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Article title: GET73 Prevents Ethanol-Induced Neurotoxicity in Primary Cultures of Rat Hippocampal Neurons

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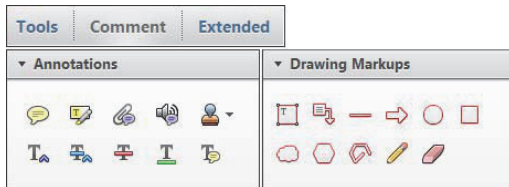
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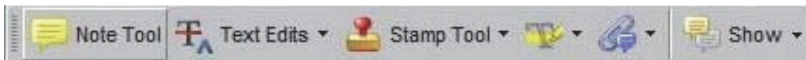
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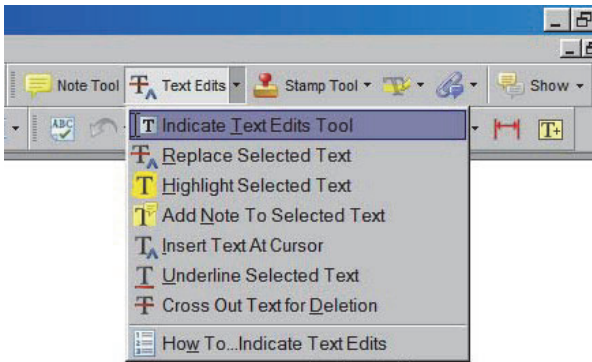
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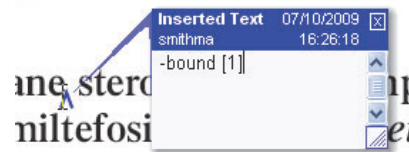
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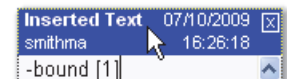
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Article

GET73 Prevents Ethanol-Induced Neurotoxicity in Primary Cultures of Rat Hippocampal Neurons

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Abstract

Aims: N-[(4-trifluoromethyl) benzyl] 4-methoxybutyramide (GET73) may be considered a promising therapeutic agent for the treatment of alcohol use disorders. The compound displayed anti-alcohol and anxiolytic properties in rat. In the present study, an *in vitro* experimental model of chronic ethanol treatment was used to investigate the ability of the compound to counteract the ethanol-induced neurotoxicity.

Methods: Primary cultures of rat hippocampal neurons were exposed to ethanol (75 mM; 4 days) and the neuroprotective effects of GET73 were assessed by evaluating cell viability, cell morphology, glutamate levels and reactive oxygen species production.

Results: The exposure to ethanol induced a reduction of cell viability, an alteration of cytoskeleton, a decrease in extracellular glutamate levels and an increase of reactive oxygen species production. The addition of GET73 (1 and 10 μ M) 1 h before and during chronic ethanol exposure prevented all the above ethanol-induced effects. Based on the proposed GET73 mechanism of action, the effects of mGlu5 receptor negative allosteric modulator, 2-methyl-6-(phenylethynyl)-pyridine (MPEP), on ethanol-induced reduction of cell viability were also assessed. The results indicated that the addition of MPEP (100 μ M) 1 h before and during chronic ethanol exposure prevented the ethanol-induced cell viability reduction.

Conclusion: The present findings provide the first evidence that GET73 shows a neuroprotective role against ethanol-induced neurotoxicity in primary cultures of rat hippocampal neurons. Together with previous findings, these results suggest that GET73 possesses multifaceted properties thus lending further support to the significance of developing GET73 as a therapeutic tool for use in the treatment of alcohol use disorders.

INTRODUCTION

The brain is a major target for ethanol and the detrimental effects of acute and chronic ethanol exposure on different functions of the central nervous system (CNS) have been well documented (Zahr *et al.*, 2011; Hermens and Logopoulos, 2013; Kim *et al.*, 2014). Chronic ethanol consumption is associated with severe and persistent changes

in brain physiology and morphology, impairment of cognition along with numerous clinical and neurological disorders (Bernardin *et al.*, 2014; Wilcox *et al.*, 2014; Zorumski *et al.*, 2014). The cognitive impairment observed in individuals affected by alcohol use disorders (AUDs) are thought to be related, at least partially, to hippocampal structural integrity disruption (Mechtcheriakov *et al.*, 2007; Geil *et al.*, 2014;

Zorumski *et al.*, 2014). There is considerable evidence that excessive ethanol intake is correlated with hippocampal damage. For example, early onset drinking has been shown to be associated with hippocampal volumetric deficits, while volume loss of hippocampus was reported in adolescent with AUDs and in older alcoholics (Nagel *et al.*, 2005; Ozsoy *et al.*, 2013). These alterations may be also due to a loss of neurons, shrinkage of neuronal cell bodies and/or reduction in the number and extent of dendrites (Kruman *et al.*, 2012). In line with this view, a direct neurotoxic effect of ethanol on the brain has been suggested as the primary cause of alcohol-related neuronal loss (Sun and Sun, 2001; Kruman *et al.*, 2012).

The pharmacological treatment of AUDs, mainly aimed at reducing craving and withdrawal symptoms (tremor, agitation and delirium) often showed a moderate efficacy. At present, only few pharmacological treatments have been approved for clinical use, including the FDA approved drugs disulfiram, naltrexone, acamprostate (O'Malley and O'Connor 2011; Wackernah *et al.*, 2014), nalmefene (Soyka, 2014) recently approved by the European Medicines Agency (EMA), and gamma-hydroxybutyric acid (GHB) employed in some European countries (Addolorato *et al.*, 2009; Leone *et al.*, 2010). The research in this field is then focused on the development of new and potentially effective pharmacological agents, through the study of alternative molecular targets involved in AUDs. Among these new substances, previous behavioral and neurochemical studies suggested that N-[(4-trifluoromethyl)benzyl]4-methoxybutyramide (GET73) could be considered a hopeful candidate. Indeed, GET73 has shown a multifaceted neuropharmacological profile, including the ability to reduce both alcohol consumption and anxiety-related behaviors in rats (Loche *et al.*, 2012; Ferraro *et al.*, 2013). It has been proposed that GET73 may partially act modulating hippocampal glutamate and GABA transmission, possibly through an allosteric modulation of metabotropic glutamate receptor 5 (mGlu5 receptor) (Ferraro *et al.*, 2011, 2013; Beggiano *et al.*, 2013). In this context, it is worth noting that negative allosteric modulators (NAMs) of mGlu5 receptors exert neuroprotective actions (Bruno *et al.*, 2000; Sarnico *et al.*, 2008; Yeganeh *et al.*, 2013). Furthermore, the mGlu5 receptor NAM 2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine (MTEP), at doses attenuating ethanol withdrawal seizures, also *in vitro* exhibited neuroprotective effects in organotypic hippocampal cultures. As it has been postulated that the recurrent transient increase in extracellular glutamate levels associated with alcohol withdrawal could eventually result in a glutamate-induced neurotoxic effect, it has been proposed that a neuroprotective effect of MTEP might contribute toward attenuating alcohol withdrawal symptoms (Kotlinska *et al.*, 2011). Thus, in view of the postulated mechanism of action of GET73 it becomes relevant to evaluate its possible neuroprotective role against the ethanol-induced neurotoxicity, an aspect that could add interesting therapeutic perspectives to the compound.

The cellular mechanisms underlying the chronic ethanol-induced disruption of neuronal outgrowth are difficult to be investigated in whole animal models, because of complexity of existing cellular networks constituted by different cell types, such as neurons and glial cells. On the contrary, ethanol's effects may be easily investigated in *in vitro* cell culture systems, such as primary cultures of rat hippocampal neurons, where the experiments can be carried out under strictly controlled conditions. Indeed, the ethanol concentrations can be easily controlled in this cell culture model and the direct actions of the compound on a selected neuronal population could be assessed, without the interference of oncoming signaling from other CNS regions (Gruol and Parsons, 1996). In view of the above, in the present study an *in vitro* experimental model of chronic ethanol treatment

was used to investigate the ability of GET73 to counteract the ethanol-induced neurotoxicity. In particular, primary cultures of rat hippocampal neurons were exposed to 75 mM ethanol for 4 days, and the neuroprotective effects of GET73 were assessed by evaluating cell viability, cell morphology and glutamate levels. Finally, as there is strong evidence showing that chronic ethanol consumption induces oxidative neuronal damages (Haorah *et al.*, 2008; Collins and Neafsey, 2012; Moon *et al.*, 2014), the effects of GET73 against ethanol-induced reactive oxygen species (ROS) production were also investigated.

METHODS

Primary cultures of rat hippocampal neurons

Primary cultures of hippocampal neurons were prepared from 1-day-old rats born from Sprague-Dawley dams (Harlan SRC, Milan, Italy). Animals were sacrificed under anesthesia by decapitation. Removed hippocampi were dissected free of meninges and dissociated in 0.025% (w/v) trypsin. The tissue fragments were mechanically dissociated by repeated gentle pipetting in culture medium (Neurobasal medium with supplements of 0.1 mM glutamine, 10 µg/ml gentamicin, and 2% B27). Cells were counted and then plated on poly-L-lysine (5 µg/ml)-coated multiwells (24 wells; Nunc A/S, Roskilde, Denmark) at a density of 400,000 cells per well and on 96-wells at a density of 50,000 cells per well. For immunocytochemistry and ROS determination, the cells were plated on glass coverslips at a concentration of 200,000 cells per well. Cultures were grown at 37°C in a humidified atmosphere, 5% CO₂/95% air. Cytosine arabinoside (10 µM; Sigma Chemical Co.) was added within 24 h of plating to prevent glial cell proliferation. After 4 days of *in vitro* incubation (DIV), cultures were used for ethanol treatment.

Experiments were carried out in strict accordance with the European Communities Council Directive (86/609/EEC) and the Guidelines released by the Italian Ministry of Health (D.L. 116/92) and (D.L. 111/94-B). A formal approval to conduct the experiments described was obtained by the local Ethics Committee (University of Ferrara, Italy). Efforts were made to minimize the number of animals used and to reduce pain and discomfort. A total of 86 pups were used.

Exposure to ethanol and pharmacological treatment

To analyze the possible neurotoxic effect of ethanol, a subset of hippocampal cell cultures were treated with 75 mM ethanol for 4 days (from 4 to 7 DIV; Kane *et al.*, 2011; Gonthier *et al.*, 2012). During the period of ethanol exposure, cultured neurons were incubated at 37°C in closed chamber equilibrated with 5% CO₂ and 75 mM ethanol in water to stably maintain the concentration of ethanol in the culture medium. In parallel, cell cultures not exposed to ethanol (control cultures) were incubated at 37°C in a humidified atmosphere with 5% CO₂/95% air. GET73 was tested either in control or in ethanol-exposed cell cultures. In the latter case, GET73 (0.1, 1 and 10 µM) was added 1 h prior to ethanol exposure and maintained in contact with neurons during ethanol exposure. The mGlu5 receptor negative allosteric modulator (NAM) 2-methyl-6-(phenylethynyl)-pyridine (MPEP; Gasparini *et al.*, 2001), was also tested in a series of cell viability experiments (MTT assay) in cultured cells. The compound (10 and 100 µM) was applied either alone or 1 h prior to ethanol exposure. GET73 and MPEP concentration ranges were chosen on the basis of previous experiments demonstrating a possible interaction between GET73 and mGlu5 receptor-mediated modulation of glutamate transmission in rat hippocampal slices (Ferraro *et al.*, 2011).

All the experimental measures were performed at the end of ethanol exposure (8 DIV).

Immunocytochemistry

On the day of the experiment, cells were rinsed in 0.1 M phosphate-buffered saline (PBS) and then fixed with 4% paraformaldehyde in Sorensen's buffer 0.1 M, pH 7.4, for 20 min. After rinsing in PBS (three times for 5 min each), the cells were incubated overnight at 4°C in 0.3% Triton X-100/PBS solution (v/v) containing the primary antibody rabbit anti-microtubule-associated protein 2 (MAP2) (1:1000 dilution, Chemicon, Temecula, CA, USA). The cells were then washed three times with PBS and incubated for 60 min at room temperature with the secondary antibody rhodamine-conjugated anti-rabbit antibody (1:100 dilution, Chemicon, Temecula, CA, USA). After three washes in PBS, the cells were mounted in glycerol and PBS (3:1, v/v) containing 0.1% 1,4-phenylenediamine and examined using a Nikon Microphot FXA microscope. For cell counts, five separate non-overlapping fields were randomly chosen in each coverslip and the images were taken using the $\times 20$ objective.

Extracellular glutamate levels

On the day of the experiment, cells were rinsed twice by replacing the culture medium with a Krebs Ringer-bicarbonate buffer (mM: NaCl 118.5, KCl 4.8, CaCl₂ 2.5, MgSO₄ 1.2, NaHCO₃ 25, NaH₂PO₄ 1.2, glucose 11, pH 7.4; 37°C). Thereafter, 400 μ l of this solution were added to each plate and, after 30 min, 200 μ l of the solution were collected. After rinsing, the procedure was repeated twice to collect a total of three consecutive 30 min fractions. Glutamate levels in each sample were quantified by using a high-performance liquid chromatography/fluorimetric detection system, including a precolumn derivatization o-phthaldialdehyde reagent and a Chromsep 5 (C18) column. The mobile phase consisted of 0.1 M sodium acetate, 10% methanol, and 2.5% tetrahydrofuran, pH 6.5. The limit of detection for glutamate was 30 fmol per sample (Tomasini *et al.*, 2012). In each cell plate, basal extracellular glutamate levels were calculated by the mean of the three collected fractions. The effects of the treatments on glutamate levels were expressed as the percentage of glutamate levels measured in control cell cultures.

MTT assay

The integrity of mitochondrial enzymes in viable neurons was determined with a colorimetric assay using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) levels. In live cells, mitochondrial enzymes have the capacity to transform MTT 0.5 mg/ml for 3 h at 37°C. The medium was removed and the insoluble product was dissolved in 100 μ l of DMSO (1 h of shaking at room temperature), and colorimetrically (absorbance at 570 nm) quantified. Neuronal viability corresponded to the value of the optical density read at 570 nm using a microplate reader. The results were expressed as the percentage of neuronal viability measured in control cell cultures (100%).

ROS formation

Intracellular ROS can be evaluated by assessment of fluorescence emission of dihydrorhodamine 123 (DHR) that is converted by ROS to rhodamine 123, a fluorescent compound that is considered as a sensitive indicator of ROS production (Brito *et al.*, 2008). Following experimental treatments, cells plated on glass coverslips were loaded with 3 μ M DHR in DMSO (0.5% final concentration) for 30 min at 37°C. At the end of the incubation period, cells were fixed with 4%

paraformaldehyde in PBS, washed with PBS and mounted in glycerol and PBS (3:1, v/v). Cellular fluorescence was measured using a Nikon Microphot FXA microscope in at least five random microscopic fields per sample with an image analyzer software. The results were expressed as percentage of ROS production measured in control cell cultures (Silva *et al.*, 2006).

Materials

The culture dishes were purchased from Nunc A/S (Roskilde, Denmark). Neurobasal medium and B27 were obtained from Gibco (Grand Island, NY, USA). Poly-L-lysine, trypsin, cytosine arabinoside, gentamicin sulfate, glutamine, L-glutamic acid and MTT were obtained from Sigma Chemical Co., St Louis, Missouri. Dihydrorhodamine 123 (DHR) was acquired from Life Technologies (ThermoFisher Scientific Waltham, MA, USA). Anti-MAP2 antibody and rhodamine-conjugated anti-rabbit antibody were purchased from Chemicon. 2-methyl-6-(phenylethynyl)-pyridine (MPEP) was purchased from Tocris-Cookson (Bristol, UK) while 4-Trifluoromethylbenzyl-4-methoxybutanamide (GET73) was a generous gift from CT laboratories (Sanremo, Italy). The compounds were dissolved in DMSO (stock solutions = 1 mg/ml) and then added to the culture medium (maximum final DMSO concentration in experiments was 0.001% v/v); by itself, DMSO did not induce any changes in glutamate efflux, mitochondrial enzymes activity, ROS production and MAP2 immunoreactivity.

Statistical analysis

Results were expressed as means \pm standard error of mean. The statistical analysis was carried out by Student's *t*-test for grouped data or analysis of variance (ANOVA) followed by the Newman-Keuls test for multiple comparisons. The level of *P* < 0.05 was defined as statistical significance.

RESULTS

MTT assay

Effect of chronic ethanol exposure on cell viability

MTT absorbance values, measured in control hippocampal cell cultures were 0.231 ± 0.012 ($n = 40$). The exposure to ethanol (75 mM; 4 days) induced an impairment of mitochondrial function in hippocampal cell cultures, as indicated by the significant decrease ($P < 0.01$) of absorbance values (Fig. 1) in respect to the control cell culture values.

Effect of GET73 and MPEP on chronic ethanol-induced reduction of cell viability

The addition of GET73 (1 and 10 μ M) 1 h before and during chronic ethanol exposure prevented the ethanol-induced MTT reduction, being the cell viability not significantly different in respect to the control group (Fig. 1). On the contrary, the lower (0.1 μ M) concentration of GET73 was ineffective (Fig. 1). By itself, GET73 (0.1–10 μ M) did not affect cellular viability in hippocampal cell cultures not exposed to ethanol (0.1 μ M: $98 \pm 8\%$ of control value, $n = 20$; 1 μ M: $97 \pm 7\%$ of control value, $n = 20$; 10 μ M: $106 \pm 5\%$ of control value, $n = 20$).

Based on the results previously obtained and the proposed GET73 mechanism of action (Ferraro *et al.*, 2011, 2013; Beggato *et al.*, 2013), the effects of mGlu5 receptor NAM, MPEP, on ethanol-induced reduction of cell viability were also assessed. As shown in Fig. 2, the addition of MPEP (100 μ M) during chronic ethanol exposure prevented the ethanol-induced MTT reduction, being the cell viability not significantly different in respect to the control group. On the contrary, the lower

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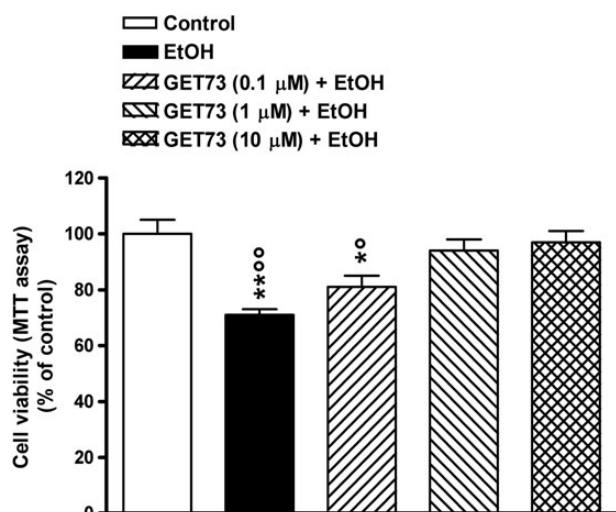
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Q5 Fig. 1. Cell viability in hippocampal cell cultures exposed to ethanol (EtOH; 75 mM, 4 days) alone or in combination with GET73. The compound was added 1 h prior to EtOH exposure and maintained in contact with neurons during ethanol exposure. Control cultures were not exposed to EtOH. Each value represents the mean \pm SEM ($n=40$). * $P<0.05$; ** $P<0.01$ significantly different from control group; ° $P<0.05$; °° $P<0.01$ significantly different from GET73 (1 μ M) + EtOH and GET73 (10 μ M) + EtOH groups, according to ANOVA followed by the Newman–Keuls test for multiple comparisons.

(10 μ M) concentration of MPEP was ineffective. By itself, MPEP (10 and 100 μ M) did not affect cellular viability in hippocampal cell cultures not exposed to ethanol (10 μ M: $99 \pm 8\%$ of control value, $n=18$; 100 μ M: $104 \pm 9\%$ of control value, $n=18$).

MAP2 immunoreactivity

Effect of chronic ethanol exposure on the MAP2 immunoreactivity

The distribution of MAP2 in the control and ethanol-exposed hippocampal neurons was analyzed by an immunofluorescence technique. Control hippocampal cell cultures presented a high number of healthy neurons, which developed a complex neuronal network characterized by highly arborized dendritic trees and MAP2 immunoreactivity homogeneously distributed in the cell bodies and dendrites (Fig. 3A). The exposure to ethanol (75 mM; 4 days) induced an alteration of the staining pattern of the cytoskeletal protein, which mainly revealed a reduction of dendrite length (Fig. 3B). Furthermore, the number of MAP2 positive cells was significantly lower in cell cultures exposed to ethanol than in control cell cultures (Fig. 4).

Effect of GET73 on chronic ethanol-induced reduction of MAP2 immunoreactivity

The addition of GET73 (10 μ M) 1 h before and during chronic ethanol exposure prevented the ethanol-induced reduction of MAP2 positive cell number (Fig. 4). By itself, GET73 (10 μ M) did not affect MAP2 positive cell number in hippocampal cell cultures not exposed to ethanol ($98 \pm 4\%$ of control value, $n=10$).

Extracellular glutamate levels

Effect of chronic ethanol exposure on the extracellular glutamate levels

Extracellular glutamate levels in control hippocampal cell cultures were 70.0 ± 5.1 nM. As shown in Fig. 5, the chronic exposure to ethanol (75 mM; 4 days) induced a significant reduction of extracellular glutamate levels.

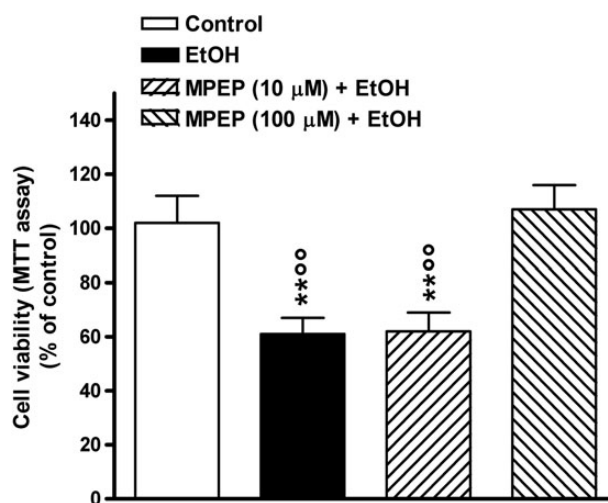


Fig. 2. Cell viability in hippocampal cell cultures exposed to ethanol (EtOH; 75 mM, 4 days) alone or in combination with MPEP. The negative allosteric modulator at the mGlu5 receptors was added 1 h prior to EtOH exposure and maintained in contact with neurons during ethanol exposure. Control cultures were not exposed to EtOH. Each value represents the mean \pm SEM ($n=40$). *** $P<0.01$ significantly different from control group; °° $P<0.01$ significantly different from MPEP (100 μ M) + EtOH group, according to ANOVA followed by the Newman–Keuls test for multiple comparisons.

Effect of GET73 on chronic ethanol-induced reduction of extracellular glutamate levels

The addition of GET73 (1 and 10 μ M) 1 h before and during chronic exposure ethanol prevented the ethanol-induced reduction of extracellular glutamate levels (Fig. 5). On the contrary, the lower (0.1 μ M) concentration of GET73 was ineffective. By itself, GET73 (0.1–10 μ M) did not affect the extracellular glutamate levels in hippocampal cell cultures not exposed to ethanol (0.1 μ M: $94 \pm 4\%$ of control value, $n=9$; 1 μ M: $97 \pm 4\%$ of control value, $n=9$; 10 μ M: $93 \pm 3\%$ of control values, $n=9$).

ROS production

Effect of chronic ethanol exposure on the ROS production

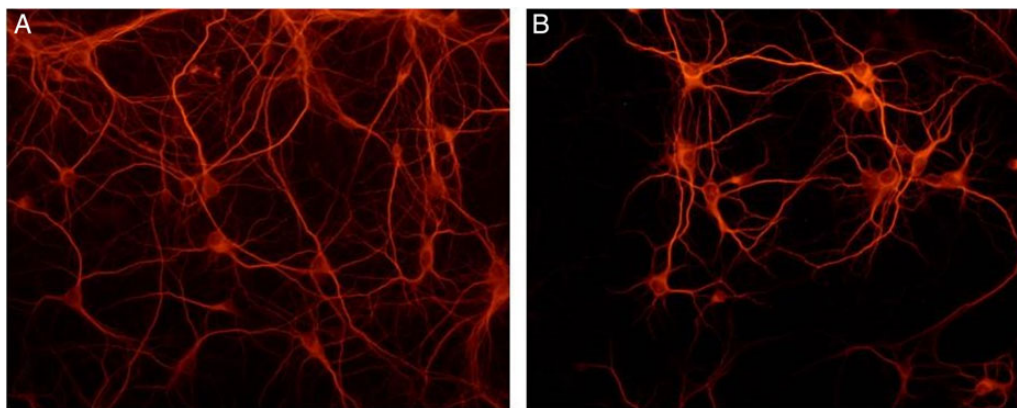
The exposure of hippocampal cell cultures to ethanol (75 mM; 4 days) induced an increase of ROS production, as revealed by the enhanced intensity in the fluorescence emission of rhodamine 123 (Fig. 6).

Effect of GET73 on chronic ethanol-induced increase of ROS production

The addition of GET73 (1 and 10 μ M) 1 h before and during chronic ethanol exposure prevented the ethanol-induced increase of ROS production (Fig. 6). On the contrary, the lower (0.1 μ M) concentration of GET73 was ineffective. By itself, GET73 (0.1–10 μ M) did not affect the ROS production in hippocampal cell cultures not exposed to ethanol (0.1 μ M: $95 \pm 10\%$ of control value, $n=8$; 1 μ M: $82 \pm 8\%$ of control value, $n=8$; 10 μ M: $88 \pm 8\%$ of control value, $n=8$).

DISCUSSION

GET73 may be considered a new promising therapeutic agent for the treatment of AUDs. In fact, the compound displays a multifaceted behavioral profile including the capacity to reduce alcohol consumption, to suppress the ‘alcohol deprivation effect’, and to reduce anxiety-related behavior in the selective bred, Sardinian alcohol-



Q5 Fig. 3. Representative photomicrographs of MAP2 immunocytochemistry in hippocampal cell cultures not exposed (A) and exposed to ethanol (75 mM, 4 days; B). Surviving neurons were stained with anti-MAP2 antibody and observed under fluorescence microscope (magnification $\times 20$).

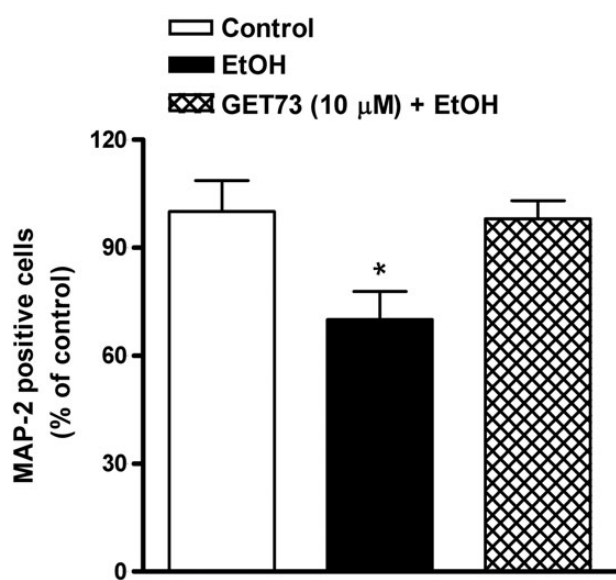


Fig. 4. MAP2 positive cell number in primary hippocampal cell cultures exposed to ethanol (EtOH; 75 mM, 4 days) alone or in combination with GET73. Neurons were stained with anti-MAP2 antibody and observed under fluorescent microscope. GET73 was added 1 h prior to EtOH exposure and maintained in contact with neurons during ethanol exposure. Control cultures were not exposed to EtOH. Data are expressed as percentage of control values. Each value represents the mean \pm SEM, $n = 8$. * $P < 0.05$ significantly different from the other groups, according to ANOVA followed by the Newman-Keuls test for multiple comparisons.

preferring (sP) rats (Loche *et al.*, 2012; Ferraro *et al.*, 2013). It has been demonstrated that GET73 is able to affect glutamate and GABA neurotransmission in the rat hippocampus, possibly through an allosteric modulation of mGlu5 receptor (Ferraro *et al.*, 2011, 2013; Beggiano *et al.*, 2013). The hippocampus may be considered an important CNS target area for the compound as this region is involved in ethanol reward, anxiety behavior and cognition. Based on promising preclinical data, the compound is currently under clinical investigation in alcoholics (Phase 1b/2a 73CT-2-03 Study; <http://clinicaltrials.gov/ct2/show/NCT01842503>).

The neurotoxic effects of alcohol on the hippocampus have been demonstrated in different preclinical models, including *in vitro* organotypic hippocampal cultures (Harris *et al.*, 2003; Moon *et al.*, 2014)

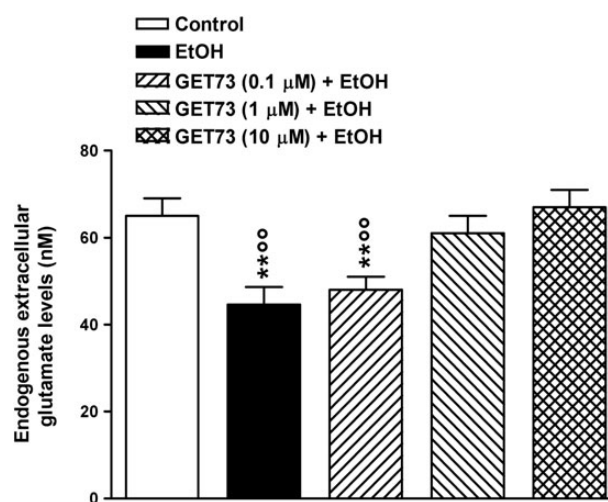


Fig. 5. Extracellular glutamate levels in hippocampal cell cultures exposed to ethanol (EtOH; 75 mM, 4 days) alone or in combination with GET73. The compound was added 1 h prior to EtOH exposure and maintained in contact with neurons during ethanol exposure. Control cultures were not exposed to EtOH. Data are expressed as absolute values (nM). Each value represents the mean \pm SEM, $n = 16$. *** $P < 0.01$ significantly different from control group; °°° $P < 0.01$ significantly different from GET73 (1 μ M) + EtOH and GET73 (10 μ M) + EtOH groups, according to ANOVA followed by the Newman-Keuls test for multiple comparisons.

or *in vivo* binge ethanol exposure model (Obernier *et al.*, 2002; Hamelik *et al.*, 2005). In the present study the possible neuroprotective properties of GET73 in hippocampal cultures exposed to ethanol have been explored in order to further characterize the 'anti-alcohol profile' of the compound. Chronic exposure (from 4 to 7 DIV) of primary cultures of rat hippocampal neurons to 75 mM ethanol induced a series of alterations in cell viability, neuronal morphology, glutamate levels and ROS production. In line with the documented direct toxic effects of ethanol on the brain (Kruman *et al.*, 2012), these findings indicate that a long term *in vitro* exposure to ethanol compromises the survival of cultured hippocampal neurons. These results are also in agreement with literature data obtained in *in vivo* animal models of alcohol dependence (4-day binge model), where neuronal loss was observed in the cortico-limbic pathway, with cellular degeneration particularly evident in the entorhinal cortex and the dentate gyrus of hippocampus (Obernier *et al.*, 2002; Kelso *et al.*, 2011). Moreover, another study in

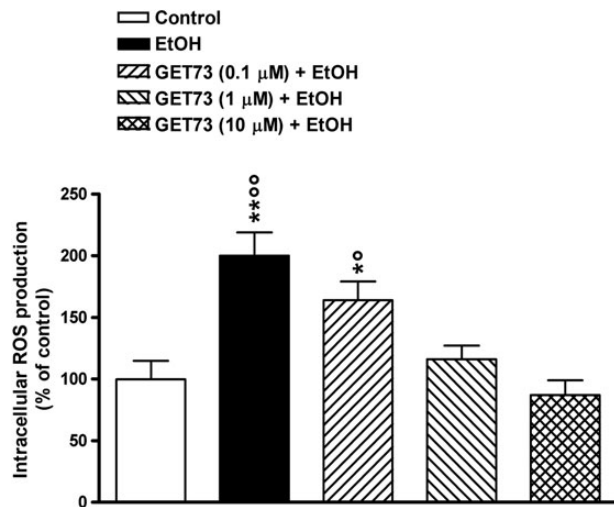


Fig. 6. Intracellular ROS production in hippocampal cell cultures exposed to ethanol (EtOH; 75 mM, 4 days) alone or in combination with GET73. The compound was added 1 h prior to EtOH exposure and maintained in contact with neurons during ethanol exposure. Control cultures were not exposed to EtOH. Data are expressed as percentage of control values. Each value represents the mean \pm SEM, $n = 16$. * $P < 0.05$, ** $P < 0.01$ significantly different from control group; ° $P < 0.05$; °° $P < 0.01$ significantly different from GET73 (1 μ M) + EtOH and GET73 (10 μ M) + EtOH groups, according to ANOVA followed by the Newman-Keuls test for multiple comparisons.

alcoholics showed significant reductions in neuron number in all regions of the hippocampus, including the dentate gyrus (Bengochea and Gonzalo, 1990).

The immunofluorescence experiments targeting MAP2, a protein associated to the cytoskeleton predominantly expressed in neurons, showed that chronic ethanol exposure significantly reduced the number of cultured hippocampal neurons, compared to control cultures. Furthermore, under these experimental conditions, survived neurons showed a reduced development of dendritic arborization and a reduction of the length of single dendrites. In line with our results, previous *in vitro* studies reported neuronal morphological alterations after alcohol exposure. Indeed, an inhibitory effect of ethanol on the assembly and organization of the cytoskeleton components was described in hippocampal neurons as a result of prenatal exposure to ethanol (Yanni and Lindsley, 2000; Romero *et al.*, 2010). In addition, chronic ethanol exposure has also been observed to produce changes in dendritic spine morphology and/or density in cultured hippocampal neurons (Carpenter-Hyland and Chandler, 2007) and in organotypic hippocampal slice cultures (Norberg and Zimmer, 1998). Finally, long-term alcohol self-administration decreased MAP2 mRNA levels in several rat brain regions (Putzke *et al.*, 1998). Interestingly, the cell loss and the alterations of hippocampal neuron morphological development by chronic ethanol exposure are associated to a decrease in extracellular glutamate levels. This effect could be a direct consequence of the reduced cell viability and the altered cell morphology induced by ethanol exposure. Another possibility is that through its inhibitory action on Ca^{2+} channels, ethanol inhibits the release of glutamate from the survived neurons. This mechanism has been previously implicated in the reduction of dopamine release from rat striatal synaptosomes (Woodward *et al.*, 1990), glutamate release in hippocampal neurons of neonate rats (Mameli *et al.*, 2005) and in the frequency of mEPSCs and mIPSCs in multipolar neurons by ethanol (Moriguchi *et al.*, 2007). This finding could have functional relevance

as glutamate is involved in synaptogenesis, determining the 'fate' of single neuron and neuronal connections (Mattson, 2008). Therefore, a reduction of synaptic availability of this excitatory neurotransmitter induced by chronic ethanol might induce altered patterns of synaptic connectivity. Thus, it could be speculated that chronic ethanol exposure may cause, through the reduction of hippocampal glutamatergic neurotransmission, alterations in synaptic remodeling processes that could affect later on the behavior observed in the whole animal. Finally, the evidence that chronic ethanol exposure significantly increases intracellular ROS production in hippocampal cell cultures suggest that, under the present experimental conditions, oxidative stress could be the potential mechanism underlying the ethanol-induced neurotoxicity. This hypothesis is in line with previous data demonstrating that chronic treatment with ethanol produces brain oxidative stress (Carpenter-Hyland 2007; Collins and Neafsey, 2012; Zorumski, 2014), characterized by an increased production of ROS and a decreased efficiency of the cellular antioxidant mechanisms (Cederbaum, 2009).

The main finding of the present study is that GET73 treatment fully prevented ethanol-induced neurotoxicity in primary cultures of rat hippocampal neurons. In fact, the compound completely counteracted the reduction in cell viability and in the number of hippocampal neuronal population along with the hippocampal neuron morphological alterations observed after chronic ethanol exposure.

Under the present experimental conditions, the pretreatment with GET73 was also able to prevent the ethanol-induced reduction of glutamate levels. This study provides the first evidence that GET73, in addition to reduce alcohol consumption and to suppress the 'alcohol deprivation effect' (Loche *et al.*, 2012), might also protect the brain from the neurotoxic effects induced by chronic ethanol exposure. This aspect assumes particular relevance in view of the experimental evidences indicating that glutamatergic efferents projecting from the hippocampus and terminating in the prefrontal cortex are implicated in the proper processing of executive functions, working memory and contextual information (Godsil *et al.*, 2013). A reduction of the neuronal function and the loss of the structural integrity of the hippocampus may therefore contribute to the impairment in the cognitive functions as observed following excessive alcohol consumption both in animal models and in alcoholics (Stephens and Duka, 2008; O'Daly *et al.*, 2012; Stavro *et al.*, 2013). Thus, it could be speculated that GET73, by preventing ethanol-induced hippocampal neuronal loss, might also prevent the detrimental effects exerted by chronic ethanol consumption on cognition. However, it is worth noting that in the present study we exposed alcohol to the cultures during synaptic development (Papa *et al.*, 1995) and not in mature neurons. Thus, a more extensive analysis by using *in vivo* animal models of alcoholism will be therefore necessary to verify whether these findings could be relevant to alcohol abuse and neurodegeneration *in vivo* in adolescent or adult brain.

In the present study we also demonstrated that the treatment with GET73 was able to prevent the intracellular ROS production induced by chronic ethanol exposure. This finding suggests that the neuroprotective effects of the compound could be possibly referred to its antioxidant properties. However, another possibility could be proposed. As previously reported, recent findings from both *in vitro* and *in vivo* studies suggest that GET73 undertakes a complex interaction with mGlu5 receptors. Indeed, this compound may act as a positive (PAM) or negative allosteric modulator at the mGlu5 receptors (NAM), depending on its concentration (Ferraro *et al.*, 2011, 2013). Although to date no studies have yet explored GET73 binding at the orthosteric and/or allosteric sites on mGlu5 receptor, this mechanism

has been proposed to explain the GET73-induced modulation of glutamate and GABA transmission in the hippocampus (Ferraro *et al.*, 2011; Beggiato *et al.*, 2013). As the mGlu5 receptor antagonists exhibited neuroprotective effects in rat hippocampus (Szydłowska *et al.*, 2007; Yeganeh *et al.*, 2013), it seems possible that the interaction with mGlu5 receptor might also be involved in the neuroprotective effects of the compound. This hypothesis is supported by the demonstration that, under the present experimental conditions, the mGlu5 receptor NAM MPEP fully prevented the ethanol-induced reduction of cell viability, thus mimicking the effect of GET73. Interestingly, MPEP displayed neuroprotective effects also against β -amyloid-induced toxicity in mouse primary hippocampal neuronal cultures (Overk *et al.*, 2014) and reduced both the production of reactive oxygen species (ROS) and cell toxicity in isolated hepatocytes (Storto *et al.*, 2003). However, some authors suggested that the neuroprotective effects of MPEP could also be mediated through its possible NMDA receptor antagonist action (O'Leary *et al.*, 2000). Thus, further experiments will be necessary to draw definitive conclusions on the possible involvement of mGlu5 receptors in the neuroprotective action of GET73.

In conclusion, the present findings provide the first evidence that GET73 possesses neuroprotective properties against ethanol-induced neurotoxicity in hippocampal cell cultures. Notably, previous studies indicate that GET73 displays a double profile of 'anti-alcohol' and anxiolytic drug which could be relevant in the treatment of AUDs, characterized by a high incidence of anxiety disorders capable of sustaining drug abuse and dependence, and increasing the risk of relapse in abstinent patients. In addition, the neuroprotective effects exerted by GET73 might represent another important therapeutic potential, given the high rate of alcohol-induced brain damage and the underlying cognitive impairments in alcoholics. Taken together, these multifaceted properties of the compound lend further support to the significance of developing GET73 as a therapeutic tool for use in the treatment of AUDs.

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CONFLICT OF INTEREST STATEMENT

A.L. and R.C. are employees of Laboratorio Farmaceutico CT, Sanremo, Italy. The other authors declare no conflicts of interest. The Company took no part in the study design, in the collection, analysis and interpretation of data, or in the decision to submit the paper for publication.

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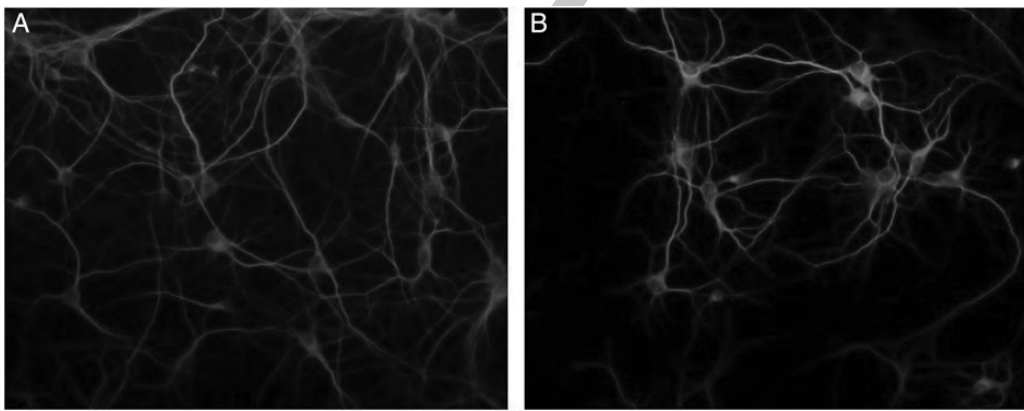
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