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Application of C_{60} Fullerene-Doxorubicin Complex for Tumor Cell Treatment *In Vitro* and *In Vivo*

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Development of nanocarriers for effective drug delivery to molecular targets in tumor cells is a real problem in modern pharmaceutical chemistry. In the present work we used pristine C_{60} fullerene as a platform for delivery of anticancer drug doxorubicin (Dox) to its biological targets. The formation of a complex of C_{60} fullerene with Dox (C_{60} + Dox) is described and physico–chemical characteristics of such complex are presented. It was found that Dox conjugation with C_{60} fullerene leads to 1.5-2-fold increase in Dox toxicity towards various human tumor cell lines, compared with such effect when the drug is used alone. Cytotoxic activity of C_{60} + Dox complex is accompanied by an increased level of cell produced hydrogen peroxide at early time point (3 h) after its addition to cultured cells. At the same time, cellular production of superoxide radicals does not change in comparison with the effect of Dox alone. Cytomorphological studies have demonstrated that C_{60} + Dox complexes kill tumor cells by apoptosis induction. The results of *in vivo* experiments using Lewis lung carcinoma in mice confirmed the enhancement of the Dox toxicity towards tumor cells after drug complexation with C_{60} fullerene. The effect of such complex towards tumor-bearing mice was even more pronounced than that in the *in vitro* experiment with targeting human tumor cells. The tumor volume decreased by 2.5 times compared with the control, and an average life span of treated animals increased by 63% compared with control. The obtained results suggest a great perspective of application of C_{60} + Dox complexes for chemotherapy of malignant tumors.

KEYWORDS: *C_{60} Fullerene, Doxorubicin, Drug Complex, Apoptosis, Treatment In Vitro, In Vivo.*

INTRODUCTION

Chemotherapy is the main approach in cancer treatment, despite a series of major disadvantages which significantly decrease its overall efficiency.[1] These are high general toxicity of conventional anticancer drugs towards cells of normal tissues and rapid development of drug resistance in tumor cells.[2,3]

Doxorubicin (Dox) is one of the most commonly used drugs in cancer chemotherapy whose application is limited by low selectivity of action and cardiac toxicity, which significantly reduce the efficiency of therapeutic effect of this antibiotic.[4,5] Hence, there is a need in developing of alternative methods of Dox delivery to tumor cells. Creation of novel pharmaceutical formulations based on using specific nanoscale carriers for targeted drug delivery to tumor cells is a principal task in modern pharmacology. Application of such delivery platforms aims on increasing drug biocompatibility and reduction of negative side effects of drug action in treated organism,[6] as well as extension of the duration of drug bioactivity[7] and circumvention of drug resistance mechanisms.[8] Novel nanocarriers are capable of not only significant lowering the negative side effects of specific chemotherapeutic agents, but also of enhancing their anticancer activity.[9]

C_{60} fullerene is a carbon nanostructure which possesses biological activity towards various types of cells both *in vitro* and *in vivo*.[10] It was established that pristine

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C_{60} fullerene is non-toxic at low concentrations,[11–13] and it penetrates through plasma membrane of cells,[14–17] and also demonstrates antioxidant action.[10, 18] Moreover, it was found that pristine C_{60} fullerene possesses anticancer activity. Recently, we reported[19, 20] that the water soluble pristine C_{60} fullerene administered once per day (during 5 days with a day interval) in a dose of 5 mg/kg of body weight of treated male mice (C57Bl/6J line) suppressed growth of transplanted Lewis lung carcinoma. Maximum therapeutic effect reached 25.1% inhibition of tumor growth, that was accompanied by 21.8% increase of animal life duration and 48% decrease in the metastasis inhibition index. It was also established[21, 22] that the antitumor effect of pristine C_{60} fullerene might be associated with its ability to modulate the oxidative stress, inhibit angiogenesis, and stimulate immune response. Recently, we found[23] that the administration of water soluble pristine C_{60} fullerene in the above mentioned dose 30 min prior to the injection of Dox (0.5 mg/kg) into Lewis lung carcinoma-bearing mice had further enhanced the antitumor activity of Dox. The maximum therapeutic effect (tumor growth inhibition) reached 34%, the metastasis inhibition index was 79%, and the increase of animal life duration was 24.4%. Taking into account these data we suggested[24] that complexation of C_{60} fullerene with Dox (C_{60} + Dox) may reduce negative side effects of this drug towards normal cells, as well as to enhance its ability to enter target tumor cells and optimize the conditions for drug release from its complex with C_{60} fullerene depending on pH in the intracellular microenvironment. Detailed investigation of the all above-reviewed facts would be of great importance for extensive use of pristine C_{60} fullerene as a novel nanocarrier of anticancer drugs.[25]

The main goal of this study was to investigate molecular mechanisms of the modulation of antitumor activity of Dox by the C_{60} fullerene *in vitro* and *in vivo*, and to find out whether C_{60} fullerene can act as an effective drug delivery platform for Dox for its further use in clinical practice.

EXPERIMENTAL DETAILS

Materials Preparation and Characterization

Preparation of C_{60} Fullerene Aqueous Colloid Solution

Pristine C_{60} fullerene aqueous colloid solution (C_{60}FAS) in 0.15 mg/ml C_{60} fullerene concentration was prepared according to protocol[26] and used in the experiments.

Atomic Force Microscopy (AFM) of C_{60} Fullerene Aqueous Solution

The state of C_{60} fullerene in aqueous solution was monitored by using the AFM on "Solver Pro M" system (NT-MDT, Russian Federation).[27] The bio-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 a semi–contact (tapping) mode, and NSG10 (NT-MDT) probes were used. AFM measurements were performed after a complete evaporation of the solvent.

Preparation of C_{60} + Dox Complex

Dox (≪Pfizer≫, Italy; 10 mg powder) was dissolved in saline at initial concentration of 0.15 mg/ml. It was immobilized on the C_{60} fullerene according to previously described method.[28] Specifically, C_{60}FAS and Dox were mixed in 1:1 weight ratio, and the resulting mixture was treated for 15 min in the ultrasonic disperser, and after that it was subjected to overnight magnetic stirring at +4 °C.

UV/VIS Spectroscopy

UV/VIS absorption spectra were recorded using a double–beam spectrophotometer SQ-4802 (UNICO, USA) in the polymethylacrylate cuvettes with 1 cm optical path (Spain).[24] The spectral measurements for both the free Dox and its complex with C_{60} fullerene were performed under similar solution conditions.

Dynamic Light Scattering (DLS)

Size distribution measurements for both the free Dox and the C_{60} + Dox complex were carried out by means of DLS at 25 °C on a DAWN® HELEOS™ 243-HHC (Wyatt Technology Corp.) instrument equipped with a Ga-As laser (60 mW) operating at 658.0 nm wavelength at 99° scattering angle.[27] The autocorrelation function of the scattered light intensity was analyzed by a DYNALS regularization analysis by using Wyatt ASTRA software package. The results were evaluated using the Smoluchowski approximation which is known to be rigorously valid only for spherical-like particles.

Calculations

The procedures for making structural analysis of the C_{60} + Dox complex corresponded to such analysis used previously for studying different complexes of small molecules in aqueous solution.[29] Briefly, the method of structure calculations was based on the following protocol. Calculations of the spatial structure of 1:*n* C_{60} + Dox complex were performed by using methods of molecular mechanics with X-PLOR software (version 3.851) and CHARMM27 force field. The modeling of aqueous environment of the interacting molecules was carried out by water molecules in the form of TIP3P placed in a cubic box with side length of 35 Å (1423 molecules). Quantum-mechanical calculations of partial atomic charges on C_{60} fullerene and Dox were performed in Gaussian 03 software within the framework of DFT (B3LYP) in 6-31G* basis set by the Merz–Kollman method.[30]

Cell Culture Experiments *In Vitro*

Human isogenic p53-null (p53-/-), Bax-null (Bax-/-) and wild-type (p53+/+, Bax +/+) human HCT-116 colon

carcinoma cells (kindly provided by Dr. Bert Vogelstein), human T-leukemia cells of Jurkat line, human leukemia cells of HL-60 line and its drug-resistant HL-60/vinc subline (over-expression of P-glycoprotein) and human breast adenocarcinoma cells of MCF-7 line were obtained from cell culture collection at Vienna Medical University, Institute of Cancer Research. Cells were cultured in RPMI-1640 medium, supplemented with 10% fetal calf serum (Sigma Chemical Co., St. Louis, USA), 50 μg/ml streptomycin (Sigma Chemical Co., St. Louis, USA), 50 units/ml penicillin (Sigma Chemical Co., St. Louis, USA) in 5% CO_2-containing humidified atmosphere at 37 °C. For experiments, the cells were seeded into 24-well tissue culture plates (Greiner Bio-one, Germany). Short-term (24 h) cytotoxic effect of anticancer drugs was studied by means of Evolution 300 Trino microscope (Delta Optical, Poland) after cell staining with trypan blue dye (0.1%).

DAPI staining of the cells was performed for studying chromatin condensation in the MCF-7 cells treated with C_{60} + Dox complexes. 24 h after addition of the drugs MCF-7 cells were washed twice with 1 × PBS, fixed for 15 min at room temperature in 4% solution of paraformaldehyde, and then permeabilized for 3 mins by 0.1% Triton X-100 in phosphate buffer saline (PBS). After that, the cells were incubated for 5 min with 1 μg/ml solution of DAPI (4′,6-diamidino-2-phenylindole) (Sigma, USA), washed twice with PBS and the cover glasses with the fixed cells were placed on slides. Cytomorphological study was performed using Carl Zeiss AxioImager A1 fluorescent microscope (Carl Zeiss, Germany).

In order to compare the effectiveness of Dox delivery into tumor cells by means of C_{60} fullerene, the Dox permeation study was carried out by means of fluorescent microscopy and flow cytometry on carcinoma and leukemia cells, correspondingly. 1, 3, 6 h after addition of the drugs the MCF-7 cells were washed twice with 1 × PBS, and the cover glasses with the alive cells were placed on slides. Cytomorphological study was performed using Carl Zeiss AxioImager A1 fluorescent microscope (Carl Zeiss, Germany), and Dox fluorescence was measured with appropriate filter.

Quantitative analysis of Dox permeation in Jurkat T-leukemia cells was accomplished using flow cytometry assay. Briefly, 1, 2, 3, 4 h after addition of the drugs Jurkat T-cells were washed twice with 1 × PBS and immediately analyzed by flow cytometry (FL2 channel) on FACSCalibur device (Becton Dickinson, USA). Autofluorescence of untreated cells was used as a control.

The content of cellular reactive oxygen species (ROS) was measured after incubating of the control or drug-treated (1, 3, 6, 12, 24 h) cells with the corresponding fluorescent dyes, dihydrodichlorofluorescein diacetate (H_2DCFDA, H_2O_2-specific) and dihydroethidum (DHE, O_2^--specific), used at 10 μM concentration for 30 min at 37 °C. After incubation with the fluorochrome the cells were washed with PBS and immediately analyzed by flow cytometry on FACSCalibur device (Becton Dickinson, USA).

Breakdown of the mitochondrial membrane potential ($\Delta\Psi_m$) was determined by FACS analysis using JC-1 dye (5,5′ tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) which allows detecting changes in the $\Delta\Psi_m$ value. The Mitochondrial Membrane Potential Detection Kit (Stratagene, La Jolla, CA, USA) was applied as described in the manufacturer's instruction. 10^6 Jurkat cells were treated for 1, 3, 6, 12 and 24 h by the drugs. After PBS washing the cells were incubated for 10 min at 37 °C in freshly prepared JC-1 solution (10 mg/ml final concentration in the medium). Spare dye was removed by PBS washing and the cell-associated fluorescence was measured with the FACS device.

Rhodamine 123 (Rh123) accumulation assay was accomplished as previously described.[31] Briefly, 5×10^5 Jurkat T-cells were pre-incubated for 30 min at 37 °C with Dox, C_{60}FAS or C_{60} + Dox complex, followed by incubation with Rh123 (0.25 mg/ml). After 1, 3, 6, 12, 24 h exposure, fluorescence of Rh123 was measured by the FACS device using 530/30 nm band-pass filter.

In Vivo Experiments

The male mice of C57Bl/6J line (20-21 g weight) were kept at 25 ± 1 °C on a standard diet in the animal facility of R. E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine (Kyiv, Ukraine). All experiments were conducted in accordance with the international principles of the European Convention for protection of vertebrate animals under control of Bio-Ethics Committee of this institution.

Lewis lung carcinoma was transplanted intramuscularly into mice limb with initial number of tumor cells equal to ~5×10^5. It is known that this type of tumor is characterized by a high degree of metastasis into lung.[20]

Group 1 (Dox). Dox was used in 1.5 mg/kg dose (0.2 ml) injected intraperitoneally to mice with transplanted tumor once per day for 5 days with a day interval.[32]

Group 2 (C_{60} fullerene). C_{60}FAS was used in 1.5 mg/kg dose (0.2 ml) injected intraperitoneally to mice with transplanted tumor once per day for 5 days with a day interval.[19, 20]

Group 3 (C_{60} + Dox complex). C_{60} + Dox complex was used in 1.5 mg/kg dose (0.2 ml) injected intraperitoneally to mice with transplanted tumor once per day for 5 days with a day interval.

Control. The mice with transplanted tumor were injected with saline (0.2 ml) once per day for 5 days with a day interval.

The injections of Dox, C_{60} fullerene or C_{60} + Dox complex were started on 2nd day after tumor transplantation. The initial number of animals was taken equal to 7 in each

experimental group including the control. The protocol of injecting of C_{60} fullerenes was based on previous work[33] reporting that C_{60} fullerenes administered intraperitoneally to rats (500 mg/kg) were subjected to clearance from the organism within 2–4 days. The C_{60} fullerene dose applied in our experiments was significantly lower than the maximum tolerated dose of C_{60} fullerene, which was found to be 5 g/kg both for oral or i.p. administration to mice.[33] The kinetics of growth of Lewis lung carcinoma in mice was characterized by changing its size from the 10th day (when the tumor was observed visually by the increased volume) up to the 22nd day (when the first tumor-bearing animals died in *control* (untreated) group).[19, 20] An average life span of animals was estimated in all experimental groups.[19, 20]

Statistical Analysis

All experiments were performed in triplicate and repeated 3 times. For statistical analysis of the obtained results, standard variation data within a group was calculated together with a statistical reliability of differences between two groups of data assessed by Student's *t*-test. The level of significance was set to 0.05.

RESULTS

C_{60} Fullerene Structure in Aqueous Solution

Pristine C_{60} fullerenes exert a distinct tendency to aggregation in aqueous solution.[27] Hence, a question arises of in which form C_{60} fullerene is bioactive, as a single molecule or as its cluster?[34] It was found[35] that aggregation keeps C_{60} fullerenes in a suspension for a long time, and the reactivity of individual C_{60} molecules is substantially altered in the aggregated form. The AFM investigation was performed in order to characterize a composition of the prepared C_{60}FAS.

The AFM images (Figs. 1(a) and (b)) clearly demonstrate that both the individual C_{60} fullerenes and their bulk aggregates (clusters) with 1.5–3 nm height are presented on the mica substrate in a 2:1 ratio. These results are in agreement with theoretical predictions and small-angle neutron scattering measurements.[26, 27, 36] 10-fold dilution of the initial C_{60}FAS in water (final concentration was 0.015 mg/ml) resulted in a corresponding decrease in surface concentration of C_{60} fullerenes (Fig. 1(c)). The ratio of the number of individual C_{60} fullerenes to the number of their clusters remained preserved. A small number of individual C_{60} fullerene aggregates with 10–100 nm sizes could be also seen in the AFM image (Fig. 1(a)).

Interaction of C_{60} Fullerene with Dox in Aqueous Solution

Direct complexation between C_{60} fullerene and Dox molecules under the condition of 1:1 mixture has been studied by UV/VIS spectroscopy. In order to exclude the

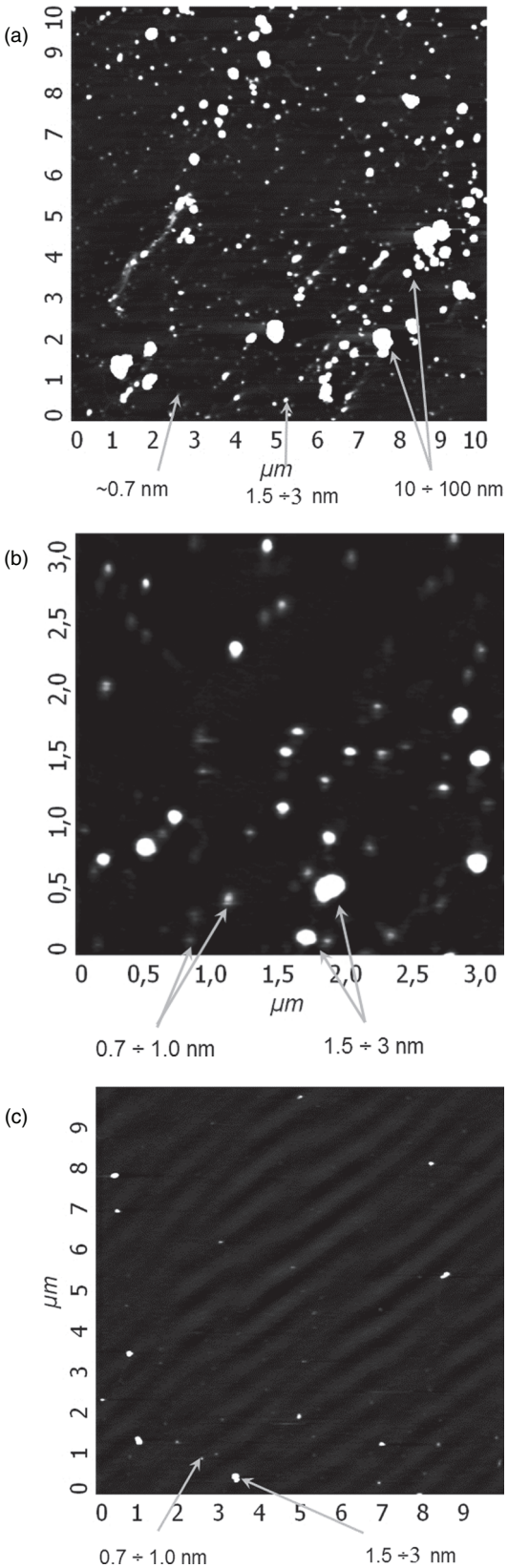

Figure 1. AFM images of C_{60} fullerenes on mica surface, obtained by precipitation from C_{60}FAS at different concentration: (a), (b) 0.15 and (c) 0.015 mg/ml. Arrows indicate the typical dimensions of the C_{60} fullerene nanoparticles.

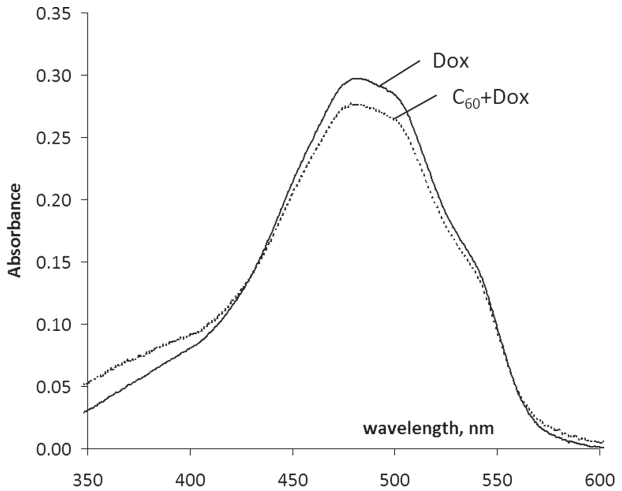

Figure 2. Electronic absorption spectra of Dox (0.26 mM) in the presence and absence of C_{60} fullerene at 1:1 composition of the mixture.

effect of scattering on the baseline in case of C_{60} + Dox spectrum and create conditions for comparative analysis, the spectra of Dox and C_{60} + Dox were referenced to zero at 650 nm. Transformation of the Dox spectrum as a consequence of drug addition to C_{60}FAS was observed (Fig. 2). The hypochromic effect is clearly seen which suggests a formation of C_{60} + Dox complexes in aqueous solution. The observed magnitude of the hypochromism under given concentration of Dox (0.26 mM) amounts to 7.02%, and is in agreement with the magnitude of this effect observed in our previous study of C_{60} + Dox interaction.[24] It should be noted that the protocol of preparation of the complex in this study was somewhat different, since the C_{60}FAS was prepared independent of Dox. However, the results show that the physico-chemical characteristics of complexation between C_{60} fullerene and Dox remained unchanged.

DLS technique was applied for further investigation of C_{60} + Dox interaction regarding a distribution of clusters by their dimensions (Fig. 3). The resultant distribution recorded for pure C_{60}FAS contains a single peak which qualitatively agrees with literature data.[37] The distribution is broad and covers cluster sizes from 40 up to 1000 nm demonstrating a poly-disperse nature of the C_{60}FAS. The latter corresponds to the results of AFM characterization of the C_{60}FAS presented above (see Fig. 1).

The most remarkable feature of the intensity–weight distribution shown in Figure 3 is a significant shift of the peak maximum from 160 to 600 nm, as well as the appearance of a shoulder on the left side of the distribution as a consequence of Dox addition. The large shift (400 nm) in distribution cannot be explained by an external binding of Dox molecules to C_{60} fullerene clusters. Apparently, it is a consequence of formation of large clusters composed of ordered mixture of Dox and C_{60} molecules inside them. In our previous work,[24] this effect termed as 'ligand-induced' fullerene aggregation, was indirectly observed by

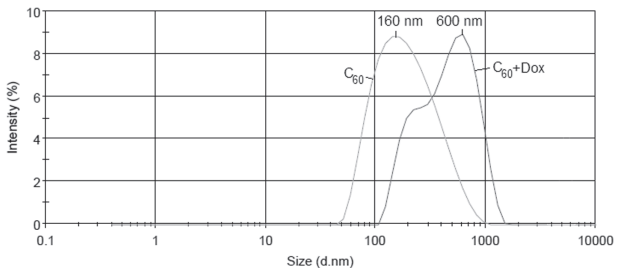

Figure 3. Size distribution by intensity of C_{60}FAS and C_{60} + Dox mixture.

means of UV/VIS spectrophotometry and deduced from a non-monotonous shape of the titration curves for binding of the C_{60} fullerene with various aromatic drugs. The possibility of formation of ordered structures containing C_{60} fullerene interleaved by the aromatic molecules is well known in fullerene chemistry.[38] It is based on the fact that flat aromatic molecule placed between two fullerenes may act as a glue pulling them together due to van der Waals attraction or by means of a reduction of the intrinsic fullerene electrostatic repulsion in solution. The effect of ligand-induced aggregation enables explaining the bimodal shape of the observed size distribution in C_{60} + Dox mixture, and assign the major peak to clusters mainly composed of ordered mixture of C_{60} + Dox. The minor peak might be assigned to clusters mainly composed of C_{60} molecules (Fig. 3). The existence of large clusters incorporating Dox molecules is important since such clusters may act as Dox carriers to target cells potentially improving a biological effect of the drug. Taking into account these data, below we shall estimate a magnitude of loading of Dox molecules into large C_{60} fullerene clusters.

Maximum filling of surface of C_{60} molecule with bound Dox molecules was estimated by means of molecular modeling. It amounts to 1:3 (i.e., three Dox molecules can bind with one C_{60} fullerene molecule without steric overlapping (see Fig. 4). Taking into account an average diameter of the C_{60} molecule as 0.7 nm and the average distance of 0.34 nm between the surface of C_{60} fullerene and the chromophores of Dox molecules in the complex (as well as between two fullerenes without Dox), the effective diameter of C_{60} + Dox_3 nanoparticle can, thus, be taken equal $D_1 \approx 1.38$ nm. We further employed the standard model assuming spherical shape of C_{60} fullerene clusters, with a link between the diameter, D_j, the cluster containing j particles, and D_1 in a form $D_j = D_1 \cdot j^{1/3}$.[24] As a result we got that at $D_j \approx 600$ nm (as estimated above from the DLS measurements) the approximate number of C_{60} + Dox_3 particles in large clusters amounts to $80 \cdot 10^6$ against the number of $3.6 \cdot 10^6$ in the C_{60} fullerene clusters ($D_j \approx 160$ nm) without Dox (NB: the calculated numbers of molecules are likely to be the upper estimate). Recalling that Dox molecules are likely acting as layers between the neighboring C_{60} fullerenes in clusters, and, thereby, are partly

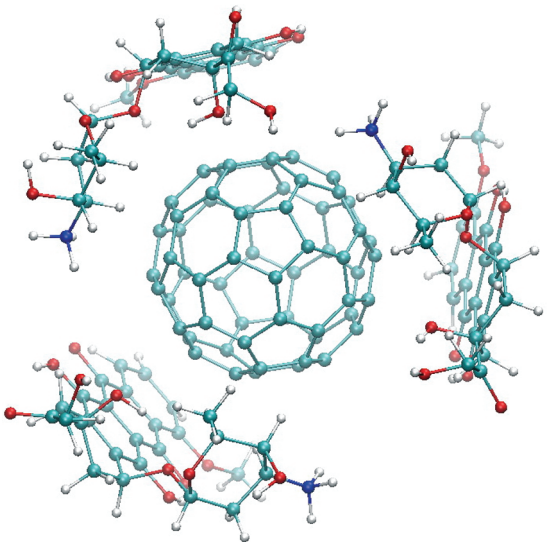

Figure 4. **Calculated structure of C_{60} + Dox complex.**

shared by them, under first approximation, the 1:2 loading of Dox molecules in large clusters might be expected. This result, as well as the 22-fold incremental (the upper estimate) in a number of Dox molecules captured by the cluster, confirms that the C_{60} fullerene clusters might act as effective carriers of Dox molecules, protecting them from the effect of water environment.

Cytotoxic Activity *In Vitro* of Dox and C_{60}+Dox Complex Targeting Drug-Resistant Tumor Cells in Comparison with Their Parental Lines

Development of multidrug resistance of tumor cells to various anticancer drugs is a principal problem in cancer chemotherapy, and is caused by variety of factors such as overexpression of ABC-transporters (P-glycorptein, MRP-1, bcrp), mutations in genes involved in cell cycle regulation and apoptosis, and others.[39]

Cytotoxic activity of C_{60} + Dox complex was studied in several tumor cell lines characterized by various mechanisms of drug resistance in comparison with C_{60} fullerene and Dox in separate. It was found that C_{60} + Dox complex possesses a 1.5–2-fold higher toxicity towards human T-leukemia cells of Jurkat line compared to the action of Dox alone (Fig. 5(A)). Similar results were obtained when the human colon carcinoma cells (HCT-116 line) were treated in the same way (Fig. 5(B)). Such increase in the cytotoxicity was shown to be linear and dose-dependent, and it was observed at low (0.1 μM), medium (0.25 μM),

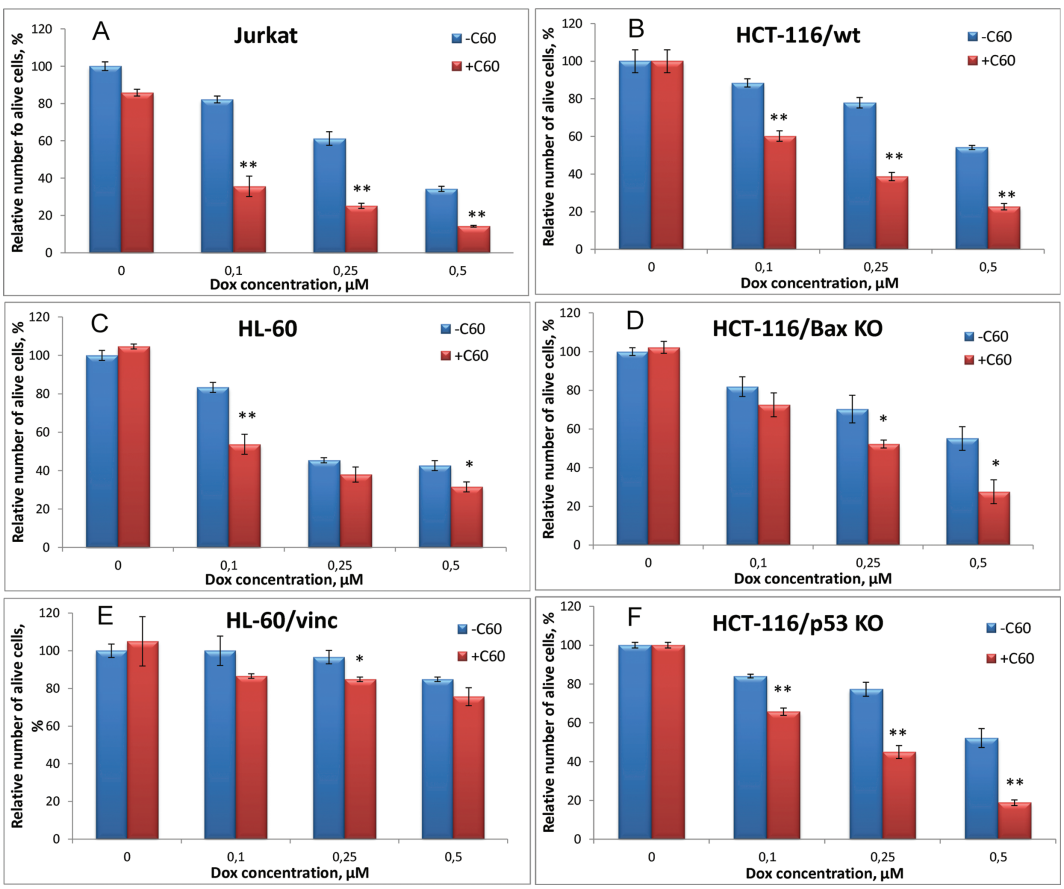

Figure 5. **Comparison of cytotoxic action of C_{60} + Dox complex and Dox alone towards carcinoma and leukemia cell lines possessing different mechanisms of drug resistance. *$P < 0.05$; **$P < 0.01$.**

and high (0.5 μM) concentrations of C_{60} + Dox complex. It should be noted that C_{60}FAS was non-toxic for the studied cells even at 1.9 μg/ml which is equivalent to 1 μM dose of Dox in the C_{60} + Dox complex (see Fig. 5 at Dox concentration marked as '0' and column #2 marked as '+C_{60}'). The observed effect of a significant increase in cytotoxic activity *in vitro* of the C_{60} + Dox complex compared to such activity of Dox alone cannot be explained by a simple additive effect of Dox and C_{60} fullerene operating in mixture individually and independent of each other.

In order to study potential ability of the C_{60} + Dox complex to circumvent multidrug resistance of tumor cells, we compared its effect towards human leukemia cells (HL-60 line) and their vincristine-resistant sub-line (HL-60/vinc). The latter one is cross-resistant to both anticancer drugs, i.e., vincristine and Dox, and is characterized by overexpression of cell membrane P-glycoprotein pumping off the drug molecules from tumor cells.[40] The HL-60/vinc cells demonstrated a 2-fold increase in their resistance to Dox action compared to the wild-type HL-60 cells, but application of the C_{60} + Dox complex additionally increased by 15–20% their sensitivity to Dox (Figs. 5(C) and (E)). This effect can be explained by an enhanced delivery of Dox into cytosol of tumor cells by means of C_{60} fullerenes, resulting in conditions when P-glycoprotein is not capable of pumping out all Dox from target cells.

For more detailed study of potential molecular mechanisms of induction of cell death under the action of C_{60} + Dox complex, we investigated isogenic knockout sub-lines of human colon cancer HCT-116 cells lacking p53 or Bax genes (HCT-116/p53-KO and HCT-116/Bax-KO, respectively). It was found that elimination of Bax gene functioning increased a resistance of HCT-116 cells to the action of C_{60} + Dox complexes by 20–35%, whereas the Bax knockout did not affect Dox action significantly (5–10% increase of the resistance) (Figs. 5(D) and (F)). Bax protein plays a crucial role in the mitochondria-mediated apoptosis, since it is responsible for formation of large pores in the outer membrane of mitochondria.[41] Taking into consideration these data, our results suggest that the C_{60} + Dox complexes might induce apoptosis of mitochondrial type.

We did not detect an impact of the C_{60} + Dox complexes on survival of HCT-116/p53 KO cells, since there was no significant difference between the action of the C_{60} + Dox complexes towards HCT-116 and HCT-116/p53-KO cells. Thus, the p53 gene, whose product is involved in DNA damage-mediated apoptosis opposite to mitochondrial Bax protein, does not play any important role in the C_{60} + Dox complex-induced apoptosis.

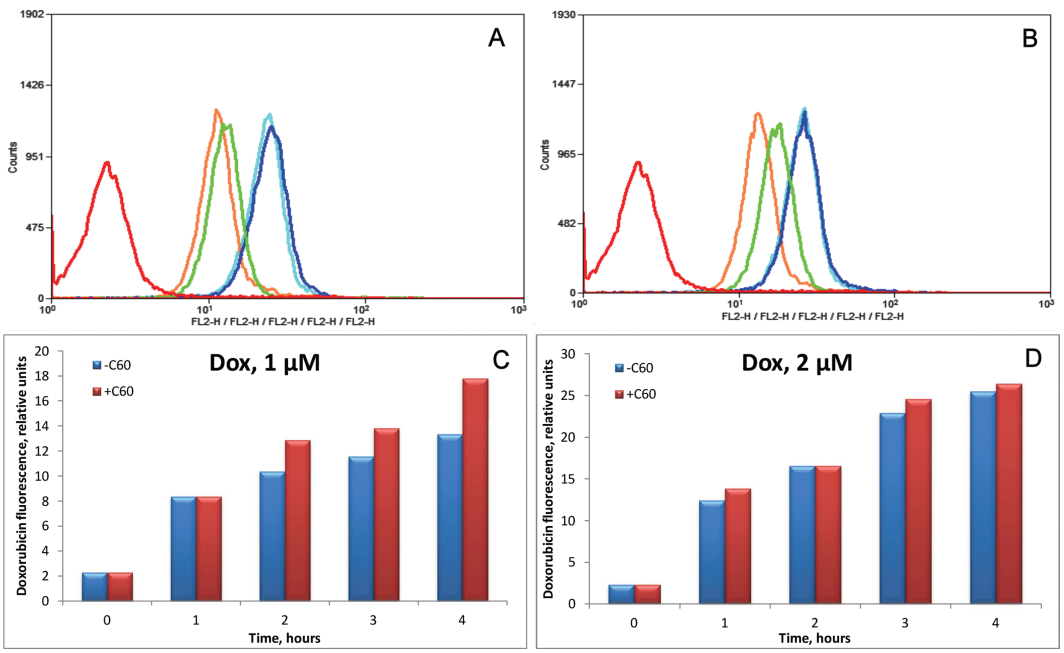

Figure 6. **Permeation of C_{60} + Dox complex and C_{60} fullerene alone into human T-leukemia Jurkat cells. Quantitative measurement of Dox fluorescence in target cells was carried out by flow cytometry in time-dependent mode: (A) comparison of permeation of Dox alone and C_{60} + Dox complex through plasma membrane of Jurkat T-cells (3 h after addition Dox or C_{60} + Dox to cultured cells); (B) comparison of permeation of Dox alone and C_{60} + Dox complex through plasma membrane of Jurkat T-cells (4 h after addition Dox or C_{60} + Dox to cultured cells); (C) quantitative analysis of the intensity of permeation of Dox alone and C_{60} + Dox complex through plasma membrane of Jurkat T-cells (1 μM concentration of Dox); (D) quantitative analysis of the intensity of permeation of Dox alone and C_{60} + Dox complex through plasma membrane of Jurkat T-cells (2 μM concentration of Dox). Control; Dox, 1 μM; C_{60}+Dox, 1 μM; Dox, 2 μM; C_{60}+Dox, 2 μM; Cell autofluorescence under treatment with C_{60} fullerene alone was the same as in control and therefore is not shown. The results of one of three independent experiments are presented.**

Increased Entry of Dox Into Target Cells is the Main Reason of Enhancement of Pro-Apoptotic Action of C_{60} + Dox Complex

Improvement of anticancer drug delivery to target cells by means of nanocarrier might be one of the reasons of enhancement of drug's cytotoxic activity towards tumor cells. For quantitative analysis of time-dependent accumulation of Dox in tumor cells, delivered by the C_{60} fullerene in comparison with Dox alone, flow cytometry was applied utilizing the Dox ability to fluorescence at 530 nm. It was revealed that immobilization of Dox on C_{60} fullerene allows to speed up its entry into Jurkast T-leukemia cells, viz. up to 20% at 3 h time-point (Fig. 6(A)) and 30% at 4 h time-point (Fig. 6(B)). This phenomenon was observed at concentrations of Dox equal to 1 μM, whereas application of C_{60}FAS had little effect when using high (2 μM) doses of Dox (Figs. 6(C) and (D)). These results highlight important carrier functions of C_{60} fullerene, which appears to be capable of increasing Dox concentration inside the cells even at low doses, whereas at high doses of Dox the speed of its diffusion through plasma membrane is insignificantly lower than its targeted delivery by C_{60} fullerene.

In order to additionally confirm this assumption a comparison of Dox and C_{60} + Dox accumulation in MCF-7 breast adenocarcinoma cells was performed by means of fluorescent microscopy (Fig. 7). One can see that at 6 h time-point the addition of C_{60} + Dox complexes to cell culture leads to more intensive Dox concentration in nuclei of MCF-7 cells (see Figs. 7(D) and (F)) compared to the action of Dox alone (Figs. 7(C) and (E)).

In order to verify whether the increased Dox accumulation in targeted cells due to its delivery by the C_{60} fullerene can intensify cell death (apoptosis) induced by this anticancer drug, the chromatin state in human breast adenocarcinoma cells of MCF-7 line was studied by DAPI staining. Hyper-condensation and disintegration of nuclear chromatin are typical hallmarks of apoptosis.[42] No significant DNA chromatin condensation was observed under the action of 0.5 μM Dox (Fig. 8(C)), whereas C_{60} + Dox complex with the same concentration of Dox had led to DNA condensation in ~50% of the MCF-7 cells (Fig. 8(D), 0.5 μM). The action of Dox at higher (1 μM) concentration (Fig. 8(E)) had led to a pronounced nuclear chromatin condensation in all treated tumor cells, whereas the application of C_{60} + Dox complexes had further enhanced this effect as evidenced from higher intensity of DAPI fluorescence (Fig. 8(F)). It follows that the C_{60} fullerene complexation with Dox did not only lead to 1.5-2-fold increase in cytotoxicity towards human tumor cells, but it was also accompanied by the enhanced induction of apoptosis.

Impact of C_{60} + Dox Complex on ROS Production and Mitochondrial Intactness in Targeted Tumor Cells

It was found that at late time points (24 h) the C_{60} + Dox complexes exerted weak protective effect towards mitochondria as revealed by cell staining with fluorescent dyes JC-1 and Rh123 (see Figs. 9(A) and (B)).

Rho123 is known to be a marker of functional status of mitochondria.[43] whereas JC-1 is a marker of mitochondrial membrane depolarization.[44] Decrease in the number of Rh123 positive mitochondria indicates a loss of their intactness, whereas increase in the number of JC-1 positive mitochondria indicates a depolarization of their mitochondrial membrane, which is one of the pre-requisites of apoptosis induction.[43,44] It was found that the C_{60} + Dox complexes lead to partial (from 70% to 50%) restoration in the number of depolarized mitochondria, and to slight (from 90% to 80%) decrease in the number of damaged

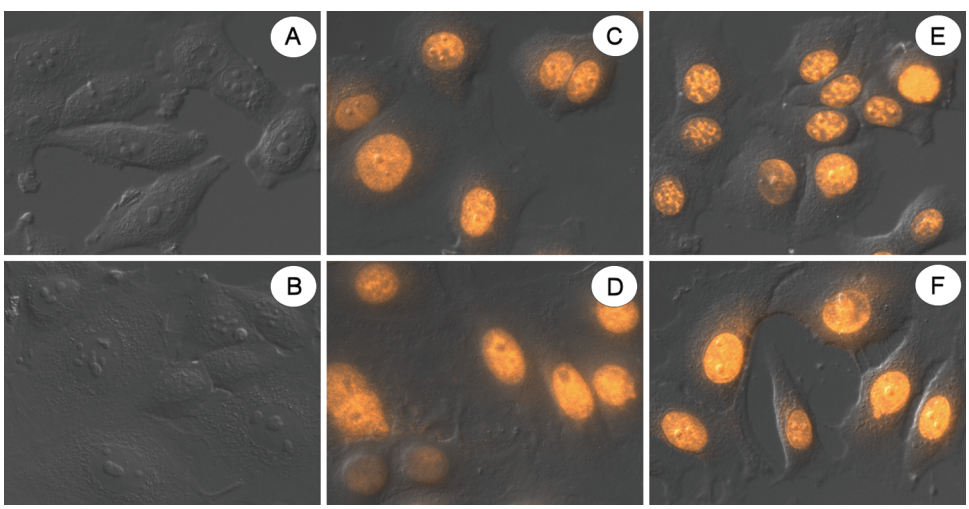

Figure 7. **Cytomorphological study of permeation of C_{60} + Dox complex and C_{60} fullerene alone into human breast adenocarcinoma cells of MCF-7 line under the action of C_{60} + Dox complex and Dox alone (6 h): (A)-control; (B)-C_{60} fullerene; (C)-Dox, 1 μM; (D)-C_{60}+Dox, 1 μM; (E)-Dox, 2 μM; (F)-C_{60}+Dox, 2 μM.**

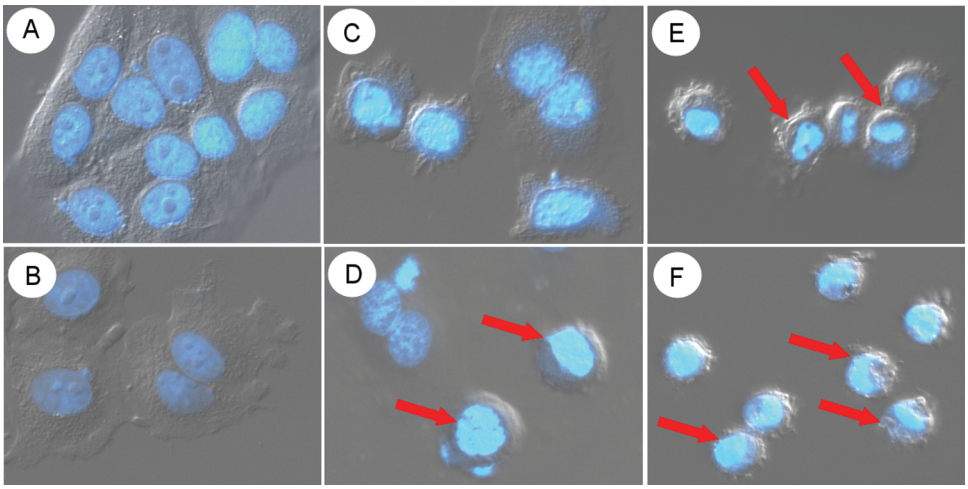

Figure 8. **Chromatin ultrastructure of human breast adenocarcinoma cells (MCF-7 line) under the action of C_{60} + Dox complex and Dox alone (24 h, DAPI staining): (A)-control; (B)-C_{60} fullerene; (C)-Dox, 0.5 μM; (D)-C_{60}+Dox, 0.5 μM; (E)-Dox, 1 μM; (F)-C_{60}+Dox, 1 μM. Arrows indicate chromatin hypercondensation (characteristic feature of apoptotic cells).**

mitochondria that were caused by Dox. We consider that such mitochondria-protecting activity of the C_{60} + Dox complex has no impact on the cytotoxic activity of Dox (see Fig. 5) which is of great importance for its further chemotherapeutic application. Taking into account strong sensitivity of cardiomyocytes to ROS action leading to mitochondrial damage and massive cell death in heart muscle, the detected mitochondria-protective effect of the C_{60} + Dox complex is considered to be very important.

The C_{60} fullerene complexation with Dox had a weak inhibiting effect on the superoxide production at early time points (1–12 h); however, it substantially increased the level of much less toxic hydrogen peroxide in 1 h after the addition of C_{60} + Dox complex to the studied tumor cells (Figs. 8(C) and (D)). One can see that such 2.7-fold increase of H_2O_2 levels under C_{60} + Dox treatment is a result of cumulative effect of Dox (2-fold increase of H_2O_2 when added alone) and C_{60} fullerene (2.5-fold increase). However, this oxidative burst had no impact on mitochondrial status in target cells treated with C_{60} + Dox complex, and, hence, the increase in H_2O_2 level does not seem to have any influence on mitochondria-protective effect of C_{60} + Dox complexes which is of high importance for decreasing negative side effects of Dox.

Both the detected and the suggested molecular mechanisms of potential anticancer action of the C_{60} + Dox

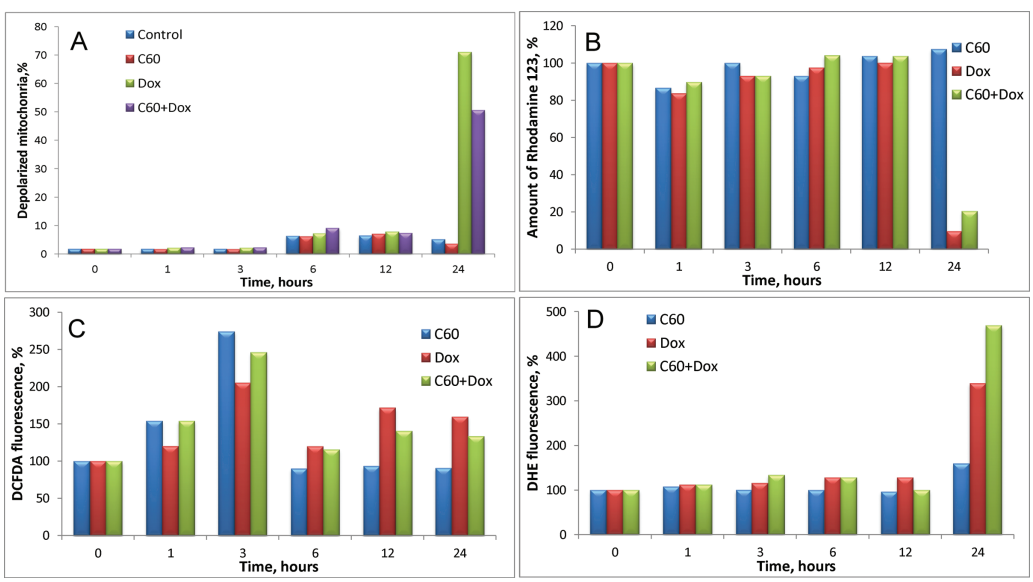

Figure 9. **Effect of C_{60} + Dox complex on production of hydrogen peroxide (A), superoxide radicals (B), depolarization of mitochondrial membrane (C) and Rhodamine 123 accumulation in mitochondria of Jurkat T-leukemia cells (D). The results of one of three independent experiments are presented. Baseline level of H_2O_2 and O_2^- in control cells is taken as 100%.**

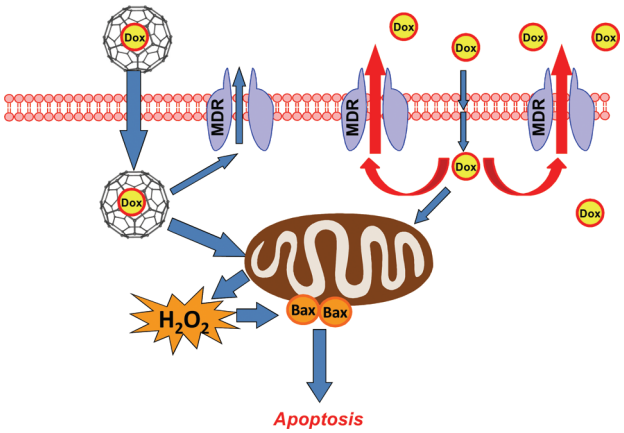

Figure 10. Proposed scheme of molecular mechanisms of anticancer action of C_{60} + Dox complex towards tumor cells.

complex were summarized in the scheme presented in Figure 10.

In Vivo Study of Antitumor Action of C_{60} + Dox Complex

Lewis lung carcinoma in mice was used as an experimental model for studying antitumor action of the C_{60} + Dox complex. This tumor was characterized by a significant growth of its size (volume) from the 10th to 22nd day of experiment (Fig. 11(A)). One can see that the all applied

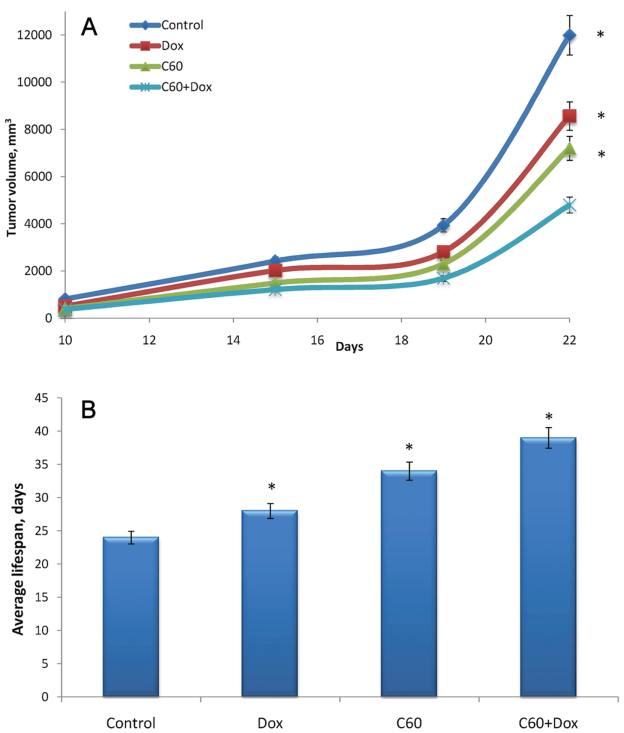

Figure 11. Changes in tumor volume (Lewis lung carcinoma) in mice (A) treated with Dox, C_{60} fullerene and C_{60} + Dox complex, and their average lifespan (B). Single dose of the corresponding drug was 1.5 mg/kg; *$P < 0.05$.

treatments (Dox, C_{60} fullerene, and C_{60} + Dox complex) caused a decrease (comparing to control, i.e., untreated mice) in tumor volume that was best observed on the 22nd day of experiment. The tumor volume decreased compared with the *control* both in the Dox *group 1* and C_{60} fullerene *group 2* by 1.4 and 1.7 times, respectively, whereas the decrease in C_{60} + Dox *group 3* was more pronounced (by 2.5 times). The potential effectiveness of applying these drugs in anticancer therapy was also confirmed by the calculated values of average life span of animals (Fig. 11(B)). In all experimental groups, this parameter demonstrated a distinct tendency to increase compared with the *control* (~24 days), namely, Dox *group 1* ~28 days (17% of increase), C_{60} fullerene *group 2* ~34 days (42% of increase) and C_{60} + Dox *group 3* ~39 days (63% of increase).

DISCUSSION

Severe toxicity of conventional anticancer drugs towards normal tissues and rapid development of tumor cell resistance to anticancer drugs are the main problems of chemotherapy in cancer treatment. Use of specific nanocarriers for addressed delivery of drugs to tumor cells is one of the most perspective ways to solve these problems, because it potentially allows to lower the overall drug toxicity and sensitizing drug-resistant tumor cells. In the present work we suggest to use C_{60} fullerene as a potential nanocarrier for Dox delivery to tumor cells. It is known that various fullerene-based materials exert toxic effects towards human cells *in vitro* and *in vivo*.[10, 13] At the same time, C_{60}FAS prepared in 0.1–1 mg/ml concentrations and containing both single C_{60} molecules as well as their aggregates with diameter up to 100 nm, did not demonstrate significant acute toxicity towards rat thymocytes within 24 h of treatment.[12]

Developing of resistance of tumor cells to anticancer drugs is a very important problem in clinical practice, since it leads to ineffectiveness of chemotherapy and endangers the life of cancer patients. It was found that resistance to anticancer drugs develops in 30–50% of cancer patients during the year after chemotherapy treatment start.[45] This is a multi-factorial and complex phenomenon,[39] which results in a significant decrease in drugs accumulation in cells by limiting their uptake, enhancing efflux, or affecting membrane lipids such as ceramide.[46] These changes lead to:

(1) inhibition of the programmed cell death (apoptosis) induced by majority of anticancer drugs;[47]

(2) activation of the mechanisms of general response that detoxify drugs and repair DNA damage;[48]

(3) alterations in cell cycle and its checkpoints that render cancer cells relatively resistant to cytotoxic effects of drugs.

MDR has been related to an increased production of ATP-binding cassette (ABC) transport proteins such as

P-glycoprotein, MRP-1 protein, and bcrp, whose high expression in human tumors is considered to be the first sign of negative prognosis for cancer patients.[40] Another mechanism of development of drug resistance is related to genetic defects in structure of Bax and p53 genes, whose products play an important role in regulation of cell cycle and apoptosis.[49] These defects lead to a synthesis of mutant and/or misfolded proteins which are unable to perform their inherent functions. As a result, tumor cells with defects in these genes exhibit higher invasive potential, increased ability to metastasize, and they are more resistant to chemotherapy than the cells containing intact copies of these genes.[50]

In the present work it was found that C_{60} + Dox complex leads to much faster accumulation of doxorubicin in nuclei of tumor cells compared to Dox alone (see Figs. 6 and 7), thus leading to enhancement of its anticancer effect as revealed by the drug toxicity studies and nuclear chromatin hypercondensation studies (see Figs. 5 and 8). In addition, application of C_{60} + Dox complexes was shown to partially overcome multidrug resistance of HL-60 leukemia cells to Dox, caused by overexpression of P-glycoprotein (Fig. 5(E)) by 20–30%. These data correlate with the results of drug permeation study which revealed 20–30% increase in delivery of Dox in C_{60} + Dox complex to tumor cells compared to Dox alone. It can be assumed that the enhanced permeation of Dox in the presence of C_{60} fullerene into P-gp overexpressing tumor cells can overcome P-glycoprotein capabilities of pumping out Dox from tumor cells, and thereby increase their sensitivity to this drug. However, in order to achieve higher MDR-overcoming capabilities of C_{60} + Dox complex, more tight conjugation of Dox on C_{60} fullerene should probably be required to ensure that it does not release from C_{60} fullerene too early and, thus, escapes from being used as a substrate for P-gp.

Mitochondria are the main energetic stations in the mammalian cells, and these organelles are essential for both life and death of cells. In normal cells they play principal role in ATP synthesis required for operation of all cellular processes, and any significant perturbation in mitochondria leads to apoptotic or necrotic cell death. Many chemotherapeutic drugs kill tumor cells via mitochondrial damaging, causing mitochondrial membrane permeability transition (MPT), membrane depolarization, osmotic swelling, and release of cytochrome *c* switching on biochemical mechanisms of cell death.[51] The ROS whose production is also enhanced under the action of many anticancer drugs, significantly impair not only tumor but also normal tissues. It is also known that intensive production of the superoxide induced by Dox is the main reason of cardiotoxicity observed on action of this anticancer drug.[52–54] Generation of ROS plays only a supplementary role in Dox anticancer activity, whereas its activity is mainly exerted via inhibition of DNA topoisomerase II.[54] Hence, effective inhibition of ROS produced under Dox treatment should decrease potential negative side effects of Dox towards normal cells while preserving little influence towards principal anticancer actions of this drug. In particular, it has already been shown that application of autophagy inhibitor rapamycin can significantly decrease ROS production in murine myoblasts under Dox action, and thereby attenuate its side effects towards heart tissue.[55, 56]

One can note that Dox alone leads to massive depolarization of mitochondria (70%, Figs. 9(A) and (B)) and loss of their intactness (only 10% of them remained intact). In contrast, C_{60} + Dox complex significantly decreases mitochondrial depolarization (up to 50%) compared to native Dox (70%), as well as increases the number of intact mitochondria (from 10% to 20%) at 24 h timepoint after drug addition to cell culture. It follows that C_{60} + Dox complex, despite increased cytotoxicity of Dox towards tumor cells due to its enhanced delivery, also decreases its effect on mitochondria depolarization.

Surprisingly, an elevated level of hydrogen peroxide at early timepoints of C_{60} + Dox application (1–3 h) was observed. Hydrogen peroxide is known to be a crucial signaling molecule which can also enhance apoptotic processes via activation of various death signaling pathways.[57] Previously we found that pristine C_{60} fullerenes can act as ROS scavengers due to unique chemical structure of fullerene molecule.[18] We consider that the increase in H_2O_2 production under the action of C_{60} + Dox complex is caused by membrane-targeting ability of the C_{60} fullerenes that was recently demonstrated by us.[17] Hence, direct penetration of the mitochondrial membrane by the C_{60} fullerene may lead to a disruption of normal mitochondrial functions, activation of superoxide dismutase enzyme (SOD), and a burst of H_2O_2 production which, in such way, modulates cytotoxic effect of Dox towards tumor cells.

In a scheme in Figure 10 we summarized both the detected and the suggested molecular mechanisms of potential anticancer action of the C_{60} + Dox complex. We consider that the membranotropic activity of C_{60} fullerene leads to an enhanced entry of the complex of C_{60} fullerene with Dox in cytosol of tumor cells, thus increasing its anticancer activity. Then, the C_{60} + Dox complexes target mitochondrial membrane leading to a loss of its integrity and early generation of the hydrogen peroxide. Oligomerization of Bax protein and further induction of apoptosis are mediated by the mitochondria. At late stages of C_{60} + Dox-induced cell death the C_{60} fullerenes may also involve their ROS-scavenging activity leading to partial restoration of the mitochondrial membrane potential. This effect does not influence Dox toxicity towards tumor cells; however, it may be protective for normal cells in the treated organism.

Simultaneous decrease of mitochondrial damage caused by Dox and increase of its targeting of tumor cells by C_{60} fullerene was confirmed by *in vivo* studies on mice bearing Lewis carcinoma. One can see that application

of C_{60} + Dox complexes resulted in extension of survival time of tumor bearing animals by 2.5-fold and led to 63% decrease of tumor volume compared to animals, treated by Dox alone. It should be stressed that such enhancement of Dox anticancer action was not accompanied by an increase of its side effects, as revealed by the morphophysiological state of tumor-bearing animals (data not presented). Hence, the observed mitochondria-protective effect of C_{60} + Dox complexes could be of crucial importance for decreasing the cardiotoxicity of this drug.

The results of the *in vivo* study demonstrated that the anticancer activity of Dox is well preserved by its complexation with C_{60} fullerene. Moreover, the use of such chemotherapy results in synergistic effect. The increased damage of tumor cells exposed to C_{60} fullerenes alone or in complex with Dox could be a consequence of their accumulation in tumor tissue due to its high membrane permeability.[14–17, 58] Possessing an antioxidant activity[18, 33] C_{60} fullerene may neutralize an excess of ROS in the treated cell and presumably block specific receptors in cell membrane, for example, the receptors of the endothelial growth factor.[59–61] Moreover, C_{60} fullerene treatment, similar to fullerol action,[62] can activate the macrophages and, therefore, be responsible for the inhibition of tumor growth. Besides, one can assume that the pristine C_{60} fullerene combined with Dox effectively reduces toxic effect of this drug similarly to fullerenol action[63] via inhibition of the oxidative stress.

CONCLUSIONS

Stable complex of pristine C_{60} fullerene with Dox was prepared and characterized by means of different physico–chemical methods. Spectroscopic and molecular modeling data confirmed the ability of C_{60} fullerene to form non-covalent complex with Dox in aqueous solution. The C_{60} fullerene complexation with Dox leads to 1.5–2-fold increase (compared to Dox alone) in its toxicity towards various human tumor cell lines, including those with different mechanisms of drug resistance. This effect of C_{60} + Dox complex was accompanied by a significantly higher induction of apoptosis in tumor cells compared with the effect of Dox alone, as well as by more pronounced stimulation of production of hydrogen peroxide, whereas the production of superoxide did not change significantly. Treatment of Lewis lung carcinoma-bearing male mice of C57Bl/6J line with the C_{60} + Dox complex inhibited tumor growth significantly (by 2.5 times) and increased an average life span of animals to higher extent (by 63%) than that detected at separate administration of Dox or C_{60} fullerene. It was suggested that tumor treatment by the complex of C_{60} fullerene with Dox can be a promising approach in developing new chemotherapies for clinical use.

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