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Effective Reduction in High Ethanol Drinking by Semi-synthetic Tetracycline Derivatives

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Abstract

Background—New pharmacotherapies to treat Alcohol Use Disorders (AUD) are needed. Given the complex nature of AUD, there likely exist multiple novel drug targets. We, and others, have shown that the tetracycline drugs, minocycline and doxycycline, reduced ethanol drinking in mice. To test the hypothesis that suppression of high ethanol consumption is a general property of tetracyclines, we screened several derivatives for anti-drinking activity using the Drinking-In-the-Dark (DID) paradigm. Active drugs were studied further using the dose-response relationship.

Methods—Adult female and male C57BL/6J mice were singly housed and the DID paradigm was performed using 20% ethanol over a 4-day period. Mice were administered a tetracycline or its vehicle 20 h prior to drinking. Water and ethanol consumption was measured daily. Body weight was measured at the start of drug injections and after the final day of the experiment. Blood was collected for ethanol content measurement immediately following the final bout of drinking.

Results—Seven tetracyclines were tested at a 50 mg/kg dose. Only minocycline and tigecycline significantly reduced ethanol drinking, and doxycycline showed a strong effect-size trend towards reduced drinking. Subsequent studies with these three drugs revealed a dose-dependent decrease in ethanol consumption for both female and male mice, with sex differences in efficacy. Minocycline and doxycycline reduced water intake at higher doses, although to a lesser degree than their effects on ethanol drinking. Tigecycline did not negatively affect water intake. The rank order of potency for reduction in ethanol consumption was minocycline > doxycycline > tigecycline, indicating efficacy was not strictly related to their partition-coefficients (LogP) or distribution constants (LogD).

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Conclusions—Due to its effectiveness in reducing high ethanol consumption coupled without an effect on water intake, tigecycline was found to be the most promising lead tetracycline compound for further study toward the development of a new pharmacotherapy for the treatment of AUD.

Keywords

Alcohol Use Disorder; Drinking-In-The-Dark; Medications Development; Tigecycline

INTRODUCTION

In the U.S., ethanol is the most commonly abused drug. Alcohol Use Disorder (AUD) is currently defined by the DSM-V (O'Brien, 2014) as a spectrum of eleven alcohol-related traits. AUD contributes between 4-8% of overall health burden (Grimm, 2008) and costs approximately 1.4 trillion dollars, or nearly \$750 for every man, woman and child, in the United States annually (CDC, 2014). Almost three-quarters of the cost is due to binge drinking, defined as >0.08 g/dL blood ethanol concentration (expressed as 0.8 mg/ml in rodent research) or generally consuming 4 or more drinks for women and 5 or more drinks for men per occasion (NIAAA, 2009). However, despite high AUD prevalence, as well as over a half-century of research devoted to the development of improved treatments, there are only three FDA approved drugs for the treatment of AUD; acamprosate, disulfiram and naltrexone (Zindel and Kranzler, 2014). Motivational interviewing, cognitive behavioral therapy and attendance of a 12-step behavioral-recovery program are currently the most effective treatments (ProjectMATCH, 1997). Unfortunately, there is no consistent standard of care and AUD patients show high rates of recurrent relapse. As a consequence, development of new AUD therapeutic strategies is warranted.

Ethanol-mediated inflammatory reactions have long been known to be associated with ethanol-related tissue damage (Opie and Meakins, 1909, Baum and Iber, 1973, McClain and Cohen, 1989, Crews and Vetreno, 2014), and accumulating evidence suggests that neuroimmune reactions are related to high ethanol consumption behaviors (Blednov et al., 2005, Pascual et al., 2007, Fernandez-Lizarbe et al., 2009, Blednov et al., 2012, Robinson et al., 2014, Crews and Vetreno, 2016, Marshall et al., 2016) and the development of Fetal Alcohol Spectrum Disorder (Drew and Kane, 2014, Chastain and Sarkar, 2014, Drew et al., 2015). Our previous studies have shown that minocycline, an FDA approved tetracycline with known anti-inflammatory characteristics, reduced ethanol consumption in the Drinking-in-Dark (DID) and Two-Bottle Choice (2BC) paradigms (Agrawal et al., 2011, Agrawal et al., 2014). Another tetracycline derivative, doxycycline, has been shown by others to reduce drinking (McIver et al., 2012).

As there is a pressing need for novel pharmacotherapies to treat AUD, we chose to follow up on our initial finding using seven structurally distinct tetracycline class compounds to test the hypothesis that anti-drinking activity is a general property of tetracyclines. Tetracyclines are a class of antibiotics with broad-spectrum activity related to their ability to prevent the association of aminoacyl tRNA with bacterial, but not mammalian, ribosomes (Brodersen et al., 2000, Pioletti et al., 2001). Minocycline, however, has extensively been shown to have

protective effects against inflammation-related conditions, presumably by multiple off-target, non-bacterial mediated mechanisms. Minocycline has been tested in numerous pre-clinical studies in animal models and humans for several diseases and disorders [for a review see: (Garrido-Mesa et al., 2013)].

Following the general screen of tetracycline class drugs, we tested a secondary hypothesis that doxycycline, minocycline and tigecycline would reduce drinking in a dose-responsive manner. Due to the fact that ethanol is known to show strong sexually dimorphic responses, we tested both female and male C57BL/6J mice.

MATERIALS AND METHODS

Mouse Husbandry

All procedures using animals were approved by the Texas Tech University Health Sciences Center Institutional Animal Care and Use Committee (Assurance #A 3056-01) and were completed in a dedicated behavior testing room within our AAALAC accredited Laboratory Animal Resource Facility. C57BL/6J (B6) mice of both sexes were obtained at 7-10 weeks of age from the Jackson Laboratory (Bar Harbor, ME) and singly housed in clear plastic shoebox cages in a dedicated animal room. Food (Harlan Teklad or Purina Lab Diet) was available ad libitum, and the room was maintained on a 12 h reverse light-cycle (lights off at 9 AM) at constant temperature and humidity.

Drugs

Ethanol (95% v/v, USP) was diluted to 20% (v/v) with rodent drinking water from the Laboratory Animal Resources Center, Texas Tech University Health Sciences Center, Lubbock TX. Tetracycline derivatives and their properties are listed in Table 1. All tetracyclines used in our study have been approved by the FDA as antibiotics in doses varying up to 500 mg, although some are no longer used (see: <http://www.accessdata.fda.gov/scripts/cder/drugsatfda/>). Drugs were obtained from commercial sources and stored according to the vendor's recommendations. Minocycline HCl (#M9511), chlortetracycline HCl (#46133-Fluka), oxytetracycline HCl (#D9811), tigecycline hydrate (#PZ0021), and tetracycline HCl (#T3383) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Doxycycline HCl (#SC337691) and demeclocycline HCl (#SC204710A) were obtained from Santa Cruz Biotechnology (Dallas, TX, USA) and tigecycline (base) (#15026) was obtained from Cayman Chemical (Ann Arbor, MI, USA). Doses of 0, 20, 40, 60, 80, 100 and 120 mg/kg were made fresh and used within 25 hours.

Ethanol Consumption: Drinking-in-the-Dark (DID) Paradigm

The DID procedure was performed as previously described (Agrawal et al., 2011, Agrawal et al., 2014). For habituation purposes, the animals were handled and injected with saline for three days prior to drug or vehicle administration. Mice were weighed the day prior to the first drug administration and after the final DID period. Ethanol (20% v/v) and water were administered from 50 ml plastic centrifuge tubes (Falcon brand, BD Biosciences, Franklin Lakes, NJ) fitted with dual ball-bearing sippers. The tubes were positioned through special in-house built cage tops to accommodate an inverted position. Fluid consumption was

determined gravimetrically. The volume of ethanol consumed was converted to grams of ethanol consumed, based on the specific gravity (0.162) of ethanol within a 20% v/v water solution at room temperature, and is expressed per kilogram of body weight.

Administration of Tetracyclines

All tetracycline derivatives were dissolved in sterile saline (0.9% NaCl in water), except for demeclocycline and chlorotetracycline which were only soluble in water at the 5 mg/ml concentration used for the screening studies. Saline or water was used as vehicle controls, as appropriate. For the screening studies, drugs were administered at a 50 mg/kg dose in 10 ml/kg body weight by intraperitoneal (i.p.) injection. For the dose response studies, the injection volume was increased to 13.3 ml/kg to accommodate the use of higher doses. The injection site (lower right or left abdomen) was alternated daily. Drugs were administered according to previously published procedures (Agrawal et al, 2014). The first injection occurred 20 h prior to initial exposure to ethanol in the DID procedure, and every 24 h afterwards, immediately following hour 4 of DID, for a total of 4 days.

Pharmacokinetics of Ethanol

To test the metabolism of ethanol following tigecycline treatment in vivo, pharmacokinetic profiling was completed. Mice ($n = 5$ / group) were injected with a single dose of tigecycline (80 mg/kg i.p.) or saline and gavaged with 4 g/kg ethanol (20% in water) 20 hr later. Food was removed from their cages 2 hours before gavage and replaced 2 hour after gavage to prevent any alteration caused by differing stomach contents. Submandibular blood samples (47.5 μ l) were taken at 135, 180, 225, 270 minutes postgavage and at 315 minutes by sacrificing the mice. The blood samples were processed and analyzed as described below for (blood ethanol concentration) BEC analysis.

Blood Ethanol Concentration (BEC) Determination

Blood (47.5 μ l) was collected either from the base of the head or submandible region and collected into heparinized, calibrated hematocrit capillary tubes (Drummond Scientific company, Broomall, PA). The hematocrit contents were emptied into a wide top, 2 ml, crimp top chromatograph vial (Agilent Technologies, Palo Alto, CA) containing 100 μ l of water. They were sealed with a rubber septum containing lid and frozen until analyzed by a gas chromatographic headspace method (Finn et al., 2007, Agrawal et al., 2014). Ethanol content was detected using an Agilent DB-ALC1 column and 7683 GC (Agilent Technologies, Palo Alto, CA) fitted with an automatic sampler, and quantified from a standard curve constructed from 47.5 μ l in 100 μ l water of 0.125, 0.25, 0.5, 1.0, 2.0 and 4.0 mg/ml ethanol. Blood ethanol concentrations were determined after the DID experiment (end of 4 hours) and throughout the metabolism study at the times listed above.

Data Analyses

Data from each animal were averaged over the 4 days of DID and the group means ($\bar{x}$) for n animals per condition were graphed and analyzed using GraphPad Prism version 6.05 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com. Variance is presented as the standard error of the mean (SEM), unless noted otherwise. Simple two

sample comparisons were analyzed by unpaired or paired, one- or two-tailed *t*-tests, as appropriate. The F-test was used to test equivalence of variance for *t*-tests. Samples with unequal variances were data transformed to provide equal variance prior to *t*-tests. One-way and 2-way ANOVA were used to compare dose response data, within and between sexes, respectively. Bartlett's test was used to test for equivalence of sample standard deviations in the 1-way ANOVA and if necessary data were transformed to provide equal standard deviations prior to analysis. Dunnett's multiple comparison test was used for post-hoc comparisons between control (0 mg/kg) and different doses of tetracyclines. Outliers (between 0-5% per experiment) were removed from the daily data set if they were greater than two standard deviations above or below the daily mean for ethanol or water consumption for each condition.

RESULTS

Screen of Tetracycline Derivatives for Anti-Drinking Activity

The amount of 20% ethanol consumed by vehicle-injected control female and male B6 mice during four days of the 4 h DID was, as expected (Agrawal et al., 2014), significantly greater for females than males ($\bar{x} = 6.9 \pm 0.3$ g/kg versus $\bar{x} = 4.8 \pm 0.3$ g/kg; $n = 20/\text{sex}$, $p < 0.0001$, two-tail *t*-test). In contrast, there was no significant difference in 20 h water consumption between females and males ($\bar{x} = 188.6 \pm 30.5$ ml/kg versus $\bar{x} = 168.1 \pm 48.3$ ml/kg; $n = 20/\text{sex}$, $p > 0.05$, two-tail *t*-test). The tetracycline analog drugs tested for anti-drinking activity are shown in Table 1 along with some of their characteristic physical properties. The results of the seven tetracycline derivatives for the ability to reduce ethanol consumption in the 4 h DID are shown in Table 2. The capacity of 50 mg/kg minocycline to reduce ethanol drinking in adult B6 mice was previously published (Agrawal et al., 2011, Agrawal et al., 2014). Four naturally occurring derivatives; tetracycline, oxytetracycline, chlortetracycline, and demeclocycline, and two semi-synthetic derivatives; doxycycline and tigecycline were used, while the previous data for minocycline is also reported. Tetracycline and oxytetracycline did not influence ethanol or water consumption in either male or female mice, and also did not affect body weight (an indicator of general health). In contrast, two naturally occurring but chlorinated derivatives, chlortetracycline and demeclocycline, reduced ethanol drinking with chlortetracycline effective only in females (Table 2). However, both derivatives also significantly reduced water intake and caused a drop in body weight, again with chlortetracycline only active in females (Table 2). The toxic signs of demeclocycline were particularly severe. In addition to weight loss, the mice appeared unkempt and two of the males succumbed within 3 days post-injection. The effect on weight loss was less severe for chlortetracycline, but it was not studied further because of a lack of efficacy in the males.

Among the two semi-synthetic derivatives tested, only tigecycline significantly reduced ethanol drinking at 50 mg/kg (Table 2). Surprisingly, doxycycline at $n = 5$, did not show a significant reduction in ethanol drinking at a 50 mg/kg dose, although mean consumption levels were lower in both males and females (data not shown). Given the strong trend in the data, effect-size was calculated and a power analysis for sample size was completed; Cohen's $d = 1.18$ for females, 0.99 for males, with $n = 10$ and 14 per group needed to adequately test the hypothesis. Doxycycline did not change water consumption or body

weight (Table 2). As a consequence, given only a small lack of power coupled to previous work demonstrating efficacy of doxycycline in the DID paradigm (McIver, et al. 2012), it was included in the dose response analyses.

Dose Response Analysis of Semi-synthetic Tetracycline Derivatives

Doses above and below the screening dose of 50 mg/kg (0, 20, 40, 60, 80 mg/kg) were tested for effects on ethanol drinking, water intake, and body weight changes in female and male B6 mice. A 2-way (dose $\times$ sex) ANOVA for doxycycline indicated there was a main effect for both dose ($F(4, 48) = 21.69, p < 0.0001$) and sex ($F(1, 12) = 95.46, p < 0.0001$). Significant 1-way ANOVA for reductions in ethanol consumption was seen in both females ($F(4, 30) = 6.97, p = 0.0004$) and males ($F(4, 30) = 9.05, p < 0.0001$), post-hoc tests showing that the reduction was significant for females only at 80 mg/kg and for males at the 60 and 80 mg/kg doses (Fig 1a). Treatment with doxycycline also significantly reduced water consumption, but only at the 80 mg/kg dose for males and at the 60 and 80 mg/kg doses for females (Fig. 1b). 1-way ANOVA also indicated reductions in body weights for both females and males (females: $p = 0.0008$; males: $p < 0.0001$), with the change at the 80 mg/kg dose (~12% reduction for both sexes) being statistically significant by Dunnett's multiple comparison test (data not shown).

The dose response (0, 20, 40, 60, 80 mg/kg) for minocycline was very similar to the response for doxycycline. A 2-way ANOVA for minocycline treatments indicated a main effect of dose ($F(4, 48) = 37.97, p < 0.0001$) and sex ($F(1, 12) = 22.08, p = 0.0005$) with no significant interaction (Figure 1). When the data were transformed to percent of control (results not shown), only a main effect of dose remained ($F(4, 48) = 28.92, p < 0.0001$). A 1-way ANOVA for ethanol consumption within sexes indicated a significant effect of dose for both females ($F(4, 30) = 12.74, p < 0.0001$) and males ($F(4, 30) = 21.65, p < 0.0001$; on data transformed as $Y=Y^2$ to provide equal variances. Post-hoc analyses indicated significant differences between the 60 mg/kg and 80 mg/kg doses and the respective controls (Figure 1c). Interestingly, mice receiving the 60 and 80 mg/kg doses of minocycline also showed significantly reduced water intake (Fig. 1d), although the percentage change was less at 80 mg/kg compared to the effect on ethanol consumption (results not shown). A 1-way ANOVA also indicated reductions in body weights for both males and females (males: $p < 0.0001$; females: $p = 0.02$), with the change at the 80 mg/kg dose (~13% reduction for both sexes) being statistically significant by Dunnett's multiple comparison test (data not shown).

Mice treated with tigecycline across the dose response (0, 20, 40, 60, 80 mg/kg) presented a somewhat different pattern of results. A 2-way ANOVA indicated a main effect of dose only ($F(4, 48) = 16.46, p < 0.0001$). Subsequent analyses revealed significant effects on ethanol drinking for both females ($F(4, 30) = 8.03, p = 0.0002$) and males ($F(4, 30) = 6.06, p = 0.001$), with significant reductions following both the 60 and 80 mg/kg doses (Fig 1e). In contrast to minocycline and doxycycline, mice treated with tigecycline showed no changes in water intake (Fig 1f) or reductions in body weight (results not shown).

To test the hypothesis that higher doses of tigecycline would reduce ethanol consumption without reduction in water drinking, 100 mg/kg and 120 mg/kg doses were added to the tigecycline dose response curve as shown in Figure 2. A 2-way ANOVA indicated that there

were dose and sex differences between males and females with tigecycline treatment (dose ($F(6,72) = 20.38, p < 0.0001$); sex ($F(1,12) = 13.68, p = 0.003$) on ethanol consumption. A 1-way ANOVA followed by a post-hoc Dunnett's multiple-comparison indicated that tigecycline was effective at reducing ethanol drinking in males and females at doses of tigecycline 60 mg/kg and above. A 2-way ANOVA was performed on water consumption as well. Again, there were sex differences between female and male groups ($F(1,12) = 50.51, p < 0.0001$) and when collapsed across sex a main effect of dose ($F(6,72) = 3.78, p = 0.003$). A 1-way ANOVA followed by a post-hoc Dunnett's multiple-comparison on water consumption indicated no differences in water consumption between treatment female groups ($F(6,42) = 1.55, p = 1.85$) and male groups ($F(6,42) = 1.035, p = 0.417$).

Effect of Tetracycline Class Drug Treatment on Blood Ethanol Concentration

DID mice treated with 50 mg/kg, i.p. doxycycline, minocycline, or tigecycline demonstrated an overall reduction in drinking and consequent reduced BEC vs. saline control mice (Table 3). An initial 2-way ANOVA revealed a significant reduction of BEC by treatment ($F(1,51) = 9.31; p < 0.005$) between treated female and male groups to concentrations below the value (0.8 mg/mL) considered "binge" drinking. However, only female mice treated with minocycline (80 mg/kg) showed a significant reduction ($p = 0.03$) with a post-hoc Dunnett's multiple comparison test. Effect sizes were calculated for each group (Table 3).

No Effect of Tigecycline on Pharmacokinetic Elimination of Ethanol

Tigecycline treatment did not affect the pharmacokinetic elimination of 4 g/kg (gavage) ethanol as there were no statistically significant differences between the linear regression line slopes of female and male treated and untreated mice ($F(3, 79) = 0.54, p = 0.66$). However, statistically significant differences were seen between the elevations of the lines formed by linear regression analyses indicating that, while overall elimination of ethanol does not differ between animals, there was a difference in blood ethanol levels achieved ($F(3, 82) = 7.54, p < 0.0003$). Linear regression analyses of female and male groups separately revealed no significant differences in elevation between female groups ($F(1,42) = 3.25, p = 0.08$, but there were differences between male groups indicating initial differences in overall BEC occurred only between treated and untreated male mice $F(1,39) = 12.16, p = 0.001$).

DISCUSSION

The initial screen of seven tetracycline drugs showed only minocycline and tigecycline significantly reduced ethanol drinking. However, a positive trend was seen with doxycycline. Therefore, a power analysis was completed, which determined potential for the completion of a dose-response. The tetracyclines with chlorinated side chains, chlortetracycline and demeclocycline, showed signs of toxicity, while oxytetracycline and tetracycline were without effect on ethanol consumption. As a consequence, dose response curves were completed on doxycycline, minocycline and tigecycline to better understand if, and how, structural differences play a role in relative effectiveness. The follow-up dose response testing showed some sex differences, and the general efficacy for reduction in ethanol drinking was minocycline > doxycycline > tigecycline. However, tigecycline, importantly,

showed no apparent thirst-related side-effects, while higher doses of minocycline and doxycycline reduced overall water consumption. As the DID model is a simple screen for high binge ethanol consumption and does not measure preference, we cannot determine whether the effective drugs change ethanol consumption through acquisition or establishment of preference mechanisms. Therefore, it is important to note that our earlier work showed that minocycline reduced 2-bottle choice consumption either before or after a history of ethanol treatment (Agrawal et al., 2011). In addition, our companion paper shows efficacy of tigecycline after ethanol exposure in both ethanol dependent and non-dependent mice (Bergeson et al., companion paper, 2016b). Taken together, these results strongly suggest that mechanisms other than taste aversion or preference acquisition contribute to the effective reduction in ethanol consumption.

Interestingly, tigecycline's ability to reduce ethanol consumption plateaued at 80 mg/kg in females, while males showed increasing responses even at higher doses. One explanation is that there are differences between male and female neuroimmune systems. For instance, in a neuropathic pain model, female mice did not have a significant reduction in apparent neuropathic pain when treated with minocycline, while males showed reduced hyperalgesia (Sorge et al., 2015). The same study showed that while microglia appear to be the key mediator of neuropathic pain in males, female mice appear to have a different effector cell. Considering the strong microglial inhibition caused by minocycline (Garrido-Mesa et al., 2013), tigecycline may share some of the actions and similarly have sexually distinct effects (Sorge et al., 2015). In fact, our own results showed that tigecycline reduced neuroinflammatory pain in males, but not in females (Bergeson et al., companion paper, 2016a). Additionally, tetracyclines may affect a variety of non-microbial processes and it is possible some of the mechanisms show sex differences at higher doses, which lead to no further decrease in ethanol consumption in the female mice.

Overall, treatment with the semi-synthetic tetracyclines reduced BEC commensurate with decreased drinking compared to controls. However, only the female minocycline treated mice reached significance in *post-hoc* analyses. Several explanations are feasible. First, the large Cohen's *d* values (Table 3) and power analyses (data not shown) indicated that larger sample size would have allowed us to adequately test our hypothesis that drugs which reduce drinking should reduce BEC. Second, the large SEM for the experimental groups indicate that the mice likely varied greatly in how much and when they consumed the last bout of ethanol. The closer drinking is to the time of termination for the experiment, a higher BEC would be expected, while lower amounts and/or longer duration from last drink would result in lower final BEC. Finally, the mice were given food *ad libitum*, and as such, different eating patterns also likely affected final BEC, with food decreasing ethanol absorption. Of importance is that all the semi-synthetic tetracycline drugs reduced the DID consumption to a level that resulted in BEC lower than considered an "at risk" binge bout compared to both the female and male, ethanol-alone, control groups, which showed BEC > 0.8 mg/kg. Tigecycline had no effect on blood ethanol pharmacokinetic elimination in C57BL/6J mice (Figure 3), which was expected, as we had not previously detected a change in ethanol elimination with treatment of minocycline in C57BL/6J mice (Agrawal et al., 2011) or tigecycline in DBA/2J mice (Martinez et al., companion paper, 2016).

Several important findings resulted from this investigation. The first is that not all tetracycline derivatives appear to possess anti-drinking activity. The reason(s) for this is currently unknown, but may indicate that the properties necessary for antibiotic activity are not required for anti-drinking activity and that the metallic taste of tetracycline drugs does not result in taste aversion. Of course, it is also possible that the lack of efficacy of the naturally occurring derivatives is due to differences in physical properties related to half-life, lipid solubility, body distribution, or capacity to cross the blood brain barrier. Only the semi-synthetic tetracycline derivatives demonstrated an ability to significantly reduce ethanol consumption in the DID paradigm without overt toxicity at the 50 mg/kg dose. Tigecycline had good activity without an effect on water consumption or body weight at the 50 mg/kg dose. The activity of minocycline was published previously (Agrawal, et al. 2014), while the ability of ~ 40 mg/kg/day doxycycline using a different treatment schedule was previously published (McIver, et al. 2012).

Tigecycline, a derivative of minocycline and also known as a glycylcycline, showed the most selectivity (reduction of ethanol, but not water consumption) of the three semi-synthetic tetracycline derivatives tested that demonstrated anti-drinking activity (compare Figures 1 and 2). Our results indicate that modification of the R6' from -OH to -H for the semi-synthetic tetracyclines may play a role in anti-drinking activity compared to the other tetracycline class compounds. Within the semi-synthetic group, efficacy was not strictly related to partition-coefficients (LogP) or distribution constants (LogD). Tigecycline, due to its ability to reduce ethanol consumption without negatively affecting overall water consumption, may be a promising lead compound for development of a novel pharmacotherapy for treatment of AUD. Further studies in humans are necessary as the drug doses given were higher than FDA approved for humans. However, the difference might reflect the generally higher overall metabolism for mice, including for ethanol. Direct comparison of the tetracycline analogs to acamprosate and formulations of naltrexone is also warranted. To date, acamprosate has shown good efficacy in dependent, but not binge-drinking animals (Mason and Heyser, 2010). In fact, in humans acamprosate had better outcomes for abstinence following detoxification, while naltrexone showed higher efficacy to reduce heavy drinking and craving (Maisel et al., 2013). Given the narrow AUD treatment options, each with their own limitations, new therapeutics are needed. Our work suggests that tetracyclines, especially tigecycline, is worth further study.

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Figure 1. Dose-response showed efficacy of the three semi-synthetic tetracycline derivatives Shown in (A, C, E) effect of 4 h ethanol consumption and (B, D, F) 20 h water consumption in male (•) and female (o) B6 mice. Average and SEM (n = 7 / group) are shown for each individual drug dose, along with the *p*-value. * and # indicate significant differences from 0.0 mg/kg dose by Dunnett's multiple comparison post-hoc test for males and females, respectively. # *p* < 0.05; **, ## *p* < 0.01; ***, #### *p* < 0.0001

Figure 2. Tigecycline dose response curve shows marked reduction in ethanol consumption with sexual dimorphic differences

Shown in (A) and (B) is the effect of tigecycline on female ethanol and water consumption respectively, while the same for males are shown in (C) and (D). Data points are presented as mean $\pm$ SEM, $n = 7$ / group. Significance is shown by * from the 0.0 mg/kg dose via Dunnett's multiple comparison post-hoc test for females and males respectively * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

C57BL6/J Pharmacokinetic Elimination

Figure 3. Tigecycline treatment (80 mg/kg i.p.) had no effect on the pharmacokinetic elimination of ethanol

Blood was sampled at 135, 180, 225, 270 and 315 minutes post gavage. Slopes represent linear regression lines. Data points are presented as mean $\pm$ SEM. Groups had $n = 4-5$, as a few time points were missing due to failed blood draws.

Table 1
Tetracycline derivative structures

Based on the simple structure of tetracycline, the naturally occurring and semi-synthetic analog structural differences are listed. Highlighted in grey is the chemical difference that exists between the effective (grey boxes) and non-effective compounds (white). The tetracycline backbone structure was made using ChemDraw (Perkin Elmer informatics, Waltham, MA) and was based on information from Chopra and Roberts (Chopra and Roberts, 2001). LogD and LogP data are predicted values from ChemSpider (The Royal Society of Chemistry) (Accessed November 16, 2015) as follows: Tetracycline, ChemSpider ID=10257122, <http://chemspider.com/chemical-structure.10257122.html>; Oxytetracycline, ChemSpider ID=10482174, <http://chemspider.com/chemical-structure.10482174.html>; Chlortetracycline, ChemSpider ID=10469370, <http://chemspider.com/chemical-structure.10469370.html>; Doxycycline, ChemSpider ID=10469369, <http://chemspider.com/chemical-structure.10469369.html>; Demeclocycline, ChemSpider ID=10482117, <http://chemspider.com/chemical-structure.10482117.html>; Minocycline, ChemSpider ID=16735907, <http://chemspider.com/chemical-structure.16735907.html>; Tigecycline, ChemSpider ID=10482314, <http://chemspider.com/chemical-structure.10482314.html>.

Drug Name	R5	R6	R6'	R7	R9	LogD	LogP
Tetracycline	H	CH ₃	OH	H	H	-3.55	-1.47
Oxytetracycline	OH	CH ₃	OH	H	H	-4.25	-1.5
Chlortetracycline	H	CH ₃	OH	Cl	H	-2.43	0.33
Demeclocycline	H	H	OH	Cl	H	-3.40	-1.07
Doxycycline *	OH	CH ₃	H	H	H	-3.29	-0.54
Minocycline *	H	H	H	N(CH ₃) ₂	H	-2.25	0.20
Tigecycline *	H	H	H	N(CH ₃) ₂	NHCOCH ₂ NHC(CH ₃) ₃	-2.73	-1.30

* Semi-synthetic tetracycline

Table 2
Screen of tetracycline derivatives at 50 mg/kg

The screen of seven tetracycline derivatives at 50 mg/kg showed differential effects on 4 h ethanol consumption, 20 h water consumption, and body weight change across 4 days on drug. The percentage of control for significant differences is given in parentheses.

Derivative	Measurement	Males	Females
Tetracycline	Ethanol Consumption	NS	NS
“	Water Consumption	NS	NS
“	Body Weight Change	NS	NS
Oxytetracycline	Ethanol Consumption	NS	NS
“	Water Consumption	NS	NS
“	Body Weight Change	NS	NS
Chlorotetracycline	Ethanol Consumption	NS	$p=0.0117$ (76.6%)
“	Water Consumption	NS	$p=0.0011$ (75.0%)
“	Body Weight Change	NS	$p=0.0155$ (95.2%)
Demeclocycline [†]	Ethanol Consumption	$p<0.0001$ (26.0%)	$p<0.0001$ (36.1%)
“	Water Consumption	$p=0.0001$ (44.9%)	$p=0.0005$ (41.1%)
“	Body Weight Change	$p=0.0001$ (80.8%)	$p=0.0008$ (85.1%)
Doxycycline [#]	Ethanol Consumption	NS	NS
“	Water Consumption	NS	NS
“	Body Weight Change	NS	NS
Minocycline [#] Data from (Agrawal et al., 2014)	Ethanol Consumption	$p<0.05$ (65.0%)	$p<0.0001$ (63.0%)
“	Water Consumption	$p<0.05$ (82.0%)	NS
“	Body Weight Change	ND	ND
Tigecycline [#]	Ethanol Consumption	$p=0.002$ (46.5%)	$p<0.0015$ (80.0%)
“	Water Consumption	$p=0.0017$ (123.1%)	NS
“	Body Weight Change	ND	ND

NS = $p > 0.05$; ND = no data collected from Agrawal et al, 2014

[†] 2 of 5 males succumbed within 3 days of termination of the experiment.

[#] semi-synthetic drug. Data are from n = 5 mice per group.

Table 3
Blood ethanol concentrations immediately following DID drinking on day 4 are generally reduced by treatment at 80 mg/kg i.p. with the three effective semi-synthetic tetracyclines

Average BEC and SEM (N = 6-10 / group) are shown for each individual drug, along with the *p*-value and calculated Cohen's *d* effect size of each treatment compared to the saline controls.

Treatment	Sex	BEC	SEM	<i>p</i> -value	Cohen's <i>d</i>
Saline	F	0.89	0.22		
Doxycycline	F	0.39	0.16	0.07	1.05
Minocycline	F	0.19	0.20	*0.03	1.33
Tigecycline	F	0.48	0.27	0.23	0.67
Saline	M	1.13	0.25		
Doxycycline	M	0.74	0.17	0.19	0.74
Minocycline	M	0.58	0.21	0.10	1.02
Tigecycline	M	0.53	0.28	0.11	0.92