Effects of Tacrolimus and Other Immune Targeting Compounds on Binge-Like Ethanol Drinking in High Drinking in the Dark Mice

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ABSTRACT: High Drinking in the Dark (HDID-1) mice represent a unique genetic risk model of binge-like drinking and a novel means of screening potential pharmacotherapies to treat alcohol use disorders (AUDs). We tested the effects of tacrolimus (0, 0.5, 1, and 2 mg/kg), sirolimus (0, 5, 10, and 20 mg/kg), palmitoylethanolamide (PEA; 0, 75, 150, and 225 mg/kg), and secukinumab (0, 5, 20, and 60 mg/kg) on binge-like ethanol intake (2-day, "Drinking in the Dark" [DID]) and blood alcohol levels (BALs) in HDID-1 mice. Tacrolimus reduced ethanol intake and BALs. Tacrolimus had no effect on water intake, but reduced saccharin intake. There was no effect of sirolimus, PEA, or secukinumab on ethanol intake or BALs. These results compare and contrast with previous work addressing these compounds or their targeted mechanisms of action on ethanol drinking, highlighting the importance of screening a wide range of models and genotypes to inform the role of neuroimmune signaling in AUDs.

KEYWORDS: Binge drinking, HDID-1, immune, pharmacotherapy

Introduction

Alcohol use disorder (AUD) is a chronically relapsing disorder, which afflicts an estimated 15 million U.S. citizens.[1,2] AUD has a polygenic basis and is comprised of several neurobiological mechanisms. A central goal of preclinical alcohol research is to understand the underlying genetic and molecular risk factors, which can in turn lead to the screening of potential pharmacotherapies in a variety of drinking paradigms, species and genetic backgrounds. Despite a growing knowledge of neural and molecular mechanisms likely to be important for treating AUD, available options have not progressed since the FDA approval of acamprosate in 2004. To optimize and expedite this process, we focus our pre-clinical research efforts on drug repurposing by testing the effects of currently Food and Drug Administration (FDA)-approved compounds on binge-like ethanol intake in a mouse model of high genetic risk for binge-like drinking (High Drinking in the Dark mice; HDID).

Selective breeding for alcohol-related behaviors has offered considerable insight into potential genetic and molecular influences on excessive alcohol use in humans. To model the intoxicating blood alcohol levels (BALs) characteristic of binge-like alcohol consumption, 2 replicate lines of HDID mice (HDID-1 and HDID-2) mice were developed. Starting with a genetically heterogenous stock of mice (HS/NPT), HDID-1 and HDID-2 were selected for reaching high BALs during the limited access drinking task, Drinking in the Dark (DID). Mice of both replicate lines reach BALs averaging over 200 mg %, suggesting these mice serve as a model of drinking to intoxication.[3] Further, they exhibit several alcohol-related behaviors relevant to human AUD, such as behavioral signs of intoxication following DID[4] and withdrawal after a single DID session,[5] despite having no physiological differences in ethanol clearance rates compared to their HS/NPT founder line.[5] Both HDID lines display marked differences in brain gene co-expression patterns compared to HS/NPT.[6-8] Compared to HDID-2, HDID-1 are further along in selection and maintain a much greater genetic distance from that of C57BL/6J, of which many of the chosen pharmacotherapies have already been tested.[6] Therefore, the following work caters to the unique genetic diversity of HDID-1.

Importantly, HDID-1 mice show reductions in drinking with clinically relevant compounds used to treat AUD. In 2017, Crabbe et al[9] showed that acamprosate and baclofen reduced HDID-1 ethanol drinking, but there was no effect of naltrexone. This contrasted with the results in C57BL6/J mice, whereby acamprosate, baclofen, and naltrexone reduced ethanol intake, suggesting the HDID background has a genetically distinct response from that of the C57BL6/J strain.[10,11] Naltrexone, while an important drug for AUD, is not effective in all individuals.[12,13] Just as humans are heterogenous, so are

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the genetic contributions to AUD and the subsequent responses of individuals with an AUD to current treatment options. Therefore, it remains important to consider potential genetic risk factors for high, binge-like drinking, as captured in the HDID mice. In all, HDID mice represent an effective behavioral genetic tool to screen and identify potential pharmacotherapies in the treatment of AUD from a polygenic basis.

Growing research supports the neuroimmune system as a central player in the progression of AUD.[14-17] Early evidence stems from microarray analysis of postmortem brain showing elevated immune-related transcriptional activity in individuals with an AUD.[18-20] It is believed that alcohol abuse initiates an innate immune response within the central nervous system, and this may in turn contribute to further alcohol use.[21] The work presented herein is part of a collaborative, multidisciplinary research effort that uses a combination of systems and genomic-level approaches to identify therapeutic drug treatments for AUD on the basis that targeting neuroimmune signaling is a promising means of reducing alcohol use.[15,21-23] Numerous preclinical and clinical studies have focused on innate immune proinflammatory pathways as potential therapeutic avenues.[15,17,24] For instance, observed increases in Toll-like receptors (TLRs) among rodent models and human patients with AUD has led to the investigation of the MyD88-dependent pathway as a key signaling component for aberrant drinking patterns and likely the development of AUD.[25] Taken together, the above evidence suggests that focusing research efforts on immune signaling and inflammatory pathways may provide valuable contributions for the development of new treatments for AUDs.

Promisingly, HDID mice are responsive to the effects of treatment with immune-related compounds. A recent transcriptome-based drug discovery study identified 2 neuroimmune compounds, terreic acid and pergolide, as effective therapies for the reduction of binge-like drinking in the High Drinking in the Dark (HDID-1) mice.[26] Similarly, additional compounds that target and reduce inflammation, ibrutinib and rolipram, reduced HDID binge-like ethanol drinking.[23] The goal of the current work is to test the ability of 4 known anti-inflammatory and immune-related compounds (tacrolimus, sirolimus, palmitoylethanolamide [PEA], and secukinumab) in reducing binge-like ethanol drinking in male and female HDID-1 mice.

Tacrolimus is a macrolide calcineurin inhibitor and has been shown to reduce ethanol consumption in a C57BL/6J mice.[27,28] Tacrolimus was initially FDA approved as an immunosuppressant for organ transplantation in 1994 and is now a commonly used immunosuppressant.[29] Sirolimus (rapamycin) is another FDA approved (in 1999) immunosuppressant that targets the mTOR complex.[30] Studies from the lab of Dorit Ron have characterized the importance of mTOR signaling in the context of preclinical ethanol drinking in both rats and mice.[31,32] For instance, Neasta et al[31] determined that systemic administration of ethanol and limited access to 20% ethanol (4 hours

every other day for 3 weeks) increased mTOR activity in the nucleus accumbens (NAc) of male C57BL/6J mice, and that this effect is blocked by inhibiting mTOR via sirolimus treatment. Similarly, this group found that intermittent access to 20% ethanol and water (for 24 hours, every other day) in male Long-Evans rats increased NAc mTOR activity.[31] Palmitoylethanolamide (PEA) belongs to a class of molecules called n-acylethanolamines (NAEs), which are known to function through several biological mechanisms. This includes the activation of proliferator activated receptor alpha (PPARα), a notably important pathway in inflammatory processes and the progression and development of AUD.[15,17,24,33] A related NAE, oleoylethanolamine (OEA), was shown to reduce binge-like ethanol intake, ethanol seeking and behavioral measures of withdrawal severity in male Wistar rats.[34] An extensive case study (Keppel-Hesselink and Hekker-2012) reported the clinical application of PEA in treating over 40 syndromes associated with chronic and neuropathic pain.[35] The use of PEA in the clinical treatment of pain is reviewed further in Gabrielson et al.[36] Secukinumab is a monoclonal antibody against interleukin-17A (IL-17A), and is currently FDA approved to treat plaque psoriasis, psoriatic arthritis, and ankylosing spondylitis. IL-17 signaling is thought to contribute to neuroimmune and neuroinflammatory-related processes, such as those underlying depression.[37,38] It was recently shown that circulating IL-17A levels are elevated in individuals with a history of excessive alcohol use and ethanol-dependent mice, suggesting IL-17A may play a critical role in the pathogenesis of AUD.[39] In the same study, treatment with an anti-IL-17A antibody decreased limited access ethanol intake in dependent mice and ameliorated chronic ethanol-induced liver fibrosis and astrogliosis, supporting IL-17A's candidacy as a molecular target in the treatment of AUD.[39] Although the above compounds function through varying mechanisms of action, they are being investigated for their abilities to reduce binge-like ethanol drinking in HDID-1 mice because of their known role in reducing inflammatory processes and responses, although these responses were not directly measured.

Materials and Methods
Animals

High Drinking in the Dark mice of the first replicate line (HDID-1) were used for all experiments.[4] All mice were bred and maintained in the Veterans Affairs Portland Health Care System Veterinary Medical Unit, on a reverse 12-hour/12-hour light/dark schedule, with lights off at 7:30 am (PST). Experimental rooms were maintained at a temperature of 21 ± 1°C. Purina 5LOD chow (PMI Nutrition International, Brentwood, MO, USA) was available *ad libitum*, except where otherwise indicated. Mice were housed in standard polycarbonate cages with stainless steel wire tops on Bed-o'cobs® bedding (The Andersons, Inc., Maumee, OH, USA), and were habituated to single housing conditions for at least 1 week

prior to experiments. All procedures were approved by the local Institutional Animal Care and Use Committee and were conducted in accordance with NIH Guidelines for the Care and Use of Laboratory Animals.

Drugs

Tacrolimus (Selleckchem, Houston, TX) and sirolimus (Selleckchem) were mixed into 1.5% Tween-80 in 0.9% saline, which served as the vehicle for both drugs. PEA (Selleckchem), was dissolved in sterile corn oil (Sigma-Aldrich, Saint Louis, MO), which also served as the vehicle for that experiment. Secukinumab (VA Inpatient Pharmacy, Portland, OR) was made in a in a vehicle of 1.75% Tween-80 in 0.9% saline. Tacrolimus and sirolimus were administered by intraperitoneal (i.p.) injection at a volume of 10 mL/kg body weight. PEA and secukinumab were administered subcutaneously (s.c.) at a volume of 10 mL/kg body weight. Ethanol (200 proof, Decon Labs, King of Prussia, PA) was dissolved in tap water to a 20% v/v ethanol solution. Saccharin sodium salt hydrate (Sigma) was dissolved in tap water to a concentration of 8.5 mM.

Experiment 1: Effects of tacrolimus on binge-like drinking in HDID-1 mice

To determine the effects of tacrolimus on binge-like ethanol drinking, we tested male and female HDID-1 mice (n = 6-7/sex/dose) in a 2-day Drinking in the Dark (DID) procedure. Mice were aged 76 to 92 days and were of selection generations (S) and filial generations S40.G42 and S41.G43. To help visualize experimental conditions, timelines for each experiment are included in corresponding data figures. Mice were singly housed 1 week prior to testing and pseudorandomized to dose group. DID testing was performed as previously described,[40] whereby water bottles were replaced with a single 10 ml tube (fitted with a metal sipper and a ball bearing) containing 20% ethanol 3 hours into the dark cycle. Meniscus levels were recorded at the beginning and end of the 2 or 4-hour drinking period. On Day 1, ethanol was offered for 2 hours, and water bottles were then returned overnight. On Day 2, mice received an injection (i.p.) of tacrolimus (0, 0.5, 1, 2 mg/kg) 30 minutes prior to ethanol access. Doses and route of administration were chosen based on previously published levels and preliminary results on HDID-1 mice.[28] Ethanol fluid levels were read at 2 and 4 hours. Immediately after the 4-hour time point on Day 2, a 20 µl blood sample was taken from the peri-orbital sinus to determine BALs using gas chromatography.[40] Animals were then allowed 6 days to rest before they were re-randomized into dose groups and tested for water DID (tap water in 10 ml tubes). One week later, animals were re-randomized again and tested for saccharin DID (8.5 mM saccharin in tap water). All procedures (including injections) for water and saccharin DID were identical to ethanol DID, except that there was no blood sampling.

Experiment: Effects of low-dose sirolimus on binge-like drinking in HDID-1 mice

To determine whether previously established doses of sirolimus (0, 1, 2, 4 mg/kg) would reduce binge-like ethanol intake, we tested male (n = 24) and female (n = 24) HDID-1 mice, aged 76 to 92 days and of selection/generation S41.G43 in a 2-day ethanol DID. These doses were determined from previous work addressing the effects of sirolimus on ethanol intake in mice.[31,32] Procedures for ethanol DID were identical to those of Experiment 1, except on Day 2, mice received an injection (i.p.) of sirolimus, 30 minutes prior to fluid access (n = 6/sex/dose). Animals then rested for 6 days.

Mice were then tested in another 2-day ethanol DID to examine the effects of timing of injection on drug efficacy. Mice were re-randomized to new dose and injection groups (n = 6-10/dose/administration time). On Day 1, ethanol was offered for 2 hours, and water bottles were then restored. Mice were then administered sirolimus (0 or 5 mg/kg, i.p.) either 20 hours, 3 hours, or 30 minutes prior to Day 2 ethanol access. On Day 2, ethanol fluid levels were read at 2 and 4 hours.

Experiment 3: Effects of high-dose sirolimus on binge-like drinking in HDID-1 mice

To evaluate the effects of higher doses of sirolimus on binge-like ethanol drinking in HDID-1 mice, we tested the effects of 0, 5, 10, or 20 mg/kg sirolimus in a 2-day ethanol DID. Male and female HDID-1 mice (n = 5-10/sex/dose), were 84 to 150 days of age and of selection/generation S40.G42 and S41.G43. Procedures for ethanol DID were identical to those used in Experiments 1 and 2, except on Day 2 mice received an i.p. injection of sirolimus (0, 5, 10, or 20 mg/kg) 30 minutes prior to ethanol access. Testing was discontinued after ethanol DID.

Experiment 4: Effects of PEA on binge-like drinking in HDID-1 mice

To determine whether PEA reduces binge-like ethanol intake, we tested male and female HDID-1 mice ([n = 11-12/sex/dose], 116-178 days, and selection/generation S42.G44 and S43.G45) in 3 sequential weeks of ethanol DID. Mice were pseudorandomized into dose groups prior to each week's testing. DID testing consisted of replacing water bottles with a single, 20% ethanol tube 3 hours into the dark cycle. The doses and routes of administration were chosen based on previously work in rodents.[36,41] In Week 1, mice were given access to ethanol during a 2-day ethanol DID. Procedural details were identical to those of Experiment 1, except mice received a s.c. injection of PEA (0, 75, 150 mg/kg) 1 hour prior to ethanol access on Day 2. In order to probe potential efficacious time periods, in Week 2, mice were again tested in a 2-day ethanol DID, but PEA (0, 75, 150 mg/kg) was administered 16-hours prior to ethanol access on Day 2. Finally, to evaluate potential chronic effects of PEA on binge-like drinking, in Week 3 mice

were tested in a 4-day, 4-hour ethanol DID. Mice received s.c. injections of PEA (0,150, 225 mg/kg) daily, 16-hours prior to ethanol access on Days 2 to 4.

Experiment 5: Effects of Secukinumab on binge-like drinking in HDID-1 mice

Male and female HDID-1 mice (n = 7-9/sex/dose), aged 64 to 93 days and of selection generation S43.G46, were tested in a 2-day ethanol DID, as in Experiment 1. Here, mice received a s.c. injection of secukinumab (0, 5, 30, 60 mg/kg) 3-days prior to Day 1 ethanol access. This timeframe was based on the relatively slow rate at which s.c. injections of secukinumab reach maximum serum concentration (Cmax), which for humans is 5 to 6 days at 150 or 300 mg/kg.[42,43] Further, comparable doses and the same route of administration have been used to test locomotor activity and short-term spatial memory function in a murine model of Multiple Sclerosis.[44] Of note, blood samples were not taken due to lack of effect, and thus BAL's were not analyzed in these mice.

Effect of route of administration on binge-like drinking in HDID-1 mice

Because different routes of drug administration (i.p. and s.c.) were used in the studies, a separate cohort of male and female HDID-1 mice were tested to determine whether delivering saline via 3 routes of administration (i.p., s.c., or per os [by mouth]) altered 4-hour ethanol intake or BALs relative to non-treated mice (Supplemental Figure 1). The above compounds were administered either i.p. (tacrolimus and sirolimus) or s.c. (PEA and secukinumab); therefore, route of administration was evaluated as a potential variable in binge-like ethanol drinking. A separate cohort of male and female HDID-1 mice ([n = 11-13/sex/route of administration]; 60-87 days of age; selection generations/generations S35.G38 and S36.G38) were tested in a 5-day, 4-hour ethanol DID. A control group received no injection (none) while the other 3 groups were administered sterile saline (10 ml/kg) via 3 routes of administration [i.p., s.c., or per os (by mouth)], 30-minute prior to the start of each DID. BALs were measured at the end of the fifth DID.

Statistics

For the ethanol DID experiments, the principal dependent variables of interest were g/kg ethanol intake during the final drinking session and, when available, BALs. Intake of other fluids during drinking sessions was analyzed as ml/kg. Two-way ANOVAs (sex × drug) were used for these analyses. Where we saw no significant interactions of sex with drug, we collapsed across sex for further analyses. We pursued planned comparisons using the Dunnett's test, to ask whether each dose group differed from the vehicle control group. To ensure that treatment groups did not significantly differ in ethanol intake

prior to drug treatment, Day 1 ethanol intake was analyzed in the manner indicated above and mice were pseudo-randomly reassigned to treatment groups when necessary to remove significant pre-treatment group differences for Experiments 1 to 3. Mice were pseudorandomized into dose groups prior to each week's testing for Experiment 4. No pseudorandomization was used for Experiment 5, as secukinumab was given 3 days prior to Day 1 ethanol access.

Results

Experiment 1: Tacrolimus reduced binge-like ethanol intake and BALs in HDID-1 mice

Tacrolimus, a calcineurin inhibitor, has been shown to reduce limited-access ethanol intake in male C57BL/6J mice.[28] Here, we examined whether tacrolimus would reduce binge-like ethanol intake in male and female HDID-1 mice. Experimental procedures are shown in Figure 1a. Ethanol intake for Day 1 and across each 2-hour time period of Day 2 is shown in Figure 1b. Ethanol intake for the first 2-hours on Day 2 revealed no main effects. After collapsing across sex, there was a main effect of dose ($F_{(3,45)} = 2.89$; P, .05), with the 2 mg/kg dose reducing ethanol intake (Supplemental Figure 2a). Analysis of total (4-hour) Day 2 ethanol intake revealed a significant main effect of drug with tacrolimus reducing ethanol intake ($F_{(3, 41)} = 11.09$, $P < .0001$). All 3 doses of tacrolimus significantly reduced ethanol intake relative to vehicle (Figure 1c, 0.5 mg/kg: $P < .001$; 1 mg/kg: $P < .001$; 2 mg/kg $P < .0001$). Analysis of BALs revealed similar results (a significant main effect of drug emerged ($F_{(3, 41)} = 11.12$, $P < .0001$), whereby, all 3 doses of tacrolimus significantly reduced BALs relative to vehicle treatment (Figure 1d, 0.5 mg/kg: $P < .001$; 1 mg/kg: $P < .001$; 2 mg/kg: $P < .0001$).

We then examined the selectivity of tacrolimus's effects by measuring the effects of the drug on water and saccharin intake. One week after ethanol DID, mice were re-randomized into dose groups and tested in water DID. Analysis of water intake during the first 2-hours on Day 2 revealed a main effect of drug ($F_{(3,36)} = 9.47$; $P < .0001$), but no significant effect of sex or sex × dose interaction, whereby all 3 doses reduced water intake (Supplemental Figure 2b). Analyses during the 4-hour water access period on Day 2 revealed no significant main effects of drug or sex and no sex × drug interaction. To determine whether tacrolimus would reduce intake of a separate rewarding fluid, mice were re-randomized again in the following week and tested in a saccharin DID. Analysis of saccharin intake for the first 2 hours of Day 2 revealed a main effect of drug ($F_{(3,44)} = 5.84$; $P < .01$), but no effect of sex or sex × drug interaction. All 3 doses were shown to reduce 2-hour saccharin intake (Supplemental Figure 2c). Analysis of saccharin intake during the 4-hour access period on Day 2 revealed a significant main effect of drug ($F_{(3, 40)} = 7.86$, $P < .0001$), but no effect of sex or sex × drug interaction. All 3 doses were shown to significantly reduce saccharin intake relative to vehicle (Figure 1f, 0.5 mg/kg: $P = .0004$, 1 mg/kg: $P < .001$, 2 mg/kg: $P < .001$).
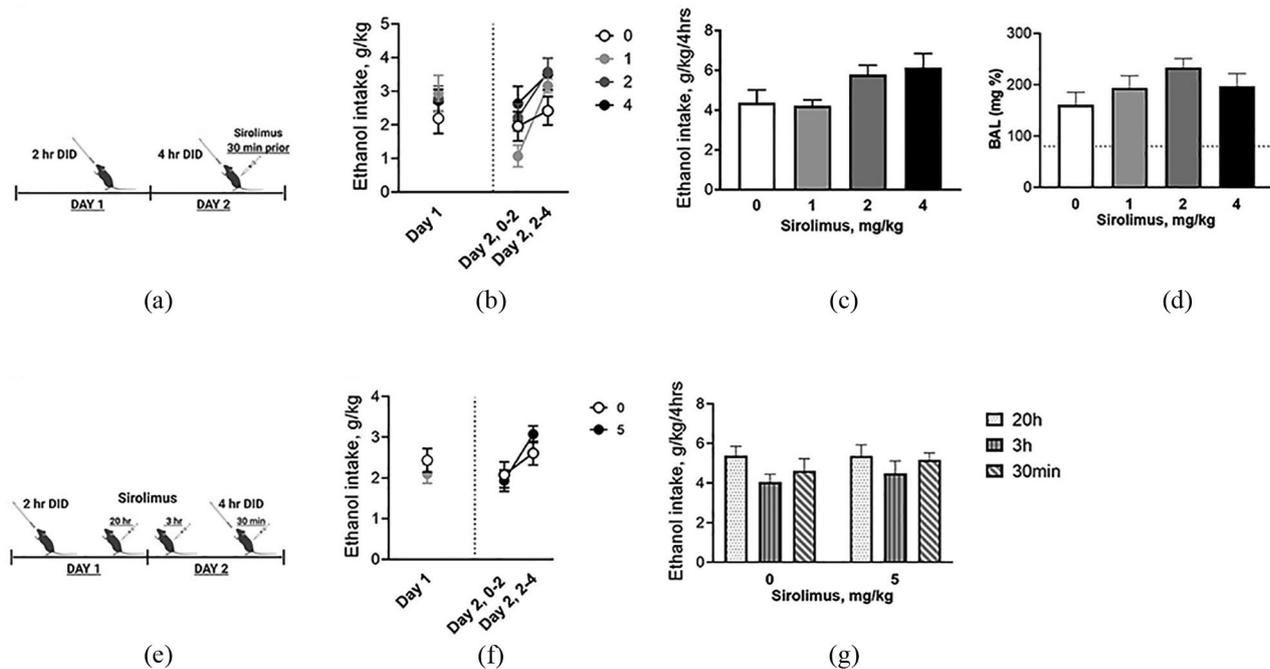
(a)

(b)

(c)

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(e)

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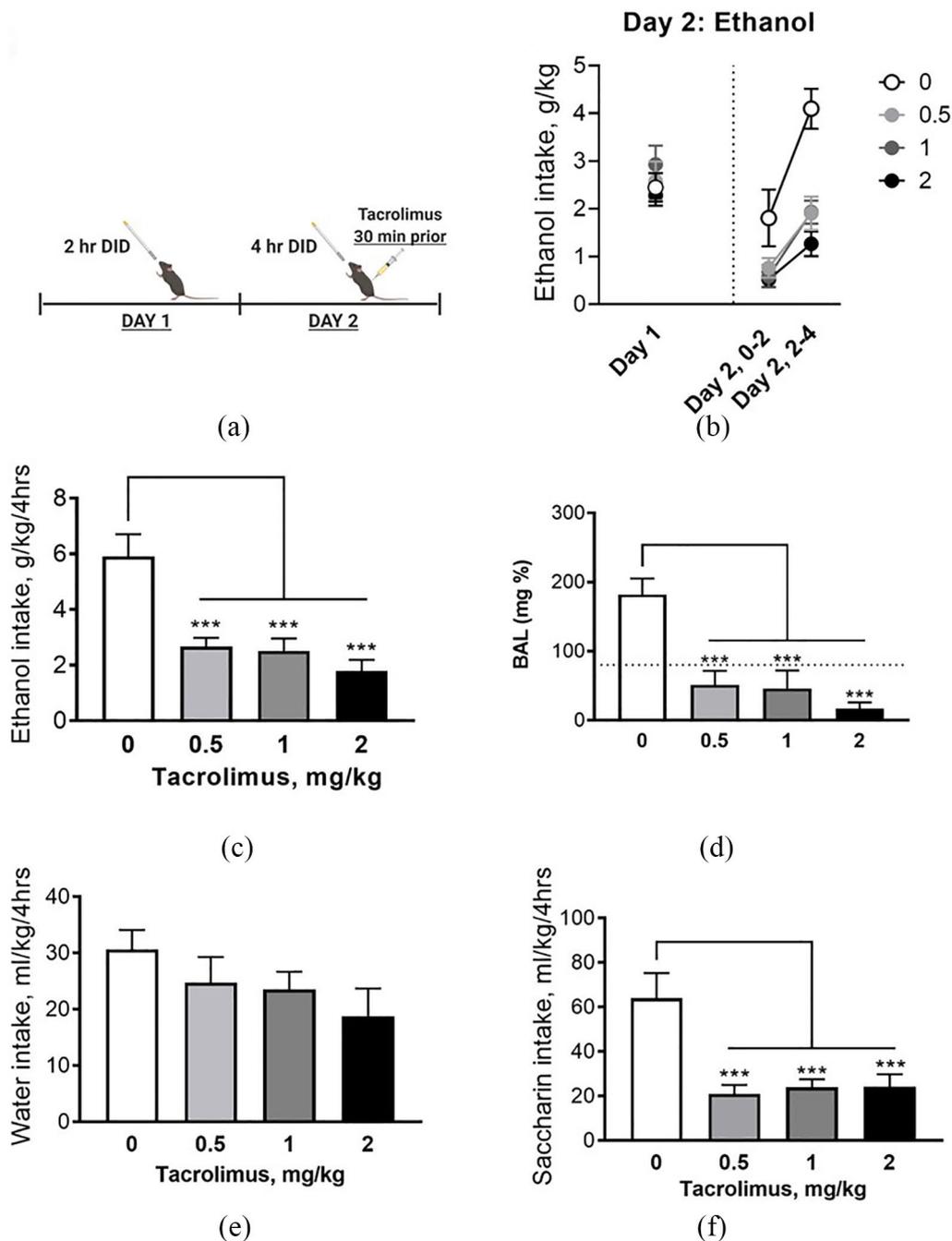
Figure 1. Tacrolimus reduced binge-like ethanol intake and BALs in HDID-1 mice. (a) Experimental timeline of tacrolimus treatment relative to DID. (b) Ethanol intake (g/kg) for Day 1 and across each 2-hour time period of Day 2. (c) Ethanol intake (g/kg/4 hours) on Day 2; main effect of dose ($F(3, 41) = 11.09$; $P < .0001$), but no effect of sex or sex by dose interaction. After collapsing across sex, there was a main effect of dose ($F(3,45) = 12.11$; $P < .0001$), with all three tacrolimus doses reducing ethanol intake. (d) Blood alcohol levels (mg %); main effect of dose ($F(3,41) = 11.12$; $P < .0001$) but no significant effect of sex or sex by dose interaction. Data was then collapsed across sex and a one-way ANOVA was conducted, there was a main effect of dose ($F(3,47) = 12.40$l; $P < .0001$). All three doses of tacrolimus significantly reduced BALs relative to vehicle. (e) Water intake (ml/kg/4 hours); no significant effects (F's(1-3,36) $<$ 2.0; P's $>$ .1). (f) Saccharin intake (ml/kg/4 hours); main effect of dose ($F(3,40) = 7.86$; $P = .0003$), with no effect of sex or a sex by dose interaction. After collapsing across sex (one-way ANOVA; $F(3,44) = 8.40$; $P = .0002$), all three doses significantly reduced saccharin intake relative to vehicle (*$P < .05$, ***$P < .001$, ****$P < .0001$).

Experiment 2: Low-dose sirolimus did not reduce binge-like ethanol intake or BALs in HDID-1 mice

We next tested the effects of sirolimus on binge-like ethanol intake and BALs in HDID-1 mice. Experimental procedures for the first week are shown in Figure 2a. Ethanol intake for Day 1 and across each 2-hour time period of Day 2 is shown in

Figure 2b. Analysis of 2-hour ethanol intake on Day 2 revealed no significant main effects (Supplemental Figure 2a). Analysis of ethanol intake during the 4-hour drinking session on Day 2 revealed a significant main effect of drug ($F(3, 40) = 3.30$, $P = .03$), but a Dunnett's multiple comparison test revealed that no dose significantly differed from vehicle (P's $> .06$).

Figure 2. Low-dose sirolimus did not reduce binge-like ethanol intake or BALs in HDID-1 mice. (a) Experimental timeline of Week 1 sirolimus treatment relative to DID. (b) Ethanol intake (g/kg) for Day 1 and across each 2-hour time period of Day 2. (c) Ethanol intake (g/kg/4 hours); main effect of dose ($F(3,40) = 3.30$; $P = .03$), but no significant effect of sex or sex by dose interaction. Data was collapsed across sex (one-way ANOVA; $F(3,44) = 3.11$; $P = .04$) and a Dunnett's test revealed that no dose differed from vehicle (P's > .07). (d) Blood alcohol levels (mg %); no significant effects were found (F's(1-3,40) < 1.7; P's > .19). Data shown has been collapsed across sex (one-way ANOVA; $F(3,44) = 1.72$; $P = .18$). (e) Experimental timeline of Week 2 sirolimus treatment relative to DID. (f) Ethanol intake (g/kg/2 hours) for Days 1 and 2. (g) Ethanol intake (g/kg/4 hours); no main effect of timing of injection ($F(2,42) = 1.95$; $P = .16$), dose ($F(1,42) = 0.51$; $P = .48$), and no interaction ($F(2,42) = .14$; $P = .87$).

When administered once daily, sirolimus has been shown to reduce ethanol intake,[28] so we next examined whether the lack of effect of sirolimus was due to the timing of administration. Mice were re-randomized into new groups and assigned to receive either sirolimus (5 mg/kg) or vehicle, either 20 hours, 3 hours, or 30 minutes prior to ethanol access. Experimental procedures for this second week of testing are shown in Figure 2e. Ethanol intake for Day 1 and across each 2-hour time period of Day 2 is shown in Figure 2f. Analysis of 2-hour intake on Day 2 showed no main effects (Supplemental Figure 3b). Analysis of 4-hour ethanol intake on Day 2 revealed no significant effect of timing of injection, drug, or interaction.
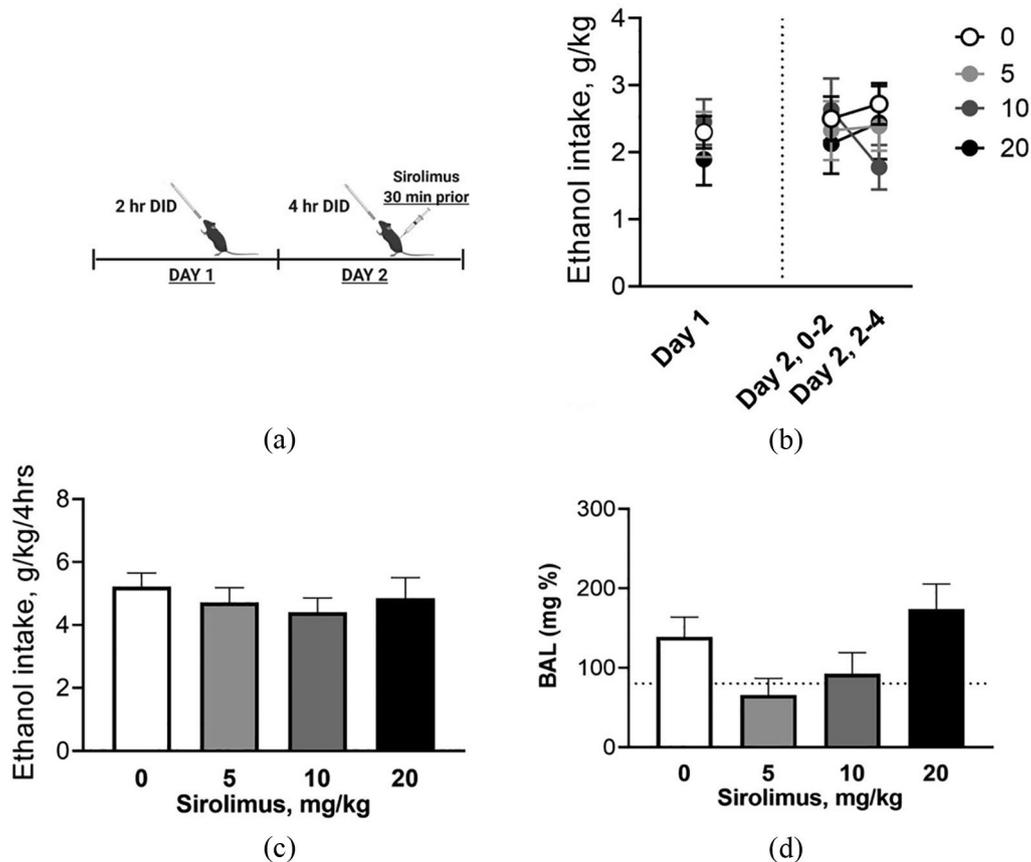
Experiment 3: High-dose sirolimus did not reduce binge-like ethanol intake or BALs in HDID-1 mice

To more thoroughly evaluate its potential to reduce binge-like ethanol intake, sirolimus was tested in a separate cohort of male and female HDID-1 mice in a 2-day DID at higher doses and a two-way ANOVA was conducted on ethanol intake (g/kg). Experimental procedures are shown in Figure 3a. Ethanol intake for Day 1 and across each 2-hour time period of Day 2 is shown in Figure 3b. Analysis of the 2-hour ethanol intake on Day revealed no main effects (Supplemental Figure 4). An analysis of ethanol intake during the 4-hour drinking session on Day 2 revealed a main effect of sex ($F(1,48) = 4.17$; $P = .047$),

with no significant effect of dose or sex × dose interaction. Data shown has been shown collapsed across sex (Figure 3c). Similarly, a two-way ANOVA was conducted on BALs and no significant effects were found. Data shown has been collapsed across sex (Figure 3d).

Experiment 4: PEA did not reduce binge-like ethanol intake or BALs in HDID-1 mice

The effects of PEA administration on HDID-1 limited access ethanol intake was tested in 3 sequential weeks as shown in Figure 4. Week 1 was a 2-day DID, in which PEA (0, 75, 150 mg/kg) was administered 1 hour prior to Day 2 ethanol access (Figure 4a). Week 2 was a 2-day DID, in which PEA (0, 75, 150 mg/kg) was administered 16 hours prior to Day 2 ethanol access (Figure 4b). Week 3 was a 4-day DID, in which PEA (0, 150, 225 mg/kg) was administered daily, 16 hours prior to Days 2 to 4 ethanol access (Figure 4c). In week 1, there was no significant main effects during the first 2-hours on Day 2 (Supplemental Figure 5a). Analysis of the 4-hour intake on Day 2 revealed a main effect of sex, with no significant effect of dose or sex × dose interaction. Data shown has been collapsed across sex (Figure 4a). Similarly, there were no main effects on BALs in Week 1. In Week 2, there were no significant effects found for Day 2 for either 2-hour (Supplemental Figure 5b) or 4-hour ethanol intake or BALs (Figure 4b). In Week 3, we found no significant effects on Day

Figure 3. High-dose sirolimus did not reduce binge-like ethanol intake or BALs in HDID-1 mice. (a) Experimental timeline of sirolimus treatment relative to DID. (b) Ethanol intake (g/kg) for Day 1 and across each 2-hour time period of Day 2. (c) Ethanol intake (g/kg/4 hours); main effect of sex ($F(1,48) = 4.17$; $P = .047$), but no significant effect of dose or sex by dose interaction. Data shown has been collapsed across sex (one-way ANOVA; $F(3,52) = 0.53$; $P = .67$). (d) Blood alcohol levels (mg %); no significant effects were found (F's(1-3,48) < 1.6; P's $> .2$). Data shown has been collapsed across sex (one-way ANOVA; $F(3,52) = 0.80$; $P = .50$).

4 for 2-hour ethanol intake (Supplemental Figure 5c), 4-hour intake, or BALs (Figure 4c).

Experiment 5: Secukinumab did not reduce binge-like ethanol intake in HDID-1 mice

Lastly, the effect of secukinumab on binge-like ethanol intake was tested in male and female HDID-1 mice, with injection of drug occurring 3 days prior to Day 1 ethanol access in a 2-day DID (Figure 5a) Mice were randomly assigned to 1 of 4 treatment groups (0, 5, 30, or 60 mg/kg) prior to ethanol exposure. Ethanol intake for Day 1 and across each 2-hour time period of Day 2 is shown in Figure 5b. There was no significant main effects on 2-hour ethanol intake on Day 2 (Supplemental Figure 6) Analysis of ethanol intake during the 4-hour drinking session on Day 2 revealed a main effect of sex ($F(1,55) = 4.25$; $P = .04$), but no effect of drug or drug $\times$ sex interaction. Data shown has been collapsed across sex (Figure 5c). BALs were not analyzed in this group of mice.

Discussion

Repurposing current FDA-approved pharmacotherapies is an efficient and expedited means of screening treatment options

for AUD. In support of this preclinical framework, we report the findings of testing 4 immune-related compounds tested in a genetic-risk model of high binge-like ethanol drinking (HDID-1). The presently used HDID-1 mice represent a unique, genetic risk model in which to screen the efficacy of promising neuroimmune and neuroinflammatory compounds. For the calcineurin inhibitor, tacrolimus, we were informed by the work of Beresford et al,[28] whereby 2.5 mg/kg of tacrolimus decreased 10% ethanol preference and intake in C75BL/6J male mice. Neasta et al[31] reported that systemic administration of the mTOR inhibitor, sirolimus (rapamycin), significantly reduced conditioned place preference to ethanol in DBA/2J male mice and reduced 20% ethanol intake in male C75BL/6J mice when given every other day, for 4 hours (beginning 2 hours into the dark cycle). There were no preclinical data on ethanol drinking available for comparison for PEA treatment. Although an anti-IL17A antibody has been shown to decrease intake in dependent mice, secukinumab itself has not been tested in preclinical alcohol drinking.[39] A key strength of our work is in testing potentially repurposeable compounds in genetically unique HDID mice that reach intoxicating BALs and show behavioral signs of intoxications after a single limited access
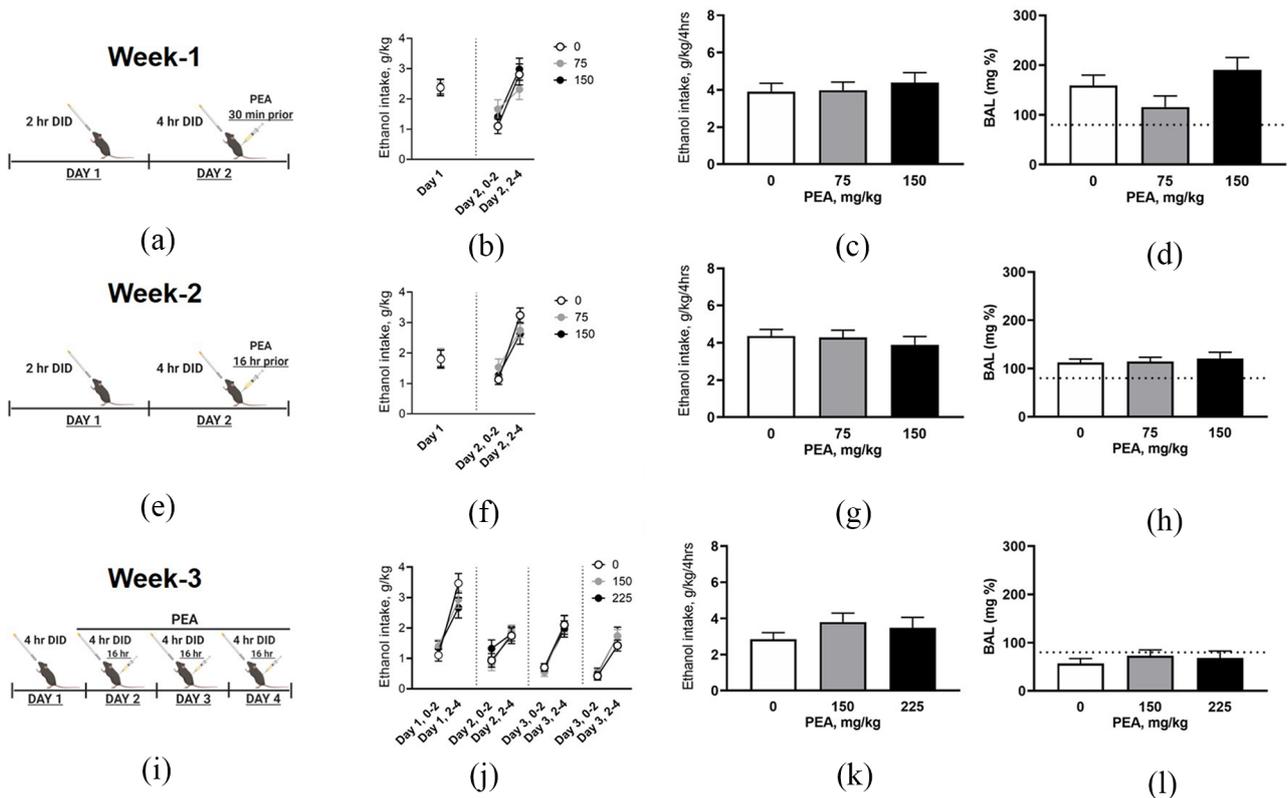
Figure 4. PEA did not reduce binge-like ethanol intake or BALs in HDID-1 mice. (a, e, and i) Experimental timelines of PEA treatment relative to DID for Weeks 1 to 3. (b) Ethanol intake (g/kg/2 hours) for Days 1 and 2 for Week 1. (c) Week 1: a two way-ANOVA (dose × sex) revealed no significant effects for 4-hour ethanol intake on Day 2 (F's(1-2,66) < 1.2; P's > .30) or (d) BALs (F's(1-2,52) < 3.4; P's > .07). Data shown collapsed across sex. (f) Ethanol intake (g/kg/2 hours) for Days 1 and 2 for Week 2. (g) Week 2: a two-way ANOVA (dose × sex) revealed no significant effects for 4-hour ethanol intake on Day 2 (F's(1-2,65) < 1.4; P's > .25) or (h) BALs (F's(1-2,62) < 2.3; P's > .11). Data shown collapsed across sex. (j) Ethanol intake (g/kg/2 hours) for Days 1 to 4 for Week 2. (k) Week 3: a two-way ANOVA (dose × sex) revealed no significant effects for 4-hour ethanol intake on Day 4 (F's(1-2,65) < 1.9; P's > .17) or (l) BALs (F's(1-2,58) < 0.42; P's < .66).
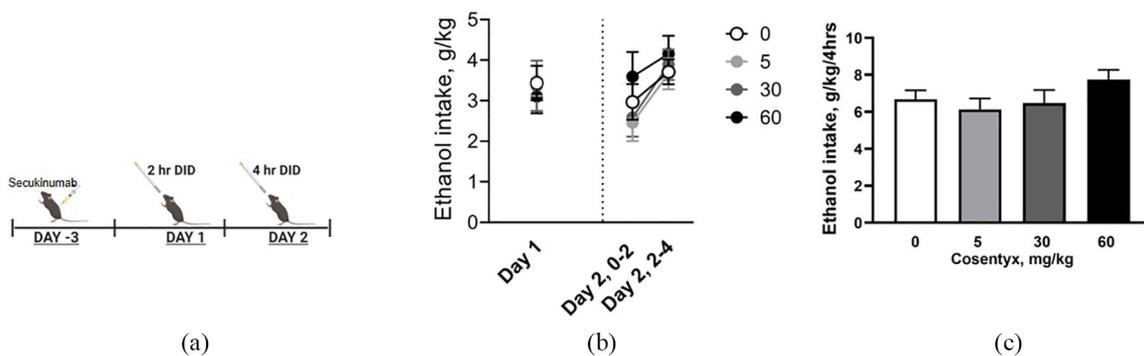
Figure 5. Secukinumab did not reduce binge-like ethanol intake in HDID-1 mice. (a) Experimental timeline of secukinumab treatment relative to DID. (b) Ethanol intake (g/kg/2 hours) for Days 1 and 2. (c) Ethanol intake (g/kg/4 hours); a main effect of sex (F(1,55) = 4.25; P = .04), but no effect of drug (F(3,55) = 1.50; P = .22) or drug by sex interaction (F(3,55) = 0.14; P = .94). Data shown collapsed across sex.

period. Importantly, this model captures the complex, polygenic nature of aberrant drinking patterns.[23,40]

Of the compounds tested, the only significant findings were that tacrolimus reduced ethanol intake and reduced BALs below the level of intoxication in both male and female HDID-1 mice at all 3 doses tested (0.5, 1, and 2 mg/kg). Previous work suggests that inhibiting calcineurin signaling may be an effective molecular means of reducing ethanol intake and preference.[28,45] Circumstantial evidence for the potential of immunosuppressants to reduce ethanol use stems from the high rates of abstinence seen in alcohol-dependent individuals after liver transplantation, many of whom receive calcineurin-based immunosuppressants, such as cyclosporin or tacrolimus.[46-48] Many immunosuppressants are known to stimulate central nervous system molecular signaling, and thereby neural networks (including dopaminergic and serotonergic brain

regions).[49-51] Therefore, it is possible that inhibiting calcineurin may play a role in promoting abstinence in transplant patients and may have therapeutic potential in reducing ethanol intake and AUD.

Tacrolimus significantly reduced 4-hour saccharin intake at all 3 doses, while having no effect on water. However, all 3 doses of tacrolimus reduced water intake at 2-hours (Supplemental Figure 2b), suggesting a potential effect on general thirst. Tacrolimus has been shown to reduce locomotor activity and causes depressive-like behaviors in diabetic rats, with no effect in controls.[52] Although tacrolimus is currently FDA approved, it remains important to test the effects of tacrolimus on locomotor activity in HDID-1 mice. It is difficult to ascertain whether these mice consume less saccharin due to tacrolimus decreasing its rewarding effects, increasing the sensitivity to reward, or because of an increased state of malaise. Alternatively, the reduction in both ethanol and saccharin may result from tacrolimus altering taste perception. In a separate group of male and female HDID-1 mice, tacrolimus was shown to reduce quinine intake at both 2 and 4 hours (Supplemental Figure 2d and e), suggesting tacrolimus may alter taste perception. The effects of tacrolimus on saccharin intake has not been tested in male C57BL/6J mice[28]; therefore, it remains to be determined whether this reduction is specific to HDID-1 mice. In all, the ability of tacrolimus to reduce ethanol drinking in the context of a polygenic, high risk model of binge-like drinking highlights its therapeutic potential as a treatment for AUD. To further test its clinical relevancy, future research efforts would benefit from screening tacrolimus and related compounds in additional strains, species, and drinking paradigms.

Sirolimus was ineffective at reducing either binge-like ethanol intake or BALs in male or female HDID-1 mice, while it is established to reduce ethanol intake in C57BL/6J mice.[31,32] The main molecular target of sirolimus (rapamycin) is mTOR, whereby a complex formed with FK-binding protein 12 (FKBP12) directly binds to and inhibits the activity of mTOR complex 1 (mTORC1).[27] mTOR signaling in the NAc and other reward-related brain regions is thought to play a role in the molecular, neuronal and behavioral adaptations induced by ethanol exposure.[31,32] When given intermittent access to 20% ethanol in a 2 bottle-choice task (when water is present continually), C57BL/6J mice show regional increases in mTOR and PI3K activity in reward related brain regions.[32] In mice that received fluid restriction prior to intermittent access to ethanol, a reduction in NAc mTOR and PI3K was seen in male, but not female, C57BL/6J mice.[53] The above evidence suggests that the role of mTOR appears in ethanol drinking to be dependent on drinking paradigm, sex, and genetic strain. Therefore, our findings in HDID-1 mice highlight the complexity of mTOR in preclinical alcohol research and the need for future research to better understand how sirolimus may be clinically beneficial for AUDs.

Of the present drugs addressed, sirolimus is supported by the most preclinically research for alcohol related behaviors and is the most characterized in terms of underlying mechanism (mTOR inhibition). Therefore, our negative findings in HDID-1 mice should be considered a contribution to the developing array of procedural and genetic conditions under which sirolimus has been tested. In a comprehensive study, Neasta et al[31] reported that i.p. injections of rapamycin reduced conditioned place preference for ethanol in DBA/2J male mice and reduced 4-hour, limited access intake of 20% ethanol in C57BL/6J mice, when presented every other day. In the same study, intra-nucleus accumbens infusion of rapamycin reduced ethanol intake in male Long Evans rats in a continual access paradigm. Similarly, Beckley et al[54] found that systemic injections of 10 mg/kg of sirolimus reduced 24-hour ethanol (20%) intake and preference in C57BL/6J mice. In contrast, systemic injections of sirolimus in the 1.0 to 5.0 mg/kg range was ineffective at reducing ethanol preference or limited access intake of 10% ethanol in male C57BL/6J mice.[28] In the present study, a comparable range of sirolimus injections (1.0-5.0 mg/kg) did not reduce binge-like 20% ethanol intake or BALs in male or female HDID-1 mice, nor did treatment with a higher dose (5-20 mg/kg) of sirolimus.

The lack of effect of sirolimus to reduce ethanol intake in these experiments could be due to the unique genetic background of the HDID-1 mice, or the procedural differences between ours and the above studies. Neasta et al[31] reported that sirolimus reduced binge-like intake of 20% ethanol when presented every other day for 4 hours (beginning 2 hours into the dark cycle). However, sirolimus did not reduce limited access 10% ethanol intake in male C57BL/6J mice when presented daily for 2 hours (2 hours into the dark cycle).[28] In the experiments described here, sirolimus failed to reduce binge-like intake of 20% ethanol in the HDID-1 mice when administered prior to a single, 4-hour DID session (3 hours into the dark cycle). Therefore, the lack of effect of sirolimus to reduce HDID-1 binge-like ethanol intake could be the result of only receiving a single injection and that only 1 day of drinking was evaluated. In all, these findings emphasize the multivariate nature of behavioral pharmacology and the need for rigorous and systematic testing of promising compounds.

To the best of our knowledge, the present findings are the first to address the effects of PEA in a preclinical model of binge drinking. PEA has gained therapeutic interest in treating binge drinking and AUD due to its anti-inflammatory and neuroprotective effects.[55] PEA is produced in response to CNS injury and is thought to be cytoprotective, acting through PPARα and TNFα-related mechanisms.[56,57] Further, in vitro studies have shown that PEA blunts neuronal cell loss.[58] Preclinical testing of PEA in a mouse model of neuropathic pain was shown to be anti-hyperalgesic.[59] Therefore, it follows that PEA may protect against alcohol-induced neuroinflammation and alleviate pain related symptoms of AUD. OEA, a

related, endogenously expressed compound, has been shown to reduce binge-like ethanol intake and other behavioral measurements associated with aberrant drinking in male rats.[34] OEA treatment has been shown to reduce impulsivity scores in both young and adult heavy drinkers, suggesting N-acyletethanolamines's may have promising efficacy in reducing symptoms of AUD.[60] In a recent study, Cristiano et al[61] reported that PEA improved central and peripheral inflammatory states in a mouse model of autistic-like behavior, suggesting that systemic PEA treatment can also potentially reduce neuroinflammatory processes.

Although PEA was presently shown to be ineffective at reducing binge-like ethanol drinking in HDID-1 mice, it is possible that PEA may have beneficial effects in blunting the neurodegeneration caused by chronic alcohol abuse. In support of this, 60 days of pretreatment with micronized PEA ameliorated behavioral deficits following an induced Parkinson's Disease model in aged mice through anti-inflammatory dependent mechanism.[62] Although PEA proved ineffective at reducing binge-like ethanol drinking in HDID-1 mice, its key role in reducing neuroinflammation may prove efficacious in preventing the neurodegeneration caused by chronic alcohol abuse. PEA's ability to potentially ameliorate alcohol induced neurodegeneration could be used in concert with established pharmacotherapies known to reduce ethanol intake, such as naltrexone.[10] In this way, PEA may enhance the effects of such compounds to reduce intake, as well as prove beneficial in reducing potential long-term effects on neurodegeneration. Future research efforts would therefore profit from testing PEA in the context of more chronic drinking and treatment paradigms.

Another investigational PPARα agonist, fenofibrate (25-150 mg/kg) reduced ethanol intake and ethanol induced conditioned place preference when orally administered to rats selectively bred for high ethanol intake (UChB).[63] When administered i.p., fenofibrate (150 mg/kg) reduced behavioral indices of ethanol seeking and withdrawal severity in C57BL/6J mice.[64] Another PPARα agonist, tesaglitizar (1.5 mg/kg), similarly reduced ethanol intake in a continual access, two-bottle choice (2BC) paradigm in male and female C57BL/6J mice.[64] When tested in HDID-1 mice, however, neither fenofibrate (150 mg/kg) nor tezaglitizar (1.5 mg/kg) reduced 2BC preference drinking or limited-access DID ethanol intake.[23] In fact, the highest dose of fenofibrate tested slightly increased DID intake, with no effect on BALs.[23] The above difference in ethanol drinking outcomes following PPARα-related treatments again highlights the importance of testing promising pharmacotherapies in several preclinical settings. Although PPARα agonists such as fenofibrate and tezaglitizar show some efficacy in reducing ethanol drinking in some strains and species, they may not be clinically feasible due to concerns over liver and kidney toxicity. The relative safety and expected tolerance of PEA makes its activity as a PPARα agonist a much safer and practical therapeutic option to treat AUD.

Secukinumab is a monoclonal antibody targeting IL-17A, a proinflammatory cytokine, and is currently FDA approved to treat plaque psoriasis, psoriatic arthritis, and ankylosing spondylitis. IL-17 and its primary source, T helper 17 cells (TH17 cells) have been identified as important contributors to the pathogenesis of several chronic inflammatory and autoimmune diseases.[65] The ability of secukinumab to treat and target inflammatory processes, as well as its relative safety and current FDA approval, make it a promising candidate to reduce harmful drinking.[39] This is the first known study to address the effects of secukinumab on binge-like ethanol drinking behavior, for which no effect in male or female HDID-1 mice was seen. IL-17A remains a viable therapeutic avenue in that elevated levels of circulating and hepatic levels are well documented in patients with alcohol-induced liver fibrosis, and IL-17A has more recently been connected with alcohol dependence in humans.[66,67] Moreover, a recent study Xu et al[39] links increases in alcohol-induced liver damage with increases in circulating IL-17A levels in humans. While we tested secukinumab in an acute, limited access paradigm because of IL-17's role in early stages of inflammation, it is reasonable to posit that secukinumab may be similarly beneficial at mitigating the inflammatory effects of alcohol in individuals with chronic alcohol exposure. In support, Xu et al[39] found that systemic inhibition of IL-17A, through either an antibody targeting IL-17A or a pharmacological inhibitor of the receptor-related orphan receptor (RORγt), which regulates the development of TH17 cells, reduced the escalated voluntary 2BC 10% ethanol of ethanol-dependent C57BL/6J male mice. It would be of immense benefit to consider the effects of targeting IL-17A following chronic binge-like ethanol drinking in male and female mice, and particularly HDID-1 mice. As mentioned earlier, targeting other proinflammatory cytokines alongside IL-17 may greatly increase the efficacy of treatment, which is something to be considered for future investigations.

Limitations

The lack of effect of sirolimus, PEA, or secukinumab on DID ethanol intake in the present study is complicated by a number of variables, namely the route of administration, the pharmacodynamics of each compound, and the heterogeneity of HDID-1 mice. To address the concern over the differences in route of administration in the present studies, a separate cohort of age matched male and female HDID-1 mice were tested for the effects of i.p., s.c. and o.p. saline administration on 20% ethanol intake (4-hour DID, for 5 days) and subsequent BALs (Supplemental Figure 1c; of note, the recorded BALs were notably low for this cohort). No effect of route of administration or sex was found, suggesting that the decision to administer tacrolimus and sirolimus i.p. and secukinumab and PEA s.c. was likely not a major variable in our observed outcomes. In support of PEA being given subcutaneously, when PEA was administered orally (100 mg/kg) to male Wistar rats, plasma

levels were reported to peak at ~15 minutes and return to baseline levels within an hour.[36] When given as a subcutaneous depot (10 mg/kg) in DBA/2 mice, however, PEA remained increased in serum, heart and brain tissue up to 48 hours after treatment.[41] The extent to which any of these investigational compounds directly influence neuroimmune or neuroinflammatory processes was not explicitly evaluated. There is known cross-talk between peripheral and central immune and inflammatory signaling, suggesting that reductions in peripheral inflammation and immune signaling may have indirect benefits at the level of the brain.[68,69] For example, while systemic sirolimus treatment was reported to not reach detectable levels at the level of the brain,[28] comparable sirolimus injections were shown to reduce ethanol intake in C57BL/6J mice.[31]

Summary

In all, we feel the results of these studies emphasize the complex and nuanced role of immune signaling in alcohol drinking. Sirolimus, a widely prescribed immunosuppressant, was shown to reduce C57BL/6J ethanol intake, while it did not reduce HDID-1 binge-like ethanol drinking. As presently shown, tacrolimus was successful at reducing HDID-1 intake, yet other previously tested compounds were not. This again highlights that perhaps some immune pathways are more critical to alcohol drinking than others, at least in the polygenic context of HDID-1 mice. Further, HDID-1 mice may capture a genetic basis for certain immune pathways having a more critical role in drinking.

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Author Contributions

All authors had continual access to the presented data and take responsibility for its integrity and accuracy. Conceptualization, K.G., A.S., P.M., and A.O.; Methodology, K.G., A.S., P.M., and A.O.; Investigation, K.G. and A.S.; Formal analysis, K.G., A.S., P.M., and A.O.; Resources, J.C. and A.O.; Writing-Original Draft, K.G., A.S., P.M., and A.O.; Writing-Review and Editing, K.G., A.S., P.M., B.M., Y.B., J.C., and A.O.; Visualizations, K.G. and A.S.; Supervision, J.C. and A.O; Funding Acquisition, B.M., Y.B., J.C., and A.O.

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Supplemental material

Supplemental material for this article is available online.

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