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Tigecycline Reduces Ethanol Intake in Dependent and Non-Dependent Male and Female C57BL/6J Mice

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Abstract

Background—The chronic intermittent ethanol (CIE) paradigm is valuable for screening compounds for efficacy to reduce drinking traits related to alcohol use disorder (AUD), as it measures alcohol consumption and preference under physical dependence conditions. Air control treated animals allow simultaneous testing of similarly treated, non-dependent animals. As a consequence, we used CIE to test the hypothesis that tigecycline, a semi-synthetic tetracycline similar to minocycline and doxycycline, would reduce alcohol consumption regardless of dependence status.

Methods—Adult C57BL/6J female and male mice were tested for tigecycline efficacy to reduce ethanol consumption using a standard CIE paradigm. The ability of tigecycline to decrease 2-bottle choice of 15% ethanol (15E) versus water intake in dependent (CIE-vapor) and non-dependent (air-treated) male and female mice was tested after four cycles of CIE vapor or air exposure using a within-subjects design and a dose response. Drug doses of 0, 40, 60, 80, 100 mg/kg in saline were administered intraperitoneally (0.01 mL/g body weight) and in random order, with a 1 hr pretreatment time. Baseline 15E intake was re-established prior to administration of subsequent injections, with a maximum of two drug injections tested per week.

Results—Tigecycline was found to effectively reduce high alcohol consumption in both dependent and non-dependent female and male mice.

Conclusions—Our data suggest that tigecycline may be a promising drug with novel pharmacotherapeutic characteristics for the treatment of mild to severe AUD in both sexes.

Keywords

Alcoholism; Alcohol Dependence; Chronic intermittent ethanol (CIE); Medications Development; Tigecycline

INTRODUCTION

Alcoholism is a complex disorder that represents an important contributor to health problems worldwide, in part due to limited effective treatment options. The development of animal models that are homologous to specific phenotypes has been crucial for our understanding of neurobiological mechanisms underlying alcoholism (Ripley and Stephens, 2011), since no single animal model recapitulates this complex phenotype.

Various models of binge ethanol exposure or consumption, in conjunction with bioinformatics analyses, provide evidence that high ethanol intoxication can dysregulate components of the neuroimmune system and that neuroimmune networks are involved in high ethanol intake (Blednov et al., 2005, Agrawal et al., 2011, Liu et al., 2011, Blednov et al., 2012, Agrawal et al., 2014). The ability of immune modulatory drugs to alter ethanol intake has provided support for the idea that drugs targeting neuroimmune pathways may represent a new therapeutic approach to treat aspects of alcoholism. For instance, recent work has shown that second generation tetracycline drugs that have immune modulatory actions, such as minocycline and doxycycline, reduce free choice and binge ethanol intake (Agrawal et al., 2011, McIver et al., 2012, Agrawal et al., 2014), and here we show that a third generation tetracycline, tigecycline, is also effective. However, these drugs have not been tested in chronic ethanol drinking models in dependent animals.

Chronic intermittent ethanol (CIE) vapor exposure has been shown to reliably produce symptoms of dependence and to produce an escalation in ethanol intake and operant self-administration in rodents (Becker and Lopez, 2004, Lopez and Becker, 2005, Finn et al., 2007, Gilpin et al., 2009, Griffin et al., 2009b, Lopez et al., 2014, O'Dell et al., 2004). Following repeated bouts of CIE vapor exposure, rats will increase ethanol self-administration during withdrawal (Roberts et al., 1996) as well as during a period of protracted abstinence (Roberts et al., 2000). In mice, the escalation in ethanol intake following repeated bouts of CIE vapor exposure was associated with tolerance to the aversive effects of ethanol (Lopez et al., 2012) and with insensitivity to the devaluation of ethanol's reinforcing properties (Lopez et al., 2014). We also found that two bouts of CIE vapor exposure significantly increased withdrawal severity (Finn et al., 2007). Since withdrawal severity has been used as an index of physical dependence in rodent models, the results suggested that repeated bouts of CIE vapor exposure produce physical dependence. Importantly, CIE vapor exposure has been shown to be a useful behavioral model of the escalation of ethanol consumption in dependent animals, and it has been used by many investigators to test pharmacological targets for their ability to decrease high ethanol consumption in dependent rodents (Roberts et al., 1996, Finn et al., 2007, Walker and Koob, 2007, Nealey et al., 2011, Bell et al., 2015, Walker et al., 2008, Richardson et al., 2008, Gomez et al., 2015, Suchankova et al., 2015).

The purpose of the present study was to examine the ability of tigecycline to decrease ethanol intake in dependent and non-dependent mice following repeated bouts of CIE vapor or Control air exposure, respectively. Both males and females were tested, based on the finding that there were sex differences in the ability of tigecycline to significantly decrease binge ethanol intake (Syapin et al., companion paper, 2016). Tigecycline is an FDA

approved drug used specifically for Methicillin-resistant *Staphylococcus aureus* (MRSA) infection (Koomanachai et al., 2009). It is structurally similar to minocycline, which has recently been shown to have neuroimmune actions and potential for use well beyond its original purpose as an antibiotic (Garrido-Mesa et al., 2013). There is an urgent need to increase the treatment options for the Diagnostic and Statistical Manual for Mental Disorders (edition V) diagnosis of Alcohol Use Disorder (AUD), with only three FDA approved therapies (Zindel and Kranzler, 2014) together with behavior modification treatments (ProjectMATCH, 1997) that have limited efficacy and high rates of repeated relapse. In fact, there is no universally accepted standard of care for AUD patients, and high heterogeneity in presentation and outcome. As a result, we tested the overarching hypothesis that tigecycline would decrease ethanol intake in both non-dependent and dependent animals. We also tested a secondary hypothesis that sex differences in ethanol intake escalation and sensitivity to tigecycline would be seen.

METHODS

Subjects

Male and female C57BL/6J mice (n=25/sex) were purchased at 8 weeks of age from Jackson Laboratories West (Davis, CA). Upon arrival at Oregon Health & Science University (OHSU), mice were separated by sex, group housed with *ad libitum* access to food and water, and acclimated to a 12:12 hr reverse light:dark cycle for a minimum of one week prior to beginning the experiment (lights off at 1130). Following acclimation, mice were individually housed in clear polycarbonate cages (28 x 18 x 13 cm) on Ecofresh bedding with nestlets and given 2 water sipper tubes. All procedures complied with the National Institute of Health *Guidelines for the Care and Use of Laboratory Animals* and were approved by the OHSU Institutional Animal Care and Use Committee.

Limited access 2-bottle choice procedure

After 3 days of individual housing and habituation to drinking from sipper tubes (25 mL graduated cylinders with sipper tubes; volumes read to the nearest 0.2 mL), mice had 2 hrs of access to one tube containing a 15% v/v ethanol solution (15E; Pharmco Products, Brookfield, CT) and one tube containing tap water, beginning 30 min before lights-out. Mice had 2 hr access to the 15E solution for 5 days per week (Mon – Fri), with access to water the remainder of the time. Mice also consumed water the weeks that they were exposed to CIE vapor or air exposure within inhalation chambers.

CIE vapor or Control air exposure

After baseline drinking was stable (< 15% variability across days), male and female mice were assigned to CIE (dependent) or air Control (non-dependent) groups that were matched for their baseline 15E intake. For each week of CIE vapor or Control air exposure, mice were placed into hanging wire mesh cages inside inhalation chambers (Flair Plastics, Portland, OR) that were housed inside climate controlled chambers (Weaver Technologies, Portland, OR). CIE or Control exposure to intermittent ethanol vapor or air occurred over 3 days (17 hrs/day x 3 days; Table 1), using routine procedures (Finn et al., 2007, Gomez et al., 2015). Food and water were freely available. During the weeks of CIE vapor or air

Control exposure, mice remained on the same light cycle as when 15E intake was measured in the home cage.

Immediately before placement into the inhalation chambers, the CIE groups were weighed and injected daily with 1.5 g/kg ethanol (20% v/v) in normal saline (0.9% NaCl) that included pyrazole HCl (68.1 mg/kg; #P56607, Sigma Chemical Company, St. Louis, MO). Pyrazole inhibits alcohol dehydrogenase and stabilizes blood ethanol concentration (BEC). The Control groups were weighed and injected with the same dose of pyrazole in saline. Before placement of the mice in the chambers, the inhalation chambers were brought to equilibration concentrations of ethanol in air of 7 – 9 mg/L and 0.0 mg/L for the CIE and Control groups, respectively. Ethanol vapor levels were adjusted daily to achieve a target BEC of 1.5 – 2.0 mg/mL following each 17 hrs of CIE exposure. The amount of ethanol vapor in the inhalation chamber was calculated hourly using gas chromatography (6890NGC, using a HP-PLOT Q column; Agilent Technologies, Wilmington, DE). Following the 17 hrs of ethanol vapor exposure in the CIE groups, mice were removed from the chambers, and a 20 μ L tail blood sample was collected. The mice were then exposed to air for 7 hrs. This procedure was repeated over 3 days (constituting a cycle of CIE exposure), and then all mice were returned to their initial home cage and limited access 15E intake was resumed three days later (see Table 1).

BEC analysis

The tail blood samples (20 μ L) were analyzed for ethanol content immediately after collection, using ambient headspace gas chromatography (6890N GC, using a DB-ALC1 column, Agilent; (Finn et al., 2007)). Six pairs of ethanol standards (0.1 – 3.0 mg/mL), which included *n*-propanol as the internal standard, were run before the samples each day.

Experimental procedures

All mice had free access to food and water throughout the experiment. Body weights were recorded at the beginning of the experiment, twice per week during the periods of home cage drinking, and daily during the bouts of CIE vapor or Control air exposure. After 3 weeks of baseline 15E intake, separate groups of male and female mice that were matched on baseline 15E intake were exposed to CIE vapor or Control air for 3 days, followed by 5 days of 15E intake (see Table 1). This pattern of alternating weeks of CIE vapor or Control air exposure (i.e., each week of CIE vapor or Control air exposure termed a “cycle” of exposure) and 15E intake was repeated 4 times, with slight differences in the number of days that animals consumed 15E after the CIE vapor or Control air exposure (Table 1 and Figure 1). A 2 week period of abstinence was incorporated in between the weeks of 15E after the 2nd cycle of CIE vapor or Control air exposure, as we had found that the abstinence period facilitates the subsequent escalation of 15E intake in male CIE mice (Finn et al., 2007).

The ability of tigecycline (0, 40, 60, 80, 100 mg/kg drug was made in sterile saline (0.9% NaCl) and used within 25 hrs; 0.01 mL/g body weight; #15026 - Cayman Chemical Company, Ann Arbor, MI) to decrease 15E intake in dependent (CIE) and non-dependent (Control) male and female mice was tested after 4 cycles of CIE vapor or Control air exposure (see Table 1 and Figure 1), using a within-subjects design. Doses were

administered intraperitoneally and in random order to all mice, with a 1 hr pretreatment time. Baseline 15E intake was re-established prior to administration of subsequent injections, with a maximum of 2 drug injections tested in a week.

Data analysis

Data are presented as mean $\pm$ SEM. Daily 15E intake and preference ratio (PR) (volume of ethanol solution /total fluid intake) were calculated for each animal, and then average 15E intake and PR were calculated for baseline (15 days of intake) and post-inhalation weeks (Tests 1 – 4; first 5 days of drinking) according to standard procedures (e.g., Finn et al., 2007; Lopez and Becker, 2005). Analysis of variance (ANOVA) examined the effect of treatment (CIE vs Control) and sex (male vs female) on 15E intake and ethanol PR, with drinking cycles (baseline & Tests 1 – 4) as a repeated measures factor. Significant interactions were pursued with 2- and 1-way ANOVAs and Tukey's post-hoc tests. Because we predicted that males and females would differ in the post-inhalation escalation in 15E intake, data for males and females were analyzed separately even in the absence of a significant interaction. A similar strategy was used for the analysis of body weight. The 3-day average BEC following each bout of CIE was analyzed by ANOVA for effects of sex and cycle of exposure. For the tigecycline portion of the study, 15E, ethanol PR, and 22 hr water intake were analyzed for effects of CIE or Control treatment, sex, and drug with 3-way ANOVA (missing values for some cells precluded use of repeated measures ANOVA across drug doses). We also predicted that males and females would differ in response to tigecycline, so data for males and females were analyzed separately in the absence of a significant interaction. For clarity of experimental groups throughout, "treatment" will refer to CIE or Control exposure and "drug" to tigecycline or saline administration. For all analyses, statistical significance was set at $p = 0.05$.

RESULTS

Effects of repeated cycles of CIE vapor or air exposure on 15E intake and PR in male and female mice

Average baseline 15E intake was 2.06 g/kg/2 hr in males and 2.17 g/kg/2 hr in females, and it did not differ significantly in the CIE versus Control groups (Figure 2). Analyses conducted on the averaged baseline and post-inhalation tests (Figure 2) documented that 15E intake was significantly higher in CIE versus Control mice [$F(1,36)=5.997$, $p<0.05$] and in female versus male mice [$F(1,36)=13.61$, $p=0.001$]. Ethanol intake increased across CIE cycles [$F(4,144)=11.32$, $p<0.001$], and there were trends for an interaction with sex [$F(4,144)=2.06$, $p<0.09$] and for an interaction with sex and ethanol treatment [$F(4,144)=2.02$, $p=0.09$]. Subsequent analyses in male mice confirmed that 15E intake increased across CIE cycles [$F(4,64)=5.00$, $p=0.001$]. Post-hoc tests indicated that 15E intake was significantly higher in the CIE versus Control group during Test 2 and tended to be higher during Test 3 and Test 4 (Figure 2A). In female mice, 15E intake also increased across CIE cycles [$F(4,80)=8.60$, $p<0.001$], with post-hoc tests confirming that ethanol intake was significantly higher in the CIE versus Control groups during Test 4 and tended to be higher during Test 1 (Figure 2B). Ethanol intake during Test 4 also was significantly higher than baseline for both the CIE and Control female mice.

Average baseline ethanol PR was approximately 0.745 in males and 0.72 in females and did not differ significantly (not shown). Analysis of the averaged baseline and post-inhalation tests indicated that there were no overall sex or group differences, but there was a significant increase in ethanol PR across time [$F(4,144)=14.44$, $p<0.001$] and a trend for an interaction between sex, ethanol treatment, and time [$F(4,144)=2.14$, $p<0.08$]. In male mice, ethanol PR increased across post-inhalation drinking tests to 0.90 in Control and 0.93 in CIE mice during Test 4. A similar result was observed in female mice, with ethanol PR increasing across post-inhalation drinking tests to 0.89 in Control and 0.91 in CIE mice during Test 4.

Table 2 depicts the BECs that were averaged across the 3 days of each CIE cycle in male and female mice. While BECs tended to be higher in the male versus the female mice [$F(1,18)=3.71$, $p=0.07$], there was a main effect of CIE cycle [$F(3,54)=3.89$, $p<0.05$] and a significant interaction between CIE cycle and sex [$F(3,54)=4.01$, $p<0.05$]. Post-hoc tests confirmed that BECs did not differ between male and female mice during CIE cycle 1, but BECs were significantly higher in male versus female mice during CIE cycles 2 and 3. As a result, we moved the female mice into a separate inhalation chamber, which enabled us to match BECs in the male and female mice during CIE cycle 4.

Upon the start of the study, mean $\pm$ SEM body weights were 17.3 ± 0.5 g (Female Control, $n=11$), 17.9 ± 0.3 g (Female CIE, $n=12$), 22.5 ± 0.6 g (Male Control, $n=10$), and 22.3 ± 0.5 g (Male CIE, $n=11$). Body weights on the last day of baseline drinking and the first day of post-inhalation drinking on Tests 1 – 4 are depicted in Table 3. As expected, body weights were lower in female versus male mice [$F(1,36)=98.57$, $p<0.001$], but there was no significant effect of CIE treatment on body weight, nor was there an interaction with sex.

Effects of tigecycline on 15E intake and PR in male and female mice

A tigecycline dose response (0, 40, 60, 80, & 100 mg/kg) was conducted during the Test 4 post-inhalation phase of ethanol drinking. Analysis of the 15E intake data revealed that 2 hr ethanol intake was significantly higher in female versus male mice [$F(1,174)=25.77$, $p<0.001$], tended to be higher in the CIE versus Control mice [$F(1,174)=2.77$, $p<0.10$], and was significantly reduced following tigecycline [$F(4,174)=11.11$, $p<0.001$] (Figure 3). Subsequent analyses in each sex confirmed the main effect of tigecycline [$F(4,75)=8.61$, $p<0.001$ for males; $F(4,99)=4.66$, $p<0.01$ for females], but there was no interaction between drug and ethanol treatment on ethanol intake in either sex. As a result, data from each sex were collapsed across ethanol treatment and reanalyzed for drug effect with 1-way ANOVA and Tukey's post-hoc tests. In male mice, tigecycline significantly reduced 15E intake [$F(4,80)=8.09$, $p<0.001$], with the 80 and 100 mg/kg doses reducing 15E intake by 57% and 42% versus vehicle injection, respectively (Figure 3B).

Ethanol intake in the mice administered the 80 and 100 mg/kg tigecycline doses also was significantly lower than intake in mice following 40 and 60 mg/kg tigecycline (Figure 3B). In female mice, tigecycline significantly decreased 15E intake [$F(4,99)=4.66$, $p<0.01$], with the 80 and 100 mg/kg doses reducing 15E intake by 42% and 30% versus vehicle injection, respectively (Figure 3D). Ethanol intake in the mice administered the 80 mg/kg tigecycline dose also was significantly lower than intake in mice following 60 mg/kg tigecycline and tended to be lower than intake in mice injected with 40 mg/kg tigecycline (Figure 3D).

Ethanol PR was not significantly influenced by sex or by ethanol treatment, although there was a slight but significant fluctuation in ethanol PR across tigecycline doses [$F(4,176)=2.45$, $p<0.05$]. Subsequent analyses in male and female mice indicated that tigecycline did not alter ethanol PR in the Control or CIE exposed mice.

Water intake during the 2 hr period of access to 15E was negligible, so the subsequent 22 hr water intake was analyzed for effects of tigecycline on water intake. Water intake did not vary across sex or treatment (Figure 4), but there was a significant effect of tigecycline dose [$F(4,180)=10.245$, $p<0.001$] and a trend for an interaction between tigecycline and sex [$F(4,180)=1.98$, $p<0.10$]. Subsequent analyses in male mice revealed that tigecycline tended to influence water intake, with a slight decrease in water intake following the 60 mg/kg dose of tigecycline [Control: $F(4,45)=2.35$, $p=0.068$; CIE: $F(4,35)=2.465$, $p=0.063$] (Figure 4A). Since the ANOVAs were not significant, post-hoc tests were not conducted. In female mice, tigecycline significantly altered water intake in both the Control and CIE groups [$F(4,50)=4.17$, $p<0.01$ for Control; $F(4,50)=5.78$, $p=0.001$ for CIE], with post-hoc tests indicating that water intake was significantly lower in mice injected with 60 mg/kg tigecycline versus the 40 mg/kg and 80 mg/kg tigecycline doses (Figure 4B). There were no significant differences in water intake following administration of each tigecycline dose versus saline.

DISCUSSION

Repeated cycles of CIE exposure significantly increased ethanol intake in male and female C57BL/6J mice, when compared with intake in air-exposed Control animals. Additionally, during Test 4 of post-inhalation drinking, ethanol intake was increased 28% and 48% over baseline in the male and female mice following CIE exposure, respectively. Because BECs were matched during CIE cycles 1 and 4 only, we are not able to comment on whether sex differences exist in the time course for the escalation in ethanol intake following repeated cycles of CIE exposure. However, it should be noted that the BECs in CIE cycles 1 and 4 (which were matched between the sexes) met the threshold criteria of 1.75 mg/ml that previously was shown to be critical to observe an escalation in ethanol intake (Griffin et al., 2009a). And during Test 1 of post-inhalation drinking, ethanol intake was increased by 11% and 23% over baseline in the male and female mice following CIE exposure, respectively. Thus, when male and female mice had similar BECs that met the threshold criteria of 1.75 mg/ml during CIE cycles 1 and 4, there was a greater increase in post-inhalation drinking in CIE-exposed female versus male mice versus their respective baseline drinking.

Previous work has shown that the escalation in ethanol intake following repeated cycles of CIE exposure was associated with a significant increase in BEC, and that there was a significant positive correlation between ethanol intake and BEC (Becker and Lopez, 2004, Finn et al., 2007). Although we did not measure BECs following the post-inhalation drinking in the present study, our prior work documented that a mean ethanol intake of 2.5 g/kg corresponded to a BEC of 1.07 mg/ml (Finn et al., 2007), which exceeds the 0.8 mg/ml criteria for binge ethanol consumption (NIAAA, 2009). As a result, we presume that the ethanol intake during Test 4 in the CIE-exposed male and female mice produced BECs that are associated with binge ethanol intake.

The primary purpose of the present study was to examine the ability of tigecycline to decrease ethanol intake in dependent and non-dependent male and female mice following repeated cycles of CIE vapor or air exposure, respectively. Consistent with the results in non-dependent mice with the “drinking in the dark” (DID) model of binge drinking (Syapin et al., companion paper, 2016), tigecycline produced a dose dependent decrease in 2 hr ethanol intake without influencing 22 hr water intake versus saline injection. Interestingly, in the present study, the 80 and 100 mg/kg tigecycline doses produced a similar suppression in ethanol intake in the dependent and non-dependent mice versus vehicle injection, and those doses were more efficacious at reducing ethanol intake in the male versus female mice. These results add to existing evidence in non-dependent mice and confirmed that the tetracycline derivative, tigecycline, significantly reduced ethanol intake in a common model of ethanol dependent and non-dependent treatment in both male and female mice.

In the present study, mice were administered tigecycline at 1 hr prior to the beginning of the 2 hr period of ethanol intake, based on the pharmacokinetic parameters for tigecycline in the mouse (van Ogtrop et al., 2000), which shows a maximal concentration within 15 minutes and a half-life of 2.34 hours. Others have reported much longer murine half-lives (>10hrs) and a large volume of distribution (Koomanachai et al., 2009). As tigecycline has been found in all tissues, including the CSF and brain (Munyeza et al., 2015), and is thought to be eliminated in unmodified form primarily in the feces and to some extent in the urine, it is hard to resolve the differences. It is unclear why we found no suppression of ethanol intake on the day after tigecycline injection, while that same approach was effective to reduce acute binge drinking in our related study (Syapin et al., companion paper, 2016).

The mechanism by which tigecycline reduces ethanol intake is not known. The primary literature predominantly describes its action on current multi-drug resistant bacteria. In fact, the drug is used as an effective alternative against MRSA infection (Koomanachai et al., 2009). Given its broad spectrum of antimicrobial action via an inhibition of tRNA binding to ribosomes (Brodersen et al., 2000), it is possible that tigecycline changes the microbiome and indirectly affects alcohol consumption. However, a microbiome effect would be more consistent with long-term treatment efficacy. In this experiment, the drug was effective when administered in a short time-frame prior to drinking. Minocycline, another semi-synthetic tetracycline to which tigecycline is structurally similar, is well known to have off-target, beneficial effects, including a reduction in ethanol consumption and protective effects against neurodegeneration (Kim and Suh, 2009, Agrawal et al., 2011, Garrido-Mesa et al., 2013). It is likely that tigecycline also has unique effects separate from its ability to bind the A-site of the bacterial 30S ribosome (Brodersen et al., 2000).

In conclusion, tigecycline significantly decreased ethanol intake in dependent and non-dependent male and female mice. While additional studies are warranted to determine the exact mechanism by which tigecycline suppresses ethanol consumption in both dependent and non-dependent animal models, the present findings, and those in a companion manuscript (Syapin et al., companion paper, 2016) suggest that tigecycline is a promising compound for development of a novel pharmacotherapy for the treatment of AUD in both males and females.

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Figure 1. General experimental time line for repeated cycles of chronic intermittent ethanol (CIE) vapor or air (Control) exposure and weeks of limited access ethanol intake

After stabilization of baseline ethanol intake (2 hr intake of 15% v/v ethanol (15E) versus water), male and female C57BL/6J mice underwent alternating weeks of CIE or air exposure followed by weeks of limited access (2 hr) 15E intake. The tigecycline dose response was examined during Test 4 in all mice. See Table 1 for additional details.

Figure 2. The change in ethanol intake following repeated cycles of chronic intermittent ethanol (CIE) vapor or air (Control) exposure in male (A) and female (B) C57BL/6J mice

Mice had a choice between a 15% ethanol solution (15E) versus water for 2 hrs, beginning 30 min prior to lights off. Data represent the average 15E baseline drinking and drinking over 5 days of each post-inhalation test (i.e., Tests 1 – 4). **Panel A:** 15E intake was significantly higher in the CIE versus Control group during Test 2 and tended to be higher during Test 3 & 4. $^+p < 0.10$, $***p = 0.001$ CIE vs respective Control group. **Panel B:** 15E intake was significantly higher in the CIE versus Control group during Test 4 and tended to be higher during Test 1. 15E intake during Test 4 also was significantly higher than baseline for both the Control and CIE groups. $^+p < 0.10$ CIE versus Control; $*p < 0.05$ Test 4 CIE versus Control, Test 4 Control versus respective Baseline; $**p < 0.01$ Test 4 CIE versus respective Baseline. Depicted are the mean $\pm$ SEM for 10 (Male Control), 8 – 10 (Male CIE), or 11 (Female Control & CIE) per time point.

Figure 3. Tigecycline significantly decreased ethanol intake in dependent and non-dependent male (A,B) and female (C,D) mice

Limited access (2 hr) ethanol intake of a 15% ethanol solution (15E) is shown. Mice were administered vehicle (saline) or tigecycline (40, 60, 80, 100 mg/kg) in a within subjects design and in a random order, with a 1 hr pretreatment time. Data are shown for the dependent mice exposed to chronic intermittent ethanol (CIE) vapor and for the non-dependent mice exposed to air (Control) in panels A & C for information only, as the analyses were conducted on the data that were collapsed across CIE and Control treatments (i.e., CIE and Control treatment groups were combined and reanalyzed) and that are depicted in panels B & D. **Panel B:** In male mice, the 80 and 100 mg/kg tigecycline doses significantly decreased 15E intake by 57% and 42% versus vehicle injection, respectively, and also significantly decreased 15E intake versus the 40 and 60 mg/kg tigecycline doses. * $p < 0.05$, *** $p < 0.001$ versus vehicle, 40 & 60 mg/kg doses. **Panel D:** In female mice, the 80 mg/kg dose significantly decreased 15E intake by 42% versus vehicle, significantly decreased 15E intake versus the 60 mg/kg dose, and tended to decrease intake versus the 40 mg/kg dose. The 100 mg/kg dose of tigecycline tended to decrease 15E intake by 30% versus vehicle. + $p < 0.10$ vehicle versus 100 mg/kg, 40 mg/kg versus 80 mg/kg; * $p < 0.05$ 60 mg/kg versus 80 mg/kg; ** $p < 0.01$ vehicle versus 80 mg/kg. Depicted are the mean $\pm$ SEM for 8 – 10 (Male Control), 8 (Male CIE), 11 (Female Control) or 10 – 11 (Female CIE) per dose.

Figure 4. Effect of tigecycline on 22 hr water intake in dependent and non-dependent male (A) and female (B) mice

Depicted are the mean $\pm$ SEM 22 hr water intake following the 2 hr measurement of 15E on the days that mice were injected with saline (0 mg/kg) or tigecycline (40, 60, 80, 100 mg/kg). Male and female mice were administered saline or tigecycline during Test 4 of the post-inhalation drinking after the 4th cycle of chronic intermittent ethanol (CIE) or air (Control) exposure. There were no significant differences in water intake following administration of each tigecycline dose versus saline. Corresponding ethanol intake and group size are depicted in Figure 3. ** p 0.01 versus the 40 mg/kg and 80 mg/kg tigecycline doses.

Table 1**Experimental Time Line**

For the weeks of CIE exposure, the intermittent vapor exposure began at 1430 and ended at 0730 the following day, with exposure to air for 7 hrs, until the next 17 hr period of intermittent vapor exposure began at 1430. Control animals were exposed to air for the entire 72 hr period. Each 72 hr period of CIE or air (Control) treatment comprised one cycle. All mice consumed only water during the weeks of CIE vapor or air exposure.

Baseline ethanol intake	3 weeks (Mon – Fri)
CIE or air exposure	3 days X 17 hr/day for CIE (Tues – Fri)
Ethanol intake (Test 1)	1 week (Mon – Fri)
CIE or air exposure	3 days X 17 hrs/day for CIE (Tues – Fri)
Ethanol intake (Test 2)	1 week (Mon – Fri)
Abstinence	2 weeks
Ethanol intake (post-abstinence)	1 week (Mon – Fri)
CIE or air exposure	3 days X 17 hrs/day for CIE (Tues – Fri)
Ethanol intake (Test 3)	2 weeks (Mon – Fri)
CIE or air exposure	3 days X 17 hrs/day for CIE (Tues – Fri)
Ethanol intake (Test 4)	5 weeks (Mon – Fri); tigecycline dose response

Table 2
Blood Ethanol Concentrations (mg/mL) During Each Cycle of Chronic Intermittent Ethanol (CIE) Vapor Exposure

Depicted are the mean $\pm$ SEM blood ethanol concentrations that were averaged across the 3 days of CIE vapor exposure for each CIE cycle. Male and female mice were placed in separate wire mesh racks in the same ethanol inhalation chamber during CIE cycles 1 – 3, but were placed in separate ethanol inhalation chambers for CIE cycle 4 to better match BECs between the male and female mice.

Sex	CIE #1	CIE #2	CIE #3	CIE #4
Female	1.78 $\pm$ 0.13 (n=12)	1.39 $\pm$ 0.05 (n=12)	1.51 $\pm$ 0.06 (n=12)	1.73 $\pm$ 0.03 (n=12)
Male	1.745 $\pm$ 0.10 (n=11)	1.66 $\pm$ 0.06 ** (n=10)	1.85 $\pm$ 0.13 * (n=9)	1.745 $\pm$ 0.08 (n=8)

*
 $p < 0.05$,

**
 $p < 0.01$ vs respective female values.

Table 3**Summary of Body Weight**

Depicted are the mean $\pm$ SEM body weights for male and female mice that were taken on the last day of baseline drinking and on the first day of each post-inhalation drinking test (i.e., Test 1 – 4). There were no significant differences between the Control and CIE groups in body weight at any of the time points for the male and female mice.

Sex/Treatment	Baseline	Test 1	Test 2	Test 3	Test 4
Female Control	17.8 $\pm$ 0.5 g	18.5 $\pm$ 0.4 g	19.95 $\pm$ 0.4 g	21.8 $\pm$ 0.5 g	21.7 $\pm$ 0.4 g
Female CIE	18.3 $\pm$ 0.5 g	19.0 $\pm$ 0.5 g	20.2 $\pm$ 0.5 g	22.1 $\pm$ 0.5 g	22.5 $\pm$ 0.4 g
Male Control	24.0 $\pm$ 0.7 g	25.5 $\pm$ 0.8 g	26.6 $\pm$ 0.8 g	28.9 $\pm$ 0.8 g	29.1 $\pm$ 0.7 g
Male CIE	23.05 $\pm$ 0.7 g	24.1 $\pm$ 0.8 g	25.2 $\pm$ 0.7 g	27.2 $\pm$ 0.7 g	28.0 $\pm$ 0.6 g