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Cytoprotective Effect of Fullerene C_{60} Derivatives with Different Structures

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ABSTRACT

Transmembrane potential of mitochondria is a sensitive biomarker of metabolic activity of cells. Here, we studied mitochondrial potential Ψm in *Yarrowia lipolytica* yeast cells treated with two fullerene C_{60} derivatives: bis-nitroxide methanofullerene and 3-phospho-pentafullerene acid. Transmembrane mitochondrial potential was measured by vital ratiometric cationic fluorochrome JC-1 using flow cytometry. The fullerene C_{60} derivatives tested in a concentration of 10 μg/ml developed cytoprotective effect in the yeast cells challenged either with non-ionic detergent tween-80, or Tris-buffer, pH 9.0. Treatment with bis-nitroxide methanofullerene resulted in a 6-fold increase in proportion of cells with high Ψm, while 3-phospho-pentafullerene acid evoked a 1,5-fold increase in this subset compared to the stressed cells. Hence, both fullerene derivatives counteract Ψm dissipation in challenged cells.

Keywords: bis-nitroxide methanofullerenes,3-phospho-pentafullerene acid, cytoprotective effect, mitochondrial potential, flow cytometry.

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INTRODUCTION

Fullerenes and their derivatives with their unique combination of physical and chemical properties are attracting the researchers' close attention as potential drugs of new generation. The spectrum of pharmacological action of these compounds is extremely wide. Antioxidant, prooxidant and membrane effects of fullerenes depend on many factors: the chemical modification of a fullerene cluster, solubility, delivery mode, affinity for other cellular structures (1-3). This allows one to control the mechanism of biological action of fullerene derivatives.

Measuring transmembrane potential of mitochondria proved as appropriate method to study energy and functional state of a cell. Mitochondrial effects of different fullerene derivatives in eukaryotic cells are subject of growing interest. Because *Yarrowia lipolytica* yeast cells are obligate aerobes and have structure of mitochondrial complex I similar to that of mammalian cells (4), we selected them as test cells.

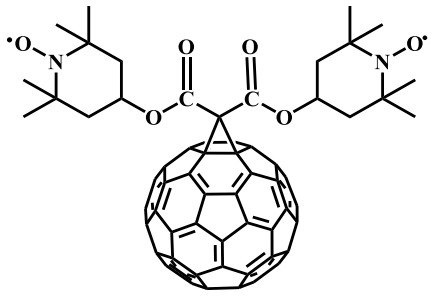
Earlier we established that water-soluble methanofullerenes C_{60} $[C_9H_{10}O_4$ $((OH)_4]_6$ and C_{60} $[C_{13}H_{18}O_4$ $((OH)_4]_6$ can dissociate respiration and oxidative phosphorylation in the mitochondria of *Yarrowia lipolytica* keeping cell size and granularity unchanged (5). This work aims to study the effects on mitochondrial potential of two other fullerene C_{60} derivatives: bis-nitroxide methanofullerene and 3-phospho-pentafullerene acid. Bis-nitroxide methanofullerene and 3-phospho-methanofullerene given in combination with anticancer agent cyclophosphamide cured up to 70% of mice inoculated with lymphocytic leukemia P-388 cells (6). The combined use of indicated C_{60} derivatives with xymedon improved therapeutic efficacy of burns treatment in rats (7). 3-phospho-pentafullerene acid is a novel derivative of fullerene, and its biological properties have not been studied. The effect of these agents on the mitochondrial potential was not investigated.

MATERIALS AND METHODS

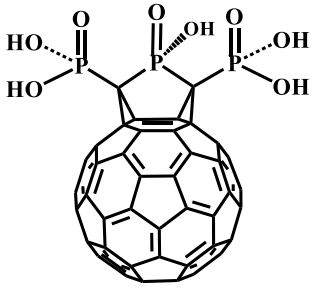
Bis-nitroxide methanofullerene (Ful1) was synthesized according to the procedure described in (6). 10 mg of the compound Ful1 was dissolved in 2 ml of chloroform and mixed with 1 ml of Tween-80 in 2 ml of chloroform. The mixture was thoroughly mixed, then chloroform was removed under vacuum for one hour at temperature of 70-80^{0}C. The residue was dissolved in 1 ml of water and used in the experiment.

The synthesis of 3-phospho- pentafullerene acid (Ful2) is described in (8). Ful2 was dissolved in 1M Tris buffer (pH 9.0).

The structures Ful1 and Ful2 are represented in Fig. 1.



Ful1



Ful2

$C_{61}[COOC_5H_5(CH_3)_4NO^{\cdot}]_2$ $(HO)_2P(O)C_{61}P(O)OHC_{62}P(O)(OH)_2$

Fig. 1. Structures of fullerene C_{60} derivatives

The *Yarrowia lipolytica* yeast strain was obtained from the American yeast collection ATCC (American typical culture collection) with number 34088. Registration number in the collection: Y-3153.

The cells were grown in Sabouraud's medium with agar (15 g/l) at 30^0C. After 24 hours cells was washed off from solid medium and flushed with the 0.9% solution of NaCl, followed by centrifugation for 5 minutes at 450 g. The precipitate suspended in 0.9% solution NaCl was diluted to 10^6 cells/ml and used in the experiment. The studies were performed with the cell culture in the phase of logarithmic growth.

Transmembrane mitochondrial potential was measured by vital ratiometric cationic fluorochrome JC-1 (Catalog No. 70011, *Biotium, Inc. USA*) using FACS Calibur flow cytometer (BD, USA), equipped with blue (488 nm) and red (635 nm) lasers. As a positive control, we used protonophoric uncoupler of oxidative phosphorylation carbonyl cyanide 3-chlorophenylhydrazone CCCP (Catalog No. ab141229, *Abcam, UK*).

The *Yarrowia lipolytica* cells, suspended in a 0.9% solution of NaCl (10^6 cells /ml) were placed in BD Falcon tubes (Cat. No. 352235) for flow cytometry. CCCP was added to the cell suspension at a final concentration of 5 μmol/L (positive control) while fullerenes were added at final concentrations of 10 μg/ml and 100 μg/ml. In addition, we set up respective solvent controls: the Tween 80 control (solvent for Ful1) and the Tris-buffer control, pH 9.0 (solvent for Ful2). In experimental and control samples, the final concentration of Tween-80 was 20 mg/ml, and that of Tris-buffer, pH 9.0 was 0.1 M. The samples were incubated for 2 hours at 27°C. Then fluorochrome JC-1 was added at a final concentration of 20 μmol/L. The cells were incubated with JC-1 at room temperature for 15 minutes in the dark, and immediately measured on a flow cytometer at a rate of 1000 cells/sec using CellQuest Pro® Software. In each experimental variant at least 30 000 cellular events were recorded. The normalized mitochondrial potential was expressed in arbitrary units as a ratio of red and green signals of fluorescence FL2/FL1, generated by dimers and monomers JC-1, respectively. The listmode files were analyzed by Weasel v2.2.3 software. The statistical data processing was performed by the Chi-square test with *Yates* correction to compare changes in percent of cell fractions, and the non-parametric Kolmogorov-Smirnov test to compare medians (STATISTICA 6.0 software package).

RESULTS AND DISCUSSION

The derivative Ful1 was dissolved in Tween-80 (pH 7.0-7.2), the derivative Ful2 was dissolved in Tris-buffer (pH 9.0). Both solvents in the tested concentrations exerted a stress effect on the *Yarrowia lipolytica* cells as judged by sharp decrease in proportion of cells with high Ψm concomitantly with Ψm downregulation (Fig. 2 and Fig. 3). The results of one representative experiment out of three independently performed experiments with similar outcomes are presented in Figure 2. Figure 3 summarizes the results of three independent experiments.

The mitochondrial transmembrane potential is a sensitive biomarker of metabolic activity of cells. One of the main features of cellular stress is the uncoupling of respiration and oxidative phosphorylation, the event drived by abnormal permeability of mitochondria membranes (10).

The non-ionic detergent Tween-80 is an effective tool of drug intracellular delivery for various organs (11, 12, 13). By acting on the membrane system in a cell, it can stimulate cellular respiration (14). Tween-80 as the surface-active substance (surfactant) has differential effects on microorganisms, depending on the concentration (15). The mechanism of action of the surfactant on the membranes of microorganisms involves several steps. First, adsorption of the molecules of surfactant on the membrane surface takes place, resulting in a change of its permeability with a subsequent dysfunctions. As a result, critical surfactant concentrations disrupt the integrity of a cell membrane and induce cell lysis. Concerning the alkali stress induced by Tris-buffer pH 9.0, it is known that *Yarrowia lipolytica* cells can effectively grow in media with alkaline pH values - up to 10.5 (16). Importantly, adaptation of *Yarrowia lipolytica* to grow at alkaline pH of the culture medium regardless of the energy source used by the cell is accompanied by the upregulation of alpha-ketoglutarate dehydrogenase gene expression (17). The data strongly indicate that alpha-ketoglutarate dehydrogenase complex is a primary site of ROS production in normally functioning mitochondria (18). In this connection, one may assume that an alkaline pH of culture medium primarily affects *Yarrowia lipolytica* mitochondrial functions.

Fullerenes Ful1 and Ful2 at concentration of 10 μg/ml significantly reduced the damaging effect of solvents (Fig. 2 and Fig. 3). Treatment with Ful1 resulted in a 6-fold increase in proportion of cells with high Ψm, while Ful2 evoked a 1,5-fold increase in those compared to the stressed solvent controls. Both fullerene

derivatives at concentration of 10 μg /ml attenuated the mitochondrial transmembrane potential drop (Fig. 3). Ful1 and Ful2 at concentration of 100 ug/ml did not counteract the drop in mitochondrial potential (P> 0.05) and less efficiently reduced the fraction of cells with high mitochondrial potential in the stressed cells (Fig. 3). Thus, both fullerene derivatives at a low concentration of 10 μg /ml display cytoprotective effect in *Yarrowia lipolytica*, challenged with alkali and Tween-80-induced stress. This effect is likely to be determined by the antioxidant activity of the agents tested. The lipophilic properties of fullerene C_{60} are well known. By computer simulation of molecular dynamics it has been shown that native fullerene C_{60} can penetrate into the membrane, and be accumulated in the lipid bilayer (19), while the functionalization of fullerene by polar groups leads to inconsistent results. In some cases, the functionalized fullerenes can cross the membrane (20), in the others, "stick" within the lipid bilayer (21). The membrane cell structures, including the mitochondrial membranes, are most sensitive to the effects of stress factors. One of the components of the rapid reaction to stress is upregulation of the non-enzymatic free radical oxidation of lipids (22, 23). In this case, the fullerene provides its antioxidant properties (24, 25) preventing or reducing the membrane damage related to free radical oxidation. Potential antioxidant activity of Ful1 and Ful2 is consistent with an electron-deficient sites on C_{60} spheroid surface and the presence of additional nitroxide radicals in Ful1 (Fig.1).

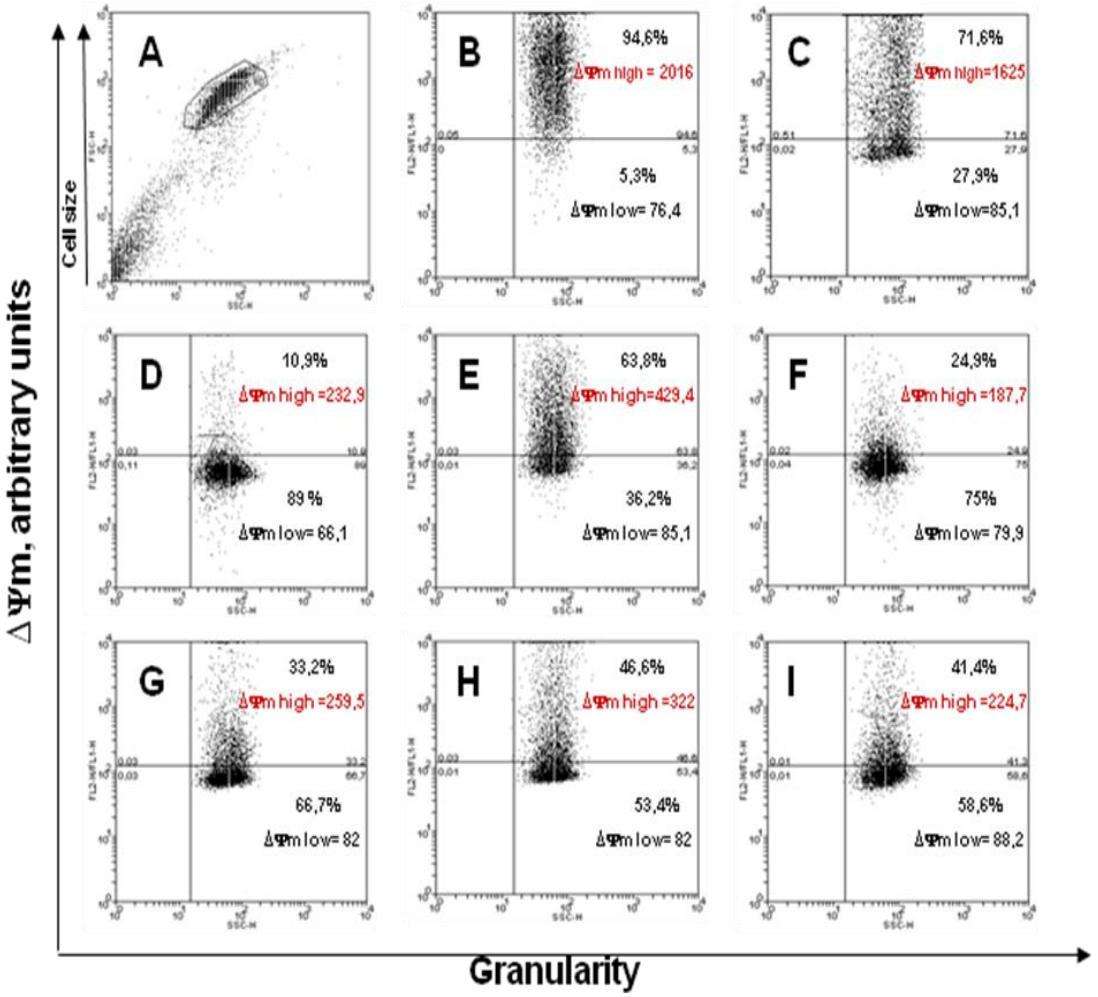


Fig. 2. Cytofluorimetric quantification of transmembrane mitochondrial potential Ψm in *Yarrowia lipolytica*.

(A) Gating of viable cells with physiological size and granularity (R0 region). (B) 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) (C) Positive control: cells treated with CCCP (5 μg/ml) and stained with JC-1 ($P < 0.001$ vs control). (D) Cells treated with tween-80 (20 mg/ml) and stained with JC-1 ($P < 0.001$ vs control). (E) Cells treated with fullerene Ful1 (10 μg /ml) and stained with JC-1 ($P < 0.001$ vs control). (F) Cells treated with fullerene Ful1 (100 μg /ml) and stained with JC-1 ($P < 0.001$ vs control). (G) Cells treated with TRIS (0,1 M) and stained with JC-1 ($P < 0.001$ vs control). (H) Cells treated with fullerene Ful2 (10 μg /ml) and stained with JC-1 ($P < 0.001$ vs control). (I) Cells treated with fullerene Ful2 (100 μg /ml) and stained with JC-1 ($P < 0.001$ vs control).

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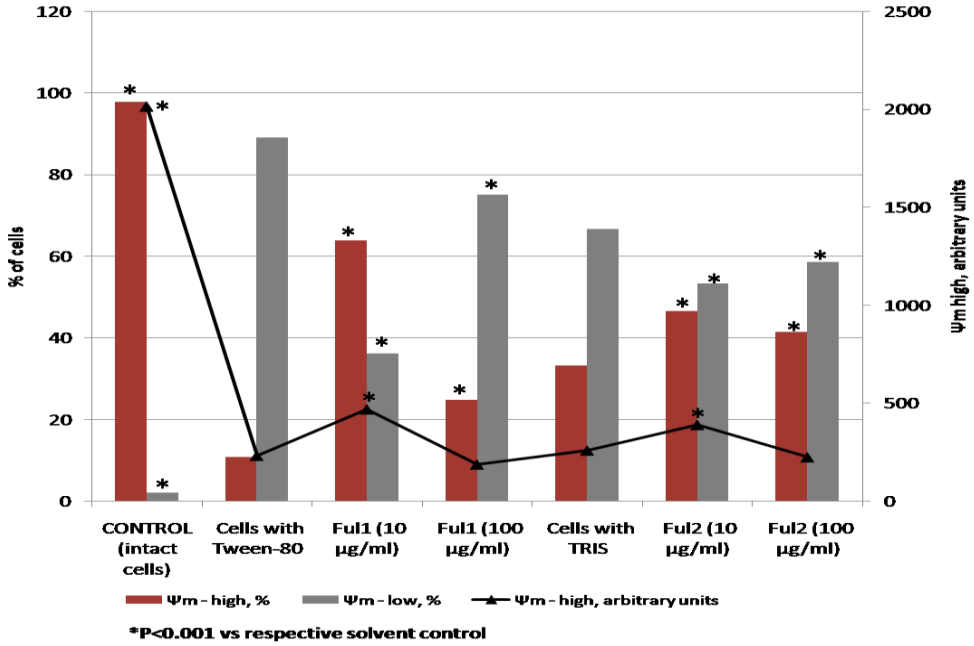

Fig. 3. Fullerene derivatives Ful1 and Ful2 increase the portion of cells with high mitochondrial potential (left side Y axis) and partially restore Ψm (right side Y axis) in stressed *Yarrowia lipolytica* cells. Data of three independent experiments are summarized. Differences between medians are tested using the Kolmogorov-Smirnov nonparametric statistics.

CONCLUSION

Bis-nitroxide methanofullerene derivative (10 µg/ml) protected *Yarrowia lipolytica* cells challenged with Tween-80, as judged by highly signifant upregulation of Ψm-high cell subset and attenuation of Ψm drop compared to unprotected cells. The cytoprotective effect of bis-nitroxide methanofullerene is consistent with the results obtained in the study of its biological activity *in vivo*.

The novel fullerene derivative - 3-phospho-pentafullerene acid (10 µg/ml) protected *Yarrowia lipolytica* cells challenged with alkali stress, as evidenced by 1,5-fold upregulation of Ψm-high cell subset and attenuation of Ψm drop compared to unprotected cells.

Both derivatives at higher concentration of 100 µg/ml lack mitochondrial-protective activity. The results obtained justify further studies of fullerene C_{60} derivatives as promising mitochondrial-targeted biological response modifiers.

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