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Novel water-soluble methanofullerenes $C_{60}[C_{13}H_{18}O_4(OH)_4]_6$ and $C_{60}[C_9H_{10}O_4(OH)_4]_6$: Promising uncouplers of respiration and phosphorylation

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ABSTRACT

Here, we report for the first time on two novel water-soluble polyol-methanofullerenes which uncouple respiration and oxidative phosphorylation. A cytofluorimetric JC-1-based ratiometric assay was used to quantify mitochondrial potential Ψm in *Yarrowia lipolytica* cells exposed to the fullerenes tested. Both methanofullerenes significantly downregulated Ψm, thereby decreasing the subset of cells with high mitochondrial potential compared with intact control cells. The Ψm-low subset of *Yarrowia lipolytica* cells resulted from methanofullerenes exposure preserved physiological cell size and granularity patterns.

Certain agents are known to accumulate in mitochondria and reduce both transmembrane mitochondrial potential Ψm and reactive oxygen species (ROS) production. Typically, these are protonophores—lipophilic substances which can accept protons and transfer them across the inner membrane of the mitochondria, bypassing the proton channel (e.g., dinitrophenol, fatty acids, derivatives of phenylhydrazone, salicylates, thyroxine, etc.). Consequently this results in uncoupling of respiration and phosphorylation in mitochondria. Once mitochondria are hyperpolarized, even a small decline (by 10–15%) of Ψm resulted in tenfold lowering of ROS production rate.[1] Therefore, substances which mildly dissipate mitochondrial transmembrane potential are promising agents to cope with pathological conditions accompanied by increased generation of free radicals (Parkinson's disease, Alzheimer's disease, aging, stroke, ischemia, etc.).[2–4]

A Skulachev's group already had created a number of such substances, the so-called «Skulachev ions», which are lipophilic cations, and successfully used them as mitochondrial-targeted antioxidants.[5–7] It is important that mitochondria can regulate intake of these substances, because the rate of their penetration into mitochondria depends on the magnitude of mitochondrial potential.

The subjects of our study are water-soluble fullerene derivatives. Fullerenes due to unique chemical structure have specific physicochemical and photophysical properties.[8,9] After getting rid of the poor solubility problem, the derivatives of fullerene attract attention as potential antioxidant, anticancer and antiviral agents.[10–14] In particular, the antioxidant properties of these molecules are intensively investigated. Fullerene C_{60} and its derivatives have been known to inactivate free radicals due to electron-acceptor properties.[9,15] Recently a novel mechanism of antioxidant action of fullerenes was postulated using computer simulation based on Density Functional Theory.[16] Namely, the fullerenes are capable of absorbing protons and after getting a positive charge can penetrate into mitochondria. Such a process will mildly uncouple respiration and phosphorylation, and decline the transmembrane mitochondrial potential. This, in turn, will reduce the production of ROS.

According to published data, water-soluble derivatives of fullerene C_{60} can penetrate through biological membranes of different cell types[17] and can be accumulated in mitochondria.[18] The effects caused by fullerene derivatives by the interaction with mitochondria are also described elsewhere. For instance, water-soluble hydroxylated fullerene derivatives inhibit some enzymes and oxidative phosphorylation in mitochondria[19] and can prevent

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mitochondrial dysfunction,[20] carboxyfullerenes are able to influence apoptosis,[21] etc.

In the present work, we studied the impact of two new water-soluble polyol-methanofullerenes $C_{60}[C_{13}H_{18}O_4(OH)_4]_6$—F1 and $C_{60}[C_9H_{10}O_4(OH)_4]_6$—F2 on the transmembrane mitochondrial potential. Analysis of individual mitochondrial responses in *Yarrowia lipolytica* yeast cells has been performed by the use of cytofluorimetric JC-1-based ratiometric assay.[22] *Yarrowia lipolytica* are obligate aerobes with a fully competent respiratory chain having all three points of energetic coupling. Therefore, *Yarrowia lipolytica* represent a valid surrogate model for the study of bioenergetic responses of human cells.[22,23]

Water-soluble methanofullerenes F1 and F2 were obtained by removing acetonide protection from the original chromatographically pure hexa-methanofullerene containing acetonide group.[24,25] Importantly, both methanofullerenes are water-soluble in a wide range of the pH (from 5 to 9) typical for intracellular compartments. In particular, mitochondria are known to be the negatively charged organelles in a cell (pH in the matrix of mitochondria is about 8, pH in the intermembrane space of mitochondria is about 4). The structures of fullerenes[24] are presented on Figure 1.

A cytofluorimetric JC-1-based ratiometric assay was used to quantify mitochondrial potential Ψm in *Yarrowia lipolytica* cells.[26] Normalized mitochondrial potential Ψm was expressed in arbitrary units as the ratio of red and green fluorescence signals FL2/FL1 generated by dimers and monomers of JC-1, respectively. The results of one representative experiment out of three independently performed experiments with similar outcomes are presented on Figure 2.

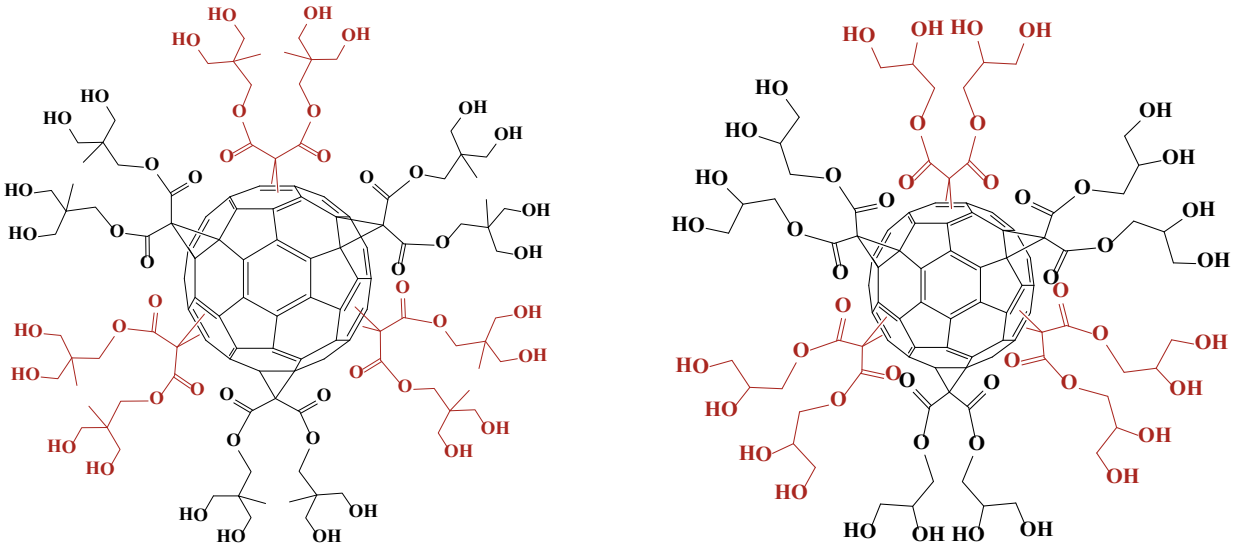
Viable cells were gated using forward scatter and side scatter (Fig. 2A, region R0). Unstained gated control cells were then plotted on SSC (Side Scatter) versus FL2/FL1 in order to set up quadrant statistics for normalized mitochondrial potential Ψm (Fig. 2B). Intact control cells stained with JC-1 were splitted into the major Ψm-high subset (upper right quadrant) and the minor Ψm-low subset (low right quadrant) (Fig. 2C). Treatment with uncoupling protonophore CCCP (carbonylcyanide-3-chlorophenylhydrazone) resulted in expected dissipation of Ψm (Fig. 2D), thereby providing proof of assay. Both F1 and F2 methanofullerenes downregulated Ψm in a concentration-dependent manner (Fig. 2E–H). Furthermore, the F2 induced more profound Ψm dissipation compared with F1 (Fig. 2F vs H).

Figure 3 summarizes the results of three independent experiments. Overall, F1 and F2 methanofullerenes significantly downregulated Ψm and decreased the subset of cells with high mitochondrial potential, that is, both methanofullerenes presented with uncoupler properties. The reason for less uncoupling effect of the F1 needs to be elucidated.

The Ψm-low subset of *Yarrowia lipolytica* cells generated by F1 and F2 methanofullerenes exposure (4 μM and 40 μM for 2 h) preserved physiological cell size and granularity patterns (Fig. 2, SSC axis reflects cell granularity, cell size pattern not shown). This phenomenon in *Yarrowia lipolytica* cells is likely explained by the presence of cyanide-insensitive terminal oxidase called «alternative oxidase». The alternative oxidase starts functioning during any disturbance of the main respiratory chain and helps cells to survive under extreme conditions.[28]

The use of substances uncoupling respiration and phosphorylation is limited by their potential toxicity because the decrease of mitochondrial potential violates the synthesis of ATP that is necessary to maintain the activities of a cell and host organism as a whole. In *Yarrowia lipolytica* yeast cells, the coexistence of the phosphorylating cytochrome chain and the oxidative pathway with alternative oxidase allows methanofullerenes to greatly reduce the transmembrane potential in mitochondria while preserving physiological size and granularity of cells. Mammalian cells (including human cells) are lacking of alternative oxidase, so that the magnitude of mitochondrial potential directly affects energy metabolism and functional status of mitochondria. Since the rate of penetration of cations into mitochondria is regulated by the proportional magnitude of mitochondrial potential, the toxicity of methanofullerenes in each case will depend on the correct choice of concentration.

In conclusion, our study proved the water-soluble methanofullerenes $C_{60}[C_{13}H_{18}O_4(OH)_4]_6$ and $C_{60}[C_9H_{10}O_4(OH)_4]_6$ as mitochondrial-targeted uncouplers. The results obtained are consistent with the in silico proposed novel mechanism of fullerene C_{60} antioxidant action,[16] and justify further studies of the methanofullerenes as promising mitochondrial-targeted biological response modifiers.



$C_{60}[C_{13}H_{18}O_4((OH)_4]_6$ - F1 $C_{60}[C_9H_{10}O_4((OH)_4]_6$ - F2

Figure 1. The structures of methanofullerenes.

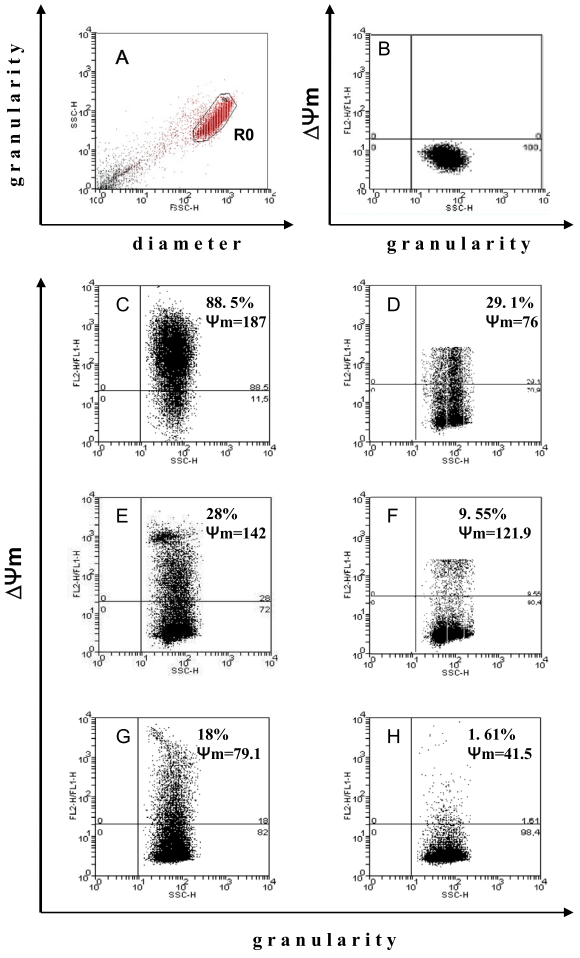


Figure 2. Cytofluorimetric quantification of transmembrane mitochondrial potential Ψm in *Yarrowia lipolytica*. (A) Gating of viable cells with physiological size and granularity (R0 region). (B) Unstained control cells gated on R0 and plotted on SSC versus FL2/FL1 ratio to set up quadrant statistics for Ψm. (C) Negative control: intact cells stained with JC-1; normalized Ψm expressed as FL2/FL1 ratio in arbitrary units. The gated R0 population is splitted into the Ψm-high subset (upper right quadrant) and the Ψm-low subset (low right quadrant) (D) Positive control: cells treated with CCCP (5 μM) and stained with JC-1 ($P < 0.001$ vs control). (E) Cells treated with fullerene F1 (4 μM) and stained with JC-1 ($P < 0.001$ vs control). (F) Cells treated with fullerene F1 (40 μM) and stained with JC-1 ($P < 0.001$ vs control). (G) Cells treated with fullerene F2 (4 μM) and stained with JC-1 ($P < 0.001$ vs control). (H) Cells treated with fullerene F2 (40 μM) and stained with JC-1 ($P < 0.001$ vs control).

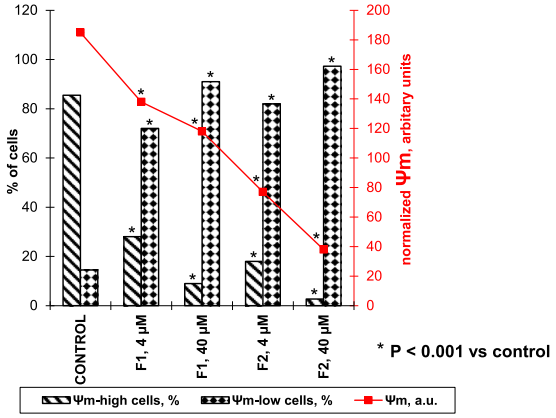


Figure 3. Fullerene derivatives F1 and F2 dissipate Ψm in the Ψm-high cell subset (right side Y axis) and decrease the portion of cells with high mitochondrial potential (left side Y axis) in a concentration-dependent manner.

Acknowledgements

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25. The original chromatographically pure hexa-methanofullerene with octahedral addition addends containing 12 acetonide groups were obtained by the Bingel–Hirsch reaction, their chemical structure correctly being proved using the complex of spectral methods. New chromatographically pure polyol methanofullerenes F1 and F2, containing 24 hydroxyl groups, are obtained by removing the acetonide protection using HCl.
F1—Mass-spectrum (MALDI): $[MH]^+$ 2556.30 $C_{138}H_{132}O_{48}$.. IR spectrum (KBr, *ν*, cm^{-1}): 527, 542, 598, 714, 826, 941, 1040, 1126, 1224, 1372, 1396, 1466, 1736 (C=O), 2880, 2932, 3359 (OH). δ_H (600 MHz; DMSO-d_6) 0.75 (br s, 36H, CH_3), 2.51 (br s, 24H, OH), 3.25 (br s, 24H, OCH_2), 4.13 (br s, 24H, CH_2), 4.56 (br s, 24H, CH_2). δ_C (150.926 MHz; DMSO-d_6) 17.63, 30.79, 33.59, 40.37, 45.44 (6C, C_{61}), 66.00, 69.10 (sp^3), 69.33, 98.00, 141.07 (sp^2), 145.65 (sp^2), 163.47 (C=O).
F2—Mass-spectrum (MALDI): $[MH]^+$ 2220.30 $C_{114}H_{84}O_{48}$. IR spectrum (KBr), *ν* (cm^{-1}): 472, 525, 565, 600, 708, 756, 840, 977, 1017, 1050, 1095, 1161, 1187, 1262, 1367, 1436, 1543, 1653, 1742 (C=O), 2926, 3423 (OH). δ_H (600 MHz; DMSO-d_6) 3.27–3.45 (br s), 3.73 (br s, 24H, OH), 4.14–4.19 (q), 4.68–4.70, 4.91–5.05 (m). δ_C (150.926 MHz; DMSO-d_6) 45.41–45.53 (6C, C_{61}), 59.27, 62.31, 63.04, 68.43, 68.94, 72.42 (sp^3), 140.62 (sp^2), 145.03 (sp^2), 162.72 (C=O).

26. The *Yarrowia lipolytica* strain (Y-3153 registration number) was obtained from the American yeasts collection ATCC. The study was performed on a synchronized yeast culture in a logarithmic growth phase. Mitochondrial membrane potential was measured using vital ratiometric JC-1 fluorochrome (Catalog No. 70011, Biotium, Inc. USA) on a FACSCalibur flow cytometer (BD, USA) according to basic experimental procedure[27] with our slight modifications. Protonophore uncoupler of oxidative phosphorylation carbonylcyanide-3-chlorophenylhydrazone—CCCP (Catalog No. ab141229, Abcam, UK) served for positive control.
Briefly, *Yarrowia lipolytica* suspended in 0.9% NaCl solution were dispensed at 10^6 cells/ml/tube in 5 ml Falcon test tubes (BD, Cat. No. 352235). The CCCP was added at final concentration of 5 μM (positive control), and the fullerenes at final concentrations of 4 μM and 40 μM. The samples were incubated for 2 h at 27 °C. 15 min prior to the end of incubation the JC-1 was given to cell suspensions at final concentration of 20 μM, and samples were incubated in the dark for 15 min at 27 °C. Immediately after the end of incubation samples were run on a flow cytometer at a flow rate no more than 1000 cells/sec using *CellQuest Pro* Software. In each variant of the experiment no less than 30,000 of cellular events were recorded. Primary listmode files were then analyzed by *Weasel 2.2.3* flow cytometry software. Normalized mitochondrial potential was expressed in arbitrary units as the ratio of red to green fluorescence signals generated by JC-1 dimers and monomers, respectively. Statistical data processing was performed by the Yates corrected Chi-square test for two dichotomous variables, that is, the Ψm-high and Ψm-low cell fractions (*STATISTICA 6.0* software), and the nonparametric Kolmogorov–Smirnov two-sample test (embedded in the *CellQuest Pro*) to compare Ψm.
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