A Pilot Study: Treatment of High Alcohol Consumption in a Novel Minipig Model of Alcohol Use Disorder

Xiaobo Liu, Ph.D.[a], **Praneetha Panthagani, M.S.**[a], **Ana G. Gutierrez, BS**[b], **Arlette Vega, BS**[b], **Abdul A. Shaik, M.S.**[b], **Monica G. Aguilera, BS**[b], **Jordan N. Sanchez, BS**[b], **Joshua O. Willms, Ph.D.**[a], **Brittany Backus, Ph.D.**[c], **Bruce Blough, Ph.D.**[d], **Elliott Pauli, BS**[d], **Ted W. Reid, Ph.D.**[e], **Thomas Benton, Ph.D.**[d], **Jeremy D. Bailoo, Ph.D.**[b], **Susan E. Bergeson, Ph.D.**[b]

[a]Department of Pharmacology & Neuroscience, Texas Tech University Health Sciences Center, Lubbock, Texas, USA;

[b]Department of Cell Biology & Biochemistry, Texas Tech University Health Sciences Center, Lubbock, Texas, USA;

[c]Department of Animal and Food Sciences, Texas Tech University, Lubbock, Texas, USA;

[d]RTI International, Research Triangle Park, North Carolina, USA;

[e]Department of Ophthalmology & Visual Sciences, Texas Tech University Health Sciences Center, Lubbock, Texas, USA

Abstract

Three medications are FDA approved in the US for treatment of Alcohol Use Disorder (AUD), and a few others are used off-label. Patient compliance and efficacy in the broader population are major hurdles for current AUD medications. As a consequence, there is an urgent need for improved pharmacotherapeutics to complement behavioral approaches. Here, we report pilot testing of a minocycline analog, 10-butylether minocycline (BEM, 10 mg/kg p.o.), in two female minipigs with free-choice drinking to intoxication for nearly two and a half years. Each pig met DSM-5 criteria for diagnosis of severe AUD, and BEM reduced both alcohol intake and preference. BEM is currently undergoing testing for approval as an Investigational New Drug by the FDA for AUD treatment.

Keywords

✉CONTACT Jeremy D. Bailoo jeremy.bailoo@ttuhsc.edu Cell Biology and Biochemistry, School of Medicine, Texas Tech University Health Sciences Center, 3601 4th St. MS6540, Lubbock, TX 79430-6540, USA; Susan E. Bergeson susan.bergeson@ttuhsc.edu.

Introduction

Nearly 2 billion people worldwide are affected by alcohol misuse annually resulting in over 5% of all deaths (Fairbanks et al., 2020). Alcohol Use Disorder (AUD) is a complex brain disorder with multifactorial genetic and environmental risk factors. In addition to recognized sex-differences in the onset, progression and severity of the disease, there are many drinking patterns and lifetime precipitating events that also complicate the disease process (Flores-Bonilla & Richardson, 2020). AUD has historically been quite recalcitrant to medication-based treatment (Fairbanks et al., 2020). While there are three FDA-approved medications in the United States and some others are used off-label, they are only prescribed for ~ 20% of the patients. FDA-approved drugs, naltrexone and disulfiram, suffer poor patient compliance due to their side-effect profiles while acamprosate has shown efficacy only in a fraction of the population. The off-label antiepileptic medications such as topiramate have a higher risk of cognitive dysfunction, numbness in extremities and taste abnormalities (Fairbanks et al., 2020; Koob, 2024). As such, there is substantial demand for new medications. Twelve-step and behavioral modification programs are currently the most common approaches to overcome AUD in the United States, often with multiple cycles of relapse prior to ultimate success (Bruce & Gross, 2023; Koob, 2024).

AUD is a complex trait with genetic and environmental influences. The basic brain neurochemistry and anatomical circuitry underlying the addiction process are reasonably well understood (Koob & Volkow, 2016; Uhl et al., 2019), yet the approved medications have been, and continue to be, selected using a classic pharmacology approach. Typically, in order to limit potential side-effects, a highly selective drug with high affinity to a single target is usually chosen. To best address current AUD efficacy challenges, we took a multimodal approach and selected targets using pathway analyses. As described in (Agrawal et al., 2014), we took a bioinformatics approach and identified several targets involved in neuroimmune function (Agrawal et al., 2014) to use as the basis for the selection of a new, potential AUD therapeutic. Based on the literature in 2010, we selected minocycline for its many positive non-antibiotic, off-target, neuroimmune, and anti-inflammatory effects, and showed that it, along with doxycycline and tigecycline significantly and dose-responsively reduced alcohol consumption (Syapin et al., 2016). We then used the differences in efficacy (a structure-function approach) and the solved X-ray crystallography structure of tigecycline bound to the bacterial ribosome (Schedlbauer et al., 2015) to modify the molecular structure to prevent A-site binding, ultimately removing any anti-microbial characteristics. Sixteen new chemically modified analogs were tested for loss of antimicrobial action and retention of efficacy to reduce alcohol consumption. Based on efficacy, safety, and pharmacokinetic parameters not detailed here, 10-butyl minocycline became our lead compound.

After positive outcomes in four different mouse AUD models (unpublished data), we tested the hypothesis that BEM would also reduce ethanol consumption in two minipigs that had been consistently drinking to intoxication for approximately two and one-half years. The selected pigs were the highest, and most consistent, ethanol consuming animals as part of a pilot study of five animals used to develop a new swine model of AUD. The model was based on specifically designed behavioral and physiological testing to best evaluate the 11

Diagnostic and Statistical Manual of Mental Disorders, fifth edition (DSM-5) criteria for diagnosing AUD [unpublished; see methods and (Liu et al., 2024)].

BEM given orally at 10 mg/kg reduced both ethanol consumption and preference in both individual minipigs. Our results, together with other strong preclinical evidence, suggest that BEM may be well poised as a new AUD medication; it is currently undergoing the FDA-required testing for Investigational New Drug (IND) approval for use in human clinical trials. BEM's multimodal actions seem advantageous such that should any individual have single genetic mutations that would have rendered other single target drugs inactive, BEM would likely continue to be efficacious, or only slightly less so. Additionally, a medication with low affinity to numerous targets in the addiction pathway may act as a rheostat to help reset normal homeostasis and overcome the allostatic changes in brain chemistry and function caused by "stress" of long-term, high ethanol exposure that results in AUD.

Methods

Animal husbandry

Five Sinclair minipigs, 7 months old, were purchased from Sinclair BioResources (Auxvasse, MO). Individually housed in the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited Texas Tech University Health Sciences Center environmentally controlled vivarium (23°C–25°C, humidity between 30%–60%, 11:13 lighting), they were provided ad libitum water and two feedings per day (LabDiet 5081, Richmond, IN). The animals were allowed to acclimate until they reached the human equivalent of adulthood (~1.5 years = 21 years for humans). Animals had intermittent ethanol drinking bouts over the course of the following two and a half years, disrupted by hoof trimming, construction, and the COVID-19 quarantine (see Figure 1). Due to a limitation of available drug, only two of the most consistent drinkers from day-to-day were tested with 10-butylether minocycline (BEM) at the end of the DSM-5 experimental model development. The study was IACUC-approved and care was provided by our institutional veterinarian (iVet) and certified laboratory animal technicians.

Two-bucket choice alcohol administration

Translucent five-gallon paint buckets with liter markings were fitted with a 1-inch Tygon tube to a hole in the bottom using dual gaskets, a shutoff valve, and sealed coupler. The other end of the tubing connected to a standard Trojan Cast-Aluminum swine water bowl fitted with an O-ring mounted stainless steel nipple bolted to an aluminum plate attached to the door of the swine kennel (see Figure 2A). All supplies were obtained from Gebo's, Lowes, and Tractor Supply Company in Lubbock, TX. Alcohol and water were filled daily and measured in approximately 1-h increments over the work day. Animals were only allowed to drink up to 3.5 L per day to avoid death by alcohol poisoning. The alcohol and water buckets were swapped in location every other day to avoid side preference influencing consumption. At the start of the experimentation, ethanol was simply dissolved in water at final concentrations of 2.5, 5.0, 7.5, and 10% v/v. The percent ethanol was progressively increased at two-week intervals. Due to construction and testing for other behavioral and physiological traits, three rounds of escalations occurred in total (see Figure 1). The 10%

ethanol concentration continued for some time in each round, and in the last round before testing BEM for efficacy to reduce drinking and/or preference, they drank for additional 6.5 months.

Testing for determination of DSM-5 modelled diagnosis of AUD

Behavioral tests created or adapted from general animal studies were used to model the 11 DSM-5 criteria used in diagnosis for AUD (mild = 2–3, moderate = 4–5, severe   6) in humans and are briefly described in quotes below (APA, 2013):

Criterion 1 – "Alcohol is often taken in larger amounts over a longer period than intended." While we cannot assess what an animal "intended," here we model Criteria 1 by assessing preference in a two-bucket choice (see Figure 1A). Our operational definition was achieving a strong preference of > 0.7.

Criterion 2 – "There is a persistent desire or unsuccessful efforts to cut down or control alcohol use." Again, we cannot expect an answer if we ask our minipigs if they wish to reduce consumption. However, we can follow their drinking over time and we set repeatedly drinking to intoxication by free-choice as our operational definition for criteria 2.

Amounts of alcohol consumed per day and the blood alcohol concentration (BAC) achieved were measured. We assigned the criteria as met by animals that drank to intoxication as that defined by NIAAA, 0.8 mg/ml in animal blood (NIAAA, 2023). Blood samples were drawn from an ear vein (50 ul) using a 0.5 inch, 26-gauge needle fitted to a 1 ml syringe, within 1 hr following the completion of the final bout at each increase in % ethanol concentration.

Criterion 3 – "A great deal of time is spent in activities necessary to obtain alcohol, use alcohol, or recover from its effects." Twenty-four-hour videos were taken and coded for time spent active or inactive during baseline testing and after alcohol consumption bouts. A significant change in baseline activity was considered to have met criteria.

Criterion 4 – "Craving, or a strong desire or urge to use alcohol." Craving was assessed using changes in the speed of consumption before and after a two-day forced restriction. An increase in time to finish their daily alcohol serving was defined as meeting criteria. This timing parameter was necessary as we limited the amount they could drink each day as to avoid death due to overdose. Otherwise, a better measure might be if they overshot the previous withdrawal consumption value.

Criterion 5 – "Recurrent alcohol use resulting in failure to fulfil major role obligations at work, school or home." The Inventory of Interpersonal Problems (Horowitz et al., 1988) is often used to assess the diagnosis of DSM-5 criterion #5, and swine cannot complete these recollection surveys. We developed a horizontal ladder test to assess their ability to ambulate using a horizontal ladder test created to mimic the "walk and turn" sobriety test done by police in the US to test for driving under the influence (Burns, 2003; Cole & Nowaczyk, 1994). The methodological details are published elsewhere test (Liu et al., 2024), and essentially consist of pre-ethanol training the animal to traverse a "horizontal ladder" made of PVC piping and five-gallon buckets filled with a bag containing 35 lbs of sand. The buckets had a series of holes so that the height of the rungs could be adjusted from 3

to 6 inches in one-inch increments to increase difficulty. Video recording of the task took place at the end of training, which was considered baseline for previous years of ethanol consumption and again at each elevation in % ethanol concentration. Operational scoring was the sum of all events that occurred over three down and back, double crossings of the ladder, briefly described as follows (for details, see test (Liu et al., 2024): 1 pt each for a kick or snout bump; 2 pts each for hind foot contact with a rung; 3 pts for anybody contact with the apparatus, while not recounting the legs; 4 pts for a slip where the animal is able to continue progressing without falling to the floor; and 5 pts for a slip that results in a fall where the animal hits the ground and comes to a temporary stop.

Criterion 6 – "Continued alcohol use despite having persistent or recurrent social or interpersonal problems caused or exacerbated by the effects of alcohol." As the pigs were housed in individual kennels as required by the TTUHSC Laboratory Animal Resources Center, we were unable to use a minipig to minipig social test due to concern about injury. However, minipigs are known to interact with humans in what could be considered a social manner. They typically show behavioral indicators of enjoyment when humans feed, groom, or pet them. It is common for them to stop activities and come to the door to "greet" familiar humans with whom they have had positive experiences. As a consequence, we used a familiar human who entered the room and stood at roughly 20 inches from the front of the kennel door. A total of 10 trials were completed using latency of the approach, and frequency and duration of interaction from captured video for scoring criteria. A baseline and scores at all increments of % ethanol increases were calculated. A score of seven out of ten was considered to have met criteria.

Criterion 7 – "Important social, occupational or recreational activities are given up or reduced because of alcohol use." Video scoring of a home pen recreational test was used to determine diagnosis of Criterion 7. A novel, cleaned soccer ball was placed in the kennel for 30 min, 2 h after the completion of drinking for the day. As our animals normally have enrichment items available, we removed them 30 min prior to testing as well as insured no humans entered the room during this time. The duration of interaction with the ball at baseline, compared to that at each % ethanol escalation was measured. A 50% decreased score was considered to meet criterion.

Criterion 8 – "Recurrent alcohol use in situations in which it is physically hazardous." Alcohol intoxication is one of the highest risks for preventable accidents, both vehicular or otherwise (Fairbanks et al., 2020). Our approach here was to use a Sportdog SD-425X [Radio Systems Corp., Knoxville, TN] waterproof shock collar as a negative reinforcer to examine whether the animals would continue to attempt to access the alcohol sipper in spite of increased level of intensity as follows: Animals were fitted with a dog shock collar set to vibrate, elicit noise and express brief shock. We previously determined that a single burst [<1 sec] at Mode 5 [low static stimulation] and level 2 intensity [of 21] did not elicit any more than modest, very brief pain in the principal investigator. Again, 10 trials were completed with > 7 attempts to continue drinking considered having met criteria.

Criterion 9 – "Alcohol use is continued despite knowledge of having a persistent of recurrent physical or psychological problem that is likely to have been caused or exacerbated by

alcohol." The sucrose preference and the home pen recreation tests were used to assess criterion #9. The home pen recreation test was described above in #7 and the sucrose test was adapted from (Figueroa et al., 2015) and was essential as follows: A choice of water or 0.5% sucrose were presented in a two-bucket choice for 30 min. Two tests in total were completed at each % ethanol of escalation and the sides were switched daily to avoid location preference. A preference of lower than 50% was considered to have met the criterion.

Criterion 10 – "Tolerance as defined by either of the following:

    a. A need for markedly increased amounts of alcohol to achieve intoxication or desired effects. *Pharmacokinetic tolerance* was measured by administering 0.8 mg/kg ethanol using 20% ethyl alcohol in water by gavage before the animals started their long drinking history and at the end of the 10% escalation and prior to BEM treatment. Blood draws from the ear vein were taken at six timepoints over approximately 8 h. Due to the difficulty of exact timing of blood draws in large, uncooperative animals, they were not evenly spaced. Ethanol content (mg/ml) was determined by gas chromatography using a standard curve as described in (Syapin et al., 2016).

    b. A markedly diminished effect with continued use of the same amount of alcohol." *Pharmacodynamic tolerance* was measured with a horizontal ladder test exacted as described in test (Liu et al., 2024). Essentially, we built an obstacle course ladder using PVC tubing and 5-gallon buckets filled with sand. The objective was to mimic the human sobriety test of walking a straight line and turning around and coming back. In this case, the animals were asked to complete the out-and-back at heights of 3-, 4-, 5- and 6-inches and were scored for the sum of total points for hitting, kicking, stepping on or body contact with the rungs or buckets or losing balance or falling. For each concentration, the test was run five times. Significant outcome from a non-parametric Friedman's test for repeated measures with a post hoc corrected Dunn's statistical test was used to determine having met criterion.

Criterion 11 – "Withdrawal, as manifested by either of the following:

    a. the characteristic withdrawal syndrome for alcohol (refer to Criteria A and B of the criteria set for alcohol withdrawal" … in DSM-5 (APA, 2013; Liu et al., 2024). Changes in blood pressure, heart rate, bowel movements, 24-hr activity (see Criterion #3) or the presence of tremors or aggressive behavior was used to assess whether the animals were experiencing physical withdrawal.

    b. "alcohol (or a closely related substance…is taken to relieve or avoid withdrawal symptoms." No test was developed to measure subset b for Criterion 11 because we needed to control intake to prevent the animals drinking themselves to death.

Drug administration

Due to limited quantity of drug, two of the highest, most consistent alcohol consuming minipigs were selected for drug testing. 10-butyl ether minocycline (BEM) was Good

Laboratory Practice (GLP) produced at > 98% purity (RTI, International, Research Triangle Park, NC). BEM has a known <15 min full absorption and a half-life of ~ 6 hrs in mice, rats, and dogs (unpublished data). BEM was administered per orally at 10 mg/kg using a syringe attached to 7-inch flexible Tygon tube to allow placement of the solution at the back of the mouth 15 min prior to two-bucket choice testing. The drug was mixed with Log Cabin™ brand pancake syrup (St. Elmo, Illinois) so that the solution was sticky and would stay in the mouth rather than be spit out. In addition, immediately after the bolus of syrup, ground chow was offered at a high angle to keep the head up, which further prevented loss from the mouth and helped initiate swallowing.

Data analysis

As this was a pilot study that included only two animals, no statistical tests were completed on their drinking. Graphs show the mean for each measurement. For the table indicating the 11 DSM-5 criteria, the discrete outcome behavioral tests were considered to have met criteria with 7 or greater positive assessments. This criterion was calculated using the Binomial Distribution Probability Model (Wypij, 2014). For tests that required a % change, we arbitrarily assigned 50% as meeting criteria. Development of pharmacodynamic tolerance was significance tested using a non-parametric Friedman's test for repeated measures and post hoc corrected with a Dunn's statistical test in GraphPad/Prism v10.0.3.

Results

The two minipigs drank to apparent intoxication daily for nearly two and one-half years. Their average daily intake for the 10% ethanol solution was 2.17 ± 0.6 g/kg/day for Pig A and 2.86 ± 0.3 for Pig B. Blood ethanol concentrations were above 0.8 mg/ml (0.08% equivalency) for both pigs at all ethanol concentrations on the days of testing. This included the initial 2.5% where other pigs in the cohort did not become intoxicated as defined at 0.8 mg ml by NIAAA (NIAAA, 2009). BEC data of each escalation concentration is shown in Figure 3.

Table 1 shows the results of the behavioral and physiological testing for each of the 11 DSM-5 criteria. Pig A was diagnosed with severe AUD by testing positive for eight criteria, including # 1–3, 5, 7, and 9–11. Pig B also met nine criteria for severe AUD, including # 1–5, 8–11.

As shown in Figure 4, the single oral administration of 10 mg/kg BEM resulted in the reduction of both ethanol consumption and preference for both pigs.

Discussion

A newly developed large mammalian swine model was used to test a novel multimodal medication developed specifically to treat AUD. Similar to reports from the 1970s (Dexter et al., 1976), the minipigs drank to apparent intoxication for weeks at a time over the course of two and one-half years. That they tested positive for severe AUD using simple behavioral and physiological tests for each of the 11 criteria is an advance in preclinical approaches to studying AUD. Swine has an ethanol metabolism closer to humans than do rodents. Mice

and rats have eliminations rates nearly 10- and 5-fold greater, respectively (Ginsburg et al., 2008; Javors et al., 2005; Stephen Pruett et al., 2021), making them convenient, but not necessarily the best fit for studies designed to lead to medications development.

In our case, we previously used mouse-based studies to better understand pathways that might be targeted for the development of a new medication that might have the capacity to bind several targets (Agrawal et al., 2014; Mulligan et al., 2006). However, prior to the large expenditure of funds needed to proceed through the FDA approval process for an IND for first use in human studies, we wanted to make sure the drug showed promise in an animal model that had closer physiological functions to humans than do rodents (Shin et al., 2020).

BEM was made based on the structure of minocycline, for which there were numerous positive, off-target effects including, but not limited to, anti-apoptotic, anti-inflammatory, anti-oxidant, and neuroprotective effects. Structure-function details from our screen of several tetracycline analogs (Syapin et al., 2016) allowed us to determine that minimally preventing binding to the bacterial ribosome should allow the positive, off-target effects to remain, while removing the negative side-effects from long-term antibiotic use. A butyl group was added to the hydroxyl on the 10-carbon of minocycline through an ether linkage. The BEM structure was confirmed by microED (micro-electron diffraction) and NMR (nuclear magnetic resonance). Furthermore, the analog was produced by FDA regulated GLP by RTI International, Research Triangle Park, NC complete with a certificate of analysis showing 98% purity. Initial safety studies showed no hERG (human ether-a-go-go related gene) binding, no mutagenic potential and no adverse effects beyond emesis at high doses of up to 750 mg/kg. Our dose of 10 mg/kg was based on conversion of the mouse effective doses of 25–150 mg/kg. We chose an oral dose rather than by intraperitoneal (i.p.) routes to better test the human route of administration.

While the study of two animals has significant limitations, the benefit was to better understand whether animals with closer physiology to humans would respond to a medications approach to reduce excessively high (risky) ethanol consumption. Due to the limitation of available drug, a dose-response was not completed, which would have given us a better idea of whether BEM is suited to be a single approach to treatment or best used as a medication assisted approach. We feel that due to the complexity of AUD, a combination approach will almost always be best. Often there are psychological injuries that either occur before or during the course of the disease that need to be dealt with for healing to proceed. We posit that BEM, as a medication, acts to reset the allostatic brain to a more normal homeostasis, which should allow an easier path to sobriety. Studies are underway to determine how BEM acts similarly or differently from minocycline and, what changes occur to reset the brain to reduce ethanol seeking.

Limitations

The clear limitation of this pilot study is that only two minipigs were tested. As more of our BEM analog is produced, it will be imperative to design an experiment sufficiently powered to test our hypothesis that BEM reduces alcohol consumption, preferably using a between subject design. The use of female animals is also a limitation as males have a slightly higher

rate of AUD, although in the recent past, females are narrowing the gap (Agabio et al., 2017). In addition, we acknowledge that high ethanol consuming females are more likely to present with AUD faster than males (Agabio et al., 2017) and it is possible if we had used boars instead, the AUD may not have met severity levels in the same time frame. However, using intact male animals has experimental limitations that include a higher risk of injury from the more aggressive animals.

Funding

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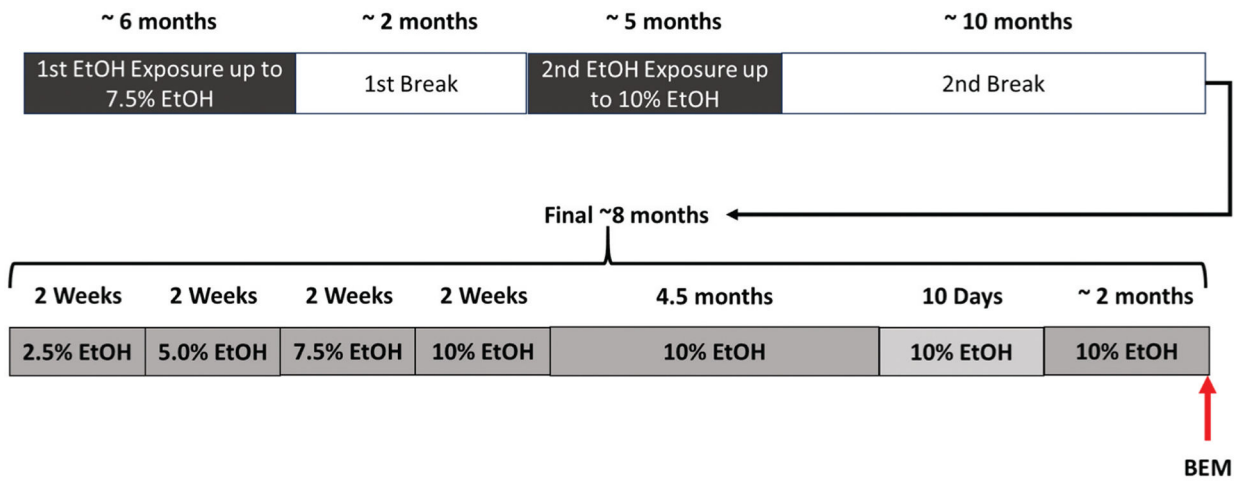
Figure 1.
Timeline of drinking. Over the course of approximately two and a half years, the pigs went for months-long stretches of time drinking to intoxication. The breaks were due to construction, hoof trimming and COVID-19 mandated working from home when only the LARC staff were allowed to complete basic care and no animal experimentation was allowed.
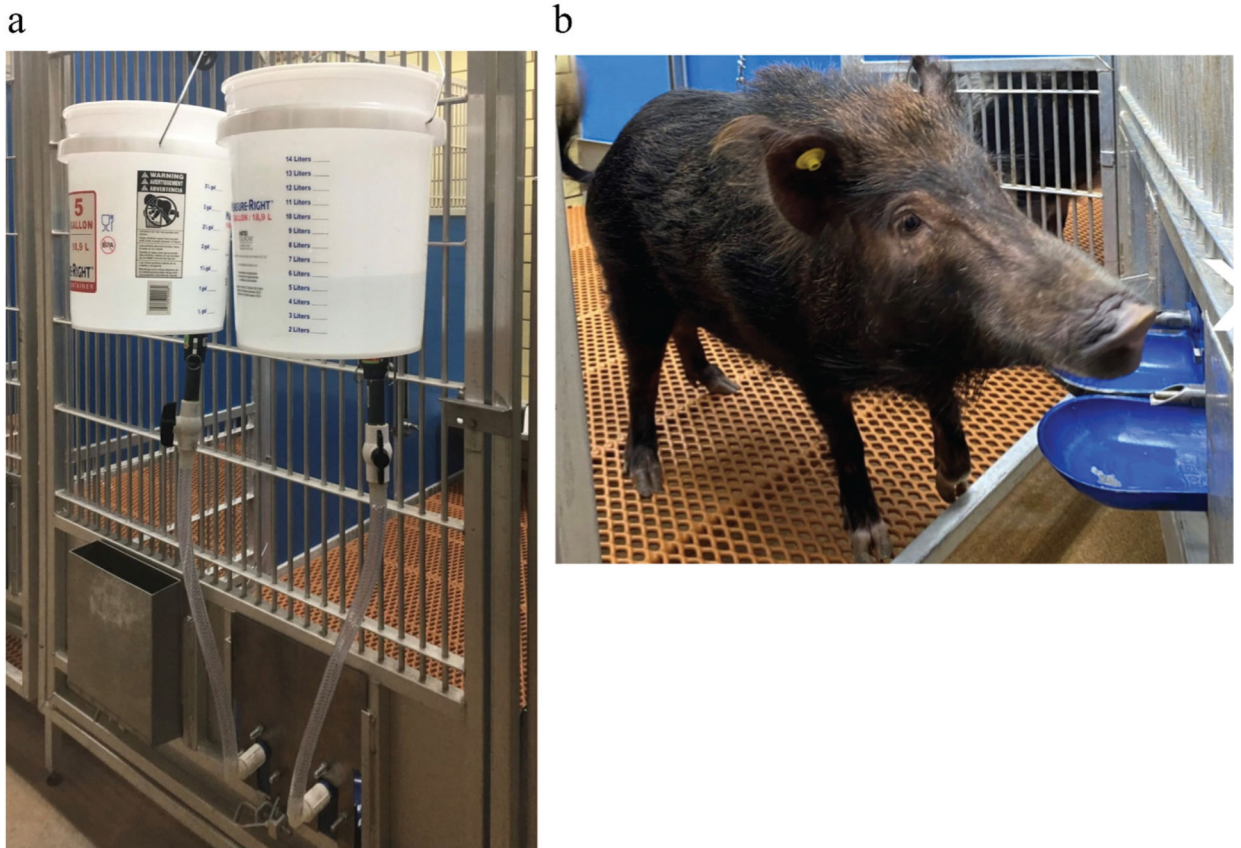
Figure 2.
The two-bucket choice set-up. (a) The outside of the pen shows the orientation of the buckets and tubes attached to the (b) Bowl and sipper apparatus located on the door inside the pen. The water and ethanol solution were changed from side to side every day.

	2.5% EtOH	5.0% EtOH	7.5% EtOH	10.0% EtOH
Pig A	0.80	1.46	1.01	1.61
Pig B	1.25	1.51	1.32	3.03

Figure 3.

Both minipigs drank to intoxication at all escalating ethanol concentrations offered for consumption. BECs, shown in mg/ml as determined by headspace gas chromatography, were not taken frequently to prevent ear vein damage. Therefore, the data approximated the course of drinking across the two and a half years. The final blood draw was for consumption of the 10% ethanol solution at the end of the experiment, just before the animals were treated with BEM.
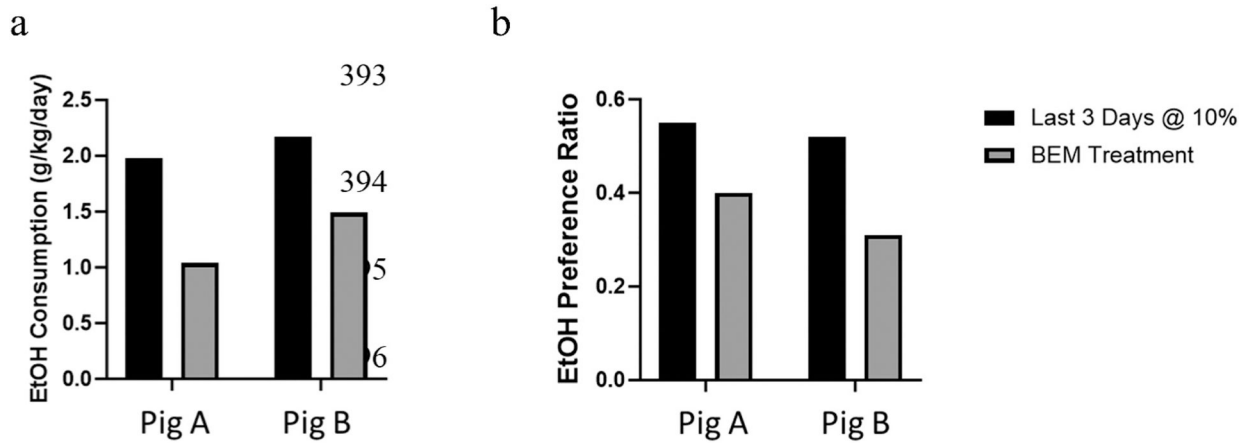
Figure 4.

10-Butylether minocycline reduced A) alcohol consumption and B) preference in both minipigs. Actual values for each of two pigs, a and B, are shown.

Table 1.

The two minipigs were diagnosed with severe AUD using behavioral tasks and physiological measurements to model the 11 DSM-5 criteria. They drank to intoxication nearly daily for two and a half years before testing. Pig a met criteria for eight out of eleven criteria while Pig B met nine. It is interesting to note the differences in the development of AUD for the two pigs.

DSM-5 Criteria	Task Description	Pig A	Pig B
1	Two-bucket, free choice alcohol consumption	✓	✓
2	Repeatedly drinking to intoxication	✓	✓
3	24 hr video of home cage activity	✓	✓
4	Speed to consume daily ethanol following 48 hr withdrawal	✗	✓
5	Horizontal ladder test	✓	✓
6	Greeting human assessment	✗	✗
7	Home pen recreation test	✓	✗
8	Shock collar test	✗	✓
9	Sucrose preference test | Home pen recreation test	✗ | ✓	✗ | ✓
10	Development of Pharmacokinetic or pharmacodynamic tolerance	✓ | ✗	✓ | ✗
11	Withdrawal: heart rate | blood pressure | video evidence	✓ | ✓ | ✗	✗ | ✓ | ✓