Multi-target iron-chelators improve memory loss in a rat model of sporadic Alzheimer's disease

Šalković-Petrišić, Melita; Knezović, Ana; Osmanović-Barilar, Jelena; Smailović, Una; Trkulja, Vladimir; Riederer, Peter; Amit, Tamar; Mandel, Silvia; Youdim, Moussa B.H.

Source / Izvornik: **Life Sciences, 2015, 136, 108 - 119**

Journal article, Accepted version
Rad u časopisu, Završna verzija rukopisa prihvaćena za objavljivanje (postprint)

https://doi.org/10.1016/j.lfs.2015.06.026

Permanent link / Trajna poveznica: https://urn.nsk.hr/urn:nbn:hr:105:013198

MULTI-TARGET IRON-CHELATORS IMPROVE MEMORY LOSS IN RAT MODEL OF SPORADIC ALZHEIMER'S DISEASE

Melita Salkovic-Petrisic[1*], Ana Knezovic[1], Jelena Osmanovic-Barilar[1], Una Smailovic[1], Vladimir Trkulja[1], Peter Riederer[2], Tamar Amit[3], Silvia Mandel[4], Moussa BH Youdim[3]

[1]Department of Pharmacology and Croatian Institute for Brain Research, University of Zagreb School of Medicine, Salata 11, HR 10 000 Zagreb, Croatia

[2]University hospital Wuerzburg, Center of Mental Health, Department of Clinical Neurochemistry,Clinic and Policlinic of Psychiatry, Psychosomatic and Psychotherapy, University of Wuerzburg, Fuechsleinstr. 15 97080 Wuerzburg, Germany

[3]Eve Topf Center for Neurodegenerative Diseases Research and Department of Molecular Pharmacology, Faculty of Medicine, Technion, Haifa, Israel

[4]Bruce Ruth Rappaport Faculty of Medicine, Technion, Haifa 31096, Israel

Corresponding author: Melita Salkovic-Petrisic, Department of Pharmacology and Croatian Institute for Brain Research, University of Zagreb School of Medicine, Salata 11, HR 10 000 Zagreb, Croatia

E-mail address: melitas@mef.hr

Phone number: +38514590219

Fax number: +38514920049

ABSTRACT

Aim: Novel effective treatment is urgently needed for sporadic Alzheimer's disease (sAD). M30 ([5-(N-methyl-N-propargylaminomethyl)-8-hydroxyquinoline]) and HLA-20 (5-{4-propargylpiperazin-1-ylmethyl}-8-hydroxyquinoline) are brain permeable, iron chelating compounds with antioxidant activity, showing also neuroprotective activity in animal models of neurodegeneration. We aimed to explore their therapeutic potential in non-transgenic (non-Tg) rat model of sAD developed by intracerebroventricular administration of streptozotocin (STZ-icv).

Main methods: Therapeutic effects of chronic oral M30 (2 and 10 mg/kg) and HLA20 (5 and 10 mg/kg) treatment on cognitive impairment in STZ-icv rat model were explored by Morris Water Maze (MWM) and Passive Avoidance (PA) test in neuropreventive and neurorescue paradigms. Data were analysed by Kruskal-Wallis and Mann-Whitney U test ($p<0.05$).

Key findings: Five-day oral pre-treatment with M30 and HLA20 dose-dependently prevented development of spatial memory impairment (MWM probe trial-time +116%/M30; +60%/HLA20) in STZ-icv rat model ($p<0.05$). Eleven-week oral treatment with M30 (3xweek), initiated 8 days after STZ-icv administration dose-dependently ameliorated already developed cognitive deficits in MWM test (reduced number of mistakes 3 months after the STZ-icv treatment – 59%; $p<0.05$) and fully restored them in PA test (+314%; $p<0.05$). Chronic M30 treatment fully restored (-47%/PHF1; -65%/AT8; $p<0.05$) STZ-induced hyperphosphorylation of tau protein and normalized decreased expression of insulin degrading enzyme (+37%; $p<0.05$) in hippocampus.

Significance: The results provide first evidence of therapeutic potential of M30 and HLA20 in STZ-icv rat model of sAD with underlying molecular mechanism, further supporting the important role of multi-target iron-chelators in sAD treatment.

Keywords: streptozotocin, Alzheimer's disease, M30 compound, HLA20 compound, memory, tau protein, insulin degrading enzyme

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder clinically characterized by a progressive memory decline. Novel and effective therapeutic approach for AD is urgently needed which should be tested pre-clinically in animals models that mimic familial and sporadic AD form (sAD). Widely exploited transgenic animal models of AD (Lithner et al. 2011) represent the familial form of AD and do not mimic the sAD condition (Balducci and Forloni 2011). Animal model which develops insulin resistant brain state and glucose hypometabolism following the intracerebroventricular application of a betacytotoxic drug streptozotocin in small rodents and cynomolgus monkey (STZ-icv model), (Agrawal et al. 2011; Grünblatt et al. 2007; Lannert and Hoyer 1998; Lee et al. 2014; Lester-Coll et al. 2006; Plaschke and Hoyer 1993; Salkovic-Petrisic et al. 2006), shares similarities with the human sAD condition (Lannert and Hoyer 1998) since insulin resistant brain state was found post-mortem in sAD patients (Correia et al. 2011; de la Monte and Wands 2005; Frölich et al. 1998). Additionally, STZ-icv model demonstrates also cognitive deficits (Mayer et al. 1990; Lannert and Hoyer 1998) and decrement in cerebral cholinergic transmission (Blokland and Jolles 1993; Hellweg et al. 1992), as well as other features of chronic neurodegeneration like oxidative stress and neuroinflammation (Saxena et al. 2011; Sharma and Gupta 2001) and in particular tau protein hyperphosphorylation (Grünblatt et al. 2007; Deng et al. 2009; Liu et al. 2014; Peng et al. 2013), pathological Aβ accumulation (Shingo et al. 2013) and cerebral amyloid angiopathy (Salkovic-Petrisic et al. 2006, 2011).

Considering the involvement of iron accumulation in AD pathophysiology (Grünblatt et al. 2010; Honda et al. 2004), metal chelation has become one of the therapeutic strategies for the treatment of AD (Budimir 2011). Deferoxamine (DFO), the natural prototype iron chelator/radical scavenger slowed the clinical progression of AD dementia (Crapper et al.

1991) and inhibited amyloidogenic processing of amyloid precursor protein (APP) and reversed iron-induced memory deficits and tau phosphorylation in mice model of AD (Guo et al. 2013a,b). Clioquinol and PBT2, 8-hydroxyquinoline bidentate ligands, attenuated cognitive loss and inhibited Aβ accumulation in AD transgenic mice (Cherny et al. 2001; Adlard et al. 2011).

Novel anti-AD therapeutic strategies focus on drugs which target multiple pathology aspects of the disease (Van der Schyf et al. 2006; Youdim and Buccafusco 2005). M30 ([5-(N-methyl-N-propargyaminomethyl)-8-hydroxyquinoline]) and HLA20 (5-{4-propargylpiperazin-1-ylmethyl}-8-hydroxyquinoline) are multi-target compounds with iron chelating potency, radical scavenging activity and features of inhibition of iron-induced lipid peroxidation (Zheng et al. 2005). Neuroprotective activity of M30 was found in vitro and in some animal models of neurodegenerative disorders (Zheng et al. 2005; Weinreb et al. 2011). Systemic treatment of transgenic (APP/presenilin 1) mice with M30 for 9 months, attenuated cognitive impairments, reduced cerebral iron accumulation, modulated glucose metabolism and reduced APP expression, Aβ accumulation and tau phosphorylation (Kupershmidt et al. 2012; Mechlovich et al. 2014a).

Using the non-transgenic STZ-icv rat model of sAD, we herein aimed to assess the effectiveness of M30 and HLA20 in improving the cognitive deficits and underlying neurochemical alterations.

MATERIALS AND METHODS

Materials

Streptozotocin was purchased from Sigma-Aldrich (Munich, Germany). 5-{N-methyl-N-propargylaminomethyl}-8-hydroxyquinoline (M30) and 5-{4-propargylpiperazin-1-ylmethyl}-8-hydroxyquinoline (HLA20) have been provided by Eve Topf Center of Excellence, Technion –Faculty of Medicine, Haifa, Israel. Nuclear fast red was a gift from Professor Camelia Monoranu from University of Würzburg, Department of Pathology, Germany.

Animals

Three to four-month old male Wistar rats weighing 280-330 g (Department of Pharmacology, University of Zagreb School of Medicine) were used throughout the studies. The rats were kept 2-3 per cage in a room with a 12 h light/12 h dark cycle (lights on 07:00 –19:00 h), and the room temperature and humidity set in the range of 21–25°C and 40–70% respectively. Standardized food pellets and water *ad libitum* were provided to all animals. Animal care and experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals (8th ed. 2011) and the protocol was approved by the institutional Ethical Committee. Animals were euthanized after cognitive testing at the end of experiments in deep anaesthesia (thiopental 50 mg/kg/6 mg/kg diazepam intraperitoneally) followed by decapitation. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny et al. 2010).

STZ-icv rat model

Rats were given general anaesthesia (chloral hydrate 300 mg/kg, ip), followed by injection of streptozotocin 1 mg /kg (single dose) or 3 mg/kg (split in two doses on day 1 and 3) administered bilaterally into the left and right lateral ventricle as previously described (Grünblatt et al. 2007; Salkovic-Petrisic et al. 2006, 2011). Drug concentration and solution volume were adjusted according to the animal body weight, and a volume of 4 μL per 300 g body weight was administered (2 μL/ventricle)**.** Control animals received bilaterally an equal volume of vehicle (0.05 M citrate buffer pH 4.5) into the lateral ventricles. Appropriate care was taken during anaesthesia and recovering period to reduce the pain and discomfort as much as possible. STZ-icv treated animals were randomly allocated to receive iron-chelator or saline.

Iron-chelator treatments

HLA20. Neuropreventive paradigm was tested by pre-treatment with saline, or HLA20 (5 mg/kg/day), administered by oral catheter (adjusted for volume of 1 mL/300 g body weight) for 5 days (9:00 to 10:00 h). On the 5^{th} day, a single STZ-icv (1 mg/kg) or vehicle-icv injection was given after the last HLA20 oral dose. Animals were sacrificed 1 month following the STZ- or vehicle-icv treatment. In additional experiment, rats were pre-treated for 5 days with HLA20 (10 mg/kg/day), similarly as in the previous experiment, but were sacrificed 3 months following the STZ-icv injection.

M30. Neuropreventive paradigm was tested by pre-treatment with saline or M30 (10 mg/kg/day), administered by oral catheter (1 mL/300 g body weight) for 5 days (9:00 to 10:00 h). On the 5th day, a single STZ-icv (1 mg/kg) or vehicle-icv injection were given after the last M30 dose and animals were sacrificed 1 month later.

M30. Neurorescue paradigm activity was tested by post-treatment with M30 administered in two doses (2 and 10 mg/kg/day, on Monday, Wednesday and Friday during each treatment week) by oral catheter (1 mL/300g body weight), for 11 weeks (9:00 to 10:00 h), starting from the 8th day following the first (out of two) STZ-icv injection (3 mg/kg). Control STZ-icv rats were orally treated by saline in a similar way. All animals were sacrificed 3 months following the first STZ-icv injection. Additional short-term experiment of a similar design was performed to test the activity of high (10 mg/kg/day) M30 dose on neurochemistry parameters (control, STZ and STZ+M30 group). Oral M30 treatment was initiated from the 8th day following the first (out of two) STZ-icv injection (3 mg/kg) and animals were euthanized a week later, after being administered with 3 M30 doses only (on Monday, Wednesday and Friday).

Cognitive performance assessment

Cognitive performance assessment was done by Morris Water Maze Swimming test (MWM, 8:00-13:00 h), which tests spatial memory, and Passive Avoidance (PA, 8:00-9:00 h) test, which tests fear-motivated avoiding memory. In the M30 neuropreventive paradigm MWM was performed once before the sacrifice, while in the M30 neurorescue paradigm, MWM was performed repeatedly after 2 weeks and 1, 2 and 3 months post- STZ-icv injection and PA was performed only once before the sacrifice. In the HLA20 -5 mg/kg neuropreventive

paradigm, MWM and PA were performed once before the sacrifice, while in another HLA20 - 10 mg/kg neuropreventive paradigm, MWM was performed repeatedly after 2 weeks and 1, 2 and 3 months post- STZ-icv injection. No cognitive testing was done in a short-term experiment in neurorsecue paradigm.

MWM Test

On the first day of adaption to the environment, rats were subjected to 1 min of freely swimming in a pool (150x60 cm, 50 cm deep), with water temperature set at 25±1 ^{o}C, and on the second day rats were allowed to freely swim in the pool divided in four compartments (I-IV). The acquisition or training phase was performed afterwards from day 1 to day 4, during which the rats were thought to escape from water by finding a hidden rigid platform submerged about 2 cm below the water surface in compartment IV. Stay on the platform was allowed for 15 s. One training trial consisted of three starts, each from a different compartments (I – III), separated by a 1-min rest period. Three consecutive trials were performed per day, separated by a 30-min rest period. Escape latency (time needed to find the platform) and number of errors (entries into the compartments without a platform) were recorded as parameters of the reference memory (memory acquisition). After the third training trial on day 4, the probe trial was performed (starts from compartments I-III) with a platform being removed from the pool, and the time spent within the compartment IV (from which the platform was removed) and the number of errors were recorded as parameters of the working memory (memory retention). The cut off time was 1 min.

PA test

The rats were placed in a light compartment of a shuttle box (Ugo Basile, Comerio, Italy) on the 1st test day for acclimatization. The light compartment has been isolated from the dark

compartment by a guillotine door which is closed automatically after entry of the rat into the dark compartment. On the 2nd test day, during the acquisition trial, animals were placed again into the light compartment and after entry into the dark compartment, received a low-intensity electric foot shock (0.5 mA; 2 s) delivered through the grid metal floor in the dark compartment. During the retention trial, animals were again placed in the light compartment and the post-shock latency time spent within the light compartment before entering the dark one was recorded.

Western blot analysis

Tissue preparation.

Hippocampal (1 animal = 1 sample) tissue samples from one half of the rat brain were homogenized with 3 volumes of lysis buffer containing 10 mM HEPES, 1 mM EDTA, 100 mM KCl, and 1% Triton X-100, pH 7.5, and protease inhibitors coctail (1:100), and the homogenates were centrifuged at 12000 rpm for 10 min at 4°C and the supernatant were frozen and stored at -80°C. Protein concentration was measured by Lowry protein assay.

Immunoblotting.

Equal amounts of total protein (35 μg *per* sample) were separated by SDS-PAGE using 9% polyacrylamide gels and transferred to nitrocellulose membranes. The nitrocellulose membranes were blocked by incubation in 5% non-fat milk added to low salt washing buffer (LSWB) containing 10 mM Tris and 150 mM NaCl, pH 7.5, and 0.5% Tween 20 for one hour at room temperature. Blocked blots were incubated overnight at 4°C with anti-IDE (1:2000), anti-AT8 (1:500), anti-total tau (1:500) or overnight at RT with primary anti-PHF1 (1:500). Following the incubation, the membranes were washed three times with LSWB and incubated for 60 min at room temperature with appropriate secondary antibody solution (anti-mouse or

anti-rabbit IgG, 1:5000). The specificity of the signal was checked on the control membranes that were not incubated with the primary antibody. After washing three times in LSWB, the membranes were immunostained using chemiluminiscence Western blotting detection reagents, signal captured and visualised with the MicroChemi 2.0 CCD camera system (DNR Bio-Imaging Systems) and after that washed three times in LSWB and incubated with loading control, anti-GAPDH (1:2000) or anti-βactin (1:5000) antibody followed by the same procedure already mentioned above.

Statistics

For all experiments based on comparisons of independent groups (N=7-10 rats per group), data were expressed as mean±SEM and analysed using non-parametric ANOVA (Kruskal-Wallis) followed by Mann-Whitney U-test, or by parametric ANOVA followed by Tukey HSD test. Data from the 3-month experiment with repeated MWM testing in the same animals were analysed by fitting generalized linear mixed models with Poisson (for the number of errors) or normal (latency times) link function. All tests were adjusted for multiple comparisons in order to keep the experiment wise alpha level at 0.05, and $p<0.05$ was considered statistically significant.

Ethics

Animal procedures, carried out at the University of Zagreb Medical School (Zagreb, Croatia), were as human as possible, as described in details in the section “Material and Methods“ in compliance with the recommendations of ARRIVE. All analyses were done in compliance

with valid institutional, national (The Animal Protection Act, NN135/06) and international (Directive 2010/63/EU) guidelines governing the use of experimental animals and have been approved by the national regulatory body responsible for issuing ethical approval, Croatian Ministry of Agriculture (licence No. UP/I-322-01/11-01/100 to MSP for research approved by Croatian Ministry of Science, Education and Sport, project 108-1080003-0020 to MSP).

RESULTS

Effect of M30 on STZ-icv-induced memory impairment

Neuroprevention

In comparison to control animals, STZ-icv treatment induced memory impairment in rats, as detected in the probe MWM trials, 1 month after the treatment (Fig. 1A). STZ-icv treated rats had a poor memory on the location of the compartment with a platform and thus had more entries into the incorrect compartments (increased number of mistakes, $p<0.05$), spending less time swimming within the targeted compartment from which the platform had been previously removed, $p<0.05$ (Fig. 1B). Five-day oral pre-treatment with **M30** (10 mg/kg/day) significantly prevented development of spatial memory impairment in STZ-icv treated rats, which was manifested by reducing the number of mistakes (Fig. 1A) and increasing the time spent within the targeted compartment (Fig. 1B) in the MWM probe trial, in comparison to the STZ-icv treatment alone. Pre-treatment with the lower M30 dose (5 mg/kg/day) was ineffective (data not shown). No significant difference was detected between M30-treated and vehicle-treated control rats in error number and the time spent within the compartment (Fig. 1).

Neurorescue

The therapeutic potential of **M30** on spatial learning and memory impairment, as well as on fear motivated escape memory deficits in STZ-icv rat model of sAD, has been also tested in a neurorescue experimental design by MWM and PA test, respectively.

MWM test

Learning and memory performance in training trails over time. A total of 4/33 animals died during the course of the experiment (Fig. 2, bottom): one animal in the control group, one in the STZ+ M30 (2 mg/kg) group and 2 animals in the STZ + M30 (10 mg/kg) group. On the 4^{th} day of each of the 4-day training cycles (after 2, 4, 8 and 12 weeks since the last STZ-icv administration), the number of errors appeared lower (Fig. 2A) and escape latency time shorter (Fig. 2C), than those on the 1^{st} day of each respective cycle, indicating the ability to learn through training (learning capacity), with following particulars: a) the largest differences (day 4 vs. day 1) appeared 2 weeks after STZ-icv administration and progressively decreased in repeated training cycles, indicating reduced space for improvement due to lower starting (day 1) values in later cycles; b) the reduction of day 1 values indicated development of memory; c) after 2 weeks, differences at day 4 vs. day 1 appeared to be greatest in control and smallest in STZ-icv treated animals, indicating impaired learning capacity in the latter, whereas M30-treated STZ-icv rats apparently performed poorer than controls, but better than STZ-icv treated animals. This relation was maintained throughout the experiment; d) similarly, reduction of day 1 values appeared most pronounced in controls, least pronounced in STZ-icv treated animals (indicating STZ-icv induced impairment), while the performance

of M30-treated STZ-icv rats appeared "intermediate". Initial formal statistical tests referred to all 16 assessments (4 in each of the 4 cycles) and demonstrated the following: a) the number of errors was markedly higher in STZ-icv-treated vs. control animals (RR=34.8, $p<0.001$) (Fig. 2B); b) M30 dose-dependently antagonized the STZ-icv-effect; differences vs. control were still significant but lower, and the number of errors was lower, as compared to STZ-icv-treated rats: RR=0.68, $p<0.001$ and RR=0.25, $p<0.001$, for M30 (2 mg/kg and 10 mg/kg), respectively (Fig. 2B). The effects of the treatments on escape latency time followed the same pattern (Fig. 2C). As shown in Fig. 2D, when escape latency was analysed with adjustment for the number of errors (as a time-varying covariate), no major effect was observed, indicating that the main effect was on number of entries into incorrect compartments (number of errors) resulting in longer time needed to escape from water onto the platform. Therefore, specific statistical evaluation of learning capacity over time and long-term acquisition/retention of memory focused on the number of errors. The main points are summarized in Table 1. Controls retained constant relative learning capacity throughout the experiment. STZ-icv-treated rats had significantly reduced learning capacity (vs. controls) in the 1^{st} ($p<0.001$) and 2^{nd} ($p=0.029$) cycles, but with repeated trainings, the capacity increased and was, in relative terms, close to that in controls, yet at a higher absolute level of the number of errors. M30 (10 mg/kg) antagonized STZ-icv-induced impairment. Day 1 values decreased over time (cycles) in all groups (development of memory), but markedly more in controls than in STZ-icv-treated rats ($p<0.001$). The decrease with M30 (2 mg/kg) was similar to that of STZ-icv alone ($p=0.480$), but less than in controls ($p=0.003$), while the decrease with M30 (10 mg/kg) was similar to that in control animals ($p=0.122$) and significantly more pronounced than in STZ-icv-treated rats ($p=0.010$) (Fig. 2E). In summary, the data illustrate an impairing effect of STZ-icv on memory acquisition/retention that is antagonized by the higher M30 dose. Furthermore, the difference between STZ-icv-treated and control rats

progressively increased across the four cycles from RR=2.03, p=0.005 to RR=18.4, p<0.001, indicating a time-dependent enhancement of STZ-icv-induced impairment. At the same time, the difference between M30 (10 mg/kg)-treated STZ-icv rats and vehicle-treated STZ-icv rats also progressively increased across the four cycles from RR=0.68, p=0.213 to RR=0.13, p=0.026, indicating a time-dependent enhancement of the beneficial effect of M30 on STZ-icv-induced impairment.

Memory performance in the probe trial over time. Performance in the MWM probe trials during 3 months after STZ-icv administration revealed a significant deficit in spatial memory of STZ-icv rats, in comparison to the controls at all time-points, demonstrated as higher number of mistakes ($p<0.05$) and shorter time spent within the targeted compartment ($p<0.05$) (Fig. 3). Time-course data, recorded in the probe trials within the STZ-icv group support the above-mentioned limited capacity of retaining the spatial memory; STZ-icv rats have retained limited memory of the pathway to the platform making less incorrect entries as the repetition of MWM trials increased in the course of 2 and 3 months after STZ-icv treatment ($p<0.05$) (Fig. 3A). In the STZ-icv-treated group, the tendency for limited improvement in the course of time is seen in the time spent in targeted compartment, which however, has not reached the significant level (Fig. 3B). Treatment with M30, initiated 8 days after STZ-icv, ameliorated STZ-induced memory deficits, affecting primarily the number of mistakes in the MWM probe trial (Fig. 3A). The number of mistakes in M30 (10 mg/kg)-treated STZ-icv group was significantly reduced ($p<0.05$) in comparison to STZ-icv treated rats, at 2 weeks and 2 and 3 months following the STZ-icv administration (Fig. 3.3A). The effect was clearly dose-dependent, as the STZ-icv group treated with M30 (10 mg/kg) demonstrated significantly reduced number of mistakes, compared to that observed in the group treated with the lower, 2 mg/kg M30 dose, which was ineffective in this respect (Fig. 3A). However, both M30 doses

were almost ineffective in increasing the time within the targeted compartment in STZ-icv treated rats, with the exception of the 2-month time-point, when a significant increase was achieved with the high M30 dose, in comparison to the STZ-icv treatment alone (Fig. 3B). Failure of the high M30 dose to increase the time spent within the targeted compartment at 3-month time point might be related to the number of animals in this group at that time point. Similar to the control group, the STZ-icv group treated with the higher M30 dose demonstrated preserved capability to retain memory over time, as shown by increased time spent in targeted compartment after 1 and 2 months in comparison to 2 weeks after STZ-icv treatment ($p<0.05$) (Fig. 3B). This beneficial effect was missing in STZ-icv group treated with a low M30 dose (Fig. 3B).

PA test

Eleven-week long treatment with M30 (10 mg/kg), initiated 8 days following the STZ-icv injection, successfully restored STZ-icv induced impairment in fear-motivated escape memory in PA test (Fig. 4), while M30 (2 mg/kg) was ineffective in this respect, as latency time remained similar to STZ-icv treatment alone (Fig. 4).

Neuropreventive effect of HLA20 on STZ-icv-induced memory impairment

Further experiments have been performed in order to examine the effect of HLA20, a chemically different multifunctional compound, which shares the iron-chelating activity with M30, on STZ-icv-induced cognitive deficits.

Five-day oral pre-treatment with **HLA20** (5 mg/kg) significantly decreased the number of mistakes in the MWM probe trial (Fig. 5A) and increased the time spent within the targeted

compartment (Fig 5B), in comparison to the STZ-icv treatment alone ($p<0.05$). HLA20 (5 mg/kg) also prevented STZ-icv-induced impairment in fear-motivated escape memory in the PA test, measured 1 month after STZ-icv treatment and increased latency time ($p<0.05$), compared to the STZ-icv treatment alone (Fig. 5C). Oral pre-treatment with a higher, HLA20 dose (10 mg/kg) exerted a neuropreventive effect in STZ-icv-treated rats in the MWM probe trial already after 2 weeks, seen both as less entries into the incorrect compartments (Fig. 6A) ($p<0.05$) and longer time (Fig. 6B) ($p<0.05$) within the targeted compartment, in comparison to the STZ-icv treated rats. The latter effect of HLA20 was persistent up to 3 months after STZ-icv treatment, while the significance of the former vanished in the course of time (Fig. 6A). HLA20 to buffer-icv-treated rats had no effects on cognitive performance, compared to control rats, with the exception of the beneficial effect (reduction of mistake number) seen of 2 weeks (Fig. 6A).

Beneficial effect of M30 on STZ-induced decrement in IDE level and on tau hyperphosphorylation

Possible molecular mechanisms underlying M30 beneficial effects on cognitive performance in the neurorescue paradigm were explored at the level of IDE and tau protein (AT8/PHF1), two parameters which build up the pathophysiological core of AD.

While IDE expression in hippocampus was found unaffected 2 weeks after STZ-icv treatment, it was significantly decreased (-21%, $p<0.05$) after 3 months (Fig. 7A, F). Long-term treatment with both low and high M30 dose in neurorescue paradigm significantly increased IDE expression in comparison to the STZ-icv treatment alone (+19% and +37%, $p<0.05$) and normalized it to the levels of controls (Fig. 7A, F).

Compared to controls, significant increase in PHF1 phospho tau expression and in PHF1 phospho/total tau ratio was detected 2 weeks (+328% and +461%, $p<0.05$) and 3 months following STZ-icv administration (+120% and +215%, $p<0.05$) (Fig. 7B, C, G, H). Long-term treatment with high M30 dose (10 mg/kg) in the neurorescue paradigm completely normalized STZ-induced increment in both PHF1 expression ($p<0.05$) and PHF1/total tau ratio ($p<0.05$) to the control levels, measured 3 months after STZ-icv administration (Fig. 7G, H). Low M30 dose was ineffective in this respect (Fig. 7G, H). This beneficial effect of high dose begun to be manifested already after a week of M30 treatment (i.e. 2 weeks after STZ-icv treatment) when significantly reduced STZ-induced increment in PHF1/total tau protein ratio (-42%, $p<0.05$) was found in comparison to STZ treatment alone (Fig. 7C). Long-term treatment with high M30 dose (10 mg/kg) significantly decreased AT8 phospho tau expression in STZ-icv treated rats (-34% and -60%, $p<0.05$ vs control and STZ), although at that time point, a tendency of STZ-induced increment in AT8 expression did not reach the level of significance (Fig. 7D, E, I, J).

DISCUSSION

Numerous clinical AD trials failed to confirm the effectiveness of drugs tested so far (Mangialasche et al. 2010) which, among other reasons, could be due to focusing on the inappropriate mechanism of drug action based on the incorrect understanding of the primary pathophysiological core of the disease. Mechanisms of action, other than those affecting primarily Aβ homeostasis or tau phosphorylation and assembly, which have all targeted a single pathological process (Salomone et al. 2012), have been rather neglected. Additionally, a growing body of evidence indicates that there might be different sAD endophenotypes (e.g.

apolipoprotein E positive and negative individuals) (Borroni et al. 2006), suggesting that choosing the animal model, which mimics a specific endophenotype (e.g. “brain insulin resistance” endophenotype) might contribute to a more successful translation of the results to the corresponding endophenotype of sAD population (Zahs et al. 2010).

Considering the diverse etiological nature of AD forms, novel therapeutic strategies should be focused on the implementation of drugs directed to various neuronal targets to address the multiple pathology aspects of the disease (Van der Schyf et al. 2006; Youdim and Buccafusco 2005). The homeostasis of brain iron is thought to be necessary for normal brain function, especially in learning and memory (Youdim et al. 1989). Disruption in iron metabolism and pathological iron accumulation in the brain have been postulated to have a role in the pathogenesis of AD (reviewed by Grünblatt et al. 2010), suggesting that iron-chelating drugs might have a beneficial therapeutic effect in AD. M30 and HLA20, have been developed as multifunctional compounds whose molecule consists of propargylamine, the active neuroprotective moiety of rasagiline, embedded in the backbone of the antioxidant-iron chelator 8-hydroxyquinoline derivative of VK-28 (Ben-Shachar et al. 2004; Zheng et al. 2005). Previously, iron-complexing agents have been suggested as a promising therapeutic strategy for the treatment of AD (Crapper et al. 1991; Ritchie et al. 2003). However, these compounds are either toxic or do not penetrate the blood brain barrier (Ritchie et al., 2003), while M30 and HLA20 have been developed as multi-targeting non-toxic, brain permeable drugs, which exert iron chelating potency, radical scavenging and inhibition of iron-induced membrane lipid peroxidation features. M30 and HLA20 were found to confer neuroprotective activity in various in vitro and in vivo animal models of neurodegenerative disorders (Ben-Shachar et al. 2004; Kupershmidt et al. 2012; Zheng et al. 2005).

The presented results have demonstrated that M30 exerted neuroprotective activity by completely preventing the development or ameliorating already developed cognitive deficits

in STZ-icv-treated rats in the preventive and neurorescue paradigms, respectively. Regardless the experimental paradigm, this beneficial effect of M30 has been clearly dose-dependent and increased in extent with the duration of drug treatment. The differences in the effectiveness of M30 on various parameters of reference and working memory measured in the MWM trials are in line with their different sensitivity to the STZ-icv injection. Thus, both the toxic effect of STZ-icv treatment and the beneficial activity of M30 were more prominent on the number of mistakes, either in training or in probe trials. M30 treatment was found to be less effective in improving the working than the reference memory, presumably as working memory seems to be more sensitive (impaired to a greater extent) following the STZ-icv treatment. However, M30 eventually restored memory acquisition in the training trials and escape latency in the PA test to the levels of controls. The reasons for that could not be elucidated by our research and are likely associated with different sensitivity of hippocampal subregions involved in processing various types of memory (Morris et al. 2012; Xavier et al. 1999); it was shown that different types of cognitive disruptions occur following STZ-icv injection with distinct time courses (Salkovic-Petrisic et al. 2012; Santos et al. 2012). Considering that similar beneficial effect on STZ-icv-induced memory impairment was observed with another multifunctional iron-chelating agent (HLA20) and also with the iron-chelator VK-28 (data not shown), iron-chelation seems to be crucial for the cognitive improvement effect of M30 in this model.

The exact mechanism by which STZ-icv treatment impairs cognitive functions still needs to be elucidated, but factors like direct neurotoxicity, brain glucose/energy and cholinergic deficits, oxidative stress and insulin resistant brain state, may all form the biological basis for the marked reduction in learning and memory capacities found in this model. STZ generates intracellular free radicals, nitric oxide and hydrogen peroxide (H_2O_2), and is selectively toxic for insulin producing/secreting cells (Szkudelski 2001). Regarding the antioxidant properties

of M30, recent studies demonstrated that the drug attenuated H_2O_2-induced mitochondrial membrane potential loss and displayed significant cytoprotective activity against cytotoxicity induced by oxidative stress in insulin producing beta-cells (Mechlovich et al. 2010). Therefore, neuroprevention in the M30 pre-treatment studies could be related to the antioxidative activity of M30 against acute STZ-icv-induced oxidative stress. Indeed, our preliminary data showed that M30 pretreatment prevented STZ-icv-induced decrement in hippocampal catalase activity (Sofic et al. 2014). However, therapeutic effects of M30 in the neurorescue design could be related, in addition to the antioxidant activity, also to the iron-chelating beneficial effect on the pathological processes developed after the STZ-icv administration. This effect might be only speculated considering the fact that there are no literature data on iron homeostasis in the brain of STZ-icv rat model, but our preliminary results of Prussian blue iron staining of brain slices obtained 3 months after icv treatment indirectly support the iron-chelating activity of M30 in this model. The analysis revealed a positive signal of ferric iron in the brain mostly in the path of the needle insertion during the icv injection with a mild to moderate intensity in the control (buffer-icv) and the very intensive, more widely distributed signal in the STZ-icv treated rats (Supplementary material). These changes, which seem to reflect the post-haemorrhage condition induced by mechanical/chemical brain damage, were significantly and dose-dependently ameliorated by a long-term oral M30 treatment in the STZ-icv treated rats.

Recent reports have indicated that M30 exerted various beneficial neuroprotective regulatory effects that might be attributed to the multimodal design paradigm of the drug; including up regulation of insulin/insulin receptor/phospho-glycogen synthase kinase β levels, activation of hypoxia-inducible factor-1 pathway, and antioxidation (Mechlovich et al. 2014a,b). It was reported that M30 significantly reduced iron accumulation in the cortex of aged mice

(Kupershmidt et al. 2012). Our results have confirmed the previous finding in transgenic mice AD model (Kupershmidt et al. 2012) that the mechanism of beneficial M30-induced cognitive effects might be related to normalization of tau protein hyperphosphorylation in the hippocampus of the non-Tg rat sAD model. This is probably an indirect effect of M30 shown to affect kinases directly involved in regulation of homeostasis of tau protein phosphorylation (Kupershmidt et al. 2012). However, we proposed here for the first time an additional mechanism of action and a new possible target for M30 by demonstrating that long-term M30 oral treatment normalizes reduced hippocampal IDE levels. IDE is a metalloprotease responsible for degradation of both insulin and Aβ, and reduced IDE levels in sAD leading to decreased Aβ degradation significantly contribute to the disease pathophysiology. Literature data indicate that iron-induced oxidative stress inactivates IDE (Shinall et al. 2005) which might provide an explanation why iron-chelators like M30 could have beneficial effect at the level of IDE, as detected in our experiments for the first time. Decreased IDE protein and gene expression has been found in STZ-icv rat model also by others (Yang et al. 2014), but our recent 9-month follow up data on staging of cognitive, neuropathological and neurochemical changes in STZ-icv rat model has shown that the order of pathology appearance after STZ-icv treatment is: tau protein/2 weeks, IDE/1 month, and amyloid β/3 months (Osmanovic Barilar et al. 2015; Knezovic et al. 2015), which supports the results presented here.

Control of iron homeostasis in the brain, as achieved by iron-chelating agents like M30, might be also important considering the fact that Aß-upregulation is inducible by two iron-dependent pathways:

(1) iron induces ROS generation, which, via stimulation of the expression of tissue inhibitor of metalloproteinase-2 (TIMP2), blocks α-secretase and thus provokes β-/γ-secretase induced

Aß production. In addition, increased ROS species shift cytoplasmatic aconitase to iron regulatory protein 1 (IRP-1), which stimulates cells to enhance iron uptake due to the wrong IRP-1 induced message of an existing iron deficiency.

(2) iron accumulation down-regulates a proconvertase furin, thus impairing the processing of ADAM 10 and TACE, two metalloproteases that show α-secretase activity and are involved in sAPP production (Silvestri and Camaschella 2008). Moreover, the proprotein convertase furin promotors reveal the presence of putative binding sites for hypoxia-inducible factor-1 (HIF-1), a transcription complex that plays a pivotal role in cellular adaptation to hypoxia (Mc Mahon et al. 2005) and thus offers another link to iron induced pathology (Crichton et al. 2011).

CONCLUSION

In summary the multifunctional iron-chelating agent M30 demonstrates neuropreventive and neurorescue activity in restoring/ameliorating cognitive deficits in a non-transgenic STZ-icv rat model of sAD. M30-induced restoration of tau hyperphosphorylation and normalization of reduced IDE level in hippocampus might be responsible for M30 beneficial cognitive effects. These data provide further support to the therapeutic potential of multi-target iron-chelating agents in sAD treatment. The advantages of using an AD model with no Aβ- or tau-related gene modification, such as the STZ-icv rat model, in comparison to the transgenic mice AD models, is additionally achieved by possibility to define the exact time of the induction of brain damage and accordingly to successfully explore the efficacy of the novel treatment initiated at different time-points before or after the STZ-icv-induced brain damage.

ACKNOWLEDGEMENT

This work was supported by a grant from the Croatian Ministry of Science, Education and Sport (MZOS project No. 108-1080003-0020 to MSP), and a grant from Deutscher Akademischer Austausch Dienst (DAAD project No. A/00/200017 to PR). M30 and HLA20 were donated by Eve Topf Center of Excellence, Technion –Faculty of Medicine, Haifa, Israel (MBHY).

CONFLICT OF INTEREST

MBHY as Chief Scientific Officer of Abital Pharma Pipeline Ltd, is part owner of the company has shares and stocks in it. Other authors claim no potential conflicts of interest.

REFERENCES

Adlard, P.A., Bica, L., White, A.R., Nurjono, M., Filiz, G., Crouch, P.J., Donnelly, P.S., Cappai, R., Finkelstein, D.I., Bush, A.I. 2011. Metal ionophore treatment restores dendritic spine density and synaptic protein levels in a mouse model of Alzheimer's disease. PloS. One. 6, e17669. doi:10.1371/journal.pone.0017669.

Agrawal, R., Tyagi, E., Shukla, R., Nath, C., 2011. Insulin receptor signaling in rat hippocampus: a study in STZ (ICV) induced memory deficit model. Eur. Neuropsychopharmacol. 21, 261-273.

Balducci, C., Forloni, G., 2011. APP transgenic mice: their use and limitations. Neuromolecular. Med. 13, 117-137.

Ben-Shachar, D., Kahana, N., Kampel, V., Warshawsky, A., Youdim, M.B., 2004. Neuroprotection by a novel brain permeable iron chelator, VK-28, against 6-hydroxydopamine lession in rats. Neuropharmacology. 46, 254-263.

Blokland, A., Jolles, J., 1993. Spatial learning deficit and reduced hippocampal ChAT activity in rats after an ICV injection of streptozotocin. Pharmacol. Biochem. Behav. 44, 491-494.

Borroni, B., Grassi, M., Costanzi, C., Archetti, S., Caimi, L., Padovani, A., 2006. APOE genotype and cholesterol levels in lewy body dementia and Alzheimer disease: investigating genotype-phenotype effect on disease risk. Am. J. Geriatr. Psychiatry. 14, 1022-1031.

Budimir, A., 2011. Metal ions, Alzheimer's disease and chelation therapy. Acta. Pharm. 61, 1-14.

Cherny, R.A., Atwood, C.S., Xilinas, M.E., Gray, D.N., Jones, W.D., McLean, C.A., Barnham, K.J., Volitakis, I., Fraser, F.E., Kim, Y., Huang, X., Goldstein, L.E., Moir, R.D., Lim, J.T., Beyreuther, K., Zheng, H., Tanzi, R.E., Masters, C.L., Bush, A.I., 2001. Treatment with a copper-zinc chelator markedly and rapidly inhibits beta-amyloid accumulation in Alzheimer’s disease trensgenic mice. Neuron. 30, 665-676.

Correia, S.C., Santos, R.X., Perry, G., Zhu, X., Moreira, P.I., Smith, M.A., 2011. Insulin-resistant brain state: The culprit in sporadic Alzheimer's disease? Ageing. Res. Rev. 10, 264-273.

Crapper McLachlan, D.R., Dalton, A.J., Kruck, T.P., Bell, M.Y., Smith, W.L., Kalow, W., Andrews, D.F., 1991. Intramuscular desferrioxamine in patients with Alzheimer's disease. Lancet. 337, 1304-1308.

Crichton, R.R., Dexter, D.T., Ward, R.J., 2011. Brain iron metabolism and its perturbation in neurological diseases. J. Neural. Transm. 118, 301-314.

de la Monte, S.M., Wands, J.R., 2005. Review of insulin and insulin-like growth factor expression, signaling, and malfunction in the central nervous system: relevance to Alzheimer's disease. J. Alzheimer's. Dis. 7, 45-61.

Deng, Y., Li, B., Liu, Y., Iqbal, K., Grundke-Iqbal, I., Gong, C.X., 2009. Dysregulation of insulin signaling, glucose transporters, O-GlcNAcylation, and phosphorylation of tau and neurofilaments in the brain: Implication for Alzheimer's disease. Am. J. Pathol. 175, 2089-2098.

Frölich, L., Blum-Degen, D., Bernstein, H.G., Engelsberger, S., Humrich, J., Laufer, S., Muschner, D., Thalheimer, A., Turk, A., Hoyer, S., Zochling, R., Boissl, K.W., Jellinger, K., Riederer, P., 1998. Brain insulin and insulin receptors in aging and sporadic Alzheimer's disease. J. Neural. Transm. 105, 423-438.

Grünblatt, E., Bartl, J., Riederer, P., 2010. The link between iron, metabolic syndrome and Alzheimer's disease. J. Neural. Transm. 118, 371-379.

Grünblatt, E., Salkovic-Petrisic, M., Osmanovic, J., Riederer, P., Hoyer, S., 2007. Brain insulin system dysfunction in streptozotocin intracerebroventricularly treated rats generates hyperphosphorylated tau protein. J. Neurochem. 101, 757-770.

Guo, C., Wang, P., Zhong, M.L., Wang, T., Huanq, X.S., Li, J.Y., Wang, Z.Y., 2013. Deferoxamine inhibits iron-induced hippocampal tau phosphorylation in the Alzheimer transgenic mouse brain. Neurochem. Int. 62, 165-172.

Guo, C., Wang, T., Zheng, W., Shan, Z.Y., Teng, W.P., Wang, Z.Y., 2013b. Intranasal deferoxamine reverses iron-induced memory deficits and inhibits amyloidogenic APP processing in a transgenic mouse model of Alzheimer's disease. Neurobiol. Aging. 34, 562-575.

Hellweg, R., Nitsch, R., Hock, C., Jaksch, M., Hoyer, S., 1992. Nerve growth factor and choline acetyltransferase activity levels in the rat brain following experimental impairment of cerebral glucose and energy metabolism. J. Neurosci. Res. 31, 479-486.

Honda, K., Casadesus, G., Petersen, R.B., Perry, G., Smith, M.A., 2004. Oxidative stress and redox-active iron in Alzheimer's disease. Ann. N. Y. Acad. Sci. 1012, 179-182.

Kilkenny, C., Browne, W., Cuthill, I.C., Emerson, M., Altman, D.G., 2010. Animal research: reporting *in vivo* experiments: the ARRIVE guidelines. Br. J. Pharmacol. 160, 1577–1579.

Knezovic, A., Osmanovic-Barilar, J., Curlin, M., Hof, P.R., Simic, G., Riederer, P., Salkovic-Petrisic, M., 2015. Staging of cognitive deficits and neuropathological and ultrastructural changes in streptozotocin-induced rat model of Alzheimer's disease. J. Neural. Transm. 122, 577-592.

Kupershmidt, L., Amit, T., Bar-Am, O., Youdim, M.B., Weinreb, O., 2012. The novel multi-target iron chelating-radical scavenging compound M30 possesses beneficial effects on major hallmarks of Alzheimer's disease. Antioxid. Redox. Signal. 17, 860-877.

Lannert, H., Hoyer, S., 1998. Intracerebroventricular administration of streptozotocin causes long-term diminutions in learning and memory abilities and in cerebral energy metabolism in adult rats. Behav. Neurosci. 112, 1199-1208.

Lee, Y., Kim, Y.H., Park, S.J., Huh, J.W., Kim, S.H., Kim, S.U., Kim, J.S., Jeong, K.J., Lee, K.M., Hong, Y., Lee, S.R., Chang, K.T., 2014. Insulin/IGF signaling-related gene expression in the brain of a sporadic Alzheiemer’s disease monkey model induced by intracerebroventricular injection of streptozotocin. J. Alzheimers. Dis. 38, 251-267.

Lester-Coll, N., Rivera, E.J., Soscia, S.J., Doiron, K., Wands, J.R., de la Monte, S., 2006. Intracerebral streptozotocin model of type 3 diabetes: Relevance to sporadic Alzheimer's disease. J. Alzheimer's. Dis. 9, 13-33.

Lithner, C.U., Hedberg, M.M., Nordberg, A., 2011. Trangenic mice as a model for Alzheiemer's disease. Curr. Alzheimer. Res. 8, 818-831.

Liu, P., Zou, L.B., Wang, L.H., Jiao, Q., Chi, T.Y., Ji, X.F., Jin, G., 2014. Xanthoceraside attenuates tau hyperphosphorylation and cognitive deficits in intracerebroventricular-streptozotocin injected rats. Psychopharmacology (Berl). 231, 345-356.

Mangialasche, F., Solomon, A., Winblad, B., Mecocci, P., Kivipelto, M., 2010. Alzheimer's disease: clinical trials and drug development. Lancet. Neurol. 9, 702-716.

Mayer, G., Nitsch, R., Hoyer, S., 1990. Effects of changes in peripheral and cerebral glucose metabolism on locomotor activity, learning and memory in adult male rats. Brain. Res. 532, 95-100.

McMahon, S., Grondin, F., McDonald, P.P., Richard, D.E., Dubois, C.M., 2005. Hypoxia-enhanced expression of the proprotein convertase furin is mediated by hypoxia-inducible factor-1: impact on the bioactivation of proproteins. J. Biol. Chem. 280, 6561-6569.

Mechlovich, D., Amit, T., Mandel, S.A., Bar-Am, O., Bloch, K., Vardi, P., Youdim, M.B., 2010. The novel multifunctional, iron-chelating drugs M30 and HLA20 protect pancreatic beta-cell lines from oxidative stress damage. J. Pharmacol. Exp. Ther. 333, 874-882.

Mechlovich, D., Amit, T., Bar-Am, O., Mandel, S., Youdim, M.B., Weinreb, O., 2014a. The novel multi-target iron chelator, M30 modulates HIF-1α-related glycolytic genes and insulin signaling pathway in the frontal cortex of APP/PS1 Alzheimer's disease mice. Curr. Alzheimer. Res. 11, 119-127.

Mechlovich, D., Amit, T., Bar-Am, O., Wienreb, O., Youdim, M.B., 2014b. Brain molecular targets of the multifunctional iron chelating drug, M30 in type 2 diabetes mellitus mouse models. Br. J. Pharmacol. doi: 10.1111/bph.12862. [Epub ahead of print]

Morris, A.M., Churchwell, J.C., Kesner, R.P., Gilbert, P.E., 2012. Selective lesions of the dentate gyrus produce disruptions in place learning for adjecent spatial locations. Neurobiol. Learn. Mem. 97, 326-331.

Osmanovic Barilar, J., Knezovic, A., Grünblatt, E., Riederer, P., Salkovic-Petrisic, M., 2015. Nine-month follow-up of the insulin receptor signalling cascade in the brain of streptozotocin rat model of sporadic Alzheimer's disease. J. Neural. Transm. 122, 565-576.

Peng, D., Pan, X., Cui, J., Ren, Y., Zhang, J., 2013. Hyperphosphorylation of tau protein in hippocampus of central insulin-resistant rats is associated with cognitive impairment. Cell. Physiol. Biochem. 32, 1417-1425.

Plaschke, K., Hoyer, S., 1993. Action of the diabetogenic drug streptozotocin on glycolytic and glycogenolytic metabolism in adult rat brain cortex and hippocampus. Int. J. Dev. Neurosci. 11, 477-483.

Ritchie, C.W., Bush, A.I., Mackinnon, A., Macfarlane, S., Mastwyk, M., MacGregor, L., Kiers, L., Cherny, R., Li, Q.X., Tammer, A., Carrington, D., Mavros, C., Volitakis, I., Xilinas, M., Ames, D., Davis, S., Beyreuther, K., Tanzi, R.E., Masters, C.L., 2003. Metal-protein attenuation with iodochlorhydroxyquin (clioquinol) targeting Abeta amyloid deposition and toxicity in Alzheimer disease: a pilot phase 2 clinical trial. Arch. Neurol. 60, 1685-1691.

Salkovic-Petrisic, M., Knezovic, A., Hoyer, S., Riederer, P., 2012. What have we learned from the streptozotocin-induced animal model of sporadic Alzheimer's disease, about the therapeutic strategies in Alzheimer's research. J. Neural. Transm. 120, 233-252.

Salkovic-Petrisic, M., Osmanovic-Barilar, J., Brückner, M.K., Hoyer, S., Arendt, T., Riederer, P., 2011. Cerebral amyloid angiopathy in streptozotocin rat model of sporadic Alzheimer's disease: a long-term follow up study. J. Neural. Transm. 118, 765-772.

Salkovic-Petrisic, M., Tribl, F., Schmidt, M., Hoyer, S., Riederer, P., 2006. Alzheimer-like changes in protein kinase B and glycogen synthase kinase-3 in rat frontal cortex and hippocampus after damage to the insulin signalling pathway. J. Neurochem. 96, 1005-1015.

Salomone, S., Caraci, F., Leggio, G.M., Fedotova, J., Drago, F., 2012. New pharmacological strategies for treatment of Alzheimer's disease: focus on disease modifying drugs. Br. J. Clin. Pharmacol. 73, 504-517.

Santos, T.D., Mazucanti, C.H., Xavier, G.F., Torrão, A.D., 2012. Early and late neurodegeneration and memory disruption after intracerebroventricular streptozotocin. Physiol. Behav. 107, 401-413.

Saxena, G., Patro, I.K., Nath, C., 2011. ICV STZ induced impairment in memory and neuronal mitochondrial function: A protective role of nicotinic receptor. Behav. Brain. Res. 224, 50-57.

Sharma, M., Gupta, Y.K., 2001. Intracerebroventricular injection of streptozotocin in rats produces both oxidative stress in the brain and cognitive impairment. Life. Sci. 68, 1021-1029.

Shinall, H., Song, E.S., Hersh, L.B., 2005. Susceptibility of amyloid beta peptide degrading enzymes to oxidative damage: a potential Alzheimer's disease spiral. Biochemistry. 44, 15345-15350.

Shingo, A.S., Kanabayashi, T., Kito, S., Murase, T., 2013. Intracerebroventricular administration of an insulin analogue recovers STZ-induced cognitive decline in rats. Behav. Brain. Res. 241, 105-111.

Silvestri, L., Camaschella, C., 2008. A potential pathogenetic role of iron in Alzheimer's disease. J. Cell. Mol. Med. 12, 1548-1550.

Szkudelski, T., 2001. The mechanism of alloxan and streptozotocin action in B cell of the rat pancreas. Physiol. Res. 50, 537-546.

Sofic, E., Salkovic-Petrisic, M., Tahirovic, I., Sapcanin, A., Mandel, S., Youdim, P., Riederer, P., 2014. Brain catalase in the streptozotocin-rat model of sporadic Alzheiemr's disease treated with the iron chelator-monoamine oxidase inhibitor, M30. J. Neural. Transm. 2014 Sep 25. [Epub ahead of print]

Van der Schyf, C.J., Geldenhuys, W.J.,Youdim, M.B., 2006. Multifunctional drugs with different CNS targets for neuropsychiatric disorders. J. Neurochem. 99, 1033-1048.

Weinreb, O., Mandel, S., Bar-Am, O., Amit, T., 2011. Iron-chelating backbone coupled with monoamine oxidase inhibitory moiety as novel pluripotential therapeutic agents for Alzheimer's disease: a tribute to Moussa Youdim. J. Neural. Transm. 118, 479-492.

Xavier, G.F., Oliveira-Filho, F.J., Santos, A.M., 1999. Dentate gyrus-selective colchicine lesion and disruption of performance in spatial tasks: difficulties in "place strategy" because of a lack of flexibility in the use of environmental cues? Hippocampus. 9, 668-681.

Yang, W., Ma, J., Liu, Z., Lu, Y., Hu, B., Yu, H., 2014. Effect of naringenin on brain insulin signaling and cognitive functions in ICV-STZ induced dementia model of rats. Neurol. Sci. 35, 741-751.

Youdim, M.B., Ben-Shachar, D., Yehuda, S., 1989. Putative biological mechanisms of the effect of iron deficiency on brain biochemistry and behavior. Am. J. Clin. Nutr. 50, 607-615.

Youdim, M.B., Buccafusco, J.J., 2005. Multi-functional drugs for various CNS targets in the treatment of neurodegenerative disorders. Trends. Pharmacol. Sci. 26, 27-35.

Zahs, K.R., Ashe, K.H., 2010. Too much good news' - are Alzheimer mouse models trying to tell us how to prevent, not cure, Alzheimer's disease? Trends. Neurosci. 33, 381-389.

Zheng, H., Weiner, L.M., Bar-Am, O., Epsztejn, S., Cabantchik, Z.I., Warshawsky, A., Youdim, M.B., Fridkin, M., 2005. Design, synthesis, and evaluation of novel bifunctional iron-chelators as potential agents for neuroprotection in Alzheimer's, Parkinson's, and other neurodegenerative diseases. Bioorg. Med. Chem. 13, 773-783.

FIGURE LEGENDS

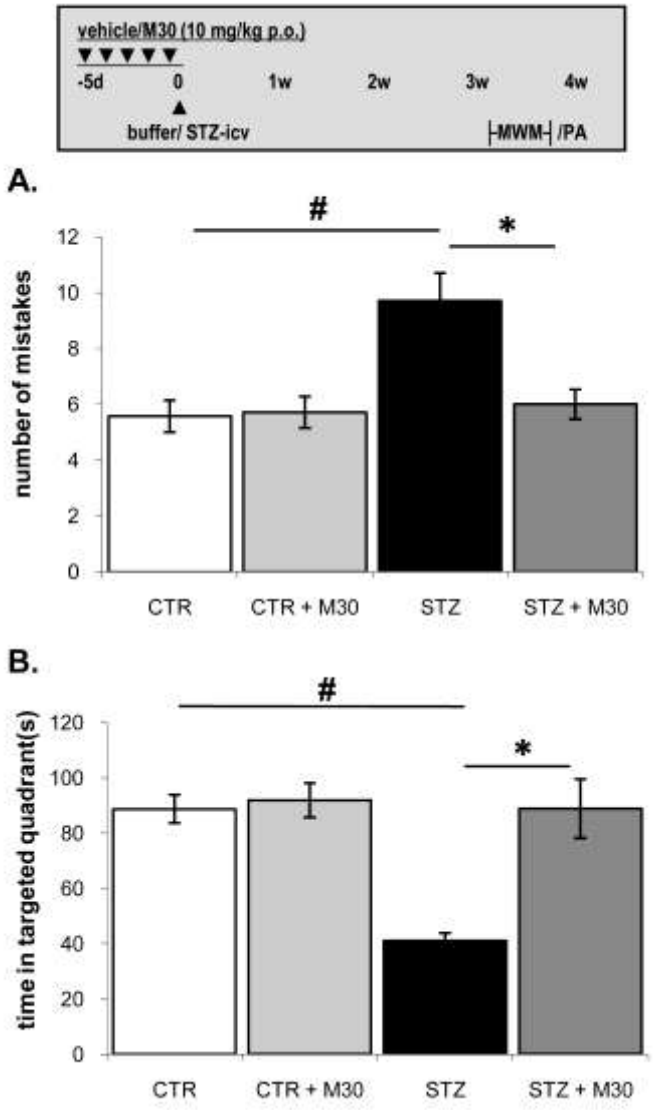

Fig. 1 Effect of pre-treatment with M30 on STZ-icv induced cognitive deficits. The top-shaded panel depicts the neuropreventive experimental design: rats were pre-treated with M30 (10 mg/kg/day), administered p.o. for 5 days. STZ-icv (1 mg/kg) or buffer-icv (CTR) injection was given after the last M30 dose on the 5th day (N = 7 / group). Number of mistakes (**A**) and time spent within the targeted quadrant (**B**) were recorded in the MWM probe trial, 4 weeks following the STZ-icv treatment. Each bar represents mean±SEM. *p<0.05 vs STZ-icv and #p<0.05 vs CTR by Kruskal-Wallis ANOVA median test followed by Mann Whitney U test.

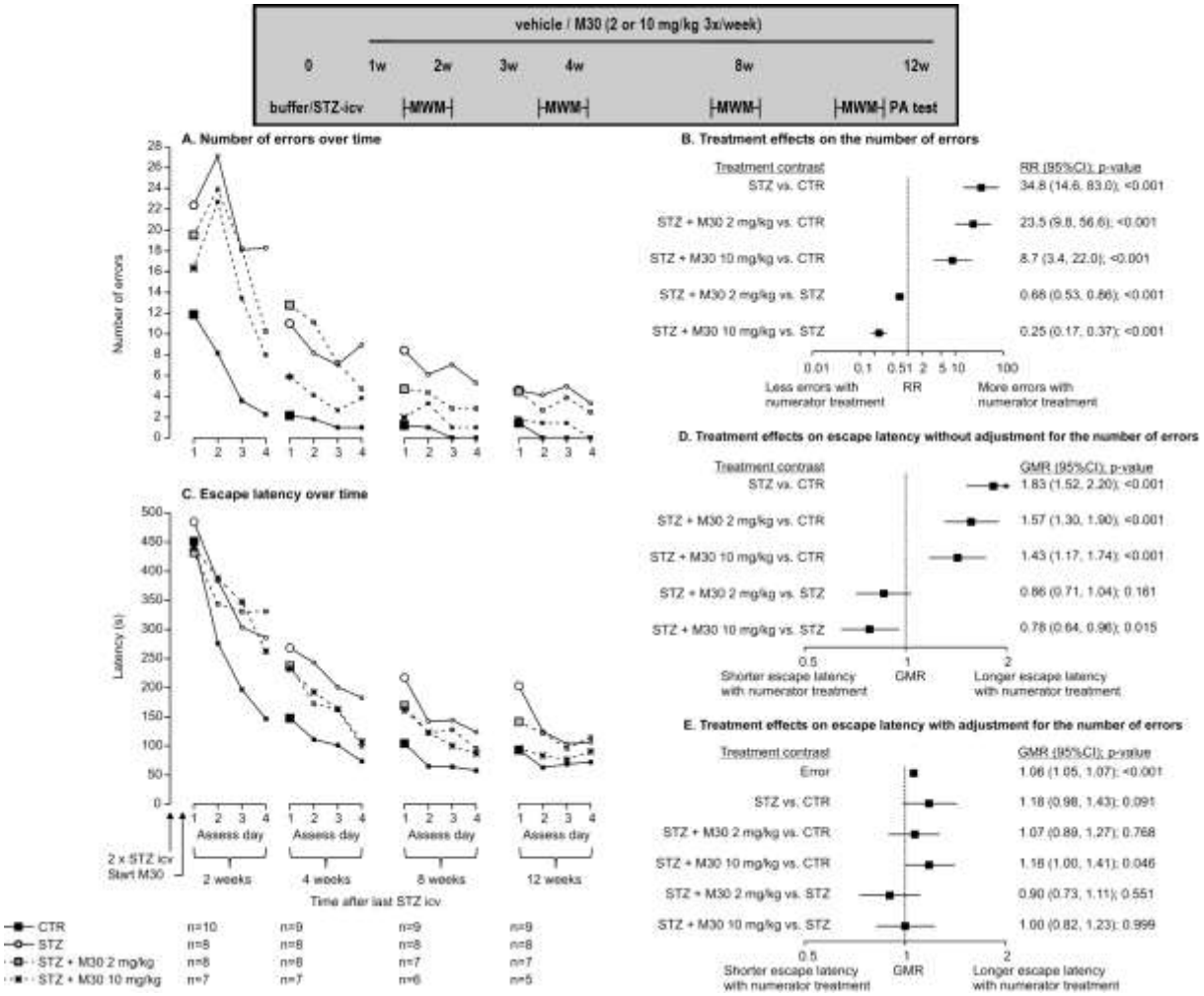

Fig. 2 Effect of M30 on STZ icv-induced cognitive deficits in the neurorescue paradigm. The top-shaded panel depicts the experimental design: rats were injected with STZ-icv (3 mg/kg) or buffer icv (CTR) on 2 consecutive days and administered with M30 (2 mg/kg or 10 mg/kg p.o. 3x week) started on the 8th day after the second icv injection and lasted for 12 weeks. Animals were evaluated in the MWM test in daily training trials (4 consecutive days, indicated as "assess day") at 2 weeks (days 14-17; all N = 8/group except 10/ group in control (CTR) group), 4 weeks (days 28-31; all N = 8/group except 9/group in CTR and 7/group in STZ+M30 (10mg/kg)), 8 weeks (days 56-59; 9/group in CTR, 8/group in STZ, 7/group in STZ+M30 (2mg/kg) and 6/group in STZ+M30 (10mg/kg)) and 12 weeks (days 84-97; 9/group in CTR, 8/group in STZ, 7/group in STZ+M30 (2mg/kg) and 5/group in STZ+M30 (10mg/kg)) after the last STZ-icv injection. A subsequent PA test was followed. (**A**) Number

of errors and (**C**) escape latency in MWM training trials over time. Each point represents the geometric mean of the cumulative number of errors/cumulative escape latency (s) of 9 trials *per* animal. The number of animals per group at the start of each training cycle is depicted at the bottom. Data for the 1st day of each cycle are shown (**B,D,E)**. A generalized linear mixed model (Poisson) (treatment, time, treatment*time interaction) was fitted to number or errors (**B**), and a linear mixed model was fitted to Ln(latency time) (treatment, time, treatment*time interaction) without (**D**) or with (**E**) further adjustment for the number of errors (a time-varying covariate). Treatment effects are presented as relative risks (RR) (errors) or geometric mean ratios (GMR) (latencies) with 95% confidence intervals and p-values. Estimates were adjusted for multiple comparisons.

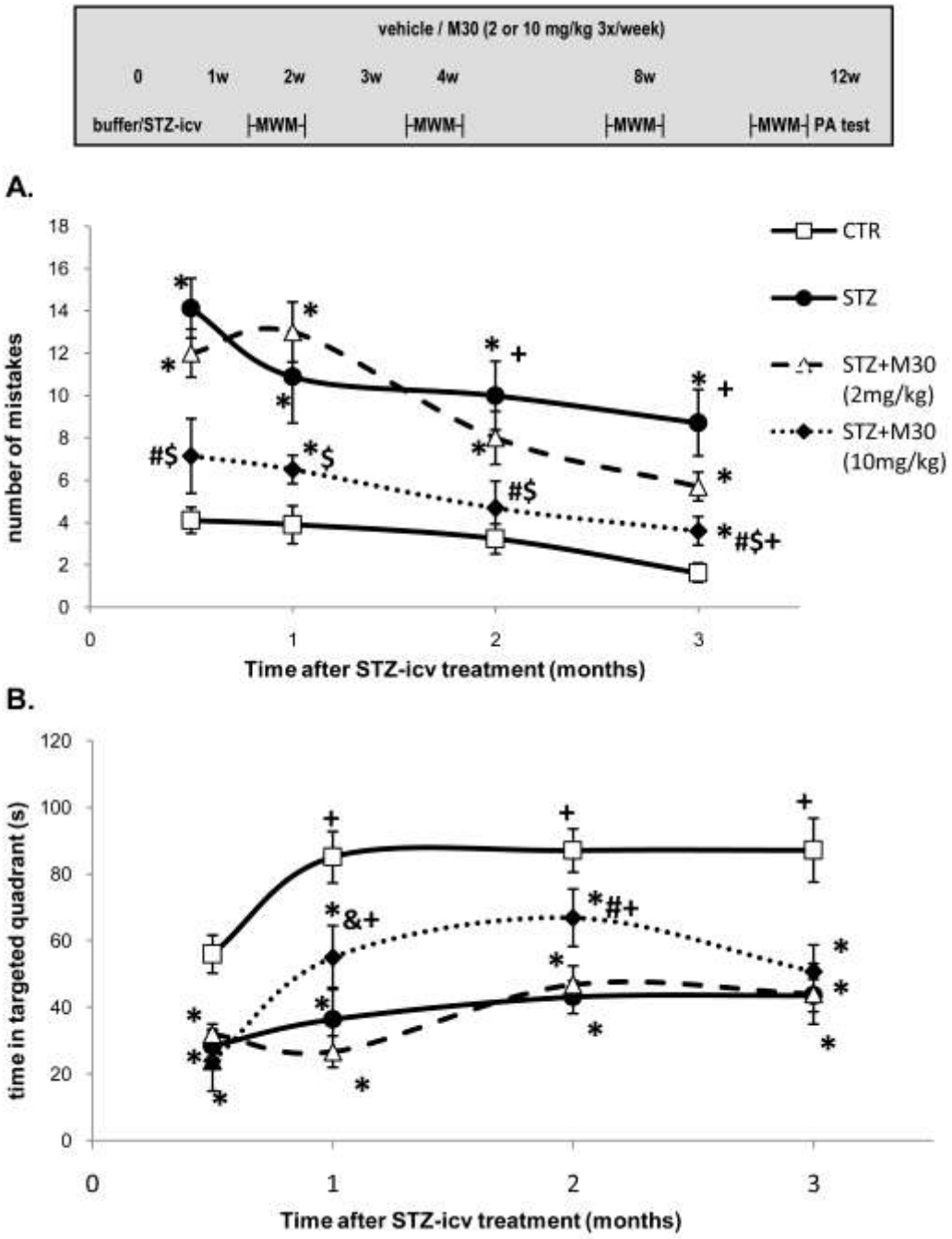

Fig. 3 Effect of M30 on memory retention in STZ-icv treated rats in the neurorescue paradigm. Treatment with M30 (2 and 10 mg/kg, p.o. 3x a week) was initiated at 8th day following the last STZ-icv (3 mg/kg) injection and continued for 11 weeks. Control animals (CTR) were treated icv with buffer. Probe trail was performed after removing of the platform on the 4th day after the last training trial, 2 weeks, 1-, 2-, and 3 months following STZ-icv treatment (N/group at particular time point are as described in Fig. 3.2), and number of

mistakes (**A**) and time spent in targeted quadrant (**B**) were recorded. Each point represents mean±SEM. *p<0.05 vs CTR at the respective time-point; #p<0.05 vs STZ-icv at the respective time-point; $p<0.05 vs STZ+ M30 (2 mg/kg) at the respective time-point; all analysis done by Kruskal-Wallis ANOVA median test followed by Mann Whitney U test. +p<0.05 vs 2 week-value of the respective group, by non-parametric Friedman test followed by Wilcoxon Signed-Ranks test.

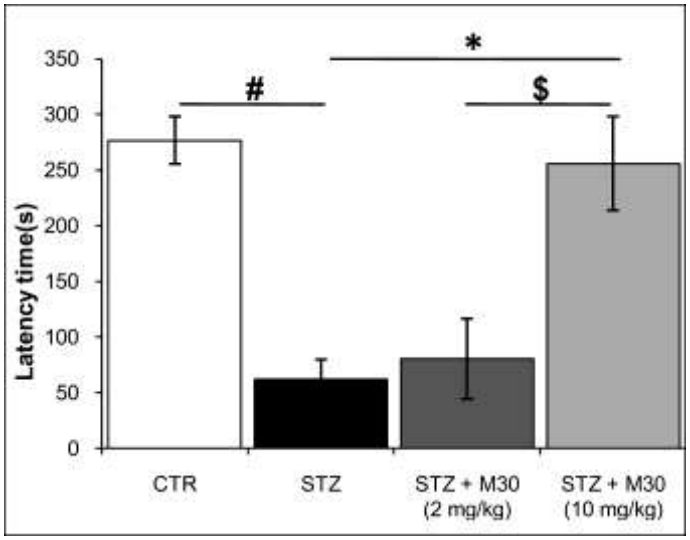

Fig. 4 Effect of M30 on cognitive performance as measured by the post-shock latency time in PA test in STZ-icv rats in the neurorescue paradigm. Treatment with M30 (2 and 10 mg/kg, p.o. 3x week) was initiated 8th day following the last STZ-icv injection (3 mg/kg) and was continued for 11 weeks. Control (CTR) animals were treated icv with buffer. Number of animals: 9/group in CTR, 8/group in STZ, 7/group in STZ+M30 (2mg/kg) and 5/group in STZ+M30 (10mg/kg). PA test was performed before sacrifice, 12 weeks following STZ-icv treatment, and escape latency (seconds) was recorded on the 3rd day of testing. Each bar represents mean±SEM value. *p<0.05 vs STZ-icv; #p<0.05 vs CTR and $p<0.05 vs STZ + M30 (2 mg/kg) by Kruskal-Wallis ANOVA median test followed by Mann Whitney U test.

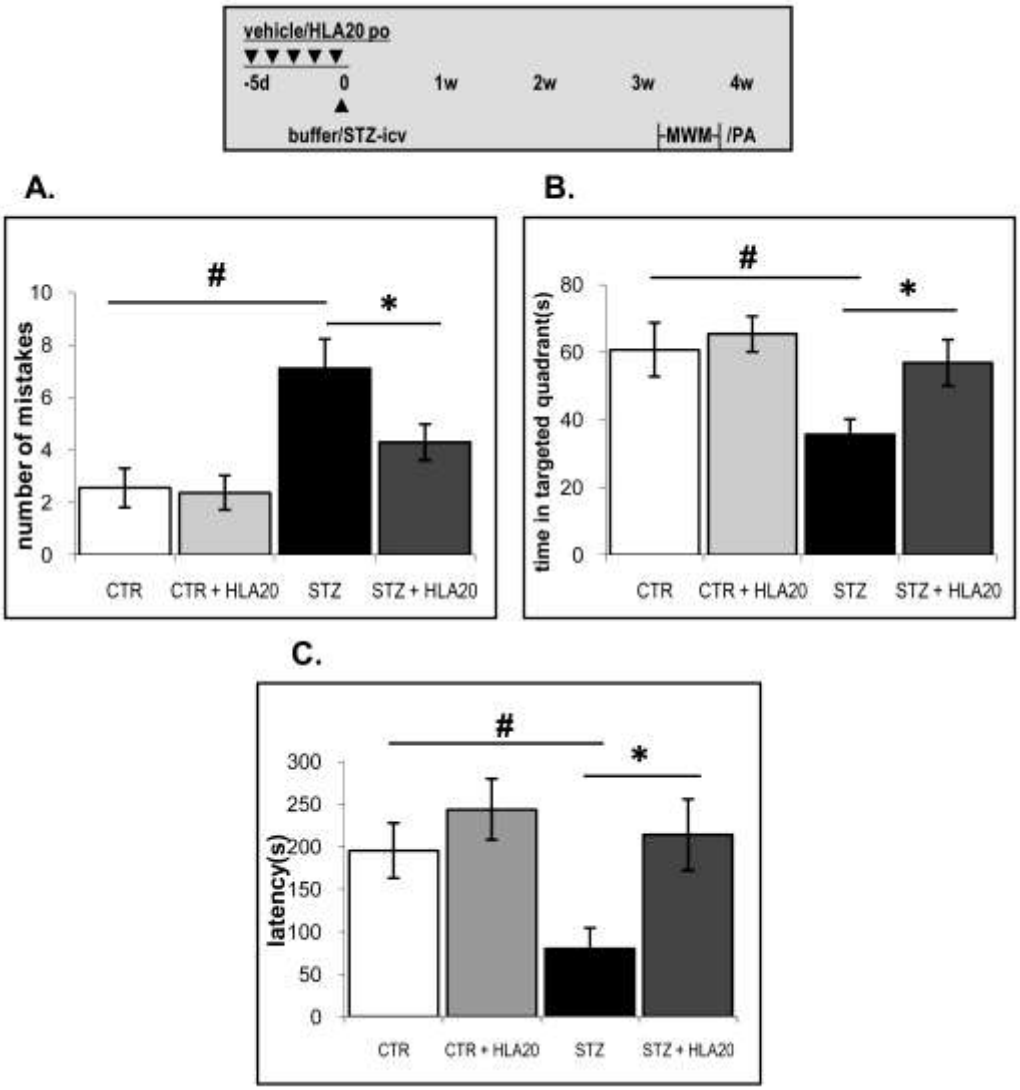

Fig. 5 Effect of pre-treatment with HLA20 on STZ-icv-induced cognitive deficits. Rats (N=9/group in CTR and CTR+HLA20, and 10/group in STZ and STZ+HLA20) were pre-treated with vehicle or HLA20 (5 mg/kg/day), p.o. administered for 5 days. STZ-icv (1 mg/kg) or buffer-icv (CTR) injection was given after the last HLA-20 dose on the 5th day. Number of mistakes (**A**), time spent within the targeted quadrant (**B**) and post-shock latency (**C**) were recorded 4 weeks following the STZ-icv injection. Each bar represents mean±SEM. *p<0.05 vs STZ-icv and #p<0.05 vs CTR by Kruskal-Wallis ANOVA median test followed by Mann Whitney U test.

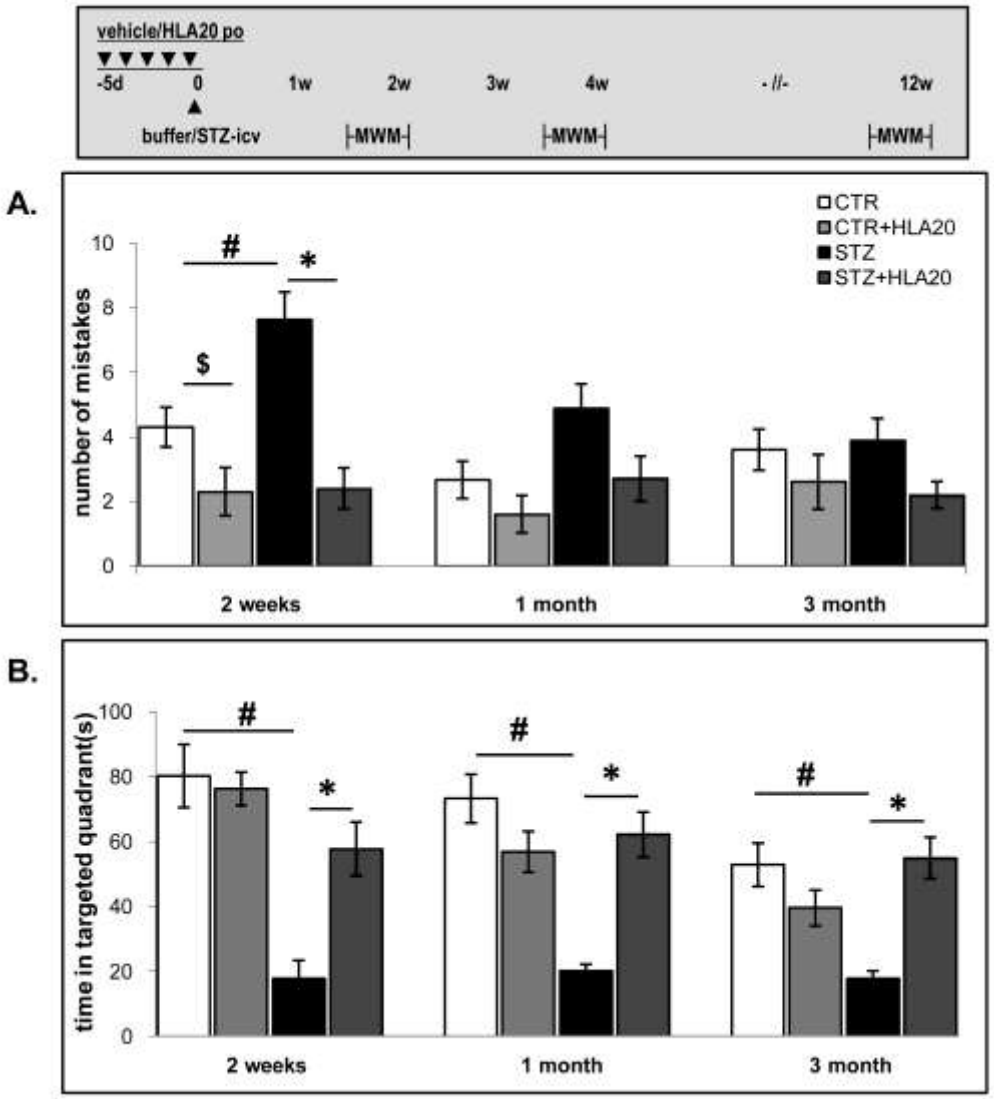

Fig. 6 Duration of the neuropreventive effect of HLA20 in the STZ-icv-induced cognitive deficits. Rats (N=9/group in CTR and STZ, and 10/group in CTR+HLA20 and STZ+HLA20) were pre-treated with HLA20 (10 mg/kg/day) p.o. administered for 5 days. STZ-icv (1 mg/kg) or buffer-icv injection (CTR) was given after the last HLA-20 dose on the 5th day. MWM test was performed 2 weeks, 1 and 3 months following STZ-icv treatment. Number of mistakes (**A**) and time spent within the targeted quadrant (**B**) were recorded in the MWM probe trial. Each bar represents mean±SEM. *p<0.05 vs STZ-icv, #p<0.05 vs CTR and $p<0.05 vs CTR + HLA20 (10 mg/kg) by Kruskal-Wallis ANOVA median test followed by Mann Whitney U test.

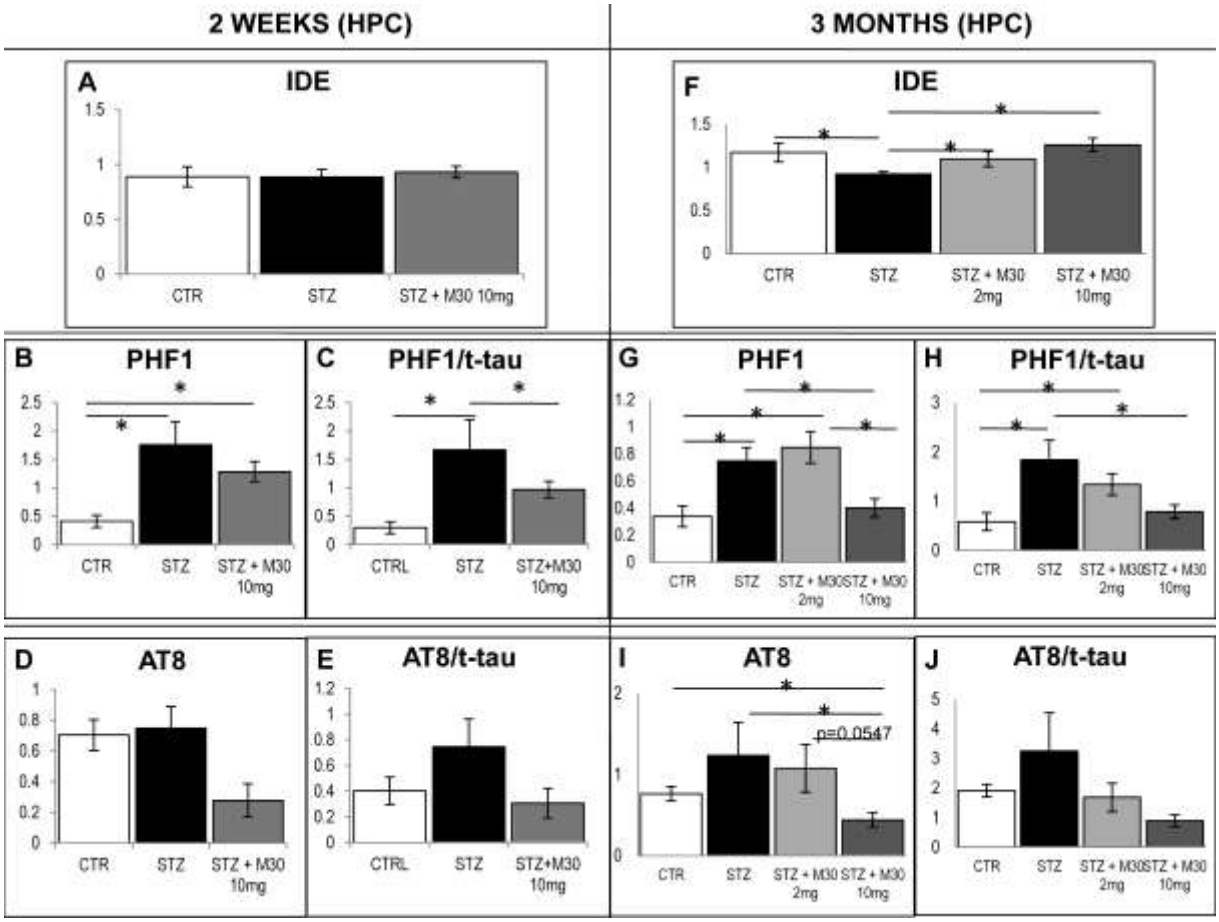

Fig. 7. Western blot analysis of IDE and phosphorylated tau protein in the hippocampus of STZ-icv treated rats following the short- and long-term administration of M30. Treatment with M30 (p.o. 3x week) was initiated 8th day following the last streptozotocin (STZ) injection (3 mg/kg) and was administered for 1 week in a 2 week-experiment, and for 11 weeks in a 3 month-experiment (neurorescue paradigm). Western blot analysis of insulin degrading enzyme (IDE) (A, F) and tau protein (B-E, G-J) expression was performed in hippocampal (HPC) tissue samples. Tau protein phosphorylation status is expressed as phospho tau level (B, D, G, I) and phospho/total tau ratio (C, E, H, J) by means of AT8 (Ser202/Thr205 phospho-tau), PHF1 (Ser 396/404 phospho tau) and anti-total tau antibodies. Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and anti-β actin were used as loading controls. Number of animals: 6/group (control /CTR/, STZ, STZ+M30). Each bar

represents mean±SEM value. *p<0.05 by Kruskal-Wallis ANOVA median test followed by Mann Whitney U test.

Table 1. Summary of the effects of M30 on long-term memory retention and learning capacity over time in STZ-icv rats (experiment depicted in Fig. 2).

Outcome	Results	Conclusions
Long-term learning capacity. Considered were differences Day 4 - Day 1 for each training cycle.*	Control: reduction of NoE in 1st cycle (Δ 9.8, p<0.001) progressively decreased (β= -3.16, p=0.021) to insignificance in further cycles. With adjustment for day 1 NoE, reduction consistently 7.0-7.5 in all cycles (all adjusted Δ p<0.001). STZ: in all cycles reduction non-significant (Δ 3.8-4.3, all p>0.05). With adjustment for day 1 NoE, Δ progressively changed from increase (by 4.4, p=0.019) to reduction (by 6.2, p<0.001) from 1st to 4th cycle (β=-4.3, p<0.001). Reduction less than in Control in 1st (adjusted Δ p<0.001) and 2nd cycle (adjusted Δ p=0.029), differences in later cycles smaller, non-significant. STZ+2 mg/kg M30: similar to STZ. STZ+10 mg/kg M30: not different vs. Control in any cycle (all adjusted Δ p>0.05). Reduction greater than in STZ in 1st cycle (adjusted Δ p<0.001), in further cycles differences smaller and non-significant.	Learning capacity (reduced NoE day 4 vs. day 1 of a training cycle) in Controls preserved over 3 months, although absolute change decreased due to lower day 1 values over time (memory). STZ reduced learning capacity in 1st and 2nd cycle, but with repeated trainings capacity increased, comparable to Control, yet at a higher level of NoE. Larger M30 dose antagonized the inhibitory effect of STZ.
Long-term memory acquisition/retention. Considered were values obtained on the 1st days of each training cycle (2 or 4 weeks apart; depicted in Figure 1).**	NoE decreased over time in all groups (all β p<0.001), but less for STZ (β=-0.020) than for Control (β=-0.081) (slopes differ p=0.001). STZ+2 mg/kg M30 (β=-0.024) close to STZ (p=0.480), different from Control (p=0.003). STZ+10 mg/kg M30 (β=-0.048) close to Control (p=0.122), different from STZ (p=0.010). Progressively more errors with STZ vs. Control: 1st cycle RR=2.03, p=0.005; 2nd RR=8.04, p<001; 3rd RR=17.6, p<0.001; 4th RR=18.4, p<0.001. STZ+2 mg/kg M30 similar to STZ at all times. STZ+10 mg/kg M30 closer to Control: 1st RR=1.37, p=0.462; 2nd RR=4.78, p=0.003; 3rd RR=3.90, p=0.213, 4th RR=2.40, p=0.812; and progressively less errors than STZ: 1st RR=0.68, p=0.245; 2nd RR=0.59, p=0.207; 3rd RR=0.22, p=0.004; 4th RR=0.13, p=0.026.	Training resulted in development of memory in all groups. Memory acquisition/retention was greatly reduced by STZ (vs. Control). The effect of STZ increased over time. The effect was largely antagonized by the higher M30 dose. Antagonizing effect also increased over time.

Data are shown for the number of errors (NoE). Escape latency times followed the same patterns (not shown). Effects are expressed as regression coefficients of outcomes on time (slopes, β), relative risks (RR) or mean differences (Δ) and were adjusted for multiple comparisons.

General linear mixed model: treatment, time, treatment*time interaction without or with further adjustment for values on day 1 of each cycle (time-varying covariate)

***Generalized (Poisson) linear mixed model: treatment, time, treatment*time interaction; for regression slopes, further adjustment for the value on day 1 of the 1sttraining cycle.*