Pyridoindole antioxidant-induced preservation of rat hippocampal pyramidal cell number linked with reduction of oxidative stress yet without influence on cognitive deterioration in Alzheimer-like neurodegeneration

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Abstract

BACKGROUND: The idea of antioxidant therapy attenuating Alzheimer disease (AD) neuropathology starts to be attractive. Animal models are often used in these studies. An AD-like model of trimethyltin (TMT)-induced neurodegeneration, targeting the hippocampus, involves neuronal cell death and cognitive impairment. OBJECTIVES: Effect of the pyridoindole SMe1EC2 (3×50 mg/kg) and vitamin C (3×50mg/kg) was analyzed in the model of TMT-induced (8 mg/kg) neurodegeneration. METHODS: The study was focused on the effect of the antioxidants tested on learning performance in the Morris water maze (MWM) on days 21–25 after TMT administration, on biochemical variables – malondyaldehyde (MDA) and lysosomal enzyme NAGA in brain cortex and blood serum, and on pyramidal cell number in the CA1 area of the hippocampus on day 31 after TMT administration in adult male Wistar rats (n=32). RESULTS: Critical deterioration of learning performance was observed due to the TMT administration in the MWM. Further, apparent reduction of pyramidal cell number to 21% in the CA1 area of the hippocampus, increased MDA and NAGA activity in serum and increased NAGA activity in the cortex were determined contrary to controls. In serum, an increase of MDA level was prevented by both antioxidants tested without any effect on NAGA activity. SMe1EC2 apparently preserved pyramidal cell viability in the CA1 area. Both substances tested failed to ameliorate the detrimental effect of TMT on spatial memory. CONCLUSION: The biochemical and morphometrical findings suggest that reduction of oxidative stress may play a role in AD-like neurodegeneration. Different doses and timing of SMe1EC2 administration might bring improvement in next learning performance.
Abbreviations:
AD - Alzheimer disease
ANOVA - analysis of variance
BAX - X protein connected with Bcl-2 protein
Bcl-2 - B-cell lymphoma protein
DMSO - dimethyl sulfoxide
HE - hematoxylin and eosin
i.p. - intraperitoneally
MDA - malondialdehyde
MWM - Morris water maze
NAGA - N-acetyl-β-D-glucosaminidase
NMDA - N-methyl-D-aspartic acid
p.o. - per os, orally
ROS - reactive oxygen species
TBA - thiobarbituric acid
TBARS - thiobarbituric acid reactive substances
TMT - trimethyltin

INTRODUCTION

Alzheimer disease (AD) is a serious medical and social problem. Progressive neurodegeneration of the brain accompanied by initial short-term memory and cognitive function impairment and in the final stage of the disorder, inability to carry out daily activities, present a burden for the patient and his/her relatives (Zverova 2012). Thus an enormous effort has been made by researchers to understand pathological mechanisms involved in AD. Neuronal dysfunction, degeneration, the presence of neurofibrillary tangles and β-amyloid peptide-containing senile neuritic plaques, morphological alterations, biochemical changes indicating inflammation and oxidative stress are manifested in AD patients in special brain areas, e.g. hippocampus, amygdala and brain cortex (Griffin et al. 1995; Retz et al. 1998; Stuchbury & Munch 2005). The loss of neurons is extensive in the entorhinal cortex and pyramidal cell layer of the CA1 region of the hippocampus (Gomez-Isla et al. 1996).

Drugs that are currently used for treatment of AD are inhibitors of acetylcholine esterase or antagonists for NMDA glutamate receptors, or combined therapy of both (Cummings 2004; Lopez et al. 2009). Recent AD therapy affects only symptoms and may prolong the time before patients require nursing care, but is not able to halt the progressive brain and personality destruction. The increases in neurological disorders may be attributed to environmental exposures to exogenous toxic chemicals (Zeliger 2013). An efficient strategy for treatment of dementia is not yet on the horizon. The non-invasive magnetic resonance technique provides the possibility to study brains of AD patients (Ibrahim et al. 2009) while otherwise only postmortem tissue is available. Thus models of Alzheimer-like neurodegeneration have been developed. There are several animal models for AD (Saraceno et al. 2013), however a model incorporating all aspects of the disease is difficult to find. One of the important research tools for the study of brain dysfunction could be the model of trimethyltin(TMT)-induced neurodegeneration. The pathology elicited by the neurotoxin TMT is common to most neurodegenerative disorders, i.e. neuronal cell death and cognitive impairment. TMT elicits neuronal death in the limbic system and causes damage particularly in the hippocampus, it has thus been found useful as experimental model especially in the investigation of Alzheimer-like diseases (Koczyn 1996; Ishikawa et al. 1997; Nilberth et al. 2002; Geloso et al. 2011).

Recent data in the scientific literature suggest that oxidative stress and an altered microenvironment around plaques may contribute to pathological changes associated with AD neurodegeneration (Hashimoto & Masliah 2003). Newly was found, that lipid peroxidation is one of the main factor triggering neurodegeneration (Sultana et al. 2013). Thus the idea that antioxidant therapy could affect the AD neuropathology starts to be attractive. In the present work, we tested the effect of the new effective pyridoindole antioxidant, coded SM1ECl2, with very low toxicity, synthetized in the Institute of Experimental Pharmacology and Toxicology, Slovak Academy of Sciences, Slovakia (Stolc et al. 2006), in the experimental model of rat hippocampal neurodegeneration induced by TMT. We compared its action with that of another water soluble antioxidant, vitamin C. We focused on the effect of the antioxidants tested in TMT-induced neurodegeneration on rat spatial memory examined in the Morris water maze (MWM). Further, we tested the effect of these two compounds on biochemical variables – malondialdehyde (MDA), the marker of lipid peroxidation, and on the activity of the lysosomal enzyme N-acetyl-β-D-glucosaminidase (NAGA) in the brain cortex and blood serum. Morphometrically, we determined the viability of neurons in the pyramidal cell layer of the CA1 area in the hippocampus. Previously published results concerning the antioxidant SM1ECl2, its scavenging and protective effect in several experimental models of diseases in which oxidative stress is supposed to be involved (Broskova & Knezl 2011; Broskova et al. 2013a, 2013b; Gasparova et al. 2009, 2010, 2014; Juranek et al. 2010; Rackova et al. 2009; Sotnikova et al. 2011; Stepek et al. 2000; Stolc et al. 2006, 2008, 2011) encouraged us to expect an ameliorative action of the pyridoindole also in the animal model of Alzheimer-like disease.

MATERIALS AND METHODS

Animals

Male Wistar rats (n=32, weighing 313±2 g, 12 weeks old) were used from the breeding station Dobra Voda (Slovak Republic, reg. No. SK CH 24011). The rats had free access to water and food pellets and were kept on a 12h/12h light/dark cycle. All procedures involving the animals were performed in compliance with the Principles of Laboratory Animal Care issued by the Ethical Committee of the Institute of Experimental Pharmacology and Toxicology, Slovak Academy of Sciences and by the State Veterinary and Food Administration of Slovakia.
Drugs and experimental groups

The animals were divided by 8 into four groups: control, TMT, TMT + vitamin C, TMT + SMe1EC2 group. TMT chloride (Sigma-Aldrich, USA) dissolved in 0.1% dimethyl sulfoxide (DMSO) and in sterile saline just prior to application, was administered in a single dose of 8 mg/kg of body weight intraperitoneally (i.p.) in the volume of 0.3 ml/100 g of body weight. Control rats received an equal volume of sterile saline with 0.1% DMSO. Further, two groups of TMT-injected rats were treated orally with antioxidants, administered 3-times orally in the volume 0.1 ml/100 g of body weight as follows: 1h before and 1h and 24 h after TMT administration. Both antioxidants tested were applied in the same dose of 50 mg/kg of body weight. Control rats and the TMT group obtained orally aqua pro injectione. All animals were tested on days 21–25 in the MWM. The rats were decapitated under short ether anesthesia on day 31 after TMT/or saline administration. Morphometrical adjustment was performed on the right hippocampus (the whole right hemisphere was cut out) of each rat. Biochemical determination of MDA level and NAGA activity were performed on the cortex of the left hemisphere and blood serum of each rat per sample.

Behavioral determination of learning performance in Morris water maze with hidden platform in constant position

The rats were trained to find the hidden platform under water (1 cm deep-seated under water level) in the pool with a diameter of 180 cm and depth of 50 cm, with water temperature of about 23°C. The inner wall of the pool and the island was black in color. The pool was virtually divided into four quadrants, a central and a peripheral zone. The camera was attached above and the movement of each animal in the pool was recorded and stored for later analysis (Software ANY-maze, Stoelting, USA). On day zero, the platform was elevated up the level, with the contrast object added, thus the rats could see the placement of the platform in the testing pool. On the first time, the rat was placed only into the opposite quadrant of the pool. The rat could orient itself due to the marked figures localized around the pool. If the animal did not reach the island within 60 s, it was gently navigated by hand onto the platform and it was allowed to stay there for 20 s. On the testing days 1–5, four training trials were carried out, thus the rat was placed subsequently into each of the four quadrants. After the final trial each animal was carefully dried with a towel and placed under a lamp.

Biochemical determination of N-acetyl-β-D-glucosaminidase activity

Tissue samples of 50–60 mg were put in ice-cold phosphate buffer pH7.4, containing Triton X-100 (0.1%), and homogenized with a knife homogenizer. Homogenates were centrifuged at 15 000×g for 20 min. The activity of NAGA and the level of proteins were assayed according to standard methods (Barret & Heath 1977; Lowry et al. 1951) used in our previous studies. All chemicals and enzyme substrates (Sigma-Aldrich, USA) were of analytical grade.

Biochemical assay of malondialdehyde

Thiobarbituric acid reactive substances (TBARS) are formed as a byproduct of lipid peroxidation. Determination of TBARS by measurement of the colored product formed upon reaction with thiobarbituric acid (TBA) is one of the most common assays used in lipid peroxidation studies (Esterbauer 1993). MDA, as a marker for lipid peroxidation, was determined by the double heating method of Draper and Hadley (1990). The principle of the method was spectrophotometric measurement of the color produced during the reaction of TBA with MDA. For this purpose, trichloroacetic acid solution was added to the homogenate of the left brain cortex or serum in a centrifuge tube and placed in a boiling water bath for 15 min. After cooling under tap water, the mixture was centrifuged at 1000×g for 10 min, and the supernatant was added to TBA solution in a test tube and placed in a boiling water bath for 15 min. The solution was then cooled in tap water and its absorbance was measured using a spectrophotometer Multiscanreader RC (Labsystems, Finland) at 550 nm. The concentration of MDA was calculated by the absorbance coefficient of MDA-TBA 1.56×10^5 M⁻¹cm⁻¹ as molar extinction coefficient.

Morphometrical determination of cell number of CA1 pyramidal cell layer in rat hippocampus

The right hemisphere of the brain was fixed in 4% formaldehyde after decapitation of the rat. The oblique sagittal section of the brain in the midline was processed, embedded in paraffin and cut to obtain slices across the hippocampus. Brain slices, 4-μm thick, were routinely stained by hematoxylin and eosin (HE). The CA1 area in the hippocampus was selected and captured by optical microscope (Leica DM 2000, Wetzlar, Germany) with attached camera (S50, Canon, Japan) in three microscopic fields, using the final magnification 400x. The number of the cells in the CA1 area was counted in each captured field and expressed as percentage compared to the control group.

Statistical evaluation

The data were statistically evaluated using the InStat software ver. 2.05 (GraphPad) and GraphPad Prism Software (GraphPad, La Jolla, USA). Data were expressed as means ± S.E.M. One-way Analysis of variance (ANOVA) was used to evaluate 1) difference among all experimental groups (Bonferroni multiple comparisons test), 2) difference compared to controls (Dunnett multiple comparisons test). The limit of p<0.05 was considered a statistically significant difference.
RESULTS

Effect of TMT and antioxidants tested on learning performance in MWM with hidden platform in constant position

Three weeks after TMT administration, development of neurodegeneration in the rat hippocampus is expected in this animal model, therefore the rats were tested in MWM on days 21–25. On day 1 of learning performance there was no difference in the test duration (in seconds) among four experimental groups (n=8 rats/group). Further, control rats (received saline i.p. and water p.o.) were able to learn and remember the position of the island hidden under water. This resulted in marked shortening of testing time, thus a significantly shorter time of test duration was observed on days 2–5 of the 5-day testing in control rats, compared to their testing time on day 1 (Figure 1). Apparent spatial memory impairment was confirmed three weeks after a single intraperitoneal TMT administration in the dose 8 mg/kg. Test duration was a significantly longer on days 2–5 compared to relevant days in the control group (Figure 1). In the experimental groups TMT, TMT + vitamin C, and TMT + SMe1EC2 no improvement of learning during the 5-day testing was found compared to day 1 in the relevant experimental group. Neither vitamin C nor pyridoindole SMe1EC2 administered orally 3 times in the dose of 50 mg/kg 1 h before, 1 h and 24 h after TMT administration did prevent extensive learning and memory loss expressed by the test duration contrary to relevant days of the control group. Further, TMT-affected rats favored to swim on the periphery and avoided the central zone of the pool (Figure 2). Neither vitamin C nor pyridoindole SMe1EC2 influenced this behavior.

Effect of the antioxidants tested on MDA level in TMT-induced neurodegeneration

All rats (n=32), undergoing MWM on days 21–25, were decapitated on day 31 after TMT/or saline i.p. administration. MDA level was significantly increased in blood serum of TMT-mediated (8 mg/kg; i.p.) rats compared to unaffected control animals (Figure 3). In both experimental groups treated with antioxidants, the amount of MDA was found to be reduced to control serum level. In the brain cortex of TMT-intoxicated rats, the level of MDA was increased only non-significantly compared to control rats (not shown).

Effect of antioxidants tested on N-acetyl-β-D-glucosaminidase activity in TMT-induced neurodegeneration

On day 31 after TMT administration, significant increase of activity of the lysosomal enzyme NAGA was found in the brain cortex as well as in blood serum compared to unaffected control animals (Figure 4). Neither of the compounds tested influenced the increased NAGA activity in the brain cortex or blood serum.

Effect of antioxidants tested on cell number of the CA1 pyramidal layer in the hippocampus of TMT affected rats

Formalin-fixed paraffin-embedded tissue of the right hemisphere, 4-μm thick slices, stained by HE, were evaluated for the number of pyramidal cells per 1 mm of length of the CA1 area of the hippocampus under optical microscope. Severity of hippocampal
damage was confirmed by massive reduction of cell number, up to 21% (Table 1), found in the CA1 area of TMT-affected rats on day 31 after its administration (Figure 5B) compared to control rats (Figure 5A). Fatal reduction of cell viability was not influenced by vitamin C administration (Figure 5D), however the compound SMe1EC2, tested in the same dosage as vitamin C (3×50 mg/kg; p.o.), significantly improved cell viability (Figure 5C, Table 1).

**DISCUSSION**

TMT application is used as a model of neurodegeneration targeting the hippocampus, a structure involved in learning and memory mechanisms. TMT-mediated spatial memory impairment was reported in Spague-Dawley rats (Earley et al. 1992; Koda et al. 2008; Park et al. 2011, 2012) but there are only a few works reporting spatial memory performance on Wistar TMT-intoxicated rats. Mignini and coworkers (1992) found that the TMT-treated Wistar rats showed impaired spatial reference memory in a MWM task compared

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**Tab. 1.** Effect of two antioxidants administered orally (3x 50 mg/kg) on TMT-induced pyramidal cell loss in the rat hippocampus.

<table>
<thead>
<tr>
<th>Group</th>
<th>Cell number/1 mm of the length of the CA1 area</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>170±6</td>
<td>100</td>
</tr>
<tr>
<td>TMT</td>
<td>37±5 ***</td>
<td>21</td>
</tr>
<tr>
<td>TMT + vitamin C</td>
<td>30±3 n.s.</td>
<td>18</td>
</tr>
<tr>
<td>TMT + SMe1EC2</td>
<td>81±9 ###</td>
<td>48</td>
</tr>
</tbody>
</table>

TMT-induced neurotoxic effect on the rat hippocampus was expressed by the pyramidal cell number per 1 mm of the length of the CA1 area. Values represent means ± S.E.M. On day 31 after TMT administration (8 mg/kg, i.p.), severe reduction of pyramidal cell number was found compared to control group, ***p<0.001, ANOVA Bonferroni multiple comparisons test. Non-significant difference was found between TMT group vs. TMT + vitamin C, thus severe loss of pyramidal cells persisted. Treatment with SMe1EC2 resulted in marked preservation of pyramidal cells contrary to untreated TMT group, ###p<0.001, ANOVA Bonferroni multiple comparisons test. On the right, values are expressed as a percentage of pyramidal cell number where the number in the control group represents 100%.
to the control group. Niittykoski and coworkers (1998) revealed that the hidden platform version of water maze was not assessed in TMT-intoxicated male Wistar rats because of severe impairment manifested even when the platform was visible. In the present work, the strong effect of TMT resulted in clear worsening of learning performance of TMT-exposed rats in MWM with hidden platform in constant position. Our measurements revealed deterioration expressed not only by long test duration and time spent in the periphery (in seconds), but by many other parameters obtained by ANY-maze software which were not shown here, i.e. longer total distance traveled (in meters), higher average speed (m/s), less distance traveled in the center zone (m), etc., compared to control animals. Therefore not only learning and memory failure, but also increased locomotor activity accompanied by suggestion of anxious behavior may be partially interpreted from these results in TMT-intoxicated male Wistar rats.

Hippocampal neurons of the CA1 area are especially vulnerable to several stressors, increasingly with advanced age, like ischemic insult (Schmidt-Kastner & Freund 1991; Back et al. 2004; Jackson et al. 2009). In the present work, pyramidal cell loss was confirmed in the CA1 area of the hippocampus of TMT-affected male Wistar rats, in contrast to male Sprague-Dawley rats, where the number of neurons was significantly lower than in the control group in the CA3 and CA4 regions (Ishida et al. 1997) and in the CA3b region but not in the CA1 or CA3a region (Koda et al. 2008). Mice are more susceptible to damage, especially in the hippocampal granular cell layer (Chang et al. 1983). Quantitative and qualitative differences were evident also in neurobehavioral screening of acute TMT neurotoxicity in Long-Evans and Fisher 344 rats (Moser 1996). Thus species-dependent and strain-specific differences are to be observed in TMT-induced neurodegeneration.

The exact mechanism of TMT action is not known, however a plethora of individual steps involved in its mechanism was found and accepted, e.g. decreased expression of anti-apoptotic Bcl-2 and increased expression of pro-apoptotic BAX proteins, lysosomal...
disruption accompanied by release of the lysosomal enzyme cathepsin-D to cytoplasm, increased production of reactive oxygen species (ROS) accompanied with increased lipid peroxidation, decreased levels of antioxidative enzymes, etc. (Geloso et al. 2011). In the hippocampus, TMT evokes increased formation of ROS resulting in disruption of the mitochondrial respiratory chain (Aldridge & Street 1971), triggers apoptosis (Gasparova et al. 2012; Quing et al. 2013) and disrupts functional circuits leading to memory impairment. This was a reason to monitor biochemical markers of oxidative stress as MDA and lysosomal enzyme NAGA in the brain cortex and blood serum of rats exposed to TMT. The increased level of MDA and NAGA, found 31 days after TMT administration in our measurements, suggests prolonged duration of oxidative stress, which could be explained by high affinity of rat hemoglobin for TMT (Rose & Aldridge 1968). Hemoglobin may therefore serve as a reservoir slowly and continuously releasing TMT into the plasma, from which it then enters the brain. The increased MDA level in blood serum found one month after TMT administration indicates sustained increase of lipoperoxidation. In the present work, prolonged increased activity of lysosomal enzyme NAGA in the brain cortex was found one month after TMT administration. Our results differ from those of Shin and coworkers (2005) who observed a rapid increase in the level of MDA and other biochemical variables indicating oxidative stress in the first 1–3 days after TMT administration, which returned to near-control levels within three weeks after TMT administration (Sprague-Dawley rats).

In neuropharmacology, the development of drugs effective for neurodegeneration of Alzheimer type is topical. On testing new prospective drugs, animal models are often used with memory impairment, since it is a major criterion of dementia. In the present experimental model, we investigated the effect of two antioxidants, vitamin C and a new pyridoindole compound with antioxidative properties, SMe1EC2, against TMT-mediated neurodegeneration since oxidative stress is widely assumed to be one of the main pathogenic mechanisms in TMT-induced neurotoxicity (Jenkins & Barone 2004). Impressive improvement in viability of pyramidal cells was obtained after SMe1EC2 treatment compared to the TMT group. This could be explained by the high anti-lipoperoxidation effect of the pyridoindole (Stolc et al. 2006), resulting probably in cell membrane preservation. Generally, it is accepted that neuronal function depends on intact cell integrity. Jenkins and Barone (2004) reported that TMT inhibited neurite outgrowth. This may explain the fact that treatment with SMe1EC2 resulted in saving almost 50% of pyramidal cells in the CA1 area, but it was still not efficient enough to preserve learning and memory, as found in MWM testing.

Based on the statement of Shin and coworkers (2005) about the protective effect of ascorbate in the TMT model of neurodegeneration, we used ascorbic acid a standard. In our experiments, vitamin C was effective in inhibiting of MDA increase in blood serum of TMT-intoxicated rats. Similarly as the pyridoindole SMe1EC2, vitamin C did not result in improvement of learning performance. In fluoride-induced neurodegeneration and oxidative stress, the ameliorative effect was found on learning and memory as a result of 15-day treatment with ascorbic acid in the dose of 100mg/kg body weight (Raghu et al. 2013). Actually, not so many compounds have as yet been found effective in the TMT model of neurodegeneration. Behavioral studies on spatial learning deficits induced by TMT in MWM include neuroprotective effect of rutin (Koda et al. 2008), Lycium chinense fruit (Park et al. 2011), the immunosuppressant and neuroprotectant FK506 (Moriga et al. 2008) and Gugijihwang-Tang with the herbal formula PM012 (Jung et al. 2013). In vitro neuroprotective effect in TMT-mediated intoxication was proved with lycopene (Qu et al. 2011), and protective effect on maintenance of glutathione homeostasis was found by ascorbate (Shin et al. 2005). According recent knowledge that lipid peroxidation is one of the main factor triggering neurodegeneration (Sultana et al. 2013), together with fact that many of the above mentioned protective compounds revealed antioxidant and scavenging effect, it can be assumed that our new pyridoindole antioxidant SMe1EC2 with the high anti-lipoperoxidation effect (Stolc et al. 2006) and along with the apparent ameliorative action on pyramidal cell viability proved in the TMT model, may provide, in different dosage, improvement also of learning in the next behavioral test.

CONCLUSIONS

The presented findings provide supportive evidence on the neuroprotective effect of the new pyridoindole antioxidant SMe1EC2 in the animal model of trimethyltin-mediated neurodegeneration of Alzheimer type. The study showed marked amelioration of neuronal viability in the pyramidal cell layer of the CA1 area of the rat hippocampus and inhibition of malondialdehyde increase in rat blood serum. Based on the high anti-lipoperoxidation effect of the pyridoindole, different dosage of SMe1EC2 administration might solve rescue of spatial learning deficits induced by TMT.

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REFERENCES


