SUPPLEMENTARY MATERIAL

Glucocorticoid Receptor Antagonism Decreases Alcohol Seeking in Alcohol Dependence

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Supplementary Materials and Methods: Preclinical studies

Animals

Adult male Wistar rats (Charles River, Raleigh, NC, USA), weighing 225-275 g at the beginning of the experiments, were housed in groups of 2-3 per cage in a temperature-controlled (22ºC) vivarium on a 12 h/12 h light/dark cycle (lights on at 8:00 AM), with *ad libitum* access to food and water except during behavioral testing. All of the behavioral tests were conducted during the dark phase of the light/dark cycle. All of the procedures adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute.

Operant self-administration

Self-administration sessions were conducted in standard operant conditioning chambers (Med Associates, St. Albans, VT, USA). The rats were trained to self-administer alcohol using a fixed-ratio 1 (FR1) schedule of reinforcement (i.e., each operant response was reinforced with 0.1 ml of solution) as previously reported (1). First, the rats were given free-choice access to alcohol (10% w/v) and water for 1 day in their home cages to habituate them to the taste of alcohol. Second, the rats were subjected to an overnight session in the operant chambers with access to one lever (right lever) that delivered water on an FR1 schedule. Food was available *ad libitum* during this training. Third, after 1 day off, the rats were subjected to a 2-h session on an FR1 schedule for 1 day and a 1-h session on an FR1 schedule on the next day, with one lever delivering alcohol (right lever). All of the subsequent sessions lasted 30 min, and two levers

were available (left lever: water; right lever: alcohol). Once stable levels of intake were reached, the animals were split into two groups matched by average of lever presses in the last three sessions: vapor-exposed (dependent, [dep]) and air-exposed (nondependent, [nondep]). All rats that did not press more than five times in the last three 30-min session of the training were excluded because they did not reach the learning criterion previously established.

Alcohol vapor chambers

The rats were made dependent by chronic, intermittent exposure to alcohol vapors as previously described (1). They underwent cycles of 14 h on (blood alcohol levels ranged between 150 and 250 mg%) and 10 h off, during which behavioral testing occurred (i.e., 6-8 h after vapor was turned off when brain and blood alcohol levels are negligible [2]). Nondependent rats were not exposed to alcohol vapor but were concomitantly tested with dependent rats. This model of alcohol dependence has been shown to produce compulsive-like alcohol drinking as indexed by increased breakpoints in a progressive-ratio schedule of reinforcement and resistance to aversive stimulus (quinine)-induced reduction of alcohol drinking (1).

Systemic drug treatment

Mifepristone was purchased from Sigma-Aldrich (St. Louis, MO, USA). CORT113176 was provided by Corcept Inc. (Menlo Park, CA, USA). Different cohorts of dependent and nondependent rats were intraperitoneally injected with mifepristone (0, 30, and 60 mg/kg) or CORT113176 (0, 10, 30, and 100 mg/kg) 90 min prior to the self-administration sessions. All of the drugs were dissolved in 10% dimethylformamide (Sigma-Aldrich, St. Louis, MO, USA)/10%

Emulphor (Rhodia, New Brunswick, NJ, USA) and diluted in saline. The volume of the injections was 3 ml/kg. The doses of each compound were administered following a within-subjects Latin-Square design.

Mifepristone injection into the central nucleus of the amygdala

Separate groups of dependent and nondependent rats were implanted with bilateral guide cannula aimed at the CeA (anterior/posterior, -2.6 mm; medial/lateral, 4.2 mm; dorsal/ventral, 6.6 mm from skull) and bilaterally infused with mifepristone (0, 10, and 30 μg/side) dissolved in 100% dimethyl sulfoxide (DMSO) 90 min prior to the operant tests during acute withdrawal in a within-subjects Latin-square design. Intra-CeA infusions of mifepristone have been shown to not cause cell death (3). Infusions (0.25 μl/side) occurred over 2 min with an additional 1 min period to allow for diffusion. The experimenter was not blind to the treatments in the injection procedures.

Saccharin self-administration

Another group of nondependent rats was trained to lever press for saccharin self-administration under an FR1 schedule using identical conditions as those described for alcohol self-administration, with the exception that a 0.004% (w/v) saccharin solution was used. Mifepristone (30 mg/kg) and CORT113176 (30 mg/kg), were injected intraperitoneally 90 min prior to saccharin self-administration in a within-subjects Latin-square design.

Western blot analysis

The quantitative analysis of total protein and phosphorylated protein densities was conducted as previously described (4). Brains were collected from groups of dependent and nondependent rats during acute withdrawal to match the time point for behavioral testing. The brains were snap-frozen and stored at -80ºC until processing. Tissue samples from the CeA and BLA were dissected on a cryostat and homogenized by sonication in lysis buffer (320 mM sucrose, 5 mM HEPES, 1 mM ethylene glycol tetraacetic acid, 1 mM ethylene diamine tetraacetic acid, 1% sodium dodecyl sulfate [SDS], with Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktails II and III, diluted 1:100; Sigma, St. Louis, MO), heated at 100°C for 5 min, and stored at -80°C until the determination of protein concentration using a detergent-compatible Lowry method (Bio-Rad, Hercules, CA). Samples of protein (15 μg) were subjected to SDS-polyacrylamide gel electrophoresis on 10% acrylamide gels using a Tris/Glycine/SDS buffer system (Bio-Rad, Hercules, CA), followed by electrophoretic transfer to polyvinylidene difluoride membranes (GE Healthcare, Pittsburg, PA). Membranes were blocked overnight in 5% nonfat milk at 4°C and then incubated in primary antibody to recognize the Ser^{211} (human)/Ser^{232} (rat) phosphorylated form of GR (1:1000, 5% nonfat milk; Cell Signaling, Danvers, MA; Antibody #4161). Membranes were washed and labeled with species-specific peroxidase-conjugated secondary antibody (1:10,000; Bio-Rad, Hercules, CA) for 1 h at room temperature. Following chemiluminescent detection (SuperSignal West Pico, Thermo Scientific, Waltham, MA), the blots were stripped for 20 min at room temperature (Restore, Thermo Scientific, Waltham, MA) and re-probed for total protein levels of GRs (1:2000; Thermo Scientific, Waltham, MA; Clone BuGR2; Product #MA1-510). Immunoreactivity was quantified by densitometry (ImageJ 1.45S, NIH) under linear exposure conditions. Density values are expressed as a percentage of the mean of control values, and individual phosphoprotein levels

were normalized to individual total protein levels to generate pGR/GR ratios for statistical comparisons.

Statistical analysis

The number of animals necessary for each study was calculated using an *a priori* power analysis based on effect sizes (Cohen's d) observed in our previous published work (1). The range of expected effect sizes varied between d=1.7 and d=2 depending on the experimental paradigm. We have determined that the sample sizes required to yield sufficient power (80%) to detect group differences at the significance level of $p = 0.05$ were $n = 5$ (d=2) and $n = 7$ (d=1.7). Note that while all the groups in this report reached the minimum required sample size for a specific d value, some groups exhibit higher sample size (up to $n = 11$) as initial sample size was higher to take into account possible loss in sample size due to failure to self-administer alcohol, and computer failure during testing.

The data are expressed as mean and SEM. Shapiro-Wilk tests indicated that all data sets were normally distributed except for water self-administration data shown in Fig S1C. This data set was log transformed to normalize the distribution. The data were then analyzed using repeated-measures analysis of variance (ANOVA), with dose (or drug) as the within-subjects factor and group (dependent *vs*. nondependent) as the between-subjects factor. When appropriate, *post hoc* comparisons were performed using Duncan's multiple comparison test. Western blot data were analyzed using Student's *t*-test. The accepted level of significance for all of the tests was $p < 0.05$.

Results

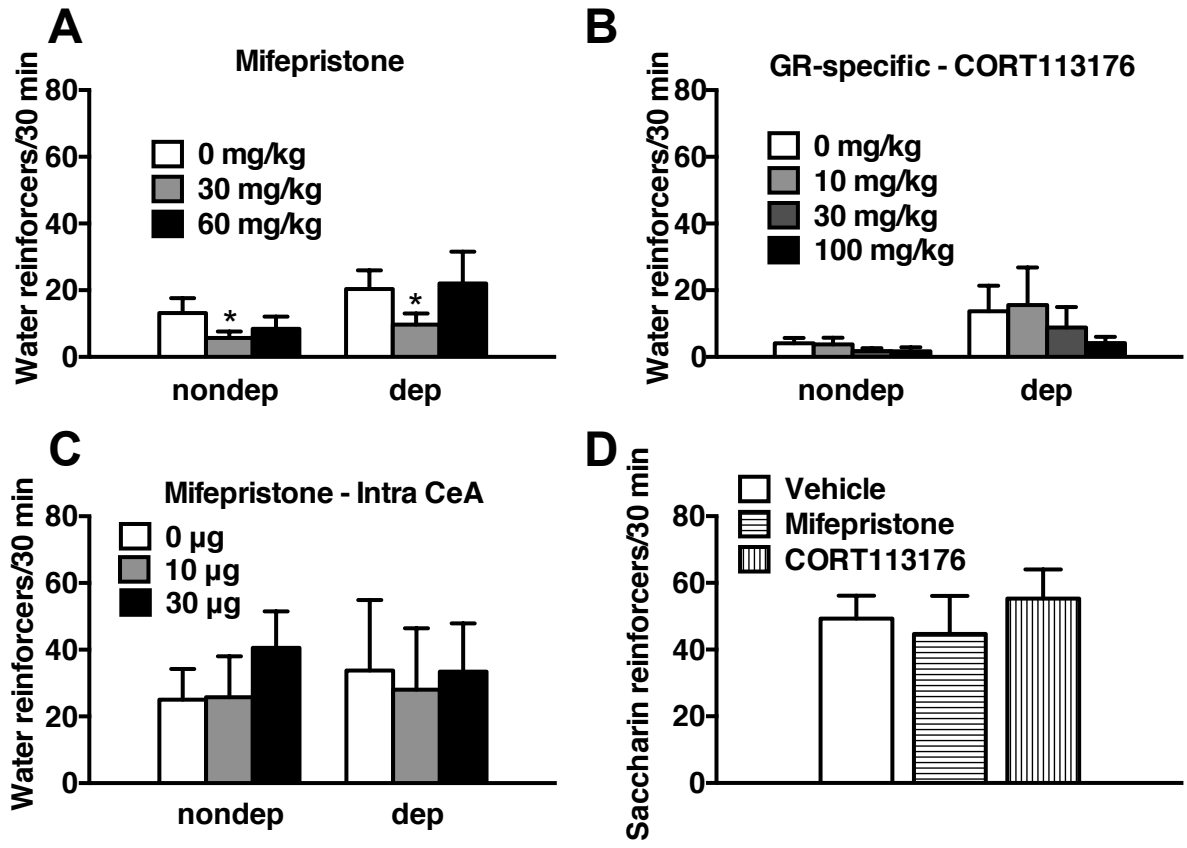


Fig. S1. Glucocorticoid receptor antagonism non-specifically alters the self-administration of water and does not change the self-administration of saccharin-sweetened water. **(A)** Mifepristone injected systemically (n = 11/group) significantly decreased water self-administration in both groups (dose effect: $F_{2,40} = 4.181$, $p = 0.0224$), with mifepristone at 30 mg/kg decreasing water self-administration compared with 0 mg/kg ($p = 0.0134$) and 60 mg/kg ($p = 0.0305$) regardless of group (*indicates significant differences from 0 mg/kg). No dose × group interaction ($F_{2,40} = 1.033$, $p = 0.3654$) or group effect ($F_{1,20} = 1.625$, $p = 0.2170$) were detected. **(B)** CORT113176 (n = 7 for dependent and n = 9 for nondependent) injected systemically did not significantly alter water self-administration in dependent or nondependent rats (dose effect: $F_{3,42} = 2.502$, $p = 0.0724$; dose × group interaction: $F_{3,42} = 1.000$, $p = 0.4024$) and no group ($F_{1,14} = 1.771$, $p = 0.2045$) differences were found for water self-administration. **(C)** Mifepristone injected directly into the central nucleus of the amygdala (CeA; n = 5/group) did not significantly alter water self-administration (dose effect: $F_{2,16} = 1.072$, $p = 0.3657$). No dose × group interaction ($F_{2,16} = 0.626$, $p = 0.5472$) or group effect ($F_{1,8} = 0.004$, $p = 0.9499$) were detected. The procedures associated with intra-CeA injections appear to increase water responding in nondependent rats. **(D)** The self-administration of saccharin-sweetened water (i.e., a nondrug reward) was not altered by systemic GR antagonist treatment in nondependent rats (treatment effect: $F_{2,12} = 0.831$, $p = 0.4593$). The data are expressed as the mean and standard error of the mean of the number of lever presses in 30 min. Every lever press resulted in the delivery of 0.1 ml of water or saccharin solution (0.004%, w/v).

Table S1. *In vitro* GR-related assays that compare binding affinity and functional activity for mifepristone and CORT113176.

Measure	Mifepristone	CORT113176
GR binding Ki (nM)	0.09	0.28
PR binding Ki (nM)	1	inactive
MR binding Ki (nM)	5495	inactive
GR reporter gene Ki (nM)	0.9	4.6
MR reporter gene Ki (nM)	not available	inactive
HepG2 TAT Ki (nM)	3	12
Rat H4 TAT Ki (nM) (max)	2.2 (100%)	4.2 (100%)
*Rat H4 TAT EC_{50} (nM) (max)	> 1,000 (54%)	No agonism
A549 IL-1 induced IL-6 Ki (nM) (max)	13 (69%)	31 (53%)
*A549 IL-1B induced IL-6 EC_{50} (nM) (max)	2.2 (51%)	390 (32%)
PBMC LPS induced TNF Ki (nM)	5.4	38

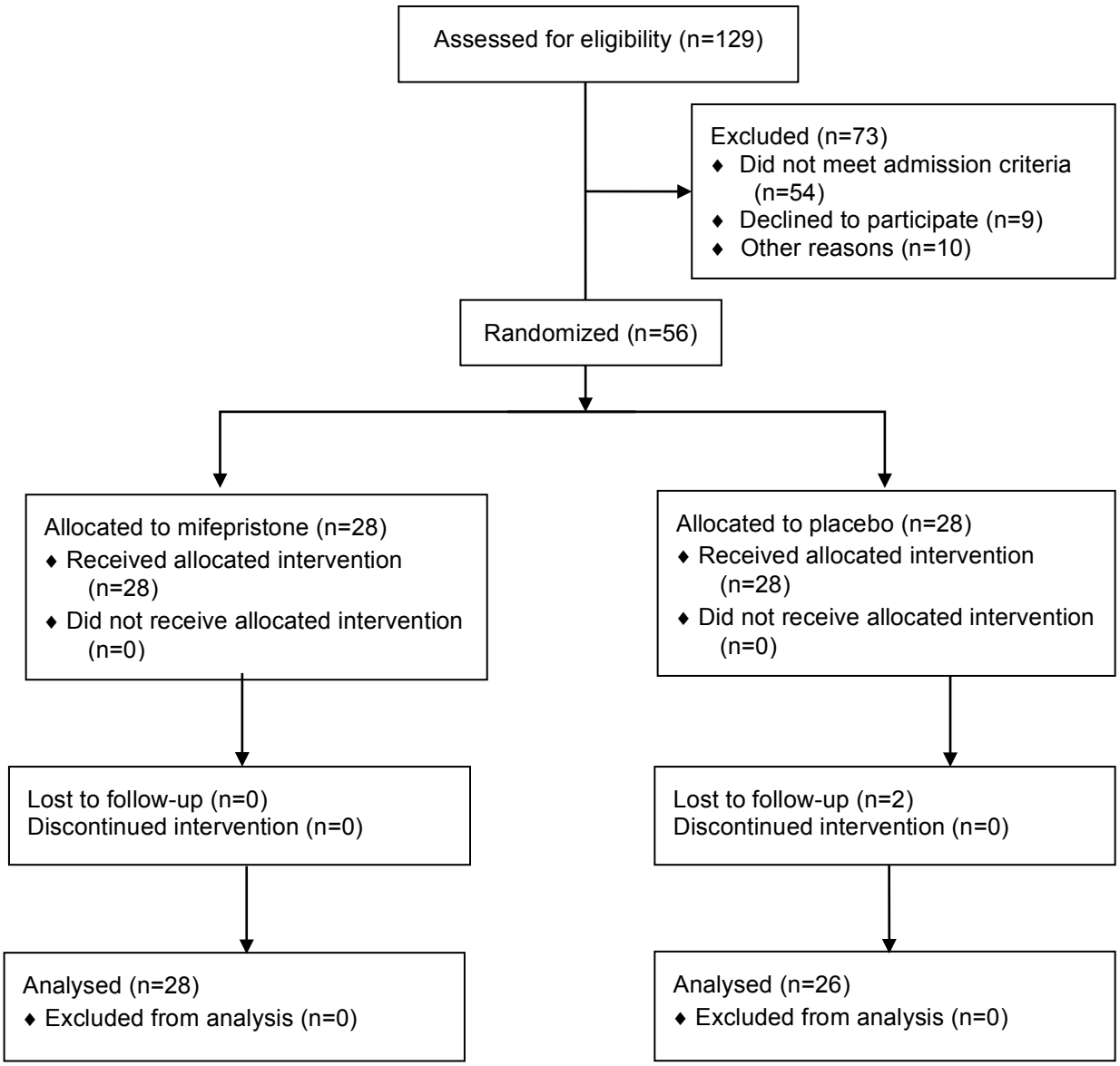
*Agonist mode. PR, progesterone receptor; MR, mineralocorticoid receptor; IL, interleukin; TNF, tumor necrosis factor; LPS, lipopolysaccharide. TAT, tyrosine amino transferase.

Supplementary Materials and Methods: Clinical studies

Evaluation of effect of mifepristone on craving and drinking in alcoholics

Our single-site study (trial registration # NCT015448417) was conducted in the Laboratory of Clinical Psychopharmacology at The Scripps Research Institute, La Jolla, CA, between 3/16/2012 (1st screened individual) and 3/14/2014 (last follow-up visit), and performed in accordance with the Declaration of Helsinki. The Scripps Institutional Review Board approved the study protocol. Written informed consent was obtained from all of the participants. Mifepristone and matched placebo tablets were provided by Corcept Therapeutics, Inc. (Menlo Park, CA, USA). This project used the human laboratory model of risk factors for relapse in abstinence that we previously developed and validated (5) and 1-week of naturalistic follow up to evaluate the therapeutic potential of mifepristone for alcohol use disorder. Medically healthy male and female non-treatment-seeking paid volunteers, aged 21-65, who met the DSM-IV criteria for current alcohol dependence were included (see flow chart). The study design was double-blind, placebo-controlled, parallel groups with simple random assignment to 1-week of treatment with oral mifepristone (600 mg daily) or placebo. The study statistician generated the randomization sequence using the open-access program found at http://randomization.com. Subjects and all personnel with subject contact (the medical assistant, the study coordinators, the principal investigator, and data entry clerks) were blind to treatment until the study outcomes were assessed. Randomization key and all medication were kept in a locked cabinet not accessible to study staff, and data were collected in a dedicated, password-protected database.

Fig. S2. Flow of Participants Through Trial



The subjects were abstinent from alcohol for 3 days prior to *in vivo* laboratory testing (verified by ethyl glucuronide testing). The primary *in vivo* laboratory outcome was rating of craving severity on four visual analogue scale (VAS) items. The subject's preferred alcoholic beverage or bottled water were presented in random order for 90 s following each mood condition (positive, negative, and neutral pictures selected from the International Affective Picture System [6]). The subject was told to view and sniff the beverage for 90 s but not to drink it, and then complete four VAS items after each of the six affect-beverage pairing: 1. Strength: How strong is your craving to drink alcohol? 2. Intent: If I could drink alcohol now, I would drink it. 3. Impulse: It would be hard to turn down a drink right now. 4. Relief: Having a drink would make things just perfect. Naturalistic assessments were performed at baseline (before treatment), on the last day of double-blind dosing and at 1-week post-treatment. The naturalistic outcomes were the daily number of standard drinks [14 g of pure alcohol] measured by the Timeline Followback Interview (7) and results of liver function tests performed on blood samples collected at the corresponding time points. (Table S1 illustrates the schedule of procedures).

Table S2. Schedule of Procedures for Human Laboratory Study

Scheduled Procedures	Week			
	-1 (Screening)	**0** (Start Drug)	**1** (Stop Drug)	**2** (Follow up)
Urinalysis, Complete Blood Count w/Differential, Blood Chemistry[1]; Urine Drug Screen	X		X	
Timeline Follow Back Interview Profile of Mood States Beck Depression Inventory Salivary Cortisol Adverse Event Assessment	X	X	X	X
Physical Exam		X		X
Dispense Study Medication		X		
Cue Reactivity Testing Mifepristone Plasma Concentration Alcohol Glucuronide			X	
Clinical Institute Withdrawal Assessment for Alcohol Addiction Research Center Inventory			X	

[1] Includes liver function tests (i.e., GGT, ALT, AST). Note: Subjects are required to abstain from alcohol (verified by alcohol glucuronide) during the three days prior to cue reactivity testing that occurs on the last day of drug administration (Week 1 visit).

Statistical analysis

Data were assessed for normal distribution and the presence of extreme values prior to analysis. Two subjects did not return after receiving medication (both placebo) and thus had no treatment data. Two subjects had extreme values in multiple outcome measures and biochemically-verified medication non-compliance (both mifepristone); their data were excluded from regression analyses. All subjects were included in baseline and safety analyses, including liver function test analysis. Between-group differences in demographics, baseline clinical/drinking characteristics, adverse events, and Addiction Research Center Inventory (8) subscale scores were analyzed using t-tests and the χ^2 test, as appropriate. Linear Mixed Effects Modeling (MEM) with Restricted Maximum Likelihood estimation was used to measure differences in alcohol-cued craving and changes in drinking. Models used to assess responses from cue exposure testing in the laboratory included treatment as a fixed, between-subjects factor

and cue condition as the repeated-measure. Results are reported as estimated marginal means and SD or SE, as indicated. For models of drinking (the number of drinks per week), treatment was considered a fixed, between-subjects factor, week a fixed within-subjects effect, and the interaction term a fixed effect. Results are reported as estimated marginal means and SE. Predictors for MEM and linear regression models were entered in a backward stepwise manner; variables that did not appreciably improve model fit or which were not independently significant predictors were removed to arrive at parsimonious models. All available data points were used, with missing values assumed missing at random. The results from laboratory tests of liver function were assessed using multivariate analysis of covariance of change scores (Week 2 minus Week 0) in the mifepristone and placebo groups separately, controlled for baseline drinking. Mean and SD are reported for each treatment group.

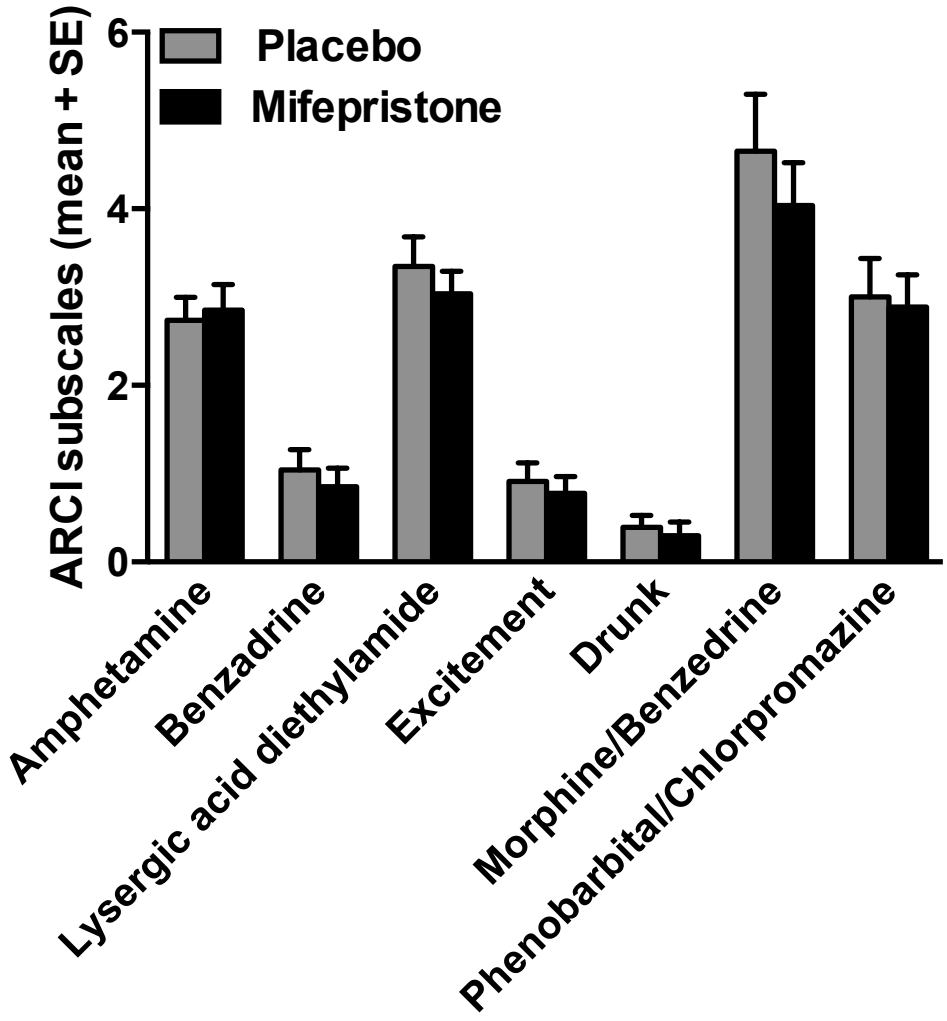


Fig. S3. Responses to questions regarding drug-specific subjective state did not differ between groups and suggest mifepristone effects did not resemble drugs of abuse on the Addiction Research Center Inventory (ARCI) subscales. ARCI subscales mean + SE.

Table S3. Baseline characteristics of human laboratory participants.

Demographic/Characteristic	Placebo (n = 28)	Mifepristone (n = 28)	p-value
Age, years	36.9 (11.2)	41.2 (11.4)	0.17
Male	20 (71%)	23 (82%)	0.34
White, non-Hispanic	20 (71%)	22 (79%)	0.54
Years of heavy drinking	14.0 (8.9)	15.0 (11.0)	0.71
DSM IV symptom count*	6.9 (2.1)	6.2 (2.3)	0.25

*Diagnostic and Statistical Manual, 4th edition (DSM-IV): requires 3 of 7 symptoms for a diagnosis of alcohol dependence.

Table S4. Adverse events reported by ≥ 5% of subjects during treatment. Mifepristone was well tolerated with no unexpected adverse events nor severity ratings > 2 (moderate) reported.

Adverse Event	Placebo (n = 26)	Mifepristone (n = 28)	p-value
Cold symptoms	3	3	0.99
Dizzy/foggy	1	3	0.61
Abrasions/bruises	1	2	0.99
Muscle pain	1	3	0.61
Fatigue	1	3	0.61
Nausea/gastrointestinal upset	1	3	0.61
Headache	2	3	0.99

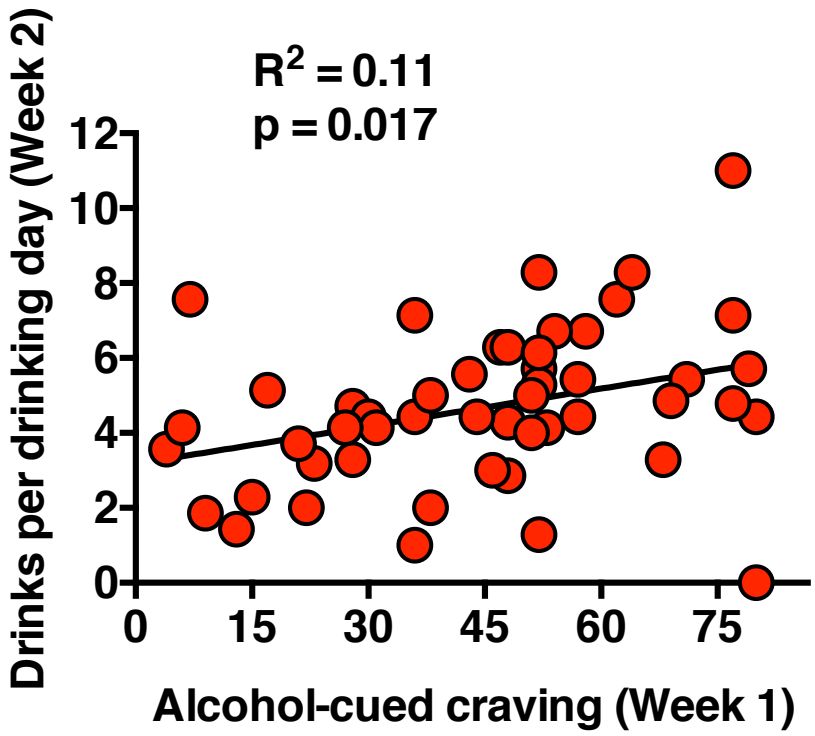


Fig. S4. Total Visual Analogue Scale craving scores elicited by alcohol cues in the laboratory on the last day of treatment (Week 1) predict the number of drinks consumed per drinking day during the post-treatment follow-up (Week 2), thereby supporting the predictive validity of the human laboratory model. Note that $R^2 = 0.11$, $p = 0.017$ excludes 1 outlier; the full dataset $R^2 = 0.15$, $p = 0.005$.

References:

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