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Water-soluble polyol-methanofullerenes as mitochondria-targeted antioxidants: Mechanism of action

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ABSTRACT

The mechanism of an antioxidant action of water-soluble polyol – methanofullerenes $C_{60}[C_9H_{10}O_4(OH)_4]_6$ and $C_{60}[C_{13}H_{18}O_4(OH)_4]_6$ as the mild uncouplers of an oxidative phosphorylation and respiration is postulated. According to this mechanism, hydroxyl group of methanofullerenols can be protonated under excess of protons in the intermembrane space of hyperpolarized mitochondria. Protonation of fullerene derivatives is confirmed by the decrease in their negative Zeta potential in the pH below 5.4. Heavily protonated methanofullerenols become positively charged and move into the mitochondrial matrix. As a consequence, the proton gradient is dissipated, which causes a decrease in mitochondrial transmembrane potential ($\Delta\Psi_m$) and reduction in ROS production.

Increased production of the reactive forms of oxygen (ROS) by mitochondria (oxidative stress) is linked to some dangerous pathological conditions (neurodegenerative diseases, cancer, aging, etc.). The oxidative stress depends on the magnitude of the mitochondrial transmembrane potential ($\Delta\Psi_m$): the higher $\Delta\Psi_m$ the more of the free radicals the mitochondria produce.[1] The damage associated with the increased production of ROS can be mitigated by the uncouplers of respiration and phosphorylation. The protonophores transport protons through the inner mitochondrial membrane that leads to a drop in the $\Delta\Psi_m$ and uncoupling of the respiration and phosphorylation. The mild uncouplers of oxidative phosphorylation – lipophilic cations capable to reversibly bind protons were patented by Skulachev et al.[2] Their ability to uncouple depends on the magnitude of the $\Delta\Psi_m$ in mitochondria. The mild uncouplers, which are able to do both: to dissipate the high $\Delta\Psi_m$ and to deactivate the already existing oxygen radicals, are of particular value.[3] In this regard, water-soluble methanofullerenols $C_{60}[C_9H_{10}O_4(OH)_4]_6$ and $C_{60}[C_{13}H_{18}O_4(OH)_4]_6$, which exhibit the properties of mitochondrial uncouplers[4] and preserve the antioxidant capacity of fullerene C_{60},[5] look very promising. In addition, both methanofullerenols are soluble at the physiological range of pH (4–8),[6] nontoxic[7,4] and retain lipophilic properties of the fullerene C_{60}, which is essential for the movement across cellular membranes. In this communication, we propose and validate a mechanism of uncoupling action of these fullerene derivatives.

Previously, the possible mechanism of an antioxidant action of C_{60} fullerene has been proposed on the basis of computer simulation by the method of the density functional theory (DFT). According to this mechanism, the protons can penetrate fullerene's surface and give the fulleren a positive charge, which allows C_{60} to cross the inner mitochondrial membrane. It is believed that these proton–confining fullerenes transfer the protons through the inner mitochondrial membrane to the mitochondrial matrix leading to a drop in the $\Delta\Psi_m$.[8] However, this mechanism of antioxidant action of unmodified fullerenes is still to be experimentally confirmed.

We had previously shown on the model of the yeast *Yarrowia lipolytica* (obligate aerobic eukaryotic cells) that water-soluble fullerene derivatives, $C_{60}[C_9H_{10}O_4(OH)_4]_6$ and $C_{60}[C_{13}H_{18}O_4(OH)_4]_6$, did reduce the $\Delta\Psi_m$, while preserving basic morphological parameters of cells (i.e. cell size and granularity), which allowed us to attribute these compounds to a potential mitochondria-targeted antioxidants.[4] In contrast to an unmodified fullerene, the methanofullerenols have the electronegative hydroxyl groups on their surface. We believe that these groups are responsible for protonophore properties of the investigated fullerene derivatives.

It is known that the fullerene surface has electron acceptor properties.[5] The oxygen of the hydroxyl groups in the structure of the tested methanofullerenols contains unshared pairs of

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electrons and carries a negative charge. The magnitude of the negative charge on the oxygen depends on the position of the hydroxyl group relative to the surface of the spheroid. These hydroxyl groups of the methanofullerenols $C_{60}[C_9H_{10}O_4(OH)_4]_6$ and $C_{60}[C_{13}H_{18}O_4(OH)_4]_6$ are distant from the surface of the spheroid and are not affected by the fullerene system of π-electrons. Therefore, the transfer of charge (unshared pairs of electrons) from hydroxyl to fullerene spheroid does not occur. We propose that raised up concentration of protons in the intermemebrane space of the hyperpolarized mitochondria stimulates substantial protonation the hydroxyl groups of methanofullerenols with the formation of oxonium cations (Fig. 1). This process is similar to protonation of the hydroxyl groups in aliphatic alcohols.[9] After receiving a positive charge, the protonated methanofullerenols move into the mitochondrial matrix and loose protons in its weakly alkaline medium (pH 8). This leads to the proton gradient dissipation, which in turn decreases the $\Delta\Psi_m$ and reduces ROS production.

The ability of water-soluble methanofullerenols to accept protons was evaluated via decrease of their negative Zeta potential in an acidic medium with the use of a dynamic light scattering (DLS) technique on a Zetasizer Nano ZS analyzer (Malvern Instruments) according to manufacturer's recommendations.

Methanofullerenols $C_{60}[C_9H_{10}O_4(OH)_4]_6$ and $C_{60}[C_{13}H_{18}O_4(OH)_4]_6$ were obtained by removing acetonide protection from the original chromatographically pure hexa- methanofullerene containing acetonide group.[6]

The Zeta potential (ζ) was measured in 0.05 M HEPES buffer (pH range 2–7) at a concentration of methanofullerenols 0.5 mg/mL at room temperature. The Table 1 presents average values of Zeta potential (ζ) of methanofullerenols $C_{60}[C_9H_{10}O_4(OH)_4]_6$ and $C_{60}[C_{13}H_{18}O_4(OH)_4]_6$, measured at the pH range 2–7. Data are presented as mean ± SD (n = 3). Representative curves of ζ of methanofullerenols at pH 2 are shown in the Fig. 2.

Table 1
Zeta potential (ζ) of fullerene derivatives (0.5 mg/mL) at different pH values (mean ± SD).

Fullerene derivatives	pH	ζ, mV
$C_{60}[C_9H_{10}O_4(OH)_4]_6$	pH 7	−27,1 ± 4,5
	pH 5,4	−28,0 ± 5,6
	pH 4	−8,8 ± 3,8
	pH 2	−5,4 ± 7,5
$C_{60}[C_{13}H_{18}O_4(OH)_4]_6$	pH 7	−21,4 ± 4,7
	pH 5,4	−21,3 ± 4,2
	pH 4	−7,1 ± 4,4
	pH 2	5,7 ± 9,5
$C_{60}(OH)_{24}$	pH 7	−46,4 ± 18,8
	pH 5,4	−47,9 ± 19,4
	pH 4	−52,5 ± 9,8
	pH 2	−46,2 ± 9,73

The reduction of pH from 7 to 5.4 did not change the Zeta potential of methanofullerenols. At the pH value of 4, in both methanofullerenols initial negative Zeta potential decreased nearly three times due to protonation (Table 1). At pH 2 the value of ζ is decreased for $C_{60}[C_9H_{10}O_4(OH)_4]_6$ to −5,4 mV, while $C_{60}[C_{13}H_{18}O_4(OH)_4]_6$ acquired a positive charge of ζ = 5.7 mV (Table 1 and Fig. 2). Evidently in the experimental conditions methanofullerene $C_{60}[C_{13}H_{18}O_4(OH)_4]_6$ is protonated better. The more effective protonation of this derivative is most likely due to the proximity an additional methyl group $-CH_3$ to each hydroxyl group (Fig. 1). The electron donor methyl group increases the negative charge of the oxygen in the hydroxyl group, thereby stimulating protonation and formation of oxonium cation.[9]

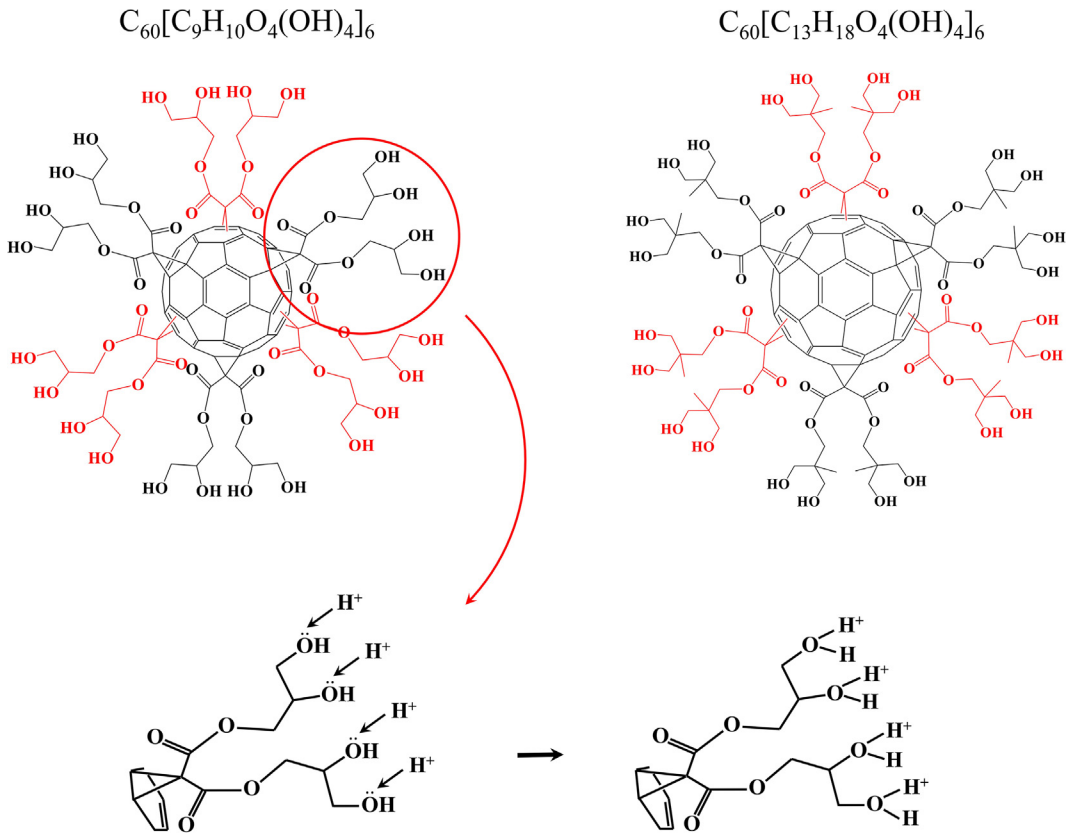

Fig. 1. Protonation of methanofullerenols.