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# *Andrographis paniculata* and Its Main Bioactive Ingredient Andrographolide Decrease Alcohol Drinking and Seeking in Rats Through Activation of Nuclear PPAR$\gamma$ Pathway

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## Abstract

**Background and aims:** *Andrographis paniculata* is an annual herbaceous plant which belongs to the Acanthaceae family. Extracts from this plant have shown hepatoprotective, anti-inflammatory and antidiabetic properties, at least in part, through activation of the nuclear receptor Peroxisome Proliferator-Activated Receptor-gamma (PPAR $\gamma$). Recent evidence has demonstrated that activation of PPAR$\gamma$ reduces alcohol drinking and seeking in Marchigian Sardinian (msP) alcohol-preferring rats.

**Methods:** The present study evaluated whether *A. paniculata* reduces alcohol drinking and relapse in msP rats by activating PPAR$\gamma$.

**Results:** Oral administration of an *A. paniculata* dried extract (0, 15, 150 mg/kg) lowered voluntary alcohol consumption in a dose-dependent manner and achieved ~65% reduction at the dose of 450 mg/kg. Water and food consumption were not affected by the treatment. Administration of Andrographolide (5 and 10 mg/kg), the main active component of *A. paniculata*, also reduced alcohol drinking. This effect was suppressed by the selective PPAR$\gamma$ antagonist GW9662. Subsequently, we showed that oral administration of *A. paniculata* (0, 150, 450 mg/kg) prevented yohimbine- but not cues-induced reinstatement of alcohol seeking.

**Conclusions:** Results point to *A. paniculata*-mediated PPAR$\gamma$activation as a possible therapeutic strategy to treat alcohol use disorder.

## INTRODUCTION

The World Health Organization (WHO) reported that harmful use of alcohol results in 3 million deaths every year at a global level (World Health Organization. Management of Substance Abuse Team, 2018). Alcohol is a rewarding substance, whose excessive use often leads to the development of alcohol use disorder (AUD), which is characterized by repeated episodes of intoxication and withdrawal symptoms when access to alcohol is prevented, heightening the risk of relapse to pathological drinking (Becker, 2008; Koob, 2013; Wackernah et al., 2014; Cui et al., 2015). The molecular mechanisms subserving AUD are not yet completely understood, but are thought to be associated with profound counteradaptive alterations of reward and stress neurocircuitries (Weiss and Porrino, 2002; Gilpin and Koob, 2008). Untangling these neuroadaptations is complex but essential to develop more efficacious therapies.

*Andrographis paniculata* is a member of the Acanthaceae family of plant widely cultivated in the Southeast Asia and has been used to prevent and treat metabolic disorders (Zhou, 1987; Thakur et al., 2016; Islam, 2017), for its hepatoprotective (Handa and Sharma, 1990; Maiti et al., 2010; Nagalekshmi et al., 2011) and anti-inflammatory properties (Chao et al., 2010; Shen et al., 2013; Low et al., 2015). *Andrographis paniculata* contains several bioactive compounds among which andrographolide is a labdane bicyclic diterpenoid lactone isolated from the stem and leaves (Reddy et al., 2003; Jayakumar et al., 2013). Evidence suggested that administration of andrographolide activates the transcription factor Peroxisome Proliferator-Activated Receptor-gamma (PPAR$\gamma$) (Islam, 2017). We have demonstrated that selective PPAR$\gamma$ agonists pioglitazone and rosiglitazone reduced alcohol drinking and stress-induced reinstatement of alcohol seeking in genetically selected Marchigian Sardinian (msP) alcohol-preferring rats (Stopponi et al., 2011, 2013; Fotio et al., 2020a). Based on this evidence, we hypothesized that administration of an extract of *A. paniculata* might reduce alcohol drinking and seeking in msP rats through a PPAR$\gamma$-dependent mechanism. To demonstrate our primary hypothesis, we conducted a series of experiments in which we tested the effect of *A. paniculata* extract and andrographolide on alcohol drinking and seeking in msP rats. We then evaluated whether it was possible to prevent the effects of andrographolide by blocking PPAR$\gamma$ with the selective receptor antagonist GW9662.

## MATERIALS AND METHODS

### Animals

Male genetically selected msP alcohol-preferring rats were used. This rat line has been genetically selected for its excessive alcohol drinking. It is highly sensitive to stress, shows anxiety and depressive-like phenotypes, and is considered a suitable animal model to study AUD (Ciccocioppo et al., 2006). At the start of the experiment the rats' body weights were between 300 and 350 g. They were bred in a room with a reverse 12:12 h light/dark cycle (lights off at 9:30 a.m.) in a temperature (20–22°C) and humidity (45–55%) controlled environment, at the University of Camerino (Camerino, Italy). Rats were offered free access to tap water and food pellets (4RF18, Mucedola, Settimo Milanese, Italy), except when noted. For the drinking experiments rats were singly housed. All the procedures were conducted in adherence with the *European Community Council Directive for Care and Use of Laboratory Animals and the National Institutes of Health Guide for the Care and Use of Laboratory Animals*.

### Drugs

*Andrographis paniculata* extract (purified by high performance liquid chromatography: andrographolide average content 95% p/p) was a generous gift of Dr. Nicotra (EPO S.r.l, Milano) and was formulated in a vehicle composed of: 1% methylcellulose, 1% Tween 80 and 98% distilled water. It was administered orally (0, 15, 150 and 450 mg/kg) at a volume of 2 ml/kg.

Andrographolide was purchased from Sigma Aldrich (Sigma Chemical Co., St. Louis, MO, USA), with purity ≥98%. It was dissolved in 5% dimethyl-sulphoxide (DMSO). GW9662, purchased from Tocris (Bristol, UK), was dissolved in 10% DMSO and 3% Tween 80 and 87%distilled water. Yohimbine, purchased from Sigma-Aldrich (Chemical Co., St. Louis, MO, USA), was dissolved in distilled water. *Andrographis paniculata* extract was administered orally (p.o.), whereas andrographolide, GW9662 and yohimbine were administered intraperitoneally (i.p.) in a volume of 1 ml/kg. Alcohol solution (10%) was prepared on a daily basis by diluting alcohol 95% (v/v) (Sigma-Aldrich, Chemical Co., St. Louis, MO, USA) in tap water.

## TWO-BOTTLE CHOICE PARADIGM

The two-bottle choice (2-BC) procedure (free choice between water and 10% alcohol) was used to measure voluntary alcohol drinking and preference (Borruto et al., 2020; Fotio et al., 2020a; Tabakoff and Hoffman, 2000). The rats were single housed in experimental chambers (30 cm length × 30 cm width × 30 cm height) for 1 week of habituation before the 2-BC test began. They were given continuous free access to water and 10% alcohol (v/v) for the next 15 days to establish a stable baseline (6.5–8 g/kg/24 h for three consecutive days) and preference for alcohol. Preference was defined as 80–90% preference for alcohol vs. water. The fluids were offered through graduated drinking tubes that were equipped with metal spouts. Fluid intake was measured by reading the volume that was consumed at specific time points (2, 8 and 24 h) following initiation of the active (dark) phase of the light/dark cycle. The drinking tubes were switched daily to avoid the development of side preference. The rats also had free access to food. Food consumption was measured by weighing the food container while considering the spillage weight. Alcohol, water and food intakes were calculated as absolute values of consumption at each time-point and are expressed as g/kg body weight.

## OPERANT SELF-ADMINISTRATION

Self-administration experiments were conducted according to our recent reports (Stopponi et al., 2018; Fotio et al., 2020a, 2020b) using standard operant chambers (Med Associate, St Albans, VT) located in sound-attenuating, ventilated cubicles. Each chamber was equipped with a drinking reservoir (volume capacity: 0.30 ml) positioned 4 cm above the grid floor in the center of the front panel of the chamber. Two retractable levers were located 3 cm to the right and left of the drinking receptacle. Auditory and/or visual stimuli were presented via a speaker and a light located on the front panel. A microcomputer controlled the delivery of fluids, the presentation of auditory and visual stimuli and the recording of the behavioral data. Further details are outlined in Experiments 2 and 3.

### Experiment 1: Effect of *A. paniculata* extract on alcohol intake in msP rats.

The effect of *A.s paniculata* on voluntary alcohol intake was assessed using the two-bottle choice paradigm. During the acquisition of stable

drinking baseline rats were trained to drug administration procedures for several days during which they received vehicles. At this point, we tested for the effect of *A. paniculata* dried extract (0, 15 and 150 mg/kg, p.o.) on alcohol intake using a between-subject design. For four consecutive days, rats received drug or vehicle twice a day 12 h (9:30 pm) and 1 h before (8:30 am) the beginning of the dark period (active phase) of the light–dark cycle. To further investigate the effect of *A. paniculata*, a new cohort of msP rats (N = 9/group), subjected to the same training described above, received a higher dose (450 mg/kg, p.o.) of the extract or its vehicle for three consecutive days. Alcohol, water and food consumption were measured after 30 min, 2, 8 and 24 h.

## Experiment 2: Effect of *A. paniculata* extract on yohimbine-induced reinstatement of alcohol seeking in msP rats.

The experiment consisted of three phases.

*Operant self-administration training phase*: A separate group of animals (N = 8) was trained to self-administer 10% alcohol in 30-min daily sessions on a fixed-ratio 1 (FR-1) schedule of reinforcement, in which each active lever response resulted in the delivery of 0.1 ml of alcoholic solution (Stopponi et al., 2018). To facilitate the acquisition of operant learning, at the beginning of training, presses on the active lever led to delivery of a solution containing 10% alcohol sweetened by 0.2% (w/v) saccharin. After this initial training, saccharin was tapered and rats continued to lever press for 10% alcohol on FR-1 with 5 s of time-out (TO 5 s). The TO period was paired to illumination of a white house light for 5 s. Self-administration training continued until stable baseline of responding for alcohol was achieved.

*The Extinction phase*: After the last alcohol self-administration session, animals were subjected to daily 30-min extinction sessions. Responding at the right lever activated the delivery mechanism and the house light but was not reinforced by alcohol delivery. The left lever, which serves as control to the active lever, was inactive throughout the self-administration training and extinction period. During the last 3 days of extinction, animals again were habituated to drug administration procedures using saline.

*Reinstatement test*: The day after the last extinction session, msP rats were subjected to the 30-min reinstatement test, conducted under the same extinction conditions, except that 30 min prior to the beginning of the operant session they received yohimbine (1.25 mg/kg, i.p.) to elicit reinstatement. To evaluate the effect of *A. paniculata* in a counterbalanced within subject design, rats (N = 8) received drug extract (0, 150 and 450 mg/kg, p.o.) 12 h and 1 h before the reinstatement test. Four days, during which animals were subjected to extinction sessions, were imposed between drug tests (Cippitelli et al., 2008; Fotio et al., 2020a).

## Experiment 3: Effect of *A. paniculata* extract on cue-induced reinstatement of alcohol seeking in msP rats

The experiment consisted of three phases.

*Conditioning phase*: Another group of rats (N = 9) was trained to lever press and discriminate between 10% alcohol and water. The discriminative stimulus for alcohol consisted of the odor of an orange extract ($S^+$), whereas water availability (no reward) was signaled by an anise extract ($S^-$). The olfactory stimuli were generated by depositing six to eight drops of the respective extract on the bedding of the operant chamber. In addition, each lever-press resulting in delivery of alcohol was paired with illumination of the chamber's house light for 5 s. The corresponding cue paired to water sessions was a 5 s white noise tone. The olfactory stimuli serving as $S^+$ or $S^-$ for alcohol availability were introduced 1 min before extension of the levers and remained present throughout the 30 min sessions. During the first 3 days of the conditioning phase, rats were given alcohol sessions only. Subsequently, alcohol and water sessions were conducted in random order across training days, with the requirement that all rats received a total of 10 alcohol and 10 water sessions.

*Extinction Phase*: After the last conditioning day, rats were subjected to 30-min extinction sessions. During this phase, both active and inactive levers were presented to animals without the discriminative stimuli. Presses at the active lever activated the syringe pumps but did not result in the delivery of liquids or the presentation of the response-contingent cues (i.e. house light or tone).

*Reinstatement:* Testing began the day after the last extinction session. This test lasted 30 min under conditions identical to those during the conditioning phase, except that alcohol and water were not made available. Sessions were initiated by the presentation of both levers and either the alcohol $S^+$ or water $S^-$ paired stimuli. The respective discriminative stimulus remained present during the entire session and responses at the previously active lever were followed by activation of the delivery mechanism and a 5 s presentation of house light in the $S^+$ condition or white noise in the $S^-$ condition. MsP rats were tested under the $S^-$ condition on Day 1 and starting from Day 2 they were tested under the $S^+$ condition. Before initiation of treatment, rats were trained to the drug administration procedure for 3 days during which they received vehicle treatments. To evaluate whether *A. paniculata* was able to prevent cue-induced reinstatement of alcohol-seeking behavior, rats (N = 9) received the extract (0, 150, 450 mg/kg, p.o.) 12 and 1 h before the reinstatement test. Animals received all drug treatments according to a counterbalanced Latin square design. A 4-day interval, during which animals remained in their home cages, was allowed between drug tests (Ciccocioppo et al., 2002; Cippitelli et al., 2008). Following presentation of water-paired cues ($S^-$) reinstatement did not occur, suggesting the behavioral specificity of relapse and making irrelevant the need to test the effect of *A. paniculata* under this condition.

## Experiment 4: Effect of andrographolide on alcohol intake in msP rats

To assess the effect of andrographolide on voluntary alcohol intake, we used the two-bottle choice test in a new cohort of msP rats (N = 27) as described in Experiment 1. Following establishment of a stable drinking baseline and habituation to the treatment procedure, msP rats (N = 9/group) received andrographolide (either 5 or 10 mg/kg, i.p.) or its vehicle using a treatment schedule identical to that of *A. paniculata* (12 h and at 30 min before the beginning of the dark period of the light–dark cycle). Alcohol, water and food consumption were monitored daily at 30 min, 2, 8 and 24 h.

## Experiment 5: Effect of PPAR$\gamma$ antagonism on andrographolide-induced reduction of alcohol intake in msP rats

To examine whether andrographolide reduces alcohol intake through recruitment of the PPAR$\gamma$, a selective antagonist (GW9662) of this receptor was administered alone or in combination with andrographolide. For this purpose, msP rats (N = 27) were treated with

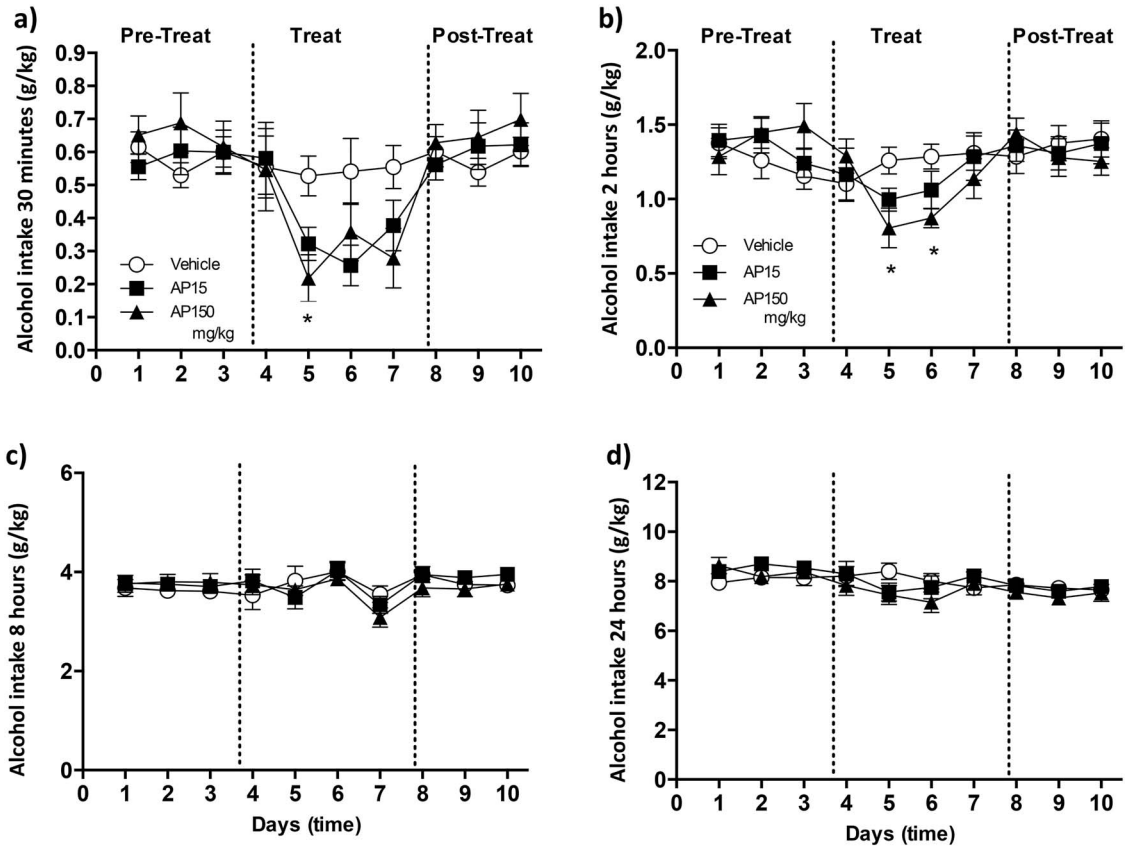


**Fig. 1.** Effect of *Andrographis paniculata* (AP) on alcohol intake in msP rats (N = 27). Voluntary 10% alcohol intake (g/kg) following treatment with AP (0, 15 and 150 mg/kg, p.o.) administered 12 and 1 h before access to alcohol. Data were recorded at 30 min (a), 2 h (b), 8 h (c) and 24 h (d) after access to alcohol. Data represent the mean ± S.E.M.: Significant difference from the vehicle treated group: $*P < 0.05$.

GW9662 (5 mg/kg i.p.) or vehicle, 10 min prior to each administration of andrographolide (0 or 10 mg/kg. i.p.). The latter was given 12 h and 30 min before the beginning of the dark phase. Rats (N = 6-7/group) received drug treatments in between-subject design for three consecutive days. Alcohol, water and food intake were recorded at 30 min, 2 and 24 h after initiation of the dark phase.

### Statistical analysis

The effects of *A. paniculata* and andrographolide on alcohol water and food consumption were analyzed at each time point (30 min, 2, 8 and 24 h) by means of a two-way repeated measures analysis of variance (ANOVA) with one between-subject factor (treatment) and one within-subject factor (days).

For reinstatement experiments, discrimination was evaluated by two-way ANOVA with one within-subject factor (time) and one between-subject factor (self-administration conditions). Differences in responses during the extinction exposure to $S^+$ and $S^-$ were analyzed in the vehicle-treated group by one-way ANOVA. The effect of *A. paniculata* on reinstatement was evaluated by means of a one-way repeated measure ANOVA. A Student's t-test was used to evaluate the effect of yohimbine on reinstatement of alcohol seeking comparing the mean of responding at the active lever during extinction to that of vehicle-treated animals following administration of yohimbine. The effects of *A. paniculata* on cue- and on yohimbine-induced reinstatements were evaluated by one-way repeated measures ANOVA. The effect of GW9662 on andrographolide-induced reduction of alcohol drinking was evaluated at each time point (30 min, 2, 8 and 24 h) using a two-way repeated measures ANOVA with one factor time (days) and one factor treatments (GW9662, andrographolide).

Where appropriate, Tukey's or Dunnett's multiple comparison tests were used for post hoc analysis. All statistical tests were two-sided, and statistical significance was set at $*P < 0.05$. Where not indicated, statistical analyses were not significant.

## RESULTS

### Result 1: Effect of *A. paniculata* on alcohol intake in msP rats

The effect of *A. paniculata* on alcohol intake in msP rats are shown in Fig. 1, ANOVA revealed a main effect of treatment (15 and 150 mg/kg, p.o.) at 30 min 30 min [$F(2,96) = 5.90$; $P < 0.001$; Fig. 1a] and at 2 h [$F(2,96) = 3.12$; $P < 0.05$; Fig. 1b], but not at 8 h [$F(2,96) = 30$ min1; $P = 0.716$; Fig. 1c] or 24 h [$F(2,96) = 1.87$; $P = 0.4825$; Fig. 1d]. A significant effect of time (days) on alcohol intake was also observed at 30 min [$F(3,96) = 3.46$; $P < 0.05$] and at 8 h [$F(3,96) = 4.86$; $P < 0.01$], but not at 2 h [$F(3,96) = 2.15$; $P = 0.6981$] and 24 h [$F(3,96) = 0.86$; $P = 0.137$]. A treatment x time interaction was not significant at any time point: [$F(6,96) = 1.03$; $P = 0.0629$], [$F(6,96) = 1.53$, $P = 0.1377$], [$F(6,96) = 0.72$; $P = 0.768$] and [$F(6,96) = 0.71$; $P = 0.3136$] for the 30 min, 2, 8 and 24 h, respectively (Figure 1a). Tukey's post hoc test revealed that at 30 min alcohol drinking was significantly reduced by 15 mg/kg

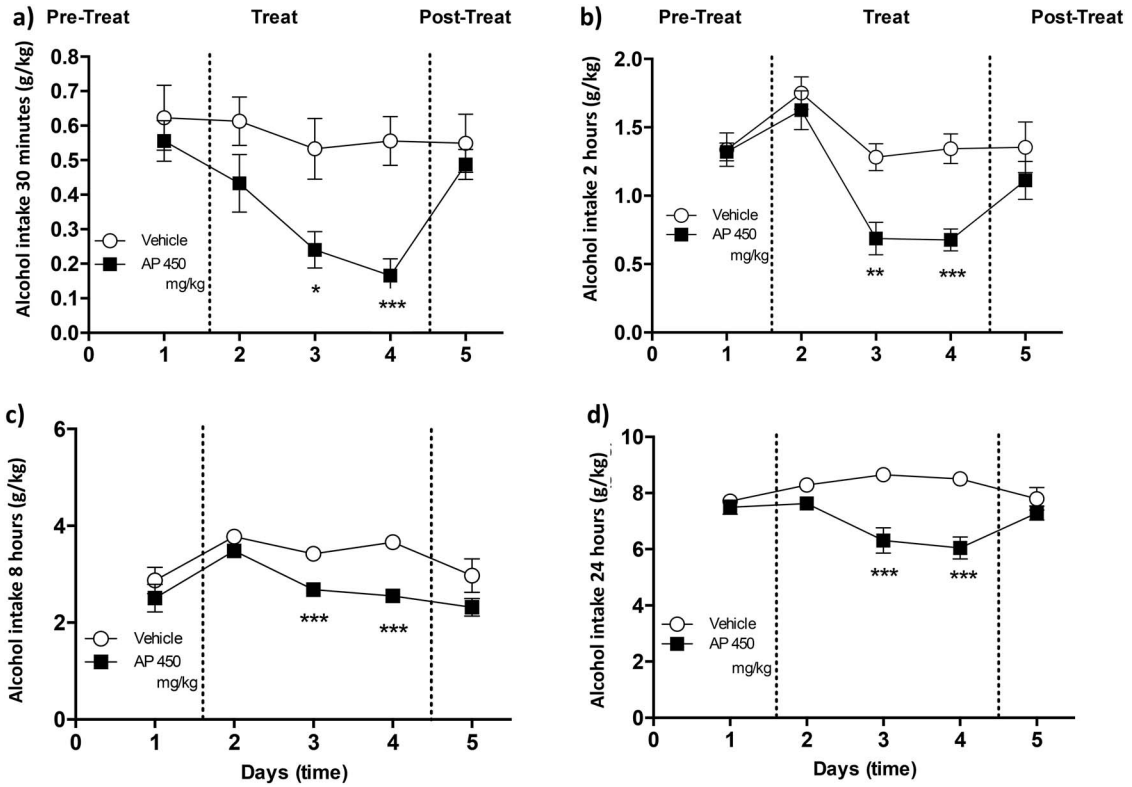


**Fig. 2.** Effect of *Andrographis paniculata* (AP) on alcohol intake in msP rats (N = 18). Voluntary 10% alcohol intake (g/kg) following treatment with AP (0 and 450 mg/kg, p.o.) administered 12 and 1 h before access to alcohol. Data were recorded at 30 min (a), 2 h (b), 8 h (c) and 24 h (d) after access to alcohol. Data represent the mean ± S.E.M. Significant difference from the vehicle treated group: $*P < 0.05$, $**P < 0.01$ and $*** P < 0.001$.

($P < 0.05$) and 150 mg/kg ($P < 0.01$) of *A. paniculata*. Additionally, post hoc tests revealed a significant reduction of alcohol drinking after administration of 150 mg/kg of the extract on the second day of treatment at 30 min ($P < 0.05$) and on the second and third treatment day at 2 h ($P < 0.05$) (Fig. 1b). Food and water intakes were not affected by treatment (data not shown).

In a subsequent experiment, rats were treated with a higher dose (450 mg/kg) of *A. paniculata* (Fig. 2a). Overall, ANOVA revealed a significant effect of treatment at 30 min [$F(1,16) = 14.26$; $P < 0.001$; Fig. 2a], 2 h [$F(1,16) = 16.14$; $P < 0.001$; Fig. 2b], 8 h [$F(1,16) = 35.87$; $P < 0.001$; Fig. 2c] and 24 h [$F(1,16) = 30.34$; $P < 0.001$; Fig. 2d]. A significant effect of time (days) at 30 min [$F(2,32) = 4.90$; $P < 0.05$], 2 h [$F(2,32) = 34.56$; $P < 0.001$], 8 h [$F(2,32) = 16.38$; $P < 0.001$], and 24 h [$F(2,32) = 6.49$; $P < 0.001$] was also detected. Treatment x time interaction was significant at 2 h [$F(2,32) = 4.68$; $P < 0.05$], 8 h [$F(2,32) = 6.72$; $P < 0.001$] and 24 h [$F(2,32) = 13.37$; $P < 0.001$], but not at 30 min h [$F(2,32) = 1.77$; $P = 0.2381$]. Post hoc comparisons revealed a significant reduction of alcohol drinking at each tested time point on the second day of treatment 30 min [30-min, ($P < 0.05$), 2 h ($P < 0.01$), 8 h, ($P < 0.001$) and 24 h, ($P < 0.001$)]. On the third day of treatment, there was a significant difference ($P < 0.001$) at each time point. When treatment was stopped, alcohol drinking rapidly returned to a baseline level (Fig. 1). Food and water consumption were not significantly affected by treatment (data not shown).

## Result 2: Effect of *A. paniculata* on yohimbine-induced reinstatement of alcohol seeking in msP rats

Stable baseline of 10% alcohol responding was established over 10 self- administration days (mean value of rewards on last 3 days of responding: 55.7 ± 4.7). Following this alcohol self-administration phase, extinction training was initiated and the responses to alcohol progressively decreased, until it was 4.0 ± 1.4 on the last 3 days of this phase. Significant reinstatement of active lever pressing was demonstrated by t-test [$t(7) = 7.02$; $P < 0.001$] comparing extinction and vehicle-yohimbine (Figure 3a). When ANOVA was used to evaluate the effect of *A. paniculata* on yohimbine-induced reinstatement, results showed a significant overall effect of treatment [$F(2,7) = 32.99$; $P < 0.001$; Fig. 3a]. As shown in Fig. 3, post hoc analysis demonstrated that at both doses tested (150 and 450 mg/kg, p.o.) *A. paniculata* significantly ($P < 0.001$) reduced reinstatement of lever pressing evoked by yohimbine. Responses at the inactive lever were very low and were not affected by yohimbine or *A. paniculata* treatments [$F(2,7) = 2.14$; $P = 0.3241$; Fig. 3b].

## Result 3: Effect of *A. paniculata* on cue-induced reinstatement of alcohol seeking in msP rats

During self-administration training, rats learned to discriminate between alcohol and water, and on the last 3 days of this phase, animals earned 52.4 ± 4.66 alcohol infusions and 13.8 ± 2.22 water

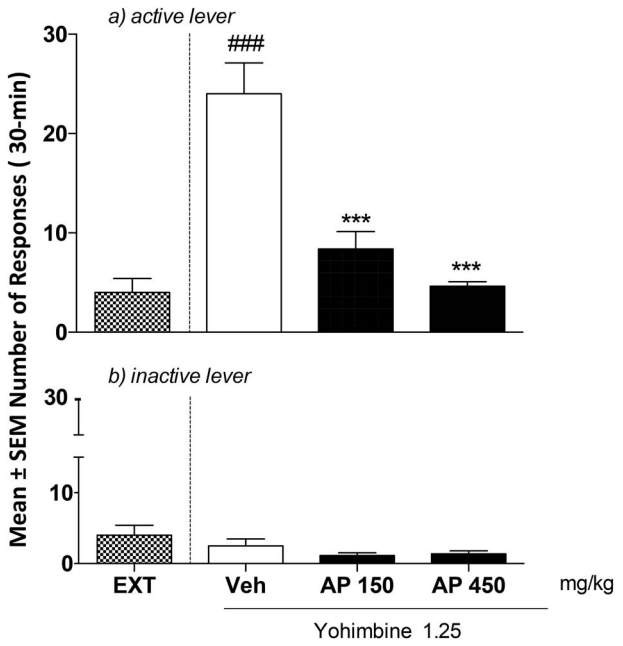


**Fig. 3.** Effect of *Andrographis paniculata* (AP) on yohimbine-induced reinstatement of alcohol seeking in msP rats (N = 8). During training phase animals reached a stable baseline of 10% (v/v) alcohol related responding. During extinction lever responding progressively decreased. Compared to extinction (EXT), yohimbine (1.25 mg/kg, i.p.) elicited a significant reinstatement of lever pressing that was significantly reduced by AP (0, 150 and 450 mg/kg, p.o.). Values represent the mean ± S.E.M number of responses at (a) alcohol active lever and (b) inactive lever. Significant difference from extinction, $^{###}P < 0.001$. Significant difference from the vehicle treated group: $^{***}P < 0.001$.

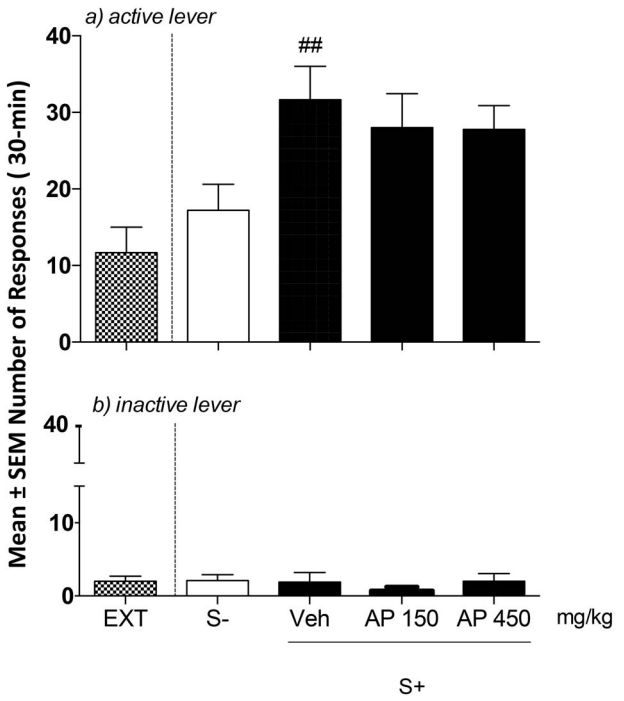


**Fig. 4.** *Andrographis paniculata* (AP) on cue-induced reinstatement of alcohol seeking in msP rats (N = 9). During training phase animals reached a stable baseline of 10% (v/v) alcohol related responding. During extinction lever responding progressively decreased. Conditioned reinstatement of alcohol seeking was evaluated after presentation of alcohol ($S^+$) or water ($S^-$) paired cues. Reinstatement in responding elicited by $S^+$ was not affected by AP (0, 150 and 450 mg/kg, p.o.) treatment. Significant difference from extinction, $^{##}P < 0.01$.

infusions. During extinction, lever pressing progressively decreased to 11.6 ± 3.36 on the last 3 days. In the reinstatement test, ANOVA revealed a significant overall effect of cue-presentation on alcohol seeking [F(2,8) = 7,01; $P < 0.05$]. Post hoc analysis showed that compared to extinction, exposure to alcohol-paired odor $S^+$ elicited a robust reinstatement of responding at the active lever ($P < 0.05$). Lever presses were not influenced by presentation of water-paired stimuli $S^-$. Conditioned reinstatement of alcohol-seeking (Fig. 4) was not modified by pretreatment with *A. paniculata* [F(2,8) = 0.44; $P = 0.1638$; Fig. 4a]. Responses at the inactive lever were very low throughout the experiment and were not influenced by the treatment [F(2,8) = 0.81; $P = 0.3422$; Fig. 4b].

## Result 4: Effect of andrographolide on alcohol intake in msP rats

Overall ANOVA showed (Fig. 5) a significant effect of treatment on alcohol intake at 30 min ([F(2,24) = 6.75; $P < 0.001$; Fig. 5a]; 2 h [F(2,24) = 7.50; $P < 0.001$; Fig. 5b]; 8 h [F(2,24) = 9.20; $P < 0.001$; Fig. 5c] and 24 h ([F(2,24) = 8.45; $P < 0.001$; Fig. 5d]. ANOVA also showed a significant effect of time (days) at 30 min ([F(2,48) = 8.20; $P < 0.001$]; 2 h [F(2,48) = 6.25; $P < 0.001$]; 8 h [F(2,48) = 4.60; $P < 0.05$] and 24 h ([F(2,48) = 7.05; $P < 0.01$]. The interaction treatment x time was a significant at 8 h [F(4,48) = 6.72; $P < 0.001$] and 24 h ([F(4,48) = 4.62; $P < 0.01$], but not at 30 min ([F(4,48) = 1.18; $P = 0.0978$] and 2 h [F(4,48) = 1.37; $P = 0.0741$].

Tukey's post hoc tests revealed a significant reduction of alcohol drinking after administration of andrographolide (5 mg/kg, i.p.) on the first treatment day at 30 min ($P < 0.05$), on the second treatment day at 30 min ($P < 0.05$) and at 2 h ($P < 0.05$) and on the third treatment day at 30 min ($P < 0.05$), 8 h ($P < 0.001$) and 24 h ($P < 0.05$). After administration of andrographolide (10 mg/kg, i.p.), alcohol drinking was significantly reduced on the first day of treatment at 2 h ($P < 0.05$) and on the second day at 30 min, 2, 8 ($P < 0.05$) and 24 h ($P < 0.001$). Finally, on the third day of treatment drinking was reduced at 30 min and 2 h ($P < 0.01$) as well as at 8 and 24 h ($P < 0.001$). Water intake was not affected by drug treatments (data not shown). ANOVA also revealed no significant overall changes in food intake (data not shown).

## Result 5: Effect of PPAR$\gamma$ antagonism on andrographolide-induced reduction of alcohol intake in msP rats

Overall ANOVA revealed a main effect of treatment with andrographolide at 30 min [F(3,23) = 6.37; $P < 0.01$; Fig. 6a], 2 h [F(3,23) = 4.90; $P < 0.01$; Fig. 6b] and 24 h [F(2,23) = 16.60; $P < 0.001$; Fig. 6c]. ANOVA also revealed a main effect of time at 2 h [F(2,46) =5.75; $P < 0.01$] and 24 h [F(2,46) =5.84; $P < 0.01$] but not at 30 min [F(2,46) = 0.09; $P = 0.194$]. Treatment x time interaction was significant at 24 h [F(6,46) = 8.28; $P < 0.001$] but not at 30 min, [F(6,46) = 0.98; $P = 0.9483$] and 2 h [F(6,46) = 1.13; $P = 0.3426$]. As shown in Fig. 6, Tukey's post hoc comparisons revealed a significant difference between animals treated with andrographolide and vehicles controls was observed at multiple time-points throughout the treatment. This effect was detected 30 min ($P < 0.01$)—2 h

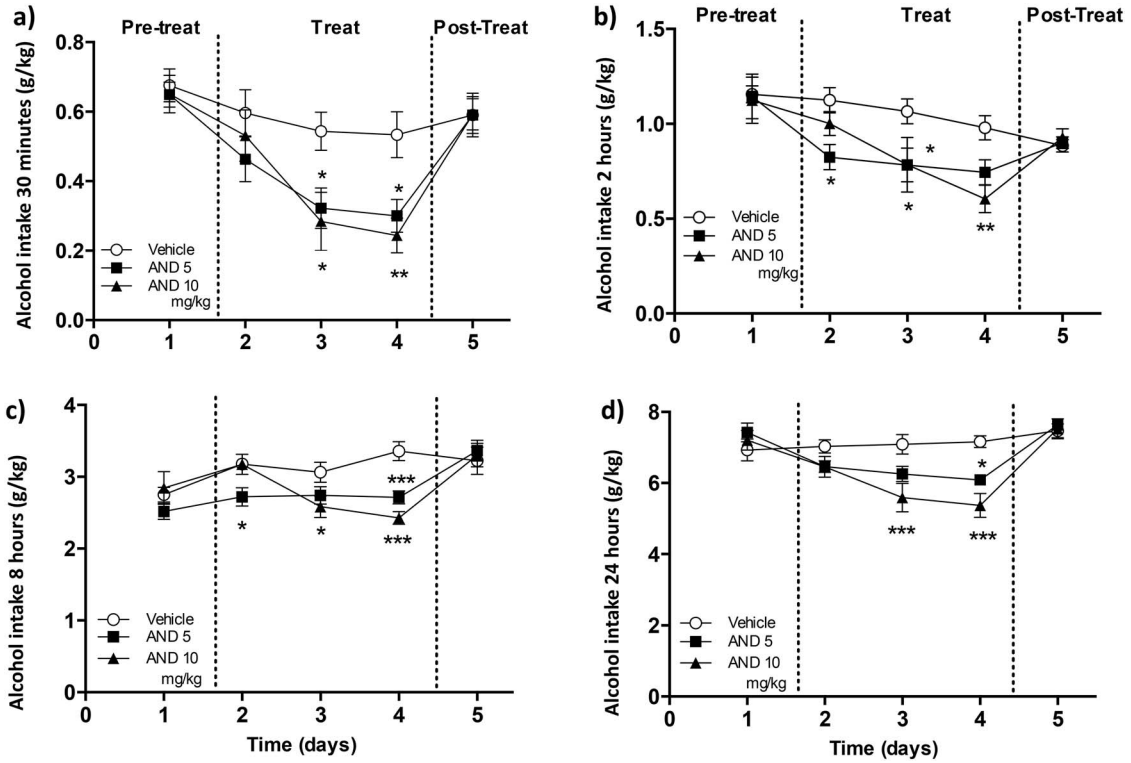


**Fig. 5.** Effect of andrographolide (AND) on alcohol intake in msP rats (N = 27). Voluntary 10% alcohol intake (g/kg) following treatment with andrographolide (0, 5 and 10 mg/kg, i.p.) administered 12 h and 30 min before access to alcohol. Data were recorded at 30 min, 2, 8 and 24 h after the availability of alcohol. Data represent the mean ± S.E.M. Significant differences from the vehicle treated group: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

($P < 0.05$)—24 h ($P < 0.001$) on day 2; at 2 h and 24 h on day 3 ($P < 0.05$). The group treated with andrographolide plus GW9662 was not different from vehicle controls at any time point. Water intake was not affected by drug treatments (data not shown). ANOVA also revealed no significant overall changes in food intake (data not shown).

## DISCUSSION

Results showed that sub-chronic treatment with *A. paniculata* significantly reduced voluntary alcohol intake in msP rats in a time- and dose-dependent manner. This effect started on the second day of administration and was maintained throughout the period of treatment. Importantly, water and food intake were not modified, indicating that the effect of *A. paniculata* is specific for alcohol and does not affect general consummatory behavior. Of note, intake of alcohol returned to the baseline level following treatment cessation. The effect on alcohol drinking was mimicked by andrographolide, one of the major secondary metabolites of *A. paniculata* (Xu and Wang, 2011; Malahubban et al., 2013). The efficacy of andrographolide was comparable to that of the parent extract, indicating that this compound is probably the most important mediator of the suppressive effect of *A. paniculata* on alcohol-seeking and -taking.

At this point we hypothesized that reduction of alcohol drinking following administration of *A. paniculata* or its main active ingredient andrographolide might have been mediated by recruitment of the nuclear transcription factor PPAR$\gamma$. This hypothesis was based on several lines of work: first, a prior report indicates that andrographolide is a partial agonist at PPAR$\gamma$ receptor (Guasch et al., 2013). Secondly, data from adult mouse and human brains have consistently reported that PPAR$\gamma$ is expressed in several mesocorticolimbic regions, including prefrontal cortex (PFC), nucleus accumbens (NAc), amygdala and ventral tegmental area (VTA), indicating an important role of this nuclear receptor in physiological control of motivation and reward processes (Sarruf et al., 2009; Domi et al., 2016; Warden et al., 2016). Thirdly, earlier studies showed that PPAR$\gamma$ agonism alleviates AUD symptoms and protects against deleterious effects of long-term alcohol consumption (Tomita et al., 2004; Stopponi et al., 2011; Drew et al., 2015; Cippitelli et al., 2017). Here, to test whether the alcohol-suppressive effects of andrographolide were mediated through a PPAR$\gamma$-dependent signaling, we pretreated rats with GW9662, a selective antagonist of this receptor (Leesnitzer et al., 2002). Consistent with our hypothesis, PPAR$\gamma$ receptor blockade completely occluded the effect of andrographolide.

PPAR$\gamma$ agonists are clinically used to control type 2 diabetes mellitus because they increase insulin sensitivity (Chiarelli and Di Marzio, 2008; Quinn et al., 2008). Given the important role of this transcription factor on hepatic metabolism (Gurnell et al., 2003; Ahmadian et al., 2013), the reduction of drinking following activation of PPAR$\gamma$ by andrographolide might be due to the effect of this compound on alcohol metabolism. In this study, we did not monitor the effects of andrographolide on blood glucose and alcohol level and therefore, we cannot theoretically exclude this possibility. However, in a previous study in which we have used the same rat model employed in this study, we have demonstrated that treatment with PPAR$\gamma$ agonists like pioglitazone and rosiglitazone neither change blood glucose nor blood alcohol levels even when given high doses (Stopponi et al., 2011).

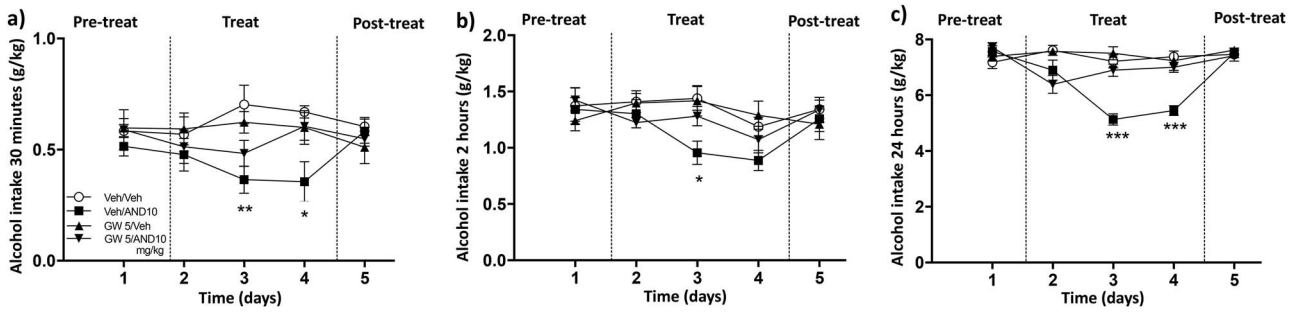


**Fig. 6**. Voluntary 10% alcohol intake following chronic administration of 5 mg/kg of GW9662 (GW 5 mg/kg, i.p.) and andrographolide (AND, 10 mg/kg, i.p.) at 30 min, 2 and 24 h in msP rats (N = 27). Data represent the mean SEM ±. Significant differences from the vehicle treated group: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$

Andrographolide, the active ingredient of *A. paniculata*, is characterized by low water solubility and a high degree of binding to plasma proteins (Panossian et al., 2000). To some extent, this may explain why the effect did not follow a clear dose–response curve, and was rather limited on the first treatment day, but increased over time. On the other hand, at the high dose (450 mg/kg) of the extract, the effect on voluntary alcohol consumption was particularly robust and long lasting, suggesting that these pharmacokinetic limitations could be at least partially overcome by increasing the dosage of the extract.

Another important feature of AUD is the elevated rate of relapse during abstinence (Hunt et al., 1971; Junghanns et al., 2005; O'Brien, 2005; Moos and Moos, 2006). Over the years, results from many human studies have consistently suggested that stress is a major causal factor of resumption of drug use in abstinent individuals (Sinha, 2001; Moos and Moos, 2006). These clinical studies have led to the development of rat models based on the operant conditioning reinstatement model to study mechanisms of relapse to alcohol use (Marchant et al., 2013; Venniro et al., 2016). Yohimbine, an $\alpha$-2 adrenoceptor antagonist, acts by increasing cell firing and release of brain noradrenalin (Aghajanian and VanderMaelen, 1982; Lee et al., 2004). Yohimbine has long been used to precipitate craving in human alcoholics following abstinence and to reinstate alcohol seeking behaviors in animals after extinction (Charney et al., 1983; Lee et al., 2004; Marinelli et al., 2007; Umhau et al., 2011; Stopponi et al., 2013; Fotio et al., 2020a). In our experimental condition, this drug was used to study the effect of *A. paniculata* on reinstatement of drug seeking. Data showed that pretreatment of msP rats with this plant extract following the extinction phase has significantly reduced alcohol seeking elicited by yohimbine. Importantly, neither yohimbine nor *A. paniculata* affected responding at the inactive lever, indicating that the effect of *A. paniculata* was specific and not a result of generalized behavioral depression. A plausible explanation of this effect is that PPAR$\gamma$ activation by andrographolide contained in the extract could act by blocking the brain's stress response and/or acting as an anxiolytic. In fact, it is known that activation of this nuclear receptor attenuates anxiety (Domi et al., 2016; Domi et al., 2019b) reduces hypothalamic–pituitary–adrenal axis activity and reduces the expression of corticotropin-releasing factor (CRF) in response to stress, especially in the paraventricular nucleus of the hypothalamus (Festuccia et al., 2008).

Environmental stimuli paired with the availability or consumption of alcohol have long been demonstrated to evoke subjective feelings of craving and to trigger episodes of relapse in abstinent alcoholics (Ludwig et al., 1974; O'Brien et al., 1990; Katner et al., 1999). In the present study, we trained msP rats to an extinction/reinstatement procedure in which re-exposure to environmental cues predictive of alcohol availability elicited a robust reinstatement of lever pressing. *Andrographis paniculata* did not show any efficacy in this model confirming previous work with other PPAR$\gamma$ agonists (Stopponi et al., 2011; Stopponi et al., 2013; de Guglielmo et al., 2017).

One potential limitation of the study is that we used a strain of rats that has a genetically determined preference for alcohol. Although widely used in alcohol research (Economidou et al., 2006; Stopponi et al., 2013, 2018; Kirson et al., 2018; Logrip et al., 2018; Domi et al., 2019a; Borruto et al., 2020; Fotio et al., 2020a, 2020b), this strain is characterized by the inherent limitation of being a genetic model that likely mimics a specific form of AUD. However, in a recent study we also reported that activation of PPAR$\gamma$ reduces alcohol drinking in nonselected rats and mice suggesting a more general role of this receptor system in AUD (Domi et al., 2020). The study of PPAR$\gamma$ agonist on alcohol abuse has been so far limited to male rats. Considering the importance of sex differences in AUD, in future studies it will be important to expand this investigation in female animals. Another aspect to consider is that in the reinstatement elicited by environmental cues we combined the presentation of context and discrete cues. Hence, we could not evaluate if activation PPAR$\gamma$ may lead to different effects if specifically tested against discriminative or discrete cues only.

Currently, three medications have been Food and Drug Administration (FDA) approved for the treatment of AUD. These include naltrexone, acamprosate and disulfiram (Littleton et al., 2004; Williams, 2005; Litten et al., 2012). These medications, however, showed significant side effects and an efficacy limited to specific subgroups of patients (Wilson, 1962; Fox, 1968; Christensen, 1973; Brahen et al., 1978; Enghusen Poulsen et al., 1992; Weinrieb and O'Brien, 2004). The development of new, possibly more effective, pharmacotherapies is therefore a priority. The results of this study strengthen our previous observations, supporting the idea that PPAR$\gamma$ agonism could represent a novel strategy to treat AUD and possibly addiction to other substances of abuse (Stopponi et al., 2011, 2013; de Guglielmo et al., 2015; Miller et al., 2018; Brami-Cherrier et al., 2020; Fotio et al., 2020a).

## AUTHORS CONTRIBUTION

R.C., G.A.D. and G.A.G. were responsible for the study concept and design. S.S., Y.F. and N.C. contributed to the acquisition of animal data. R.C., S.T. and Y.F. wrote the manuscript. C.C., H.L. and C.H.K. assisted with the data analysis and interpretation of findings. C.H.K. and G.A.D. provided critical revision of the manuscript. All authors critically reviewed the content and approved the final version for publication.

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## CONFLICT OF INTEREST STATEMENT

Dr. Demopulos is the Chairman & CEO of Omeros Corporation (Omeros) and Dr. Gaitanaris is Chief Scientific Officer of Omeros. Dr. Ciccocioppo is the inventor on several patent applications relating to the therapeutic use of PPAR$\gamma$ agonists in addiction. Omeros, through agreements with the University of Camerino and with Dr. Ciccocioppo, exclusively controls the intellectual property rights directed to Dr. Ciccocioppo's inventions related to the use of PPAR$\gamma$ receptor agonists for the treatment of addiction and addictive behaviors. Under these agreements, Dr. Ciccocioppo may be entitled to receive payments and royalties from Omeros. The other authors have no conflict of interest.

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