

APOPTOSIS PHOTOINDUCTION BY C₆₀ FULLERENE IN HUMAN LEUKEMIC T CELLS

K. O. PALYVODA^{1,2}, I. I. GRYNKYUK², S. V. PRYLUTSKA², A. A. SAMOYLENKO¹,
L. B. DROBOT¹, O. P. MATYSHEVSKA²

¹*Palladin Institute of Biochemistry, National Academy of Sciences of Ukraine, Kyiv;*

²*Taras Shevchenko Kyiv National University, Ukraine;*

e-mail: pksenii@yahoo.com

The viability of normal (Wistar rat thymocytes) and transformed (human leukemia Jurkat cells) T cells after UV/Vis irradiation in the presence of pristine C₆₀ fullerene was studied. The data obtained have shown that C₆₀ fullerene exhibits cytotoxic effect against transformed T lymphocytes when combined with UV/Vis irradiation using mercury-vapor lamp (320-600 nm). C₆₀ fullerene photocytotoxicity was not detected in thymocytes. C₆₀-dependent photoinduced apoptosis of Jurkat cells was confirmed by DNA fragmentation and caspase-3 activation. No substantial increase of caspase-3 activation was observed in thymocytes treated with C₆₀ fullerene plus irradiation, while antileukemic agent cytosine arabinoside was shown to induce caspase-3 activation both in Jurkat cells and thymocytes. The data obtained may be useful for development of photosensitizers for photodynamic therapy with selective action on leukemia cells.

Key words: C₆₀ fullerene, thymocytes, Jurkat cells, apoptosis, cell viability, caspase-3, DNA ladder, Ara-C, photodynamic therapy.

Recently it became evident that modulation of signal transduction pathways rather than interference at genetic level is a promising strategy for anticancer chemotherapy. It is well recognized that tumor cells are characterized by significant genome instability and great variety of potential gene targets. The genetic defects that promote the growth and survival of tumor cells may also contribute to their resistance against various anticancer agents. That is why intense research has been devoted to understanding the molecular events involved in apoptotic cell death to develop strategies that can selectively restore the apoptotic potential of tumor cells and avoid injury of normal cells.

Photodynamic therapy (PDT) is one of non-invasive treatments with reduced side effects for various types of tumors. Although the majority of photosensitizing compounds are based on the tetrapyrrole backbone, found particularly in porphyrins, other molecular structures have been studied for medical purposes in PDT [1, 2]. Recent progress in nanobiotechnology have arised interest in biomedical application of a new class of nanostructures made exclusively of carbon atoms – C₆₀ fullerenes, spheroidally shaped molecules (0.72 nm in diameter), which demonstrate unique physicochemical properties and are able to interfere into

cell signal transduction events [3, 4]. Due to extended π -conjugated system of molecular orbitals C₆₀ fullerene absorbs UV/Vis light and generate reactive oxygen species (ROS) with almost 100% quantum yield. The studies of C₆₀ fullerene biological activity were focused mainly on water-soluble chemically modified derivatives [2, 5]. But substantial modification of fullerene core appears to cause a perturbation of its electronic structure and hence to reduce the photodynamic potential of the molecule [6]. Therefore pristine (nonmodified) C₆₀ fullerene is suggested to be perspective photosensitizer.

Although no selective uptake of C₆₀ by tumor cells has been demonstrated yet, the phototoxicity of C₆₀ fullerene against HeLa cells [7] and a panel of mouse cancer cells [2] was demonstrated. In a previous research we have demonstrated that C₆₀ fullerene reduced the viability of transformed T lymphocytes when combined with UV/Vis irradiation [8]. But the mechanisms involved in cell death induction as well as selectivity of C₆₀ damaging effects still remain unclear and need better understanding. The aim of this study was to evaluate the determinants of apoptosis in normal (Wistar rats thymocytes) and transformed (human leukemia Jurkat cell line) T lymphocytes treated with C₆₀ fullerene after UV/Vis irradiation.

Materials and Methods

Materials. Rabbit polyclonal antibody against cleaved caspase-3 (Cell Signaling, USA), antibodies against β -actin (Sigma, USA) and horseradish peroxidase-conjugated secondary antibodies (Promega, USA) were used.

A stable water colloidal solution of pristine C_{60} fullerene was prepared at Ilmenau Technical University (Germany) by C_{60} transfer from toluene to water using prolonged ultrasound sonication [9]. Both hydrated individual C_{60} molecules and C_{60} clusters (12-50 nm) were present in colloidal solution.

Cell culture and treatments. Primary rat thymocytes were isolated from female Wistar rats (150-180 g). Thymus was removed, passed through nylon mesh into RPMI 1640 medium, the cells were washed by centrifugation (600 g) and resuspended in RPMI 1640 medium. The human T leukemia Jurkat cell line was obtained from the Bank of Cell Lines of R. E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine. Jurkat cell line was maintained in suspension culture using RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 μ g/ml streptomycin, 100 μ g/ml penicillin.

Cells (thymocytes – 1-2·10⁶/ml, Jurkat cells – 0.5·10⁶/ml) were preincubated for 1 h in RPMI 1640 medium with or without C_{60} fullerene followed by illumination with mercury-vapor lamp (320-600 nm light, irradiance 200 mW/cm², distance 2 cm). Cells were further incubated for different time periods in RPMI 1640 medium at 37 °C in a 5% CO₂ humidified atmosphere. In other experiments cells were incubated in the presence of 10 μ M cytosine arabinoside (Sigma, USA) for 24 h.

Cell viability was assessed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] reduction assay [10]. After treatment, 10 μ M MTT, which is metabolized into formazan by intact mitochondrial dehydrogenases, was added to cells. After 2 h reaction period, the precipitates were dissolved with 150 μ l DMSO and absorbance was measured at 570 nm using automatic plate reader (mQuant, BioTek, USA).

Immunoblot analysis [11]. Cells were washed with PBS and lysed in a buffer containing 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 50 mM NaF, 1 mM PMSF and the Complete Protease Inhibitor Cocktail Tablet (Roche, Germany) on ice for 15 min. Cell lysates were clarified by centrifugation at 14,000 g for 15 min. Cell lysates (about 30 μ g of total cell protein) were loaded onto a 15% SDS-polyacrylamide gel. After electrophoresis the proteins were transferred onto

PVDF membranes. The PVDF membranes were then incubated in blocking solution (5% skim milk in PBST) for 1 h followed by overnight incubation with antibody against cleaved caspase-3 at a dilution of 1:2500 at 4 °C. The membranes were washed with PBST and incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000 dilution) for 1 h. After additional washing, immunoreactive bands were detected by enhanced chemiluminescence with an ECL plus Western blotting detection system (Amersham, USA). Equal loading of samples was confirmed by using anti- β -actin antibodies (1:2000 dilution).

Detection of DNA fragmentation [12]. For DNA ladder assay the cells (3·10⁶ for each sample) were lysed in a buffer, containing 0.5% sarkosyl, 50 mM Tris-HCl, 10 mM EDTA, proteinase K (0.5 mg/ml), pH 8. After 1 h incubation RNase A (0.25 mg/ml) was added to each sample. The DNA was analyzed by electrophoresis on a 1.5% agarose gel in the presence of 0.5 μ g/ml ethidium bromide. A DNA markers (Fermentas, USA) were used to size DNA fragments.

Protein concentration was determined using DC Protein Assay Kit (Bio-Rad, USA).

Statistical analysis. Paired Student's *t*-tests were performed. Values *P* < 0.05 were considered to be significant.

Results and Discussion

As we have shown previously irradiation with applied light (320-600 nm) *per se* does not affect cell survival – the viability of thymocytes was at the level of control and that of Jurkat cells was not less than 83% at 24 h of incubation [8]. These control indices (viability of cells preincubated without C_{60} fullerene but irradiated as described above) were taken as 100%.

The effect of C_{60} in different concentrations plus UV/Vis irradiation on the viability of thymocytes incubated for 6 h was evaluated and compared with that of transformed T-lymphocytes (Fig. 1). No appreciable effect on thymocytes was revealed in the range of applied C_{60} concentrations. At the same time substantial phototoxicity of C_{60} fullerene against transformed T lymphocytes was detected. Photocytotoxic effect of fullerene C_{60} was shown to be concentration dependent – viability of Jurkat cells was diminished to approximately 50% when C_{60} concentration was increased up to 100 μ M.

To study the dynamics of photoexcited C_{60} effects the experiments were done with short- and long-term incubation of T lymphocytes irradiated in the presence of 50 μ M C_{60} . Fig. 2 shows that up to 24 h the viability of thymocytes was comparable to that of control. Only a slight change of Jurkat

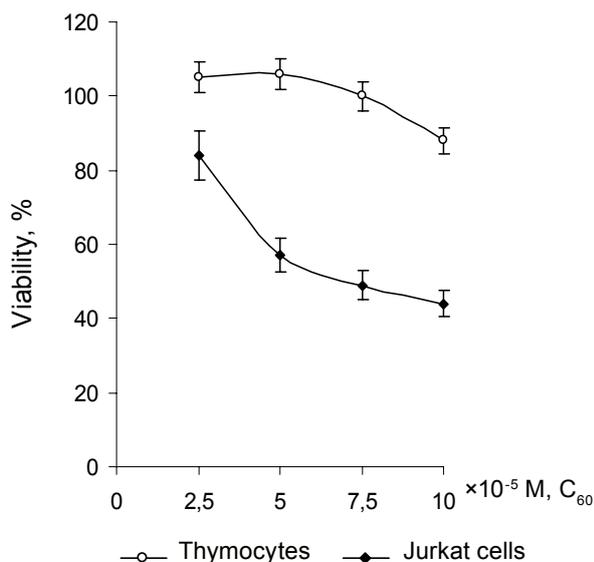


Fig. 1. Viability of cells after UV/Vis irradiation in the presence of C₆₀ fullerene at different concentrations (viability of cells irradiated in the absence of C₆₀ fullerene was set as 100%)

cells viability was observed at 3 h, but a substantial decrease was observed after subsequent 6 and 24 h of incubation.

The results of MTT test demonstrate an evident photodamage of Jurkat cells incubated with C₆₀ fullerene. These data do not provide information regarding the type of cell death. The decrease of cell viability can be mediated by different cell death mechanisms such as necrosis, apoptosis, autophagy, etc. Therefore the formation of DNA ladder was monitored as a hallmark of apoptosis.

Agarose gel electrophoresis of DNA extracted from control untreated thymocytes incubated for 24 h revealed the characteristic ladder pattern of fragments with a spacing of about 200 bp (Fig. 3). Internucleosomal DNA cleavage is considered to be typical for thymocytes. Immature lymphocytes precursors with unstable genome undergo spontaneous apoptosis as a mechanism of negative selection during differentiation [13]. After UV/Vis irradiation either without or in the presence of 50 μM C₆₀ fullerene no appreciable difference in the amount of fragmented DNA was revealed as compared to untreated thymocytes.

In contrast only large scale DNA fragmentation was detected in control untreated Jurkat cells at 24 h. A slight increase of DNA fragmentation was observed after UV/Vis irradiation, while irradiation in the presence of C₆₀ fullerene was followed by internucleosomal DNA cleavage and appearance of DNA fragments of 200 and 400 bp in ladder pattern (Fig. 3).

So the data of DNA ladder assay confirm that Jurkat cells death induced by C₆₀ after UV/Vis irradiation was of apoptotic type.

It is generally assumed that proteases of caspase family are rate limiting for the development of apoptosis. Executive caspase-3 and caspase-6 are responsible for the terminal phase of apoptosis by cleaving various protein substrates thereby disabling cellular structural and repair processes [14]. Caspase-3 is expressed in cells as an inactive precursor with molecular weight of 35 kDa and is cleaved upon activation into two fragments of 17 and 19 kDa [15]. Therefore our next step was to assess the status of caspase-3 during PDT-induced apoptosis in Jurkat cells.

As it is shown at Fig. 4 UV/Vis irradiation alone is followed by caspase-3 activation in Jurkat cells at 6 and 24 h. But in cells, irradiated in the presence of 50 μM C₆₀ fullerene, caspase-3 processing was dramatically intensified – the content of 17 and 19 kDa intermediate cleavage products was increased already at 6 h (Fig. 4). After 24 h caspase-3 activation was increased times 20 as compared to control (Fig. 4 and Fig. 5).

The correlation between caspase-3 activation and DNA fragmentation under the same experimental conditions was observed, supporting the role of caspase-3 as executable protease of apoptosis in C₆₀-treated Jurkat cells after UV/Vis irradiation.

We have done more detailed analysis of photoexcited C₆₀ fullerene apoptosis-inducing potential and selectivity against transformed lymphocytes in a comparative study of caspase-3 activation in thymocytes and Jurkat cells treated with cytosine arabinoside (1-β-d-arabinofuranosylcytosine, Ara-C).

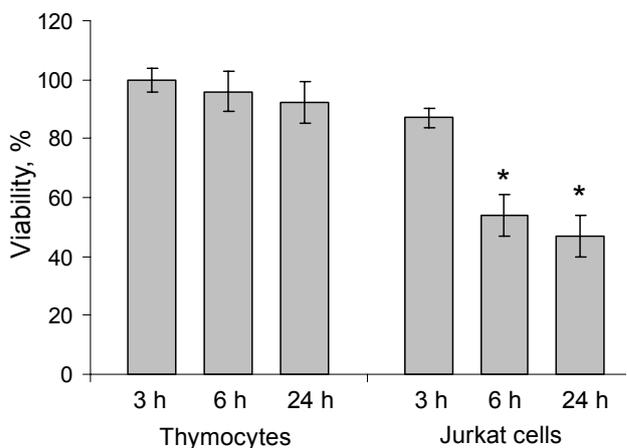


Fig. 2. Viability of cells incubated for different time periods after UV/Vis irradiation in the presence of C₆₀ fullerene. *P ≤ 0.05 in comparison with cells irradiated in the absence of C₆₀ fullerene

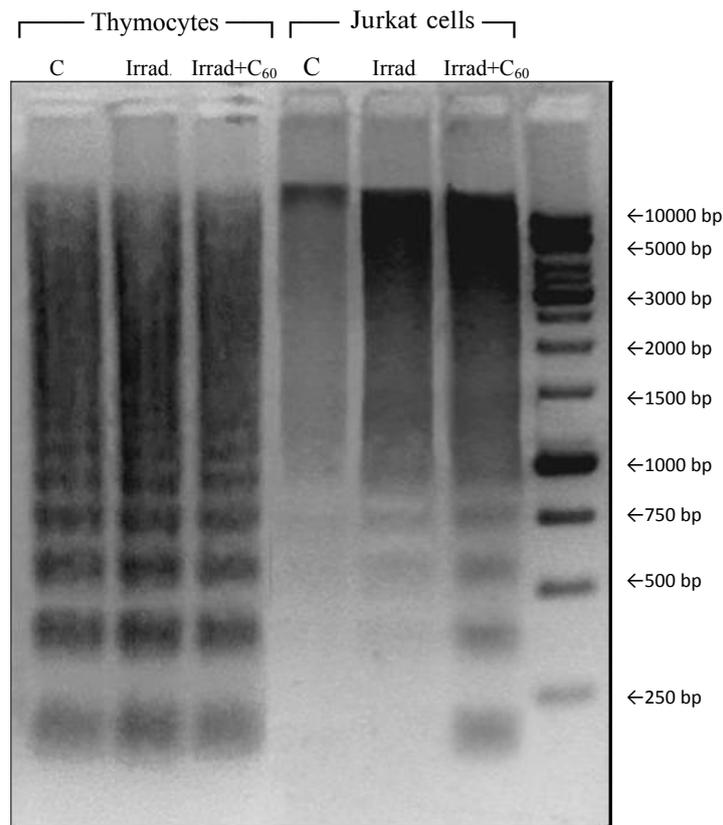


Fig. 3. DNA fragmentation in thymocytes and Jurkat cells at 24 h incubation after UV/Vis irradiation in the absence or in the presence of C_{60} fullerene

Ara-C is a cytidine analogue containing a modified sugar moiety (arabinose instead of ribose) and one of the most important antimetabolite used to induce remission in children and adults with acute leukemia. This agent inhibits DNA polymerase resulting in a decrease in DNA replication and repair. The major mechanisms of Ara-C-induced apoptosis are incorporation of the drug into DNA

during replication, increase of ROS generation and p53-dependent cytotoxicity [16].

Figure 5 shows caspase-3 status presented as a fold of enzyme activation at 24 h in cells subjected to treatment with light alone, with light plus C_{60} fullerenes or with 10 μ M Ara-C as compared to control (caspase-3 activation in untreated cells). As we showed previously [17], Ara-C in this concen-

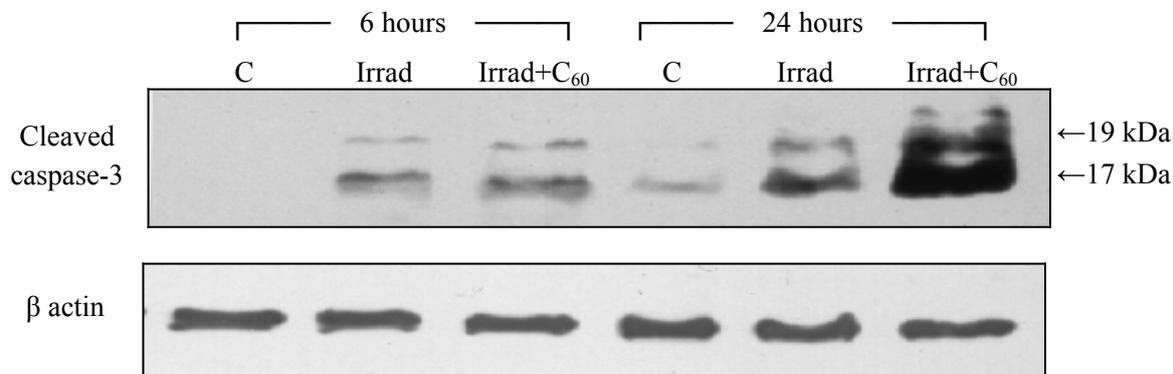


Fig. 4. Western blot analysis of caspase-3 activation in Jurkat cells after UV/Vis irradiation in the absence or in the presence of C_{60} fullerene

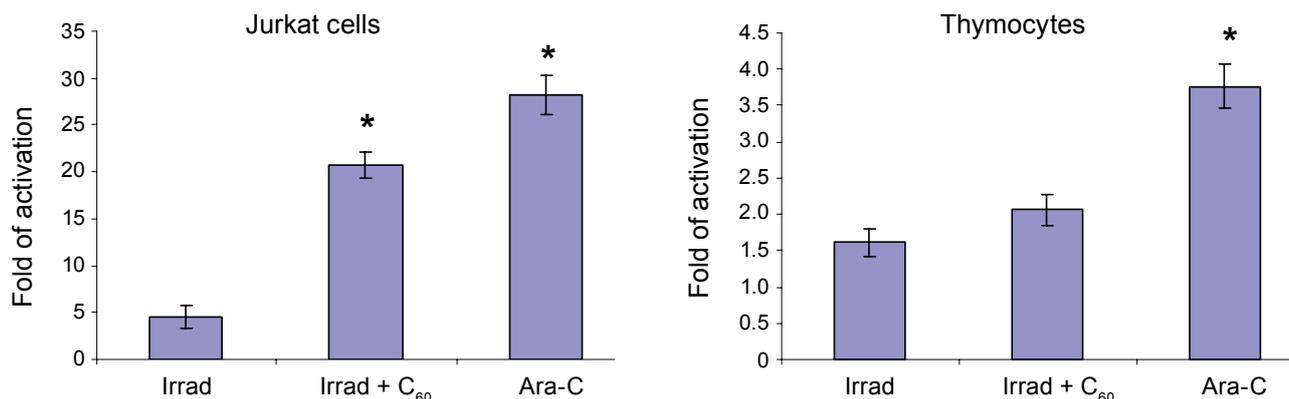


Fig. 5. Caspase-3 activation at 24 h in cells treated with light alone, with light plus 50 μM C₆₀ fullerene or with 10 μM Ara-C. * $P \leq 0.05$ in comparison with caspase-3 activation in irradiated cells

tration induces 50% decrease of both thymocytes and Jurkat cells viability.

Although in Ara-C-treated Jurkat cells the greater fold increase of caspase-3 activation was observed than in cells subjected to treatment with light plus C₆₀ fullerene it is obvious that in general photosensitizing cytotoxic effect of C₆₀ fullerene is comparable with that of Ara-C in apoptosis induction. The data observed are in agreement with susceptibility of a number of typical human leukemia cell lines to Ara-C-induced cell death via apoptosis [16]. It should be stressed that 10 μM Ara-C appears to induce apoptosis via caspase-3 activation not only in Jurkat cells but in thymocytes as well (Fig. 5), while no substantial increase in caspase-3 activation was observed in thymocytes subjected to treatment with C₆₀ fullerene plus irradiation (Fig. 5). These results correspond to data that photoexcited C₆₀ fullerene does not influence thymocytes viability and the level of DNA fragmentation. So the selectivity of photoexcited C₆₀ cytotoxic effect against transformed T cells but not T cells from thymus is demonstrated in contrast to Ara-C nonspecific cytotoxicity.

All experiments with irradiation in this study were done after 1 h incubation of cells with C₆₀ fullerene. During this period of time fullerene should be incorporated into the cell membrane or taken up inside the cells [18], because generation of ROS outside the cell will not be sufficient to produce cell death. Fullerene has inherent photoluminescence but it allows to detect intracellular uptake only at high C₆₀ concentrations. In recent report [18] non-toxic pristine C₆₀ preparation (200 $\mu\text{g}/\text{ml}$) was used and intracellular uptake of C₆₀ in malignant cancer breast cells was demonstrated by fluorescence microscopy.

We suggest that transformed T cells death after C₆₀ illumination is mediated mainly by ROS

influence on targets in cytoplasmic membrane involved in cellular signaling, but subcellular organelles can also be involved. It was shown recently [2] that C₆₀ modified by single pyrrolidinium group can mediate killing of lung and colon adenocarcinoma and reticulum sarcoma cells at low concentration (2 μM) by induction of apoptosis after very modest exposure to white light. In addition, the increase of intracellular ROS probe dichlorofluorescein fluorescence was demonstrated.

Studies carried out on animal tumor models demonstrate that an apoptotic response can be achieved when mitochondria [19] or lysosomes [20] are targeted for photodamage. The kinetics of apoptotic response following lysosomal photodamage appear to be slower than that observed in cell culture following mitochondrial photodamage [21, 22]. We demonstrated the substantial induction of caspase-3 activation by PDT mediated by C₆₀ fullerenes in Jurkat cells at 24 h after irradiation. The relatively slow induction of apoptosis allows to suggest that the release of lysosomal proteases and subsequent activation of cytoplasmic caspase or damage of mitochondria leading to release of cytochrome *c* may be involved in cell response.

In conclusion, our investigation has revealed that pristine C₆₀ fullerene exhibits cytotoxic effect against transformed T lymphocytes when combined with UV/Vis irradiation. Photoinduced apoptosis of Jurkat cells was confirmed by DNA fragmentation and caspase-3 activation. C₆₀ fullerene photocytotoxicity was not detected in normal T lymphocytes (thymocytes) in contrast to Ara-C cytotoxicity detected in cells of both types. Our results may be useful for search of new photosensitizers for PDT with selective action on leukemia cells.

**ИНДУКЦИЯ АПОПТОЗУ ЗА ДІЇ
ФОТОЗБУДЖЕНОГО ФУЛЕРЕНУ
C₆₀ У ЛЕЙКЕМІЧНИХ Т-КЛІТИНАХ
ЛЮДИНИ**

*К. О. Паливода^{1,2}, І. І. Гринюк²,
С. В. Прилуцька², А. А. Самойленко¹,
Л. Б. Дробот¹, О. П. Матишевська²*

¹Інститут біохімії ім. О. В. Палладіна
НАН України, Київ;

²Київський національний університет
імені Тараса Шевченка, Україна;
e-mail: pkseniiia@yahoo.com

Досліджено життєздатність нормальних (тимоцити щура лінії Вістар) та пухлинних (клітини лінії Jurkat лейкемії людини) Т-клітин після опромінення в ультрафіолетовому та видимому діапазоні за присутності фулерену C₆₀. Одержані дані свідчать, що комбінована дія фулерену C₆₀ та опромінення ртутно-дейтерієвою лампою (320–600 нм) спричиняє цитотоксичний ефект на лейкемічні Т-лімфоцити. Фулерен C₆₀ не виявляє фотоцитотоксичності по відношенню до тимоцитів. Фотоіндукований апоптоз клітин лінії Jurkat за дії C₆₀ підтверджується міжнуклеосомною фрагментацією ДНК та активацією каспази-3. Вірогідної зміни рівня активації каспази-3 у тимоцитах за дії фотозбудженого фулерену C₆₀ порівняно з опроміненням не спостерігалось, тоді як цитозинарабінозид спричиняє активацію каспази-3 не лише в клітинах Jurkat, але і в тимоцитах. Одержані дані можуть бути корисними для розробки нових фотосенсибілізаторів для фотодинамічної терапії із селективною дією на лейкемічні клітини.

Ключові слова: фулерен C₆₀, тимоцити, клітини Jurkat, цитозинарабінозид, апоптоз, життєздатність клітин, каспаза-3, міжнуклеосомна фрагментація ДНК, фотодинамічна терапія.

**ИНДУКЦИЯ АПОПТОЗА
ПРИ ДЕЙСТВИИ
ФОТОВОЗБУЖДЕННОГО ФУЛЛЕРЕНА
C₆₀ В ЛЕЙКЕМИЧЕСКИХ Т-КЛЕТКАХ
ЧЕЛОВЕКА**

*К. О. Паливода^{1,2}, И. И. Гринюк²,
С. В. Прилуцкая², А. А. Самойленко¹,
Л. Б. Дробот¹, О. П. Матишевская²*

¹Інститут біохімії ім. А. В. Палладіна
НАН України, Київ;

²Киевский национальный университет
имени Тараса Шевченко, Украина;
e-mail: pkseniiia@yahoo.com

Исследовано жизнедеятельность нормальных (тимоциты крысы линии Вистар) и опухолевых (клетки линии Jurkat лейкемии человека) Т-клеток после облучения в ультрафиолетовом и видимом диапазоне в присутствии фуллерена C₆₀. Полученные результаты свидетельствуют о том, что комбинированное действие фуллерена C₆₀ и облучения ртутно-дейтериевой лампой (320–600 нм) вызывает цитотоксический эффект на лейкемические Т-лимфоциты. Фуллерен C₆₀ не проявляет фотоцитотоксичность по отношению к тимоцитам. Фотоиндуцированный апоптоз клеток линии Jurkat при действии C₆₀ подтверждается межнуклеосомной фрагментацией ДНК и активацией каспазы-3. Достоверных изменений уровня активации каспазы-3 в тимоцитах при действии фотозбудженного фуллерена C₆₀ по сравнению с облучением не наблюдалось, тогда как цитозинарабинозид вызывал активацию каспазы-3 не только в клетках Jurkat, но и в тимоцитах. Полученные результаты могут быть полезными для разработки новых фотосенсибилизаторов для фотодинамической терапии с селективным действием на лейкемические клетки.

Ключевые слова: фуллерен C₆₀, тимоциты, клетки Jurkat, цитозинарабинозид, апоптоз, жизнеспособность клеток, каспаза-3, межнуклеосомная фрагментация ДНК, фотодинамическая терапия.

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Received 21.07.2010