Electronic supplementary information for the manuscript:

Water-soluble fullerene-based nanostructures with high antiviral and myogenic activity

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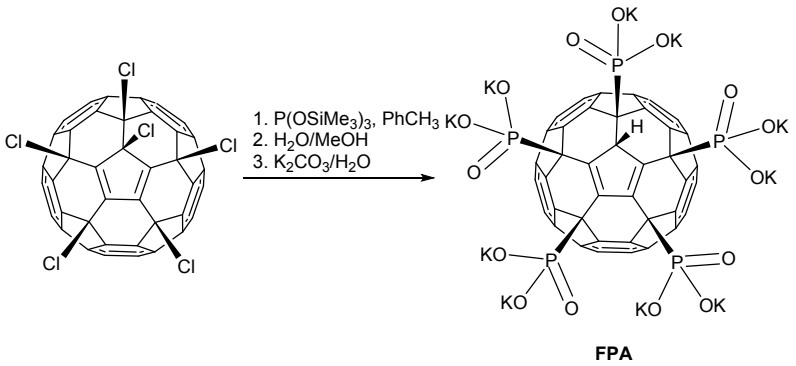
1. One-step synthesis of the water-soluble fullerene derivative FPA



FPA

Chlorofullerene $C_{60}Cl_6$ was prepared as described in P. A. Troshin et al., *Fullerenes, Nanotubes, Carbon Nanostruct.*, **2003**, *11*, 165. Tris(trimethylsilyl) phosphite (>95%) was used as obtained from Sigma-Aldrich. Toluene was distilled and dried over sodium flakes (water content – 23 ppm).

Chlorofullerene $C_{60}Cl_6$ (500 mg, 0.536 mmol) was dissolved in dry toluene (150 mL). After 1 hour of vigorous stirring solution was filtrated through tight paper filter to single-neck round-bottom flask, and tris(trimethylsilyl) phosphite (2.4 g, 8.04 mmol, 15 eq) was added dropwise. Reaction mixture was stirred for additional 15 min, and then concentrated at the rotary evaporator. Product of the reaction was precipitated with heptane (100 mL). Suspension was centrifuged and residue was washed with heptane c.a. 10 times (10*20 mL) and dried. Obtained residue was dissolved in water-MeOH mixture, filtered through tight paper filter and concentrated using rotary evaporator. Compound FPA (acidic form, $C_{60}[PO(OH)_2]_5H$ was obtained as bright-orange powder (512 mg, 85%). Fullerene-based acid (500 mg, 0.44 mmol), distilled water (50 mL) and stoichiometric amount of anhydrous potassium carbonate (306 mg, 2.22 mmol) were placed into a single-necked flask and the reaction mixture was intensively stirred until complete dissolution. Than the solution was filtered through a PES syringe filter (average pore size 0.45 μm) to single-neck round-bottom flask and freeze-dried for 24 h to afford an orange powder of FPA (potassium salt form, $C_{60}[PO(OK)_2]_5H$) with quantitative yield. Spectral characteristics of **FPA** (acidic form) were similar to those reported before (A. Yurkova, E. Khakina, S. Troyanov, A. Chernyak, L. Shmygleva, A. Peregudov, V. Martynenko, Y. Dobrovolskiy and P. Troshin, *Chem. Commun*., 2012, **48**, 8916). **FPA** (acidic form).^{1}H $\{^{31}P\}$ NMR (500 MHz, DMSO-d_6, δ, ppm): 6.05 (s, 1H). ^{29}Si $\{^{31}P\}$ NMR (99 MHz, DMSO-d_6, δ, ppm): no signals (glass).

2. DFT calculations

Optimization of the molecular structures was performed using the PBE exchange-correlation functional (J. P. Perdew, K. Burke and M. Ernzerhof, *Phys. Rev. Lett.,* 1996, **77**, 3865) and Λ2 basis set of cc-pVTZ quality (D. N. Laikov, *Chem. Phys. Lett.*, 2005, **416**, 116): H {3,2,1}/{8s,4p,2d}; C, O

{4,3,2,1}/(12s,8p,4d,2f); P {5,4,2,1}/{18s,13p,5d,2f}; K {8,7,4,2}/{23s,18p,9d,6f} implemented in the **PRIRODA** package (D. N. Laikov, *Chem. Phys. Lett.,* 1997, **281**, 151). NMR chemical shifts were computed at the GIAO-PBE/Λ2 level using the **PRIRODA** code. The Hirschfeld method (F. L. Hirshfeld, *Theor. Chim. Acta*, 1977, **44**, 129) was used to calculate atomic charges. All calculations were performed at Joint Supercomputer Center of the Russian Academy of Sciences.

To test the method, the structure of a related molecule $C_{60}[P(O)(OEt)_2]_5H$ characterized by X-ray single crystal diffraction (A. Yurkova, E. Khakina, S. Troyanov, A. Chernyak, L. Shmygleva, A. Peregudov, V. Martynenko, Y. Dobrovolskiy and P. Troshin, *Chem. Commun*., 2012, **48**, 8916) was calculated (Fig. S1).

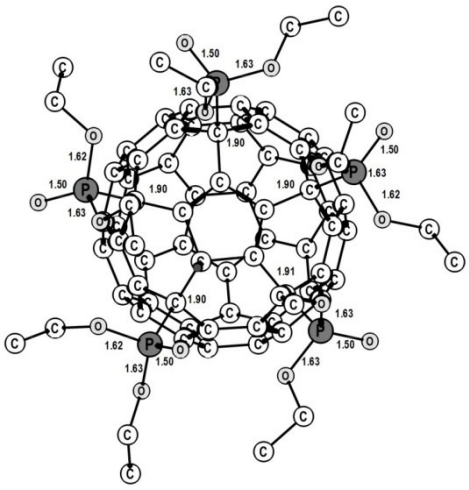


Figure S1. Calculated structure of $C_{60}[P(O)(OEt)_2]_5H$ molecule.

The selected method reproduces well the spatial arrangement of ethyl substituents and the bond lengths involving P atoms: P=O 1.490 (1.462), P-O 1.613 (1.575), P-C 1.890 (1.841) Å. The average values from the single crystal XRD data are given in parentheses. Due to the disordered arrangement of the ester groups, the data for the $-P(O)(OEt)_2$ fragment without disorder was used. The calculated chemical shift for the hydrogen atom attached to the fullerene cage is 6.27 ppm, which is a bit higher than the experimental value (5.65 ppm).

The calculated structure of the **FPA** molecule is shown in Fig. S2. All P-O bonds are equivalent due to two O-K coordination interactions and have almost the same length of 1.55 Å.

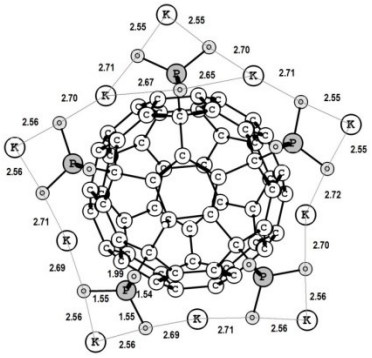


Figure S2. Calculated structure of $C_{60}[P(O)(OK)_2]_5H$ fullerene derivative.

Potassium ions are symmetrically arranged in two belts - the outer one (five potassium ions) around the fullerene molecule and the inner one (five potassium ions) over the C_{60} molecule (Fig. S3). The potassium atoms have a charge of +0.51 in the outer layer and +0.41 in the inner layer. Due to the strong Coulomb

interactions, a slight stretching (0.1 Å) of the C-P bonds occurs. The calculated chemical shift for the hydrogen atom is 7.37 ppm. While applying the correction based on the comparison of theoretical and experimental chemical shifts for $C_{60}[P(O)(OEt)_2]_5H$, we estimated the theoretical shift for H atom in $C_{60}[P(O)(OK)_2]_5H$ as 6.75 ppm, which is in satisfactory agreement with the experimental value of 6.05 ppm.

Dimerization of **FPA** molecules leads to the formation of van der Waals complexes. The opposite orientation of the dipole moments of **FPA** molecules allows the formation of ten K-O intermolecular coordination bonds (2.80–2.83 Å), which decreases the system energy by 51.0 kcal/mol. As can be seen from the structure of the dimer on Fig. S3, potassium ions of the second belt are coordinatively unsaturated. Thus, one should expect a further association of dimers into lateral structures.

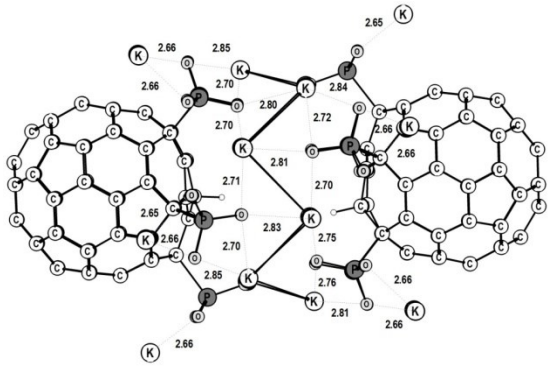


Figure S3. Calculated structure of the dimer of $C_{60}[P(O)(OK)_2]_5H$ molecule.

3. Biological assays

3.1. Antiviral activity

Screening of the antiviral activity of the fullerene derivative against Feline coronavirus, Influenza virus (A, B), Varicella virus, Cytomegalovirus, Herpes simplex virus (HSV-1, HSV-2), Cowpox virus, Feline herpes virus, Human immunodeficiency virus (HIV-1, HIV-2) was performed as described previously (A. B. Kornev, A. S. Peregudov, V. M. Martynenko, J. Balzarini, B. Hoorelbeke, P. A. Troshin, *Chem. Commun.*, 2011, **47**, 8298; O. A. Troshina, P. A. Troshin, A. S. Peregudov, V. I. Kozlovskiy, J. Balzarini and R. N. Lyubovskaya, *Org. Biomol. Chem*., 2007, **5**, 2783).
HEL (Human Erythroleukemia cells), CRFK (Crandell Rees Feline Kidney cells), MDCK (Madin-Darby Canine Kidney cells), CEM cell lines were used to evaluate the cytotoxicity and antiviral activity of the compound.

3.2. Myogenic activity

3.2.1. Cell Culture

MSCs were derived from adipose tissue. To obtain stromal cells, minced adipose tissue was digested with collagenase as described previously (S. Kostyuk, T. Smirnova, L. Kameneva, L. Porokhovnik, A. Speranskij, E. Ershova, S. Stukalov, V. Izevskaya, N. Veiko, *Oxid. Med. Cell. Longev.,* 2015, 782123). Tissue samples were mechanically disrupted in Dulbecco's Modified Eagle medium (DMEM) (Paneko, Moscow) with 250 μg/mL gentamycin, 60 U/mL penicillin, and 60 U/mL streptomycin (Paneko). Cells were dissociated by incubation with 0.04% collagenase (Sigma) in DMEM with 10% fetal bovine serum (FBS) (PAA, Austria) at 37°C for 16 h. Cells were centrifuged at 200 g for 10 min, transferred into slide

flasks and cultivated at 37°C in AmnioMax Basal Medium with AmnioMax Supplement C100 (Gibco). Cultures were split no more than four times before experiments.

MSCs were characterized by standard markers using fluorescence-activated cell sorting (FACS): MHC molecules (HLA-ABC+) and adhesion molecules (CD44+, CD54 (low), CD90+, CD106+, CD29+, CD49b (low), and CD105). However, they were negative for hematopoietic markers (CD34−, CD45−, and HLA-DR−) and the marker CD117 (M. Dominici, K. Le Blanc, I. Mueller, I. Slaper-Cortenbach, F. Marini, D. Krause, R. Deans, A. Keating, Dj. Prockop, E. Horwitz, *Cytotherapy,* 2006, **8**, 4, 315). Moreover, cells differentiated into adipocytes in the presence of inducers in a kit for adipogenic differentiation (STEMCELL Technologies). Ethical approval for the use of MSCs was obtained from the Regional Committees for Medical and Health Research Ethics (approval number 5).

3.2.2. MTT assay

Cells were grown in a 96-well plate for 72 h. Survival was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as described previously (E. S. Ershova, V. A. Sergeeva, V. J. Tabakov, L. A. Kameneva, L. N. Porokhovnik, I. I. Voronov, E. A. Khakina, P. A. Troshin, S. I. Kutsev, N. N. Veiko, S. V. Kostyuk, *Oxid. Med. Cell. Longev.,* 2016, 9895245; E. S. Ershova, V. A. Sergeeva, A. I. Chausheva, D. G. Zheglo, V. A. Nikitina, L.V. Kameneva, L. N. Porokhovnik, S. I. Kutsev, P. A. Troshin, I. I. Voronov, E. A. Khakina, N. N. Veiko, S. V. Kostyuk, *Mut. Res.* 2016, **805**, 46.). The plates were read at 550 nm (EnSpire reader).

3.2.3. Flow Cytometry Analysis (FCA)

Cells were washed with Versene solution, then treated with 0.25% trypsin, washed with culture medium, and suspended in PBS. Paraformaldehyde (PFA, Sigma-Aldrich, Saint Louis, USA) treatment at 37°C for 10 min was performed to fix the cells. Cells were washed three times with 0.5% BSA-PBS and permeabilized with 0.1% Triton X-100 in PBS for 15 min at 20°C or with 90% methanol at 4°C, then washed with 0.5% BSA-PBS (3 times) and stained with antibodies MYF6, MYOG, MYF5, CEBPB, AP2, RUNX2, OCN (1 μg/mL) for 2 h at 4°C, and washed three times with 0.5% BSA-PBS. The cells were then incubated for 2 h (20°C) with FITC goat anti-rabbit IgG (1 μg/mL) and analyzed.

Table S1. Expression levels of the proteins that determine the differentiation pathway of MSCs in the presence of the **FPA**: average values ± SD, a.u. Control values (for MSCs cultivated without **FPA**) are assigned as 1 a.u. (*) - statistically significant differences, $p < 0.01$.

Myogenic differentiation	
MYF6	1,7 ± 0,3*
MYF5	6,7 ± 0,6*
MYOG	2,0 ± 0,4*
Adipogenic differentiation	
CEBPB	1,2 ± 0,3
AP2 (FABP4)	0,7 ± 0,4
Osteogenic differentiation	
RUNX2	1,1 ± 0,3
OCN	1,1 ± 0,4

3.2.4. Fluorescence Microscopy

The Axio Scope.A1 microscope (Carl Zeiss) and the confocal microscopy platform Leica TCS SP8 (Germany) were used for fluorescent microscopy of stained cells.

3.2.5. Immunocytochemistry

Cells were grown in slide flasks (25 cm^3, Thermo Fisher Scientific, Waltham, USA), fixed in 3% paraformaldehyde at 4°C for 20 min, washed with PBS, and then permeabilized with 0.1% Triton X-100

in PBS for 15 min at room temperature, followed by blocking with 0.5% BSA in PBS for 1 h and incubation overnight at 4°C with the antibodies. After washing with 0.1% Triton X-100 in PBS, fibroblasts were incubated for 2 h at room temperature with the FITC goat anti-mouse IgG, washed with PBS, and then stained with DAPI as described in (V. Sergeeva, O. Kraevaya, E. Ershova, L. Kameneva, E. Malinovskaya, O. Dolgikh, M. Konkova, I. Voronov, A. Zhilenkov, N. Veiko, P. Troshin, S. Kutsev, S. Kostyuk, *Oxid. Med. Cell. Longev*., 2019, 4398695).

3.2.6. Reactive Oxygen Species (ROS) Assays

Cells were grown in 96-well plates, incubated with **FPA**, washed with PBS, and treated with 10 μM solution of H2DCFH-DA in PBS (Molecular Probes/Invitrogen, Carlsbad, USA) for 10 min. Cells were analyzed at 37°C using the total fluorescence assay in a plate reader at λex = 488 nm and λem = 528 nm (EnSpire Equipment, Turku, Finland). The reaction rate constant for the formation of DCF (k) was calculated using the dependence of the DCF signal intensity on the time of cell incubation with H2DCFH-DA. The data are presented as the k_i/k_0 ratio, where k_i and k_0 are the rate constants in the exposed and unexposed cells, respectively. The average value of the DCF signal for 16 wells ± standard deviation is reported.