-25). All recordings were performed at 37°C .

Figure 3. Comparative effects of HZ-166, KRM-II-81, KRM-II-82, MP-III-080 and diazepam against electroshock-induced convulsions in mice. Quantal data were analyzed by Fisher's Exact Probability test (*: $p<0.05$). Each point represents the effects in 10 mice. Baseline values across studies (effects of drug vehicle) was $94 \pm 2.5\%$

Figure 4. Comparative effects of HZ-166, KRM-II-81, and diazepam against clonic convulsions induced by pentylenetetrazole (35 mg/kg, s.c.) and on motor performances on an inverted screen in rats. Each point represents the effect in groups of 5 -8 rats. Quantal data were analyzed by Fisher's Exact Probability test (*: $p<0.05$). For motor scores, each point represents the mean $\pm$ SEM in groups of 5 (diazepam, 3 mg/kg) or 8 (all other data) rats. Data were analyzed by ANOVA followed by Dunnett's test with * signifying statistically-significant separation from vehicle control values ($p<0.05$). PTZ alone produced convulsions in $96 \pm 4\%$ of the rats. The baseline motor scores were 0.12 ± 0.8 .

Figure 5. Comparative effects of KRM-II-82, MP-III-080, and valproate against clonic convulsions induced by pentylenetetrazole (35 mg/kg, s.c.) and on motor performances on an inverted screen in rats. Each point represents the effect in groups of 5 (3 mg/kg dose groups) or 8 (all other groups) rats. Quantal data were analyzed by Fisher's Exact Probability test (*: $p < 0.05$). PTZ alone produced convulsions in $97 \pm 2\%$ of the rats. The baseline motor scores was 0.08 ± 0.1 .

Figure 6. Comparative effects of HZ-166, KRM-II-81, KRM-II-82, and diazepam against convulsions induced by pentylenetetrazole (PTZ, i.v.) in rats. Data show dose of PTZ required to induce convulsions as a function of drug dose. Each point represents the mean $\pm$ SEM effect in groups of 8 rats. Data were analyzed by ANOVA followed by Dunnett's test with * signifying statistically-significant separation from vehicle control values ($p < 0.05$). Each point represents the effects in 8 mice. Baseline values across studies (effects of drug vehicle) was 35.1 ± 1.2

Figure 7. Comparative effects of HZ-166, KRM-II-81, KRM-II-82, and diazepam in rats that were seizure kindled to daily electrical stimulations of the basolateral amygdala. Each point represents the mean $\pm$ SEM effect in groups of 8 rats. Data were analyzed by ANOVA followed by Dunnett's test with * signifying statistically-significant separation from vehicle control values ($p < 0.05$). Seizure free scores (seizure severity = 0) were 0/8 for HZ-166, 1/8 for KRM-II-82, 2/8 for diazepam, and 7/8 for KRM-II-81. Additional non-parametric analysis was conducted on the seizure severity data with essentially comparable results and is reported in Results.

Figure 8. Dampening effects of KRM-II-81 firing rate frequency (Hz) in tissue resected from a juveniles with epilepsy. Data were collected for 1 hour under each control conditions (no KRM-II-81 (unfilled circles) or in the presence of $30\mu\text{M}$ (KRM-II-81) using either picrotoxin (left panel) or AP-4 (right panel) as a stimulant of neuronal activity.

Table 1. Comparative potencies of HZ-166, KRM-II-81, and diazepam across multiple seizure models.

Assay	HZ-166 ^a	KRM-II-81	KRM-II-82	MP-III-080	Diazepam ^g
Electroshock ^a	30	10	30	---	3
Electroshock to Criteria ^b		>30	30	30	5.6
PTZ Clonus Potency ^a	>30	30	10	10	10
PTZ Clonus to Criteria 1 ^b		>30	30	10	10
PTZ Threshold Potency ^a	>60	10	10	---	1
PTZ Threshold to Criteria ^c	>60	30	30	---	>1
After-Discharge Threshold Potency ^a	>60	10	10	---	10
After-Discharge Threshold to Criteria ^d	>60	10	30	---	>10
After-Discharge Duration Potency ^a	>60	10	>30	---	>10
After-Discharge Duration to Criteria ^e	>60	10	>30	---	>10
Seizure Severity Potency ^a	>60	10	10	---	10
Seizure Severity to Criteria ^f	>60	10	>30	---	10
6Hz Potency ^a	---	50	---	---	---
6 Hz to Criteria ^b	---	50	---	---	---

^aValues are minimal effective doses (MED) in mg/kg. MED was defined as the lowest dose administered that produced statistically significant protection compared to vehicle control. Doses tested are shown in the figures and tables. The compounds were generally dosed in ½ log increments from 3 mg/kg to 30 mg/kg with the exception of diazepam that was given at doses beginning at 0.1 mg/kg (PTZ seizure threshold), 0.3 mg/kg (amygdala kindling), and 1 mg/kg (maximal electroshock). HZ-166 was dosed up to 60 mg/kg, and the doses in the 6Hz model were 10-50 mg/kg. Valproic acid was given at 300 mg/kg.

^bMED producing maximal protection (0% seizures) in mg/kg

^cMED for producing $\geq 2x$ PTZ seizure threshold at baseline in mg/kg

^dMED for producing after-discharge threshold ≥ 3 in mg/kg

^eMED for producing after-discharge duration ≤ 30 sec in mg/kg

^fMED for producing seizure severity score ≤ 2

^gBold values in the table represent doses not producing full efficacy comparable to valproic acid (300 mg/kg, i.p.) as defined by the efficacy criteria in b,c,d,e and f above

--- Data not collected

Table 2. Effects of orally-administered KRM-II-81 in the 6Hz seizure model and or observed motor effects.

<u>Dose</u>	<u>Number protected/number tested</u>	
10	0/8	
25	3/8	
50	7/8	
<u>Dose</u>	<u>Number with observed motor effects</u>	<u>Observation</u>
50	0/8	
100	3/8	tremors
150	5/8	tremor, unable to grasp
200	8/8	more severe tremor' loss of righting

Mice were tested at 2 hr post dosing for seizures post 6Hz stimulation and another group was evaluated at 4 hr post dosing for motor side effects.

Table 3. Protective indices (PI) for diazepam, KRM-II-81, KRM-II-82 and MP-III-080^a

Assay	KRM-II-81	KRM-II-82	MP-III-080	Diazepam
PTZ Clonus	5	15	>3	1
PTZ Threshold	15	15	---	10
After-Discharge Threshold	15	15	---	1
After-Discharge Duration	15	<5	---	<1
Seizure Severity	15	15	---	1
6Hz	4	---	---	---

^aPI values were calculated as the minimal effective dose producing motor impairment /minimal effective doses producing efficacy. Electroshock values were not computed since motor impairment at higher doses was not evaluated. Values for HZ-166 could not be calculated for any measure due to lack of efficacy. Values of ≤ 1 are highlighted in bold. Values $>x$ could not be assigned a definitive value as the highest dose tested did not significantly impair motor performance.

Table 4. Unbound Plasma and brain concentrations of KRM-II-81 (nM) after i.p. dosing in male, Sprague-Dawley rats (n=3).

<u>Dose (mg/kg, i.p.)</u>		
17.3	Plasma	3290 $\pm$ 107
	Brain	1640 $\pm$ 141
121	Plasma	10000 $\pm$ 2147
	Brain	6250 $\pm$ 1652
121/17.3 Ratio	Plasma	3.04
	Brain	3.81

Values are means $\pm$ S.E.M. of 3 rats in nM.

KRM-II-81

Figures

Fig 1.

HZ-166

KRM-II-81

KRM-II-82

MP-III-080

Fig 2.

Fig 3. - Electroshock Mouse

Fig 4. PTZ Rat

Fig 5. - PTZ Rat

Fig 6. - PTZ Seizure Threshold

Fig 7.

Fig 8.

Picrotoxin Stimulation

AP-4 Stimulation