

Dark and Photoinduced Cytotoxicity of Cationic Chlorin e_6 Derivatives with Different Numbers of Charged Groups

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Dedicated to the memory of talented scientists
Professor G.V. Ponomarev and Professor A.F. Mironov

Dark and photoinduced toxicity of several novel and previously described derivatives of chlorin e_6 with tetraalkylammonium substituents differing from each other in number of cationic groups and their location in the macrocycle were studied in HeLa cells and mammalian erythrocytes. The optimal design of cationic derivatives of chlorin e_6 that provides the most efficient photodynamic effect was shown to require the localization of cationic substituents in the same part of the molecule. In combination with the hydrophobicity of the chlorin macrocycle, it increases the amphiphilic properties of the whole molecule and enhances its interaction with biomembranes.

Keywords: Methylpheophorbide *a*, chlorin e_6 , cationic derivatives, chlorin macrocycle, dark and photoinduced toxicity, HeLa cells, erythrocytes.

Темновая и фотоиндуцированная цитотоксичность катионных производных хлорина e_6 с различным количеством заряженных групп

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Изучена темновая и фотоиндуцированная токсичность по отношению к клеткам HeLa и эритроцитам млекопитающих ряда новых и ранее описанных производных хлорина e_6 с тетраалкиламмониевыми группами,

отличающихся друг от друга количеством катионных групп и их расположением в макроцикле. Показано, что оптимальный дизайн катионных производных хлорина e_6 , обеспечивающий наиболее эффективное фотодинамическое действие, заключается в локализации катионных заместителей в одной и той же части молекулы, что, в сочетании с гидрофобностью хлоринового макроцикла, придает молекуле в целом амфифильные свойства и усиливает взаимодействие с биомембранами.

Ключевые слова: Метилфеофорбид a , хлорин e_6 , катионные производные, хлориновый макроцикл, темновая и фотоиндуцированная токсичность, клетки *HeLa*, эритроциты.

Introduction

Porphyrins containing cationic groups are considered to be promising photosensitizers (PSs) for photodynamic therapy (PDT) of oncological diseases and photoinactivation of pathogenic organisms.^[1–10] The presence of cationic groups in the porphyrin molecule can contribute to its preferential intracellular localization in the plasma membrane or mitochondria, resulting in their photoinduced damage and consequently the death of tumor cells via necrosis or apoptosis.^[11] An essential issue arising in the molecular design of such PSs is the number and location of cationic groups in the molecule which would provide the maximum efficiency of the photodynamic action. Therefore, the aim of this work was to study dark and photoinduced cytotoxic activities of cationic derivatives of chlorin e_6 , differing in the number of cationic groups and their location in the macrocycle, against *HeLa* cells and erythrocytes.

Experimental part

¹H NMR spectra (δ , ppm; J , Hz) were recorded on a Bruker Avance II 300 spectrometer (Germany) in CDCl₃ at an operating frequency of 300 MHz; internal standard was chloroform and DMSO- d_6 . Mass spectra were recorded on a Thermo Finnigan LCQ Fleet Mass Spectrometer System (Thermo Scientific, USA). The progress of the reaction was monitored by TLC on Sorbfil plates (IMID, Russia). For column chromatography, we used 0.06–0.2 mm silica gel (Alfa Aesar, USA). Tetrahydrofuran (pur.) and bis(*N,N*-dimethylamino)methane (99 %, Sigma Aldrich) were preliminarily distilled. The rest of the solvents and amines were used without further purification.

Cationic chlorins **8**, **10–12** were synthesized according to previously described procedures.^[9, 12–14]

3(2)-(N,N-Dimethylaminomethyl)chlorin e_6 13(1)-N-(2-N',N'-dimethylaminoethyl)amide-15,17-dimethyl ester (**4**). A solution of 97.8 mg (0.14 mmol) of compound **2** in 5 mL of CH₂Cl₂ was mixed with 105 mg (0.58 mmol) of Eschenmoser's salt. The resulting mixture was left for 23 h; the progress of the reaction was monitored by TLC on Sorbfil plates at the CHCl₃:C₂H₅OH ratio of 9:1. The reaction mixture was diluted with chloroform (50 mL), rinsed with water, dried with anhydrous Na₂SO₄, and the resulting solution was evaporated to dryness under reduced pressure. The residue after evaporation was chromatographed on silica gel (eluent: CCl₄-acetone, 50:1 – 1:1, then CHCl₃-C₂H₅OH, 30:1 – 1:1), the fractions containing the main reaction product were collected and evaporated. Yield: 32.2 mg (30 %) of chlorin **4** as a dark blue-green crystalline powder. Mass spectrum (ESI) m/z : 752.5 (MH⁺), 707.4 (MH-HN(CH₃)₂)⁺. UV-Vis (CHCl₃) λ_{\max} nm: 661, 607, 554, 527, 493, 390. ¹H NMR spectrum (CDCl₃): -1.76 (1 H, br.s, HN(III)), -1.56 (1 H, br.s, HN(I)), 1.75 (3 H, d, J = 7.3 Hz, H18(I)), 1.76 (3 H,

J = 7.3 Hz, H8(2)), 2.65–2.51, 2.44–2.11 (1 H, 3 H, both m, H17(1), H17(2)); 2.36 (6 H, s, H13(4)), 2.61 (6 H, s, 3-CH=CHCH₂N(CH₃)₂), 2.75 (2 H, t, J = 6.4 Hz, H13(3)), 3.53 (3 H, s, H7(1)), 3.37 (3 H, s, H2(1)), 3.64–3.60 (2 H, m, 3-CH=CHCH₂N(CH₃)₂), 3.62 (3 H, s, H12(1)), 3.65 (3 H, s, H17(4)), 3.80 (3 H, s, H15(3)), 3.84 (2 H, q, J = 7.3 Hz, H8(1)), 4.08–3.93, 3.92–3.82 (by 1 H, both m, H13(2)), 4.43 (1 H, br.d, J = 9.2 Hz, H17), 4.51 (1 H, q, J = 7.3 Hz, H18), 5.35 (1 H, d, J = 19.3 Hz, H15(1)_A), 5.63 (1 H, d, J = 19.3 Hz, H15(1)_B), 6.91 (1 H, dt, J = 15.6 Hz, 6.4 Hz, 3-CH=CHCH₂N(CH₃)₂), 6.99 (1 H, br.t, J = 4.7 Hz, 13-CONH), 7.93 (1 H, d, J = 16.5 Hz, 3-CH=CHCH₂N(CH₃)₂), 8.84 (1 H, s, H20), 9.65 (1 H, s, H5), 9.74 (1 H, s, H10).

3(2)-(N,N,N-trimethylaminomethyl iodide) chlorin e_6 13(1)-N'-(2-N'',N''',N'''-trimethylammonioethyl iodide)amide-15(2),17(3)-dimethyl ester (**9**). A solution of 50 mg of chlorin **4** in 5 mL of methylene chloride was mixed with 0.1 mL of iodomethane. The resulting mixture was kept for 1 h at room temperature, then methylene chloride and iodomethane were evaporated under reduced pressure. Yield: 64.7 mg (94 %) of chlorin **9** as a dark blue-green crystalline powder. Mass spectrum (ESI), m/z : (M²⁺I₂) (MI): 908.4 (M²⁺I)⁺, 849.1 (M²⁺I-N(CH₃)₃)⁺, 781.6 (M²⁺+e)⁺, 722.7 (M²⁺+e-N(CH₃)₃)⁺, 721.3 (M²⁺-H⁺-N(CH₃)₃)⁺, 666.3 (M²⁺+4H-H⁺-2N(CH₃)₃-H⁺)⁺, 662.42 (M²⁺-2N(CH₃)₃-H⁺)⁺, 619.13 (M²⁺-H-2N(CH₃)₃-H⁺-NHCHCH₂)⁺, 390.7 M²⁺, 361.5 (M²⁺-N(CH₃)₃)₂²⁺, 332.0 (M²⁺-2N(CH₃)₃)₂²⁺. UV-Vis (CHCl₃) λ_{\max} nm: 660, 602, 549, 530, 498, 395. ¹H NMR spectrum (DMSO- d_6): -2.03 (1 H, br.s, HN(III)), -1.71 (1 H, br.s, HN(I)), 1.68 (3 H, d, J = 6.4 Hz, H18(1)), 1.71 (3 H, t, J = 7.3 Hz, H8(2)), 2.44–2.07 (4 H, m, H17(1), H17(2)), 2.81–2.66 (2 H, m, H13(2)), 3.14 (2 H, t, J = 5.5 Hz, H13(3)), 3.39, 3.36 (9 H, both s, H13(4), 3-CH=CHCH₂N⁺(CH₃)₃I), 3.59 (3 H, s, H7(1)), 3.57 (3 H, s, H2(1)), 3.65 (3 H, s, H12(1)), 3.76 (3 H, s, H17(4)), 3.87 (3 H, s, H15(3)), 3.94–3.80 (2 H, m, H8(1)), 4.47 (1 H, br.d, J = 8.3 Hz, H17), 4.75–4.56 (1 H, m, H18), 5.36 (1 H, d, J = 18.3 Hz, H15(1)_A), 5.50 (1 H, d, J = 18.3 Hz, H15(1)_B), 7.10 (1 H, br.t, J = 5.5 Hz, 13-CONH), 7.11 (1 H, dt, J = 16.5 Hz, 6.4, 3-CH=CHCH₂N(CH₃)₂), 8.58 (1 H, d, J = 15.6 Hz, 3-CH=CHCH₂N⁺(CH₃)₃I), 9.23 (1 H, s, H20), 9.79 (1 H, s, H5), 9.85 (1 H, s, H10).

Fluorometric Cytotoxic Microculture Assay (FMCA).^[15] *HeLa* cells were cultured in DMEM/F12 nutrient medium (PAA Laboratories, Austria) supplemented with 10 % fetal bovine serum (FBS) (Thermo Scientific HyClone, UK) without antibiotics at 37 °C and 5 % CO₂. Re-seeding was carried out using 0.05 % trypsin-EDTA solution with Hank's salts (PanEko, Russia) 2 times a week.

Stock solutions of the test substances were prepared by dissolving them in DMSO (Amresco, USA) at different concentrations. 1 μ L of a stock solution of the corresponding concentration was added to 199 μ L of culture medium containing 5,000 cells per well of a sterile culture plate. The final concentrations of the substances ranged from 1 to 100 μ M at the DMSO concentration of 0.5 % (v/v). Examination of solutions of all compounds in a nutrient medium containing 0.5 % DMSO under a microscope showed that the formation of a solid phase (sediment or large colloidal particles) does not occur up to a concentration of 100 μ M. The control suspension was mixed with DMSO at the same concentration. In the study of dark cyto-

toxicity, HeLa cells with the test substances were incubated for 72 h at 37 °C, 100 % humidity and 5 % CO₂. In the study of photoinduced cytotoxicity, HeLa cells with the test substances were incubated for 2 h in the dark, then the cells were exposed to light with a wavelength of 660 nm (light source – LEDs, dose of light exposure – 12 J/cm²) for 20 min, then the cells were again incubated in the dark for 69 h 40 min at 37 °C, 100 % humidity and 5 % CO₂. After that, the culture medium was removed and the monolayer culture was rinsed with 200 µL of phosphate-buffered saline. 100 µL of fluorescein diacetate solution (Sigma, USA) was added to the wells and incubated at 37 °C and 5 % CO₂ for 40 min, after which the fluorescence intensity was measured on a Fluorat-02-Panorama liquid analyzer (Lumex, Russia) at a wavelength of 485 (excitation) nm / 520 (registration) nm. The cell survival index was calculated as the percent ratio of the fluorescence intensity of the cells in the well with a test substance to the fluorescence intensity of the cells in the control well (containing DMSO at a concentration of 0.5 %). The obtained results are provided in Table 1. The experiments were carried out in nine replicates. Statistical data processing was performed using the Student's *t*-test in the Statistica 6.0 software package. The verification of the samples for artifacts was carried out using the Grubbs' test.

For assessment of dark and photoinduced hemolysis, we used a 0.5 % suspension of erythrocytes of laboratory mice in phosphate-buffered saline, pH 7.4. Solutions of the test compounds in DMSO were introduced into the suspension of erythrocytes at a working concentration of 10 µM and incubated for 3 h at 37 °C in an ES-20 thermostatic shaker (Biosan, Latvia). Control samples contained the corresponding volume of 0.2 % DMSO. The presence of photodynamic activity was inferred based on the degree of erythrocyte hemolysis after 1 and 3 h of incubation at constant irradiation of cells with the red light at wavelength of 660 nm (light source – LEDs with a power of 60 mW each). The presence of cytotoxicity (hemolytic activity) was inferred based on the degree of hemolysis after 1 and 3 h of incubation of erythrocytes in the dark. Hemolysis was assessed by the release of hemoglobin from erythrocytes using a Spectronic Genesys 20 spectrophotometer (Thermo Scientific, USA), according to the formula $A = B/C \cdot 100\%$, where *A* is the percentage of hemolysis, *B* is the optical density of the supernatant of the test sample, *C* is the optical density of the supernatant of the sample subjected to complete hemolysis. All experiments were carried out in four replicates. The experimental data in Table 2 are presented as arithmetic mean with the standard error of the sample. Statistical data processing was carried out using the Microsoft Office Excel 2007 software package.

Determination of the quantum yield of singlet oxygen. The quantum yields (γ) of the photoproduction of singlet oxygen (¹O₂) were determined based on the intensity of its luminescence in pyridine by the relative method using 5,10,15,20-tetraphenylporphyrine (H₂TPP) as a standard according to a previously described procedure.^[4,14] The value of γ was calculated according to the general formula:^[14] $(\gamma/\gamma_{st}) = (A \cdot I \cdot n^2)/(A \cdot I \cdot n^2)_{st}$, where *A* is the optical density of the sensitizer at an excitation wavelength of 337 nm; *n* is the refractive index of the solvent; *I*₀ is the fluorescence in-

tensity at $t \rightarrow 0$. Parameters with a subscript “st” refer to H₂TPP. The γ_{st} value was taken to be 0.62 in benzene, which was found by the chemical method^[16] and which is in good agreement with the result of averaging the γ_{st} values obtained by the luminescence method in CCl₄, toluene, and benzene. The luminescence of ¹O₂ was measured on a Lif-200 laser pulsed fluorometer (excitation wavelength – 337.1 nm; pulse duration, energy, and repetition rate – 2 ns, 20 µJ/mol, and 30 Hz, respectively).

Results and Discussion

The synthesis of cationic derivatives of chlorin *e*₆ studied in this work is based on the chemical modification of methylpheophorbide *a* (**1**)^[8,9,12,13] (Scheme 1) with the aim of introducing dimethylamino groups (compounds **2**, **4–7**) to the periphery of the macrocycle. Alkylation of the later with methyl iodide results in cationic derivatives of chlorin *e*₆ (**8–12**) which differ in the number of cationic groups and their location in the macrocycle. A previously undescribed chlorin **9** containing cationic substituents in different parts of the macrocycle (in the vinyl group and at position 13) was synthesized by aminomethylation of the vinyl group of chlorin **2** by the action of Eschenmoser's salt similarly to a previously described procedure^[17] with a subsequent quaternization of dimethylaminomethyl groups present in the product of reaction of **4** with methyl iodide. The structure of the newly synthesized chlorin **9**, as well as its synthetic precursor **4**, was proven using ¹H NMR, electronic (UV-Vis) spectroscopy, and mass spectrometry. Aminomethylation with Eschenmoser's salt, as in the case of chlorins described in the literature,^[17] occurs with the formation of the only isomer with the *trans*-arrangement of the macrocycle and the dimethylaminomethyl group: the constant of the spin-spin interaction of the protons of the substituted vinyl group corresponds to the *transoid* arrangement of the two remaining in it protons both in the aminomethylation product **4** and in the dicationic chlorin **9**. Thus, using Eschenmoser's salt, we synthesized chlorin **9** with two cationic groups in different parts of the molecule (at positions 3 and 13), complementing the series of previously described cationic chlorins **8**, **10–12**.

The study of the dark cytotoxicity of cationic chlorins **8–12** in the *HeLa* cell culture showed that this characteristic is most influenced by the location of cationic groups in the molecule (Table 1).

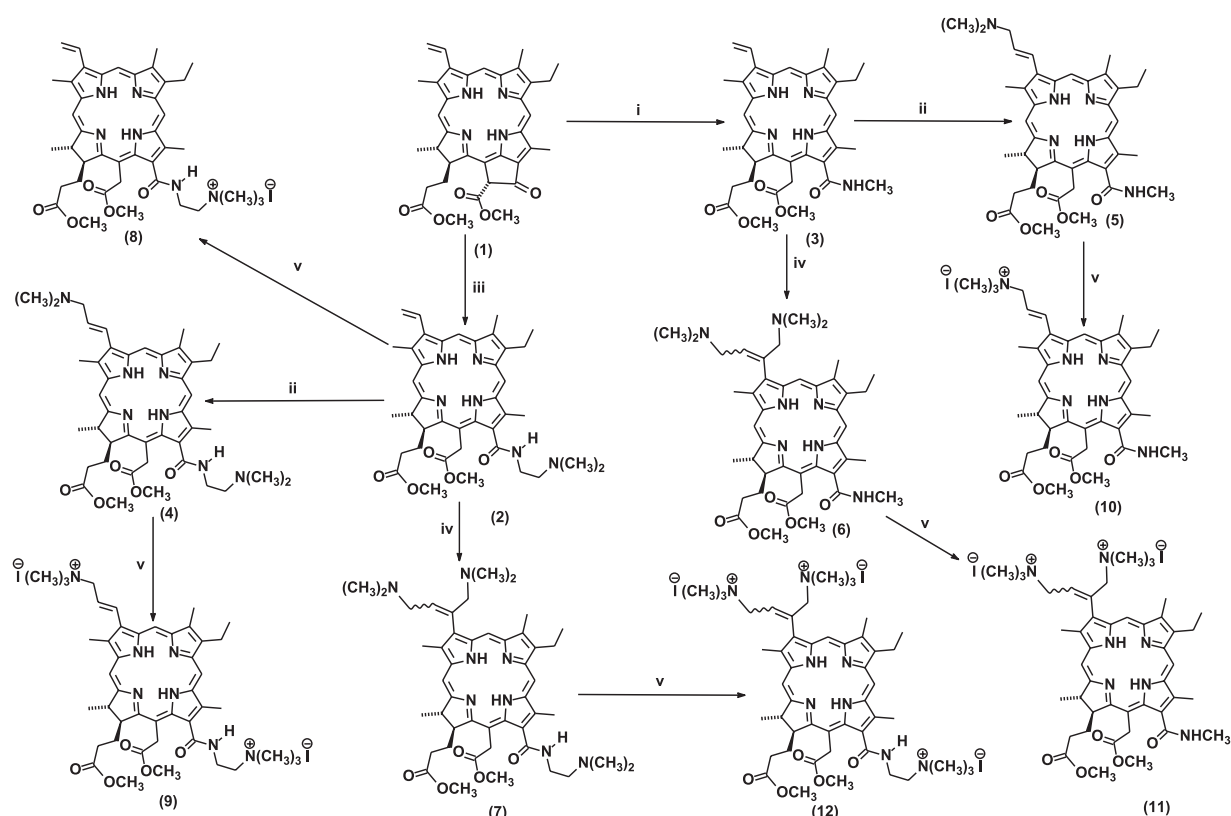
For instance, chlorins **9** and **12** which have, respectively, two and three cationic groups located in different parts of the macrocycle do not exhibit significant dark cytotoxic-

Table 1. Dark and photoinduced cytotoxic activities of cationic derivatives of chlorin *e*₆ **8–12** in experiments on *HeLa* cells and quantum yields of singlet oxygen generation.

Compound	Survival index, %			Photoinduced activity, 1 µM	Quantum yield of singlet oxygen generation, γ
	Dark activity				
	1 µM	10 µM	100 µM		
8	93.90 ± 2.54	24.16 ± 2.34	3.71 ± 0.11	3.17 ± 0.04	0.56
9	113.40 ± 3.52	93.87 ± 3.68	67.42 ± 2.72	88.38 ± 2.84	0.55
10	100.64 ± 3.49	82.39 ± 3.37	3.10 ± 0.04	28.02 ± 2.61	0.62
11	75.78 ± 2.66	52.31 ± 3.25	3.37 ± 0.36	3.28 ± 0.06	0.64
12	118.96 ± 5.82	112.24 ± 6.98	90.06 ± 4.28	84.09 ± 2.95	0.59

Table 2. Degree of erythrocyte hemolysis after 1 and 3 h incubation with cationic derivatives of chlorin e_6 **8–12** at a concentration of 10 μM in the dark and upon the exposure to light.

Compound	Degree of erythrocyte hemolysis, %			
	Dark activity		Photo-induced activity	
	1 h	3 h	1 h	3 h
Control	0.6 \pm 0.1	1.3 \pm 0.1	0.9 \pm 0.1	1.2 \pm 0.1
8	54.1 \pm 2.0	79.7 \pm 2.2	79.8 \pm 0.4	80.1 \pm 0.2
9	4.5 \pm 0.2	5.3 \pm 0.3	10.7 \pm 0.7	83.8 \pm 2.6
10	3.7 \pm 0.1	4.7 \pm 0.1	15.0 \pm 1.4	82.2 \pm 2.1
11	3.3 \pm 0.1	4.3 \pm 0.2	9.9 \pm 1.6	90.7 \pm 3.0
12	2.3 \pm 0.1	2.9 \pm 0.0	4.9 \pm 0.2	83.9 \pm 1.6



Reaction conditions: i:^[9] CH_3NH_2 - H_2O /THF, room temp., 3 h, yield of **3** – 85 %; ii:^[9] $[(\text{CH}_3)_2\text{N}=\text{CH}_2]^+\text{I}^-$, CH_2Cl_2 , r.t., 12 h, yield of **5** – 35 %, yield of **4** – 30 %; iii:^[12] $\text{H}_2\text{NCH}_2\text{CH}_2\text{N}(\text{CH}_3)_2$, CH_2Cl_2 , r.t., yield of **2** – 74 %; iv:^[13,14] $\text{CH}_2\text{N}(\text{CH}_3)_2$, AcOH-THF, boiling for 30 min, yield of **6** – 65 %, yield of **7** – 45 %; v:^[13] CH_3I , CH_2Cl_2 , r.t., yields of **8–12** – 93–96 %.

Scheme 1.

ity at the maximal investigated concentration of 100 μM , while monocationic chlorins **8** and **10** with cationic groups at positions 13 and 3, respectively, as well as dicationic chlorin **11** with both cationic groups localized in the substituent at position 3, have a noticeable cytotoxic effect. It should be noted that monocationic chlorin **8** showed a high dark cytotoxicity even at a concentration of 10 μM . The obtained data allow us to conclude that dark cytotoxicity is higher when cationic groups are located in one part of the molecule, which ensures the presence of a charged hydrophilic part and an uncharged hydrophobic part in the molecule. This suggests that one of the leading mechanisms of the toxic action of these compounds is damage to biomembranes.

Photoinduced cytotoxic activity of chlorins on *HeLa* cells was studied at a chlorin concentration of 1 μM (Figure 1). At this concentration, all the studied chlorins (**8–12**) practically do not have a dark toxic effect on *HeLa* cells (Table 1), which makes it possible to assess the photodynamic effect: a decrease in the proportion of surviving cells in the presence of these compounds under the exposure to light is caused almost exclusively by a photoinduced toxic effect. As in the case of dark cytotoxicity, the chlorins **8**, **10** and **11** had the greatest effect. In contrast to dark cytotoxicity, differences in the photoinduced toxic effect may be due not only to the difference in the distribution of the compound in the cell, but also to the ability to generate singlet oxygen

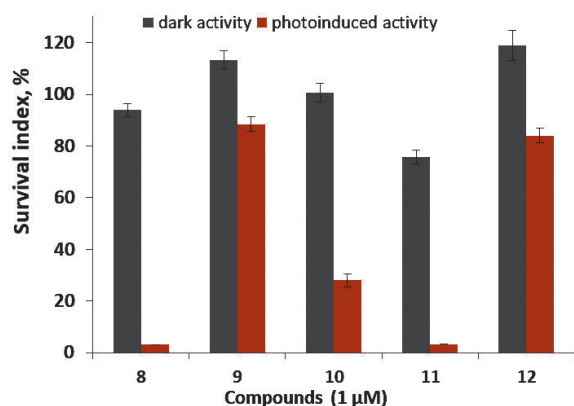


Figure 1. Dark and photoinduced cytotoxic activities of cationic derivatives of chlorin e_6 **8–12** in experiments on *HeLa* cells.

which is the main cytotoxic agent in photosensitized cell damage.^[18–19] The measure of this ability is the quantum yield of singlet oxygen generation. We established that chlorins **8–12** have close values of this indicator (Table 1). The obtained results allow us to conclude that, as in the case of dark cytotoxicity, the photoinduced cytotoxicity is higher when cationic groups are located in one part of the molecule, which facilitates its interaction with cell membranes. This suggests that the main target of photo exposure are also biomembranes.

Mammalian erythrocytes are known to be a good model for studying various influences on biological membranes.^[20] The results of a study of the effect of cationic chlorins **8–12** on dark and photoinduced hemolysis of erythrocytes showed that, as in the experiments with *HeLa* cells, the maximum photoinduced toxicity (minimum period of hemolysis induction) was observed for chlorin **8** (Figure 2, Table 2), the presence of which in the incubation medium within an hour caused the death of about 80 % of blood cells. However, after 3 h of incubation, the degree of photohemolysis for all studied compounds **8–12**, regardless of the number and location of charged groups in the macrocycle of chlorins, was more than 80 %. The rapid destruction of erythrocytes in the presence of monocationic chlorin **8** is due not only to photodynamic activity, but also to high dark cytotoxicity (Figure 2). This supports the assumption that cell membranes are the main target of the cytotoxic effect of this compound. It is interesting, however, that other chlorins investigated in this work, regardless of the location of the cationic groups in the macrocycle, did not have significant hemolytic activity under dark conditions.

The high dark and photoinduced activities of the monocationic chlorin **8** may be due to the features of the design of the molecule that contains a charged hydrophilic part and an uncharged hydrophobic part, which allows this compound to easily and quickly interact with the plasma membrane of erythrocytes,^[20] causing these effects.

Conclusion

Thus, based on the above results, we can conclude the following. Out of all the studied cationic chlorins

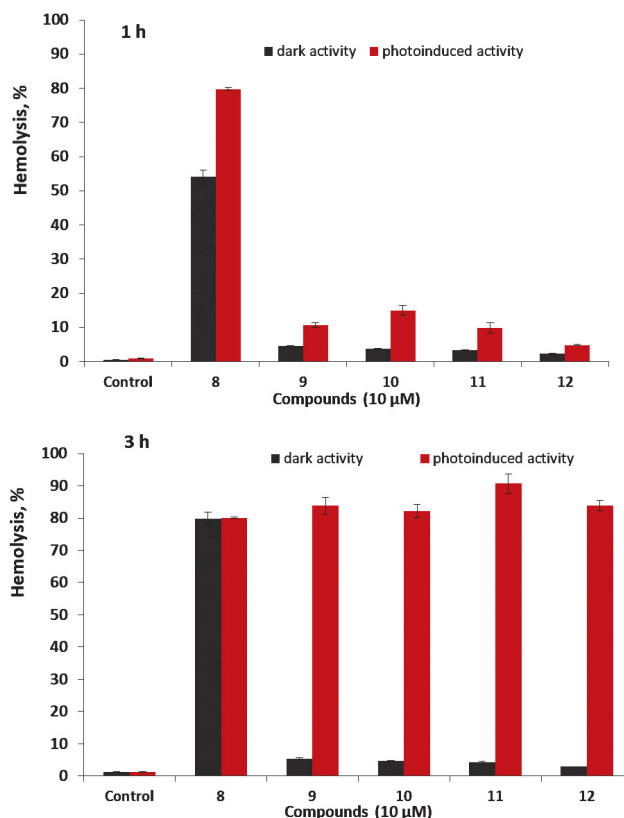


Figure 2. Degree of erythrocyte hemolysis after 1 and 3 h incubation with cationic derivatives of chlorin e_6 **8–12** (at a concentration of 10 μM) in the dark and under exposure to light.

8–12, the highest dark cytotoxic activity in experiments on *HeLa* cells and erythrocytes was shown by monocationic chlorin **8**. This is probably due to the ability of chlorin **8** to interact with biomembranes and at high concentrations cause their destruction, which is supported by the ability of this compound to cause hemolysis of erythrocytes without exposure to light. The same compound also has the highest photodynamic activity against both *HeLa* cells and erythrocytes, which, most likely, is also due to its high affinity for biomembranes – targets of the damaging effects of singlet oxygen. This assumption is in good agreement with the absence of significant differences between the chlorins under study in terms of the quantum yield of singlet oxygen generation. The high membranotropy of chlorin **8** may be due to the remoteness of the cationic group from the macrocycle.

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from the scientific collection of experimental animals of the Institute of Biology, KSC UB RAS. The animals were kept in accordance with the “Regulations on the vivarium of experimental animals” (protocol No. 1 of 24.01.2017) taking into account the sanitary-hygienic and bioethical aspects. The authors are grateful to Dr. E.A. Venediktov (G.A. Krestov Institute of Solution Chemistry of Russian Academy of Sciences) for carrying out luminescence measurements.

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