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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 molecular mechanisms responsible for the neuroprotective activity of water-soluble [6o]fullerene derivatives (WS[6o]FDs). It has been shown that WS[6o]FDs influence the therapeutically important targets of the Alzheimer disease: inhibit catalytic activity of the monoaminooxidase B in vitro, decrease the level of free radical species and behave as positive modulators of AMPA receptors of Purkinje neurons in rats cerebellum. Cognitive stimulation effects of WS[6o]FDs were revealed in *in-vivo* using behavioural experiments with mice.

1. Introduction

Alzheimer disease (AD) is one of the severest problems for the modern society. Patients suffering from Alzheimer disease face constantly progressing dementia leading to a complete loss of memory and intelligence. Nowadays about 0.4-0.5% of the world population are suffering from AD and this number is growing rapidly with a prospect to reach 1% by 2030-2050.[1] There are no pharmaceuticals which can be used successfully for 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 which 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 results of our comparative study of few different WS[60]FDs as pharmaceutically promising lead compounds which 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 which were reported previously.[19-21] Details are given in the Electronic Supplementary Information (ESI) section.

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

The lipid peroxidation (LPO) level in mice brain homogenate subcellular fractions was quantified by determining the concentration of malonic dialdehyde (MDA), which is known to be specific intermediate product of the oxidation of polyunsaturated lipids.[22] The subcellular fractions were obtained by homogenizing of the mice 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.

Radical scavengering activity of WS[60]FDs was investigated *in vitro* using homogenized mice 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 hydroperoxyde (tBuOOH) as a source of peroxide free radicals.[23]

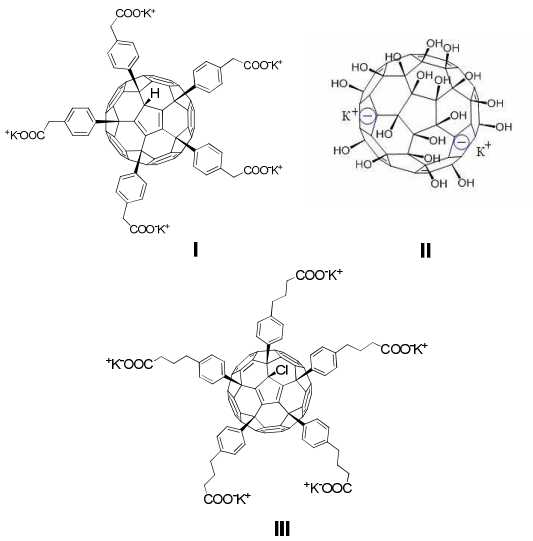

Figure 1. Molecular structures of investigated compounds.

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 which reacted with luminol within a certain timeframe. Introducing the antioxidants performing 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 mice brain tissue with the final protein concentration in sample of 0.1 mg/ml, 0.2 ml of luminol at a final concentration in sample of $5 \cdot 10^{-5}$ M, 0.2 ml of WS[60]FDs at a final concentration in sample of 10^{-5} M, 0.2 ml of tBuOOH at a final concentration in sample of 0.073 M and 1.2 ml of tris-HCl buffer solution at a final concentration in 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 was registered using Luminometr-1250 LKB Wallak instrument for 15 minutes. All measurements were performed in a temperature-controlled cell with a continuous purging of the sample with air. The protein concentration was determined using standard Lowry protein assay. [25]

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

The influence of WS[60]FDs on AMPA receptors (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors) was investigated using patch-clamp method in a «whole cell» configuration. AMPA receptors are known as one of the types of glutamate receptors which 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 EPC-9 instrument (HEKA, Germany) operated with original Pulse software (HEKA). Each compound was investigated on the five neurons. The results were treated using Pulsefit software (HEKA).

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

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

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

where t_1 and t_2 correspond to the times of the object exploration in a new and previous (known) localizations, respectively. The total time required for exploring 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.

Results and discussion

It is known that the ability of substances to penetrate through the cell membranes influences significantly their biological activity. Moreover, some compounds can interact with the components of the membrane modifying catalytic activity of some enzymes, 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 oxidize 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 the 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 a 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 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 peroxidaton is one of the main targets for developing pharmaceuticals for 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 also an additional compound **III** as a reference since it caused no effect on the MAO-B activity. According to our initial hypothesis, compound **III** cannot induce cognitive stimulation in animals in contrast to the much more promising compounds **I** and **II**.

Table 1. The influence of WS[60]FDs **I-III** on the malondialdehyde production, tBuOO· radical scavengering activity and inhibition of the catalytic activity of MAO-B in the subcellular fraction of the homogenized mice brain tissue; $p<0.05$ relative to control.

WS[60]FD, concentration	Relative concentration of MDA,%	S*, %	MAO-B activity, mM of NH_3 /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

* S is an integral intensity of the luminol chemoluminescence proportional to the tBuOO· radical concentration in the medium (see Experimental). All compounds were investigated in concentration of 10^{-5} M.

In order to compare the radical scavengering activity of the selected WS[60]FDs we studied kinetics of the luminol chemoluminescence in the subcellular fraction of the homogenized mice brain tissue in the presence of the tBuOOH as a radical source. It is seen from the Table 1 that all investigated WS[60]FDs **I-III** decrease the integral chemoluminescence intensity S (see Experimental). At the same time, WS[60]FDs **I-III** decrease significantly the production level of malondialdehyde which suggests that they inhibit lipid peroxidation (Table 1). This observation evidences that all investigated compounds possess radical scavengering and antioxidant activities. However, only the 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 earlier[5] that the fullerene derivative **II** demonstrates a strong anti-amyloid activity. It destroyed efficiently the preformed β-amyloid fibrils and also prevented their formation *in vitro*. It has been shown that compounds **I, II** and **III** possess low acute toxicity in mice with LD_{50} values of 600, 1800 and 300 mg/kg, respectively.[36]

Thus, WS[60]FDs **I** and **II** possess a combination of properties which make promising their application as neuroprotectors and investigation of their action of 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 a mice brain tissue has been investigated. It is known that a positive modulation of AMPA receptors response in mammals is one of the most important criteria for selection of compounds which are expected to show a cognitive stimulation effect. Positive modulators of AMPA receptors enhance the synaptic signal transduction (increase the amplitude and duration of stimulating postsynaptic potentials) and improve formation and amplification of the long-term potentiation, which is physiologically related to a 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 treatment of AD. Here we applied electrophysiological patch-clamp method in the "whole cell" configuration for studying the influence of the selected WS[60]FDs on the functioning of ionotropic AMPA receptors obtained from a mice brain tissue (Fig. 2).

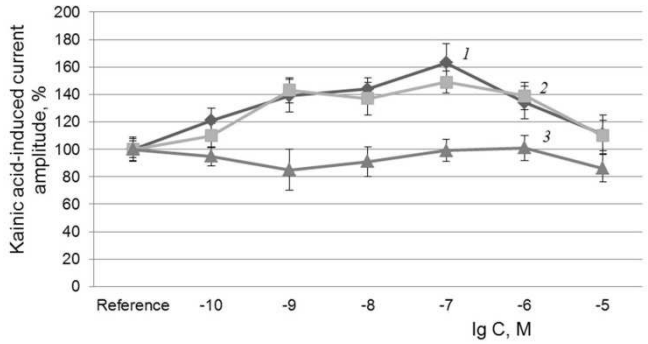

Figure 2. The influence of the WS[60]FDs **I-III** *(1-3,* respectively) on the kainic acid-induced current amplitude of the AMPA receptors in Purkinje neurons isolated from the rat cerebellum.

It is seen from the figure that compounds **I** and **II** applied in the 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 correlate with their ability to stimulate the cognitive processes in mammals.

The cognitive stimulation 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 a 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 which do not remember so well a previous location of the object start exploration of the field almost immediately. Consequently, they find a new location of the known object faster than animals with a good spatial memory.

Figure 3 shows that mice in a control group appeared to have not so good spatial memory since finding the object in a new location requires just about 30% more time than finding it in a known location. Very similar results were obtained when compound **III** was administrated in mice. This result suggests that WS[60]FD **III** has virtually no influence on the spatial memory of mice.

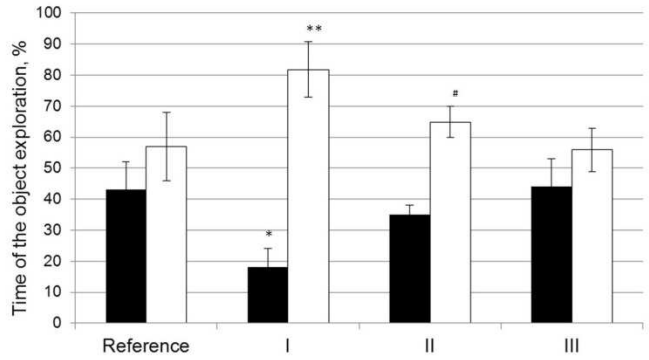

Figure 3. The influence of the WS[60]FDs **I-III** on a spatial memory of mice. Compounds **I** and **II** induce considerable changes in the time of the object exploration in a known location (■) and new location (□) compared to the reference (control). *- p<0.05; ** - p<0.01 relative to control, #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

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 two-fold decrease in the time of finding the object in a known location. At the same time, the time of searching the object in a new location is increased by a factor of ~1.5. Other compound, WS[60]FD **II**, induced very similar effects though they were smaller in magnitude (Fig. 3). Thus it was revealed that WS[60]FDs **I** and **II** improve significantly spatial memory of mice.

Conclusion

In this work we have revealed a correlation between the MAO-B inhibition, positive modulation of AMPA receptors and cognitive stimulation activity for the investigated WS[60]FDs. This result agrees well with the previous publications.[39,40] The obtained experimental data evidenced that some water-soluble fullerene derivatives can be used as rather efficient neuroprotectors improving long-term memory of 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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Notes and references

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