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C_{60} FULLERENE PREVENTS GENOTOXIC EFFECTS OF DOXORUBICIN IN HUMAN LYMPHOCYTES *IN VITRO*

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The self-ordering of C_{60} fullerene, doxorubicin and their mixture precipitated from aqueous solutions was investigated using atomic-force microscopy. The results suggest the complexation between the two compounds. The genotoxicity of doxorubicin in complex with C_{60} fullerene (C_{60}+Dox) was evaluated in vitro with comet assay using human lymphocytes. The obtained results show that the C_{60} fullerene prevents the toxic effect of Dox in normal cells and, thus, C_{60}+Dox complex might be proposed for biomedical application.

K e y w o r d s: C_{60} fullerene, doxorubicin, human lymphocytes, genotoxicity, comet assay.

The biomedical application of pristine C_{60} fullerene requires a comprehensive study of possible displays of its toxic effect in the whole organism, as well as in the isolated cells. This allotropic form of nanocarbon possesses a unique structure, physical and chemical properties and biological activity *in vitro* and *in vivo* [1-3]. However, there are several contradictory reports on C_{60} fullerene genotoxicity [4-8].

Numerous studies with using different cell types revealed that C_{60} fullerenes possess genotoxic activity. Data obtained from comet assay indicate that aqueous suspension of colloid C_{60} fullerenes induced DNA strand breaks and oxidative DNA damages in concentration-dependent manner [9–11]. Genotoxicity of C_{60} fullerene was also confirmed by micronuclei test *in vitro* [12]. It is assumed that basic mechanisms of its toxic effect are lipid peroxidation, oxidative stress dissemination and genotoxicity [13].

On the other hand, there are few studies showing no mutagenic effect of C_{60} fullerene *in vivo* and *in vitro*. For example, C_{60} fullerene did not increase the level of DNA strand breaks, but significantly increased the level of FPG sensitive sites/oxidized purines determined by a comet assay in lung epithelial cell line [10]. No increase in the level of *in vitro* chromosomal aberrations and *in vivo* micronuclei was observed in the cytogenetic test at any C_{60} fullerene nanoparticle dose regardless of metabolic activation and irradiation [14].

It was found that the toxicity of C_{60} molecules depends on their concentration in the medium, surface modifications, synthesis and processing conditions [15, 16]. The toxic effect of C_{60} fullerene and its derivatives differs significantly with respect to various cell lines [17]. Thus, the genotoxicity of C_{60} fullerene *in vitro* and *in vivo* strongly depends on the size of its aggregates, dose administration, type of cells and duration of exposure.

At the same time, there are data suggesting that C_{60} fullerene possesses an ability to prevent oxidative stress dissemination in thymocytes [18, 19]. Due to the nanosize, pristine C_{60} fullerene is unable to penetrate cell membranes [20, 21]. Some special biological effects of pristine C_{60} fullerenes, such as anticancer activity, were detected [22, 23].

Doxorubicin (Dox), the antibiotic of anthracycline class, is one of the most common therapeutic agents in cancer chemotherapy [24]. Its main drawbacks are cardiotoxicity and low specificity which considerably limit the effectiveness of the therapeutic action. Therefore, to improve the effectiveness of Dox therapeutic action the alternative cancer treatments are to be developed, including purposeful search for new agents such as targeted carriers and agents which would promote minimization of Dox side effects. One can assume that immobilization of Dox on C_{60} fullerene [25] prevents its toxic action towards normal cells and enhances its uptake by the target cells that is important for the biomedical application of C_{60} fullerene-drug conjugates [26].

The aim of this study was to evaluate and compare *in vitro* genotoxic effects of C_{60} fullerene, doxopubicin, and their complex (C_{60}+Dox) towards normal cells (human lymphocytes) using the comet assay technique.

Materials and Methods

Material preparation and characterization. A highly stable reproducible pristine C_{60} fullerene aqueous colloid solution (C_{60}FAS) in concentration 0.15 mg/ml was prepared according to protocol described in [27, 28].

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

Dox was immobilized by the C_{60} fullerene according to protocol based on recent findings that C_{60} fullerene may act as an effective carrier of the antibiotic molecules (three Dox molecules per C_{60} fullerene), protecting them from water environment [25]: C_{60}FAS (0.15 mg/ml) and Dox solution (0.15 mg/ml) were mixed in 1:2 (volume ratio). The resulting mixture was treated for 20 min in the ultrasonic disperser, and then left for 12 h of magnetic stirring at room temperature. The absorption spectra of Dox solution and C_{60}+Dox mixture were measured in the wavelength range from 400 to 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 [25].

The structural state of C_{60} fullerene, Dox, as well as C_{60}+Dox complex, in aqueous solutions was monitored using the atomic-force microscopy (AFM) on “Solver Pro M” system (NT-MDT, Russian Federation). A sample was deposited onto a cleaved mica substrate (V-1 Grade, SPI Supplies) by precipitation from a droplet of aqueous solution. The sample visualization was carried out in semi-contact (tapping) mode, and NSG10 (NT-MDT) probes were used. AFM measurements were performed after a complete evaporation of the solvent.

Comet assay. Human lymphocytes were obtained from finger-prick blood of healthy donors by separation of cells in a density gradient Histopaque 1077 (Sigma, USA) according to instructions of the manufacturer and then washed in 0.15 M NaCl twice. After washing, 200 µl of cell suspension were shared equally into four parts and each aliquot was mixed with 250 µl of RPMI 1640 medium. C_{60} fullerene, anticancer drug Dox, and complex of C_{60} fullerene with Dox were added at different concentrations to the lymphocyte suspensions in RPMI 1640 medium. The cells were incubated in the presence of these agents for 1 hour at 37 ºC and then washed once in 0.15 M NaCl. The suspension in the amount of 50 µl was mixed with 100 µl of 1% low-melting point agarose (Sigma, USA) at ~37 ºC; 20 µl of the mixture were used to prepare a microscope slide covered with 1% high-melting point agarose.

Slides were kept for 3-5 min at room temperature until agarose polymerization and then placed in the lysis solution: 2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl (pH 8.0), and 1% Triton X-100 (Ferak, Germany) which was added before use. Cells were exposed to lysis solution for 2 hours at 4 ºC. After the lysis, slides were washed with TBE buffer (89 mM Tris-Borat, 2 мМ EDTA, pH 7.5) and subjected to electrophoresis in the same buffer for 20 min at 4 ºC (1 V/cm, 300 mA).

After electrophoresis, slides were stained with 1.3 µg/ml of DAPI (Sigma, USA) and immediately analyzed under fluorescence microscope (LOMO, Russia) connected with Canon A570 camera (a total 100 to 200 cells on each slide were analyzed). The relative amount of DNA in the comet tail, the parameter that reflects the level of DNA damages, was determined using image analysis softwares Comet Assay IV (Perspective Instruments, UK) and CometScore (TriTec Corp., USA). Statistical analysis of the experimental data was performed using Student *t*-test (the level of significance was $P \leq 0.05$).

Results and Discussion

Structure of C_{60} fullerene, Dox and their complex precipitated from aqueous solution. AFM study of C_{60} fullerenes precipitated from their aqueous solution revealed that C_{60} molecules arranged singly (~0.7 nm in diameter) or in the form of their volume aggregates up to 60 nm in diameter (Fig. 1, *a*).

From the solution containing 0.15 M NaCl, C_{60} fullerenes precipitate in the form of 'island-like' aggregates, with the height no more than 0.7 nm spread on the surface (Fig. 1, *b*). NaCl crystals can be seen in Fig 1, *b* as white area, i.e. they have much larger height.

At water evaporation from the Dox solution and from the mixture of Dox with C_{60} fullerene containing NaCl, a non-homogeneous distribution of the precipitated material on the mica surface was observed. NaCl crystals were localized in the ‘salt’

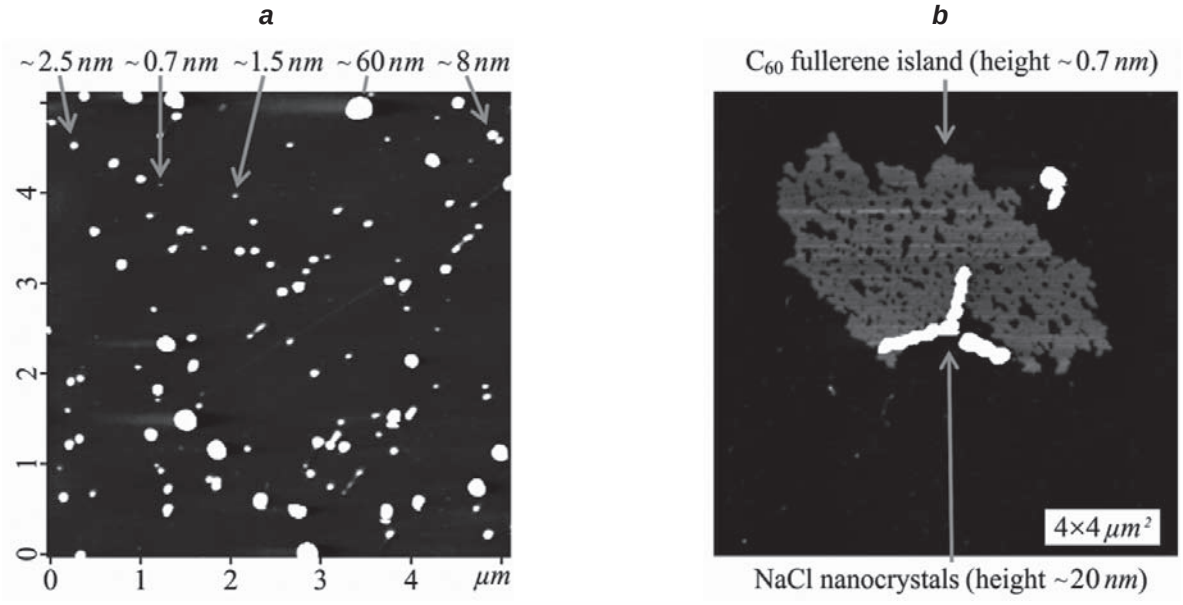

Fig. 1. AFM image of C_{60} fullerenes (0.15 mg/ml concentration) precipitated on the mica substrate from the aqueous solution (a) and from the aqueous solutions containing 0.15 M NaCl (b)

spot, which was clearly seen under optical microscope. The area of the spot occupies ~50% of the whole surface initially covered by the solution. It was established that the main fraction of NaCl and the studied compounds, i.e. Dox or C_{60} fullerene, is localized within the 'salt' spot (the range of high concentration). In this region Dox molecules form ordered long-chain branched nanostructures with 6-20 nm height (Fig. 2, region I). In the vicinity of Dox, one can notice the nanocrystals of salt from physiological solution which are seen on the picture as white points (Fig. 2, region II). The height of these nanocrystals is approximately ~35 nm.

Study of the layer of C_{60} fullerene with Dox mixture showed that in the range of high concentration its structure is similar to that of the Dox layer (see Figs. 2 and 3). In the region of the surface away of the 'salt' spot (the range of low concentration), the structure of the layer of C_{60}+Dox containing system is seen as an island-like structure (Fig. 4), which is quite similar to the structure of C_{60} fullerene precipitated alone from the salt solution (Fig. 1, *b*). In contrast to C_{60} fullerene alone, the height of the observed islands formed in the presence of Dox is larger than 1 nm (Fig. 4). This implies the formation of molecular complexes of C_{60} fullerene with Dox. Recent studies [25] have shown that three Dox molecules may simultaneously bind to one C_{60} molecule without sterical overlapping so that the diameter of such complex should be about 1.38 nm.

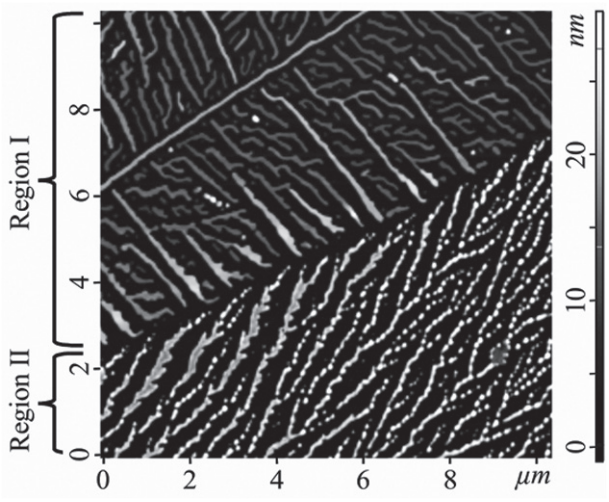

Fig. 2. AFM image of Dox (0.15 mg/ml concentration) precipitated from the aqueous solution containing 0.15 M NaCl. High concentration range (inside the salt spot) is shown. Two regions with nanocrystals formed preferentially by Dox (region I) and salt (region II) are indicated

Genotoxicity of C_{60} fullerene, Dox and their complex. Genotoxicity of C_{60} fullerene and Dox was tested using the comet assay. Several representative images of comets obtained after 20 min of electrophoresis of intact lymphocytes and cells treated with C_{60} fullerene, Dox, or their complex are shown in Fig. 5. In control experiments, when isolated cells were incubated in RPMI 1640 medium containing

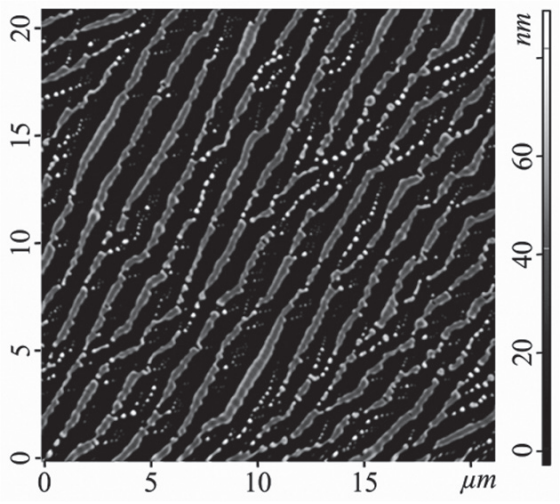

Fig. 3. AFM image of C_{60}+Dox mixture (0.15 mg/ml of C_{60} fullerene per 0.15 mg/ml of Dox) precipitated on the mica substrate from the aqueous solution containing 0.15 M NaCl. High concentration range (inside the salt spot) is shown

Dox concentration (0.01 mg/ml), Dox-treated cells showed the DNA damage level comparable with such level in control cells, but a significant increase of the average relative amount of DNA in comet tails up to 0.15 was observed after treatment with Dox at 0.03 mg/ml (Fig. 6). Anticancer effect of Dox implies two mechanisms of its action [31, 32]. As an antibiotic of anthracycline class Dox intercalates in DNA and, in this way, blocks DNA replication in cells undergoing division and, thus, causes their death [31]. Obviously, such effect is essential for dividing cells (e. g. cancer cells), not for non-dividing lymphocytes. On the other hand, Dox induces an appearance of the reactive oxygen species (ROS) in treated cells and, thus, may provoke DNA damage [31]. This mechanism of DNA damage should be valid for both dividing and non-dividing cells. We assume that concentration-dependent influence of Dox upon lymphocyte DNA is related to the ability of the drug to induce generation of ROS. Thus, ROS

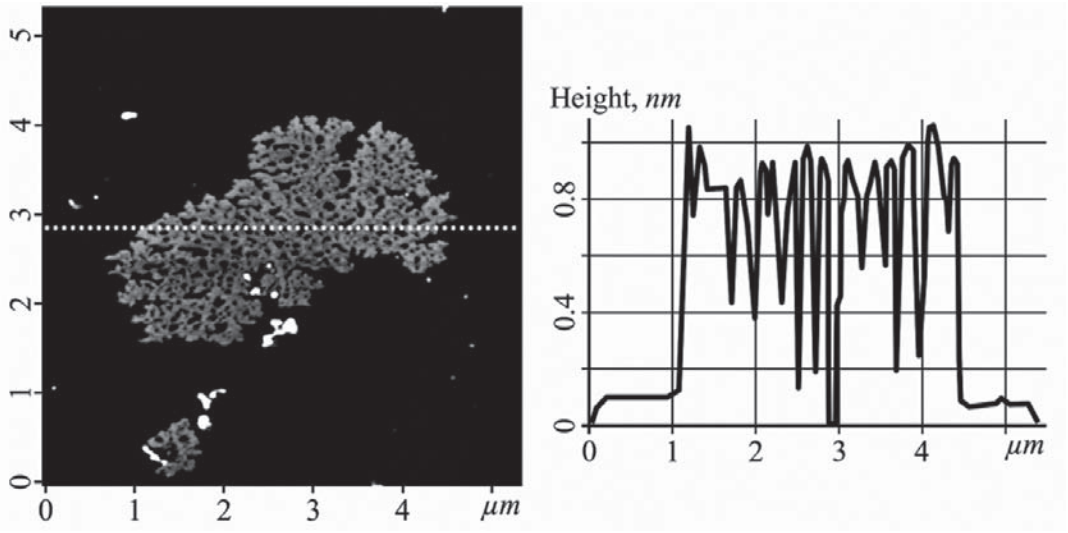

Fig. 4. AFM image of C_{60}+Dox mixture (0.15 mg/ml of C_{60} fullerene per 0.15 mg/ml of Dox) precipitated on the mica substrate from the aqueous solution containing 0.15 M NaCl (low concentration range). Right: cross-section along the indicated line

common used antibiotics kanamycin and streptomycin, the average relative amount of DNA in the comet tails was 0.06 ± 0.01. This value corresponds to the DNA damage rate typically observed in intact differentiated cells [29, 30].

Culturing of lymphocytes with C_{60} fullerene at 0.005 mg/ml or 0.015 mg/ml did not change the rate of DNA damage, thus, C_{60} fullerene nanoparticles do not possess genotoxic effects in cells (Fig. 6). At low concentration in cells depends on Dox concentration in the medium.

Numerous studies argue that C_{60} fullerene nanoparticles possess an antioxidant activity [2, 19], and one may expect that they can reduce mutagenic effects of Dox associated with generation of ROS induction. To test this possibility, we investigated the DNA damage rate in cells treated with the C_{60}+Dox complex (Fig. 6). In the cells treated with the com-

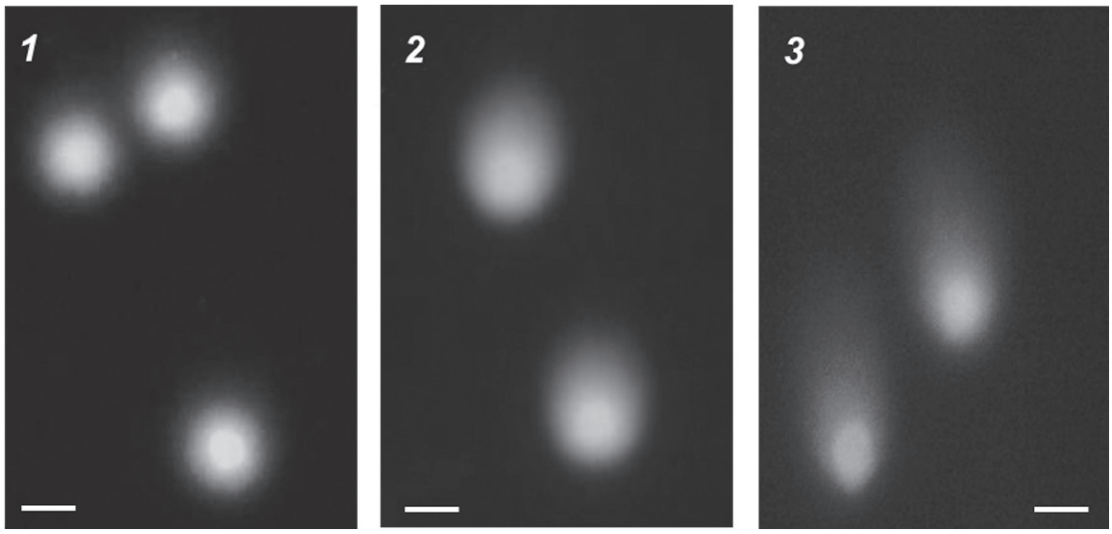

Fig. 5. The representative comet images obtained after 20 min of electrophoresis of control cells (1); cells treated with Dox (0.03 μg/ml) in presence of C_{60} fullerene at 0.015 μg/ml (2); and with free Dox at 0.03 μg/ml (3)

plex, a significant decrease of the relative amount of DNA in the comet tails in comparison with free Dox was observed (in both cases – free Dox and Dox in the complex with C_{60} fullerene – drug concentrations were the same). It should be noted that the average relative amount of DNA in the comet tails in cells treated with C_{60}+Dox complex was comparable with control cells despite a difference in comet morphology (Fig. 5). This difference implies that the co-incubation with C_{60}+Dox complex induces slight DNA fragmentation in treated cells that leads to comet tail formation. Nevertheless, the quantitative analysis

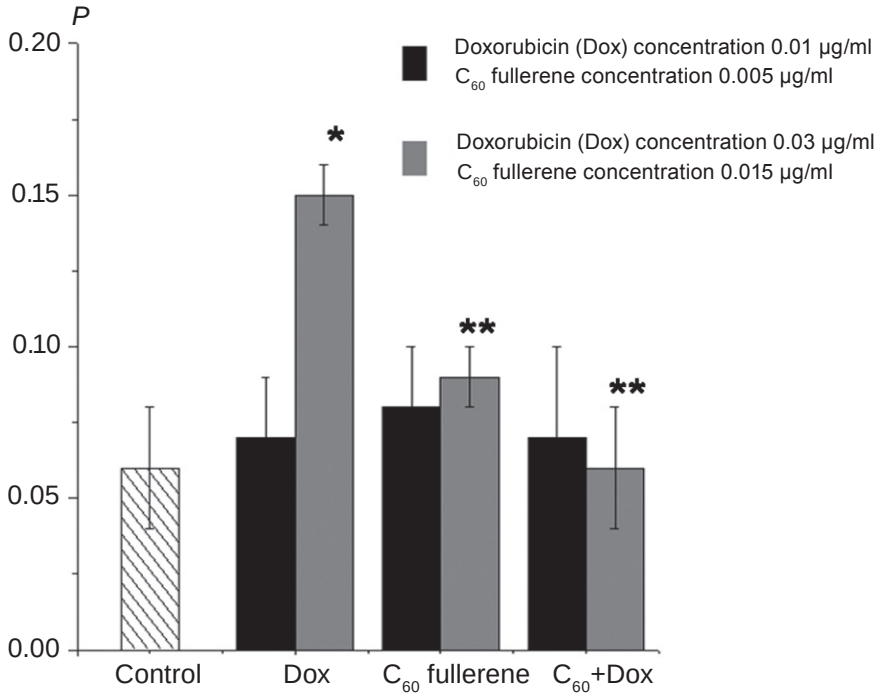

*Fig. 6. The relative amount of DNA in the comet tails (P) after 20 min of electrophoresis of control cells and cells treated with Dox, C_{60} fullerene or C_{60}+Dox complex. The average values for three independent experiments are presented. * Statistically significant ($P \leq 0.05$) with regard to control cells; ** statistically significant ($P \leq 0.05$) with regard to Dox treated cells*

shows the amount of this “migrated DNA” relative to all DNA in cells to be very small, i.e. approximately on the level of control (Fig. 6). Thus, the results of this study show that the C_{60} fullerene may prevent the toxic effects of Dox in normal cells.

Specific characteristics of structural self-organization of C_{60} fullerene in the presence of Dox were studied by using the AFM technique. The obtained results demonstrate the formation of C_{60}+Dox complexes. The genotoxicity of the complex was estimated *in vitro* using the comet assay. It was found that C_{60} fullerene nanoparticles do not possess genotoxic effect towards human lymphocytes. Moreover, the results indicate that C_{60} fullerene in such complex prevents the toxic effect of Dox towards normal cells. Thus, the C_{60}+Dox complex can be a promising agent used in antitumor therapy.

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ФУЛЕРЕН C_{60} ЗАПОБІГАЄ ГЕНОТОКСИЧНІЙ ДІЇ ДОКСОРУБІЦИНУ НА ЛІМФОЦИТИ ЛЮДИНИ *IN VITRO*

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Самоорганізація фулерену C_{60}, доксорубіцину та їх суміші, осаджених із водних розчинів, була досліджена з використанням методу атомно-силової мікроскопії. Результати передбачають комплексоутворення між цими двома препаратами. Генотоксичність комплексу фулерену C_{60} із доксорубіцином (C_{60}+Докс) оцінювали *in vitro* на лімфоцитах людини за допомогою методу ДНК-комет. Одержані результати демонструють, що фулерен C_{60} запобігає токсичній дії Докс на нормальні клітини і, відповідно, комплекс C_{60}+Докс може бути використаний у біомедичних цілях.

Ключові слова: фулерен C_{60}, доксорубіцин, лімфоцити людини, генотоксичність, метод ДНК-комет.

ФУЛЛЕРЕН C_{60} ПРЕДОТВРАЩАЕТ ГЕНОТОКСИЧЕСКОЕ ДЕЙСТВИЕ ДОКСОРУБИЦИНА НА ЛИМФОЦИТЫ ЧЕЛОВЕКА *IN VITRO*

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Самоорганизация фуллерена C_{60}, доксорубицина и их смеси, осажденных из водных растворов, была исследована с использованием метода атомно-силовой микроскопии. Результаты предполагают комплексообразование между этими двумя препаратами. Генотоксичность комплекса фуллерена C_{60} с доксорубицином (C_{60}+Докс) оценивали *in vitro* на лимфоцитах человека с помощью метода ДНК-комет. Полученные результаты показали, что фуллерен C_{60} предотвращает токсическое действие Докс на нормальные клетки и, следовательно, комплекс C_{60}+Докс может быть использован в биомедицинских целях.

Ключевые слова: фуллерен C_{60}, доксорубицин, лимфоциты человека, генотоксичность, метод ДНК-комет.

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