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Toxic effect of C_{60} fullerene-doxorubicin complex towards tumor and normal cells *in vitro*

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Creation of new nanostructures possessing high antitumor activity is an important problem of modern biotechnology. ***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 λ = 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 ± 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 (ρ = 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 ~$3 \cdot 10^5$ 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: $(1 - \varepsilon/\varepsilon_0) \cdot 100$ %, where ε_0 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 $p \leq 0.05$).

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 C_{60} + Dox mixture and 33 nm for C_{60} fullerene dissolved in saline (for comparison). Thus, one can assume that C_{60} + Dox mixture contains individual C_{60} + 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 C_{60} 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 C_{60} fullerene (0.15 mg/ml) towards normal cells (Table) have shown that C_{60} fullerene does not have any toxic effect towards macrophages and hepatocytes. On the contrary, it stimulates lymphocytes growth by ~ 15 %, which is probably caused by enhancing metabolism leading to an increase in their proliferative activity.

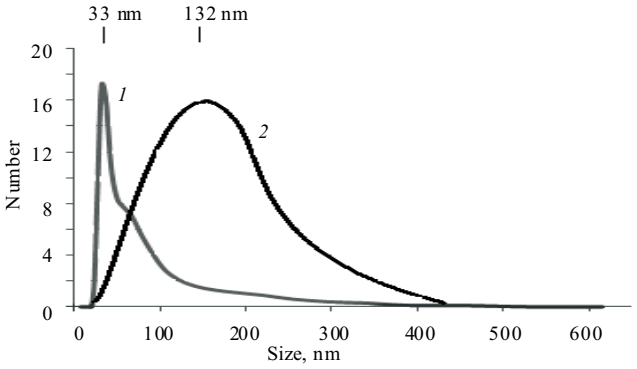

Fig. 1. Light scattering distribution of the number of particles according to their hydrodynamic diameters for C_{60} fullerene dissolved in saline (*1*) and C_{60} + Dox mixture (*2*)

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

The toxic effect of the C_{60} + 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 C_{60} fullerene [3, 4].

The results obtained on the basis of evaluating the cytotoxic activity of C_{60} fullerene (0.15 mg/ml) towards tumor cells (Table) have shown that C_{60} fullerene displays 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 C_{60} + 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 C_{60} + 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

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)
		Normal cells	
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*
		Tumor cells	
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*

N o t e. The results are statistically significant compared to the corresponding control (without any compound); *$p \leq 0.05$.

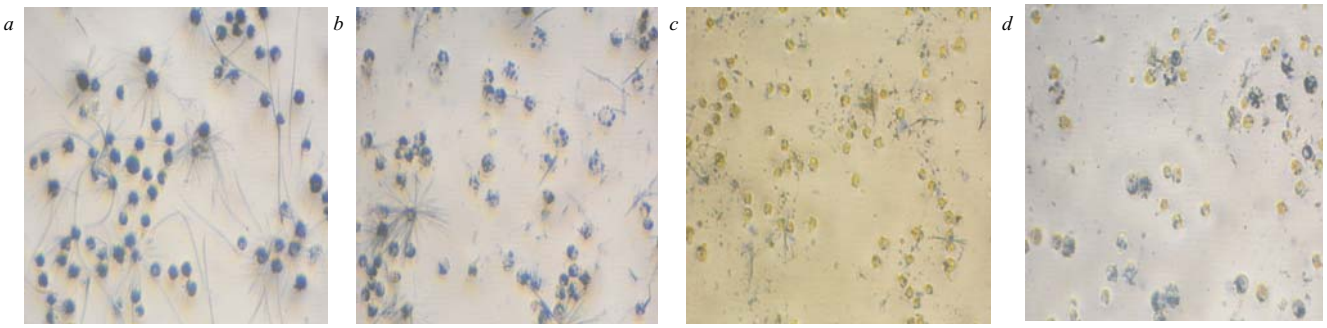

Fig. 2. Toxic effect of C_{60} fullerene (*b*), Dox (*c*) and C_{60} + Dox complex (*d*) towards cells of Ehrlich ascite carcinoma after 18 h treatment compared with the control (*a*) (× 160)

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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Токсичний ефект комплексу фулерен C_{60}–доксорубіцин на нормальні і пухлинні клітини *in vitro*

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Резюме

Створення нових наноструктур з високою протипухлинною активністю є важливою проблемою сучасної біотехнології. ***Мета****. Оцінити цитотоксичність створеного комплексу фулерену C_{60} з антибіотиком антрациклінового ряду доксорубіцином (Докс) на різні типи клітин (пухлинні, імунокомпетентні і гепатоцити) та порівняти одержані результати з токсичною дією вільного фулерену C_{60} і Докс за умов in vitro.* ***Методи****. Розподіл за розміром частинок у C_{60} + Докс суміші вимірювали методом динамічного розсіювання світла (ДРС). Токсичний ефект комплексу C_{60} + Докс щодо нормальних і пухлинних клітин вивчали in vitro з використанням МТТ-тесту.* ***Результати****. Встановлено, що в суміші C_{60} + Докс в основному реєструються частинки з діаметром 132 нм. Токсична дія комплексу C_{60} + Докс щодо нормальних (лімфоцити, макрофаги, гепатоцити) і пухлинних (асцитна карцинома Ерліха, лейкоз L1210, карцинома легені Льюїс) клітин виявилася меншою приблизно на 10–16 і 7–9 % відповідно порівняно із впливом вільного Докс.* ***Висновки****. Розроблений комплекс C_{60} + Докс можна розглядати як новий фармакологічний препарат, що за умов in vitro здатний ефективно знищувати пухлинні клітини і одночасно запобігати побічним токсичним ефектам, притаманним традиційному протипухлинному препарату Докс щодо нормальних клітин.*

Ключові слова: комплекс фулерен C_{60}–доксорубіцин, нормальні і пухлинні клітини, цитотоксичність, МТТ-тест, динамічне розсіяння світла.

Токсический эффект комплекса фуллерен C_{60}–доксорубицин на нормальные и опухолевые клетки *in vitro*

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Резюме

Создание новых наноструктур с высокой противоопухолевой активностью является важной проблемой современной биотехно-

логии. ***Цель****. Оценить цитотоксичность созданного комплекса фуллерена C_{60} с антибиотиком антрациклинового ряда доксорубицином (Докс) на разные типы клеток (опухолевые, иммунокомпетентные, гепатоциты) и сравнить полученные результаты с токсическим действием свободного фуллерена C_{60} и Докс в условиях in vitro.* ***Методы****. Распределение по размеру частиц в смеси C_{60} + Докс измеряли методом динамического рассеивания света (ДРС). Токсический эффект комплекса C_{60} + Докс на нормальные и опухолевые клетки in vitro изучали с использованием МТТ-теста.* ***Результаты****. Установлено, что в смеси C_{60} + Докс регистрируются в основном частицы с диаметром 132 нм. Токсическое действие комплекса C_{60} + Докс по отношению к нормальным (лимфоциты, макрофаги, гепатоциты) и опухолевым (асцитная карцинома Эрлиха, лейкоз L1210, карцинома легкого Льюис) клеткам оказалось меньшим на ~ 10–16 и 7–9 % соответственно по сравнению с действием свободного Докс.* ***Выводы****. Разработанный комплекс C_{60} + Докс можно рассматривать как новый фармакологический препарат, способный в условиях in vitro эффективно уничтожать опухолевые клетки и одновременно предотвращать побочные токсические эффекты, присущие традиционному противоопухолевому препарату Докс относительно нормальных клеток.*

Ключевые слова: комплекс фуллерен C_{60}–доксорубицин, нормальные и опухолевые клетки, цитотоксичность, МТТ-тест, динамическое рассеяние света.

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