

PREVENTION OF CISPLATIN TOXICITY AGAINST NORMAL CELLS BY COMPLEXATION WITH C₆₀ FULLERENE

S. V. Prylutska¹
I. I. Grynyuk¹
T. D. Skaterna²
L. B. Drobot²
N. S. Slobodyanik¹
O. P. Matyshevska²

¹Taras Shevchenko National University of Kyiv, Ukraine

²Palladin Institute of Biochemistry
of the National Academy of Sciences of Ukraine, Kyiv

E-mail: psvit_1977@ukr.net

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The aim of this study was to evaluate the toxicity of noncovalent nanocomplex of C₆₀ fullerene with cisplatin (C₆₀-Cis-Pt) against normal cells. The toxicity of the C₆₀-Cis-Pt nanocomplex compared to the free Cis-Pt was studied by estimating kidney human embryonic (HEK293) cells viability using MTT assay and rat erythrocytes resistance to acid haemolysis. It was shown that free 40 μM Cis-Pt changed the morphology and reduced the viability of HEK293 cells, as well as increased the number of haemolyzed erythrocytes compared to the control. According to the investigated parameters analysis no cytotoxic effects of C₆₀-Cis-Pt nanocomplex was observed at Cis-Pt equivalent concentration. The prevention of Cis-Pt toxic action against normal cells by its complexation with C₆₀ fullerene opens the prospect of nanostructure usage as an effective cytoprotector and a target carrier in tumor cells.

Key words: C₆₀ fullerene, cisplatin, nanocomplex, HEK293 cells, cytotoxicity, erythrocytes, haemolysis.

The use of biologically active nanomaterials for targeted drug delivery, enhancement of the traditional anticancer drugs therapeutic efficacy and prevention of its side effects is a significant and complex problem of modern biotechnology. The representative of carbon nanostructures C₆₀ fullerene is promising in this direction. It is a chemically stable, nanosized (0.72 nm), almost spherical and hydrophobic molecule that penetrates through biological membranes, localizes within cells [1–3]. As is known, chemical modification affects the physical, chemical and biological properties of C₆₀ fullerene. Pristine C₆₀ fullerene and its water-soluble derivatives do not cause toxic effects [1, 4, 5]. The accumulation of C₆₀ fullerene in tumors of the liver, stomach, intestine, lungs, bones and its selective damaging effect on malignantly

transformed cells was detected [6–8]. The surface structure of C₆₀ molecule with a system of double π-conjugated electron-deficient bonds is unique and determines the properties of this nanostructure as an antioxidant (free radical scavenger) [9, 10] as well as its ability to generate reactive oxygen species (ROS) after UV-Vis light irradiation [11–14] that can be used in photodynamic therapy of tumors. Besides, C₆₀ fullerene can form stable complexes with chemotherapeutic drugs [15–17], that can be used to optimize their action.

The traditional broad-acting anticancer drug is cisplatin (cis-diaminodichloroplatinum, cis-[Pt(II)(NH₃)₂Cl₂], Cis-Pt), the cytotoxic effect of which is caused by DNA damage and oxidative stress induction [18–22]. Despite the negative side effects of Cis-Pt, it is widely used in antitumor therapy. Clinical usage of

Cis-Pt might be increased with improving its selectivity, overcoming drug resistance and reducing toxicity.

To increase the effectiveness of Cis-Pt antitumor effect and minimize its side effects, a noncovalent nanocomplex of C₆₀ fullerene with Cis-Pt (C₆₀-Cis-Pt) was created [23]. Estimation of C₆₀-Cis-Pt nanocomplex toxicity is an important prerequisite of its usage for biomedical purposes. Thus, the aim of this work was to estimate the toxic effect of free Cis-Pt against normal cells in comparison with C₆₀-Cis-Pt nanocomplex.

Materials and Methods

Creation of C₆₀-Cis-Pt nanocomplex. C₆₀ fullerene aqueous colloid solution (C₆₀FAS) (150 µg/ml, 2–10⁻⁴ M, purity 99.95%) was prepared at the Technical University of Ilmenau (Germany) as described in [24, 25]. C₆₀ FAS is characterized by a high C₆₀ fullerene concentration and stable up to 12 months at +4 °C.

To generate C₆₀-Cis-Pt nanocomplexes, the C₆₀ solution (150 µg/ml) and Cis-Pt (Sigma, USA) solution in 0.9% NaCl saline (150 µg/ml) were mixed in 1:1 volume ratio. The mixture was sonicated with ultrasound (22 kHz, 20 min) and stirred (400 rpm, 18 h). Final concentrations of C₆₀ fullerene and Cis-Pt were 75 µg/ml (104 µM) and 75 µg/ml (250 µM), respectively. The stability of C₆₀-Cis-Pt nanocomplexes in the aqueous medium was confirmed by the results of the dynamic light scattering technique [26]. The calculated dissociation constant for the obtained noncovalent C₆₀-Cis-Pt nanocomplex is ~ 2 mM [27].

Cell culture. Non-tumor HEK293 (human embryonic kidney 293) cells were kindly supplied by the Bank of Cell Cultures and Transplantable Experimental Tumors of Kavetsky Institute of Experimental Pathology, Oncology, and Radiobiology of the National Academy of Sciences of Ukraine (Kyiv, Ukraine). Cells were maintained in DMEM (Sigma-Aldrich Co, Ltd, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich Co, Ltd, USA), 50 U/ml penicillin and 100 µg/ml streptomycin at 37 °C in a humidified atmosphere with 5% CO₂. Cells were incubated for 24 h with or without free Cis-Pt or C₆₀-Cis-Pt nanocomplex in Cis-Pt equivalent concentration.

Cell viability was assessed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma-Aldrich Co, Ltd, USA) reduction assay [28]. At indicated time

points of incubation 100 µl aliquots (10×10³ cells) were placed into the 96-well microplates Greiner (Sigma-Aldrich Co, Ltd, USA), 10 µl of MTT solution (4 mg/ml in phosphate-buffered saline (PBS)) was added to each well and the plates were incubated for another 2 h at 37 °C. Precipitates were dissolved with 100 µl of dimethyl sulfoxide (DMSO) (Sigma-Aldrich Co, Ltd, USA). Diformazan formation was determined by measuring absorption at 570 nm with a microplate reader µQuant (BioTEK, USA).

Curve fitting and calculation of the half-maximal inhibitory concentration (IC₅₀ value) were done using GraphPad Prism 7 (GraphPad Software Inc., USA). Briefly, individual concentration-effect curves were generated by fitting the logarithm of the tested compound concentration versus corresponding normalized percent of cell viability values using nonlinear regression.

Cells morphology was investigated using phase-contrast microscopy (Olympus CKX41SF, Japan). For light microscopy images an Olympus SP-500UZ (Indonesia) camera was used.

Erythrocytes haemolysis. Erythrocytes isolated from the heparinized rat blood, were incubated at 37 °C with or without C₆₀-Cis-Pt nanocomplex. Erythrocytes haemolysis was induced by addition of hydrochloric acid to the final concentration of 0.001 N [29]. Measurements of the haemolysis dynamics were carried out for 2 min with a 10 s interval on the spectrophotometer (Scinco, Germany) at λ = 630 nm.

All experiments with animals in this study were performed according to the Bio-Ethics Committee of the abovementioned institution.

Statistical analysis was performed using two-way ANOVA followed by post Bonferroni tests. The IC₅₀ value was represented as M ± SD of more than four independent experiments. A value of *P* < 0.05 was considered statistically significant.

Results and Discussion

Viability and morphology of HEK293 cells. Cytotoxic activity of C₆₀-Cis-Pt nanocomplex against HEK293 cells in Cis-Pt equivalent concentrations of 5–40 µM in comparison with the free drug was studied by MTT test at 24 h of incubation. The viability of cells incubated without additions of C₆₀ fullerene, Cis-Pt, or C₆₀-Cis-Pt nanocomplex was taken as 100% (control).

No effect of C₆₀ fullerene used alone in the range of 2.8–16.6 µM concentrations,

equivalent to those in C₆₀-Cis-Pt nanocomplex, on HEK293 cells viability during the incubation period was detected (data are not presented). The calculated IC₅₀ values for C₆₀ fullerene (IC₅₀ = 530 μM) (Table 1) action on HEK293 cells showed that it is a low toxic compound. These results are in a good agreement with the literature data. Thus, the toxic effect of pristine C₆₀ fullerene against normal baby hamster kidney BHK-21 cells were observed only at high 440 μM concentration [30].

We have detected the cytotoxic effect of Cis-Pt against HEK293 cells at 40 μM concentration. Under the action of the drug at this concentration cell viability at 24 h was reduced by 28% compared to the control (Fig. 1). The decrease of HEK293 cells viability by 43% compared to the control under the action of 50 μM Cis-Pt was also demonstrated in [31]. The calculated IC₅₀ value for Cis-Pt was shown to be 75 μM (Table 1).

Table 1. IC₅₀ values for C₆₀ fullerene, Cis-Pt and C₆₀-Cis-Pt nanocomplex in HEK293 cells ($M \pm m, n = 6$)

Compounds	IC ₅₀ (μM), 24 h
C ₆₀ fullerene	530 ± 43
Cis-Pt	75 ± 5.6
C ₆₀ -Cis-Pt nanocomplex	90 ± 6.8*

Note: * — $P < 0.05$ in comparison with Cis-Pt.

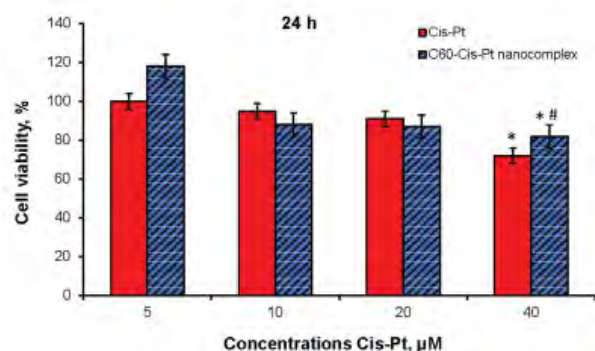


Fig. 1. Viability of HEK294 cells treated with free Cis-Pt or C₆₀-Cis-Pt nanocomplex in Cis-Pt equivalent concentrations at 24 h of incubation ($M \pm m, n = 6$):

* $P < 0.05$ in comparison with control (untreated cells); # $P < 0.05$ in comparison with Cis-Pt

With the action of 5 μM C₆₀-Cis-Pt nanocomplex the viability of HEK293 cells was increased by 20% compared to the control (Fig. 1), probably due to the initial adaptive response to the compound. C₆₀-Cis-Pt nanocomplex at 40 μM Cis-Pt equivalent concentration inhibited HEK293 viability, but the toxic effect appeared to be only 18% as compared to control (Fig. 1). The calculated value of IC₅₀ for C₆₀-Cis-Pt nanocomplex was higher (90 μM) than that for free Cis-Pt (Table 1) confirming the decreased cytotoxicity of Cis-Pt against non-tumor cells at complexation with C₆₀ fullerene.

Morphological studies showed that untreated (control) HEK293 cells formed elongated epithelioid structures and dense monolayer in some areas, a large number of intercellular contacts were observed. The cytotoxic effect of 40 μM Cis-Pt on HEK293 cells was evidenced by morphological changes of cells (Fig. 2, Table 2).

Most of HEK293 cells treated with 40 μM Cis-Pt were characterized by atypical morphology and smaller size (Table 2). No evident effect of C₆₀-Cis-Pt nanocomplex in equivalent 40 μM Cis-Pt concentration on cells morphology was detected.

Therefore, the toxic effect of Cis-Pt on normal cells at complexation with C₆₀ fullerene was reduced. The protective effects of C₆₀ fullerene against the toxic effects of Cis-Pt may be due to the antioxidant properties of the carbon nanostructure [32, 33, 34]. We have previously shown that C₆₀ fullerene prevented ROS production in thymocytes and prevented the decrease of thymocytes viability induced by Cis-Pt [35, 36].

Erythrocytes haemolysis. The use of platinum-based drugs in chemotherapy is limited due to its high haematotoxicity, that is the cause of haemolytic anemia and bone marrow disease [37]. So the search for erythrocyte protection pathways against drug damage is of current interest.

The effect of Cis-Pt and C₆₀-Cis-Pt nanocomplex at the level of cells plasma membrane was estimated by the dynamics of erythrocytes haemolysis, which reflects the dynamics of erythrocyte plasma membrane destruction and the release of haemoglobin into the environment. Erythrocytes haemolysis of the control (untreated red blood cells) was accelerated at 40 s after the treatment with haemolytic and reached the maximum at 60 s, the number of haemolyzed cells was 30 ± 2% (Fig. 3).

Haemolysis of erythrocytes treated with 40 μM Cis-Pt slightly slowed, however, the

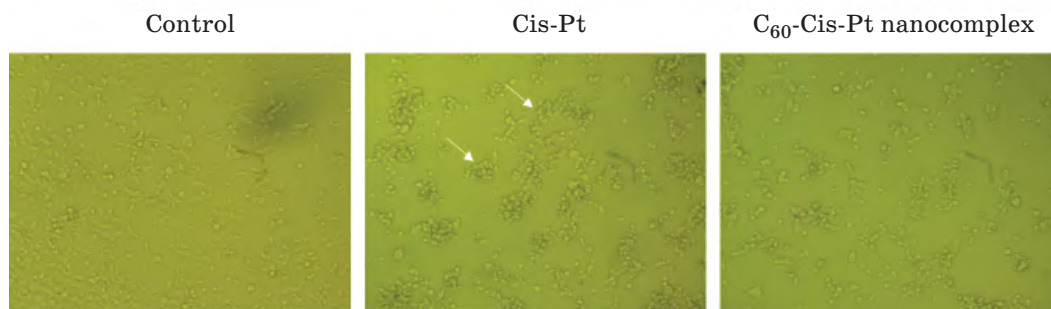


Fig. 2. Microphotographs of HEK293 cells incubated for 24 h in the presence of free 40 μM Cis-Pt or C_{60} -Cis-Pt nanocomplex (phase-contrast microscopy, $\times 400$)

Table 2. Morphological features in HEK293 cells at 24 h after action 40 μM Cis-Pt or C_{60} -Cis-Pt nanocomplex ($M \pm m, n = 6$)

Compounds	Changes		
	Atypical cell morphology	Smaller cell size	Roud shape and nonadherent pattern
Cis-Pt	++++	++	++++
C_{60} -Cis-Pt nanocomplex	+	-	+

Note: + few, ++ moderate, +++ severe, ++++ many.

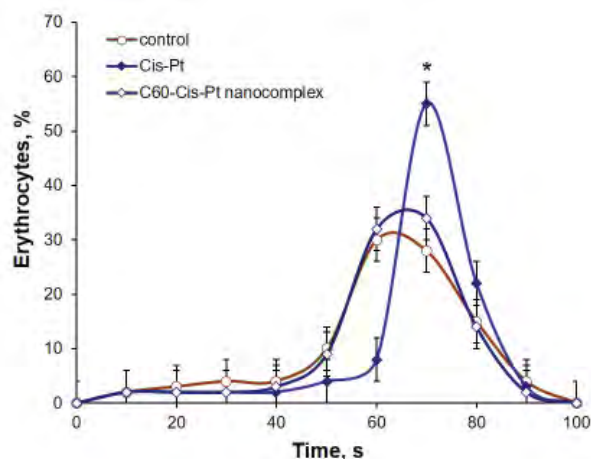


Fig. 3. Erythrocytes haemolysis after action 40 μM Cis-Pt or C_{60} -Cis-Pt nanocomplex ($M \pm m, n = 6$): * $P < 0.05$ in comparison with control (untreated cells)

number of haemolyzed cells reached $55 \pm 2\%$ (Fig. 3), indicating on Cis-Pt-induced decrease of cells resistance to haemolysis. As it was shown in [38], Cis-Pt at 35 μM concentration caused morphological changes of human erythrocyte membrane by embedding into the inner layer of cells. The extranuclear mechanism of Cis-Pt cytotoxic action is associated with its high affinity to phosphatidylserine the inner monolayer of the erythrocyte plasma membrane [39].

C_{60} fullerene at 16.6 μM concentration equivalent to that in C_{60} -Cis-Pt nanocomplex did not change parameters of erythrogram, indicating on the absence of haemolytic effect of carbon nanostructure (data are not presented). As it was shown by us previously [40, 41], the slight haemolytic activity of C_{60} fullerene was detected at concentrations $> 50 \mu\text{M}$.

No haemolytic effect was detected when erythrocytes were treated with C_{60} -Cis-Pt nanocomplex at 40 μM Cis-Pt equivalent concentration. The parameters of erythrogram were the same as in control (Fig. 3) indicating that C_{60} fullerene weakened Cis-Pt interaction with erythrocyte membrane and was able to increase the resistance of erythrocyte membrane to Cis-Pt induced haemolysis.

The data obtained showed that the cytotoxic effect of Cis-Pt against normal cells was prevented at complexation with C_{60} fullerene and that C_{60} -Cis-Pt nanocomplex has a potential to be used for minimization of anticancer drug side effects.

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The authors declare that they have no conflicts of interest.

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ЗАПОБІГАННЯ ТОКСИЧНІЙ ДІЇ ЦИСПЛАТИНУ ЩОДО НОРМАЛЬНИХ КЛІТИН ЗА КОМПЛЕКСООУТВОРЕННЯ ІЗ C₆₀ ФУЛЕРЕНОМ

С. В. Прилуцька¹, І. І. Гринюк¹,
Т. Д. Скатерна², Л. Б. Дробот²,
М. С. Слободяник¹, О. П. Матишевська²

¹Київський національний університет імені Тараса Шевченка, Україна

²Інститут біохімії ім. О. В. Палладіна НАН України, Київ

E-mail: psvit_1977@ukr.net

Метою роботи було оцінити токсичність нековалентного наноконкомплексу C₆₀ фуллерену з цисплатином (C₆₀-Cis-Pt) щодо нормальних клітин. Токсичність C₆₀-Cis-Pt наноконкомплексу, порівняно з вільним Cis-Pt, вивчали на клітинах ембріональної нирки людини (НЕК293), оцінюючи їх життєздатність за допомогою МТТ-тесту та на еритроцитах щура за їхньою стійкістю до кислотного гемолізу. Було показано, що вільний 40 мкМ Cis-Pt змінював морфологію та знижував життєздатність клітин НЕК293 на 28%, а також збільшував кількість гемолізованих еритроцитів на 25% порівняно з контролем. C₆₀-Cis-Pt наноконкомплекс за еквівалентної концентрації Cis-Pt не впливав на досліджувані показники і не спричиняв цитотоксичних ефектів. Запобігання токсичній дії Cis-Pt на нормальні клітини за його комплексоутворення із C₆₀ фуллереном відкриває перспективу використання наноструктури як ефективного цитопротектора і таргетного носія у пухлинні клітини.

Ключові слова: C₆₀ фуллерен, цисплатин, наноконкомплекс, НЕК293 клітини, цитотоксичність, еритроцити, гемоліз.

ПРЕДОТВРАЩЕНИЕ ТОКСИЧЕСКОГО ДЕЙСТВИЯ ЦИСПЛАТИНА НА НОРМАЛЬНЫЕ КЛЕТКИ ПУТЕМ КОМПЛЕКСООБРАЗОВАНИЯ С C₆₀ ФУЛЛЕРЕНОМ

С. В. Прилуцкая¹, И. И. Гринюк¹,
Т. Д. Скатерна², Л. Б. Дробот²,
Н. С. Слободяник¹, О. П. Матишевская²

¹Киевский национальный университет имени Тараса Шевченко, Украина

²Институт биохимии им. А. В. Палладина НАН Украины, Киев

E-mail: psvit_1977@ukr.net

Целью работы было оценить токсичность нековалентного наноконкомплекса C₆₀ фуллерена с цисплатином (C₆₀-Cis-Pt) относительно нормальных клеток. Токсичность C₆₀-Cis-Pt наноконкомплекса, в сравнении со свободным Cis-Pt, изучали путем оценки жизнеспособности клеток эмбриональной почки человека (НЕК293) с помощью МТТ-теста и на эритроцитах крысы по их устойчивости к кислотному гемолізу. Было показано, что свободный 40 мкМ Cis-Pt вызывал морфологические изменения и снижение жизнеспособности клеток НЕК293 на 28%, а также увеличение количества гемолізованных эритроцитов на 25% по сравнению с контролем. C₆₀-Cis-Pt наноконкомплекс в эквивалентной концентрации Cis-Pt не влиял на исследуемые показатели и не вызывал цитотоксических эффектов. Предотвращение токсического действия Cis-Pt на нормальные клетки при комплексообразовании с C₆₀ фуллереном открывает перспективу применения наноструктуры как эффективного цитопротектора и таргетного носителя в опухолевые клетки.

Ключевые слова: C₆₀ фуллерен, цисплатин, наноконкомплекс, НЕК293 клетки, цитотоксичность, эритроциты, гемоліз.