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ORIGINAL INVESTIGATION

Interest of new alkylsulfonylhydrazide-type compound in the treatment of alcohol use disorders

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Abstract

Rationale Recent preclinical research suggested that histone deacetylase inhibitors (HDACIs) and specifically class I HDAC selective inhibitors might be useful to treat alcohol use disorders (AUDs).

Objective The objective of this study was to find a new inhibitor of the HDAC-1 isoenzyme and to test its efficacy in an animal model of AUDs.

Methods In the present study, we prepared new derivatives bearing sulfonylhydrazide-type zinc-binding group (ZBG) and evaluated these compounds in vitro on HDAC-1 isoenzyme. The most promising compound was tested on ethanol operant self-administration and relapse in rats.

Results We showed that the alkylsulfonylhydrazide-type compound (ASH) reduced by more than 55% the total amount of ethanol consumed after one intracerebroventricular microinjection, while no effect was observed on motivation of the animals to consume ethanol. In addition, one ASH injection in the central amygdala reduced relapse.

Conclusions Our study demonstrated that a new compound designed to target HDAC-1 is effective in reducing ethanol intake and relapse in rats and further confirm the interest of pursuing research to study the exact mechanism by which such inhibitor may be useful to treat AUDs.

Keywords Alkylsulfonylhydrazide · Histone deacetylase · Zinc-binding group · Inhibition · Ethanol self-administration · Rats

Abbreviations

ASH	Alkylsulfonylhydrazide
AUD	Alcohol use disorder
aCSF	Artificial cerebrospinal fluid
CeA	Central nucleus of amygdala
FDA	Food Drug Administration
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HDACI	Histone deacetylase inhibitor
i.c.v.	Intracerebroventricular

Jérôme Jeanblanc and Erika Bourguet contributed equally to this work.

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PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
SAHA	Suberoylanilide hydroxamic acid
TSA	Trichostatin A
ZBG	Zinc-binding group

Introduction

Alcohol use disorder (AUD) is a chronic disorder characterized by the loss of control over ethanol intake, compulsive use of ethanol, and development of a negative emotional state during ethanol withdrawal. A large body of evidence has shown that epigenetic mechanisms and especially those involving histone acetylation/deacetylation may play a role in the development of AUD (Botia et al. 2012; Jangra et al. 2016; Barbier et al. 2017; Berkel and Pandey 2017; Pandey et al. 2017). New strategies for the treatment of AUDs are a pressing need, and it has been suggested that targeting histone deacetylase (HDAC) may have great potential (Jangra et al. 2016; Bourguet et al. 2018; Warnault et al. 2013; Legastelois et al. 2017).

Histone acetyltransferases (HATs) and HDACs regulate gene transcription by acetylation-deacetylation of histones, respectively (Mottet and Castronovo 2008). Up to date, 18 human isoforms of HDAC have been identified and are listed in four classes based on their sequence homology with the yeast (*Saccharomyces cerevisiae*) transcriptional regulator RPD3, their location, and their molecular weight. All classes of HDACs contain a zinc ion in their active site except for class III, whose activity is regulated by NAD^+. Members of the classical HDAC family are differentiated into two phylogenetic classes, called class I and class II. The HDACs of class I (HDAC-1, HDAC-2, HDAC-3, and HDAC-8) are mostly located in the nucleus, whereas HDACs from class II (HDAC-4, HDAC-5, HDAC-6, HDAC-7, HDAC-9, and HDAC-10) sharing homologies with the yeast Hda1 protein can shuttle between the cytoplasm and the nucleus. Class III HDACs have been identified to belong to the Sir2 family, requiring NAD^+ co-factor for deacetylation, while HDAC-11 is the only isoenzyme of the class IV (de Ruijter et al. 2003).

Preclinical studies demonstrated that HDAC inhibitors (HDACIs) could be useful tools to treat AUDs. Non-specific HDACIs such as sodium butyrate, TSA, and the FDA-approved SAHA (Fig. 1a) have been shown to reduce ethanol intake and ethanol-withdrawal-induced anxiety (Sakharkar et al. 2012; Pandey et al. 2008; Warnault et al. 2013). In mice and rats, SAHA (50–100 mg/kg) inhibited ethanol intake and the motivation of rats to seek ethanol (Warnault et al. 2013). The same authors showed that both TSA (0.1–0.4 mg/kg) and the more selective HDAC class I inhibitor MS-275 (5–20 mg/kg) are also effective in reducing ethanol intake in mice. The reduction of ethanol intake is lasting as long as mice are treated with SAHA and the effect is still present only 1 day post-treatment. Warnault et al. (2013) also demonstrated that all the HDACIs are selective of ethanol since they did not observe any effect on saccharin intake. Sodium butyrate has been shown to be very effective in reducing ethanol intake in a model of ethanol intake escalation. Simon-O'Brien et al. (2015) have demonstrated that sodium butyrate can either prevent ethanol intake escalation or reduce intake once rats display escalation of ethanol intake. The same authors have also demonstrated that sodium butyrate is very effective in reducing ethanol intake in dependent rats that have been exposed to chronic and intermittent ethanol vapor. The effect of sodium butyrate on ethanol dependent animals was mimicked by MS-275 (10 mg/kg once a day, via the intraperitoneal way) since it decreased the operant ethanol self-administration after the second injection in ethanol-dependent rats (Simon-O'Brien et al. 2015). In addition, the effect of intracerebroventricular (i.c.v.) injections of MS-275 was also tested in a model of voluntary heavy drinking in which rats self-administered excessive ethanol intake for 2 months (Jeanblanc et al. 2015). MS-275 decreased operant ethanol self-administration (75% decrease) after the second i.c.v. injection (one injection per day) at the dose of 500 μM, the motivation to consume ethanol (25% decrease) and relapse after a period of abstinence (50% decrease). Altogether, the results demonstrated that both non-selective HDACIs and more selective inhibitors targeting the class I HDACs are effective in reducing ethanol intake and relapse in rats displaying excessive ethanol intake and/or ethanol dependence. Attention has now turned to more potent and selective compounds that may prove to be more effective.

We previously demonstrated that HDAC-1 mRNA levels are increased after either acute or repeated ethanol injections in the striatum (Legastelois et al. 2013). Interestingly, HDAC-1 is predominantly localized in the nucleus and is part of stable transcriptional complexes that are recruited to gene promoters by DNA-binding proteins thus suggesting gene-specific rather than global and unspecific transcriptional regulation (Kazantsev and Thompson 2008). Results obtained with MS-275, which has been described as more selective inhibitor of HDAC-1 (Khan et al. 2008 but see Glaser et al. 2004), on ethanol-related behaviors are encouraging in terms of targeting specifically HDAC-1 to treat AUD, but this molecule may suffer from its poor brain penetration (Hooker et al. 2010). Despite, its poor brain penetration, we demonstrated its efficacy to reduce ethanol intake after one daily peripheral injection for 2 days (Simon-O'Brien et al. 2015). In this context, it seems of great interest to develop new molecules targeting specifically HDAC-1 in order to potentially achieve higher efficacy (potential effect after only one administration and less side effects).

All current HDACIs are characterized by a common pharmacophore with three key-elements for inhibitor-enzyme interactions: a metal-binding domain that interacts

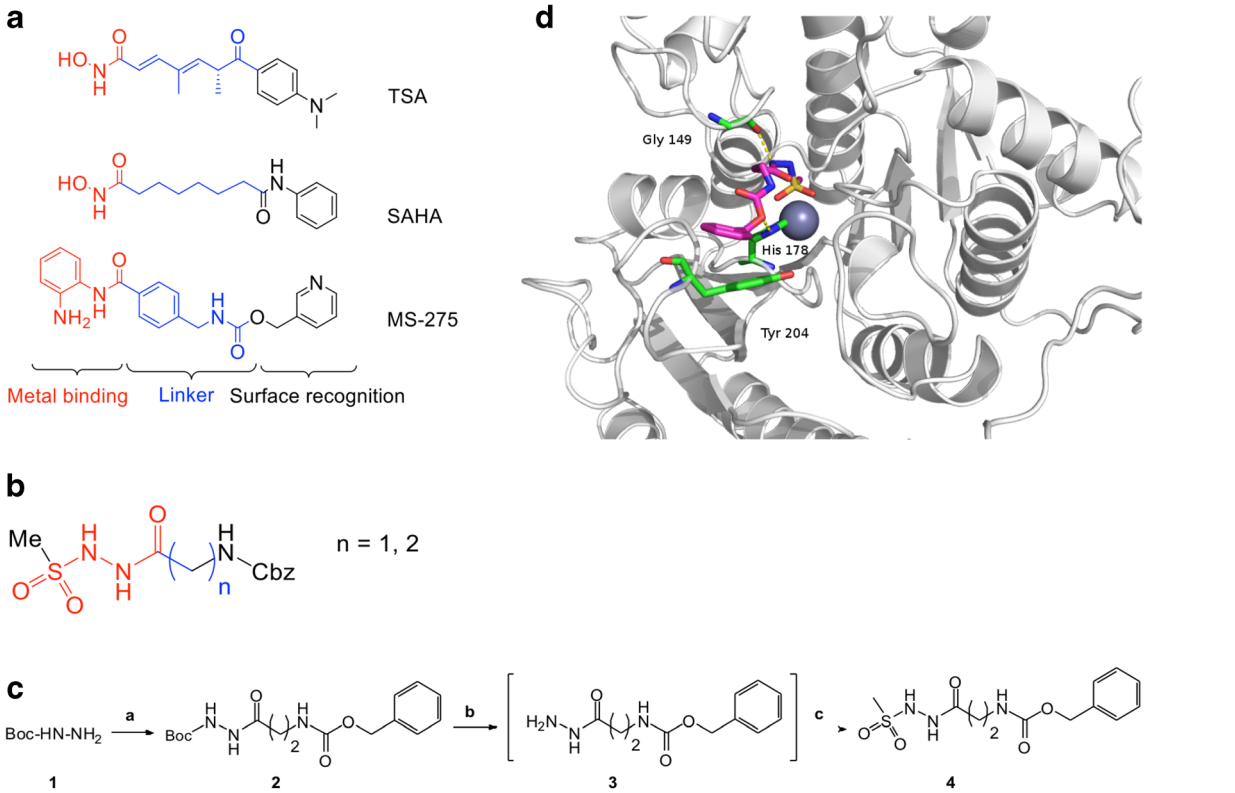

Fig. 1 **a** Pharmacophore of different HDACIs. **b** Structures of molecules envisioned as HDACIs. **c** Reagents and conditions: (a) Cbz-beta-Ala-OH, HBTU, 2,4,6-collidine, CH_2Cl_2, RT, 18 h, 48%; (b) TFA, CH_2Cl_2, RT, 1 h 100%; (c) Me-SO_2-Cl, Et_3N, THF, RT, 2 h, 50%. **d** detailed binding mode of the compound 4 with HDAC-1. HDAC-1 is in white cartoon representation. Compound 4 and residues implicated in the interaction are in stick representation. The carbon atoms of 4 are colored in magenta, and those of HDAC-1 are in green. The Zn atom is in gray sphere

with the active site, a linker domain, and a surface recognition group interacting with the residues on the rim of the active site (Fig. 1a) (Chavan and Somani 2010). In 2003, Auge et al. (2003) introduced a sulfonylhydrazide group (R–SO_2–NH–NH–CO–), as new zinc-binding group (ZBG) replacing the hydroxamate function and docking experiments of sulfonylhydrazide type inhibitors evidenced a NH and CO chelation with the Zn^{2+} ion similarly to hydroxamate ZBG. In the present work, we sought to evaluate whether sulfonylhydrazide ZBG-bearing derivatives may be considered as a novel class of HDACIs that may target HDAC-1 based on our structure-activity relationships and molecular docking experiments. To test this hypothesis, alkylsulfonylhydrazide chelators were introduced (Fig. 1b). Here, our primary aim was to test our new alkylsulfonylhydrazide-type compound (ASH), the benzyl 3-(2-(methylsulfonyl)hydrazinyl)-3-oxopropylcarbamate compound, targeting HDAC-1, on ethanol operant self-administration and relapse in rats. We also wanted to test whether the injection of the molecule directly in the central amygdala (CeA) could reduce relapse after an extinction of the behavior. The role of the CeA in the control of emotional behavior, anxiety, and relapse for drugs of abuse is well described in the literature (Roberto et al. 2012; Gilpin et al. 2015). For example, Pandey and colleagues (Pandey et al. 2008) demonstrated that the anxiolytic effect of acute exposure to ethanol is associated to an increase in the expression of Arc and BDNF within the central but not the basolateral amygdala. On the opposite, during withdrawal, when anxiety increases, expression of both genes is decreased and microinfusion of BDNF into CeA is able to attenuate anxiety-like behaviors during withdrawal. Moreover, several reports and reviews already implicated epigenetic mechanisms occurring within the CeA during alcohol exposure, consumption, and withdrawal (Moonat et al. 2013; Simon-O'Brien et al. 2015; Pandey et al. 2017). Thus, in order to evaluate the effect of a new HDACI on relapse after extinction, CeA was an obvious neurobiological substrate candidate to test our compound.

Materials and methods

Chemistry

All the reagents and the experiments carried out for chemistry and the synthesis of the compounds are detailed in supplementary informations.

HDAC assays

All details regarding HDAC assays are detailed in supplementary informations.

Docking

All details regarding docking studies are detailed in supplementary informations.

Binge drinking behavior in the operant self-administration paradigm

Animals

Male Long-Evans rats (320–345 g at the beginning of the experiment) were obtained from Charles River (L'Arbresle, France). Animals were individually housed under a light/dark cycle of 12 h (lights on at 7:00 am) with food and water available ad libitum. Experiments were carried out in accordance with the guidelines for Care and Use of Laboratory Animals (National Institutes of Health) and the European community regulations for animal use in research (CEE no. 86/609) and were approved by the local research ethics committee (CREMEAP; no. 260912-10). The total number of animals used in this study was 40 and we had to exclude a total of 10 rats (misplacement of the cannula or no acquisition of the operant task).

Reagents

ASH compound was dissolved to a concentration of 125, 250, and 500 μM in an artificial cerebrospinal fluid (aCSF) solution (CMA Microdialysis, Solna, Sweden). Ethanol 96% was purchased from VWR (Fontenay-sous-Bois, France). Phosphate-buffered saline (PBS) was made with NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, and KH_2PO_4 1.8 mM purchased from Sigma-Aldrich (Saint-Quentin Fallaviers, France). Paraformaldehyde (Sigma-Aldrich, Saint-Quentin Fallaviers, France) was dissolved in PBS at 4%. Sodium pentobarbital sodium (54.7 mg/100 mL) and buprenorphine (Buprecare) were purchased from Centravet (Dinan, France).

Ethanol self-administration

Naïve rats were exposed to a 20% intermittent access paradigm with access to two bottles: one containing tap water and the other a 20% ethanol solution, every other day in the homecage. This procedure induces escalation of ethanol consumption in several strains of rats. After 4 weeks of 20% intermittent access, rats were trained to self-administer ethanol. The self-administration chambers contained two levers: an active lever for which presses resulted in delivery of a 0.1 mL of fluid reward (a 20% ethanol solution) and an inactive lever, for which presses were counted, but no programmed events occurred. The effort required to obtain the reward was progressively increased (from one press to three consecutive presses to obtain the reward), and then the duration of the sessions were progressively decreased (from 1 h to 15 min). Animals were trained for at least 5 weeks before the beginning of the experimental manipulations. During the self-administration sessions, number of lever presses and number of ethanol deliveries were recorded using PackWin software (Bioseb, Vitrolles, France). This paradigm allows us to observe ethanol intake reaching a level near 1 g/kg/15 min which are above the intoxication levels.

Sucrose self-administration

Ten naïve rats were submitted to a procedure of self-administration of a 4% sucrose solution in operant chambers as described for the ethanol self-administration group. After two overnight sessions under a fixed ratio one schedule (one press on the active lever leads to a delivery of 0.1 mL of a 4% sucrose solution), rats underwent daily sessions of 1 h for 3–4 days under a FR3 schedule, 30 min and then 15 min for at least 2 weeks before the surgery aiming at the implantation of guide cannula targeting the lateral ventricle. Four rats had to be excluded from the study due to a lack of acquisition of the operant task.

Surgery

Stereotaxic surgery was used to implant each rat with one cannula (i.c.v. group) or two cannulae (central nucleus of amygdala, CeA group). Rats were continuously anesthetised with isoflurane during the surgery. Four holes were drilled for screws, one or two other holes were drilled for the placement of cannulae (26GA, 12 mm, Phymep). The coordinates for the lateral ventricle were −0.8 mm posterior to bregma, + 1.4 mm lateral to the medial suture, and −3 mm from the skull surface. The coordinates for the CeA were −1.80 mm from bregma, ± 4.0 mm laterally from median suture, and −6.0 mm from skull surface. The cannulae were fixed with dental cement. The injectors used for the microinfusions extended 1 mm below the tip of the cannula for the i.c.v. group and 2 mm for the CeA group. Subject weights were monitored daily after the surgery for five consecutive days to ensure recovery. One week after recovery, subjects returned to self-administration training and were habituated to the microinjection procedure with two sham injections. The experimental microinjections began upon acquisition of stable responding for ethanol.

Microinfusions

The intracerebral route of administration (i.c.v. and intra CeA) was preferred because of the synthetized quantity available, to avoid potential peripheral side effects and because we have previously shown that MS-275 has the same behavioral effect when injected either via the i.c.v. or the i.p. route (Simon-O'Brien et al.

2015). ASH compound (0, 150, 250, 500, or 1000 μM) was microinfused i.c.v. and intra-CeA 3 h before the beginning of the self-administration sessions (as we previously done in Jeanblanc et al. 2015). For the test of ASH on relapse, only the dose of 500 μM was microinfused. Microinfusions were performed with a Harvard pump 11 Plus Advanced (Phymep, France) and 25-mL Hamilton syringes (1702 N, Bonaduz, Switzerland). Injectors were built using stainless steel tubing (outer diameter 0.229 mm, inner diameter 0.127 mm, Phymep). ASH compound was microinfused at a speed of 1 μL/min for the i.c.v. group (2 μL total) and at 0.5 μL/min for the CeA group (0.3 μL). All the injections were performed following a Latin square design.

Progressive ratio

To evaluate the effect of ASH compound on the motivation to consume alcohol, we performed a progressive ratio test. In this test, the delivery of each reward requires an increased number of active lever presses for still the same volume of 0.1 mL of the 20% ethanol solution. The schedule of increase is as follows: 3, 4, 5, 7, 9, 12, 15, 17, 20, 22, 25, 28, 30, 33, and 35. The breaking point, i.e., the maximum effort rats are willing to make to obtain a single reward reflects the level of motivation to consume the drug. ASH compound (500 μM) was i.c.v. microinfused 3 h before the session.

Relapse after extinction

After several weeks of self-administration, rats were trained to extinguish their pressing behavior during sessions in which both levers were present, but the presses on any of them led to no delivery of ethanol. After 1 week, the number of active lever presses decreased, and for each animal, the threshold determining the extinction was set to 10% of the baseline for three consecutive days. The relapse test is based on the reacquisition paradigm in which ethanol is available again (under a FR3 schedule) after a free prime of 0.1 mL. ASH compound was microinfused into the CeA 3 h prior to the test.

Histology

Rats were deeply anesthetized with pentobarbital and were submitted to a transcardiac perfusion with 50 mL of PBS and 50 mL of PFA 4%. Brains were stored in a sucrose 20%/PFA 4% solution for 48 h. Brains were then cut in slices of 100 μm of thickness for histological verification of the cannulae placements.

Statistical analysis

Data were analyzed by one-way ANOVA with repeated measures followed by a Tukey post hoc test when indicated by significant effects of treatments or interactions. For simple comparisons, data were analyzed by a Student's *t* test. Significance for all tests was set at $p < 0.05$.

Results

Chemistry

The coupling was carried out between *tert*-butylcarbazate and Cbz-beta-alanine with HBTU as coupling agent in the presence of 2,4,6-collidine. The product **2** was obtained with a yield of 48%. Deprotection of the Boc protective group was performed with TFA and the compound was obtained in quantitative yield. Then, the reaction with methanesulfonyl chloride in THF with Et_3N allowed us to obtain compounds **4** (alkylsulfonylhydrazide-type compound (ASH) for benzyl 3-(2-(methylsulfonyl)hydrazinyl)-3-oxopropylcarbamate) in 50% yield (Fig. 1c).

HDAC assays

The inhibitory activity of compound **4** (ASH) towards HDACs from a total nuclear extract using a HDAC colorimetric assay kit (Active Motif, Inc., Belgium) was performed. Half maximal inhibitory concentration (IC_{50}) of ASH was estimated to 8 mM. The ASH compound decreased the emitted fluorescence displaying more than 51% of HDAC-1 inhibitory activity.

Docking

Based on the blind docking experiments, compound **4** has the best affinity for HDAC-1 in the vicinity of the catalytic site. Further docking experiments with a better accurately were therefore focused on the catalytic site to identify the most likely binding mode between HDAC-1 and compound **4**. The analysis of docking results shows that the lowest energy models are stabilized by the chelation of Zn atom by the sulfonyl group (Fig. 1d) and by insertion of the methyl group of the sulfonylhydrazide moiety into a hydrophobic pocket formed by Met 30, Gly 138, Leu 139, Phe 150, Gln 260, Gly 300, Gly 301, and Tyr 303. Two hydrogen bonds are observable, the first one between the imidazole ring of His 178 and the oxygen atom of the carbamate function and the second one between the carbonyl group of Gly 149 and the NH group close to the carbonyl of the sulfonylhydrazide moiety. Moreover, the binding mode is stabilized by a π-stacking interaction between the phenyl group of **4** and the aromatic ring of Tyr 204 sidechain.

Histology

No rats were excluded from the i.c.v. study due to misplacement of the cannulae, while two rats had to be excluded from the analysis of the intra-CeA study. Schematic representation of the

placements of the different cannula is shown in Supplementary Fig. 1 (a) for the i.c.v. group and (b) for the intra-CeA group.

ASH compound decreases ethanol self-administration

We tested the effect of ASH compound on operant ethanol self-administration in an original model of voluntary binge drinking in rats. We used a specific behavioral paradigm to achieve high voluntary ethanol intake to mimic binge drinking. In this model, rats drink more than 0.8 g of pure ethanol per kilogram of body weight in only 15 min and display clear signs of intoxications such as sedation and ataxia at the end of the 15-min session. Intracerebroventricular injections of ASH compound were performed 3 h prior to the self-administration sessions as we have already performed in our previous studies with MS-275 (Jeanblanc et al. 2015). This delay was chosen because it allows changes in gene expression to occur. Each rat ($n = 12$) received a microinfusion of each ASH compound concentration with a 1-week period of washout between two microinfusions. As shown in Fig. 2a, we observed a dose-dependent decrease in the number of active lever presses to obtain the delivery of alcohol. A one-way ANOVA with repeated measures indicated a main effect of the factor treatment ($F(4, 44) = 5.24$, $p = 0.002$). The post hoc test (Tukey) conducted on the data revealed significant differences between the group aCSF and the dose of 500 μM ($p < 0.05$). No other doses were significantly different from aCSF (all p's > 0.05). Significant differences were observed between the group 125 μM and both 250 and 500 μM groups. The total amount of alcohol consumed during these 15-min sessions was analyzed using a one-way ANOVA with repeated measures (Fig. 2b). This analysis indicated a significant effect of the factor treatment ($F(4, 44) = 5.46$, $p < 0.001$). The post hoc test revealed a significant difference between the aCSF group and the 500 μM group ($p < 0.05$). No other group was significantly different from the aCSF group (all p's > 0.05). Significant differences were observed between the group 125 μM and both 250 and 500 μM groups. The test also revealed a significant difference between the 500 and the 1000 μM groups. Further analysis of the pattern of drinking for the aCSF group and the 500 μM group indicates that there is no difference between both groups as regards with the latency to obtain the first reward ($p > 0.05$, Fig. 2c). On the opposite, the latency to the delivery of the last reward is significantly shorter for the group 500 μM as compared to the aCSF group ($p < 0.05$, Fig. 2d). In addition, and as a consequence, the duration of the consummatory episode is shorter for the 500 μM group ($p < 0.05$, Fig. 2e). The analysis of drinking pattern after the 500 μM dose of ASH compound microinfusion was also conducted using the cumulative deliveries. For this analysis, only rats showing at least one delivery during the session were included ($n = 10$). As shown in Fig. 2f, the initiation of the drinking episode is similar in both

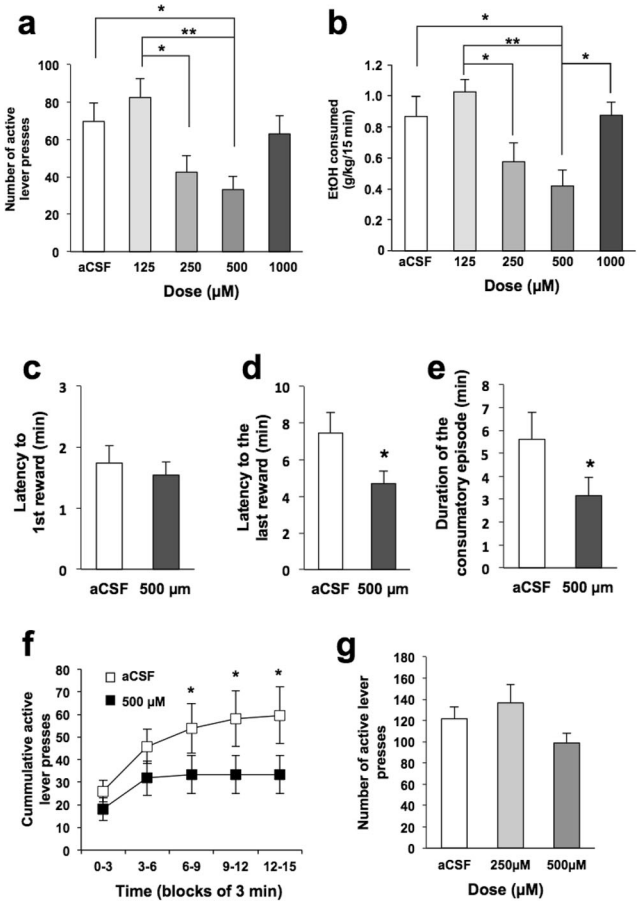

Fig. 2 The ASH compound reduces alcohol self-administration. **a** The number of active lever presses is dose-dependently reduced after the i.c.v. microinfusion of the ASH compound. Results are expressed as mean ± SEM of active lever presses. $n = 12$, $^*p < 0.05$, $^{**}p < 0.01$. **b** The total amount of alcohol consumed during the 15-min session is dose-dependently reduced after the i.c.v. microinfusion of the ASH compound. Results are expressed as mean ± SEM of pure ethanol consumed in g/kg/15 min. $n = 12$, $^*p < 0.05$, $^{**}p < 0.01$. The most efficient dose of ASH compound (500 μM) does not alter the latency for the first press but leads to a premature end **d** and a reduced duration of the drinking episode. **e** Results are expressed as mean ± SEM in minutes. $n = 11$, $^*p < 0.05$. One rat was removed from this analysis because it did not obtain any reward over the 15-min session. **f** The ASH compound modifies the pattern of drinking over the 15-min session. **g** Number of active lever presses for 4% sucrose. Results are expressed as mean ± SEM of cumulative active lever presses. $n = 12$, $^*p < 0.05$

groups (aCSF and ASH compound at 500 μM). It is only after few deliveries that ASH compound blocked the increase in ethanol consumption along the self-administration session. The two-way repeated measures ANOVA conducted on the data revealed a main effect of the factor treatment ($F(1,36) = 5.11$, $p = 0.050$), of the factor time ($F(4,36) = 12.40$, $p < 0.001$), and indicated an interaction between both factors ($F(4,36) = 2.79$, $p < 0.05$). The post hoc test indicated a significant difference between groups aCSF and ASH 500 μM for the time points 6–9, 9–12, and 12–15 min (all p's < 0.05). ASH compound (250 and 500 μM) was i.c.v. microinfused in rats trained to self-administer 4% sucrose and show no effect

on the level of sucrose self-administration (Fig. 2g). A one-way ANOVA with repeated measures analysis revealed no effect of the factor treatment ($F(2,10) = 1,88$, $p > 0.05$).

Rats underwent to a regular 15-min session of ethanol self-administration the day after the microinfusion (Fig. 3a), and the results indicate that there is no more differences between the groups ($F(4, 44) = 0.16$, $p > 0.05$).

ASH compound does not alter the motivation to consume ethanol

Motivation to consume was evaluated during a progressive ratio protocol in which the effort to obtain a delivery of ethanol increases progressively after each delivery. We observed (Fig. 3b) that the microinfusion of the dose of 500 μM has no effect (Student's t test, $p > 0.05$) on the value of the breaking point.

ASH compound microinfused within the CeA does not alter ethanol consumption but reduces relapse after extinction

The dose of 500 μM was microinfused 3 h prior to the self-administration sessions and had no effect on the total amount of ethanol consumed (Fig. 4a, $p > 0.05$). Rats were then submitted to seven sessions of extinction (Fig. 4b) and re-exposed to ethanol during a re-acquisition session. We found that the aCSF group relapsed moderately to about 50% of their previous baseline of ethanol consumption (0.53 g/kg/15 min vs. 0.91 g/kg/15 min). The ASH compound group relapsed significantly less than the aCSF group with an average of 0.22 g/kg/15 min ($p < 0.05$, Fig. 4c).

Discussion

Acute and chronic ethanol intake has been shown to change HDAC gene expression within the peripheral blood in both rats and humans (López-Moreno et al. 2015). A large body of evidence suggests that epigenetic regulation of gene expression, including histone acetylation and deacetylation, contributes to the development of ethanol reward-related behaviors linked to ethanol addiction (Pandey et al. 2008; Bourguet et al. 2018; Pandey et al. 2017). Recent studies have also suggested that targeting a specific HDAC class and isoenzyme such as HDAC-1 (Jeanblanc et al. 2015; Bourguet et al. 2018) or HDAC-2 (Sakharkar et al. 2014) can reduce ethanol-related behaviors. Numerous studies that have investigated the role of HDAC in ethanol-related behaviors used non-specific HDACIs, and thus further studies are needed in order to decipher which HDAC isoenzyme may play a role in particular components of ethanol addiction. Finding new epigenetic drugs targeting specific HDAC isoenzyme could be useful in order to avoid toxic side effects that are currently seen with available HDACIs. Because MS-275, suggested as more selective inhibitor of HDAC-1, has been shown to have promising potential to treat ethanol-related behaviors but may suffer from its poor brain penetration capacity, there is an interest to further develop new compounds targeting HDAC-1. In the present study, using docking and enzyme activity tests, we identified an alkylsulfonylhydrazide-type compound as a good inhibitor of HDAC-1. We found that administration of ASH via the i.c.v. route, but not directly in the CeA, decreased ethanol operant self-administration. As shown with other HDACIs, the effect of ASH lasted only the first day of administration. Moreover, when injected directly in the CeA, ASH decreased resumption of ethanol self-administration after abstinence.

After the identification of the ASH compound as a preferentially inhibitor of HDAC-1 isoform, we evaluated its efficacy on ethanol-related behaviors. We used the operant self-administration procedure in an animal model of binge-like consumption, i.e., intake of large quantity of ethanol during a short period of time. First, we tested the dose-response effect of the ASH compound on operant self-administration when microinfused 3 h prior to the sessions via the i.c.v. route, and

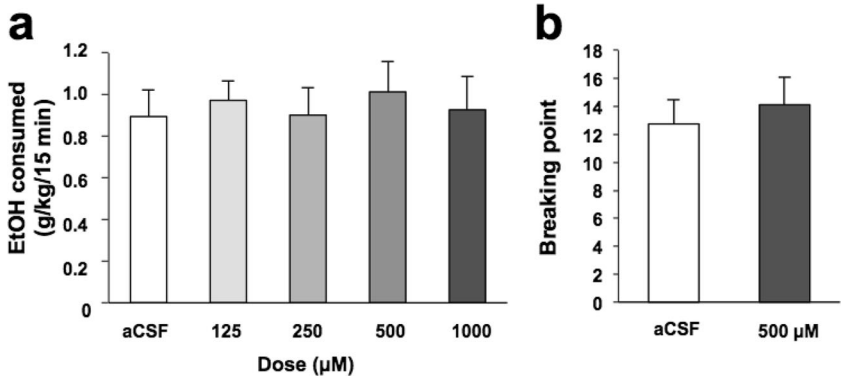

Fig. 3 **a** The ASH compound has no more effect on alcohol self-administration 27 h after its i.c.v. microinfusion. Results are expressed as mean ± SEM of pure ethanol consumed in g/kg/15 min. $n = 12$. **b** The ASH compound, at the dose of 500 μM i.c.v. administrated, does not modify the motivation to consume alcohol. Results are expressed as mean ± SEM of the breaking point meaning the maximum effort a rat is willing to make to obtain one single reward (0.1 mL of 20% ethanol). $n = 8$

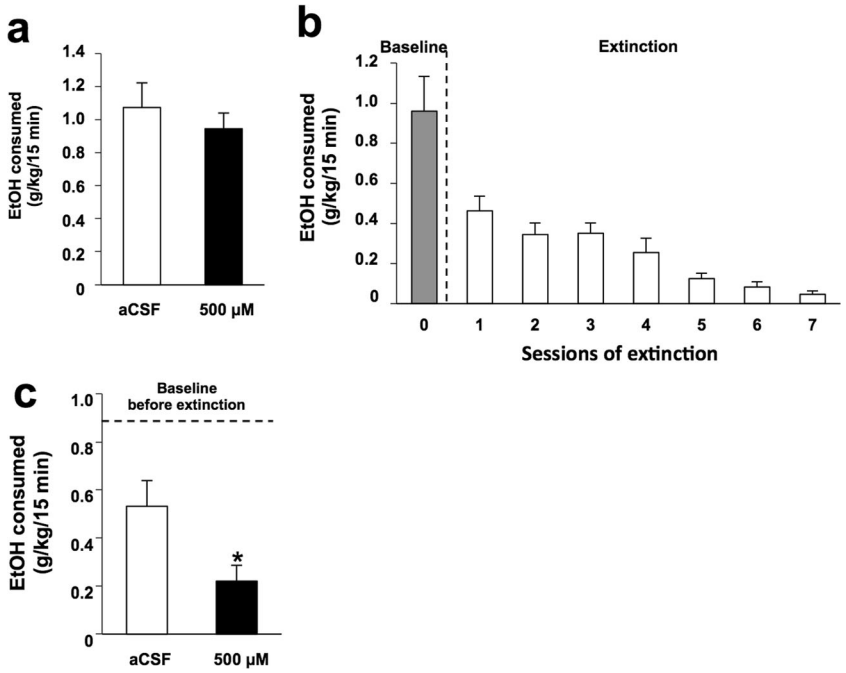

Fig. 4 **a** Intra-CeA microinfusion of ASH compound (500 µM) does not alter alcohol self-administration. Results are expressed as mean ± SEM of pure ethanol consumed in g/kg/15 min. $n = 12$. **b** Rats extinguished their pressing behavior after seven consecutive days of extinction sessions in which the levers were available, but no alcohol was delivered at any time. The extinction criterion was set to three consecutive sessions with a level of consumption below 10% of the baseline level when alcohol is available. Results are expressed as mean ± SEM of pure ethanol consumed in g/kg/15 min. $n = 12$. **c** ASH compound microinfused into the CeA 3 h prior to the test reduces the relapse after a period of abstinence (extinction). Results are expressed as mean ± SEM of pure ethanol consumed in g/kg/15 min. $n = 10$, $^*p < 0.05$

we observed a *U* shape curve with the maximum efficacy (−55% of ethanol consumed) at the dose of 500 µM, while an absence of effect was also observed at the highest dose (1000 µM). All the different HDACIs so far identified are not specific to only one HDAC isoform but have different affinity for several isoforms. It cannot be ruled out that the absence of effect at the highest dose of our ASH compound is due to its action through other HDAC isoforms than the HDAC-1. By analyzing the pattern of drinking, by the mean of the latencies to the first reward, the delay to obtain the last reward and the profile of the cumulative ethanol deliveries, we can conclude that the ASH compound does not alter the initiation of the consumption episode but reduces its total duration by inducing its premature termination. Unlike MS-275 that postponed the initiation of the drinking episode (Jeanblanc et al. 2015), ASH compound produces its effect only after a certain amount of ethanol consumed. In addition, we found that the ASH compound has no effect on the motivation to consume ethanol in the progressive ratio schedule. All together, these results suggest that the ASH compound does not affect the motivation to consume ethanol and that either there is an aversive effect due to the ASH compound in the presence of ethanol which makes the rats stop pressing to obtain more ethanol or there is a substitutive effect of our ASH compound since ethanol exposure leads to a hyperacetylation of various proteins including histones (Shepard and Tuma 2009).

Interestingly, the overall effect of our ASH compound is similar to the one observed with the MS-275 which is described as a HDAC-1, HDAC-3, HDAC-8, and HDAC-9 on the reduction of alcohol self-administration (Jeanblanc et al. 2015). Here, with the ASH compound, the effect occurs after the first administration, whereas with the MS-275, two consecutive daily microinfusions were necessary to significantly decrease ethanol self-administration. However, the mechanisms leading to the reduction of ethanol consumption of these two drugs seem different. Indeed, MS-275 reduces ethanol consumption by a reduction of the motivation to consume ethanol, whereas the ASH compound has no effect on the motivation but stop the drinking episode after a certain amount of consumed ethanol. These differences could be due to a difference in the inhibitory spectrum towards HDACs isoforms. In addition, we found that our ASH compound has no effect on sucrose self-administration suggesting that it does not have any locomotor effects. Moreover, this absence of effect may suggest a specific effect to alcohol or drug of abuse and not a general effect on all the rewarding substances.

One of the major issues in the treatment of patients with AUDs is the maintenance of the abstinence period and thus to avoid the occurrence of relapse. The withdrawal-induced anxiety is often described as a main reason for the relapse (Becker 2008; Simioni et al. 2012), and several studies have implicated

epigenetic mechanisms in a specific brain structure, namely the central amygdala, as a key factor for relapse (Silberman et al. 2009; Gilpin et al. 2015). We tested here the efficacy of the ASH compound on both ethanol self-administration and on relapse after a period of abstinence. Interestingly, the microinfusion directly into the CeA has no effect on the total amount of ethanol consumed in a regular self-administration session. This results suggest either that epigenetic modifications induced by ASH within the CeA has no role in the control of ethanol consumption or that the final quantity of ASH is high for a small structure such as CeA and thus that we are on the ascending arm of the "*U* shape" curve observed in Fig. 2a, b (the total amount of ASH compound is about 315 ng when i.c.v. microinfused, while it is of about 45 ng for the intra CeA microinfusion).

On the other hand, after a week of abstinence conducted in a behavioral extinction paradigm, we performed a relapse test using a re-acquisition protocol in which one delivery of ethanol is given "for free" before the animals can again get as much ethanol as they want by pressing the active lever three times during the 15-min session. Control animals relapse to half of their pre-extinction baseline level, whereas animals treated with ASH only relapse at 20% of their pre-extinction baseline level.

Thus, we can conclude that the ASH compound has a rapid but transient effect on ethanol consumption after a single administration. This decrease in ethanol consumption is not mediated by a decrease in the motivation to consume ethanol but appears after a certain amount of ethanol consumed as if there was a "STOP" signal leading to the early termination of the drinking episode. Although the overall effect on ethanol use appears to be identical, the underlying mechanisms are certainly different as the ASH compound and the MS-275 does not have the same effects on the motivation to consume ethanol and the consumption profile. It is possible that, due to the difference of the two compounds regarding HDAC specificity, they do not target the same molecular mechanisms. The question of specificity supports the interest of pursuing research in the synthesis of new ultra-specific compounds of each of the different HDAC isoforms in order to really succeed from a purely fundamental point of view to identify the role of each HDAC in addictive behaviors and on the other hand, from a clinical point of view, choosing the treatment to the patients according to their addictive symptomatology (therapeutic objective oriented either towards the reduction of the consumption, or the maintenance of abstinence, or the reduction in craving, or the decreased in motivation to consume drugs, etc.). Moreover, we show that the administration of an HDAC-1 inhibitor directly into the CeA nucleus makes it possible to reduce relapse.

Our current observations raise a number of interesting questions for future study. The mechanism by which an HDAC inhibitor can reduce ethanol self-administration 3 h after a single injection needs to be further investigated and especially regarding the genes whose expression is change and that may be involved in gating ethanol intake. In the same vein, the effect of the HDAC inhibitor does not last 1 day after treatment, and thus, it could be very interesting to explore the effect of a chronic treatment. The mechanistic knowledge will help develop new therapeutic interventions targeting specific components of ethanol addiction.

Conclusion

In conclusion, we report for the first time the interest of new alkylsulfonylhydrazide compound in the treatment of AUDs. In the animal model of voluntary operant ethanol binge drinking, only one i.c.v. microinjection of the ASH compound significantly inhibited ethanol intake, whereas two consecutive injections were necessary with other HDACIs (Jeanblanc et al. 2015; Simon-O'Brien et al. 2015). Our ASH compound is not only able to reduce ethanol self-administration but also it significantly reduced relapse when directly injected into the CeA. In addition, we demonstrate that the mechanism of action of our ASH is different to the MS-275 one despite the chemical similarity suggesting a difference in the molecular targets. On the basis of these results, the design of next generation of alkylsulfonylhydrazide will be engaged to unravel the exact mechanism of action of this type of molecules.

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Compliance with ethical standards

Experiments were carried out in accordance with the guidelines for Care and Use of Laboratory Animals (National Institutes of Health) and the European community regulations for animal use in research (CEE no. 86/609) and were approved by the local research ethics committee (CREMEAP; no. 260912-10).

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