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Influence of water-soluble derivatives of [60]fullerene on therapeutically important targets related to neurodegenerative diseases†

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We report the investigation of the molecular mechanisms responsible for the neuroprotective activity of water-soluble [60]fullerene derivatives (WS[60]FDs). It has been shown that WS[60]FDs influence the therapeutically important targets of the Alzheimer disease by inhibiting the catalytic activity of the monoaminooxidase B *in vitro*, decreasing the level of free radical species and behaving as positive modulators of AMPA receptors of Purkinje neurons in the cerebellum of rats. The cognitive stimulatory effects of WS[60]FDs were revealed *in vivo* through behavioural experiments in mice.

1. Introduction

Alzheimer disease (AD) is one of the most severe problems for the modern society. Patients suffering from Alzheimer disease face constantly progressing dementia leading to a complete loss of memory and intelligence. Currently, about 0.4–0.5% of the world's population is suffering from AD, and this number is growing rapidly and predicted to reach 1% by 2030–2050.[1] There are no pharmaceuticals that can be used for the successful treatment of AD. It is known that [60]fullerene and many of its functional derivatives exhibit a range of promising neuroprotective activities.[2–10] It was also shown that some water-soluble carboxyfullerenes behave as superoxide dismutase mimics, and this activity correlates well with their neuroprotective efficacy.[11,12] Fullerene derivatives and their nanoclusters are also studied as promising drug carriers that can be used for delivering pharmaceutically important compounds to many therapeutic targets.[13–16] WS[60]FDs are known to penetrate easily through the cell membranes, which supports their use as advanced drug carriers.[17,18]

Here we report the results of our comparative study of a few different WS[60]FDs as pharmaceutically promising lead compounds that influence therapeutic targets of Alzheimer disease and prevent neuronal disorders in animals.

2. Experimental

2.1. Synthesis of WS[60]FDs

The investigated WS[60]FDs **I–III** shown in Fig. 1 have been prepared using general synthetic procedures that were reported previously.[19–21] Details are given in the ESI.†

2.2. Investigation of biological activity of WS[60]FDs

The lipid peroxidation (LPO) level in mouse brain homogenate subcellular fractions was quantified by determining the concentration of malonic dialdehyde (MDA), which is known to be a specific intermediate product of the oxidation of

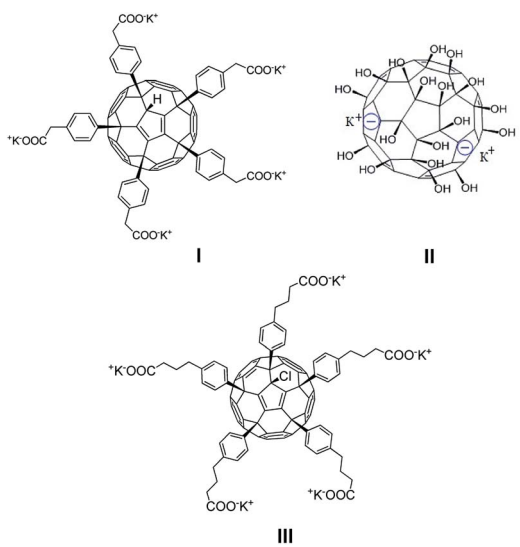

Fig. 1 Molecular structures of investigated compounds.

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polyunsaturated lipids.[22] The subcellular fractions were obtained by homogenizing the mouse brain tissue (1.0 g) with 0.1 M Tris–HCl buffer solution (pH = 7.4, 8.0 ml) in a Potter homogenizer at 4 °C.

The radical scavenging activity of WS[60]FDs was investigated *in vitro* using homogenized mouse brain tissue. Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) was applied as a dye to carry out the chemoluminescence assay using *tert*-butyl hydroperoxide (*t*BuOOH) as a source of peroxide free radicals.[23]

Quantification of the results was performed by integrating the chemoluminescence kinetic function $I(t)$ (calculating the area under the kinetic curve), where I is the intensity of the chemoluminescence and t is a registration time. The resulting integral S is proportional to the number of peroxide free radicals that reacted with luminol within a certain timeframe. Introducing the antioxidants that perform as radical scavengers to the system decreases the intensity of the chemiluminescence and leads to a smaller S value.[24]

The test samples contained 0.2 ml subcellular fraction of the homogenized mouse brain tissue with a final protein concentration in the sample of 0.1 mg ml^{-1}, 0.2 ml of luminol at a final concentration in the sample of 5×10^{-5} M, 0.2 ml of WS[60]FDs at a final concentration in the sample of 10^{-5} M, 0.2 ml of *t*BuOOH at a final concentration in the sample of 0.073 M and 1.2 ml of Tris–HCl buffer solution at a final concentration in the sample of 0.06 M (pH = 7.4). The reference samples were prepared in the same way with except for WS[60]FDs, which were not introduced in this case. The chemoluminescence kinetics were examined using a Luminometr-1250 LKB Wallak instrument for 15 minutes. All measurements were performed in a temperature-controlled cell with continuous purging of the sample with air. The protein concentration was determined using the standard Lowry protein assay.[25]

The catalytic activity of monoamine oxidase B (MAO-B) in the subcellular fraction of the homogenized mouse brain tissue was evaluated according to the method reported previously.[26] This method is based on spectrophotometric detection of ammonia formed in the benzylamine deamination reaction catalyzed by MAO-B enzyme integrated in the membranes. The protein concentration was determined using the Lowry assay.

The influence of WS[60]FDs on AMPA receptors (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors) was investigated using the patch-clamp method in a whole cell configuration. AMPA receptors are known as one of the types of glutamate receptors that mediate fast synaptic transmission in the central nervous system of mammals.[27] The single Purkinje neurons were isolated from the cerebellum of the 12–16 day-old Wistar rats using a modified Kaneda method.[28] The signal acquisition was performed using an EPC-9 instrument (HEKA, Germany) operated with original Pulse software (HEKA). Each compound was investigated on five neurons. The results were analysed using Pulsefit software (HEKA).

The effects of WS[60]FDs on the memory of animals were studied using an object recognition test described previously.[29] All compounds were administered once in the C57B1/6 male mice at a dose of 1 mg kg^{-1}. The test is based on recognition of a known object in a new location. Animals (mice) spontaneously explore the new location of the object for a longer time than a known location that they remember.

To obtain a recognition index k for each animal trial the experimental data were evaluated using the following equation:

$$k = [t_1/(t_1 + t_2)] \times 100\%$$

where t_1 and t_2 correspond to the times of the object exploration in a new and previously known location, respectively. The total time required to explore both objects is taken as 100%. Statistical treatment of the results was performed using Student's t-distribution. The results were considered to be reliable if they fall within the 95% confidence interval ($p < 0.05$).

All experiments were carried out in accordance with the European Communities Council Directive for the care and use of laboratory animals following approval by the local governmental bodies for animal care and welfare.

3. Results and discussion

It is known that the ability of substances to penetrate through the cell membranes significantly influences their biological activity. Moreover, some compounds can interact with the components of the membrane, modifying the catalytic activity of some enzymes and the functional activity of receptors or just inhibiting dangerous free radical processes. It was shown that WS[60]FDs interact with hydrophilic sites of the phospholipid membranes, and the character of this interaction is defined by the charge state of WS[60]FDs.[30]

Known therapeutic targets for the treatment of Alzheimer's disease are the membrane-integrated enzyme monoamine oxidase B (MAO-B), free radical oxidation, and recently, β-amyloidal fibrils.[31,32] It is known that MAO-B is one of the key enzymes metabolizing dopamine in the brain to a final product, homovanillic acid. Inhibition of this enzyme leads to a prolonged action of the synaptic dopamine, which has highly positive therapeutic effects in the treatment of some psychiatric disorders.[33] It was also demonstrated that MAO-B inhibitors might be used to treat patients suffering from Alzheimer disease (AD).[31]

Unbalanced lipid peroxidation, particularly, the formation of excessive amounts of free radicals are supposed to be the main factors responsible for the development of AD.[34] A correlation was revealed between the decreased level of superoxide dismutase and increased production of the superoxide radicals in AD patients.[35] The protective effect of the superoxide dismutase is directly related to its ability to inactivate the superoxide anion-radicals. Therefore, inhibition of lipid peroxidation is one of the main targets for developing pharmaceuticals for the treatment of AD.

As a background for this work, we evaluated nineteen different WS[60]FDs as potential inhibitors of MAO-B and lipid peroxidation. These studies showed that WS[60]FDs **I** and **II** (Fig. 1, Table 1) are the most promising lead compounds. In addition, we selected an additional compound **III** as a reference, because it had no effect on the MAO-B activity. According to our initial hypothesis, compound **III** cannot induce cognitive

Table 1 The influence of WS[60]FDs **I–III** on the malondialdehyde production, *t*BuOO˙ radical scavenging activity and inhibition of the catalytic activity of MAO-B in the subcellular fraction of the homogenized mouse brain tissue; $p < 0.05$ relative to control

WS[60]FD, concentration	Relative concentration of MDA, %	S^a, %	MAO-B activity, mM of NH_3 per mg of enzyme
Control	100.0 ± 7.0	100.0 ± 4.5	3.20 ± 0.17
I	43.3 ± 5.2	85.2 ± 5.0	1.82 ± 0.21
II	61.4 ± 4.0	72.1 ± 6.9	1.89 ± 0.23
III	54.2 ± 6.5	78.3 ± 4.5	3.49 ± 0.26

[a] *S* is an integral intensity of the luminol chemoluminescence proportional to the *t*BuOO˙ radical concentration in the medium (see Experimental). All compounds were investigated at a concentration of 10^{-5} M.

stimulation in animals, in contrast to the much more promising compounds **I** and **II**.

In order to compare the radical scavenging activity of the selected WS[60]FDs, we studied the kinetics of the luminol chemoluminescence in the subcellular fraction of the homogenized mouse brain tissue in the presence of *t*BuOOH as a radical source. It is seen from the results in Table 1 that all investigated WS[60]FDs **I–III** reduce the integral chemoluminescence intensity *S* (see Experimental). At the same time, WS[60]FDs **I–III** significantly reduce the production of malondialdehyde, which suggests that they inhibit lipid peroxidation (Table 1). This observation shows that all investigated compounds possess radical scavenging and antioxidant activities. However, only compounds **I** and **II** suppress MAO-B, while WS[60]FDs **III** has virtually no effect on the catalytic activity of this enzyme (Table 1).

It was reported previously[5] that the fullerene derivative **II** demonstrates a strong anti-amyloid activity. It efficiently destroys the preformed β-amyloid fibrils and also prevents their formation *in vitro*. It has been shown that compounds **I**, **II** and **III** exhibit low acute toxicity in mice with LD_{50} values of 600, 1800 and 300 mg kg^{-1}, respectively.[36]

Thus, WS[60]FDs **I** and **II** possess a combination of properties that make them promising for application as neuroprotectors and support investigation of their action on the AD therapeutic targets. They were shown to perform as antioxidants, inhibitors of lipid peroxidation and MAO-B enzyme and also as anti-amyloid agents.

The influence of WS[60]FDs **I–III** on the ionotropic AMPA glutamate-type receptors from rat brain tissue has been investigated. It is known that positive modulation of AMPA receptor responses in mammals is one of the most important criteria for the selection of compounds that are expected to show a cognitive stimulatory effect. Positive modulators of AMPA receptors enhance the synaptic signal transduction (increase the amplitude and duration of stimulating postsynaptic potentials) and improve the formation and amplification of the long-term potentiation, which is physiologically related to long-term memory.[9,37,38] Therefore, the AMPA receptors are considered as one of the most important therapeutic targets in the screening of potential lead compounds for the treatment of AD. Here, we applied the electrophysiological patch-clamp method in the "whole cell" configuration to study the influence of the selected WS[60]FDs on the functioning of ionotropic AMPA receptors obtained from rat brain tissue (Fig. 2).

It is seen from the figure that compounds **I** and **II** applied in concentrations from 10^{-10} to 10^{-5} M induce positive modulation of the AMPA receptor responses in the Purkinje neurons. On the contrary, WS[60]FDs **III** does not influence the functioning of the AMPA receptors. These results suggest that the observed positive modulation of AMPA receptors by compounds **I** and **II** might be correlated with their ability to stimulate the cognitive processes in mammals.

The cognitive stimulatory activity of the investigated WS[60]FDs was assessed using the object recognition test described previously.[29] According to a general theory,[29] the animals with good spatial memory find an object in an expected location rather quickly. However, if the object is moved to a new location, they spend more time near the previous location before starting random exploration of the field. On the contrary, the animals that do not remember well a previous location of the object start exploration of the field almost immediately. Consequently, they find the new location of the known object faster than animals with good spatial memory.

Fig. 3 shows that mice in a control group appeared to have not so good spatial memory, since finding the object in a new location required about 30% more time than finding it in a known location. Very similar results were obtained when compound **III** was administered in mice. This result suggests

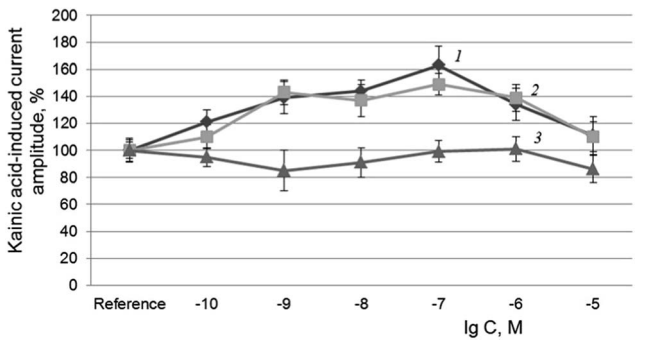

Fig. 2 The influence of the WS[60]FDs **I–III** (1–3, respectively) on the kainic acid-induced current amplitude of the AMPA receptors in Purkinje neurons isolated from the rat cerebellum.

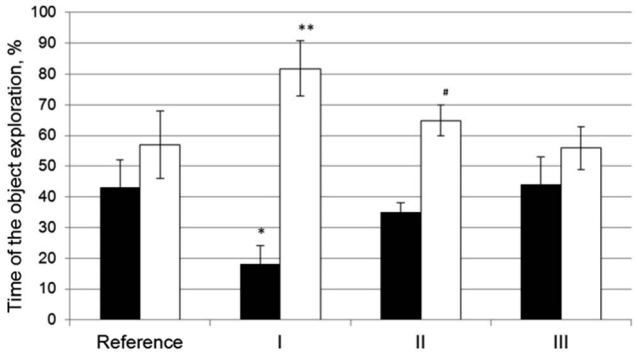

Fig. 3 The influence of the WS[60]FDs I–III on the spatial memory of mice. Compounds I and II induce considerable changes in the time of object exploration in a known location (■) and new location (□) compared to the reference (control). * – $p < 0.05$; ** – $p < 0.01$ relative to control, # – $p < 0.05$ relative to a known localization.

that WS[60]FD **III** has virtually no influence on the spatial memory of mice.

On the contrary, administration of compounds **I** and **II** in mice provided sharply distinct results. For instance, the application of WS[60]FD **I** leads to a more than 2-fold decrease in the time to find the object in a known location. At the same time, the time of searching for the object in a new location is increased by a factor of ~1.5. Other compound, WS[60]FD **II**, induced very similar effects, although they were smaller in magnitude (Fig. 3). Thus, it was revealed that WS[60]FDs **I** and **II** significantly improve the spatial memory of mice.

4. Conclusion

In this work, we have revealed a correlation between MAO-B inhibition, positive modulation of AMPA receptors and cognitive stimulatory activity for the investigated WS[60]FDs. This result agrees well with those of previous publications.[39,40] The obtained experimental data demonstrate that some water-soluble fullerene derivatives can be used as rather efficient neuroprotectors to improve long-term memory in mammals. These findings suggest that the investigated WS[60]FDs **I** and **II** can be considered as potentially promising lead compounds for treatment of Alzheimer disease.

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