Exp Oncol 2015
37, 3, 187–191

Experimental ONCOLOGY

ENHANCED CYTOTOXICITY OF PHOTOEXCITED FULLERENE C_{60} AND CISPLATIN COMBINATION AGAINST DRUG-RESISTANT LEUKEMIC CELLS

D.V. Franskevych, S.V. Prylutska, I.I. Grynyuk, D.M. Grebinyk, O.P. Matyshevska*
Taras Shevchenko National University of Kyiv, Kyiv 01601, Ukraine

Aim: **To evaluate the viability of leukemic cells sensitive (L1210S) and resistant (L1210R) to cisplatin, ROS production and free cytosolic Ca^{2+} concentration under treatment with cisplatin or its combination with photoexcited fullerene C_{60}.** ***Methods:*** **Cell viability was assessed by the MTT reduction assay. Light-emitting diode lamp (2.45 J/cm^2) was used for photoexcitation of intracellular accumulated fullerene C_{60}. Free cytosolic calcium concentration ($[Ca^{2+}]_i$) and ROS production in cells were estimated with the use of fluorescent probes Indo-1 and 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA), respectively.** ***Results:*** **It is shown that viability of L1210R cells wasn't changed under treatment with cisplatin in concentration range 0.1–10 μg/ml. 50% and 30% decrease of L1210S cells were observed after 24 h of incubation with cisplatin at concentrations 5 and 1 μg/ml, respectively. Intensification of extranuclear cytotoxic effects (ROS production and $[Ca^{2+}]_i$ increase) after treatment with 1 μg/ml was detected in L1210S, but not in L1210R cells. The most strongly pronounced increase of ROS production and $[Ca^{2+}]_i$ in both L1210 cell lines was revealed in dynamics after combined treatment with cisplatin (1 μg/ml) and photoexcited fullerene C_{60} (10^{-5} M) and was followed by decreased viability of not only L1210S, but of L1210R cells as well.** ***Conclusion:*** **Combined treatment with photoexcited C_{60} and cisplatin allowed to decrease effective concentration of cisplatin against parental L1210 cells and to increase sensibility of resistant cells to the drug.**

Key Words: **cisplatin, L1210 cells, photoexcited fullerene C_{60}, ROS, cytosolic Ca^{2+}, cell viability.**

Despite the fact that the most commonly used cytotoxic agents — cisplatin (CP), doxorubicin and paclitaxel, are highly efficient in current cancer therapy; their therapeutic efficiency is lowered by high toxicity and easy development of drug resistance. CP displays clinical activity against variety of human malignancies and is generally recognized as the DNA-damaging drug. Nevertheless CP is shown to induce apoptosis independently of DNA damage; and the ability to activate several different rather than a single apoptotic pathway is thought to be a distinction, which makes CP highly efficient as an anticancer drug. Cytoplasmic reactive oxygen species (ROS) production, free cytosolic Ca^{2+} concentration increase, modification of membrane ion channels and transport proteins, endoplasmic reticulum stress are supposed to be the pathways of CP-induced cell death signaling [1].

Because CP has multiple cellular targets and many different routes of cell entry, resistance to this drug is very complex and requires numerous mechanisms including reduced accumulation by impaired influx or active efflux, defective endocytosis, detoxification by GSH system, inactivation of apoptotic pathways, increased DNA repair, alterations in the expression of tumor suppressor gene p53 [2–6]. To increase the therapeutic index and to minimize CP resistance combined therapies with the use of nanotechnologies are developing now.

The recent studies have showed that combination or conjunction of anticancer drugs with the representatives of carbon nanostructures (single-walled carbon nanotubes, hydroxylated fullerene C_{60}, metallofullerenes with gadolinium [Gd@$C_{82}(OH)_{22}$]n, etc.) could be promising for targeted drug delivery, overcoming of tumor cells drug resistance and reduction of toxic effects in normal cells [7–11].

To estimate the probability of enhancing CP pro-apoptotic effects we have studied the effect of combined treatment with CP and photoexcited fullerene C_{60} on cancer cells. Fullerene C_{60} demonstrates unique physicochemical properties and biological activity: spherical form, nanosize (0.72 nm in diameter), allocation inside hydrophobic regions of cell membranes and ability to penetrate into cytoplasm, compatibility with biological molecules [12, 13]. Due to extended π-conjugated system of molecular orbitals fullerene C_{60} absorb UV/Vis light efficiently and is able to generate cytotoxic ROS with almost 100% quantum yield. Modification of fullerene core appears to cause a perturbation of its electronic structure and hence to reduce the photodynamic potential of the molecule [14]. Using pristine fullerene C_{60} we have demonstrated its accumulation inside leukemic cells and apoptosis induction after photoexcitation [15].

The aim of this study was to evaluate the viability of leukemic cells sensitive (L1210S) and resistant (L1210R) to CP, ROS production and free cytosolic Ca^{2+} concentration under CP treatment or combined treatment with CP and photoexcited fullerene C_{60}.

MATERIALS AND METHODS

Two leukemic L1210 cell lines — sensitive (L1210S) and resistant (L1210R) to CP were used, which were kindly provided by Dr. Liudmyla Drobot (V.A. Palladin Institute of Biochemistry, the NAS of Ukraine). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich,

Submitted: June 22, 2015.
*Correspondence: E-mail: dashaqq@gmail.com
Abbreviations used: CP – cisplatin; DCF-DA – 2′,7′-dichlorodihydrofluorescein diacetate; ROS – reactive oxygen species.

Germany), 50 μg/ml penicillin and 100 μg/ml streptomycin at 37 °C in a 5% CO_2 humidified atmosphere. Cells were treated with CP (Sigma, USA) in concentrations 0.1–10 μg/ml.

Stable water colloid solution of fullerene C_{60} (10^{-4} M, purity >99.5%, nanoparticle average size 50 nm) was synthesized in Technical University of Ilmenau (Germany) as described in [16, 17]. Cells in RPMI 1640 medium were incubated for 2 h with or without fullerene C_{60} (10^{-5} M). Photoactivation of accumulated fullerene C_{60} was done by probes irradiation with light-emitting diode lamp (410–780 nm light, irradiance 100 mW (2.45 J/cm^2), during 2 min).

Cell viability was assessed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide] reduction assay. At indicated time points of incubation 200 μl aliquots were removed from cell suspensions into the 96-well microplates (L1210S/R cells — 1×10^5/well), 20 μl of MTT solution (2.5 μg/ml) was added to each well and the plates were incubated for another 2 h. The culture medium was then replaced with 100 μl of DMSO, diformazan formation was determined by measuring absorption at 570 nm with a plate reader (μQuant, BioTek, USA).

ROS production was measured using fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (Sigma, USA), which was added to the cell incubation medium (1×10^6 cells/ml) in concentration 5 mM. The fluorescent intensity was measured in real time on spectrofluorimeter Shimadzu 150 RF (Japan) λ excitation — 480 nm, λ emission — 520 nm [18].

The concentration of free cytosolic Ca^{2+} was measured using fluorescent probe Indo-1 (Sigma, USA). Cells (3×10^7/ml) in buffer A consisting of (mM): KCl — 5, NaCl — 120, $CaCl_2$ — 1, glucose — 10, $MgCl_2$ — 1, $NaHCO_3$ — 4, HEPES — 10, pH 7.4 were loaded with Indo-1AM (in concentration 1 mM) in the presence of 0.05% Pluronic F-127 (Sigma, USA) for 40 min at 25 °C. Indo-1 fluorescence in cells was recorded using spectrophotometer (Shimadzu RF-510, Japan), λ excitation — 350 nm, λ emission — 410 and 495 nm. The concentration of free cytosolic Ca^{2+} was calculated as described in [19].

Data processing and plotting were performed by IBM PC using specialized applications Excel 2010 and Origin 7.0. Statistical analysis was performed using *Statistica 6.0* computer program (StatSoft Inc.). Paired Student's *t*-tests were performed. Differences values $p < 0.05$ were considered to be significant.

RESULTS

To estimate the sensitivity of two L1210 cell lines to CP, the viability of cells treated with the drug in concentration range 0.1–10 μg/ml was studied by MTT test. The viability of parental L1210 cells treated with CP appeared to be decreased in a dose and duration dependent manner (Fig. 1, *a*). At 24 h of incubation the viability of cells treated with 1 μg/ml was decreased by 30% while of those treated with 5 μg/ml — by 50%. No L1210S cell survival was detected at 72 h after treatment with 5 or 10 μg/ml of CP.

Treatment with CP in a range 0.1–10 μg/ml was virtually nontoxic to L1210 resistant cells. A slight decrease of viability (to approximately 80%) was observed at 48 h after the treatment with 5 and 10 μg/ml CP, but at 72 h the indexes were normalized (Fig. 1, *b*).

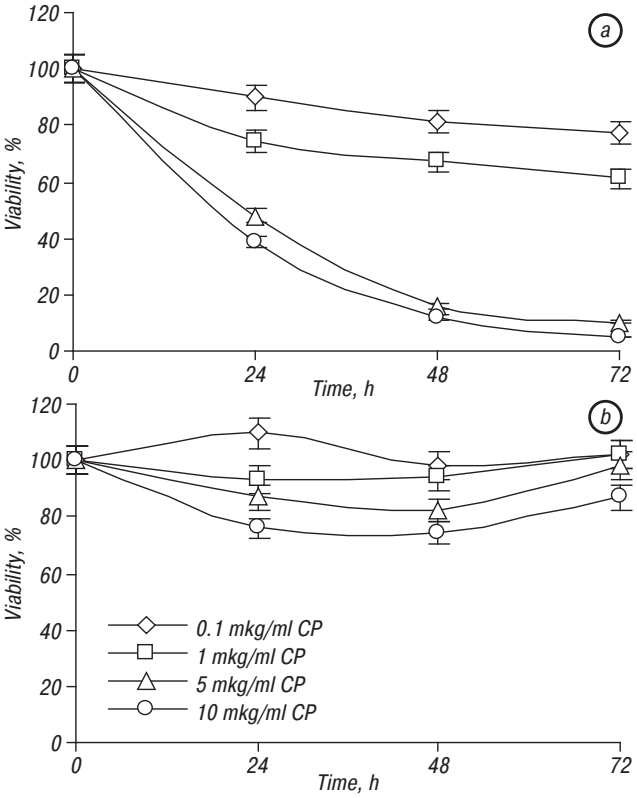

Fig. 1. Cell viability kinetics of L1210S (*a*) and L1210R (*b*) cells treated with CP in different concentrations (n = 4)

To investigate the effect of combined treatment with photoexcited fullerene C_{60} and CP on susceptibility of L1210 cells to the drug, we used in further experiments with long-term cell incubation the CP concentration — 1 μg/ml. Cells were preincubated with C_{60} nanoparticles for 2 h, irradiated for photoexcitation of accumulated fullerene C_{60} and incubated for 72 h. In the case of combined treatment CP was added just before C_{60} photoexcitation.

Irradiation of cells with or without CP did not change the viability of either L1210S or L1210R cells (data not shown), while treatment with fullerene C_{60} was followed by slight decrease of both cell lines viability (Fig. 2, *a*, *b*).

The cytotoxic effect of CP against L1210S appeared to be comparable with the effect of photoexcited C_{60}; both agents cause the 40% decrease of cell viability at 72 h. However, after combined treatment with CP and photoexcited C_{60} the cytotoxic effect was increased and CP concentration of 1 μg/ml was proved to be sufficient to reach 50% decrease at 48 h as distinct from CP treatment alone (see Fig. 2, *a*).

We have demonstrated that cytotoxic effect of photoexcited fullerene C_{60} was realized not only against parental, but against CP resistant cells as well. Moreover while treatment with 1 μg/ml CP alone did not change the viability L1210R cells the combined treatment with photoexcited C_{60} and CP allowed to intensify cytotoxic

effect and to decrease viability of resistant cells by 68% at 48 h of incubation.

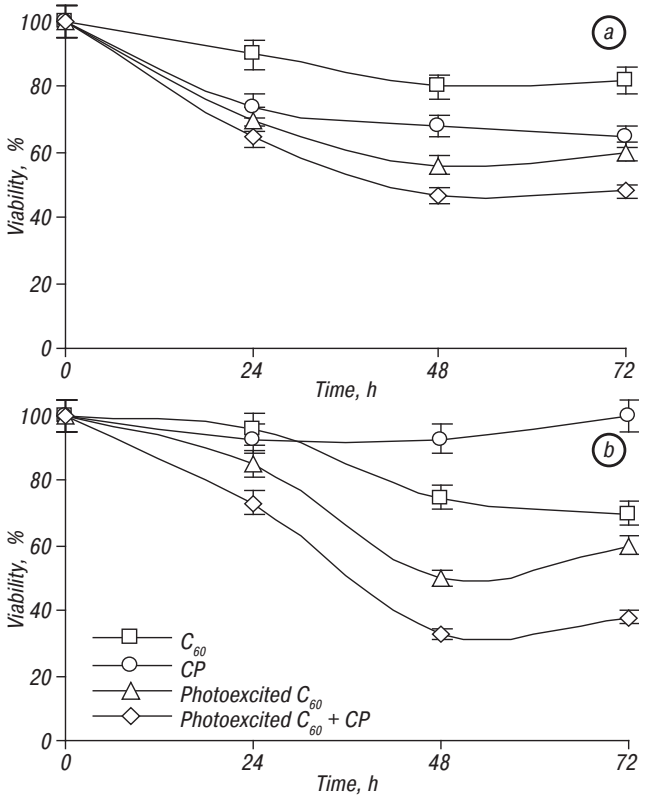

Fig. 2. Cell viability kinetics of L1210S (*a*) and L1210R (*b*) cells exposed to fullerene C_{60} (10^{-5} M; n = 4), CP (1 μg/ml; n = 4), photoexcited C_{60} (n = 5) and combined treatment with CP and photoexcited fullerene C_{60} (n = 5)

We supposed that enhancement of cytotoxicity after combined treatment with CP and photoexcited C_{60} could be caused by ability to activate early cytoplasmic events leading to cell death independently of DNA damage. It has been shown, that CP requires cytoplasmic generation of ROS and $[Ca^{2+}]_i$ increase to induce apoptosis [20–22].

As we have demonstrated earlier that fullerene C_{60} is accumulated in leukemic Jurkat cell lines, increased ROS production and free cytosolic calcium concentration at early period (3 h) after photoexcitation with further substantial activation of caspase-3 and cell death by mitochondrial-dependent pathway at 24 h [15, 23–25]. To confirm this assumption the experiments with CP addition to both cell lines at 3 h after photoexcitation of accumulated fullerene C_{60} were carried out.

Sensitive and specific method for ROS production evaluation in cells is the use of fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA). When DCF-DA was added to untreated cells, the increase of fluorescence after 10 min incubation was detected corresponding to the endogenous ROS level (Fig. 3). The values of free cytosolic calcium concentration in sensitive and resistance cells in control were 125 ± 6 and 130 ± 4 nM, respectively, and weren't changed during cells incubation (taken for 100%) (Fig. 4).

Treatment of L1210S with CP alone was shown to be followed by increasing of both ROS generation and $[Ca^{2+}]_i$ at early period after CP addition while after treatment of L1210S with the drug neither ROS nor $[Ca^{2+}]_i$ levels were changed (see Fig. 3, *a*, 4, *a*). In contrast to CP photoexcited fullerene C_{60} induced ROS production and $[Ca^{2+}]_i$ elevation not only in L1210S, but in L1210R cells as well (see Fig. 3, 4).

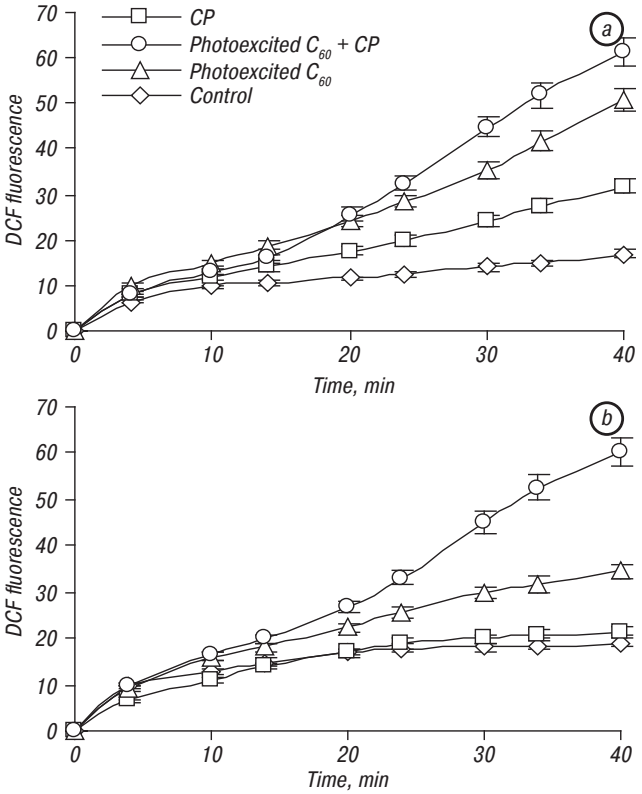

Fig. 3. The kinetics of ROS production in L1210S (*a*) and L1210R (*b*) cells after exposition to CP (n = 3), photoexcited fullerene C_{60} (n = 4) and combined treatment with CP and photoexcited fullerene C_{60} (n = 4)

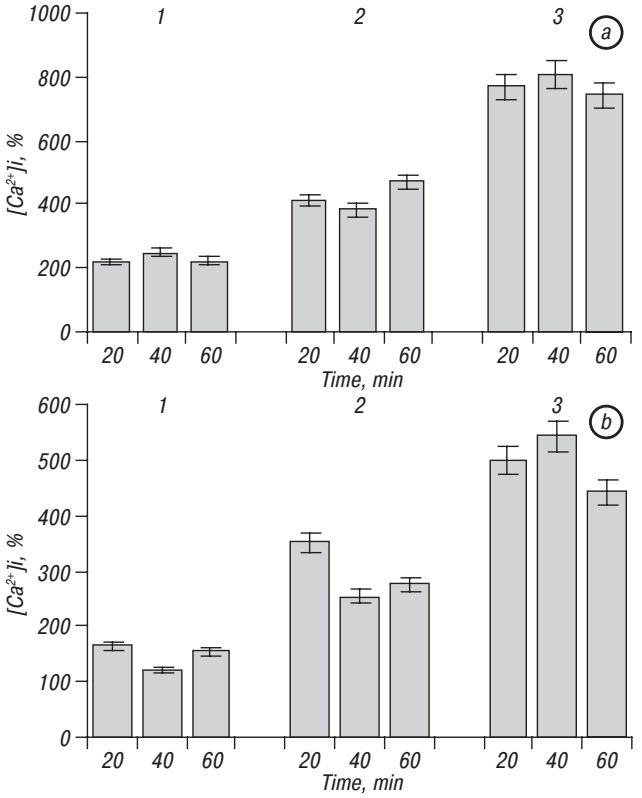

Fig. 4. Ca^{2+} concentration in L1210S (*a*) and L1210R (*b*) cells exposed to CP — 1 (n = 3), photoexcited fullerene C_{60} — 2 (n = 4) and combined treatment with CP and photoexcited fullerene C_{60} — 3 (n = 4)

But the most strongly pronounced increase of DCF and indo-1 fluorescent signals in both L1210S and L1210R cells were detected in dynamics after combined treatment with CP and photoexcited fullerene C_{60} indicating on substantial disturbance of intracellular redox status and calcium homeostasis (see Fig. 3, 4).

The obtained data demonstrate the synergic cytotoxic effect of CP and photoexcited fullerene C_{60} against L1210R leukemic cells. The combined treatment with these agents is followed by intensification of ROS dependent pathways of cell death and allows not only to decrease effective concentration of CP against parental L1210 cells, but to restore sensibility of resistant cells to the drug.

DISCUSSION

Recent studies [3, 6, 21, 22] demonstrate that activation of the signaling pathways to apoptosis which occurs in cytoplasm independently of nuclear is the alternative to DNA damaging mechanism of CP toxicity against cancer cell. Searching for the ways to enhance CP extranuclear cytotoxic effects with the use of nanotechnology opens the possibility to increase the therapeutic efficiency of the drug.

We studied the effect of CP at a range 1–10 μg/ml against parental and drug-resistant leukemic L1210 cells. Early intensification of ROS production and Ca^{2+} increase after treatment with 1 μg/ml was detected in L1210S cells. No decrease of L1210R cells viability and no changes in ROS or $[Ca^{2+}]_i$ levels at the early period of treatment with 1 μg/ml CP were detected.

These data are in agreement with the studies which reported that CP-induced extranuclear effects involve intensification of ROS generation due to CP upregulation of nicotinamide adenine dinucleotide phosphate oxidases (NOX-1 and NOX-4) and ER stress induction [20, 26]. It is also shown that CP could provoke elevation of cytosolic calcium concentration by an increased Ca^{2+} uptake from the extracellular space (mediated through plasma membrane calcium channel) or by Ca^{2+} release through IP_3 receptors of endoplasmic reticulum [21, 27].

To overcome drug resistance in L1210R cells the combined treatment with photoexcited fullerene C_{60} and CP was used. The results indicated that combined treatment improved the cytotoxic effect of CP in L1210R cells by increase of ROS production and free cytosolic calcium concentration and decrease of cells viability. We suggested that this alteration in L1210R cells sensitivity to CP could be explained by the ability of fullerene C_{60} to affect and modify some mechanisms of cell drug resistance development: platinum compounds transport through the plasma membrane, antioxidative system activity (detoxification by glutathione conjugates), induction of apoptotic pathways, calcium homeostasis maintenance [3, 28–33]. Thus, fullerene derivatives are able to reactivate endocytosis in cancer cells and to promote CP accumulation [8]. Fullerene C_{60} is shown to be located in the plasma membrane and intermembrane space of mitochondria with further intensification of ROS in mitochondrial electron transport chain [24, 34]. It is also shown that cancer cells have genetically remodulated system of calcium homeostasis: underfilled ER calcium pool and reduced of SOCE (store-operated calcium entry through plasma membrane) directed to prevent Ca^{2+}-dependent way of apoptosis. We have earlier shown that photoexcited C_{60} induced the increase of SOCE, depletion of mitochondrial Ca^{2+}-pool, cytochrome *c* release from mitochondria and activate Ca^{2+}-dependent apoptotic pathway [23].

Consequently, we supposed that sensitivity enhancement of L1210R cells to CP induced by combined treatment was connected with properties of photoexcited fullerene C_{60} to affect on auxiliary intracellular targets beside that which were involved in CP cytotoxic effect.

This suggests that combined treatment with CP and photoexcited fullerene C_{60} is followed by synergistic anticancer effect of both compounds and allows to increase cytotoxicity against leukemic L1210 cells and to restore sensibility of L1210 resistant cells to CP by enhancing extranuclear proapoptotic effects of the drug.

The proposed model of combined treatment can be helpful in developing the approaches to decrease anticancer drug's toxic dose and to optimize the methods of photodynamic therapy.

REFERENCES

1. Chung T-W, Choi H-J, Kim S-J, ***et al.*** The ganglioside GM3 is associated with cisplatin-induced apoptosis in human colon cancer cells. PLoS One 2014; **9**: doi: 10.1371/journal.pone.0092786.

2. Shen DW, Pouliot LM, Hall MD, ***et al.*** Cisplatin resistance: a cellular self-defense mechanism resulting from multiple epigenetic and genetic changes. Pharmacol Rev 2012; **64**: 706–21.

3. Florea A-M, Büsselberg D. Cisplatin as an anti-tumor drug: cellular mechanisms of activity, drug resistance and induced side effects. Cancers 2011; **3**: 1351–71.

4. Kartalou M, Essigmann J. Mechanisms of resistance to cisplatin. Mutat Res 2001; **478**: 23–43.

5. Siddik ZH. Cisplatin: mode of cytotoxic action and molecular basis of resistance. Oncogene 2003; **22**: 7265–79.

6. Mandic A, Hansson J, Linder S, ***et al.*** Cisplatin induces endoplasmic reticulum stress and nucleus-independent apoptotic signaling. J Biol Chem 2003; **278**: 9100–6.

7. Abhimanyu S, Shivani S, Kenneth T, ***et al.*** Harnessing structure-activity relationship to engineer a cisplatin nanoparticle for enhanced antitumor efficacy. PNAS 2010; **28**: 12435–40.

8. Xing-Jie L, Huan M, Yingze W. Metallofullerene nanoparticles circumvent tumor resistance to cisplatin by reactivating endocytosis. PNAS 2010; **107**: 7449–54.

9. Li YY, Tian YH, Nie GJ. Antineoplastic activities of $Gd@C_{82}(OH)_{22}$ nanoparticles: tumor microenvironment regulation. Sci China Life Sci 2012; **55:** 884–90.

10. Uchino H, Matsumura Y, Negishi T, ***et al.*** Cisplatin-incorporating polymeric micelles (NC6004) can reduce nephrotoxicity and neurotoxicity of cisplatin in rats. Br J Cancer 2005; **93**: 678–87.

11. Kim JH, Kim YS, Park K, ***et al.*** Antitumor efficacy of cisplatin-loaded glycol chitosan nanoparticles in tumor-bearing mice. J Control Release 2008; **127**: 41–9.

12. Troshin PA, Lyubovskaya RN. Organic chemistry of fullerenes: the major reactions, types of fullerene derivatives and prospects for practical use. Russ Chem Rev 2008; **77**: 323–33.

13. Hotze E, Labille J, Alvarez P, ***et al***. Mechanisms of photochemistry and reactive oxygen production by fullerene suspensions in water. Environ Sci Technol 2008; **42**: 4175–80.

14. Orlova MA, Trofimova TP, Orlov AP, ***et al.*** Perspectives of fullerene derivatives in PDT and radiotherapy of cancers. BMJ Med Res 2013; **3**: 1731–56.

15. Palyvoda KO, Grynyuk II, Prylutska SV, ***et al.*** Apoptosis photoinduction by C_{60} fullerene in human leukemic T cells. Ukr Biokhim Zh 2010; **82**: 121–7.

16. Scharff P, Risch K, Carta-Abelmann L, ***et al***. Structure of C_{60} fullerene in water: spectroscopic data. Carbon 2004; **42**: 1203–6.

17. Grynyuk II, Prylutska SV, Slobodyanik NS, ***et al.*** The aggregate state of C_{60}-fullerene in various media. Biotechnologia Acta 2013; **6**: 71–6 (in Russian).

18. Myhre O, Andersen J, Aarnes H, ***et al.*** Evaluation of the probes 2,7-dichlorofluorescin diacetate, luminol, and lucigenin as indicators of reactive species formation. Biochem Pharmacol 2003; **65**: 1575–82.

19. Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 1985; **260**: 3440–50.

20. Berndtsson M, Hägg M, Panaretakis T, ***et al.*** Acute apoptosis by cisplatin requires induction of reactive oxygen species but is not associated with damage to nuclear DNA. Int J Cancer 2007; **120**: 175–80.

21. Florea AM, Büsselberg D. Anti-cancer drugs interfere with intracellular calcium signaling. Neurotoxicology 2009; **30**: 803–10.

22. Brozovic A, Ambriović-Ristov A, Osmak M. The relationship between cisplatin-induced reactive oxygen species, glutathione, and BCL-2 and resistance to cisplatin. Crit Rev Toxicol 2010; **40**: 347–59.

23. Grebinyk SM, Palyvoda KO, Prylutska SV***, et al.*** Photoactivated fullerene C_{60} induces store-operated Ca^{2+} entry and cytochrome *c* release in Jurkat cells. Ukr Biokhim Zh 2012; **84**: 58–63.

24. Matyshevska OP, Palyvoda KO, Prylutska SV, ***et al.*** Induction of apoptotic leukemic cells death with the use of fullerene C_{60}. In: Nanoscale systems and nanomaterials research in Ukraine. AG Naumovets, ed. board. NAS of Ukraine. K: Akademperiodyka, 2014: 524–9 (in Russian).

25. Prylutska SV, Burlaka AP, Matyshevska OP, ***et al***. Effect of the visible light irradiation of fullerene-containing composites on the ROS generation and the viability of tumor cells. Exp Oncol 2006; **28**: 160–2.

26. Kim HJ, Lee JH, Kim SJ***, et al.*** Roles of NADPH oxidases in cisplatin-induced reactive oxygen species generation and ototoxicity. J Neurosci 2010; **30**: 3933–46.

27. Hong JY, Kim GH, Kim JW, ***et al.*** Computational modeling of apoptotic signaling pathways induced by cisplatin. BMC Syst Biol 2012; **11**: 122.

28. Shen DW, Pouliot LM, Hall MD. Cisplatin resistance: a cellular self-defense mechanism resulting from multiple epigenetic and genetic changes. Pharmacol Rev 2012; **64**: 706–21.

29. Melendez-Zajgla J, Cruz E, Maldonado V, Espinoza AM. Mitochondrial changes during the apoptotic process of HeLa cells exposed to cisplatin. Biochem Mol Biol Int 1999; **47**: 765–71.

30. Takuya Tachikawa, Yoshio Hori, Toshio Yamashita. Intracellular calcium changes and chemosensitivities of human epidermoid carcinoma cell lines after exposure to cisplatin. Ann Otol Rhinol Laryngol 1998; **107**: 611–8.

31. Marullo R, Werner E, Degtyareva N, ***et al.*** Cisplatin induces a mitochondrial-ROS response that contributes to cytotoxicity depending on mitochondrial redox status and bioenergetic functions. PLoS One 2013; **8**: doi: 10.1371/journal.pone.0081162.

32. Costantini P, Jacotot E, Decaudin D. Mitochondrion as a novel target of anticancer chemotherapy. J Natl Cancer Inst 2000; **92**: 1042–53.

33. Muscella A, Calabriso N, Fanizzi FP, ***et al.*** [Pt(O,O'-acac)(gamma-acac)(DMS)], a new Pt compound exerting fast cytotoxicity in MCF-7 breast cancer cells via the mitochondrial apoptotic pathway. Br J Pharmacol 2008; **153**: 34–49.

34. Santos SM, Dinis AM, Peixoto F, ***et al.*** Interaction of fullerene nanoparticles with biomembranes: from the partition in lipid membranes to effects on mitochondrial bioenergetics. Toxicol Sci 2014; **138**: 117–29.