SELECTIVE PHOTOTOXICITY OF CHLORIN–E_6 DERIVATIVES TOWARD LEUKEMIC CELLS

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ИЗБИРАТЕЛЬНАЯ ФОТОТОКСИЧНОСТЬ ПРОИЗВОДНЫХ ХЛОРИНА–E_6 ПО ОТНОШЕНИЮ К ЛЕЙКОЗНЫМ КЛЕТКАМ

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The processes of accumulation and removal of some clorin-e6 derivatives – perspective sensitizers for photodynamic therapy – in normal and tumor cells of bone marrow have been studied. It has been shown that the cells in acute myelogenous leukemia accumulate significantly higher amounts of those photosentisizers (PS) and remove them much slower than normal mononuclear cells and, respectively, are stained with PS with higher contrast. The differences in PS distribution strongly determine the effectivity of photodynamic treatment of the cells.

***Key Words*: photodynamic therapy, chlorin-e6, leukosis, accumulation, flow cytofluorometry.**

Исследованы процессы накопления и выведения ряда производных хлорина е6, являющихся перспективными сенсибилизаторами для фотодинамической терапии, в нормальных и опухолевых клетках костного мозга. Установлено, что лейкозные клетки накапливают большие количества пигментов по сравнению с нормальными клетками. Клетки больных с острым миелобластным лейкозом характеризуются существенно меньшей скоростью выведения хлоринов после удаления пигментов из среды инкубирования, в результате чего существенно возрастает контрастность окрашивания клеток. Обнаруженные отличия в процессах распределения исследованных пигментов в значительной степени определяют эффективность фотосенсибилизированного воздействия на клетки.

***Ключевые слова*: фотодинамическая терапия, хлорин е6, лейкоз, накопление, проточная цитофлуориметрия.**

For extracorporal (*ex vivo*) antitumor treatment of bone marrow, immune and gene therapy methods as well as photodynamic therapy (PDT) using photosensitizers (PS) are applied [1]. At present time photodynamic therapy is used for the treatment of some solid tumors. The data of experimental studies point to the possibility that the efficacy of PDT is strongly dependent on the selectivity of PS binding to malignant but not normal cells. However, the mechanisms of selective action of PS remains poorly studied yet [1, 2].

The present work was aimed on the study of interaction of chlorin–e6 (Cle6) and its derivatives (dimethyl–ester and trimethyl–ester — DME and TME, respectively) with normal and leukemic cells of bone marrow. As it was shown earlier [3, 4], those PS compounds are able to accumulate in the cells and tissues at different degree.

MATERIALS AND METHODS

Photosentisers. In the research, Cle6, DME and TME sintetized by N.D. Kochubeeva (Institute of Molecular and Atomic Physics, National Academy of Sciences of Byelorussia, Byelorussia) were used. The stock PS solutions (10^{-3} M in acetone or ethanol) were stored in the dark at 4 °C.

Cells. The samples of bone marrow of patients with acute myelogenous leukemia or from healthy donors were placed in the silicone tubes with heparin (20 U/ml). Mononuclear cells (MNC) were isolated by centrifugation in the density gradient of Histopaque–1077 (Sigma, USA). Cell suspension was placed in RPMI–1640 medium containing fetal calf serum (Sigma, USA), and Cle6 derivatives.

Flow cytofluorometry. The effectivity of PS accumulation in the cells was studied with the use of flow fluorometer FACScan (Becton Dickinson, USA). The cell suspension was omitted through the 100 μm nozzle at the rate 500 cells per s in the flow solution (phosphate buffer). As excitation source the argon laser (wave length 488 nm, 15 mW) was used. The intensity of PS fluorescence was registered with the use of LP560 filter; DF660 filter was used for exclusion of basal signal. For exclusion of cell conglomerates and injured cells the frontal (FSC) and side (SSC) light scattering were registered. In each sample the amount of analyzed cells was not less than 10^4. The intensity of fluorescence and light scattering was expressed in arbitrary units (channels). The analysis of the data was carried out with the use of statistical packet of cytometer LYSIS II.

Photoirradiation of the cells. After incubation of the cells in Cle6–containing medium in the dark, cells were twice washed with RPMI and irradiated with the light of halogen lamp through the system of filters (λ = 630–680 nm, 20 mW/cm^2). The determination of relative content of damaged cells in the samples was performed by cytofluorometry with the use of fluorescent probe propidium iodide.

RESULTS AND DISCUSSION

The evaluation of relative effectivity of Cle6, DME and TME accumulation was performed by analysis of cell distribution in dependence of fluorescence intensity (FI) in the region of PS emission. The typical histo–

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Abbreviations used: Cle6 – clorin–e6; DME — clorin–e6 dimethyl–ester; FI — fluorescence intensity; MNC — mononuclear cells; PS — photosensitizer; TME — clorin–e6 trimethyl–ester.

grams of flurescence of leukemic cells incubated for 2 h in PS–containing medium as well as the histograms of MNC of healthy donors are presented on Fig. 1.

The samples without PS are characterized by low (basal) values of FI. The incubation of the cells in the PS–containing medium results in the shift of histograms to the region of higher FI values. As we have shown (see Fig. 1), FI values for leukemic cells were significantly higher than those for normal MNC. At the same time the histogrammes of the cells treated with DME and TME are shifted to the regions with higher FI values in comparison with Cle6–treated cells.

The Cle6 derivatives under study have nearly equal spectral and photophysical characteristics in monomeric form. The values of quantum fluorescence output in different media vary not more than by 1–3% [3]. That's why the values of FI for cells in the region of chlorin emission may be used for comparison of the levels of intracellular accumulation of those compounds. The results of statistical analysis for cell fluorescence values are presented in Table. As one may conclude, the tumor cells accumulate PS 2.8–6.1–fold more effectively than MNC of healthy donors; such selectivity of PS accumulation doesn't seem to be caused by systemic non–specific alterations in PS distribution between plasma proteins and the blood cells. Our additional study has shown that normal non–transformed MNC of patients with leukemia are stained on the same level as MNK of healthy donors. The comparison of fluorescence level of normal MNC and tumor cells has shown that stain contrast for DME is significantly higher than that of Cle6 and TME: the ratio of the PS amount binded during 2 h of incubation with leukemic cells to that in normal MNC was 6.1, 4.5 and 2.8 for DME, Cle6 and TME respectively.

It's necessary to note that the level of clorin accumulation strongly depends on the duration of staining. Cle6 and DME are entering the cells in 2 stages (Fig. 2). As it was shown earlier, the 1–st stage occuring in few seconds corresponds to the binding of PS with plasma membrane [4]. The second phase is more prolonged and corresponds to transmembrane transfer of PS and its accumulation in the itracellular compartments. Higher membrane permeability of DME results in more quick (in comparison with Cle6) attainment of equilibrial distribution in cell suspension. For TME, the stages of accumulation are less pronounced due to the low rate of diffuse redistribution of this pigment from proteins to membranes [5].

Thus, equilibrial distribution in cell suspension during studied duration of treatment was found for DME only; the duration of incubation of cells with Cle6 and TME should be much longer.

The withdrawal of PS from the medium results in the shift of equilibrial distribution and removal of studied PS from the cells. As it was demonstrated (Fig. 3), TME has the lowest rate of removal from the cells. After 2 h of incubation in PS–free medium the concentration of TME decreased by a factor 1.7 and 1.1 in normal MNC and malignant cells, respectively. The rates of removal of Cle6 and DME were significantly higher: after 2 h of incubation in PS–free medium the concentration of Cle6 and DME decreased by a factor 9.2 and 19.7 in normal MNC and by a factor 4.1 *и* 2.4 in malignant cells, respectively. As a result, 49–fold excess for tumor cell staining with DME and 10.2–fold — for Cle6 was registered.

The differences in PS accumulation and removal from the cells possibly reflect the existence of structural and morphological peculiarities of transformed MNC which

Table. FI values of MNC of healthy donors and tumor cells treated with Cle6 derivatives

PS	MNC of healthy donors	Tumor cells
Cle6	83 ± 7	377 ± 30
DME	276 ± 58	1696 ± 155
TME	307 ± 35	855 ± 100

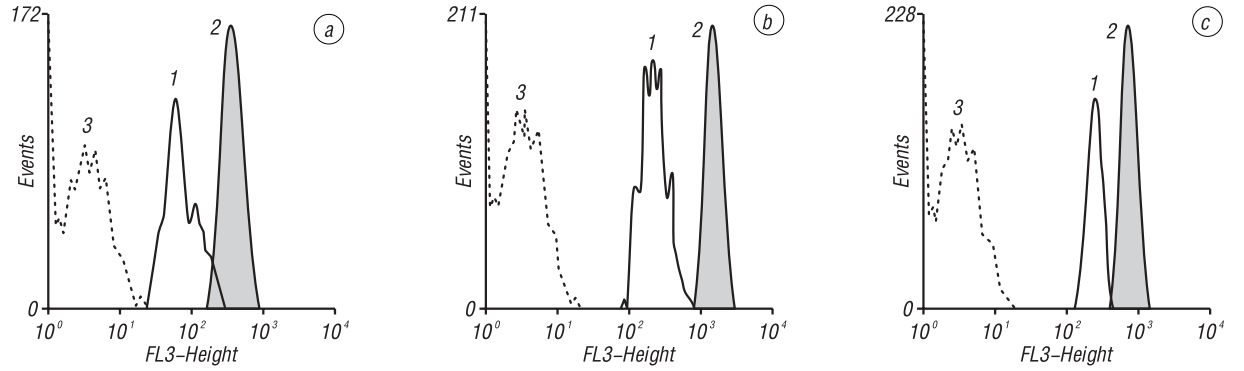

Fig. 1. Histogrammes of Cle6 (*a*), DME (*b*) and TME (*c*) accumulation in the cells. 1 – MNC of healthy donors, 2 – leukemic cells, 3 – control

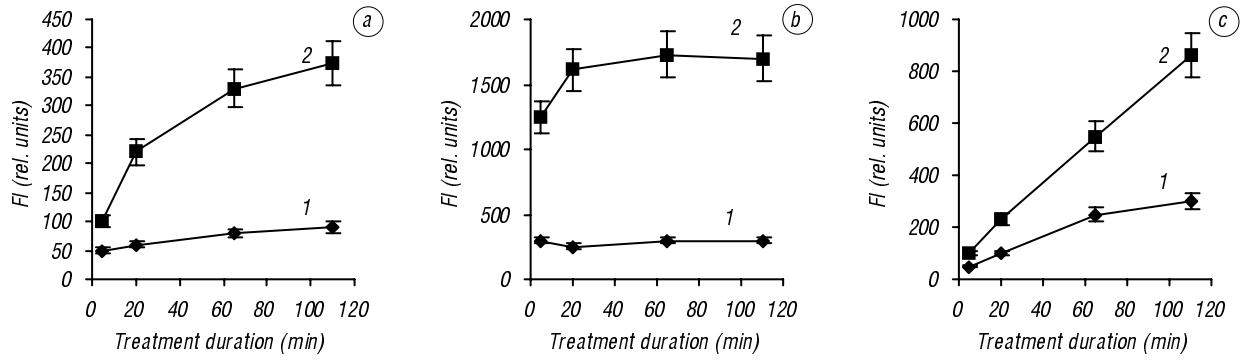

Fig. 2. The time course of Cle6 (*a*), DME (*b*) and TME (*c*) accumulation in MNC of healthy donors (1), and in leukemic cells (2)

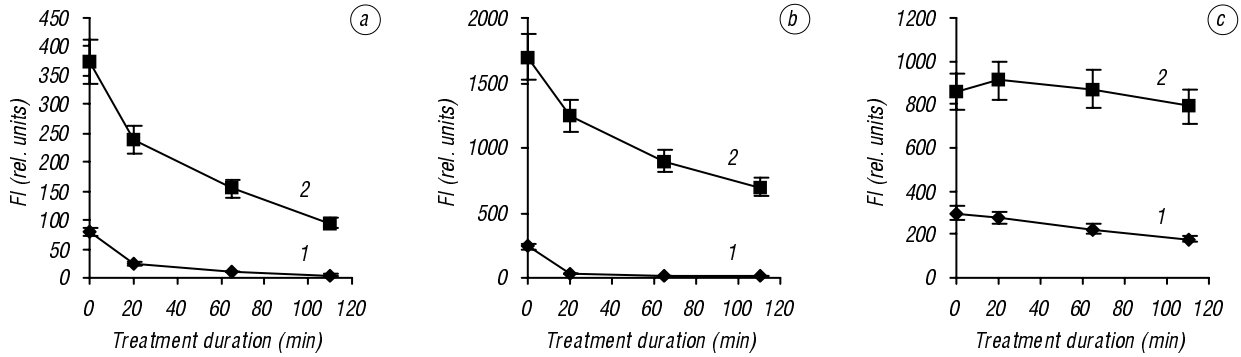

Fig. 3. The time course of Cle6 (*a*), DME (*b*) and TME (*c*) accumulation in MNC of healthy donors (1), and in leukemic cells (2) after the removal of PS from culture medium

are bigger and more granulated than normal MNC and differ in biochemical content of plasma membranes. As it was shown earler with the use of luminiscent microscopy, porphyrin sensitizers are localized preferentially in plasma membranes and membranes of Golgi apparatus and endoplasmic reticulum [6, 7] but Cle6 derivatives has different distribution between cell compartments due to lipid composition of membranes. According to the data [8], viscosity of cellular membranes in leukemic cells decreased by a factor 1.2–1.9 due to the decreased cholesterol content. As it was reported in [9, 10], the relative content of cholesterol in plasma membrane influence the affinity of porphyrins to biomembranes.

Earlier it was shown that the decrease of polarity of chlorin molecule caused by etherification of its side carboxyl groups could affect distribution of PS between plasma proteins and the cells by alteration of PS affinity to membrane lipids [10]. The level of PS accumulation in the cells depends on the rate of its redistribution and ability to permebealize cell membrane [5]. Moderately non–polar DME possessing high diffuse motility in plasma protein environment as well as maximal rate of diffusion through plasma membrane, accumulates in the cells in few fold higher concentration than polar Cle6. Relatively slow accumulation of non–polar TME, possibly, is caused by its low rate of redistribution between biomolecules and low solubility in the water causing aggregation of TME molecules [5, 10].

For quantitive evaluation of chlorin photocytotoxicity toward cells under study the test with fluorescent probe propidium iodide was applied. The damage of membrane integrity initiates significant (nearly 100–fold) increase of propidium iodide staining of injured cells. We studied the relative number of damaged cells in the samples obtained from healthy donors and patients with leukemia both laser–irradiated at 660 nm (Fig. 4). Our data pointed to the correlation between the effectivity of PDT–induced injury of normal and malignant cells and the level of intracellular accumulation of PS. Also it was demonstrated that the level of PDT–induced blast cell damage in AML patients is 5.9–8.5 higher than that in MNC of healthy donors and the highest level is registered after DME application. So, the PS binding is playing a central role in determination of the rate of photocytolysis.

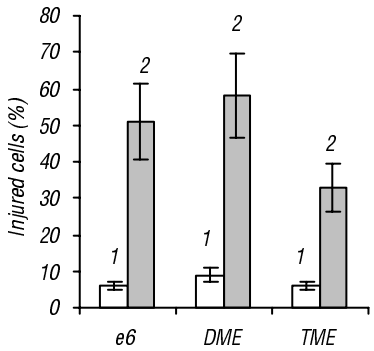

Fig. 4. Relative content of injured cells after Cle6–PDT: 1 – normal MNC; 2 – leukemic cells

In conclusion, the presented data demonstrated that photocytotoxicity of porphyrin PSs depends on their intracellular concentration; our results may be useful for design of sensibilizators with selective action against leukemic cells.

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