Dark and Photoinduced Cytotoxicity of Cationic Chlorin e₆ Derivatives with Different Numbers of Charged Groups

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Dedicated to the memory of talented scientist Professor G.V. Ponomarev

Dark and photoinduced toxicity of several novel and previously described derivatives of chlorin e₆ 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 e₆ 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 e₆, cationic derivatives, chlorin macrocycle, dark and photoinduced toxicity, HeLa cells, erythrocytes.
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Introduction

Porphyrins containing cationic groups are considered to be promising photosensitizers (PSs) for photodynamic therapy (PDT) of oncological diseases and photoinactivation of pathogenic organisms.[11] 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.[13] An essential issue arising in the molecular and consequently the death of tumor cells via necrosis or apoptosis.

Experimental part

The progress of the reaction was monitored by TLC on Sorbfil plates at the CHCl₃:CH₂Cl₂:MeOH ratio – 10:1:1. The fractions containing the main reaction product were collected and dried. The rest of the solvents and amines 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.[8, 11–14]

3(2)-(N,N,N-trimethylaminomethoxy)chlorin e₆ 13(1)-(N,N,N,N′-trimethylammonioethyl iodide)amide/15(2),17(3)-dimethyl ester (4). A solution of 97.8 mg (0.14 mmol) of compound 2 in 5 mL of CHCl₃ was mixed with 105 mg (0.58 mmol) of Eschenmoser’s salt. In 5 mL of CH₂Cl₂, 2.44–2.11 (1 H, 3 H, both m, H17(1), H17(2)); 2.36 (6 H, s, H13(4)); 2.75 (2 H, s, J = 6.4 Hz, H13(3)); 3.53 (3 H, s, H17(1)); 3.37 (3 H, s, H2(1)); 3.64–3.60 (2 H, m, 3-CH₂=CH(NH)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, k (Авторам - ?), 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, H8), 5.35 (1 H, d, J = 19.3 Hz, H15(1)), 5.63 (1 H, d, J = 19.3 Hz, H15(2)), (Авторам - ?), 7.3 Hz, H8(1)).

3(2)-(N,N,N-trimethylaminomethoxy)chlorin e₆ 13(1)-(N,N,N,N′-trimethylammonioethyl iodide)amide/15(2),17(3)-dimethyl ester (4). 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. We obtained 64.7 mg (94 %) of chlorin (9) as a dark-blue-green crystalline powder. Mass spectrum (ESI), m/z (М+): 908.4 (М+I); 816.5 (М+2-I); 727.0 (М+3-I); 638.5 (М+4-I); 549.0 (М+5-I); 460.0 (М+6-I); 371.5 (М+7-I); 282.0 (М+8-I); 193.0 (М+9-I); 104.0 (М+10-I).

Fluorimetric 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 μL of a stock solution of the corresponding concentration was added to 199 μL 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 μM 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 μM. The control suspension was mixed

Ключевые слова: Метилфосфорбид α, хлорин e₆, кационные производные, хлориновый макроцикл, темновая и фотоиндуцированная токсичность, клетки HeLa, эритроциты.
with DMSO at the same concentration. In the study of dark cytotoxicity, 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 600 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 and 40 min at 37 °C and 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 Genesyss 20 spectrophotometer (Thermo Scientific, USA), according to the formula \( A = B/C \times 100 \ % \), 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 (\( \gamma \)) of the photoproduction of singlet oxygen were determined based on the intensity of its luminescence in pyridine by the relative method using 5,10,15,20-tetraphenylporphine (H₃TPP) as a standard according to a previously described procedure.\(^\text{8-14}\) The value of \( \gamma \) was calculated according to the general formula:\(^\text{14}\) \( \gamma = (A-I_{\gamma})/(A-I_{\gamma})_{\text{st}}, \) where \( A \) is the optical density of the sensitizer at an excitation wavelength of 337 nm; \( I_{\gamma} \) is the fluorescence intensity at \( t=0 \). Parameters with a subscript “st” refer to H₃TPP. The \( \gamma \) value was taken to be 0.62 in benzene, which was found by the chemical method\(^\text{15}\) and which is in good agreement with the result of averaging the \( \gamma \) values obtained by the luminescence method in CCl₄, toluene, and benzene. The luminescence of \( ^{1}O_{2} \) 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 methylphleophorbide \( a (1) \)\(^\text{8-13}\) (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 (similar to a previously described procedure\(^\text{17}\) with a subsequent quaternization of dimethylaminomethyl groups present in the product of the reaction 4 with methyl iodide. The structure of the newly synthesized chlorin 9, as well as its synthetic precursor 4, was proven using \(^1\)H NMR, electronic (UV-Vis) spectroscopy, and mass spectrometry. Aminomethylation with Eschenmoser’s salt, as in the case of chlorins described in the literature,\(^\text{16}\) 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 dicaticionic 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).

| Compound | Survival index, % | Photoinduced activity, 1 μM | Quantum yield of singlet oxygen generation, \( \gamma \) |
|----------|-------------------|-----------------------------|----------------------------------|
|          | 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       | 118.56 ± 5.82     | 112.24 ± 6.98               | 90.06 ± 4.28                     | 84.09 ± 2.95                     | 0.59 |

For instance, chlorins 9 and 12 which have, respectively, two and three cationic groups located in different parts
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Table 2. Degree of erythrocyte hemolysis after 1 and 3 h incubation with cationic derivatives of chlorin e6 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 ± 0.1 | 1.3 ± 0.1 | 0.9 ± 0.1 | 1.2 ± 0.1 |
| 8        | 54.1 ± 2.0 | 79.7 ± 2.2 | 79.8 ± 0.4 | 80.1 ± 0.2 |
| 9        | 4.5 ± 0.2  | 5.3 ± 0.3  | 10.7 ± 0.7 | 83.8 ± 2.6 |
| 10       | 3.7 ± 0.1  | 4.7 ± 0.1  | 15.0 ± 1.4 | 82.2 ± 2.1 |
| 11       | 3.3 ± 0.1  | 4.3 ± 0.2  | 9.9 ± 1.6  | 90.7 ± 3.0 |
| 12       | 2.3 ± 0.1  | 2.9 ± 0.0  | 4.9 ± 0.2  | 83.9 ± 1.6 |

Reaction conditions: i: [9] CH₃NH₂·H₂O/THF, room temp., 3 h, yield of 3 – 85 %; ii: [9] [(CH₃)₂N=CH]⁺I⁻, CH₂Cl₂, room temp., 12 h, yield of 5 – 35 %, yield of 4 – 30 %; iii: [12] H₂NCH₂CH₂N(CH₃)₂, CH₂Cl₂, room temp., yield of 2 – 74 %; iv: [13,14] CH₃(N(CH₃)₂)₂, AcOH-THF, boiling for 30 min, yield of 6 – 65 %, yield of 7 – 45 %; v: [13] CH₃I, CH₂Cl₂, room temp., yields of 8–12 – 93–96 %.

Scheme 1.

of the macrocycle do not exhibit significant dark cytotoxicity 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 which is the main cytotoxic agent in photosensitized cell damage.\cite{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.

**Figure 1.** Dark and photoinduced cytotoxic activities of cationic derivatives of chlorin e$_6$ 8–12 in experiments on HeLa cells.

Mammalian erythrocytes are known to be a good model for studying various influences on biological membranes.\cite{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,\cite{20} causing these effects.

**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.

**Conclusion**

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