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Mode of photoexcited C_{60} fullerene involvement in potentiating cisplatin toxicity against drug-resistant L1210 cells

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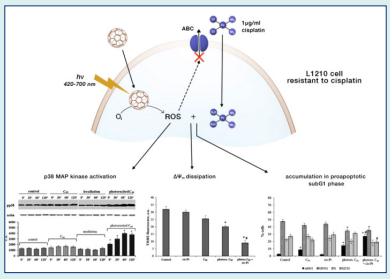
Abstract



Introduction: C_{60} fullerene has received great attention as a candidate for biomedical applications. Due to unique structure and properties, C_{60} fullerene nanoparticles are supposed to be useful in drug delivery, photodynamic therapy (PDT) of cancer, and reversion of tumor cells' multidrug resistance. The aim of this study was to elucidate the possible molecular mechanisms involved in photoexcited C_{60} fullerene-dependent enhancement of cisplatin toxicity against leukemic cells resistant to cisplatin.

Methods: Stable homogeneous pristine C_{60} fullerene queous colloid solution (10^{-4} M, purity 99.5%) was used in the study. The photoactivation of C_{60} fullerene accumulated by L1210R cells was done by irradiation in microplates with light-emitting diode lamp (420-700 nm light, 100 mW·cm^{-2}). Cells were further incubated with the addition of Cis-Pt to a final concentration of 1 μg/mL. Activation of p38 MAPK was visualized by Western blot analysis. Flow cytometry was used for the estimation of cells distribution on cell cycle. Mitochondrial membrane potential ($\Delta\psi_m$) was estimated with the use of fluorescent potential-sensitive probe TMRE (Tetramethylrhodamine Ethyl Ester).

Results: Cis-Pt applied alone at 1 μg/mL concentration failed to affect mitochondrial membrane potential in L1210R cells or cell cycle distribution as compared with untreated cells. Activation of ROS-sensitive proapoptotic p38 kinase and enhanced content of cells in subG1 phase were detected after irradiation of L1210R cells treated with 10^{-5}M C_{60} fullerene. Combined treatment with photoexcited C_{60} fullerene and Cis-Pt was followed by the dissipation of $\Delta\psi_m$ at early-term period, blockage of cell transition into S phase, and considerable accumulation of cells in proapoptotic subG1 phase at prolonged incubation.

Conclusion: The effect of the synergic cytotoxic activity of both agents allowed to suppose that photoexcited C_{60} fullerene promoted Cis-Pt accumulation in leukemic cells resistant to Cis-Pt. The data obtained could be useful for the development of new approaches to overcome drug-resistance of leukemic cells.

Introduction

Multidrug resistance (MDR) is a major problem in anticancer therapy and approximately 70-90% of patients do not respond to initial chemotherapy.[1] Mechanisms of MDR including reduced drug uptake, active drug efflux by transporters of ATP-binding cassette (ABC) superfamily, decreased intracellular drug concentration, altered cell cycle checkpoints, and induced expression of genes for impairing apoptotic pathways of cell death are well studied.[2,3] Nevertheless the problem of MDR reversion in cancer still remains. Thus, the chemical development of ABC transporter inhibitors was found to increase the toxicity associated with chemotherapy.[4]

Unique properties of C_{60} fullerene nanoparticles with the size of 10-100 nm distinguish them from other cancer therapeutics; they are non-toxic for normal cells,[5,6] can bypass traditional drug resistance mechanisms,[7,8] have therapeutic properties themselves as photosensitizers in photodynamic therapy (PDT),[9-11] and enable combinatory treatment with anticancer drugs.[12,13]

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Due to the extended π-conjugated system of molecular orbitals, C_{60} fullerene is able to generate toxic reactive oxygen species (ROS) in polar solvents after UV/Vis light absorption. Photoexcited C_{60} molecule is reduced from a long-lived triplet state ($^3C_{60}^*$) to radical anion $C_{60}^{-\bullet}$ which subsequently reduces O_2 to $O_2^{-\bullet}$; thereby initiating radical chain reactions with the generation of hydroxyl radical and hydrogen peroxide.[9, 14] Photoexcited C_{60} fullerene or its derivatives have been shown to evoke oxidative stress and to induce apoptosis in cancer cells of different origins.[14-18]

In this study, we used the photodynamic potential of C_{60} to enhance the cytotoxic effect of chemotherapeutic drug cisplatin towards murine leucosis cell line resistant to cisplatin. Cisplatin (cis-[$Pt(NH_3)_2Cl_2$], Cis-Pt) belongs to the first-line highly efficient cytotoxic agents in current cancer therapy. It is generally accepted, in that the main Cis-Pt target is nuclear DNA, but recent studies have demonstrated that activation of apoptosis signaling pathways in the cytoplasm is the mechanism of Cis-Pt toxicity alternative to DNA damage.[3, 19, 20] Therapeutic efficiencies of Cis-Pt are substantially limited by the development of cancer cells' MDR.

In the previous studies, we confirmed fullerene C_{60} nanoparticles penetration into leukemic L1210 cells and demonstrated photoinduced cytotoxicity of accumulated C_{60} determined by ROS production.[21] The possibility to decrease substantially the viability of cisplatin-resistant L1210R cells by combined treatment with photoexcited C_{60} fullerene and cisplatin was also shown,[22] but the mechanisms of this phenomenon still need further investigation.

The aim of this study was to elucidate the possible molecular mechanism of photoexcited C_{60} fullerene-dependent enhancement of cisplatin toxicity against leukemic cells resistant to cisplatin.

Materials and Methods

Materials

The materials used included: RPMI 1640 liquid medium (Sigma-Aldrich Co, Ltd, USA), fetal bovine serum (FBS) (Sigma-Aldrich Co, Ltd, USA), penicillin/streptomycin and L-glutamine (Merck KGaA (Darmstadt, Germany)), propidium iodide (Sigma-Aldrich Co, Ltd, USA), RNase A (Sigma, USA), tetramethylrhodamine ethyl ester perchlorate (TMRE) (Sigma, USA), antibodies against β-actin (1:2000 dilution) (Sigma, USA), anti-phospho-p38 kinase antibodies (1:1000 dilution) (Cell Signaling, USA), DC Protein Assay kit (Bio-Rad, USA), cisplatin (cis-Pt, Sigma-Aldrich Co, Ltd, USA).

Preparation and characterization of pristine C_{60} fullerene aqueous colloid solution

C_{60} fullerene aqueous colloid solution was synthesized and characterized in the Ilmenau Technical University (Germany) as described by Scharff et al.[23] In brief, the toluene extract was obtained after graphite combustion and after toluene evaporation, C_{60} fullerene was transferred to the water phase followed by prolonged ultrasound sonication. An aqueous colloid solution of C_{60} fullerene (concentration 10^{-4} M, purity 99.5%) was highly stable for 12 months when stored at room temperature.[24] The average hydrodynamic diameter of C_{60} fullerene nanoparticles was 50 nm, and no changes in their size were detected in RPMI-1640 medium containing 5% FBS.[25]

Cell culture

The murine cancer cell line of leukemic origin resistant to cisplatin L1210R was obtained from the Bank of Cell Lines from Human and Animal Tissues, R. E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine (Kyiv, Ukraine). Cells were incubated in RPMI 1640 medium supplemented with 10% FBS, 50 μg·mL^{-1} penicillin and 100 μg·mL^{-1} streptomycin at 37°C in a humidified atmosphere with 5% CO_2.

Photodynamic treatment

Cells were incubated for 2 hours with or without 10^{-5} M C_{60} fullerene in a medium described above. Photoactivation of accumulated C_{60} fullerene was done by probes irradiation in microplates with light-emitting diode lamp (420-700 nm light, irradiance 100mW·cm^{-2}). Cells were further incubated for an indicated time period without or with the addition of cisplatin to a final concentration of 1 μg/mL in the incubation medium.

Immunoblot analysis

Cells were washed with PBS and lysed with ice-cold lysis buffer containing protease inhibitor. Cell lysates were centrifuged (14 000 g, 15 minutes) and 30 μg of cell lysate protein was loaded onto a gradient 8%-15% SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred onto polyvinylidene difluoride membrane[26] and incubated overnight at 4°C with monoclonal antibody against phospho-p38 kinase (dilution 1:1000). The membranes were washed and incubated for 1 hour with anti-rabbit peroxidase-linked secondary antibody. Immunoreactive bands were visualized by enhanced chemiluminescence plus western blotting detection system (Amersham, USA). Then, the membranes were incubated with antibodies against β-actin to provide the loading control. Finally, protein concentration was determined using DC Protein Assay kit (Bio-Rad, USA).

Cell cycle analysis

For cell cycle analysis, cells (1x10^6) were resuspended in 0.1 mL PBS (pH 7.4), fixed by adding 0.9 mL of 90% ethanol at -20°C overnight and centrifuged at 13000 g for 1 minute. The fixed cells were rinsed twice with PBS and resuspended in propidium iodide solution (10 μg/mL) containing RNase A (100 μg/mL) in PBS. The stained cells were analyzed by a COULTER EPICS XL™ (Beckman

Coulter, USA) and FCS Express 3 Flow Cytometry Software (DeNovo Software, USA).

Mitochondrial transmembrane potential assay

Mitochondrial membrane potential ($\Delta\psi_m$) was estimated with the use of fluorescent potential-sensitive probe TMRE. Cells (10^7/mL) were suspended in buffer A containing (mM): KCl – 5, NaCl – 120, $CaCl_2$ – 1, glucose – 10, $MgCl_2$ – 1, $NaHCO_3$ – 4, HEPES – 10, pH 7.4, incubated with 100 nM TMRE for 40 minutes at 25°C with the addition of 0.05% Pluronic F-127 and washed from the excess of probe. 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 the addition of protonophore FCCP (carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone) (1 μM).[27]

Statistics

The data were represented as Mean (M) ± standard deviation (SD) of more than four independent experiments. Mean and SD were calculated for each group. Statistical analysis was performed using two-way ANOVA followed by post Bonferroni test. A value of $P<0.05$ was considered statistically significant. Data processing and plotting were performed by IBM PC using specialized applications GraphPad Prism 7 (GraphPad Software Inc., USA) and Gel-Pro Analyzer 6.3 (Media Cybernetics Inc., USA).

Results

Activation of p38 MAPK in L1210R cells after C_{60} fullerene photoexcitation

Mitogen-activated p38 kinase (MAPK) is one of the important redox-sensitive and stress-activated targets involved in apoptosis induction by phosphorylation of proapoptotic proteins p53 and Bax.[28, 29] We examined p38 MAPK activity in L1210R cells by estimation of the level of its active phosphorylated form (pp38) using Western blot analysis. As shown in Fig. 1, no statistically valid changes in the level of active p38 kinase were detected at 2-hour incubation of cells loaded with C_{60} fullerene or irradiated with 420-700 nm light alone, though photoexcitation of C_{60} fullerene accumulated with L1210R cells was followed by an increase of p38 MAPK level which was found to be 3 times higher than that in the control at 1 hour of incubation and remained at enhanced level at 2 hours.

This finding is in agreement with data presented by Li et al,[30] where substantial activation of p38 MAP kinase was detected after light irradiation of MCF-7 cells loaded with C_{60} derivatives C_{60}-phe or C_{60}-gly. This increase was prevented by antioxidant N-acetyl-L-cystein and thus proved to be ROS dependent. Activation of p38 MAP kinase as a result of H_2O_2-induced oxidation of its thiol groups was also shown by Olson and Hallahan.[29]

A growing body of evidence suggests that p38 MAPK is able to control the p53-mediated response to several genotoxic stimuli and could be specific to cancer therapy.[31, 32] The data on increase in the viability of HaCaT cells pretreated with p38 MAPK specific inhibitors before incubation with cisplatin as well as the data obtained in experimental head and neck cancer model indicating that lower activation or lack of activation of p38 MAPK correlates with a more resistant phenotype[33] suggested that the inhibition of p38 MAPK is a potential mechanism of resistance and that activation of this pathway could help to overcome cancer cells drug resistance.

Interference into cell cycle transition is suggested to be one of the mechanisms of p38 MAP kinase involvement into apoptosis induction. To elucidate if ROS dependent effects of photoexcited C_{60} fullerene could disturb cell cycle checkpoints in cisplatin-resistant L1210R cells, we

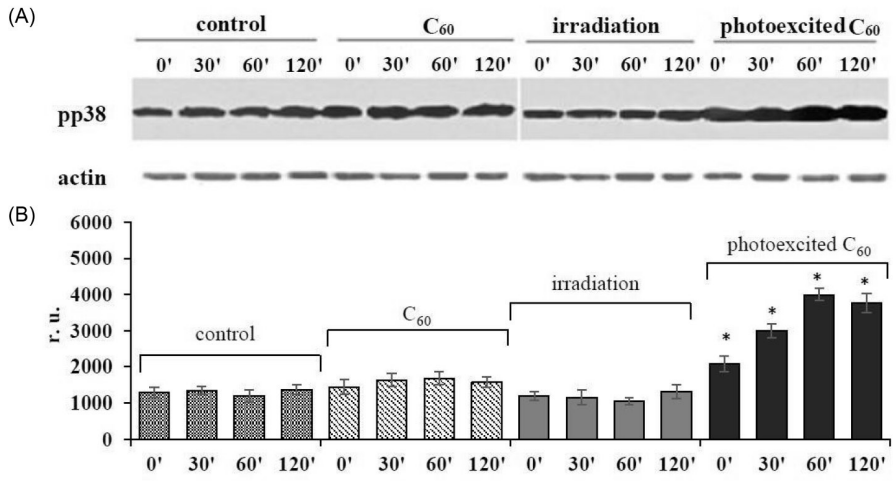

Fig. 1. Activation of p38 MAP kinase in L1210R cells treated with 10^{-5} M C_{60} fullerene and irradiated with 420-700 nm light: (A) Western blot analysis of pp38 MAPK level (typical blotogram); (B) quantitative analysis of the fold increase of pp38 MAPK level. (M±m, n=3); *P<0.05 in comparison to control.

further studied the cells cycle distribution after combined treatment with photoexcited C_{60} and cisplatin.

Cell cycle distribution of L1210R cells after the combined action of cisplatin and photoexcited C_{60} fullerene

Flow cytometric analysis showed that at 48 hour incubation of L1210R cells in control, the most significant content of cells (47.6±4.6%) was detected in the G0/G1 phase (Figs. 2A, B). Accumulation of cisplatin-resistant cancer cells of different origins in the G0/G1 phase of cell cycle is believed to ensure the transition from G1 to S phase, DNA doubling, and mitosis.[34, 35]

No effect on cell cycle profile was detected after L1210R cells light irradiation (data not shown) or treatment with cisplatin alone, while treatment with C_{60} fullerene was followed by an increase of cells content in the subG1 phase (8.7±3.5% vs. 2.7±1.5% in control) (Figs. 2A, B). After C_{60} fullerene photoactivation, this effect was enhanced (14.4±2.9% vs. 2.7±1.5% in control) with simultaneous decrease of cells content in the G0/G1 phase. After combined treatment with photoexcited C_{60} fullerene and cisplatin, a decrease of cells content in the G2/M phase was shown, while the number of cells accumulated in subG1 phase was found to be substantially higher than that after C_{60} fullerene photoexcitation alone (28±4% vs. 14.4±3% respectively) (Fig. 2B). Cells accumulation in the subG1 phase is considered to be the marker of the blockage of cell transition into S phase and transition to apoptotic pathway.[8]

Activation of proapoptotic MAP kinases in L1210R cells after C_{60} fullerene photoexcitation could be the initial step of the cell death program, but its realization needs reinforcement of apoptotic signals particularly at the level of mitochondria. Since acute apoptosis induced by cisplatin is shown to be associated with mitochondrial ROS response,[20, 36] we tested whether combined treatment of L1210R cells with photoexcited C_{60} fullerene and cisplatin had an impact on mitochondrial redox status.

Mitochondrial membrane potential in L1210R cells after the combined action of cisplatin and photoexcited C60 fullerene

The relative value of mitochondrial membrane potential ($\Delta\psi_m$) in L1210R cells incubated for 2 hours after the treatment with either cisplatin or photoexcited C_{60} fullerene alone or their combination was estimated with the use of fluorescent probe TMRE.

No effect of light irradiation (data not shown) or 1 μg/mL cisplatin alone on the $\Delta\psi_m$ value in L1210R cells was detected, while the tendency to its decrease after cells treatment with C_{60} fullerene was revealed (Fig. 3).

C_{60} fullerene photoexcitation was followed by 1.7 fold decrease of $\Delta\psi$ relative value in L1210R cells as compared with control. Combined treatment with photoexcited C_{60} fullerene and cisplatin was followed by further substantial decrease of TMRE fluorescent signal, the $\Delta\psi$ relative value was decreased 3.9 folds as compared with the control and 2 folds as compared with the effect of photoexcited C_{60}

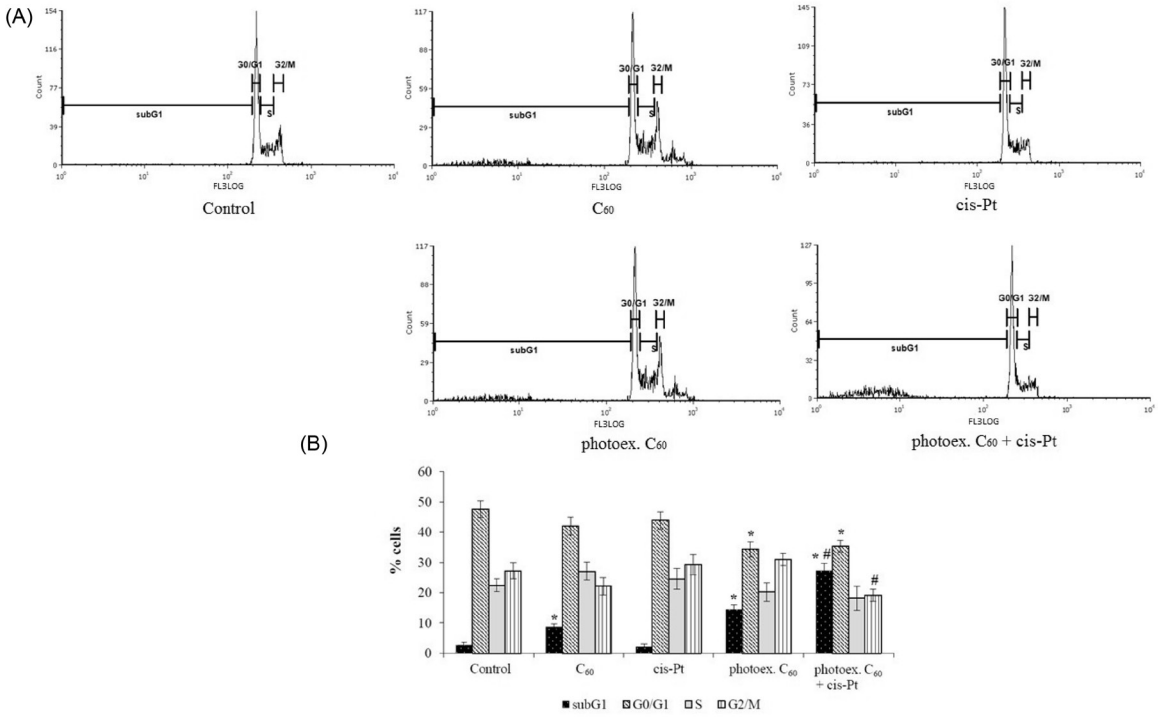

Fig. 2. Cell cycle distribution of L1210R cells at 48 hour time point after combined treatment with photoexcited C_{60} fullerene and cisplatin. (A) Representative histograms of a typical experiment. (B) % of total cell population in phases. (M±m, n=3); *P<0.05 in comparison with control, #P<0.05 in comparison with photoexcited C_{60} fullerene.

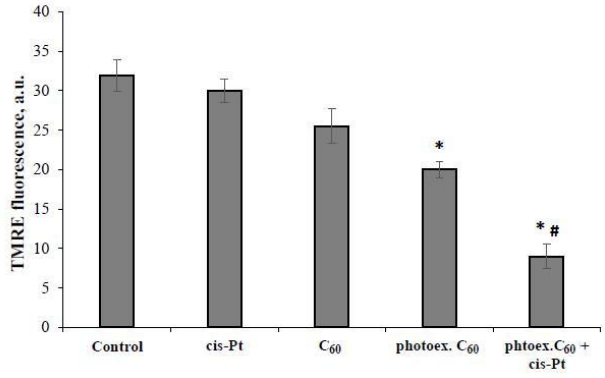

Fig. 3. Relative value of mitochondrial membrane potential in L1210R cells at 2 hour time point after combined treatment with photoexcited C_{60} fullerene and cisplatin. (M±m, n=5); **P*<0.05 in comparison with control, #*P*<0.05 in comparison with photoexcited C_{60} fullerene.

fullerene alone, indicating the cisplatin involvement in mitochondria redox status disturbance.

Discussion

The ability of photoexcited C_{60} fullerene to induce apoptosis in human leukemic cells was confirmed in our previous studies, where the depletion of mitochondrial Ca^{2+}-pool, cytochrome *c* release from mitochondria to the cytosol, caspase - 3 activation and DNA fragmentation with the formation of the "ladder pattern" after UV/Vis irradiation (320-600nm) of cells treated with 10^{-5} M C_{60} fullerene were demonstrated.[37, 38]

As we have shown earlier, the treatment of cisplatin resistant L1210R leukemic cells with cisplatin in a range of 0.1-10 μg/mL had no effect on cell viability, while substantial C_{60}-mediated photodamaging effect was detected with 50% decrease of cell viability at 48 hour time point after photoexcitation (420-700 nm) of accumulated carbon nanostructure.[21] The intense ROS production detected at 3 hour time point after irradiation of L1210R cells loaded with C_{60} fullerene confirmed the ability of photoactivated C_{60} fullerene to generate $O_2^{-\bullet}$ in intracellular space.[22]

In this study we demonstrated the activation of p38 MAP kinase in L1210R cells after treatment with C_{60} fullerene and light irradiation. These data indicate that p38 MAPK could be the target of ROS produced by photoexcited C_{60} and thus be involved in the molecular mechanisms of photoexcited C_{60} toxic effect against leukemic cells resistant to cisplatin. This suggestion was confirmed by the data indicating the ability of photoexcited C_{60} to evoke L1210R cells accumulation in proapoptotic $subG_1$ phase of cell cycle.

We showed that under combined treatment with photoexcited C_{60} and cisplatin in a low 1 μg/mL concentration, the synergic effect of both agents became apparent both in dropping the mitochondrial membrane potential and inducing L1210R cells accumulation in $subG_1$ phase of cell cycle.

The synergic cytotoxic activity of photoexcited C_{60} and cisplatin could be realized on condition that cisplatin enters L1210R cells and is accumulated in intracellular space. The ability of C_{60} fullerene derivatives to reactivate cisplatin endocytosis in cancer cells and thus to circumvent tumor resistance to cisplatin[8] as well as incapability of P-gp type of ABC transporters to recognize pristine C_{60} fullerene nanoparticles and to prevent their accumulation in drug-resistant K562R leukemic cells[7] confirm this assumption. Additionally, the expression of ABC family transporters responsible for drug efflux from cancer cells is shown to be ROS-regulated. Thus, ROS induced down-regulation of both P-glycoprotein in prostate tumor cells[39] and MDR-associated protein (MRP1) expression in urinary bladder cells[40] were demonstrated. The results of our study allow suggesting that photoexcitation of C_{60} fullerene may affect the components of the system controlling cisplatin influx and accumulation in L1210R cancer cells, thereby promoting overcoming of drug resistance.

Research Highlights

What is the current knowledge?

√ C60 fullerene is potential for PDT and phenomenon of cancer cells MDR.

What is new here?

√ Activation of ROS-sensitive proapoptotic p38 kinase and enhanced content of cells in proapoptotic subG1 phase were detected when leukemic cell line L1210 resistant to Cis-Pt was treated with 10-5M C60 fullerene and irradiated with visible light.

√ Combined treatment of L1210R cells with photoexcited C60 fullerene and Cis-Pt in low concentration was followed by the intensification of proapoptotic effects.

√ The effect of the synergic cytotoxic activity of both agents allowed us to suppose that photoexcited C60 fullerene promoted Cis-Pt accumulation in leukemic cells resistant to Cis-Pt.

Conclusion

Series of genetic and metabolic rearrangements allow cancer cells to prevent the cytotoxic effects of anticancer drugs. The phenomenon of cancer cells MDR substantially reduces the effect of anticancer therapy and the efficiency of cisplatin as the commonly used drug. In this respect, application of C_{60} fullerene nanoparticles seems to be perspective as they penetrate into cancer cells, avoid efflux by transporters of ABC family and facilitate drug delivery, produce toxic ROS after photoexcitation and enable combinatory treatment with anticancer drugs.

In this study the activation of p38 MAP kinase and the decrease of $\Delta\psi_m$ value as the inducing markers of ROS-dependent apoptotic pathways in resistance to cisplatin leukemic L1210R cells treated with 10^{-5} M C_{60} fullerene

and irradiated with visible light was shown. The data obtained indicated that combined treatment of L1210R cells with photoexcited C_{60} fullerene and cisplatin in a low 1 μg/mL dose was followed by more intense proapoptotic effect as compared with treatment with photoexcited C_{60} fullerene alone. Dissipation of $\Delta\psi_m$ at early term period, the blockage of cell transition into S phase and mitosis with accumulation in the proapoptotic subG1 phase of the cell cycle at long-term period after combined treatment of L1210R cells were detected. The effect of synergic cytotoxic activity allowed us to suppose that photoexcited C_{60} fullerene promoted cisplatin accumulation in L1210R cells. The data obtained could be useful for the development of approaches to overcome drug-resistance of leukemic cells and to extend the methods of PDT.

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Ethical Statement
Not applicable.

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Competing interests
The authors declare that they have no competing interests.

Authors' contribution
OM, LD, UR: the conceptual idea and design of the experiments; SP, IG, GP, DF: realization of experiments, data handling, and data analysis; OM, DF, SP: writing the manuscript.

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