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MODULATION OF CISPLATIN-INDUCED REACTIVE OXYGEN SPECIES PRODUCTION BY FULLERENE C_{60} IN NORMAL AND TRANSFORMED LYMPHOID CELLS

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The early response of normal (Wistar rat thymocytes) and transformed (mice lymphoid leukemia L1210) cells to treatment with anticancer drug cisplatin or to combined treatment with cisplatin and carbon nanostructure fullerene C_{60} was studied. We demonstrated with fluorescent probes DCFH-DA and TMRE that cisplatin at concentration 1 μg/ml induced reactive oxygen species (ROS) production and decreased the value of mitochondrial membrane potential in both cell types. The combined treatment with cisplatin (1 μg/ml) and fullerene C_{60} (7.2 μg/ml) was shown to be followed by oppositely directed modulation of ROS production in thymocytes and L1210 cells. Cisplatin-induced ROS production was intensified in L1210 cells, while in thymocytes it was decreased. It is supposed that the different effects of combined treatment are associated with peculiarities of fullerene C_{60} accumulation and localization in normal and cancer cells.

K e y w o r d s: cisplatin, fullerene C_{60}, ROS, mitochondrial membrane potential, thymocytes, L1210 cells.

Cisplatin (CP) is one of the primary chemotherapeutic agents used for treatment of malignant tumors. It is a metal-containing compound and an alkylating agent that covalently binds to DNA and exerts cytotoxic, bacteriostatic and mutagenic effects. The toxicity of the compound is either due to its DNA-platinum adduct products [1], or due to extranuclear effects mediated by initiation of apoptosis via increased reactive oxygen species (ROS) production, changes in calcium signaling, or depolarization of mitochondrial membrane [2, 3].

Along with its positive chemotherapeutic effect, cisplatin exhibits noticeable side effects (i.e. nephrotoxicity, hepatotoxicity, and cardiotoxicity) that limits its application in therapeutic dosage [2]. Thus, it is currently of importance to identify compounds that, combined or in complex with antitumor drugs, may potentiate the cytotoxic effect in cancer cells and limit it in normal cells. Carbon nanostructures, and fullerene C_{60} in particular, are promising objects of study in this respect. Fullerene C_{60} can permeate plasma membrane, accumulate within cell and bind free radicals due to a network of conjugated double bonds on its surface, thus acting as an antioxidant [4, 5]. It can also produce ROS if photoexcited [6]. C_{60} application as a modulator of antitumor drugs cytotoxic effect is promising for modifying approaches in anticancer therapy.

The aim of the present study was to evaluate the rate of ROS production and the value of mitochondrial membrane potential as the early effects of fullerene C_{60}, cisplatin, and their combination in normal (rat thymocytes) and transformed (mouse lymphocytic leukemia L1210) cells.

Materials and Methods

The thymocytes were isolated from thymus of Wistar rats (150-180 g). Thymus (200-300 mg) was removed, cleaned from blood and connective tissue and passed through nylon mesh into buffer A of the following composition (in mM): Na_2HPO_4 – 3, KCl – 5, NaCl – 120, $CaCl_2$ – 1, glucose – 10, $MgSO_4$ – 1, $NaHCO_3$ – 4, HEPES – 10; pH 7.4. The cell suspension was centrifuged (5 min, 600 g) in the same medium, the sediment was resuspended to a concentration of 2-5×10^8 cells per ml. L1210 cells (lymphocytic leukemia) had been obtained from cell bank of RE Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology of NAS of Ukraine. The ascitic form of L1210 cancer cells was obtained after 8-10 day of intraperitoneal injection of cells from donor animals into mice hybrids F_1 DBA2 with body mass of 20 g. The L1210 cells were washed from ascitic fluid by centrifugation (10 min, 600 g) in buffer A, and used in the experiments.

The animal experiments were conducted in accordance with guidelines of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes.

The cell count was performed with Biolam "LOMO" P12 in Goryaev hemocytometer with 0.4% solution of trypan blue.

Cell viability was assessed by rate of MTT reduction (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) [7]. The test was performed in 96-well plates, cell count per well was 1-5×10^6 in 200 μl of RPMI medium. 20 μL of MTT solution was added to each well and incubated for 2 h at 37 °C. The plates were then centrifuged for 7 min at 600 g. Formazan sediment was dissolved with 150 μL of concentrated dimethyl sulfoxide and assayed in digital spectrometer (μQuant, BioTEK, USA) at λ = 570 nm. Cell viability was calculated as percentage to control.

A stable water colloid solution of pristine fullerene C_{60} was prepared in Ilmenau Technical University (Germany) [8]. The fullerene C_{60} samples used in the experiments were over 99.5% pure; the average hydrodynamic diameter of nanoparticles was 50 nm [9]. L1210 cells were preincubated with fullerene C_{60} (7.2 μg/ml, 10^{-5} M) for 1.5 h to load them with nanoparticles [10], and then cisplatin was added (Sigma, USA).

ROS production was measured using fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma, USA), which was added to the cell incubation medium (1×10^6 cells/ml) in concentration 5 μM. The probe's fluorescent signal was assayed in real time by Shimadzu RF-1501 spectrofluorometer (Japan), λ_{exc} = 480 nm, λ_{em} = 520 nm [11].

Mitochondrial membrane potential was determined with fluorescent potential-sensitive probe tetramethylrhodamine ethyl ester perchlorate (TMRE, Sigma, USA). Cells suspended in buffer A (10^7 per ml) were loaded with the probe for 40 min at 25 °C with addition of Pluronic F-127 (0.05%) to facilitate probe dissolution in hydrophilic medium. The cells loaded with probe (1×10^6 per ml) were incubated at 25 °C. TMRE fluorescence was registered with Shimadzu RF-1501 spectrofluorometer (Japan), λ_{exc} = 540 nm, λ_{em} = 595 nm. Relative values of mitochondrial potential were determined as changes in probe fluorescence after addition of protonophore FCCP (1 μM) [12].

Data analysis was performed in MS Excel 2010. Statistical analysis of the results was done with conventional methods of variance statistics using Student's *t*-test [13].

Results and Discussion

We investigated effects of CP in various concentration on viability of L1210 leukemic cells after 24 h incubation.

The viability of the cells after treatment with CP in concentration range from 0.1 to 10 μg/ml decreased in dose-dependent manner (Fig. 1). Cell viability after treatment with 0.1 μg/ml of CP remained within control limits, decreased by 25% after treatment with 1 μg/ml CP, further increase of CP concentration (up to 10 mg/ml) caused more pronounced drop in the values of this parameter.

In order to study the capability of fullerene to potentiate the effects of CP in a low doses, we evaluated the relative value of mitochondrial membrane potential as an indicator of early influence of these compounds on leukemic and normal cells. We used rat thymocytes as a relative control in these model experiments to compare the effects of the compounds.

The relative values of mitochondrial potential in L1210 cells are higher than those in thymocytes, as shown on Fig. 2. These results are in good agreement with data concerning increased activity of electron-transport chain and higher hyperpolarization of mitochondrial inner membrane in cancer cells [14, 15].

The mitochondrial potential changes were comparable in both leukemia and normal lymphoid cells (approx. 50% decrease), which indicates gene-

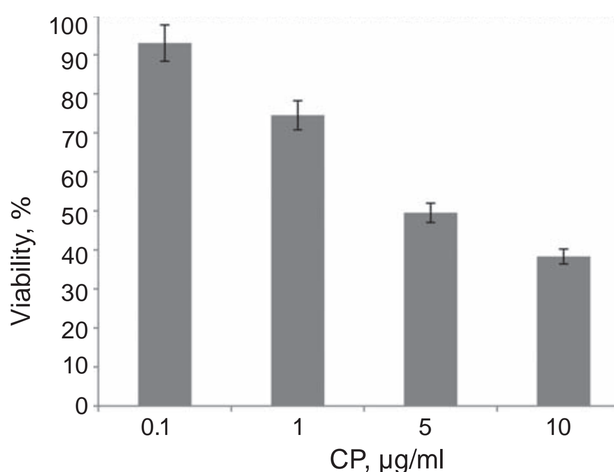

Fig. 1. Viability of L1210 cells after 24 h incubation with cisplatin (CP) in different concentrations, (n = 6)

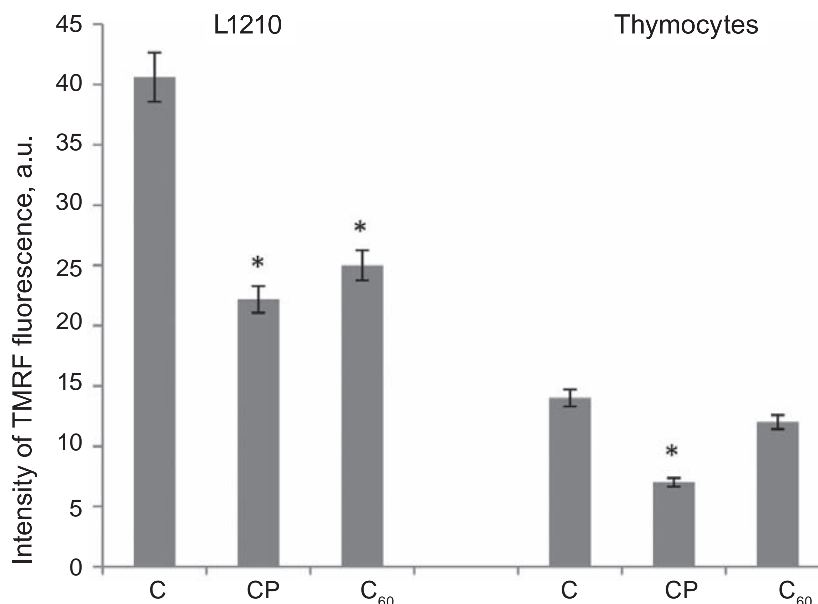

*Fig. 2. Relative value of mitochondrial membrane potential in L1210 cells and thymocytes treated with CP (1 μg/ml) and fullerene C_{60} (7.2 μg/ml) (n = 4); * denotes $P \leq 0.05$ in comparison to control (C)*

ral cytotoxicity of antitumor drug and its potential to cause early disturbance of mitochondrial status. An unspecific induction of mitochondrial apoptotic pathway in breast cancer cells (MCF-7) as well as in non-transformed kidney cells (LLC-PK1) under effect of CP had been demonstrated in [3, 16]. The authors had demonstrated that CP induced Bax translocation to mitochondria and decreased mitochondrial membrane potential in both cell types.

We found differences in response of mitochondria in L1210 cells and thymocytes to treatment with fullerene C_{60}. The relative values of mitochondrial potential was not changed in thymocytes preincubated with the nanostructure, while was decreased in leukemic cells (Fig. 2).

These differences in fullerene C_{60} effect can be linked to modified properties of plasma membrane, enhanced uptake of compounds, particularly of fullerene C_{60}, in cancer cells.

Fullerene C_{60} is accumulated by transformed cells (epithelial Hep-2, breast cancer MCF10A, leukemic L1210, and keratinocytes HaCaT). The capacity of C_{60} and its derivatives to bind to mitochondrial membranes and permeate into intermembrane space had been demonstrated with FITC-labeled monoclonal antibodies [17, 18]. It has been proposed that the negative surface charge of fullerene nanostructure promotes its binding to mitochondrial membranes, dissipation of proton gradient and mitochondrial membrane depolarization [19-21].

Since we did not find significant decrease in L1210 cell viability after 24 h treatment with 1 μg/ml of CP (Fig. 1), as well as after fullerene C_{60} loading [22], we can assume that a decrease of mitochondrial potential in L1210 cells treated with these effectors may be of adaptive nature and does not affect cells' survival after longer period of incubation.

Next task was to evaluate ability of fullerene C_{60} in combination with CP in low concentrations to modulate the cytotoxic effect of the drug. The role of intensified ROS production in apoptosis induction in cancer cells if overcoming their antioxidant defense is currently widely accepted [23, 24]. As there is a correlation between mitochondrial functional state and level of ROS production, the effect of combined treatment with C_{60} and CP on ROS generation in normal and leukemic cells was studied.

The results presented in Fig. 3. (*A*, *B*) demonstrate that CP at concentration 1 μg/ml increased ROS production in L1210 cells as well as in thymocytes, which is in accordance with decrease of mitochondrial potential in these cells. The intensification of ROS production after treatment with CP at concentration 5 μg/ml was observed, more pronounce effect was in thymocytes than in leukemic cells. Such differences in dynamics of ROS produc-

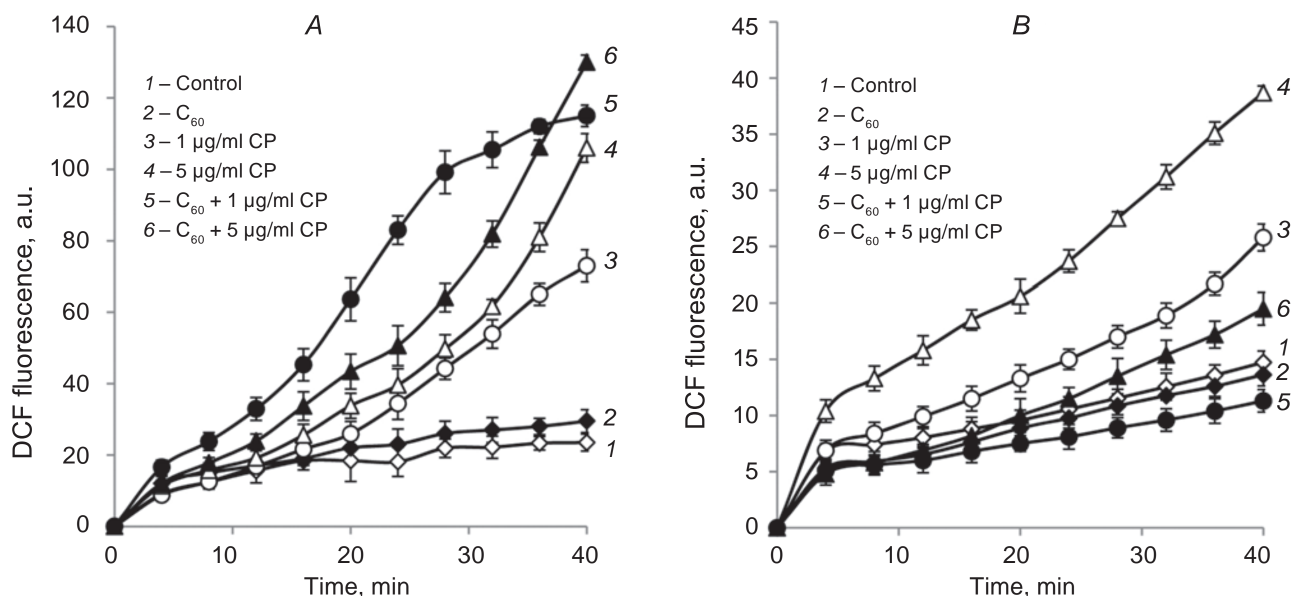

Fig. 3. The dynamics of ROS production in L1210 cells (A) and thymocytes (B) under effect of fullerene C_{60}, CP and their combination; DCF, dichlorofluorescein (n = 4)

tion may be due to higher activity of antioxidant enzymes in L1210 cells in comparison to that in thymocytes [25].

The prooxidative effect of CP can be explained by its influence not only upon mitochondria, but also upon endoplasmic reticulum as another extranuclear target. Cisplatin has been demonstrated to cause time-dependent increase of ROS production in HTC116 (colon cancer), MCF-7 (breast cancer), and HeLa (cervix cancer) cells [26-28], presumably due to activation of NADPH-oxidases NOX-1 and NOX-4 [29]. Induction of oxidative stress by CP via inhibition of antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase) has been also demonstrated in non-transformed kidney cells (LLC-PK1, RTE) and in hepatocytes [16].

Preincubation with fullerene C_{60} did not affect ROS production in L1210 cells and in thymocytes, yet it did modulate the prooxidative effect of CP in normal and leukemia cells in opposite directions. In L1210 cells combined treatment with C_{60} and 1 μg/ml CP is followed by more pronounce intensification of ROS production in comparison to the effect of the drug alone in this dose. We also observed a synergistic effect of fullerene C_{60} and CP in dose 5 μg/ml on ROS generation in leukemic cells. This effect of combined treatment on ROS production can be connected with ability of fullerene C_{60} to enhance endocytosis in cancer cells. The metallofullerene in complex with CP has been demonstrated to increase intracellular CP accumulation in human prostate cancer cells by activation of endocytosis, the suppression of which is one of the possible mechanisms of antitumor drug resistance [30].

According to the results presented in Fig. 3 (*B*), in thymocytes fullerene C_{60} modulates CP-induced ROS production by exhibiting of antioxidant effect. In thymocytes ROS production after combined treatment with fullerene C_{60} and CP at concentration 1 μg/ml is not higher than in control, and under fullerene with CP in a dose of 5μg/ml is significantly weaker than the effect of the drug alone.

It is supposed that our results on opposite directed effects in thymocytes and L1210 cells after combined treatment is connected with differences in fullerene C_{60} nanoparticles interaction with plasma membrane, rate of its uptake and distribution inside normal and cancer cells. For instance, we found that incubation of thymocytes with fullerene C_{60} leads to inhibition of plasma membrane ecto-ATPase activity in thymocytes, but not in MT-4 leukemic cells [17, 31]. It is possible that suppression of CP-induced prooxidative effect by fullerene C_{60} in thymocytes is due to its accumulation in plasma membrane and adjacent of endoplasmic reticulum. The mechanism underlying the antioxidant activity of C_{60} is known to be the interaction between ROS and conjugated double bonds system on the surface of C_{60}, which results in e^- acceptance, transition of unstable 4n π-electron

system to stable (4n+2) system with production of stable C_{60} radical. This mechanism may be realized as well if the nanostructure is localized within cellular membranes [32].

The protective effect of fullerene C_{60} and its derivatives has been confirmed also under hydrogen peroxide treatment of thymocytes [33] and under treatment of non-malignant transformed cells (LLC-PK1, kidney columnar epithelial cells) with anticancer drug [34, 35].

Therefore, our results indicate the possibility of potentiation of CP cytotoxicity in low concentrations against leukemic cells after combined treatment with fullerene C_{60}. The detected intensification of ROS production in leukemic cells under combined treatment may indicate that fullerene C_{60} can reinforce extranuclear mechanisms of CP action, leading to induction of cancer cell death. The positive side to this is also a protective effect of fullerene C_{60} towards CP-induced ROS production in normal cells.

МОДУЛЯЦІЯ ІНДУКОВАНОГО ЦИСПЛАТИНОМ ПРОДУКУВАННЯ АКТИВНИХ ФОРМ КИСНЮ ФУЛЕРЕНОМ C_{60} У НОРМАЛЬНИХ ТА ТРАНСФОРМОВАНИХ ЛІМФОЇДНИХ КЛІТИНАХ

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Досліджено ранні прояви дії протипухлинного препарату цисплатину та його комбінованої дії із представником вуглецевих наноструктур фулереном C_{60} на нормальні (тимоцити щура Wistar) та трансформовані (лімфоїдна лейкемія миші L1210) клітини. З використанням флуоресцентних зондів DCFH-DA та TMRE показано, що цисплатин (1 мкг/мл) спричиняв продукування активних форм кисню (АФК) та знижував величину мітохондріального потенціалу в клітинах обох типів. Комбінована обробка цисплатином (1 мкг/мл) та C_{60} (7,2 мкг/мл) призводила до різноспрямованої модуляції продукування АФК у тимоцитах та клітинах L1210. Індуковане цисплатином продукування АФК у клітинах L1210 посилювалось, тоді як у тимоцитах зменшувалось. Припускається, що виявлені різноспрямовані ефекти пов'язані з відмінностями в акумуляції та локалізації фулерена C_{60} у нормальних та злоякісних клітинах.

К л ю ч о в і   с л о в а: цисплатин, фулерен C_{60}, АФК, мітохондріальний мембранний потенціал, тимоцити, клітини L1210.

МОДУЛЯЦИЯ ИНДУЦИРОВАННОГО ЦИСПЛАТИНОМ ПРОДУЦИРОВАНИЯ АКТИВНЫХ ФОРМ КИСЛОРОДА ФУЛЛЕРЕНОМ C_{60} В НОРМАЛЬНЫХ И ТРАНСФОРМИРОВАННЫХ ЛИМФОИДНЫХ КЛЕТКАХ

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Исследованы ранние проявления действия противоопухолевого препарата цисплатина и его комбинированного действия с представителем углеродных наноструктур фуллереном C_{60} на нормальные (тимоциты крысы Wistar) и трансформированные (лимфоидная лейкемия мыши L1210) клетки. С использованием флуоресцентных зондов DCFH-DA и TMRE показано, что цисплатин (1 мкг/мл) усиливал продуцирование АФК и снижал величину митохондриального потенциала в клетках обоих типов. Комбинированная обработка клеток цисплатином (1 мкг/мл) и C_{60} (7,2 мкг/мл) приводила к разнонаправленной модуляции продуцирования АФК тимоцитов и клеток L1210. Индуцированное цисплатином продуцирование АФК в клетках L1210 усиливалось, тогда как в тимоцитах ослабевало. Предполагается, что обнаруженные разнонаправленные эффекты связаны с различиями в аккумулировании и локализации фулерена C_{60} в нормальных и злокачественных клетках.

К л ю ч е в ы е   с л о в а: цисплатин, фуллерен C_{60}, АФК, митохондриальный мембранный потенциал, тимоциты, клетки L1210.

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