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APOPTOSIS PHOTOINDUCTION BY C_{60} FULLERENE IN HUMAN LEUKEMIC T CELLS

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The viability of normal (Wistar rat thymocytes) and transformed (human leukemia Jurkat cells) T cells after UV/Vis irradiation in the presence of pristine C_{60} fullerene was studied. The data obtained have shown that C_{60} fullerene exhibits cytotoxic effect against transformed T lymphocytes when combined with UV/Vis irradiation using mercury-vapor lamp (320-600 nm). C_{60} fullerene photocytotoxicity was not detected in thymocytes. C_{60}-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 C_{60} 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.

K e y w o r d s: C_{60} 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 develope strategies that can selectively restore the apoptotic potential of tumor cells and avoid injury of normal cells.

Photodynamic therapy (PDT) is one of noninvasive 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 – C_{60} 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 C_{60} fullerene absorbs UV/Vis light and generate reactive oxygen species (ROS) with almost 100% quantum yield. The studies of C_{60} fullerene biological activity were focused mainly on water-soluble chemically modified derivatives [2, 5]. But substantial modification of fullerene core appears to cause a pertubation of its electronic structure and hence to reduce the photodynamic potential of the molecule [6]. Therefore pristine (nonmodified) C_{60} fullerene is suggested to be perspective photosensitizer.

Although no selective uptake of C_{60} by tumor cells has been demonstrarted yet, the phototoxicity of C_{60} fullerene against HeLa cells [7] and a panel of mouse cancer cells [2] was demonstrared. In a previous research we have demonstrated that C_{60} 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 C_{60} 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 C_{60} 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 μg/ml streptomycin, 100 μg/ml penicillin.

Cells (thymocytes – $1\text{-}2\cdot10^6$/ml, Jurkat cells – $0.5\cdot10^6$/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/cm^2, distance 2 cm). Cells were further incubated for different time periods in RPMI 1640 medium at 37 °C in a 5% CO_2 humidified atmosphere. In other experiments cells were incubated in the presence of 10 μM 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 μM 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 μl 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 μg 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 ($3\cdot10^6$ 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 pg/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 μM.

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 μM 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

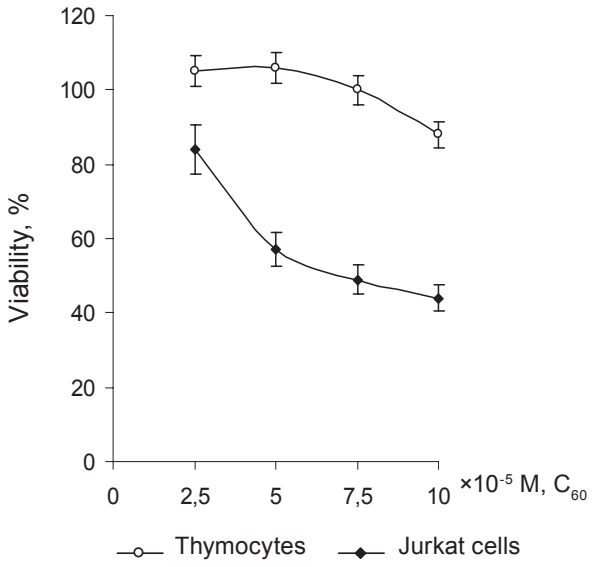

Fig. 1. Viability of cells after UV/Vis irradiation in the presence of C_{60} fullerene at different concentrations (viability of cells irradiated in the absence of C_{60} fullerene was set as 100%)

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 C_{60} 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 C_{60} 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 C_{60} 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 C_{60} after UV/Vis irradiation was of apoptotic type.

Is 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 C_{60} 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 C_{60}-treated Jurkat cells after UV/Vis irradiation.

We have done more detailed analysis of photoexcited C_{60} 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).

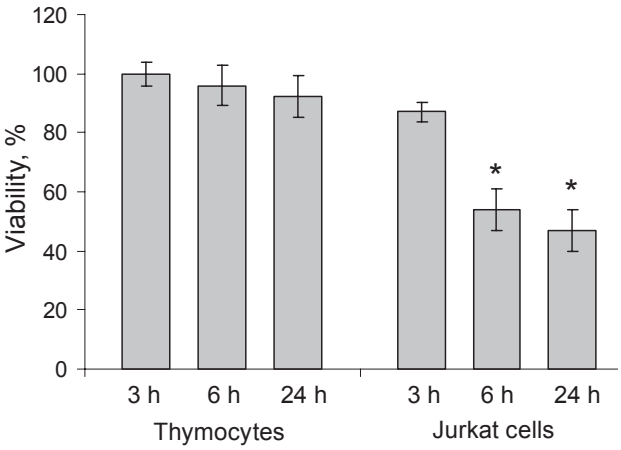

*Fig. 2. Viability of cells incubated for different time periods after UV/Vis irradiation in the presence of C_{60} fullerene. *$P \leq 0.05$ in comparison with cells irradiated in the absence of C_{60} fullerene*

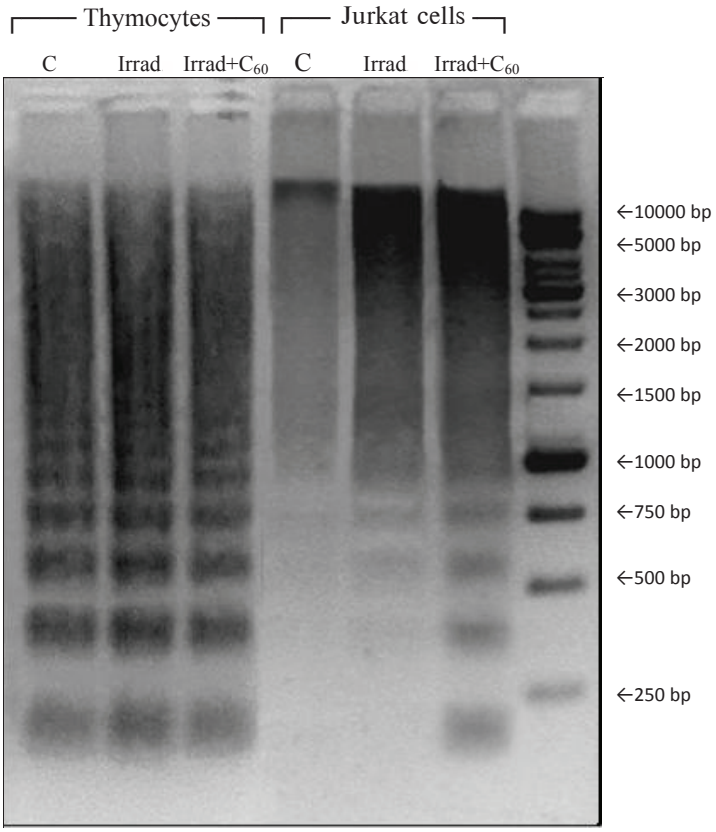

Fig. 3. DNA fragmentation in thymocytes and Jurkat cells at 24 h incubation after UV/Vis irradiation in the absence or in the presence of C_{60} fullerene

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 μM Ara-C as compared to control (caspase-3 activation in untreated cells). As we showed previously [17], Ara-C in this concen-

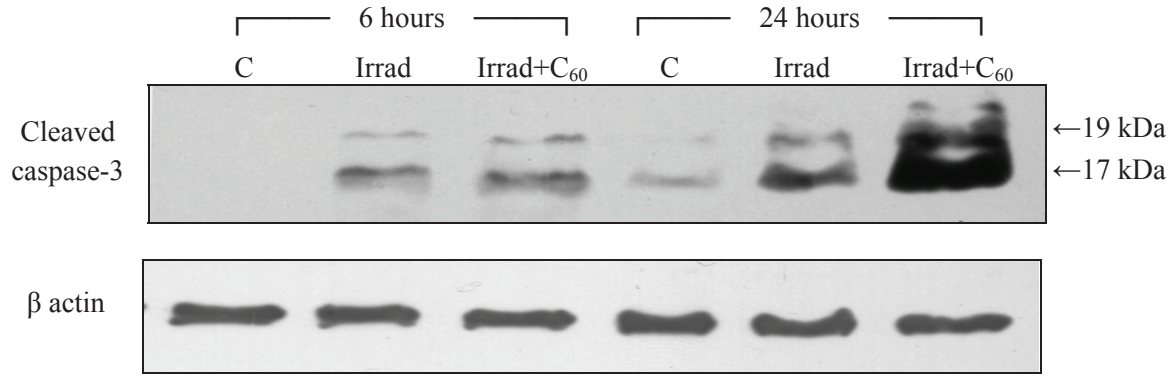

Fig. 4. Western blot analysis of caspase-3 activation in Jurkat cells after UV/Vis irradiation in the absence or in the presence of C_{60} fullerene

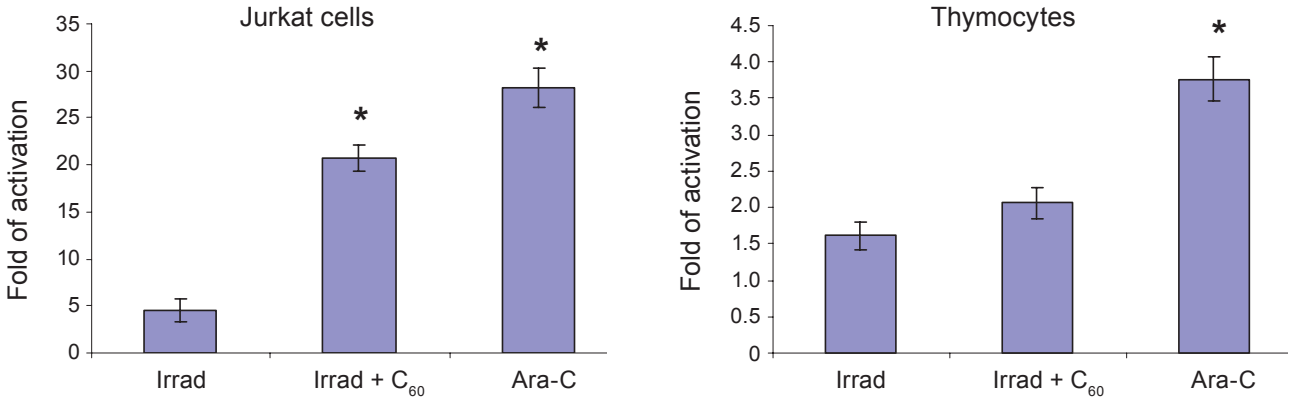

*Fig. 5. Caspase-3 activation at 24 h in cells treated with light alone, with light plus 50 μM C_{60} fullerene or with 10 μM Ara-C. *P ≤ 0.05 in comparison with caspase-3 activation in irradiated cells*

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 C_{60} fullerene it is obvious that in general photosensitizing cytotoxic effect of C_{60} 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 C_{60} fullerene plus irradiation (Fig. 5). These results correspond to data that photoexcited C_{60} fullerene does not influence thymocytes viability and the level of DNA fragmentation. So the selectivity of photoexcited C_{60} 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 C_{60} 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 photoluminiscence but it allows to detect intracellular uptake only at high C_{60} concentrations. In recent report [18] non-toxic pristine C_{60} preparation (200 μg/ml) was used and intracellular uptake of C_{60} in malignant cancer breast cells was demonstrated by fluorescence microscopy.

We suggest that transformed T cells death after C_{60} illumination is mediated mainly by ROS influence on targets in cytoplasmic membrane involved in cellular signaling, but subcellular organells can also be involved. It was shown recently [2] that C_{60} 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 C_{60} 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 C_{60} 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. C_{60} fullerene photocytotoxicity was not detected in normal T lymphocytes (thymocytes) in contrast to Ara-C cytotoxocity detected in cells of both types. Our results may be useful for search of new photosensitizers for PDT with selective action on leukemia cells.

ІНДУКЦІЯ АПОПТОЗУ ЗА ДІЇ ФОТОЗБУДЖЕНОГО ФУЛЕРЕНУ C_{60} У ЛЕЙКЕМІЧНИХ Т-КЛІТИНАХ ЛЮДИНИ

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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 C_{60}. Одержані дані свідчать, що комбінована дія фулерену C_{60} та опромінення ртутно-дейтерієвою лампою (320–600 нм) спричиняє цитотоксичний ефект на лейкемічні Т-лімфоцити. Фулерен C_{60} не виявляє фотоцитотоксичності по відношенню до тимоцитів. Фотоіндукований апоптоз клітин лінії Jurkat за дії C_{60} підтверджується міжнуклеосомною фрагментацією ДНК та активацією каспази-3. Вірогідної зміни рівня активації каспази-3 у тимоцитах за дії фотозбудженого фулерену C_{60} 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 C_{60}, тимоцити, клітини Jurkat, цитозинарабінозид, апоптоз, життєздатність клітин, каспаза-3, міжнуклеосомна фрагментація ДНК, фотодинамічна терапія.

ИНДУКЦИЯ АПОПТОЗА ПРИ ДЕЙСТВИИ ФОТОВОЗБУЖДЕННОГО ФУЛЛЕРЕНА C_{60} В ЛЕЙКЕМИЧЕСКИХ Т-КЛЕТКАХ ЧЕЛОВЕКА

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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 C_{60}. Полученные результаты свидетельствуют о том, что комбинированное действие фуллерена C_{60} и облучения ртутно-дейтериевой лампой (320–600 нм) вызывает цитотоксический эффект на лейкемические Т-лимфоциты. Фуллерен C_{60} не проявляет фотоцитотоксичность по отношению к тимоцитам. Фотоиндуцированный апоптоз клеток линии Jurkat при действии C_{60} подтверждается межнуклеосомной фрагментацией ДНК и активацией каспазы-3. Достоверных изменений уровня активации каспазы-3 в тимоцитах при действии фотовозбужденного фуллерена C_{60} 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 C_{60}, тимоциты, клетки Jurkat, цитозинарабинозид, апоптоз, жизнеспособность клеток, каспаза-3, межнуклеосомная фрагментация ДНК, фотодинамическая терапия.

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