WATER-SOLUBLE FULLERENE $C_{60}(OH)_{24}$ MODULATES GROWTH AND PROLIFERATION OF K562 HUMAN ERYTHROLEUKEMIA CELL LINE

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Abstract: - Fullerenes and their water-soluble derivatives have recently become a topic of interest in biomedicine. Here we report on growth and proliferation activity of fullerenol $C_{60}(OH)_{24}$ on K562 human erythroleukemia cells. Capability of K562 cells to synthesize a DNA by incorporation of radiolabeled thymidine, their mitotic activity and cell cycle distribution has been evaluated 3, 24 and 48 hours after fullerenol treatment. Viability of fullerenol treated K562 cells was up to 5% lower in all samples in comparison to control, regardless $C_{60}(OH)_{24}$ concentration or incubation period of cells with fullerenol. No significant differences were found in morphological appearance between control and fullerenol treated cells analyzed by light microscopy. A mitotic index of fullerenol treated cells was decreased in comparison to control regardless the period of cell incubation with fullerenol. Synthesis of DNA of fullerene treated K562 cells was inhibited, but only at the highest fullerenol concentration. The cell cycle distribution pattern of K562 cells treated with fullerenol at concentration of 0.041umol/l was comparable to that of the control but increasing number of cells in G2M phase was observed during the treatment period.
Water-soluble [C_{60}] fullerene derivative, $C_{60}(OH)_{24}$, modulates cell cycle of the K562 cell influencing both synthetic and mitotic cell cycle phase

Key-Words: - Fullerene; Cell line; Cell cycle; DNA synthesis; Mitosis.

1 Introduction

Fullerenes are relatively new group of compounds and represent a class of sphere-shaped molecules made exclusively of carbon atoms. Since they were discovered in 1985 [1], many aspects of both fullerene [C_{60}] and its analogous have been intensively studied to reveal their physical and chemical reactivity [2-7]. However, mainly due to high hydrophobicity and poor solubility of the compounds in aqueous media, their biological activities have not been fully investigated.
The chemical modification of fullerene [C_{60}] molecule toward water-soluble adducts resulted in compounds exhibiting a variety of biological activities [2,3,6,8-10]. [C_{60}] fullerene derivatives cleave double-strand DNA upon visible light irradiation [7,9,11]. Cytotoxicity to various tumor cell lines [9-10] and distinct inhibiting activities against various enzymes (some proteases and reverse transcriptases) were also reported [12-14]. Anti-apoptotic, anti-bacterial and virocidal activity of [C_{60}] derivative, as well as neuroprotective effects of polyhydroxylated [C_{60}] fullerene derivatives and polar carboxy acid [C_{60}] derivatives were reported [15-20]. Several *in vitro* and *in vivo* studies have shown that fullerenols, polyhydroxylated [C_{60}] fullerene derivatives, are free radical scavengers and potent antioxidative agents in biological systems [15,17,21-24]. Antiproliferative effect of fullerenols, as well as their enzyme inhibition activity, can be attributed to their antioxidative property [3,15,25-28].
Our preliminary results showed that fullerenol $C_{60}(OH)_{24}$, continuously present in culture up to 48

hours, at nanomole concentrations, induced low but transient growth inhibition of several human tumor cell lines [10]. A mechanism of growth-inhibitory activity of the fullerenol might be explained by inhibition of mitotic spindle formation [34].
The aim of this study was to investigate the influence of nanomolar concentrations of fullerenol $C_{60}(OH)_{24}$ on growth and proliferation activity of K562 cells estimating their synthetic and mitotic cell cycle phase.

2 Materials and Methods

2.1 Cell line

The human erythroleukemia cell line K562 grows in RPMI 1640 medium (Sigma) supplemented with 2 mM glutamine, 10% FCS (fetal calf serum, NIVNS) and antibiotics: 100 IU/ml of penicillin and 100 μg/ml of streptomycin (ICN). The cells were sub-cultured twice a week at the concentration of 50,000-100,000 cell/ml. Cells were cultured in flasks (Costar, 25 cm^2) and incubated at 37 ^{0}C in the 100% humidity atmosphere and 5% of CO_2.

2.2 Fullerene derivative

Polyhydroxylated fullerene derivative, fullerenol $C_{60}(OH)_{24}$, was synthesized and characterized in the laboratories of the Chemistry Department, Faculty of Sciences, University of Novi Sad. The fullerenol $C_{60}(OH)_{24}$ was yielded by complete substitution of bromine atoms from $C_{60}Br_{24}$ with hydroxyl groups [4-5].
The fullerenol was dissolved in 1 ml of distilled water ("stock" solution). Dissolved substance was further diluted with RPMI 1640 medium to set an array of "working" solutions of proper concentration, which were added in volume of 10 μl/culture to achieve final concentrations of 0.04, 0.18 and 0.71 μmol/l.

2.3 Cell treatment

Samples of control and treated K562 cells were seeded in 10ml tubes (10^5 cells/ 4 ml of RPMI 1640 medium with 10% FCS) for mitotic index determination or in 96-well microtitar plates ($1x10^5$ cells /180 μl RPMI 1640 medium with 10 % of FCS) for DNA synthesis evaluation. Cells were incubated at 37 ^{0}C in the 100% humidity atmosphere and 5% of CO_2, for 3, 24 and 48h.Three different concentrations of the fulerenol were added into culture medium: 0.04, 0.18 and 0.71 μmol/l.

2.4 Viability and cell density

Population cell density (a number of cells per volume unit) and percentage of viable cells were performed by DET (dye exclusion test) as previously described [35].

2.5 Determination of mitotic index

Slide smears (cytocentrifugue preparations) were prepared from both control and fullerenol treated cells ($3x10^4$ cells / 100 μl from each sample in duplicate) on the cytospin centrifugue (Shandon). Staining of dried specimens were performed by May Grunwald - Giemsa method (MGG). The slides were observed under the light microscope with 400x magnification. At least 500 cells were counted on each specimen, within five different view fields per specimen, and at least two specimens were observed for each sample.
Cells were counted in the mitosis (all fases) and mitotic index (MI) was estimated as follows:
MI = [No. of cells in mitosis] / [total No. of cells] x100

2.6 ^{3}H-Thymidine incorporation assay

Aqueous solution of ^{3}H-Thymidine (^{3}H-Thy) (specific activity of 185 GBq/mmol, Amersham) was diluted with RPMI 1640 culture medium in ratio 1:20. Two hours before the cells harvesting, 20 μl of diluted ^{3}H-Thy was added in every well of the plates (1 μCi / well). After the incubation, the K562 cells were harvested on filter-paper disks (Scatron Titertek), dried overnight at 40^{0}C, transferred into vials with 5 ml of scintillation liquid and counted in the liquid scintillation beta-counter (TM Analytic BetaTrac 6895). Radioactivity of the samples was expressed as DPM (Desintegrations Per Minute) and is proportional to the amount of ^{3}H-Thy incorporated into DNA of cultured cells. Results were presented as a proliferation index i.e. DPM ratio of treated and control samples.

2.7 Cell cycle analysis by flow cytometry

Cell suspension ($1x10^6$) was treated with 1ml of 0.1% Triton-x-100 for five minutes at 4^{0}C, followed by 20 μl RNA-ase (1mg/ml) in PBS and stained with propidium iodide (PI). The cellular DNA content was measured using FACScan flow cytometer equipped with CellQuest software (Becton Dickinson).

3 Results

A viability of fullerene treated K562 cells was comparable to control (96.3-98.1) and remained high regardless of fullerene concentration or incubation period of cells with fullerene (92.6 - 99.1). Small (by 10 to 14 %) but persistent growth inhibition of fullerenol, at concentration of 0.04 μmol/l, on K562 cells was found. The same growth pattern was observed in 72-hour culture previously treated with fullerenol for 48 hours (Fig 1).

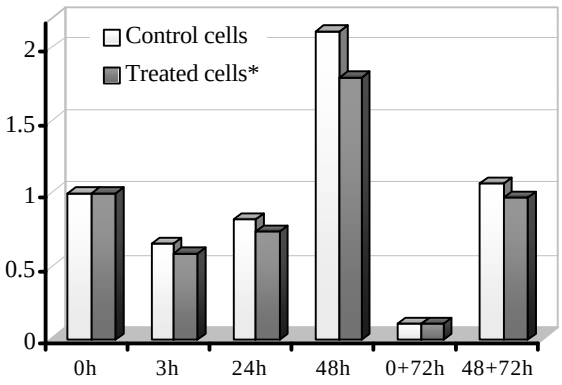

Fig. 1: Total number (x10^6) of K562 cells: untreated, fullerenol treated (0.04 μmol/) during 3, 24 and 48 hours and 72 hours after 48-hour fullerenol treatment.

No significant differences were found in morphological appearance between control and fullerenol treated cells analyzed by light microscopy, concerning cell size, shape and cytoplasm/nucleus ratio.

A mitotic index of fullerenol treated (0.04 μmol/l) cells was decreased during the incubation period up to 30 percent in comparison to control (Table 1).

Table 1: Mitotic index of K562 cells.

Fullerenol conc. (μmol/l)	Mitotic index (%)		
	3h	24h	48h
0	3.85%	3.77%	3.71%
0.4	2.70%	2.76%	2.36%

K_{562} cells were treated for 3, 24 or 48 hours with 0.04 μmol/l of fulerenol $C_{60}(OH)_{24}$, Each value (MI) was obtained by counting at least 1000 cells per sample

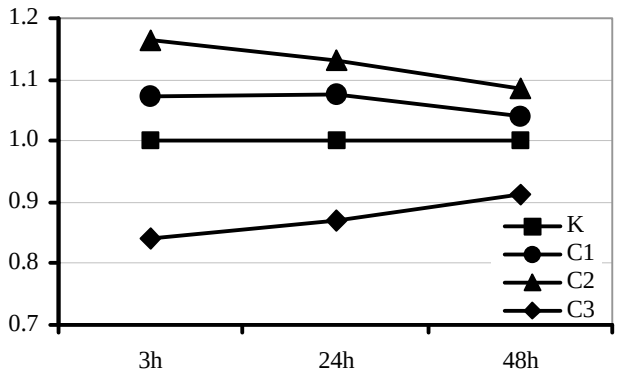

Fig. 2**:** DNA synthesis of K562 cells treated for 3, 24 or 48 hours with 0.04 (C1), 0.18 (C2) and 0.71(C3) μmol/l of fulerenol $C_{60}(OH)_{24}$. Each value (proliferation index) is the mean of six replicates.

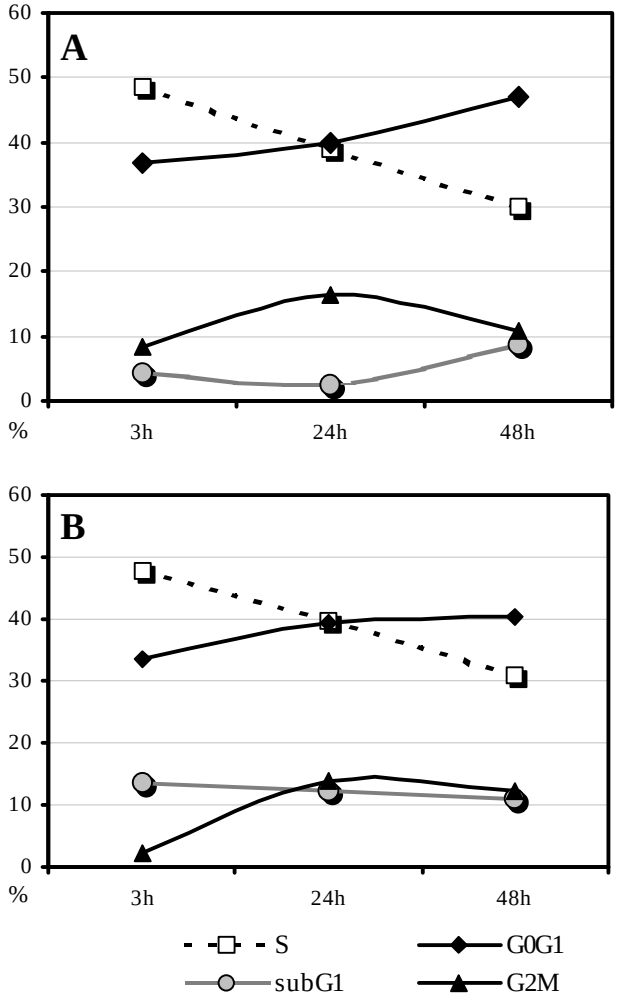

Fig. 3: Flow cytometry analysis of the cell cycle distribution of K562 cells after 3, 24 or 48 hours of culture. A = control K562 cells; B = fullerenol (0.04 μmol/l) treated K562 cells.

DNA synthesis of fullerenol treated K562 cells was also inhibited but only at the highest fullerenol concentration (Fig. 2).
The cell cycle distribution pattern of K562 cells treated with fullerenol at concentration of 0.041umol/l was comparable to the control but increasing number of cells in G2M phase was observed during the treatment period. The proportion of sub-G1 cells was higher in comparison to control (11-13 *vs.* 3 -8%) (Fig. 3).

4 Discussion

The results from previous studies on polyhydroxylated fullerene [C_{60}] derivative activity in human tumor cell lines showed that fullerenol $C_{60}(OH)_{24}$ induced low but transient cell growth inhibition during 48-hour cell treatment [10].
This study confirmed low growth-inhibitory activity of fullerenol $C_{60}(OH)_{24}$ on K562 cell. High cell viability (above 92%) of all fullerenol treated samples additionally confirms low cytotoxicity of water-soluble [C_{60}] derivative. Synthetic and mitotic cell cycle phases of K562 cells were evaluated by determination of the mitotic index and the index of proliferation. Decreased mitotic index of treated cells in relation to control ones (i.e. smaller number of cells in various mitotic phases during 48 hours), as well as changes in DNA synthesis indicates that fullerenol modulates cell cycle of K562 cells.
The finding of decreased MI supports previously mentioned theory that $C_{60}(OH)_{24}$ inhibits the formation of mitotic spindle and microtubule assembly during cell division [34].
DNA synthesis of K562 cells was inhibited - proliferation index is lower for each time point but only for the highest fullerenol concentration.
The cell cycle analysis using flow cytometry with PI showed that cell cycle distribution pattern of K562 cells treated with nanomolar fullerenol concentration, was similar to that of control. However, increasing number of cells in G2M and G1 phases were observed during the treatment period being almost unchanged during last 24 hours of culture. In fullerenol treated cells a proportion of sub-G1 cells were remained during treatment period indicating that fullerenol induced apoptosis in K562 cells. To verify this last observation it would be necessary to analyze both the cell cycle and apoptosis induction using a range of fullerenol concentration and some other methods for apoptosis measurement.
Recently, some studies reported on antiproliferative effects of fullerenols containing 18-20 OH-groups per C_{60} molecule on normal vascular smooth muscle cells and tumor cell lines as well.[4,20] The results related to the study of Nakamura et al. [9] can be explained by interaction of $^1O_2$, (generated upon light excitation of fullerene), with cells or by direct interaction between the fullerene excited state and cells. In the second study inhibitory effect on cell growth was explained by the fullerenol antioxidative properties and partly by inhibition of signal transduction through tyrosine kinase pathway [10].
The persistent decrease of cell number might be a result of the fullerenol that entered the cells and induced growth inhibition by mechanism unexplained so far. It is already confirmed, by *in vitro* and *in vivo* studies, that fullerenes can be taken up by cells and be accumulated in some organs, such as liver, bone tissue and hair.[11,21] There are scarce data on their metabolism and their chronic effects in biological systems as well [11] Small but sustained growth inhibition of K562 cells even 72 hours after 48 hour fullerenol treatment is in accordance with the assumption that it is witheld by the cells.
The results obtained - small but persistent decrease in cell number with high cell viability, small changes in cell cycle distribution, DNA synthesis and mitotic activity indicate that fullerenol $C_{60}(OH)_{24}$ exerts a cytostatic rather than a cytotoxic activity against K562 cells.
The examinations of the biological properties of water-soluble fullerens are still insufficient. Further investigations of their effects on other different cell line types and explanation of the mechanism of their action on cell level are needed.

5 Conclusion

Water-soluble [C_{60}] fullerene derivative, $C_{60}(OH)_{24}$, modulates cell cycle of the K562 cell influencing both synthetic and mitotic cell cycle phase. The results obtained indicate that fullerenol $C_{60}(OH)_{24}$ exerts a cytostatic rather than a cytotoxic activity against K562 cells.

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