Enhancement of anticancer action of traditional (doxorubicin and cisplatin) and experimental (landomycin A) drugs by their delivery *in vivo* with novel C_{60}-fullerene-based nanocarrier possessing innate ROS-modulating activity

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ABSTRACT

Novel nanocomposites based on C_{60} fullerene were prepared for delivery of anticancer drugs doxorubicin (Dox), cisplatin (Cis), and experimental drug landomycin A (LA). It was demonstrated that these nanocomposites possess 1,5-3-fold higher cytotoxic activity towards various drug-resistant tumor cell lines *in vitro* compared to aforementioned drugs in free form. This process is accompanied by stronger induction of apoptosis in target cells. *In vivo* studies of these nanocomposites in mice have shown similar tendencies leading to 2-fold higher growth inhibition of Lewis lung carcinoma compared to action of Cis and Dox alone. The ability of C_{60}-drug complexes to overcome drug resistance was also confirmed by molecular docking studies, showing that C_{60} fullerene inhibits P-glycoprotein and MRP-1 by generating a significant amount of Van-der-Waals interactions with their binding sites. Thus, tumor treatment by C_{60} fullerene complex with Cis, Dox or LA could be a promising approach in developing new chemotherapies for clinical use.

Keywords: cancer drug resistance, C_{60} fullerene, apoptosis, nanocarriers, *in vivo*

1 INTRODUCTION

Chemotherapeutic agents used in clinical oncology have a series of disadvantages which substantially diminish their therapeutic effect in tumor treatment. Thus, a development of novel drug delivery systems possessing enhanced therapeutic effect, decreased side effects and ability to circumvent cancer drug resistance, is of key importance for current pharmacology and oncology. In this study, we addressed C_{60} fullerene – an allotropic carbon modification, which is non-toxic for normal cells and possesses strong antioxidant activity. C_{60} fullerene was used as a platform for immobilization of "gold standards" of chemotherapy – Dox and Cis that are widely used in clinics, as well as novel angucycline antibiotic landomycin A (LA) possessing a unique feature of circumventing cancer drug resistance (1). The main goal of present study was to analyze the actions of Dox, Cis, LA and their complexes with C_{60} fullerene *in vitro* and *in vivo*.

2 MATERIALS AND METHODS

The pristine C_{60} fullerene aqueous colloid solution (C_{60}FAS) in 0.5 mg/ml concentration of C_{60} fullerene were prepared according to the protocol (2), and used in the experiments. For preparation of drug-fullerene complexes, initial solutions of C_{60}FAS and Dox/Cis/LA were mixed in 1:1 weight ratio. The resulting mixture was treated for 10 min with the ultrasonic disperser, and after that it was subjected to overnight magnetic stirring at room temperature.

In silico studies of C_{60} fullerene interactions with ABC-transporters were done using ClustalW software and most representative structure 4M1M of P-glycoprotein, 2CBZ structure of MRP-1 and 2GHI model of MRP-2, homologuously simulated to human organism (3). Water molecules were removed from the selected proteins, and Arg and Lys residues were protonated. The procedure was

performed using flexible ligand model employing systematic docking algorithm (sdock+).

Human HeLa cervix cancer cell line and human Jurkat T-leukemia cell line were derived from the ATCC. Human cervix carcinoma KB-3-1 cell line and its colchicine-selected KBC-1 subline (P-gp overexpressing) were a generous gift of Dr. Shen (NIH, Bethesda, USA). Human leukemia HL-60 cell line and its drug-resistant sublines HL-60/adr (MRP-1 overexpression) and HL-60/vinc (P-glycoprotein overexpression) were donated by Dr. M. Center (Kansas State University, Manhattan, USA). For cell death analyses, cells were stained with annexin-V-FITC and propidium iodide (PI) using the apoptosis detection kit (BD Biosciences, San Jose, CA), according to the manufacturer's instructions, and were further analyzed on BD FACScan flow cytometer. For analysis of nuclear chromatin condensation, cells were stained with DAPI (4',6-diamidino-2-phenylindole, Sigma Aldrich, St. Louis, USA) and further studied under Carl Zeiss AxioImager A1 fluorescent microscope (Carl Zeiss, Gottingen, Germany).

In vivo studies were performed on male mice (18-19 g) of C57BL/6J line bearing Lewis lung carcinoma (LLC), according to the protocol (4). Mice were bred and maintained on a standard diet at 25 ± 1 °C in the animal facility of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine (Kyiv, Ukraine). Experiments were conducted in accordance with the international regulations of the European Convention for protection of vertebrate animals under the control of the Bio-Ethical Committee of mentioned Institute.

3 RESULTS AND DISCUSSION

3.1 Interaction of C_{60} fullerene with Dox in aqueous solution

A direct complexation between C_{60} fullerene and Dox molecules under the condition of their 1:1 mixture has been studied by the UV/VIS spectroscopy. In order to exclude the 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. A transformation of the Dox spectrum as a consequence of drug addition to the C_{60}FAS was found (Fig. 1A). The hypochromic shift of the absorption maximum from 481.5 (Dox) down to 479.0 nm (C_{60}+Dox), as well as the hypochromic effect, were 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%, that is in agreement with the magnitude of this effect observed in our previous study of C_{60}+Dox interaction. DLS technique was applied for further investigation of C_{60}+Dox interaction regarding a distribution of clusters by their dimensions (Fig. 1B). The most remarkable feature of the intensity-weight distribution shown in Fig. 1 is a significant shift of the peak maximum from 160 to 600 nm, as well as an appearance of a shoulder on the left side of the distribution under the action of Dox. A large shift (400 nm) in distribution cannot be explained by an external binding of Dox molecules to C_{60} fullerene clusters. It could be caused by the formation of large clusters composed of ordered mixture of Dox and C_{60} molecules.

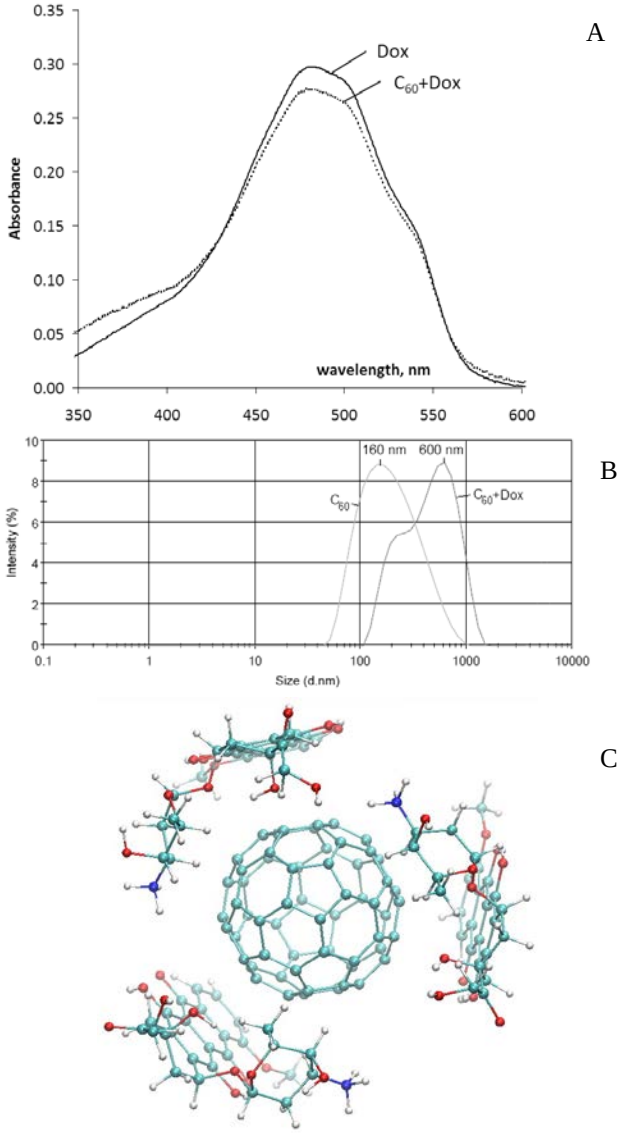

Fig. 1. Electronic absorption spectra of Dox (0.26 mM) in the presence and absence of C_{60} fullerene in 1:1 composition of the mixture (A), size distribution by intensity of C_{60}FAS and C_{60}+Dox mixture (B), and calculated structure of C_{60}+Dox complex (C)

3.2 Studies of i*n vitro* toxicity of C_{60}-drug complexes

Based on promising results of the molecular docking studies, the potential ability of C_{60}+Cis/Dox/LA complexes to circumvent drug resistance was studied *in vitro* using cell

lines with acquired multidrug resistance phenotypes. Several tumor cell lines differing in drug resistance mechanisms (overexpression of P-gp, MRP-1, knockout of key genes involved in cell cycle regulation and apoptosis) were addressed. They belong to three cancer types commonly treated with Cis, namely colon and cervix cancer, and leukemia. The highest difference (over 2-fold) in the cytotoxic activity between the C60-drug complexes and aforementioned drugs alone was observed in human HL-60 leukemia cells and its drug-resistant sublines, characterized by the expression of various ABC-transporter proteins (Fig. 2).

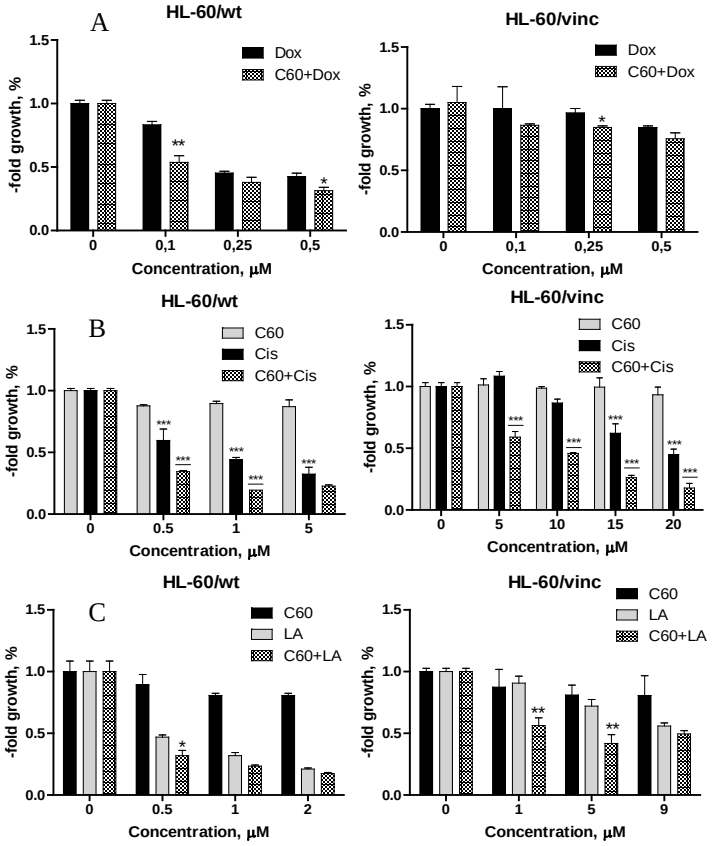

Fig. 2. Cytotoxic activity of C_{60} fullerene and its complex with Dox (A), Cis (B) and LA (C) towards leukaemia cell lines. The graphs present the effects of the treatment with C_{60} fullerene (presented in gray), drug (presented in black) and C_{60}+drug complex (presented in crossed gray). Cell viability was estimated using trypan blue exclusion assay. The effect of drugs and C_{60}-drug complexes on cell growth was plotted relative to the untreated control. Data are given relative to the untreated control samples and represent the mean +/- SD of three independent experiments. *p < 0.05 relative to control, ** p < 0.01 relative to control, *** p < 0.0001 relative to control, unpaired t-test. Significance levels indicated directly above bars refer to the comparison with the respective vehicle-treated controls.

In order to study potential mechanism underlying increased cytotoxic activity of C_{60}-Dox/Cis/LA complexes, their impact on apoptosis induction and cell cycling in tumor cells was studied using flow cytometry and fluorescent microscopy. C_{60}-Cis complex caused a significant increase in number of annexin V-positive HL-60/adr (MRP-1+) cells compared to single Cis action at all used concentrations (Fig. 3A). The most prominent effect was observed for 15 μM dose of these compounds (19.16% vs 10.11%, p<0.01). The same effects were observed also for C_{60}-LA complex targeting Jurkat T-leukemia cells (Fig. 3B), as well as for C_{60}-Dox complex analyzed by DAPI assay (Fig. 3C). Thus, in all studied cases C_{60} fullerene enhanced cytotoxic activity of used drugs *in vitro* by increasing their proapoptotic potential.

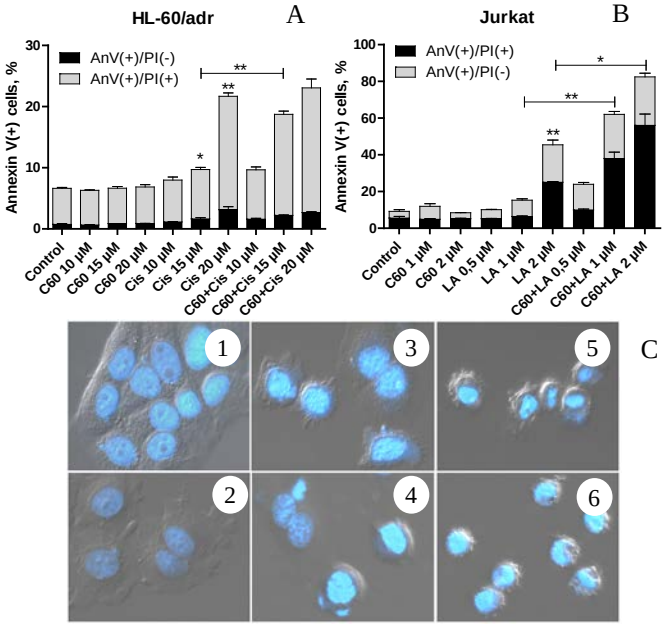

Fig. 3. Flow cytometry analysis of apoptosis induction in human leukemia cells of HL-60/adr (MRP-1+) and Jurkat lines under the action of complex of C_{60} fullerene with Cis (A) or LA (B), and cytomorphological study of chromatin ultrastructure of human breast adenocarcinoma cells of MCF-7 line under the action of C_{60}-Dox complex (C). Apoptosis analysis was conducted using Annexin V-FITC/PI assay and DAPI staining, respectively. Data are given relative to the untreated control samples and represent the mean +/- SD of three independent experiments. *p < 0.05 relative to control, ** p < 0.01 relative to control, unpaired t-test. Significance levels indicated directly above bars refer to the comparison with the respective vehicle-treated controls. 1 – control, 2 – C_{60}FAS, 3 – Dox, 0.5 μM, 4 – C_{60}+Dox, 0.5 μM, 5 – Dox, 1 μM, 6 – C_{60}+Dox, 1 μM

3.3 *In vivo* studies of C_{60}-drug complexes

Studies of therapeutic activity of C_{60}-Dox and C_{60}-Cis complexes were performed on an establieshed solid tumor model – Lewis lung carcinoma (LLC).

All treatments resulted in a decrease of tumor volume, when compared to the control (untreated group) (Fig. 4A). At the 20th day of the experiment, the tumor volume in mice of group 1 (treated with FC_{60}) and group 2 (treated with Cis alone) was reduced by 15% compared to the control. However, the most prominent antitumor effect was observed in the case of mice of group 3 (treated with C_{60}+Cis complex), where the decrease in tumor volume reached 75% of the control (Fig. 4A). Similar tendencies were observed for the action of C_{60}-Dox complex (Fig. 4B).

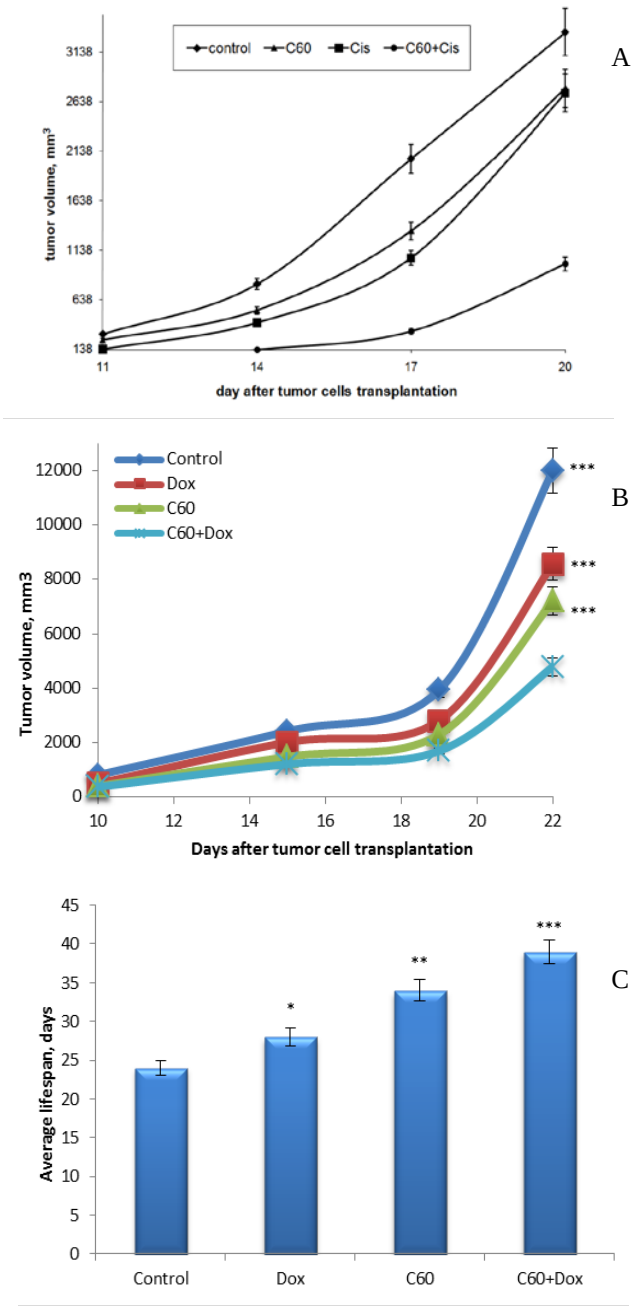

Fig. 4. Changes of tumor volume in Lewis lung carcinoma bearing mice treated with C_{60}-Cis (A) and C_{60}-Dox complexes (B), and mean of average lifespan of tumor-bearing animals (C).

Tumor volume decreased compared with the control in both Dox group 1 and C_{60} fullerene group 2 by 1.4 and 1.7 times, respectively, while such decrease in C_{60}+Dox group 3 was more pronounced – 2.5 times. The effectiveness of applying these drugs in the anticancer therapy was also confirmed by the calculated values of average life span of animals (Fig. 4C). In all experimental groups, this parameter demonstrated a distinct tendency to increase comparing with the control one (~24 days), namely, Dox group 1 – ~28 days (17% increase), C_{60} fullerene group 2 - ~34 days (42% increase) and C_{60}+Dox group 3 - ~39 days (63% increase).

4 CONCLUSIONS

Stable complexes of Dox, Cis and LA with pristine C_{60} fullerene were prepared and characterized by means of different physico-chemical methods. The spectroscopic and molecular modeling data confirmed the ability of C_{60} fullerene to form the non-covalent complex with Dox, LA and Cis in aqueous solution. Complexation of these drugs with the C_{60} fullerene leads to 1.5-2-fold increase in their toxicity towards various human tumor cell lines, including those with different mechanisms of drug resistance. This effect of C_{60}+Dox/Cis/LA complexes was accompanied by a significantly higher induction of apoptosis in tumor cells compared with the effects of these drugs alone. Treatment of Lewis lung carcinoma-bearing male mice of C57Bl/6J line with the C_{60}+Dox and C_{60}+Cis complexes inhibited tumor growth significantly (by 2.5 times) and increased an average life span of animals to a higher extent (by 63%) than that detected at separate administration of Dox, or Cis, or C_{60} fullerene. Thus, using novel nanocomposites based on C_{60} fullerene might be a promising approach in developing new chemotherapies for clinical use.

Approval of BioEthics Committee. This study and the experimental procedures performed on animals were approved by the Ethical Committee at R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine (Kyiv, Ukraine), Protocol N1 from 01.09.2014, and Protocol N4 from 1.02.2016.

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