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Toxic effect of C$_{60}$ fullerene-doxorubicin complex towards tumor and normal cells 	extit{in vitro} 

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Aim. To evaluate cytotoxicity of created complex of pristine C$_{60}$ fullerene with the anthracycline antibiotic doxorubicin (Dox), as well as of free C$_{60}$ fullerene and Dox, towards different cell types – tumor, normal immunocompetent and hepatocytes. Methods. Measurement of size distribution for particles in C$_{60}$ + Dox mixture was performed by a dynamic light scattering (DLS) technique. Toxic effect of C$_{60}$ + Dox complex in vitro towards tumor and normal cells was studied using the MTT assay. Results. DLS experiment demonstrated that the main fraction of the particles in C$_{60}$ + Dox mixture had a diameter in the range of about 132 nm. The toxic effect of C$_{60}$ + Dox complex towards normal (lymphocytes, macrophages, hepatocytes) and tumor (Ehrlich ascites carcinoma, leukemia L1210, Lewis lung carcinoma) cells was decreased by ~10–16 % and ~7–9 %, accordingly, compared with the same effect of free Dox. Conclusions. The created C$_{60}$ + Dox composite may be considered as a new pharmacological agent that kills effectively tumor cells in vitro and simultaneously prevents a toxic effect of the free form of Dox on normal cells.

Keywords: C$_{60}$ fullerene-doxorubicin complex, normal and tumor cells, cytotoxicity, MTT assay, dynamic light scattering.

Introduction. The important problem in nanobiotechnology is to solve a complex task at the intersection of chemistry, physics, materials science, biology and medicine, which involves the targeted design, synthesis and study of functional properties of nanomaterials (with at least one of the dimensions in the size range up to 100 nm), which are characterized by low toxicity and high specific bioactivity, for their application in the treatment of common diseases. The proposed unique nanobiotechnology is expected in the nearest future to solve the problem of early diagnosis of various pathologies with the identification of their localization and selective delivery of drugs to the target organs. C$_{60}$ fullerene has an eminent position among the available promising and effective biomedical compounds [1]. Due to their nanoscale dimension, combination of strength with low weight [2], strong antioxidant properties [3, 4], accessibility for cellular uptake [5–7], the pristine C$_{60}$ fullerenes are considered as pharmaceutically valuable compounds of a new class [8–10].

However, along with significant prospects of use of these substances for the prevention and treatment of diseases, there are also some problems and cautions. Thus, some results of biological studies of C$_{60}$ fullerene aqueous dispersions indicate their possible toxic effects on the human organism [11]. On the other hand, the pristi-
ne C_{60} fullerenes were demonstrated not to possess any toxicity at low concentrations, or at least they showed no acute toxic effect in the in vitro systems and in vivo [12, 13]. The toxicity of C_{60} molecules was identified to depend strongly on their surface modification, synthesis and treatment conditions, concentration of nanoparticles in solvent medium and, consequently, a size of the formed aggregates (clusters) [11]. It is assumed that the main mechanisms of cytotoxic action of the C_{60} fullerene derivatives are the evoked lipid peroxidation and consecutive progress of oxidative stress and related effects, including DNA damage and necrosis [11, 14].

Doxorubicin (Dox) is an anthracycline antibiotic that is one of the most common therapeutic agents in cancer chemotherapy [15]. Dox binds non-covalently to DNA, blocks the synthesis of nucleic acids, demonstrates high anti-mitotic activity and pronounced mutagenic effects, but also exerts toxic effects towards normal tissues and cells [16]. The free radicals which are formed during Dox chemotherapy, inactivate enzymes of antioxidant protection and invoke immediate oxidative damage of cells with high oxidative metabolism and activity of the mitochondrial respiratory chain, particularly cardiac myocytes and hepatocytes.

One of the ways in protection against Dox-induced chemical insults of normal tissues is a combined use of cytostatics together with antioxidants of different nature [17]. One can assume that immobilization of Dox on C_{60} fullerene [18] will prevent its toxic action on normal cells and enhance its uptake by the target cells that is important for the biomedical application of C_{60} fullerene-drug conjugates [19, 20].

The aim of this study was to evaluate the in vitro toxicity of the C_{60} fullerene with doxorubicin (C_{60} + Dox) complex towards different cell types (tumor, immunocompetent, hepatocytes) and compare it with the effect of C_{60} fullerene and free Dox under in vitro conditions.

**Materials and methods.** Material preparation and characterization. A highly stable reproducible C_{60} fullerene aqueous colloid solution (C_{60}FAS) was prepared according to protocol [21, 22]. In our experiments the C_{60}FAS sample with 0.15 mg/ml concentrations of C_{60} fullerene was used. The resulting probe microscopy images clearly indicate the presence in water of individual C_{60} fullerenes and their aggregates with a typical size up to 100 nm [21–23].

Dox («Doxorubicin-TEVA», «Pharmachemie B. V.», Netherlands, 10 mg of lyophilized powder), dissolved in saline (0.9 % NaCl), with an initial concentration 0.15 mg/ml was used in experiments.

Dox was immobilized on the C_{60} fullerene according to the following protocol: C_{60}FAS (0.15 mg/ml) and Dox (0.15 mg/ml) were mixed in 1:2 volume ratio, the resulting mixture was treated in the ultrasonic disperser for 20 min, and afterwards left for 12 h magnetic stirring at room temperature. The absorption spectra of native Dox and C_{60} + Dox mixture were measured in the wavelength range $\lambda = 400–600$ nm at room temperature. The pronounced hypochromic effect observed in the experiment indicates the formation of a stable complex between Dox and C_{60} fullerene [18].

Measurement of the size distribution of particles in C_{60} + Dox mixture was performed by a dynamic light scattering (DLS) at $T = 298$ K on a Zetasizer Nano-ZS90 (UK). DLS instrument equipped with a He-Ne laser (max 5 mW) operating at the wavelength of 633 nm was used.

**Cell culture experiments in vitro.** Immunocompetent cells and hepatocytes were isolated from intact mice (males of Balb/c line 2.0–2.5 months of age, weight 20–25 g), who were kept at $25 \pm 1$ °C on a standard diet of vivarium of the R. E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine (R. E. Kavetsky IEPOR, Kyiv). All experiments were conducted in accordance with the standards of the European Convention for the Protection of Vertebrate Animals under supervision of bioethical committee of the abovementioned institution.

Lymphocytes and hepatocytes were obtained by centrifugation (1,500 rpm, 40 min) of cell suspensions of spleen and liver, respectively, in Ficoll-Hypaque density gradient ($\rho = 1.077$) [24]. Peritoneal macrophages were obtained from the abdominal cavity of animals by the treatment with 89 % RPMI 1640 medium supplemented with 10 % fetal calf serum and 1 % heparin (5 U/ml) and subsequent centrifugation (1,000 rpm, 10 min).

For comparative evaluation of the cytotoxic effect of C_{60} + Dox complex and free C_{60} fullerene and Dox, the cells of Ehrlich ascites carcinoma, L1210 leukemia and Lewis lung carcinoma were used. The cells were obtained from the bank of R. E. Kavetsky IEPOR. 10 µl of C_{60} + Dox mixture or C_{60}FAS, or Dox was added to 100
μl of cell suspension in the amount of ~3·10⁵ cells and incubated for 18 h. The toxic effect of studied drugs was evaluated using the MTT assay [25], based on the ability of the mitochondrial respiratory chain dehydrogenases to convert 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazol bromide (MTT) to formazan, which crystallizes in cells. Cell suspension containing the studied compound was incubated in the presence of MTT for 3.5 h at the temperature of 37 °C. Formazan precipitate was dissolved in a concentrated solution of DMSO.

Extinction measurement was performed on a digital spectrophotometer («μQuant, BioTEK», USA) at the wavelength of 540 nm. Cytotoxic activity of the studied compound was calculated using the formula: (1 – ε/ε₀) · 100 %, where ε₀ and ε are the extinctions of control and test sample, respectively. The MTT-test was repeated triple for different experiments.

MTT dye was used for visualization of viable tumor cells.

Statistics. Statistical analysis of the experimental data was performed using a Student t-test (the level of significance was p ≤ 0.05).

Results and discussion. A typical result of DLS experiment is presented in Fig. 1 and shows the distribution of the number of light scattering particles according to their hydrodynamic diameters in the studied system. As one can see, the main fraction of the particles had diameters in the range of 132 nm for C₆₀ + Dox mixture and 33 nm for C₆₀ fullerenes dissolved in saline (for comparison). Thus, one can assume that C₆₀ + Dox mixture contains individual C₆₀ + Dox complexes as well as their clusters. Previously, based on a detailed analysis of quantum-chemical calculations, we have shown [18] that three Dox molecules may simultaneously bind with one C₆₀ fullerene without sterical overlapping; a diameter of such complex is 1.38 nm.

Table present the data on survival of different cell types (in % of control) under the action of studied compounds.

The results obtained from the evaluation of cytotoxic activity of C₆₀ fullerenes (0.15 mg/ml) towards tumor cells (Table) have shown that C₆₀ fullerenes display the maximum toxic effect on cells of Ehrlich ascite carcinoma (~26 %), does not have this effect on leukemia L1210 cells at all, and conversely, stimulates the growth of Lewis lung carcinoma cells by ~17 %.

The toxic effect of Dox sample (0.15 mg/ml) is high for all types of normal cells (~ 48–66 %; Table).

The toxic effect of the C₆₀ + Dox (0.05 + 0.1 mg/ml) complex is kept in respect of all types of normal cells (~ 38–50 %; Table).

However, in this case the number of dead cells was lower by ~10–16 % compared with the same effect of free Dox that is a very important applied result. We believe that reducing the toxic effect of this complex on normal cells compared with the effect of free Dox might be associated with the antioxidant activity of the pristine C₆₀ fullerene [3, 4].

The results obtained on the basis of evaluating the cytotoxic activity of C₆₀ fullerenes (0.15 mg/ml) towards tumor cells (Table) have shown that C₆₀ fullerenes display the maximum toxic effect on cells of Ehrlich ascite carcinoma (~26 %), does not have this effect on leukemia L1210 cells at all, and conversely, stimulates the growth of Lewis lung carcinoma cells by ~17 %.

The toxic effect of Dox sample (0.15 mg/ml) is high for all types of tumor cells (~ 69–93 %; Table).

The toxic effect of C₆₀ + Dox (0.05 + 0.1 mg/ml) complex is kept in respect of all types of tumor cells (~ 62–84 %; Table). However, in this case the toxic effect of C₆₀ + Dox complex compared with the same effect of free Dox is weaker, namely: the number of dead tumor cells was lower by ~7–9 %.

As an example, the microscopic images shown in Fig. 2 illustrate the result of toxic action of studied compounds towards Ehrlich ascite carcinoma cells after 18 h of incubation.

The cancer cells are known to develop resistance to traditional chemotherapy drugs, but it is less likely that
they can develop resistance when multiple drugs, for example, C_60 fullerene and Dox, are delivered simultaneously as a C_60 + Dox complex. It is important that one of the potential drugs, for example, C_60 fullerene can be a carrier of Dox to the nucleus. Finally, C_60 fullerene can act as a strong antioxidant effectively reducing the toxic side effects of anticancer drug Dox towards normal cells.

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The survival of normal and tumor cells (in %) under the action of various compounds (MTT-test)

| Cells                  | C_{60} fullerene (0.15 mg/ml) | Dox (0.15 mg/ml) | C_{60} + Dox complex (0.05 + 0.1 mg/ml) |
|------------------------|-------------------------------|------------------|----------------------------------------|
| Lymphocytes            | 114.67 ± 1.37*                | 43.20 ± 4.61*    | 56.22 ± 2.02*                          |
| Macrophages            | –                             | 51.52 ± 3.92*    | 62.38 ± 3.72*                          |
| Hepatocytes            | –                             | 34.43 ± 3.22*    | 49.53 ± 4.97*                          |
| Ehrlich ascite carcinoma | 74.19 ± 2.39*                | 21.39 ± 2.39*    | 29.19 ± 2.99*                          |
| L1210 Leukemia         | –                             | 7.47 ± 0.56*     | 16.44 ± 0.44*                          |
| Lewis lung carcinoma   | 117.47 ± 1.36*                | 31.09 ± 3.13*    | 38.42 ± 3.47*                          |

Note. The results are statistically significant compared to the corresponding control (without any compound); *p ≤ 0.05.

**TOXIC EFFECT OF C_{60} FULLERENE-DOXORUBICIN COMPLEX TOWARDS TUMOR AND NORMAL CELLS IN VITRO**

![Fig. 2. Toxic effect of C_{60} fullerene (b), Dox (c) and C_{60} + Dox complex (d) towards cells of Ehrlich ascite carcinoma after 18 h treatment compared with the control (a) (×160)](image-url)
логии. Цель. Оценить цитотоксичность созданного комплекса фуллераена C₆₀ с антибактериальным агентом и изучить его влияние на рост и миграцию клеток L1210, HaCaT, SF-1, HeLa, C66 и HeLa S3.

Методы. Разработаны методы определения влияния синтезированного комплекса на рост и миграцию клеток. С использованием MTT-теста проводилось измерение роста и миграции клеток.

Результаты. Установлено, что синтезированная система фуллераена C₆₀ + Докс оказывает противораковое действие на рост и миграцию клеток.

Ключевые слова: фуллерен C₆₀, Докс, цитотоксичность, MTT-тест, рост и миграция клеток.

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