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ORIGINAL ARTICLE

Pyridoindole SMe1EC2 as cognition enhancer in ageing-related cognitive decline

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ABSTRACT

Synthetic pyridoindole-type substances derived from the lead compound stobadine represent promising agents in treatment of a range of pathologies including neurological disorders. The beneficial biological effects were suggested to be likely associated with their capacity to ameliorate oxidative damage.

In our study, the effect of supplementation with the derivative of stobadine, SMe1EC2, on ageing-related cognitive decline in rats was investigated. The 20-months-old male Wistar rats were administered SMe1EC2 at a low dose, 0.5 mg/kg, daily during eight weeks. Morris water maze test was performed to assess the spatial memory performances. The cell-based assays of capacity of SMe1EC2 to modulate proinflammatory generation of oxidants by microglia were also performed.

The rats treated with SMe1EC2 showed significantly increased path efficiency, significantly shorter time interval of successful trials and exerted also notably lower frequencies of clockwise rotations in the pool compared to non-supplemented aged animals. Mildly improved parameters included test durations, distances to reach the platform, time in periphery of the pool and overall rotations in the water maze. However, the pyridoindole SMe1EC2 did not show profound inhibitory effect on production of nitric oxide and superoxide by activated microglial cells.

In conclusion, our study suggests that pyridoindole SMe1EC2, at low doses administered chronically, can act as cognition enhancing agent in aged rats. The protective mechanism less likely involves direct modulation of proinflammatory and prooxidant state of microglia, the prominent mediators of neurotoxicity in brain ageing and neurodegeneration.

KEY WORDS: brain ageing; cognitive decline; antioxidants; pyridoindoles

Introduction

Cognitive decline manifested by spatial learning and memory dysfunction has been shown to be associated with normal ageing in both humans and animals (Foster *et al.*, 2012). Besides of other factors, chronic oxidative stress in all brain regions including hippocampus, is thought to be important contributing factor to the fall in cognitive and motor performance seen in ageing as well as major neurodegenerative diseases (Devi & Satpati, 2017; Shukitt-Hale, 1999; Berr, 2000). Oxidative stress conditions cause the structural damage to neurons and other brain cells and also contribute to alterations in the redox-sensitive signaling mechanisms including the insulin receptor signaling pathway (Dröge & Schipper, 2007). However, in contrast to neurodegenerative diseases, the cognitive decline in normal ageing is not associated with a significant loss of neurons (Rapp & Gallagher, 1996).

The most widely accepted Free Radical Theory of Aging introduced by Denham Harman in 1956 (Harman, 1992), proposes that ageing may be related to the deleterious side attacks of free radicals, produced during metabolic processes, on cell components and inability to counterbalance these processes by endogenous antioxidant defense systems. The brain is particularly vulnerable to the effects of reactive oxygen species and neurons as long-lived postmitotic cells are at higher risk and build up greater amounts of damaged waste than short-lived mitotic cells (Friedman, 2011). The brain cells exhibit higher (about 10-fold) oxygen consumption compared to other tissues and they contain high levels of readily peroxidisable polyunsaturated fatty acids such as arachidonic acid and docosahexaenoic acid. On the other hand, brain is relatively poor in antioxidant enzyme capacity compared to other organs (Halliwell & Gutteridge, 1989) and its ageing is accompanied by further decline in performance

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of the antioxidant defence. For instance, intracellular glutathione concentrations and GSH:GSSG ratio were found to decrease in all brain regions in various animal models (Zhu *et al.*, 2006; Calabrese *et al.*, 2004). In addition, elevation of content of transition metals in specific brain regions including hippocampus, in particular Fe, Cu, Zn, and Al prone to generate prooxidant environment and increase oxidative stress-induced neuronal vulnerability, can be also associated with brain ageing (Braidy *et al.*, 2017). Increase in concentration of iron, a catalyst of the conversion of hydrogen peroxide into highly reactive hydroxyl radicals by the Fenton reaction, has been found in aged human brain tissue (Martin *et al.*, 1998) as well as in aged rodent brains (Donahue *et al.*, 2006). In humans, a raise in iron levels and ferritin immunoreactivity was detected in aged microglia and astrocytes in various brain regions including hippocampus (Zecca *et al.*, 2004).

Increasing evidence associates brain ageing with ongoing inflammation with microglia, the resident immune cells of the central nervous system, as the key players in this process (Norden & Godbout, 2013). Under normal state, microglia provide a prompt protective innate immune response resolving cellular damage. This defensive mechanism involves activation of cytokine, chemokine and complement receptors, major histocompatibility complex and others, leading to transient elevation of inflammatory mediators as well as neurotrophic factors (Aloisi, 2001; Batchelor *et al.*, 1999). However, chronic inflammatory state in brain ageing and neurodegeneration is associated with prolonged overactivation of these cells and elevated production of neurotoxic mediators such as proinflammatory cytokine including interleukin-6 (IL-6), interleukin-1β (IL-1β) and tumor necrosis factors-α (TNF-α). Studies on animals have confirmed that elevated levels of IL-6 in hippocampus and cerebral cortex of the aged brains originated predominantly from microglia (Ye & Johnson, 1999). Moreover, activated microglia represent one of the main sources of oxidative damage in the brain arising from production of superoxide and nitric oxide leading to generation of more reactive secondary species, such as peroxynitrite and H_2O_2, playing a critical role in oxidative and nitrosative damage in neuropathologies (Dringen, 2005; Guix *et al.*, 2012).

Accumulating evidence suggests that a diet rich in plant-derived phenolic antioxidants can play a role in delaying the onset of ageing-related cognitive declines as seen in Alzheimer's disease and dementia, possibly through their anti-inflammatory properties and alleviation of oxidative stress. However, synthetic antioxidants can provide efficient neuroprotection and reversal of brain ageing-related cognitive changes as well (Socci *et al.*, 1995). The pyridoindole stobadine has shown efficient antioxidant properties in a variety of experimental models including those of pathologies of the central nervous system (Juranek *et al.*, 2012; Juranek *et al.*, 2010). A number of its structural analogues have been designed, synthesized and characterized (Juranek *et al.*, 2010). SMe1EC2 (Figure 1A) is one of the most studied derivatives which was shown to possess improved intrinsic free radical scavenging efficacy compared to stobadine, mediated by electron donating methoxy-substituent in *para* position with regard to indolic nitrogen, an established center of antioxidant reaction of stobadine-related substances (Rackova *et al.*, 2006; Rackova *et al.*, 2002). On the other hand, biological availability may be also optimally modulated due to replacement of methyl substituent on piperidine nitrogen present in stobadine by ethoxycarbonyl moiety, resulting in elimination of its basicity and suppression of ionization processes.

Given these considerations, we investigated the potential efficacy of supplementation with SMe1EC2 at a single dose 0.5 mg/kg in reversing ageing-related deficits in behavioral function in 20-month-old Wistar rats. We chose this dose based on reported pro-cognitive effects of low doses of Dimebon (Latrepirdine, Figure 1B) (Giorgetti *et al.*, 2010), a drug that failed in Phase III Alzheimer's disease trials despite exceedingly promising Phase II data (Bezprozvanny, 2010, Pfizer). Behavioral testing by measuring cognitive performance was started at age 22 months, following 8 weeks on the supplementation. In addition, potential modulation of proinflammatory production of superoxide and nitric oxide by SMe1EC2 in BV-2 microglial cell line was investigated.

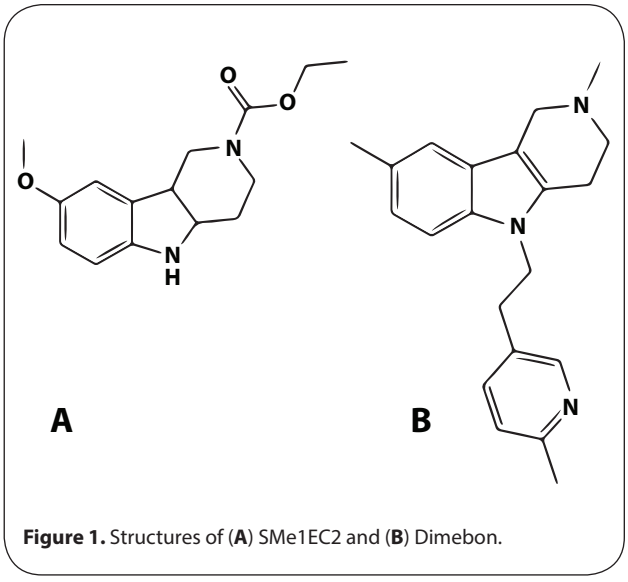

Figure 1. Structures of (**A**) SMe1EC2 and (**B**) Dimebon.

Material and Methods

Materials

All reagents were of analytical grade or the highest possible purity. SMe1EC2 (Figure 1A) was synthesized at the Institute of Experimental Pharmacology and Toxicology, Slovak Academy of Sciences in the form of hydrochloride.

Phosphate buffered saline (PBS) tablets were obtained from Sigma-Aldrich (Bratislava, Slovakia). Cryomount medium, microscopic glass slides and coverslips were purchased from BD Bamed s.r.o. (Veľké Leváre, Slovakia).

Paraformaldehyde and sucrose (p.a.) were obtained from Lachema, o.p. (Brno, Czech Republic).

Thiazolyl blue tetrazolium bromide (MTT), nitroblue tetrazolium (NBT), lipopolysaccharide (LPS) from Escherichia coli 0111:B4 (L4391), phorbol 12-myristate 13-acetate (PMA), and other chemicals were obtained from Sigma-Aldrich (Bratislava, Slovakia).

Animal model

The subjects were male Wistar rats, 20 months and 1 month of age from the breeding station Dobra Voda (reg. No. SK CH 24011). The rats had free access to water and food pellets and were kept on a 12h/12 h light/dark cycle. All procedures involving 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 (Act No. Ro-2590/11-221).

Voluntary oral drug administration was used to minimise the stress and pain levels for the animals according to Atcha *et al*. with some modifications (Atcha *et al*., 2010). Seven animals received SMe1EC2 daily at a dose 0.5 mg/kg. The substance was administered in 0.5 ml of 10% sucrose solution instilled on a piece of biscuit to improve palatability of a drug. Supplementation with SMe1EC2 started following 3 initial training days when each animal received sucrose on a biscuit only. Control animals (aged, n=7 and young, n=3) continued with the training diet until the end of experiment. The feeding duration was 8 weeks, the animals were supplemented also 30 min prior to behavioral test.

Behavioral test

The standard Morris water maze test was used to screen memory function of the aged rats. 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 escape platform was placed in the Northeast quadrant of the pool. The training was carried out in course of 5 consecutive days. At Day 0, the rats underwent initial training period as the first phase of the experiment with placement only into the quadrant opposing the platform quadrant. The platform was elevated above the water level, with the contrast object added, thus the rats could see the position of the platform in the testing pool. On each testing day 1–5, the animals passed four training trials during which they were placed subsequently into each of the four quadrants. If the animal did not find the platform within 60 seconds, it was removed from the water and placed on it. The animal was left on the platform for 20 seconds. The rats were removed from the maze, toweled dry, and placed under a heat lamp (60 W, 20 cm above). The test was recorded by video camera and evaluated with ANY-Maze videotracking software (Stoelting Europe, Ireland).

Lipofuscin analysis in hippocampus

Animals were decapitated under short ether anaesthesia, the brain was removed quickly by gross dissection of the dorsal surface of the skull. Intact brains were post-fixed in 4% paraformaldehyde overnight and then moved to 30% sucrose in 0.1 M PBS solution at 4 °C until no longer buoyant, then snap frozen in liquid nitrogen and stored at –70 °C until further use. Frozen brains were let to warm up to –20 °C in a Zeiss Hyrax C50 cryostat chamber (Zeiss International, Oberkochen, Germany) and mounted with cryomount medium onto the specimen disc. Tissue sections of 40 μm width were cut and thaw-mounted on gelatine-coated glass slides, allowed to dry briefly and cover slipped and stored at 4 °C until imaging. Endogenous hippocampal cellular autofluorescence was detected under the FITC and TRITC filters by Leica DM IL fluorescence microscope.

Cellular model

The immortalized mouse microglial cell line BV-2 was developed in the laboratory of Dr. Blasi at the University of Perugia (Blasi *et al*., 1990). The cells were cultured in DMEM, supplemented with 10% FBS (Sigma-Aldrich, Bratislava, Slovakia), and 1% P/S (100 U/ml penicillin, 100 μg/ml streptomycin, K-Trade, s.r.o., Bratislava, Slovakia) in 5% CO_2 at 37 °C. Cells were used for 10 passages at maximum.

MTT assay

The cells were grown in 96-well microplates, in complete DMEM. At the end of the 24h incubation with or without SMe1EC2, the cells were incubated with MTT (0.5 mg/ml) in DMEM in 5% CO_2 at 37 °C for 120 min. Subsequently, 100 mL of 100% DMSO was added and the cells were thoroughly resuspended. The absorbance was spectrophotometrically recorded at 570 nm using the reference at 690 nm.

Production of NO by BV-2 microglia

The BV-2 cells were treated with LPS (10 μg/ml) and/or SMe1EC2 for 16 h. Aliquots of conditioned media were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphtylethylenediamide in 2.5% phosphoric acid) in clean 96-well plates. Absorbance was measured at 540 nm with reference (690 nm) using a microplate reader.

Production of $O_2^{\cdot-}$ by BV-2 microglia

The superoxide anion radical production was assessed using the nitroblue tetrazolium (NBT) reduction method as described by (Rook *et al*., 1985). Briefly, after exposure of the cells to LPS (1 μg/ml) for 16 h in serum-free media, cells were treated with 2 μmol/l PMA and/or the compounds tested for 1.5 h in the presence of NBT (0.75 mg/ml) in DMEM in 5% CO_2 at 37 °C. At the end of the incubation, the cells were washed twice with 70% methanol and the precipitate of reduced NBT was dissolved with mixture of KOH/DMSO. The absorbance was recorded at 630 nm by using a microplate reader.

Statistical analysis

Data were expressed as means ± SEM in case of animal studies and as means ± SD in case of cell culture studies. One-way Analysis of variance (ANOVA) was used to evaluate difference among all experimental groups (Tukey's pairwise comparisons test), difference compared to controls (Dunnett multiple comparisons test). The level of $p<0.05$ was considered as a statistically significant difference.

Results

Voluntary treatment of animals

All animals voluntarily and readily learned to receive biscuit containing either the sucrose solution only or sucrose and SMe1EC2 (0.5 mg/kg) (Atcha *et al.*, 2010). After 3 days of training, all of the rats lined up at the cage edge to receive their respective treatments once the cage lid was removed.

The supplementation based on sucrose/biscuit did not cause any significant increase in body weight of the treated aged animals with respect to their initial weights (Figure 2). Moreover, there were no statistically significant differences in body weight increase between the groups of aged animals supplemented by sucrose vehicle or sucrose with SMe1EC2 and the non-supplemented group ($p>0.05$; average weight increase ± SEM: control aged group fed with sucrose vehicle, 7.64±1.20%; SMe1EC2 group, 7.94±0.93%, aged non-supplemented group, 5.33±3.11%).

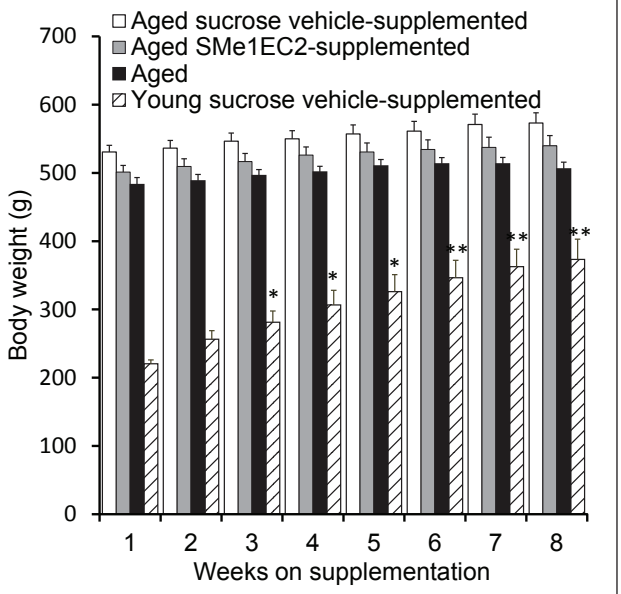

Figure 2. Changes in body weight of Wistar rats in course of supplementation period. Values are means ± SEM; n=7 rats in aged 20-months-old group supplemented with sucrose/biscuit vehicle; n=3 rats in young 1-month-old group supplemented with sucrose/biscuit vehicle; n=7 rats in aged 20-months-old group supplemented with SMe1EC2; *$p<0.05$, **$p<0.01$ *vs* initial weight.

However, the young rats showed significant weight gain starting at the third week of the supplementation linked likely to their normal age-dependent body growth (Shoji *et al.*, 2016).

Learning performance in MWM with hidden platform in constant position

The data obtained from the Morris water maze (MWM) showed that learning capability of aged group was decreased as early as after the first day of training as shown by the evaluated parameters: path efficiency, duration, distance, average duration of successful trials, time in periphery, overall body rotations and clockwise body rotations (Figure 3). The most significant difference in acquisition and performance of the task by aged animal with respect to young control was seen on the fourth day of training. The increased values of the parameters duration, average duration of successful trials and time in periphery in the aged group can be associated with lower locomotoric capacity reflected by swimming speed of the aged animals compared to young rats ($p<0.001$ at the 4th day of training). The group of animals supplemented with SMe1EC2 over eight weeks showed mildly improved spatial learning and memory (Figure 4). The most remarkable differences were observed on the fourth day of training, as well. In comparison with the non-supplemented animals, the SMe1EC2-treated rats showed significantly increased path efficiency (0.42±0.07 and 0.17±0.06, for the aged SMe1EC2 group and vehicle-treated group, respectively, $p<0.05$), significantly shorter time interval of successful trials (15.37±2.38 s and 24.47±3.37 s, for the aged SMe1EC2 group and vehicle-treated group, respectively, $p<0.05$) and exerted also notably lower frequencies of clockwise rotations in the pool (0.32±0.15 and 1.21±0.15 for the aged SMe1EC2 group and vehicle-treated group, respectively, $p<0.001$). The SMe1EC2-supplemented animals showed also mildly shortened latencies to platform (test durations), took slightly shorter distances to reach the platform, spent slightly shorter time in periphery of the pool and exerted lower number of overall rotations in the water maze (Figure 4). These differences were not attributed to swimming speeds as both groups swam with comparable speeds (0.014±0.001 m/s and 0.016±0.0005 m/s for the aged SMe1EC2 group and vehicle-treated group, respectively, $p>0.05$).

Autofluorescence in brain slices

In the prepared brain slices, hippocampal region showed particularly increased autofluorescence, as visualised by using fluorescence microscopy and red and green emission filter cubes (Figure 5). Notably lower autofluorescence was seen in the slices prepared from the brains of the SMe1EC2-supplemented group of rats.

Cellular model of microglia

SMe1EC2 showed markedly lower impact on viability/proliferation parameter in BV-2 microglial cell line as assessed by MTT compared to semisynthetic derivative of quercetin, showing its improved anti-neuroinflammatory

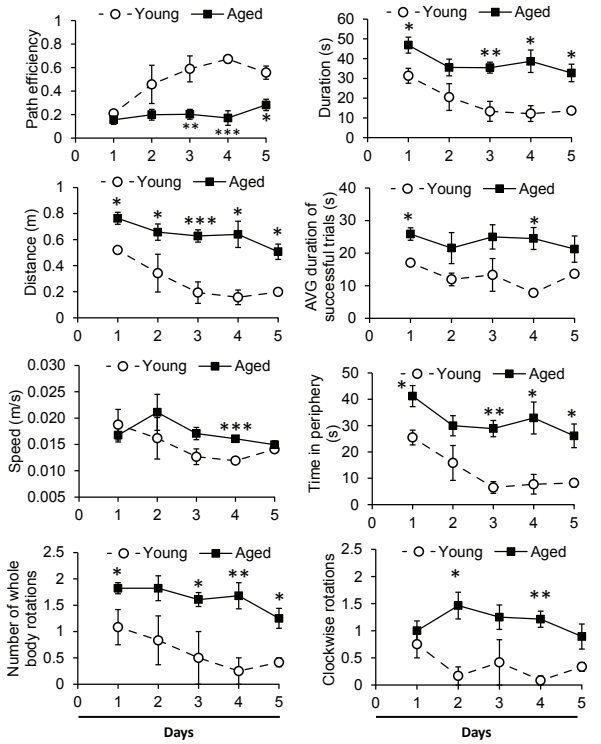

Figure 3. Impairment of spatial learning and memory in aged Wistar rats. Path efficiency, duration, distance, average duration of successful trials, swimming speed, time in periphery, overall body rotations and clockwise body rotations. Values are means ± SEM; n=7 rats in aged 20-months-old group supplemented with sucrose/biscuit vehicle; n=3 rats in young 1-month-old group supplemented with sucrose/biscuit vehicle; *p<0.05, **p<0.01, ***p<0.001 *vs* young group.

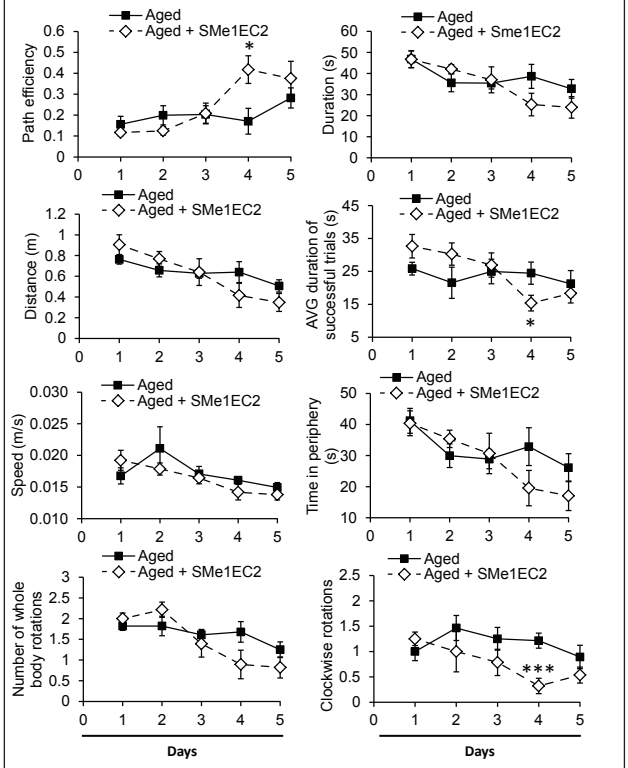

Figure 4. Effect of eight-week supplementation with SMe1EC2 (0.5 mg/kg) on the spatial learning and memory in aged Wistar rats. Path efficiency, duration, distance, average duration of successful trials, swimming speed, time in periphery, overall body rotations and clockwise body rotations. Values are means ± SEM; n=7 rats in aged 20-months-old group supplemented with sucrose/biscuit vehicle; n=7 rats in aged 20-months-old group supplemented with SMe1EC2; *p<0.05, ***p<0.001 *vs* SMe1EC2 group.

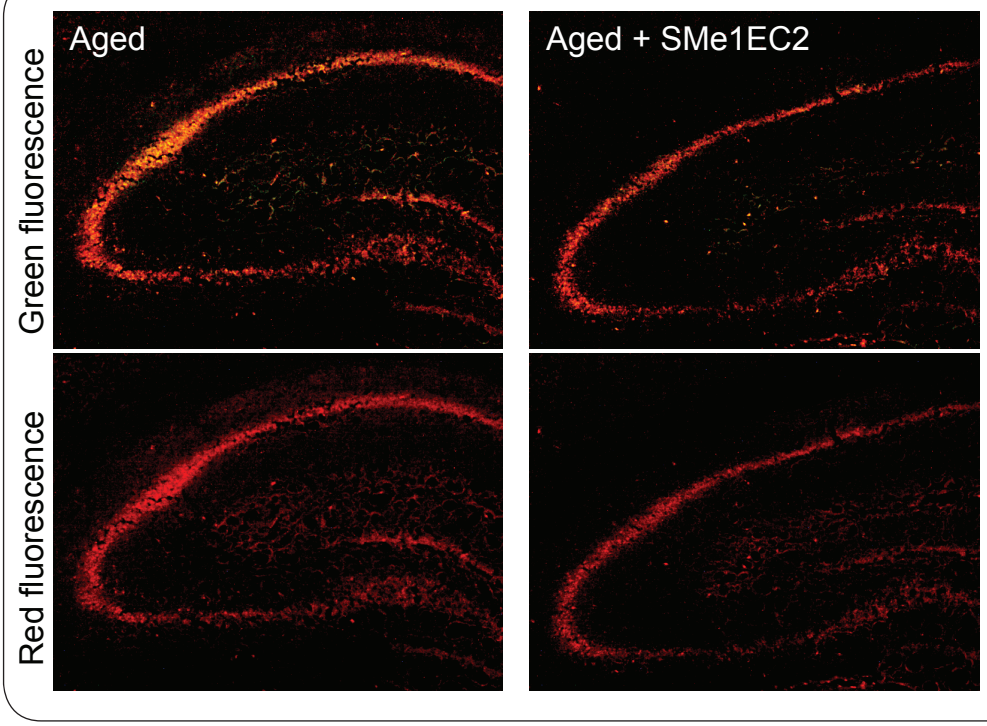

Figure 5. Representative photomicrographs of autofluorescent region in hippocampus of aged rats and effect of 8-week treatment with SMe1EC2 (0.5 mg/kg) visualized by using fluorescence microscopy and red and green emission filter cubes. Scale bar, 200 µm.

potential in our previous study (Mrvova *et al.*, 2015, Figure 6A). However, unlike quercetin and its derivative in a similar concentration range tested, co-treatment of lipopolysaccharide-stimulated microglia with SMe1EC2 did not downregulate nitrite levels in media of the activated cells (Figure 6B). Moreover, SMe1EC2 at 25 µmol/l concentration tested even enhanced the nitrite levels by 118.6±6.47% *vs* LPS control, $p<0.01$. The enhancement of nitrite production appeared to correlate with upregulation of viability/proliferation parameter (117.6±5.15% *vs* control, $p<0.01$). Unlike novel derivative of quercetin (Mrvova *et al.*, 2015) and anti-depressant drug venlafaxine (Dubovicky *et al.*, 2014), SMe1EC2 did not downregulate superoxide generation by LPS-primed phorbol myristate acetate (PMA)-stimulated cells at 25 µmol/l concentration and only mildly downregulated $O_2^{\cdot -}$ production at 100 µmol/l concentration (84.6±6.1% *vs* PMA control, $p<0.05$, Figure 6C,D). Addition of superoxide dismutase (0.5 mg/ml) suppressed formazan development confirming thus the specificity of the method.

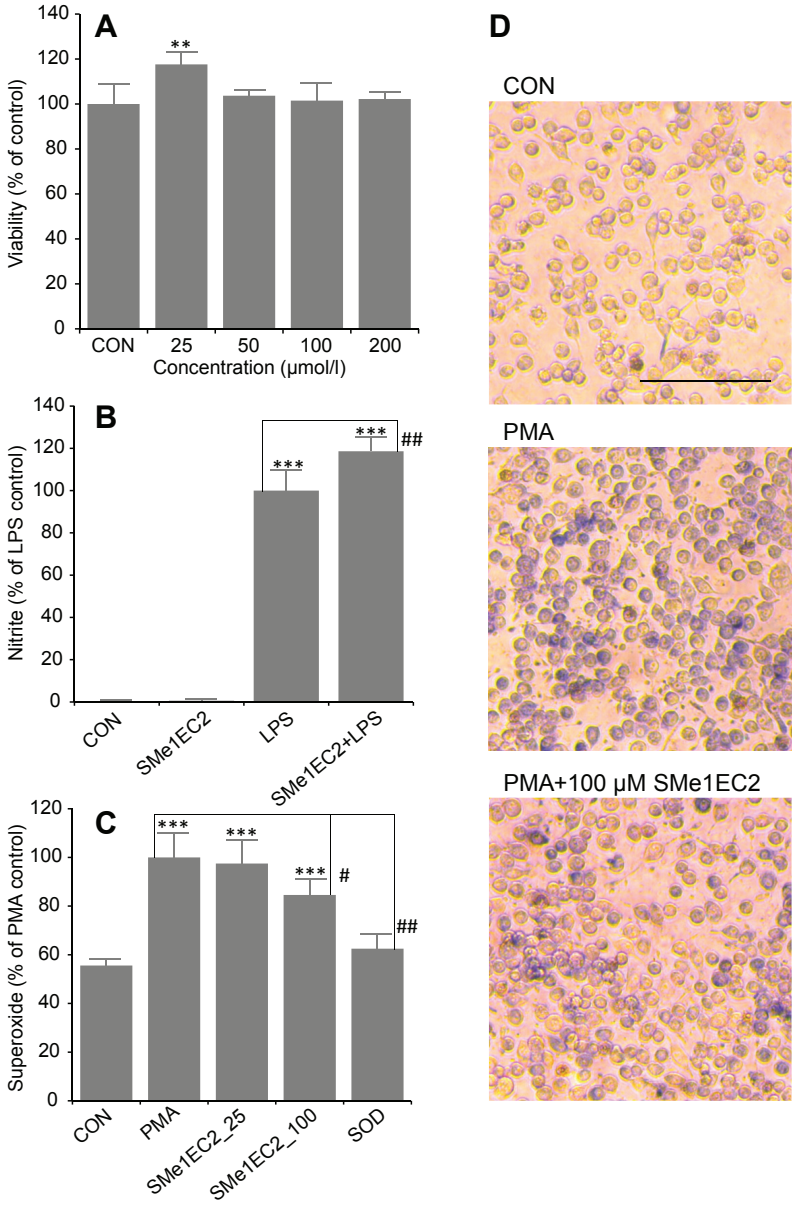

Figure 6. Effect of SMe1EC2 on viability/proliferation, nitric and superoxide production in BV-2 microglial cells. (A) Viability/proliferation was measured by standard MTT assay in the cells incubated for 24 h with or without SMe1EC2. (B) Nitric oxide production was measured in cell culture medium following 24 h stimulation with lipopolysaccharide (LPS) using Griess reagent. (C) For assessment of superoxide generation, BV-2 cells were stimulated with 2 µmol/l PMA and co-treated with SMe1EC2 and nitroblue tetrazolium salt for 1.5 h. Specificity of reaction was measured by addition of SOD (0.5mg/ml). (D) Light microscopy of development of NBT-derived formazan by PMA-stimulated BV-2 cells and effect of SMe1EC2. Results are representative means ± SD of 3–6 replicates from 2 independent experiments. **$p<0.01$, ***$p<0.001$ *vs* control, #$p<0.05$, ##$p<0.01$ *vs* stimulated cells. MTT – thiazolyl blue tetrazolium bromide; PMA – phorbol-12-myristate-13-acetate; LPS – lipopolysaccharide-stimulated cells; NBT – nitro blue tetrazolium; SOD – superoxide dismutase. Scale bar, 200 µm.

Discussion

A range of preclinical studies suggested that SMe1EC2 can be a promising agent alleviating pathological processes involved in neurological disorders. SMe1EC2 showed neuroprotective efficacy *in vitro* in rat hippocampal slices exposed to transient hypoxia/reoxygenation (Gasparova *et al.*, 2009, 2010, 2011, 2014a). Moreover, significant neuroprotective effects of SMe1EC2 were shown *in vivo* in the murine model of acute head trauma (Stolc *et al.*, 2006, 2008). SMe1EC2 exerted also protection of HT22 neuron-like cells against high glucose toxicity and oxidative stress (Rackova *et al.*, 2009). Although administration of SMe1EC2 (3×50 mg/kg) did not improve cognitive impairment in AD-like model of trimethyltin-induced neurodegeneration, it apparently preserved pyramidal cell viability in the CA1 area and downregulated serum levels of malondialdehyde, a biomarker of oxidative damage to lipids (Gasparova *et al.*, 2014b).

Dimebon, an inhibitor of cholinesterase and NMDA receptors structurally related to pyridoindoles, failed to improve cognitive capacity compared to the control cohort in a Phase III multinational trial initiated after Phase II studies showed positive clinical efficacy in addressing

cognitive and behavioral decline (Bezprozvanny, 2010, Pfizer). Dimebon was shown to enhance cognition in the rodent model of short-term memory at low acute oral doses of 0.05, 0.5, and 5.0 mg/kg (Giorgetti *et al*., 2010). As the measured brain concentrations associated with cognition enhancement at lower doses (e.g., 1.7 and 14 nM after 0.05 and 0.5 mg/kg doses, respectively) are well below the Ki for many receptors associated with cognitive function, cognition-enhancing effect of Dimebon in the rats performing novel object recognition task was suggested to be mediated by its positive effect on mitochondrial function in neurons (Kumar & Singh, 2015). In analogy with Dimebon, administration of SMe1EC2 at the dose 0.5 mg/kg resulted in mild enhancement of cognition capacity in the trained aged animals. Moreover, comparably to the acutely administered Dimebon, eight-week repetitive supplementation with SMe1EC2 appeared to improve rather the parameters of spatial learning and memory than the parameters reflecting locomotoric function (i.e. swimming speed, residence time in periphery, travelled distance and test duration).

The aged animals showed enhanced overall and clockwise rotation in the pool compared to young group, while the treatment with SMe1EC2 almost restored the clockwise turning behaviour. Besides deficits in spatial learning and memory, unilateral damage to neurons in substantia nigra pars compacta (SNpc), a condition typically seen in development of Parkinson's disease, might be associated with one-directional rotation behavior of animals (Da Cunha *et al*., 2008). The direction of the turns was shown to be determined by the site of lesion in the SNpc, its size or magnitude or by dopamine receptor supersensitivity in the striatum and unilaterally SNpc-lesioned rats were suggested to rotate toward the side with the weaker activation of dopamine receptors.

Lipofuscin is a heavily oxidized material, containing damaged lipids and proteins and low amounts of sugars that accumulates during ageing as a long-term product of oxidative stress, particularly in postmitotic aging cells such as neurons (Jung *et al*., 2007). It sequesters redox-active transition metals (mainly Fe^{2+}) catalyzing radical formation via Fenton-reaction, again increasing the rate of biomolecules oxidation and lipofuscin-formation (Brunk, 1989). Lipofuscin has autofluorescent properties which arise probably from reactions between carbonyls and primary amino groups of proteins resulting in formation of lipofuscin-like fluorophore (Yamada *et al*., 2001). Both lipofuscin accumulation and mitochondrial damage were suggested to have common underlying mechanisms, likely including imperfect autophagy and ensuing lysosomal degradation of oxidatively damaged mitochondria and other organelles (Terman & Brunk, 2006). Lipofuscin in brain accumulates with age as a result of oxidative stress, appearing first and accumulating fastest in the hippocampus (Oenzil *et al*., 1994). Similarly, Spitzer et al. identified subset of neurons in hippocampus with cellular autofluorescence resistant to treatment with $NaBH_4$ followed by $CuSO_4$ which was suggested to be a subpopulation of cells with high lipofuscin content (Spitzer *et al*., 2011). The increased level of lipofuscin along with lowered vitamin E concentration were shown to be associated with cognitive dysfunction in animal models of dementia (Skoumalova *et al*., 2003). Thus, improvement of cognitive capacity in aged animals treated with SMe1EC2 can be correlated with its lipofuscin lowering effect in the brain observed as decrease of autofluorescence in the hippocampal region. It may be also speculated that SMe1EC2 can exert other defensive effects with regard to lipofuscinogenesis-related mechanisms, such as protective effects on mitochondria functions.

The improved free radical scavenging efficacy of SMe1EC2 (Rackova *et al*., 2002, Rackova *et al*., 2006) might be responsible for lipofuscin diminution as well as for other neuroprotective efficacies reported elsewhere (Gasparova *et al*., 2009, 2010, 2011, 2014, Stolc *et al*., 2006). Nevertheless, in our study, SMe1EC2 did not show any significant efficacy in suppression of the proinflammatory production of primary reactive species, superoxide anion radical and nitric oxide, by activated BV-2 microglia, measured as tetrazolium salt reduction and nitrite concentration in media, respectively. This is in agreement with previously reported observation that SMe1EC2, lacking the ability to scavenge superoxide radical, had no influence either on basal tone or on acetylcholine-induced relaxation of rat arterial endothelium (Broskova *et al*., 2013). Also, stobadine showed lack in efficacy to inhibit NO production by LPS-stimulated BV-2 microglia along with deficiency to profoundly suppress other inflammatory markers, TNF-α release and iNOS expression (Račková *et al*., 2014). Therefore, SMe1EC2 might be less effective in diminution of oxidative and nitrosative stress in ageing brain through direct modulation of chronically activated microglia, a critical source of neuronal oxidative damage. However, SMe1EC2 was reported to effectively reduce oxidation of H2R123 and H2DCF DA, fluorogenic probes sensitive to secondary reactive species such as hydroxyl and peroxyl radicals, in LPS-primed PMA-stimulated RAW264.7 macrophages (Balcerczyk *et al*., 2014). Furthermore, other mechanisms than direct scavenging of free radicals can be responsible for beneficial modulation of redox homeostasis in the brain by SMe1EC2 following its oral administration (Forman *et al*., 2014).

In conclusion, our study suggests that pyridoindole SMe1EC2, at low doses administered chronically, can act as cognition enhancing agent in aged rats. Although the detailed mechanism of this effect warrants further investigation, this preliminary study points to protective effect of SMe1EC2 against accumulation of oxidative damage accompanying ageing. Furthermore, the protective mechanism less likely involves direct modulation of proinflammatory and prooxidant state of microglia, the prominent mediators of neurotoxicity in brain ageing and neurodegeneration.

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