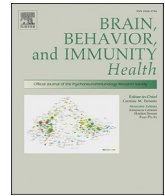


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## Full Length Article

## Tetracycline derivatives reduce binge alcohol consumption in High Drinking in the Dark mice

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## ABSTRACT

Alcohol use disorders (AUDs) are prevalent, and are characterized by binge-like drinking, defined by patterns of focused drinking where dosages ingested in 2–4 h reach intoxicating blood alcohol levels (BALs). Current medications are few and compliance with the relatively rare prescribed usage is low. Hence, novel and more effective medications are needed. We developed a mouse model of genetic risk for binge drinking (HDID: High Drinking in the Dark mice) by selectively breeding for high BALs after binge drinking. A transcriptional analysis of HDID brain tissue with RNA-Seq implicated neuroinflammatory mechanisms, and, more specifically extracellular matrix genes, including those encoding matrix metalloproteinases (MMPs). Prior experiments from other groups have shown that the tetracycline derivatives doxycycline, minocycline, and tigecycline, reduce binge drinking in inbred C57BL/6J mice. We tested these three compounds in female and male HDID mice and found that all three reduced DID and BAL. They had drug-specific effects on intake of water or saccharin in the DID assay. Thus, our results show that the effectiveness of synthetic tetracycline derivatives as potential therapeutic agents for AUDs is not limited to the single C57BL/6J genotype previously targeted, but extends to a mouse model of a population at high risk for AUDs.

## 1. Introduction

Prevalence of binge like drinking is increasing in the US. A binge is defined by the National Institute on Alcohol Abuse and Alcoholism as a period of temporally-focused drinking that leads to a blood alcohol level (BAL) > 80 mg%, as that exceeds the threshold for demonstrable behavioral intoxication (NIAAA, 2004). Binge drinking is a strong predictor of alcohol use disorders (AUD)s. AUDs are comorbid with many other psychiatric conditions and AUD diagnosis has deleterious health consequences (Esser et al., 2014). We propose that the persistent effects of AUD must reflect underlying changes in brain gene expression, a fundamental molecular mechanism of brain adaptation and maladaptation. Preclinical studies are essential for efficient screening of novel therapeutic drugs to treat AUD. The need for new drugs remains acute, as fewer than 15% of AUD individuals receive any treatment, and only three compounds are approved by the US Food and Drug Administration for this purpose: disulfiram, naltrexone, and acamprosate. However, all have limitations (e.g., side effects, limited efficacy, compliance), and

additional compounds are still needed (Farokhnia et al., 2019).

Elucidating the associated risk genes is key to understanding AUDs. Selective breeding has been exploited by many groups since the 1970s using both rats and mice to produce animals differing in ethanol (EtOH) sensitivity, tolerance, dependence and self-administration [for reviews, see (Crabbe, 2014; Becker, 2013; Bell et al., 2017)]. Genetic animal models have been a major staple of alcohol research since the late 1940s. Most high-drinking rodent models have targeted genetic influences on risk using long-term directional selective breeding for 2-bottle choice preference for ethanol vs water with continuous access. However, even after many generations, these highly-preferring animals generally reach BALs >80 mg% only under certain circumstances (Matson and Grahame, 2013).

To model binge-like drinking, we developed the drinking in the dark (DID) assay, where mice consume enough alcohol to reach intoxicating BALs (Rhodes et al., 2005). The DID assay offers a 20% EtOH solution to mice for a limited period each day, during the early hours of the circadian dark cycle. Starting from HS/NPT founders [formed by crossing 8 inbred

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mouse strains (Hitzemann et al., 1994)], we selectively bred HDID-1 and HDID-2 mouse lines for high BALs after a 4 h DID session. These mice drink to BALs averaging 160–230 mg% (Crabbe et al., 2009, 2014). HDID mice exhibit behavioral impairment after drinking and withdrawal convulsions after a single binge. They do not exhibit altered tastant preference or EtOH clearance rates (Barkley-Levenson and Crabbe, 2014; Crabbe et al., 2014). One clear limitation of the DID model is that EtOH is not offered as a choice vs water; when it is, EtOH intake and BALs are somewhat lower, but still exceed NIAAA guidelines for a binge rate (Barkley-Levenson and Crabbe, 2014; Crabbe et al., 2014).

Many peripherally administered drugs can reduce drinking in rodent preclinical tests (Fritz and Boehm, 2016). However, most of such drugs that progress to human clinical trials fail in the clinic (Egli, 2005). One limitation of DID studies is that they have been conducted nearly exclusively in C57BL/6 (B6) inbred mice. This may contribute to translational failures from preclinical to clinical studies. B6 mice have long been known to drink more alcohol than other inbred strains (Wahlsten et al., 2006), but they represent a single genotype, which may limit the generalizability of findings (Crabbe, 2014). Also, B6 mice do not drink as much in the DID test as HDID mice and reach lower BALs (Crabbe et al., 2009).

In a transcriptional analysis with RNA-Seq of HDID-2 brain tissue vs the non-selected HS/NPT controls, the patterns of differential gene expression implicated neuroimmune pathways and extracellular matrix (ECM) (Iancu et al., 2018). Many of the features of the transcriptome found to differ significantly between HDID-2 and HS mice could be traced to genes associated with the basement membrane, the interstitial ECM, perineuronal nets, and the turnover of ECM constituents such as matrix metalloproteinases (MMPs) and their tissue inhibitors (MMPis). Genes and pathways associated with immune and synaptic functions were also affected by selection (Iancu et al., 2018). These effects may be related, as the remodeling of the ECM (e.g., metabolism of collagens) produces ligands that initiate microglial inflammatory responses (Lasek, 2016; Khokha et al., 2013). Neuroimmune pathways have long been implicated in risk and consequences of excessive EtOH consumption (Robinson et al., 2014). The FDA-approved drug doxycycline is a broad spectrum MMPi with anti-inflammatory effects. Prior experiments from other groups have shown that tetracycline derivatives (doxycycline, minocycline, and tigecycline), reduce binge drinking in inbred C57BL/6J (B6J) mice (Agrawal et al., 2011, 2014; McIver et al., 2012; Bergeson et al., 2016a, 2016b; Syapin et al., 2016; Martinez et al., 2016). These studies have been reviewed (Oliveros and Choi, 2017). To determine whether tetracycline derivatives were effective treatments for binge-like drinking in our genetically susceptible population, we tested these three compounds in female and male HDID-2 mice and found that all three reduced DID and BAL. They had drug-specific effects on intake of water or saccharin in the DID assay.

## 2. Methods

### 2.1. Animals and husbandry

All mice were bred in the VA Portland Health Care System Veterinary Medical Unit, an AAALAC approved facility, and all procedures were approved by the Institutional Animal Care and Use Committee and were conducted in compliance with the NIH Guidelines for the Care and Use of Laboratory Animals. Mice were group housed until experiments in standard polycarbonate cages with stainless steel lids on Bed-o'-cobs® bedding (The Andersons, Inc., Maumee, OH, USA) changed weekly. Animals were maintained on a reversed 12hr:12hr light:dark cycle with lights off at 08:30 a.m. Purina 5LOD chow was available at all times, and rearing and testing environments were maintained at  $21 \pm 1$  °C. At least one week before the start of behavioral testing, mice were acclimated to single housing. HDID-2 mice were from Selected Generations S32 – S37 and ages ranged from 11 to 27 weeks at the start of testing. Males and females were used in all experiments in order to consider the potential role of sex as a biological variable.

### 2.2. Drugs

Doxycycline hyclate, minocycline HCl and tigecycline were obtained from Selleck Chemicals, Houston, TX, USA. EtOH (200 proof, Decon Labs, King of Prussia, PA, USA) was used in all experiments. For some studies, saccharin sodium salt hydrate (Sigma-Aldrich, St. Louis, MO, USA) was used. Vehicle for all drugs was physiological saline and drugs were given by intraperitoneal injection (ip) in a volume of 10 ml/kg body weight.

### 2.3. Drinking in the dark test

Our general method has been described in detail elsewhere (Crabbe et al., 2017). Mice were weighed on the first day of each week that a new fluid was offered. Mice were assigned randomly to dose groups within each sex. In Week 1, on Days 1, 2, and 3, starting 3 h after lights off, the water bottle was replaced with a tube containing 20% EtOH in tap water (v/v) and volume was recorded. Two hr later, the volume consumed was recorded and the EtOH tube was replaced with the water bottle. As we patterned our dosing schedule on that used by Syapin et al. (2016), drug or vehicle was administered 2 h after the DID session each day. On Day 4, mice were offered EtOH for 4 h, and a blood sample (20  $\mu$ l) was taken from the periorbital sinus for analysis of BAL attained. BALs were assessed using a previously published chromatographic method (Finn et al., 2007). Mice then had free access to water for the next 3 days. All volume readings were performed by an experimenter blind to the specific drug dose assigned to that mouse.

In Week 2, the same procedure was followed, except that the fluid offered by tube for 2 (or 4) hr was tap water. The same dose of drug (or vehicle) was administered to each mouse after DID on Days 1–3. This procedure is intended to differentiate drug effects on general fluid intake (e.g., possibly due to reduced thirst, or general malaise) from those specific to EtOH. Water bottles were again restored for 3 days. In Week 3, the fluid offered during the DID test was 8.5 mM sodium saccharin in tap water. The saccharin data also addressed the specificity of the drug's effects to EtOH, as a reduction in saccharin intake might indicate a reduction in hedonic value of EtOH. This 3 week serial testing schedule was used for Experiments 2, 3, and 4.

In the initial doxycycline study (Experiment 1, described below), a two-day DID test was employed with a single drug injection on Day 1. This was followed by a 2-day test of the same dose in Week 2 with water only offered. Saccharin was not tested in this initial experiment.

### 2.4. Statistical analyses

Our general strategy uses analysis of variance considering the between-subjects factors of drug dose and sex, and for some analyses, the within-subjects factor of days. There were usually significant sex differences as main effects, which reflected the well-known higher intake levels of females than males regardless of fluid offered, and we do not report these statistics in the Results. [Supplementary Table 1](#) provides means and standard errors of all baseline intake, day 2 or 4 intake, and BAL data separately for each sex and drug dose group for all fluids, by Experiment, and notes statistically significant effects of sex. Since there was only a single instance where sex interacted significantly with drug doses (see water drinking results in Experiment 4), we generally pooled subjects of both sexes and repeated the ANOVAs. All data are therefore shown in the Figures combined across approximately equal numbers of males and females. The significant sex X dose effect is reported in the text and shown in the Supplementary material. Significant effects were pursued using *post hoc* analyses with Tukey's HSD. We analyzed the data for each offered fluid (i.e., Week) separately, as has always been our practice (Ferguson et al., 2018; Crabbe et al., 2017). We generally started by analyzing the 2-hr intake data on the first day of each experiment for each fluid to determine whether drug treatment groups began with equivalent intakes, before drug treatments started. Our principal variables of interest were the total intake across 4 h access on Day 4, and

during the first (EtOH) week of each experiment, the associated BAL. We also analyzed the 2-hr intake data across the 3 (i.e., post-drug) days of the experiment with repeated-measures ANOVA to assess the time-course of emergence and/or disappearance of response to repeated treatments, as well as the possibility of short-duration effects; however, as shown in the figures the results were not further informative.

Occasionally, we record a physiologically impossible intake level. For example, a 25 g mouse that “drank” 2.6 ml of 20% EtOH during a 2 h test would have ingested a dose of 21.1 g/kg body weight, while the 100% lethal dose for ip injected EtOH in male mice is between 7 and 9 g/kg depending on genotype, and death often occurs 24–36 h after injection (Tsibulsky and Amit, 1993). We eliminated 7 out of 1232 EtOH data points on all days for exceeding 11 g/kg/2 h (2 in Expt 2, 5 in Expt 3), 1 for complete leakage (Expt 1), and threw out all data from one mouse in Expt 4 that exceeded 13 g/kg at multiple 2 h reads. Two instances of misreading tube menisci occurred (1 EtOH in Expt 1, 1 saccharin in Expt 3), and each mouse’s data on those days were removed. Additionally, 2 water tubes that were emptied in 2 h were presumed leaks, as were 20 saccharin tubes (6 in Expt 2, 4 in Expt 3, and 10 in Expt 4). Lastly, 6 mice died either between the EtOH and water weeks (3), during the water week (2), or between the water and saccharin weeks (1). No more than 2 deaths occurred in a single experiment, and no reason was readily apparent.

In sum, we eliminated 31 data points from the 3580 collected (0.9%).

### 2.5. Experiment 1. Acute doxycycline

The same 2-day DID test we use for selective breeding was employed (Crabbe et al., 2009). After the 2 h Day 1 EtOH drinking test, mice were given doxycycline ip 2 h later at a dose of 0, 80 or 120 mg/kg. We tested 7–8 mice/sex/dose (total N = 48 at the start of the test). In week 2, water was offered in place of EtOH, and mice were administered doxycycline as described for Week 1.

### 2.6. Experiment 2. Doxycycline

Because doxycycline reduced both EtOH and water intakes in Experiment 1 (see Results), we employed lower doses in Experiment 2. For this and the other experiments, we also gave three drug injections and tested DID for 4 days. Groups of 10–12 mice/sex/dose (total N = 96 at the start of the test) were given 0, 20, 40, or 60 mg/kg doxycycline. DID tests and drug doses were repeated in Week 2 with water offered, and Week 3 with saccharin offered.

### 2.7. Experiment 3. Minocycline

We followed the same procedure described for Experiment 2. Doses of minocycline were 0, 40, 60, or 80 mg/kg ip. Groups of 11–12 mice/sex/dose (total N = 96 at the start of the test) were tested for 3 weeks.

### 2.8. Experiment 4. Tigecycline

We followed the same procedure described for Experiment 2. Doses of tigecycline were 0, 30, 60, or 90 mg/kg ip. Groups of 10–12 mice/sex/dose (total N = 92 at the start of the test) were tested for 3 weeks.

## 3. Results

### 3.1. Experiment 1. Acute doxycycline

Average EtOH intake on Day 1 (Fig. 1a) did not differ significantly across groups [F(2,44) = 1.45,  $P > 0.20$ ]. Intake over 4 h on Day 2 (Fig. 1b) showed a main effect of Treatment [F(2,43) = 47.4,  $P < 0.0001$ ]. *Post hoc* analyses showed that both drug doses significantly reduced drinking vs vehicle ( $P$ s < 0.0001). BALs (Fig. 1c) were also significantly reduced [F(2,45) = 48.2,  $P < 0.0001$ ], by either dose ( $P$ s < 0.0001). In Week 2, water intake did not differ significantly across groups on Day 1 (not shown). After doxycycline treatment, 4 h intake (Fig. 1d) was reduced on Day 2 [F(2,44) = 6.04,  $P < 0.01$ ], but only in the 120 mg/kg dose group ( $P < 0.01$ ), although there was a tendency toward a reduction in the 80 mg/kg group ( $P = 0.063$ ).

### 3.2. Experiment 2. Doxycycline

Average EtOH intake on Day 1 (Fig. 2a) differed significantly across groups prior to treatment [F(3,91) = 2.86,  $P < 0.05$ ]. *Post hoc* analysis showed that only the 40 mg/kg group drank significantly less than the 0 mg/kg group before drugs were given ( $P < 0.05$ ). EtOH intake over 4 h on Day 4 (Fig. 2b) showed a main effect of Treatment [F(3,91) = 8.64,  $P < 0.0001$ ]. *Post hoc* analyses showed that both the 40 mg/kg ( $P < 0.05$ ) and the 60 mg/kg ( $P < 0.001$ ) doses significantly reduced drinking vs 0 mg/kg. BALs (Fig. 2c) were also significantly reduced [F(3,92) = 10.03,  $P < 0.0001$ ]. A significant reduction was seen in the 60 mg/kg group ( $P < 0.001$ ) and there was a trend in the 40 mg/kg group ( $P = 0.06$ ).

On the first day of week 2 drinking, water intake differed significantly across groups [F(3,90) = 3.44,  $P < 0.05$ ; data not shown]. However, no group differed significantly from the vehicle group (all  $P$ s  $\geq 0.4$ , data not shown). After 3 daily doxycycline injections, no significant reductions in water intake vs vehicle were seen [Fig. 3a, F(3,90) = 1.0]. In the saccharin week, groups again showed a significant difference in intake on the initial day [F(3,88) = 4.91,  $P < 0.01$ ]. This resulted from lower intake by the 60 mg/kg group ( $0.97 \pm 0.17$  mls/20 g body weight) than by the vehicle group ( $1.68 \pm 0.14$  mls/20 g body weight;  $P < 0.01$ ). After 3 injections (Fig. 3b), groups differed significantly in saccharin intake [F(3,89) = 11.05,  $P < 0.0001$ ]. Both the 60 mg/kg ( $P < 0.001$ ) and the 40 mg/kg doses ( $P < 0.01$ ) drank less saccharin than the 0 mg/kg group.

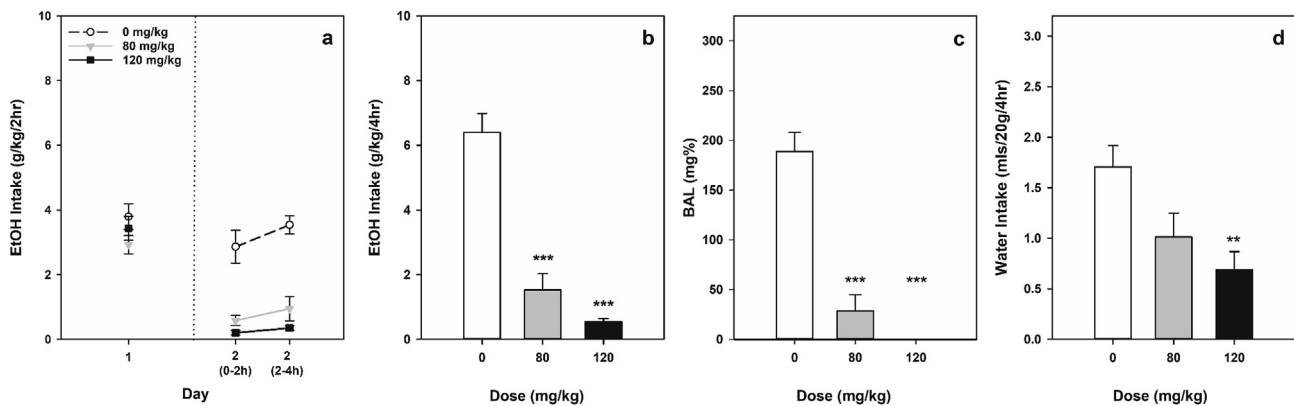


Fig. 1. Doxycycline reduced drinking in male and female HDID-2 mice. A single injection of doxycycline after Day 1 DID reduced EtOH intake on Day 2 (Panels a and b) as well as BAL (Panel c). In Week 2, a single injection of 120 mg/kg doxycycline after Day 1 DID reduced water intake after 4 h on Day 2 (Panel d) and tended to do so at the 80 mg/kg dose. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.0001$  vs 0 mg/kg group.

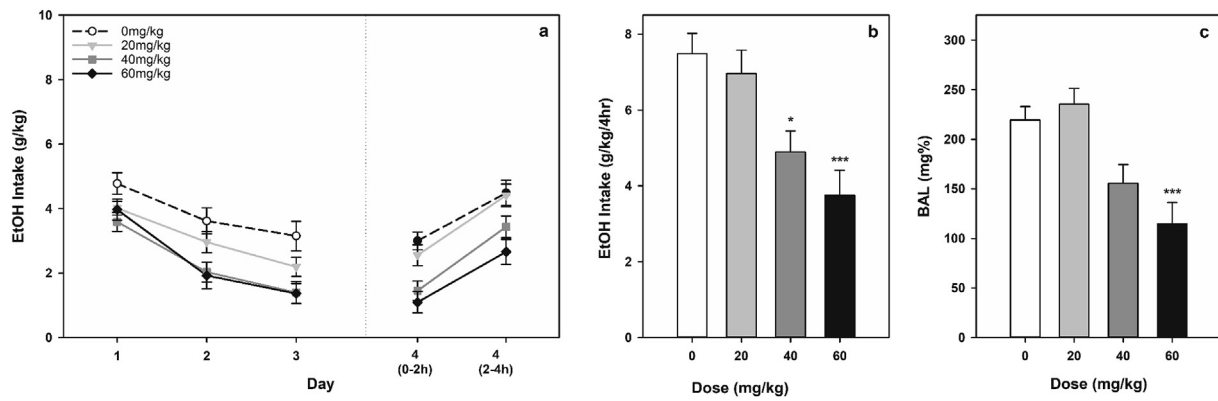


Fig. 2. Doxycycline reduced EtOH intake after 2 h on Days 2 and 3 (Panel a) as well as intake (Panel b) and BAL (Panel c) after 4 h on Day 4. See legend to Fig. 1.

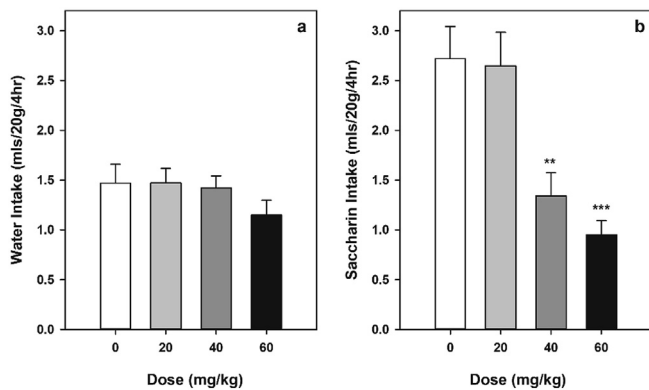


Fig. 3. Doxycycline had no effect on water intake on Day 4 (Panel a), while the two higher doses reduced saccharin intake (Panel b). See legend to Fig. 1.

### 3.3. Experiment 3. Minocycline

Average EtOH intake on Day 1 (Fig. 4a) did not differ significantly across groups prior to treatment [ $F(3,88) < 1$ ]. EtOH intake over 4 h on Day 2 (Fig. 4b) showed a main effect of Treatment [ $F(3,91) = 12.50, P < 0.0001$ ]. *Post hoc* analyses showed that both the 60 mg/kg ( $P < 0.05$ ) and the 80 mg/kg ( $P = 0.0001$ ) doses significantly reduced drinking vs vehicle. BALs (Fig. 4c) were also significantly reduced [ $F(3,92) = 13.60, P < 0.0001$ ]. The reduction was seen in both the 60 mg/kg ( $P < 0.05$ ) and the 80 mg/kg groups ( $P < 0.0001$ ).

On the first day of water drinking, groups did not differ significantly ( $F < 1$ ). However, after 3 daily minocycline injections, significant reductions in water intake vs vehicle were seen [Fig 5a,  $F(3,81) = 15.27, P$

$< 0.001$ ]. Both the 60 and 80 mg/kg groups drank less water than vehicle-treated mice ( $P_s \leq 0.01$ ). On the first day of saccharin drinking, groups did not differ significantly [ $F(3,89) = 1.44, P > 0.20$ , data not shown]. After 3 injections (Fig. 5b), groups differed significantly in saccharin intake [ $F(3,88) = 8.35, P < 0.0001$ ]. As with EtOH and water, both the 60 mg/kg ( $P < 0.01$ ) and the 80 mg/kg doses ( $P < 0.001$ ) drank less saccharin than the 0 mg/kg group.

### 3.4. Experiment 4. Tigecycline

EtOH intake on Day 1 (Fig. 6a) did not differ significantly across groups prior to treatment [ $F(3,85) < 1$ ]. Intake over 4 h on Day 4 (Fig. 6b) showed a main effect of Treatment [ $F(3,85) = 25.4, P < 0.0001$ ].

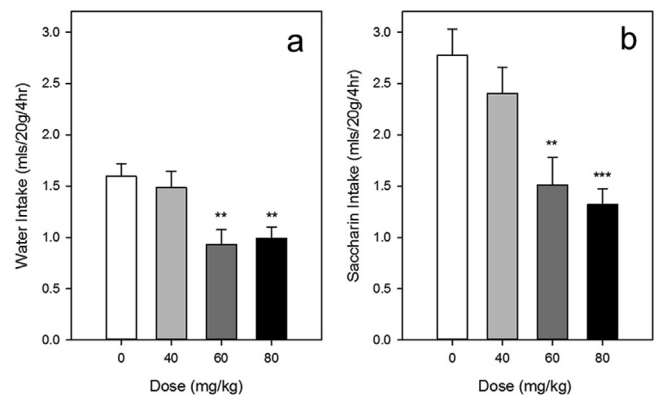


Fig. 5. Minocycline at the two higher doses reduced water (Panel a) and saccharin intake (Panel b). See legend to Fig. 1.

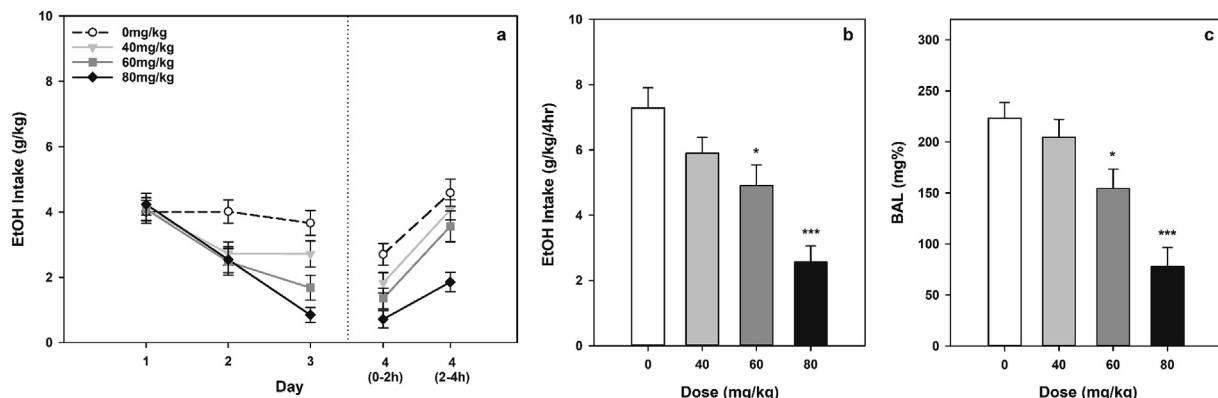


Fig. 4. Minocycline reduced EtOH intake after 2 h on Days 2 and 3 (Panel a) as well as intake (Panel b) and BAL (Panel c) after 4 h on Day 4. See legend to Fig. 1.



Post hoc analyses showed that all 3 doses of tigecycline significantly reduced drinking vs vehicle ( $P$ s < 0.001). BALs (Fig. 6c) were also significantly reduced [ $F(3,85) = 22.2$ ,  $P < 0.0001$ ] by all 3 doses ( $P$ s < 0.0001).

On the first day of water drinking, dose groups did not differ significantly [ $F(3,85) = 1.51$ ,  $P > 0.20$ , data not shown]. However, after 3 daily tigecycline injections, significant reductions in water intake vs vehicle were observed [Fig 7a,  $F(3,85) = 8.70$ ,  $P < 0.0001$ ] [ $F(3,81) = 15.27$ ,  $P < 0.001$ ]. Both the 30 and 60 mg/kg groups drank somewhat less water than vehicle-treated mice ( $P$ s < 0.05), and the 90 mg/kg dose group drank even less ( $P < 0.0001$ ). In addition to the usual main effect of sex ( $F > M$ :  $F(1,81) = 15.27$ ,  $P < 0.001$ ) there was a significant sex  $\times$  treatment interaction [ $F(3,81) = 3.91$ ,  $P < 0.05$ ]. Females showed reductions in water intake [ $F(3,40) = 10.36$ ,  $P < 0.0001$ ] due to drug doses 30 and 90 [ $P$ s < 0.01], but not 60. Although a significant main effect of dose was observed in males [ $F(3,41) = 3.40$ ,  $P < 0.05$ ], no significant effects of any single dose on water intake were observed [ $P$ s > 0.058]. Data for female and male mice are shown in Supplementary Table 1. On the first day of saccharin drinking, groups did not differ significantly [ $F(3,83) = 1.76$ ,  $P > 0.10$ , data not shown]. Even after 3 injections in Week 3 (Fig. 7b), groups did not differ significantly in saccharin intake [ $F(3,82) = 1.77$ ,  $P > 0.10$ ].

#### 4. Discussion

We report that the effectiveness of synthetic tetracycline derivatives as potential therapeutic agents for binge-like alcohol drinking is not limited to the single B6J genotype previously targeted by most research, but extends to a mouse model of a population at high risk for AUDs. Because we have carefully avoided inbreeding during the development of the HDID selected lines (Crabbe et al., 2009, 2014), these animals remain genetically diverse at many if not most loci that do not contribute to their risk for binge-like drinking (Iancu et al., 2013, 2018).

Our overall results generally confirmed the prior findings of the Bergeson and Syapin groups at Texas Tech University Health Sciences Center, i.e., that tetracycline derivatives could effectively reduce binge-like drinking. However, our experiments also revealed some novel findings. We summarize the outcomes of our studies in Table 1. The drug showing closest agreement with the Texas group's data was minocycline; both our groups saw dose-dependent decreases in binge drinking. However, these were accompanied by reductions in water drinking, so the drug may have been reducing overall thirst through an unknown effect on fluid balance, or by inducing general malaise. We saw these reductions also during testing of the highly preferred tastant saccharin, but no comparison is available as the Texas group did not probe with a sweet tastant. Although the Texas group found modest sex specificity of minocycline's effects, we saw no differences between males and females. Thus, minocycline's EtOH reducing effects may be non-specific.

For doxycycline, the HDID mice may be more sensitive than B6J to the drug's EtOH drinking effects (although no direct comparison is available). Furthermore, at the highest doxycycline dose tested, we differ with respect to both effects on water drinking (we saw none) and in that we saw no hints of sex differences in responding. Doxycycline was also unusual in that in our data, animals showed dose-dependent reductions in saccharin intake even though there had been no effect on water drinking. This could reflect a blunting of sensitivity to either the sweet taste of saccharin, to its rewarding effects, or both. Further tests would be required to discriminate among these possibilities. For tigecycline (a minocycline derivative), our animals appear again to possibly be more sensitive than B6J, but we agree that this drug also reduces water intake (in females only), but it did not reduce saccharin intake in either sex, and there are no apparent sex differences in the drug's effect to reduce EtOH drinking. The lack of efficacy of doxycycline and tigecycline to reduce intake of all fluids offered shows clearly that a general malaise was not the cause of the reduced drinking of the fluids (of most interest, the reduced EtOH drinking).

The differences between our laboratory's findings and those of the Texas group are not easily attributed to procedural differences. Both groups are highly experienced with studies of murine drinking. The Texas group used the DID procedure that was developed in Oregon (Rhodes et al., 2005), as did we. We intentionally modeled our drug administration parameters (e.g., doses and times of administration) closely on their publication (Syapin et al., 2016). Both groups used sufficient numbers of male and female mice to be able to detect signs of sex differences in response to the drugs. However, they used B6J mice, as noted, while we used HDID-2 mice based on the neuroimmune dysregulation suggested by transcriptomics analyses (Iancu et al., 2018). They showed all their drinking data averaged across 4 daily 4-hr DID sessions, while we showed results from the original DID paradigm (2 h/day for 3 days, 4 h on the last day). We consider this to be a minor difference. Finally, they assessed the potential drug effects on water drinking based on total daily intake during the 20 h following drug injection across the 4 day EtOH test, while we measured 2–4hr intakes of water (and then saccharin) in two separate further experiments starting after the completion of the EtOH studies. Each such test used the same limited-access sessions as for EtOH. Therefore, it is possible that the reductions we observed may be due to additional administrations of the compounds or from some other unknown carryover effect from the previous week or weeks of testing. For example, we cannot be sure about HDID response to 40 mg/kg doxycycline on EtOH intake given the lower intake of that group than the vehicle group on Day 1, before drug was administered, but the lack of response to this dose on water drinking the following week we deem unambiguous. Each such test used the same limited-access sessions as for EtOH. Neither assessment method for water seemed generally more sensitive to drug effects, and we have no other plausible explanations for the drug-specific differences reported in Table 1.

These experiments do not address the potential mechanism(s) of action of the drugs. Tetracyclines are broad-spectrum antibiotics in wide clinical use against *acne vulgaris*, sexually transmitted, and many other diseases. In recent years, anti-inflammatory, anti-metastatic and other non-microbial effects have emerged as clinically useful effects. For example, doxycycline and minocycline have emerged as broadly neuroprotective therapeutic agents (Garrido-Mesa et al., 2013; Santa-Cecilia et al., 2019). We do not believe that the binge-reducing effects of the drugs reported here are due to their antimicrobial effects, but further studies would need to be performed to evaluate that possibility. In part, our belief is supported by an unpublished study where the same doses of minocycline used in Experiment 3 were administered to HDID-2 mice 30 min before testing EtOH DID. Minocycline dose-dependently reduced binge drinking. This is much more quickly than the development of antimicrobial effects, which occur several days later in mice (Batty et al., 2007). For an extended consideration of the potential mechanisms of action of these three compounds to reduce EtOH intake, see Oliveros and Choi (2017).

Overall, our data suggest that further exploration of synthetic tetracycline derivatives as a promising direction for potential AUD therapeutics, a conclusion reached by others examining prior work [Oliveros and Choi (2017)]. They are consistent with the growing evidence in support of anti-inflammatory and ECM targeting therapies for excessive alcohol drinking (Savarese and Lasek, 2018; Lasek, 2016). Clinical studies with these generally very safe drugs would be very useful. One such study has been conducted by the Petrakis group at Yale (Clinicaltrials.gov, NCT02187211). This was an outpatient study of male and female heavy social drinkers given minocycline (200 or 400 mg/day) or placebo for 10 days. Alcohol vs placebo was administered intravenously on Days 8 or 10 targeting 100 mg %. Outcome measures included subjective, motor, and cognitive measurement with the Biphasic Alcohol Effects Scale and plasma cytokine levels. No results from this study have as yet been posted. Clinicaltrials.gov also lists a study by the Haass-Koffler group at Brown University initiated in Fall 2019) (NCT04135846) intending to enroll 184 male or female subjects who meet the DSM-5 criteria for AUD. Subjects will receive placebo or 16 mg

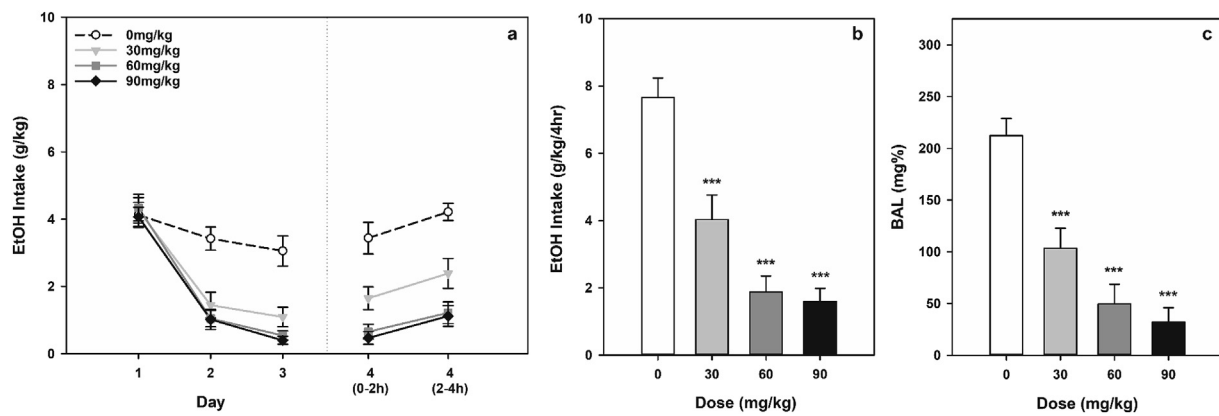


Fig. 6. All 3 doses of tigecycline reduced EtOH intake after 2 h on Days 2 and 3 (Panel a) as well as intake (Panel b) and BAL (Panel c) after 4 h on Day 4. See legend to Fig. 1.

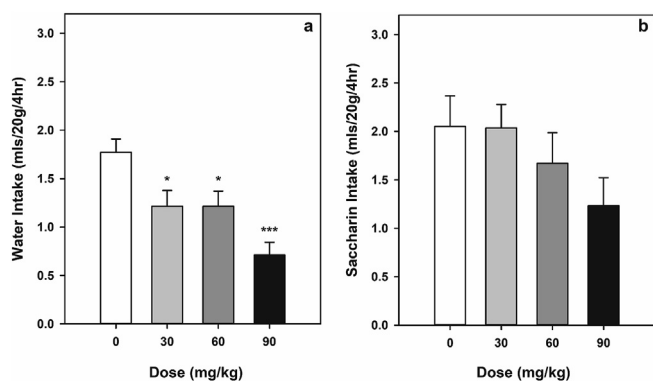


Fig. 7. Tigecycline reduced water intake on Day 4 (Panel a), but it had no significant effect on saccharin intake (Panel b). See legend to Fig. 1.

Table 1  
Comparison of outcomes from current studies and Texas studies.

Our studies	Syapin/Bergeson/Agarwal					
Drug/Dose mg/kg, ip	EtOH	BAL	Water	Sacch	EtOH	Water
Doxycycline 20	-	-	-	-	-	-
Doxycycline 40	↓	-	-	↓↓	-	-
Doxycycline 60	↓↓↓	↓↓↓	-	↓↓↓	↓ M	↓↓ F
Minocycline 40	-	-	-	-	-	-
Minocycline 60	↓	↓	↓↓	↓↓	↓↓ M, ↓F	↓↓
Minocycline 80	↓↓↓	↓↓↓	↓↓	↓↓↓	↓↓↓	↓↓↓F, ↓↓ M
Tigecycline 30	↓↓↓	↓↓↓	↓ F, -M	-	- 20 & 40	- 20 & 40
Tigecycline 60	↓↓↓	↓↓↓	↓ F, -M	-	↓	-
Tigecycline 90	↓↓↓	↓↓↓	↓↓↓ F, -M	-	↓↓↓	-

EtOH: consumption (g/kg) of 20% EtOH in DID test. BAL: BAL at end of Day 4, 4 h DID test. Water: consumption of water (ml/g) in DID test. Sacch: consumption of 8.5 mM sodium saccharin in DID test. Arrows indicate reduced intake (or BAL) vs animals treated with saline. ↓↓ - P < 0.001 (↓ - 0.01; ↓ - 0.05). - No significant reduction vs saline.

M = males, F = females where sex differences were detected.

(or a maximum tolerated dose) of doxycycline for 6 weeks and report number of drinks per week and craving using the timeline follow-back method.

Lastly, our studies reinforce the idea that multiple preclinical models will continue to be useful as screening tools given that they each prove to be sensitive, but to different sorts of compounds. While our data did not

find evidence for sex-specific responses to the tested drugs, they support the potential for more general utility of these compounds in populations representing different genetic risk factors.

Declaration of competing interest

Each author states that he or she has no conflicts of interest, financial or otherwise, to report.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbih.2020.100061>.

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