Fullerenol - a new nanopharmaceutic?

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Abbreviations:
DOX – doxorubicin; DPPH - 2,2-diphenyl-1-picrylhydrazyl; ESR- electron spin resonance; GR – glutathione reductase; GSH-Px - glutathione peroxidase; γ-GT - γ-glutamyltransferase; HCT – hematocrit; Hgb - hemoglobin; LT_{50} – lethal time 50, time need for death of 50% of animals; NO - nitrogen oxide; ROS - reactive oxygen species; SNP - sodium nitroprusside; SOD - superoxide dismutase

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SUMMARY

Chemical modification of fullerenes to hydrosoluble cluster molecules made fullerenes interesting for biological investigation. Among them, polycarbonated and polyhydroxylated fullerene C_{60} derivatives showed the most interesting biological activities. In this paper, we present the most important recent results of in vitro and in vivo biological studies with fullerenol $C_{60}(OH)_{24}$. Fullerenol $C_{60}(OH)_{24}$ was strong antioxidant: it reacted with superoxide anion radical, hydroxy radical and nitrous oxide radical in chemical and biological systems. Fullerenol $C_{60}(OH)_{24}$ did not inhibit human breast cancer cell growth at concentrations from 0.8 to 3.45 μM, but strongly modulated cytotoxic effects of doxorubicin and cis-platinum after 24 and 48 hours of treatment. Radioprotective effects of fullerenol $C_{60}(OH)_{24}$ were shown in different in vitro and in vivo models. Fullerenol $C_{60}(OH)_{24}$ (100 mg/kg) protected rat heart from doxorubicin toxicity. Biodistribution studies of fulelrenol were also investigated. Accumulating data from the literature and from our studies suggest that fullerenol, as a nanoparticle might be a new promising pharmaceutical in the near future.

Key words: *Fulleren; Antioxidants; Doxorubicin; Drug Toxicity; Protective Agents; Radiation-Protective Agents*

INTRODUCTION

Fullerenes are the third pure crystal form of carbon in addition to diamond and graphite. In September 1985, Robert F. Curl, Jr., Richard E. Smalley, and Sir Harold W. Kroto discovered fullerene C_{60} during laser spectroscopy experiments at Rice University (1). Fullerenes were named after Richard Buckminster Fuller. Carbon atoms in fullerenes can be organized in spherical (C_{60}), ellipsoid (C_{70}), or cylindrical (carbon nanotubes) form Figure 1. Fullerene C_{60} possesses geometry of truncated icosahedrons fulerene-60-I_h. Diameter of the molecule C_{60} is 0.710±0.007 nm. The outer and inner diameters of π electron cloud can be estimated as 0.340 nm and 0.350 nm respectively (2). Pure fullerenes are black crystals or powder. Fullerene C_{60} is practically insoluble in water and proton-acceptor solvents but it is soluble in halogen and alkyl-substituted benzene. Using combinations of nucleophilic and electrophilic additions, cycloadditions and radical additions, it is possible to covalently bond any class of organic compounds to a fullerene core. These structural and electronic characteristics of fullerene C_{60} provide possibility for conducting various types of chemical transformations (2-4) that resulted in many water-soluble fullerenes with wide variety of biological activity.

The chemical modification of fullerene C_{60} molecule by attachment of hydroxyl groups was an easy and straightforward method to synthesis water-soluble fullerenes, named fullerols or fullerenols ($C_{60}(OH)n$) (5-15). Structure of fullerenol with 24 hydroxyl groups, $C_{60}(OH)_{24,}$ is shown in Figure 2.

Fullerenol $C_{60}(OH)_{24}$ has diameter of around 1 nm with symmetrically arranged hydroxyl groups on the C_{60} sphere (3). Fullerenol is a dark brown amorphous substance, soluble in water and DMSO. Fullerenol dissolved in water (pH interval from 3.5 to 9.8), at a range of concentrations (10^{-3} mol/dm^3 to 5 10^{-3} mol/dm^3) forms polyanion nano aggregates, whose size is mostly between 20 nm and 100 nm. The presence of aggregates larger than 400 nm is biologically insignificant (16,17). Fullerenol may react rapidly and irreversibly with a variety of metal salts under ambient aqueous conditions to produce insoluble metal-hydroxyfullerene cross-linked polymers (M-fullerenol). Interaction of fullerenols with bio-metal is important with regard to fullerene-based pharmaceutical agents and waste treatment as well (18,19).

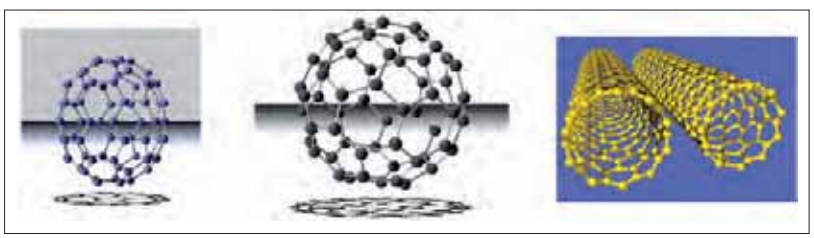

Figure 1. Fullerene C_{60}, C_{70} and carbon nanotube

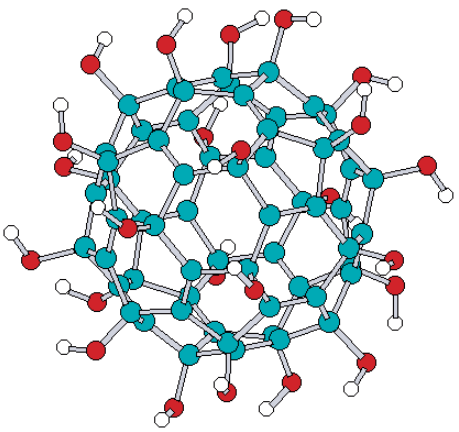

Figure 2. Fullerenol $C_{60}(OH)_{24}$

Studies on biological properties of fullerene C_{60} and its derivatives started a decade ago and now are important area of trandisciplinary research. The most important characteristics of fullerenols regarding mechanisms of their biological activity are photosensitizing property and free radical scavenging activity. Antioxidative and free radical scavenging activity of fullerenols are widely exploited properties in biomedical studies (20-22). Antiproliferative and cytotoxic effects of various water-soluble fullerenol $C_{60}(OH)n$ have been observed in different experimental models (23-26). Fullerenols may produce both singlet oxygen and superoxide under visible and UV light (27). On photoexcitation, fullerenol can generate free radical species (ROS) and induce significant oxidation of membrane lipids and proteins that can be prevented by endogenous or natural antioxidants (27). Yamawaki and Iwai (28) evaluated direct effects of nanomaterial on endothelial toxicity by treatment of human umbilical vein endothelial cells (ECs) with fullerenol $C_{60}(OH)_{24}$ for 24 h. They found that only

fullerenol at dose of 100 μg/ml induced cytotoxic injury (i.e. cytosolic vacuole formation and increased level of lactate dehydrogenase), cell growth inhibition or cell death by activation of ubiqutin-autophagy cell death pathway. However, chronic treatment with low-dose $C_{60}(OH)_{24}$ (10 μg/ml /ml for 8 days) only inhibited cell attachment and delayed EC growth. Dugan et al showed that fullerenols $C_{60}(OH)_{12}$ and $C_{60}(OH)_{18\text{-}20}$ might reduce excitotoxic and apoptotic death of cultured cortical neurons (29). Huang et al. (30) evaluated the effect carboxylate C_{60} (fullerenol-1) on amyloid β peptide-induced cytosolic free calcium *in vitro*. They found that fullerenol-1 reduced the amyloid β peptide-induced $[Ca^{2+}]$ in PC 12 cells at a range nanomolar and picomolar concentrations.

In this paper, we present the most important recent results of *in vitro* and *in vivo* biological studies with fullerenol $C_{60}(OH)_{24}$.

ANTIOXIDATIVE PROPERTIES OF FULLERENOL $C_{60}(OH)_{24}$

The antioxidative activity of fullerenol $C_{60}(OH)_{24}$ was tested against stable (DPPH) and unstable free radicals (·OH) by ESR spectrometry. The addition of fullerenol (0.18 mmol/dm^3 to 0.88 mmol/dm^3) to the reaction systems resulted in a dose-dependent inhibition of the ESR signal intensity of both DPPH free radical and DMPO-OH spin adduct. Higher fullerenol concentrations were more effective in reducing the ESR signal of DMPO-OH spin adducts than DPPH radical. There are two possible mechanisms of antioxidative activity of fullerenol. The first possibility is radical-addition reaction of 2n ·OH radicals to remaining olefinic double bonds of fullerenol core to yield $C_{60}(OH)_{24}+2n$OH (n=1-12) The second proposed mechanism is the possibility of hydroxyl radical to abstract a hydrogen from fullerenol, including the formation of relatively stable fullerenol radical $C_{60}(OH)_{23}O$ (31). These mechanisms are not mutually excluded. Superoxide radicals, generated through enzymatic activity of xanthine oxidase, reduce cytochrome c. Application of fullerenol at nanomolar and micromolar concentrations into xanthine/xanthine oxidase system decreased a rate of cytochrome c reduction by 5 % to 40 % compared to the control (32). Fullerenol hypothetical mechanism of action with superoxide anion radical is presented in Figure 3.

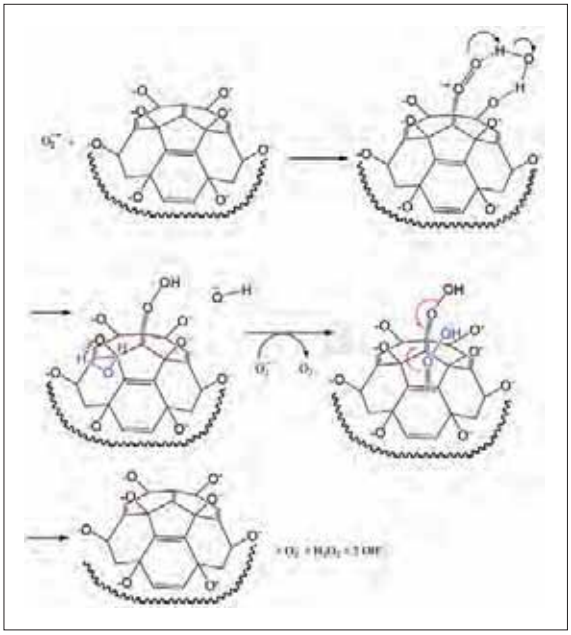

Figure 3. Fullerenol hypothetical mechanism of action with superoxide anion radical

NO-scavenging activity of fullerenol was estimated in the system with sodium nitroprusside (SNP) as NO donor. Coincubation of SNP (1, 2 or 5 mmol/dm^3) with fullerenol (0.05 or 0.1 mmol/dm^3) resulted in a dose-dependent decrease of the nitrite level, in comparison to the control without fullerenol. However, the percentage of the nitrite decrease was the same disregarding of SNP concentration in the solution and average ED_{50} was 10^{-4} mol/dm^3 of fullerenol (32).

EFFECT OF FULLERENOL ON THE GROWTH OF TUMOR CELL LINES

Effects of fullerenol $C_{60}(OH)_{24}$ on cell proliferation were tested in the system of antitumor drug-induced cytotoxicity against human breast cancer cells T47, MCF-7 and MDA-MB-231 (33). Doxorubicin, cisplatin, paclitaxel, and thiazofurin were used at their IC_{50} concentrations in different schedules of fullerenol and antitumor drugs. Fullerenol alone, at a range of nanomolar concentrations, induced weak (≤20%), but concentration dependent cell growth inhibition at different time points (from 2 h to 72 h). However, fullerenol strongly modulated cytotoxic effects of antitumor drugs and the rate inhibition depended on fullerenol concentration, type of antitumor drug and cell line. Protective effect of fullerenol was found to be more pronounced against doxorubicin and cisplatin, antitumor drugs whose general toxicity is related to free radical formation. Fullerenol also decreased toxicity of tizofurin but only for the period of 24 h post treatment, while toxicity of fullerenol-taxol combination, at all time points, was very low against both MCF-7 and MDA-MB-231 cells. Reduced toxicity of taxol and tiazofurin indicated that fullerenol protective effects might be mediated by mechanisms other than antioxidative activity (33).

In combination with doxorubicin, fullerenol inhibited doxorubicin cytotoxicity against human breast cell lines by more than 50% for most concentrations at each time point (34). The best cell protection was obtained when fullerenol was added to culture one hour prior to doxorubicin, but there was no significant difference if it was administered simultaneously with doxorubicin or one hour later. Generally, protective effect of fullerenol in the model of doxorubicin-induced cytotoxicity on human breast cancer cell lines could be explained by fullerenol localization close to the mitochondria (35), its free radical scavenger activity, and by the ability to act as an artificial electron acceptor leading to inhibition of monooxygenase activity (34).

RADIOPROTECTIVE EFFECTS OF FULLERENOL *IN VITRO* AND *IN VIVO*

We investigated radioprotective effects of fullerenol *in vitro* and *in vivo* models. Human erythroleukemia cells K562 pretreated with fullerenol (10nM) were x-ray irradiated (24Gy, IC_{50}/24h). After 24h, total cell number, changes in cell morphology, reproductive ability, and antioxidative enzyme status were evaluated (36,37). Irradiation of K562 with 24Gy decreased cell number and significantly changed cell morphology - larger cell area and numerous vacuoles throughout the cytoplasm were noticed; colony formation ability was decreased and activity of three antioxidative enzyme was increased. Fullerenol pretreatment significantly changed all parameters in irradiated K562 cells: total cell number increased, cell area was smaller and colony-forming activity was partially prevented. Administration of fullerenol before irradiation induced significant increase in the activities of both superoxide dismutase (SOD) and glutathion peroxidase (GSH-Px) that was followed by preserved cell number and colony forming capacity. Increased level of total SOD were

attenuated 24 and 48 hours after irradiation in fullerenol pretreated cells that was in accordance with fullerenol ROS scavenging activity (37, 38). Increased SOD activity in fullerenol pretreated K562 cells could resulted from fullerenol $NO^{\cdot}$ scavenging activity: scavenging $NO^{\cdot}$ prevents formation of peroxynitrite anion, concomitantly increasing the $O_2^{\cdot -}$ concentration and increase of SOD activity because of superoxide excess. The fullerenol pretreatment significantly reduced the level of γ-glutamyl transferase (γ-GT) in irradiated cells indicating important role of fullerenol in maintenance of redox homeostasis (36). GSH-Px level was significantly elevated in fullerenol pretreated cells 1h after irradiation with 24 Gy. Consecutive activation of GSH-Px in fullerenol-pretreated cells may be due to increased SOD activity as well as glutathione accumulation in cell cytosol, which inhibits the GSH-Px enzyme active center (39).

It is possible to propose that fullerenol pretreatment prevented deleterious effects of ROS by increasing the antioxidant enzyme activities of irradiated cells.

After *in vitro* investigations, we extended studies of radioprotective efficiency of fullerenol to whole-body irradiated mice and rats. The mice were pretreated with 10 mg/kg and 100 mg/kg of fullerenol I.P. 30 minutes before irradiation and than irradiated with 6, 7 or 8 Gy. Mice protected with 100 mg/kg of fullerenol had better body mass gain than those pretreated with 10 mg/kg of fullerenol. In addition, 100 mg/kg of fullerenol significantly increased of LT_{50} of mice irradiated with 8 Gy, compared to both control group and group pretreated with 10 mg/kg of fullerenol. These results clearly showed radioprotective effects of higher fullerenol dose (40,41).

Protective efficacy of fullerenol (10 and 100 was compared mg/kg, IP with amifostine (300 mg/kg, I.P.) in irradiated Wistar rats. The results of hematological investigation showed that fullerenol better then amifostine prevented radiation induced reduction of the granulocytes and lymphocytes particularly in the first 7 days after irradiation. Similar results were observed in thrombocytes count, but increase in the number of erythrocytes was observed in all groups of rats. Histopathological changes in tissues of protected animals were significantly less pronounced 30 days after irradiation compared to unprotected rats. Fullerenol better than amifostine protected spleen, intestine and lung, while amifostine gave better radioprotection of the heart, liver and kidney (40). Tissue damage by X-ray irradiation in both unprotected and protected animals was less expressed after 7 days compared to 30 days after irradiation, implying aggregation of radiation-induced damaging processes in subsequent post radiation course. In order to evaluate the general radioprotective efficacy of fullerenol and amifostine pretreatment, rats were irradiated with an absolutely lethal dose (8 Gy) of X-rays. Thirty days after irradiation, 60% of rats protected with 100 mg/kg of fullerenol had longer survival, while only 10% survived if protected by 10 mg/kg of fullerenol. Besides, in animals pretreated with 100 mg/kg of fullerenol body mass gain had the same trend as in the control group and was more prominent in comparison with rats pretreated with amifostine (41).

INFLUENCE OF FULLERENOL ON DOXORUBICIN-INDUCED CARDIOTOXICITY IN RATS

Acute toxicity of fullerenol was investigated on Wistar rats. LD_{50} was 349 mg/g (95% confidence limits) after I.P: application. We also examined the influence of fullerenol (50, 100 and 200 mg/kg, I.P. 30 minutes before doxorubicin, DOX) as a potential antioxidative protector on doxorubicin-induced toxicity in adult Wistar rats, after single dose of DOX (8 mg/kg, I.V. Heart function, tissue damage, different blood count parameters and antioxidative enzymes activity were followed 2 and 7 days after treatment.

Reflex bradycardia appeared later in DOX treated rats compared to the control group suggesting myocardial damage. Rats pretreated with fullerenol had electrocardiogram parameters comparable to the control**.** Histopathological analysis of myocardial tissue confirmed the heart damage (vacuolization of myocardial cells) after DOX administration. In hearts of fullerenol-protected animals, there were intracellular edema and discrete myofibrillar lysis, and intensity of changes depended on fulleronol dose. All morphological changes were reversible (42,43).

Hemoglobin (Hgb) and hematocrit (HCT) values in rats treated with fullerenol only, were comparable with those in the control group. However, on seventh day after application of DOX and fullerenol combination, statistically significant depletion of Hgb and HCT values were found compared to both control and fullerenol treated group (42).

DISTRIBUTION STUDY OF $^{99M}TC(CO_3)_3(H_2O)_3$-$C_{60}(OH)_{22}$

Several radiopharamaceuticals containing fullerenol and 99m technetium (^{99m}Tc) were successfully synthesized and their biodistribution was investigated on Wistar rats. ^{99m}Tc-carbonil – fullerenol was less attached to proteins compared to its precursor molecule, $^{99m}Tc(CO)_3(H_2O)_3]^+$. Liposolubility is one of the most important characteristics of the molecule that can help in prediction of molecule tissue accumulation and in its elimination pathway (44). Distribution of the complex between organic and nonorganic phase indicated that the whole amount of fullerenol radiopharmaceutical, $^{99m}Tc(CO_3)_3(H_2O)_3]$-$C_{60}(OH)_{22}$, existed in aqueous phase (i.e. distrubution coefficient was approximately zero) and pH values did not influence extractability. Distribution of precursor molecule, $^{99m}Tc(CO)_3(H_2O)_3]^+$ was different compared to $^{99m}Tc(CO_3)_3(H_2O)_3]$-$C_{60}(OH)_{22}$. Precursor molecule was found in liver in 20.1 % compared to 14.1% for fullerenol radiopharmaceutical. Both compounds accumulated in kidneys and intestine that indicated the routes of elimination (44).

In conclusion, our investigations of biological effects of fullerenol confirmed its strong antioxidative activity in various chemical and biological systems. Special interest for further investigation with fullerenol will be focused on fullerenol radioprotective activity in animal models, and on cardioprotective effects in doxorubicin-induced cardiotoxicity. Biodistribution studies of fullerenol derivatives will help to understand its pharmacokinetic and pharmacodynamic behavior. Accumulating data suggest that fullerenol as a nanoparticle might be a new promising pharmaceutical in the near future.

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Conflict of interest

We declare no conflicts of interest.

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