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Synthesis of Dioxidine-Conjugated and/or Cationic Chlorin e_6 Derivatives and Study of Their Dark and Photoinduced Cytotoxicity *in vitro*

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The work describes the synthesis and biological assays of a conjugate of a chlorin e_6 cationic derivative with the reserve antimicrobial drug "Dioxidine". The influence of the alternate and joint introduction of the cationic N,N-dimethylpiperazinio and "Dioxidine" fragments into the macroheterocycle molecule on dark and photoinduced toxicity of the photosensitizers towards malignant HT-29, A549 and HeLa cells was studied. The introduction of the "Dioxidine" fragment into the cationic 13(1)-N,N-dimethylpiperaziniochlorin molecule is found to significantly reduce both the solubility of the photosensitizer in water and its photoinduced cytotoxicity. Our results clearly indicate that phototoxicity of the chlorins towards different tumor cells has a multidirectional nature.

Keywords: Combined photodynamic therapy, photosensitizer, cationic chlorins, dioxidine conjugates, dark and photoinduced cytotoxicity.

Синтез диоксидин-конъюгированных и/или катионных производных хлорина e_6 и исследование их темновой и фотоиндуцированной цитотоксичности *in vitro*

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В работе описаны синтез и биологические испытания конъюгата катионного производного хлорина e_6 с резервным антимикробным препаратом «Диоксидин». Изучено влияние попеременного и совместного введения катионного N,N-диметилпиперазинильного и «диоксидинового» фрагментов в молекулу макрогетероцикла на темновую и фотоиндуцированную токсичность фотосенсибилизаторов по отношению к злокачественным клеткам опухолей HT-29, A549 и HeLa. Установлено, что введение фрагмента «Диоксидина» в катионную молекулу 13(1)-N,N-диметилпиперазинилхлорина существенно снижает как растворимость фотосенсибилизатора в воде, так и его фотоиндуцированную цитотоксичность. Полученные результаты однозначно свидетельствуют о том, что фототоксичность хлоринов по отношению к различным опухолевым клеткам имеет разнонаправленный характер.

Ключевые слова: Комбинированная фотодинамическая терапия, фотосенсибилизатор, катионный хлорин, конъюгаты с диоксидином, темновая и фотоиндуцированная цитотоксичность.

Introduction

Cancer and multi-resistant microbial infections are a rapidly growing social problem in the world.[1] Owing to new antibiotics and antiseptics, antitumor immune vaccines, methods of radioisotopic, hyperthermal and target therapies, the progress of treating these pathologies became obvious in last decades.[2-8] However, mortality caused by oncological diseases or antibiotic-resistant pathogens remains rather high.

Photodynamic therapy (PDT) is an efficient high-tech low invasive method of treating both problems mentioned above which can be successfully used as a single modality or in the combination with a traditional surgical intervention, chemo- or antibiotic therapy.[9-17] Simultaneously, with the local elimination of tumor tissue or microbial cells, this technique allows physicians to perform fluorescent navigation and induces an appreciable immune response activating macrophages and dendritic cells.

The perspective direction of the development of new light-activated drugs involves target synthesis of multifunctional agents containing biologically active fragments with the chemotherapeutic or antimicrobial activity on the periphery of the macrocycle.[8,16-19] The release of these fragments with cytostatic action inside the human body, as a result of photo- or biodegradation of the photosensitizer (PS), may essentially enhance the total antitumor effect of PDT. The combination of PDT with chemotherapy proved to be highly efficient both *in vitro* and *in vivo* studies.[12,16] It is believed that an appropriate cytotoxic agent and a PS conjugated in one molecule may result in a synergistic antitumor effect to overcome drug resistance and reduce the therapeutic dose required.

"Dioxidine" or 2,3-dihydroxymethylquinaxaline-1,4-dioxide is better known as a broad-spectrum antibacterial drug from the quinoxaline group with pronounced activity towards the series of infections.[8,20,21] However, the pharmacological profile of the quinoxaline class of compounds is rather diverse including anti-inflammatory, antineoplastic, enzyme inhibitory and other activities.[22,23] Indeed, the authors[23] observed the anti-tumor effect of "Dioxidine" using both adenocarcinoma cell lines Colo MT *in vitro* and sarcoma M1-bearing rats *in vivo*. Apparently, this compound covalently bound to a macrocyclic PS can be released from the conjugate after its photodegradation during PDT, thereby, enhancing the antitumor effect. Hence, synthesis of such dual agents, where a PS is responsible for the photodynamic treatment and a target transport of the drug into tumor tissues is of particular interest.

Several years ago, we described synthesis of two dioxidine esters of the chlorin and phorbine types and studied their thermal stability in a condensed state,[24] aggregation in water-alcohol[25] and water-surfactant[26] solutions as well as the PS affinity towards a lipid-like medium.[18,19,27,28] Additionally, some results of biophysical studies associated with accumulation and intracellular distribution of compound **1** inside K562 tumor cells have been recently reported.[29]

The cationic group on the periphery of the macrocycle increases overall hydrophilicity of a chlorin, may contribute to a greater bioavailability of the conjugate with dioxidine and provide a universal therapeutic effect on both malignized and infected tissues. The chlorin PSs with a single cationic trialkylammonium group studied before demonstrated high photodynamic activity towards tumor cells and antibiotic-resistant bacteria both *in vitro* and *in vivo*.[30,31] With this information in mind, we synthesized a covalently bound conjugate **3** (Figure 1) containing the monocationic fragment like compound **2** and "Dioxidine" residue common for PS **1** on the chlorin e_6 platform, and investigated its dark and photoinduced antitumor activity *in vitro*. Compound **4** free of both the cationic group and the "Dioxidine" fragment was chosen as a reference PS.[18]

Results and Discussion

Synthesis

To synthesize the target compound **3**, a conjugate **6** of pheophorbide *a* with a "Dioxidine" molecule, where the macrocycle and the drug fragment were linked through the ester bond, was obtained initially. The reaction catalyzed by the Mukayama reagent (CMPI)[32] was performed by the interaction of equimolar amounts of pheophorbide *a* (**5**) and dioxidine (Scheme 1).[19] Subsequently, the formation of the cationic substituent was achieved through the chemical modification of the chlorin fragment of the compound **6**.

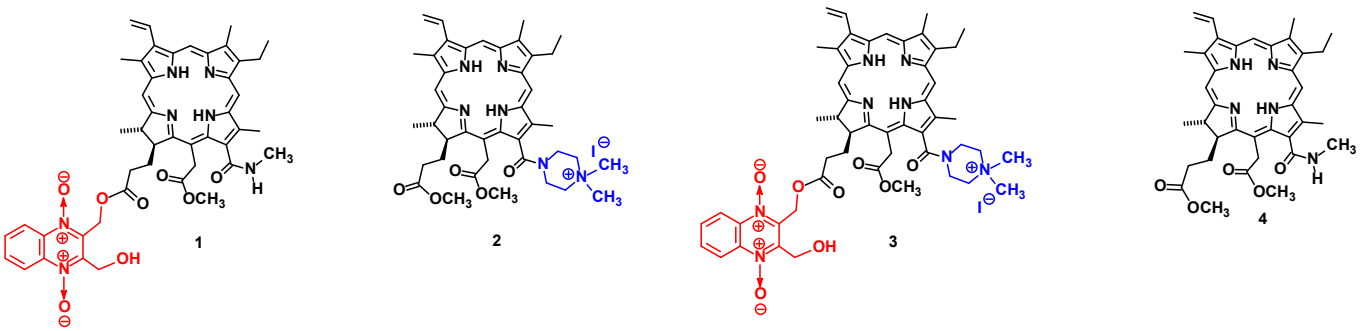
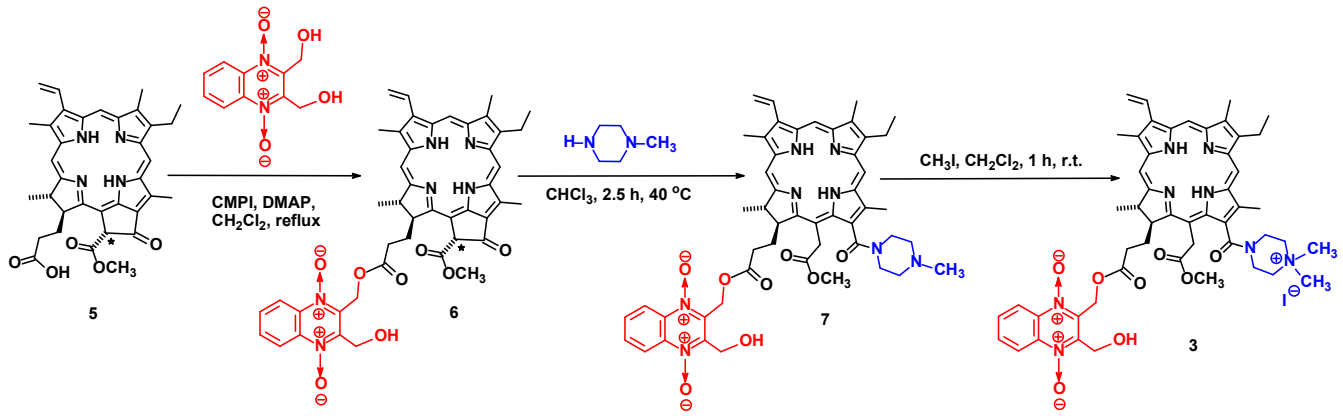


Figure 1. Structures of the compounds studied.



Scheme 1. Synthetic procedure of preparation of cationic chlorin **3** from methylpheophorbide *a* (**5**).

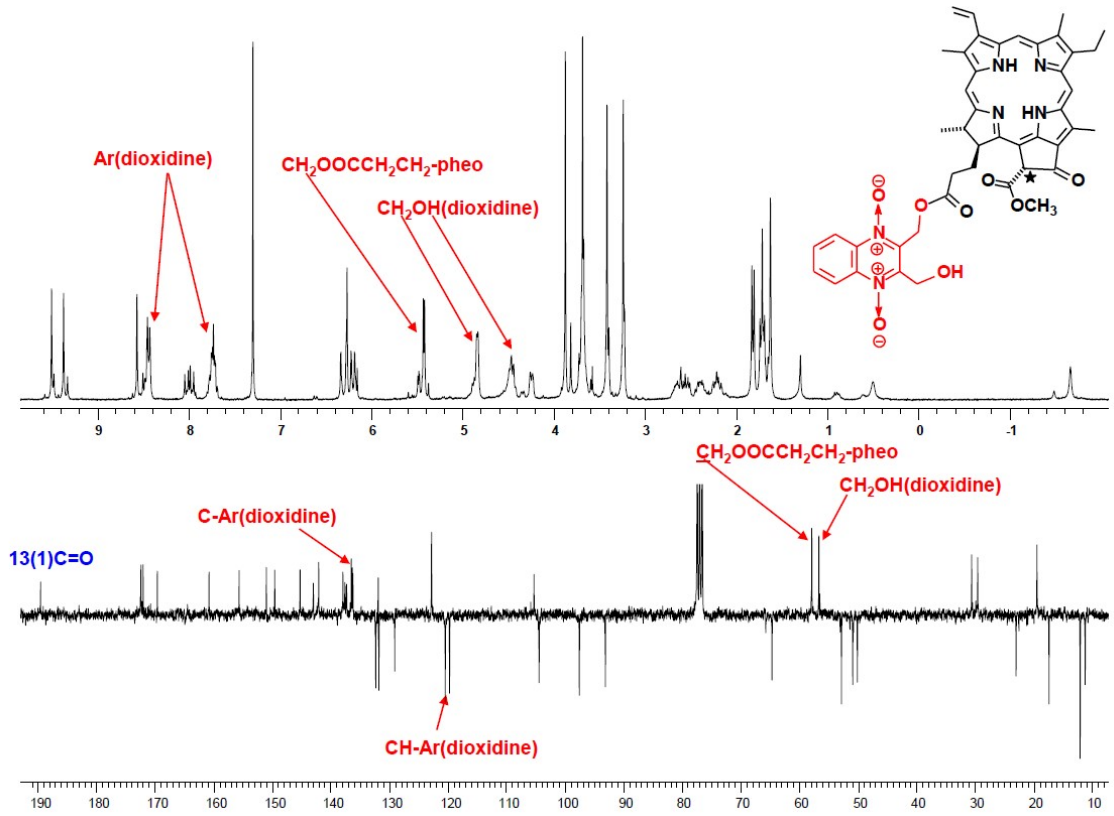


Figure 2. ^{1}H and ^{13}C NMR spectra of the conjugate **6** of pheophorbide *a* and "Dioxidine" ($CDCl_3$, 300 MHz).

Chlorin e_6 derivative **7** substituted in the 13(1)-position of the macrocycle was prepared under treating of the compound **6** with N-methylpiperazine followed by the alkylation of tertiary nitrogen of heterocyclic fragment with methyl iodide yielding in corresponding cationic compound **3** (Scheme 1).

The structure of the target conjugate **3** and intermediate compounds **6-7** was confirmed by IR and NMR spectroscopy as well as mass spectrometry data. Two peaks corresponding to the protonated molecular ion (MH^+) and macrocycle adduct with a sodium cation (MNa^+) were observed in electrospray mass spectra (ESI-MS) of the conjugate **6** of pheophorbide *a* and dioxidine. ^{1}H NMR spectrum of the compound **6** (Figure 2) was represented by two sets of the signals clearly demonstrating the presence of both pheophorbide *a* and "Dioxidine" fragment in the molecule with the 1:1 ratio of integral intensities. Further evidence for the presence of a hydroxyl group from the "Dioxidine" fragment in the conjugate **6** was provided by the exchange peak in the NOESY spectrum (Figure 3). The signals of the ^{1}H and ^{13}C NMR spectra corresponding to the protons and the carbon atom of the methylene group adjacent to the ester oxygen are evidenced by the presence of the ester bond between two parts of the conjugate. The IR spectrum of the "Dioxidine" fragment is characterized by stretching vibrations of the C-H methylene groups adjacent to the alcohol hydroxyl and ester oxygen atoms at 2733 cm^{-1} as well as by stretching vibrations of C-H bonds of the aromatic fragment at 3094 cm^{-1}.

Since the exocyclic moiety of pheophorbide *a* **5** undergoes enolization in the course of the esterification reaction, the product **6** obtained is represented by the mixture of diastereomers with the chiral center located at position 13(2) of the macrocycle. One of the diastereomers is predominant and, consequently, another isomer is appeared in the ^{1}H and ^{13}C NMR spectra as an additional low intensity set of signals differing from the major one in a chemical shift. The cleavage of the exocyclic moiety of pheophorbide *a* at the second stage of the synthesis (Scheme 1) is accompanied by elimination of the chiral center and the same product is formed regardless of the isomer stereo configuration. Therefore, diastereomers separation of the phorbine macroheterocycle **6** was not expedient.

Under reaction of N-methylpiperazine with the "Dioxidine" conjugate of pheophorbide *a* **6** the exocyclic moiety of the pheophorbide fragment is disclosed resulting in the amide group formation located at the position 13(1) of the corresponding chlorin e_6 derivative **7**. Such a structural modification of the molecule is confirmed by IR and NMR spectroscopy, as well as mass spectrometry. Similar to the phorbine compound **6** the mass spectrum of the chlorin **7** contains signals of the protonated molecular ion (MH^+) and the adduct with a sodium cation (MNa^+).

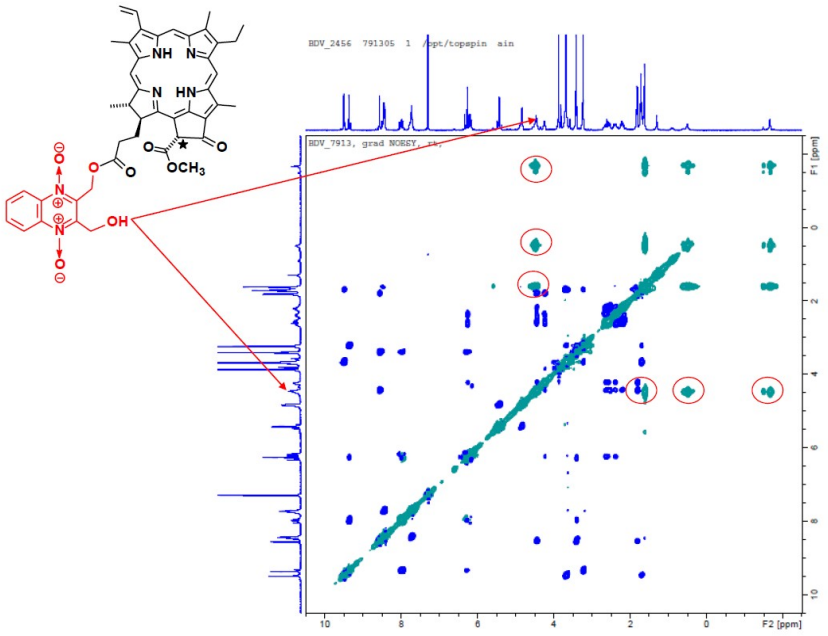


Figure 3. 2D NMR (NOESY) spectrum of the conjugate **6** of pheophorbide *a* and "Dioxidine" ($CDCl_3$, 300 MHz); cross peaks corresponding to the exchange interactions of the hydroxyl group of the dioxidine fragment are highlighted by circles.

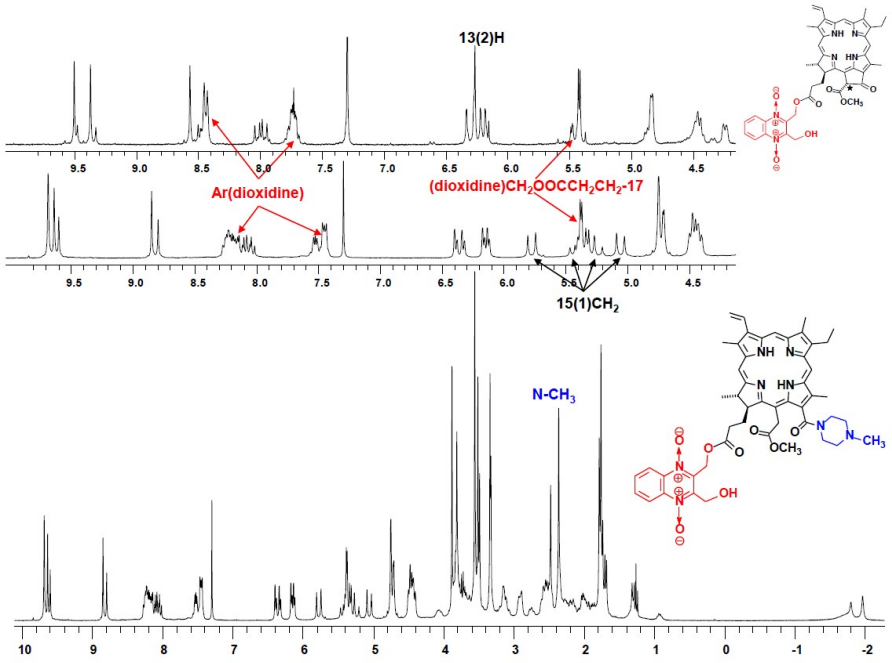


Figure 4. ^{1}H NMR spectra of the conjugate of pheophorbide *a* with "Dioxidine" **6** and the product of its interaction with N-methylpiperazine (**7**) ($CDCl_3$, 300 MHz).

The cleavage of the exocyclic moiety and the formation of the 13(1)-amide group are evident from both the IR and NMR spectra. Thus, the band of the exocyclic carbonyl group ν(C=O) of the pheophorbide fragment at 1699 cm^{-1} observed in the IR spectrum of the compound **6** is disappeared in the case of the intermediate substance **7**, indicating exocyclic cleavage. Besides, the formation of the amide fragment in the chlorin **7** molecule is confirmed by the presence of the band at 1627 cm^{-1}, corresponding to the ν(C=O) of the tertiary amide group ("amide I-band").

The singlet in the ^{1}H NMR spectra attributed to the CH proton at position 13(2) of the exocyclic moiety (6.15 ppm) of the phorbine compound **6** is lost in the spectrum of the chlorin product **7** (Figure 4). At the same time AB-type doublets of the methylene group at position 15(1) of **7**, formed upon exocycle cleavage, is acquired. In the ^{13}C NMR spectrum of the chlorin product **7** (Figure 5) signals corresponding to the carbon atom of the exocyclic carbonyl group and the carbon atom at position 13(2) observed at 189.54 ppm and 64.69 ppm, respectively, are absent in the spectrum of the phorbine compound **6**. The appearance of new signals which are referred to the carbon atom of the methylene group at position 15(1) and carbonyl carbon of the amide group at position 13(1) clearly demonstrates the exocyclic cleavage and formation of chlorin instead of phorbine macrocyclic structure. These data are consistent with conclusions drawn from the ^{1}H NMR measurements, mass spectrometry and IR spectroscopy data. Signals of the N-methylpiperazine and dioxidine fragments are observed in both the ^{1}H and ^{13}C NMR spectra. Thereby, the structure of the synthesized 13(1)-amide chlorin e_6 conjugate with dioxi-dine is confirmed reliably by the set of spectroscopic data.

Like all other known 13(1)-amide derivatives of chlorin e_6 bearing tertiary amide group, chlorin product **7** is represented by two atropisomers, distinct in mutual spatial arrangement of the amide group and the chlorin macrocycle. Two sets of signals are observed in the ^{1}H and ^{13}C NMR spectra, which are similar in numbers and shape but different in chemical shifts. The largest distinctions are observed for the signals of protons and carbon atoms most affected by the presence of the 13(1)-amide group.

The formation of the final cationic chlorin **3** due to the treatment of the macroheterocycle **7** by methyl iodide is confirmed by NMR spectroscopy and mass spectrometry. In the mass spectrum (ESI) of the cationic derivative **3** two peaks corresponding to the chlorin cation M^+ and doubly charged cation MH^{2+} of this salt-mimicking molecule M^+I^- are observed. The ^{1}H and ^{13}C NMR spectra of the final compound **3** are characterized by significant shifts of the signals of the N-methyl groups compared to the starting compound **7** from 2.36 ppm to 3.06 ppm for the major isomer, indicating downfield effect owing to the positive charge on the quaternized nitrogen atom (Figure 6).

All the compounds studied demonstrate UV-Vis spectra typical for natural chlorins with the intensive B- or Soret band at 400 nm and several Q-bands between 500–660 nm (see Experimental) originated from π-π*-electron transfers in the macrocyclic chromophore. The B- and Q-band positions and their intensity are not strongly affected by the substitution in the 13(1)- and 17(3)- positions of a macrocycle because this process does not induce π-electron cloud polarization in the dye molecule.

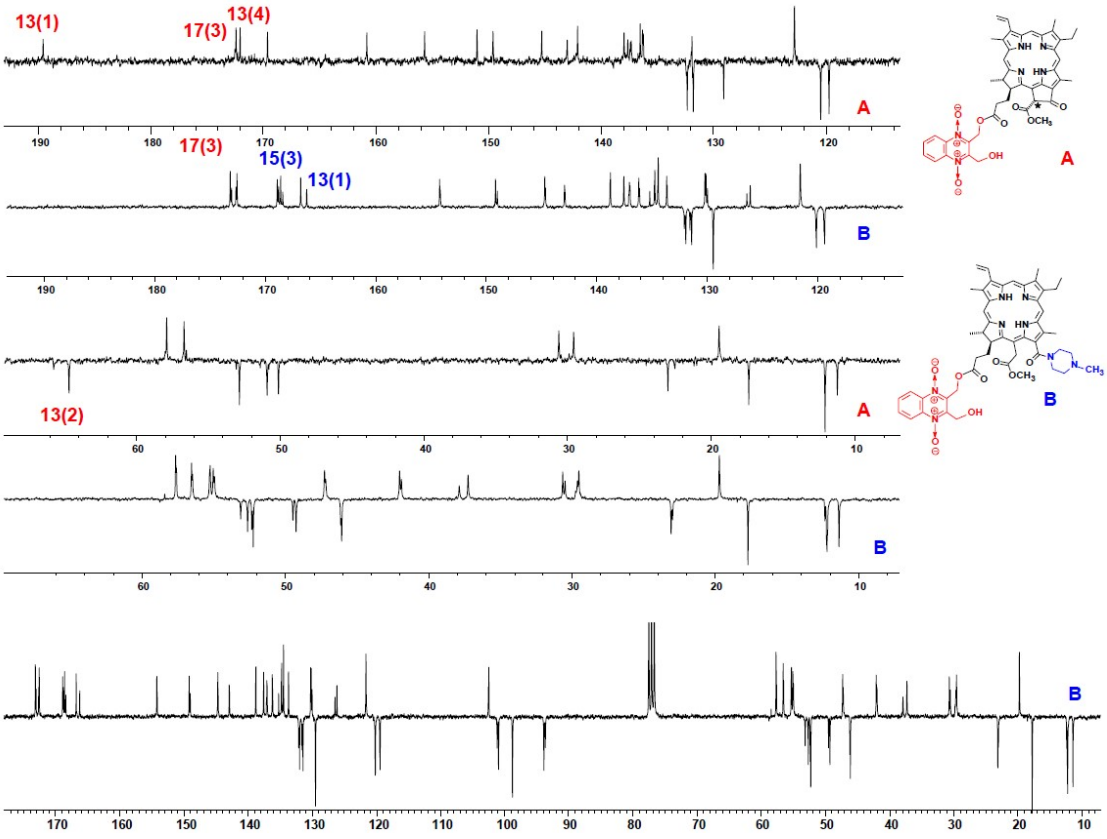


Figure 5. ^{13}C NMR spectra of the conjugate of pheophorbide *a* with "Dioxidine" (**6**) and the product of its interaction with N-methylpiperazine (**7**) ($CDCl_3$, 300 MHz).

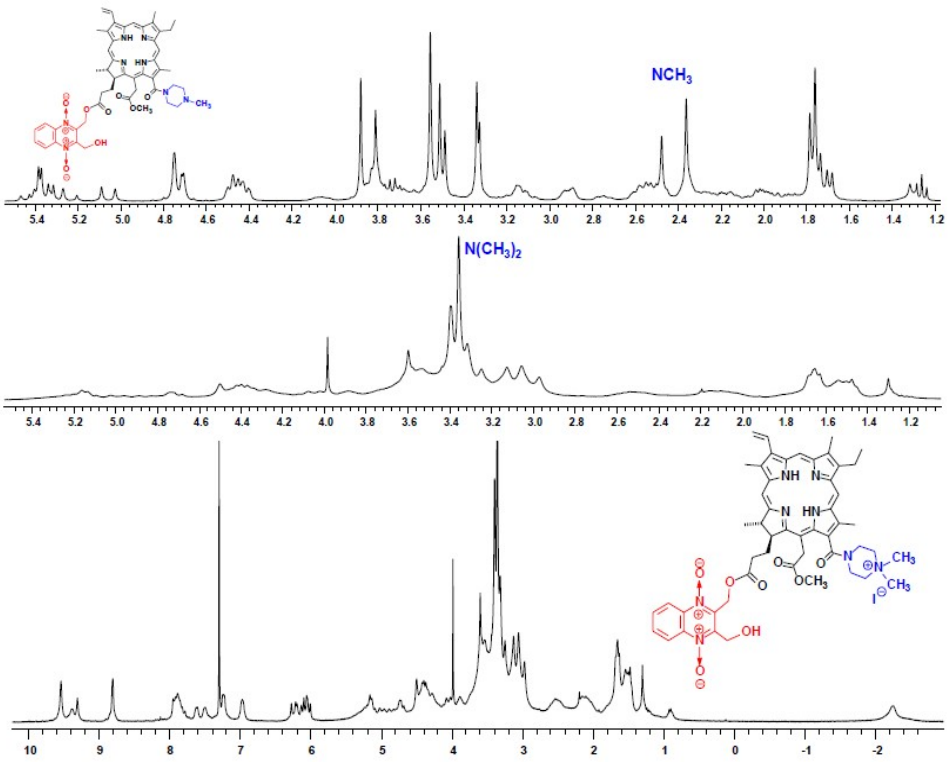


Figure 6. ^{1}H NMR spectra of 17(3)-dioxidine conjugate of the 13(1)-(N-methylpiperazinyl)amide of chlorin e_6 (**7**) and the product of its interaction with methyl iodide (**3**) ($CDCl_3$, 300 MHz).

Table 1. Values IC_{50}±SE (μmol/L) for *HeLa*, *A549*, and *HT-29* cell lines exposed to the dark ($IC_{50(d)}$) and photoinduced ($IC_{50(ph)}$) impact of the compounds (**1**-**4**).

Compound	*HeLa*		*A549*		*HT-29*	
	$IC_{50(ph)}$	$IC_{50(d)}$ ($IC_{50(d)}/IC_{50(ph)}$)	$IC_{50(ph)}$	$IC_{50(d)}$ ($IC_{50(d)}/IC_{50(ph)}$)	$IC_{50(ph)}$	$IC_{50(d)}$ ($IC_{50(d)}/IC_{50(ph)}$)
1	0.56±0.18	8.9±1.9 (16)	0.152±0.042	>50 (>329)	0.0202±0.0017	5.20±0.75 (257)
2	0.79±0.29	>50 (>63)	0.302±0.057	23.9±6.9 (79)	0.0308±0.0064	31.8±7.3 (1032)
3	1.44±0.33	3.9±1.8 (2.7)	2.24±0.35	21.8±4.9 (9.7)	1.2±1.0	13.9±1.5 (11.6)
4[19,33]	0.025±0.003	4.01±0.80 (160)	0.060±0.004	5.82±0.35 (97)	0.180±0.006	>50 (>278)

Dark and photoinduced cytotoxicity investigations

The second part of our work was devoted to the study the effect of the "Dioxidine" fragment and the cationic group covalently bounded to the chlorin macrocycle on biological activity of the conjugate **3**. To explore these effects, the dark and photoinduced toxicity of three compounds as the zwitter-ionic 13(1)-methylamide conjugate of chlorin e_6 with "Dioxidine" (**1**),[18] the cationic 13(1)-amide derivative of chlorin e_6 with a quaternized N-methylpiperazinyl group and without "Dioxidine" fragment (**2**)[31] and the newly synthesized conjugate of cationic chlorin **3** containing both zwitter-ionic and cationic groups in one molecule was evaluated. The data obtained were compared with those for the 13(1)-methylamide chlorin e_6 derivative **4** without charged groups. The cytotoxicity studies were performed using *HeLa*, *A549*, and *HT-29* tumor cell lines (Table 1).

The comparison of the half-maximal inhibitory concentration values (IC_{50}, μmol/L) for compounds **1** and **4** demonstrates that the introduction of the "Dioxidine" fragment to the PS molecule causes a slight decrease in dark toxicity for *HeLa* and a significant drop for *A549* cells. Meantime, an opposite trend is observed for *HT-29* cells (Table 1). Similarly, photo-induced toxicity of these chlorins decreases significantly for *HeLa* and *A549* cells, while it notably grows up in the case of HT-29 cells. It is worth noting that due to relatively low dark toxicity towards *A549* cells, the "Dioxidine" conjugate of chlorin e_6 (**1**) reveals a remarkable photodynamic effect with the $IC_{50(d)}/IC_{50(ph)}$ ratio > 300. Despite relatively high phototoxicity of compound **1** the comparable value of $IC_{50(d)}/IC_{50(ph)}$ equal to 257 is observed for *HT-29* cells. Surprisingly, "Dioxidine"-free 13(1)-methylamide **4** also demonstrates the comparable values of $IC_{50(d)}/IC_{50(ph)}$ (see Table 1). Hence, the conjugation of the "Dioxidine" fragment can either increase or decrease both inherent and photoinduced toxicity of the chlorin PSs. The 3-fold efficiency improvement of the compound (**1**) photodynamic action defined by the higher value of ($IC_{50(d)}/IC_{50(ph)}$) is observed only for *A549* cells, while for *HeLa* cells this ratio is ten times smaller.

Comparing the biological activity of compounds **2** and **4** we may draw a tentative conclusion that the introduction of a charged group into the PS molecule results in the decrease in both inherent and photoinduced toxicity towards *HeLa* and *A549* cells with comparable $IC_{50(d)}/IC_{50(ph)}$ values. However, for *HT-29* tumor cells there is a slight increase in dark toxicity and a significant enhancement of photoinduced one, with the $IC_{50(d)}/IC_{50(ph)}$ ratio > 1000 (see Table 1).

Unfortunately, the expectations related to the combined therapeutic action of compound **3** associated with its photodynamic and drug effects were not met in the course of its toxicological *in vitro* investigation. The simultaneous introduction of the cationic group and the "Dioxidine" fragment into the chlorin macrocycle (**3**) leads to a notable decrease in photoinduced toxicity across all tested cell lines compared to the model PS **4**. The double substitution in compound **3** results in the much larger $IC_{50(ph)}$ values which are approximately 7, 37 and 58 times higher for *HT-29*, *A549* and *HeLa* cells, respectively. This reduction in phototoxicity can be attributed to the decreased membrane affinity of the PS molecule due to the presence of several polar groups on the periphery of the macrocycle. Unlike the cationic 13(1)-amide chlorin e_6 with the N,N-dimethylaminopiperazinio fragment in the 13-amide group (**2**), its conjugate with "Dioxidine" (**3**) seems to be poorly soluble in water due to the combined effect of the N,N-dimethylpiperazinio and zwitter-ionic N-oxide groups.

In contrast, compounds **1** and **2** containing only one polar group do not exhibit a significant decrease in photoinduced toxicity for *HeLa* and *A549* cell lines, while for *HT-29* cells it increases significantly. The dark toxicity of compound **3** depends on the type of the cell line, decreasing for *HT-29*, slightly decreasing in the case of *A549*, and remaining nearly constant for *HeLa* cells. Due to the lower photoinduced toxicity of the compound **3** compared to the unsubstituted chlorin **4** its $IC_{50(d)}/IC_{50(ph)}$ ratio significantly worsens for all cell lines studied.

Experimental

Synthesis

^{1}H NMR spectra (δ, ppm; *J*, Hz) were recorded on a Bruker Avance II 300 spectrometer (Germany) in $CDCl_3$ at an operating frequency of 300 MHz; internal standard was chloroform or 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). IR spectra were recorded in KBr tablets on a Shimadzu IR Prestige 21 device. For column chromatography 0.06–0.2 mm silica gel (Alfa Aesar, USA) was used. All the reagents and solvents were used without further purification. The synthesis of compounds (**1**, **2**, **4**, **6**) was carried out according to the procedures described in [18,19,34].

*Chlorin e_6 13(1)-N-methylamide 15(2)-methyl, 17(3)-dioxidine ester (**1**)* was synthesized according to the protocol described in [18]. UV-Vis (EtOH) λ_{max} nm (lgε): 399 (5.02), 500 (4.19), 526 (3.97), 555 (3.91), 661 (4.56). Mass spectrum (ESI) *m/z*: for MH^+ ($C_{46}H_{50}N_7O_8$) found 828.7, calculated 828.4; for MNa^+ ($C_{46}H_{49}N_7NaO_8$) found 850.3, calculated 850.4. ^{1}H NMR (300 MHz, $CDCl_3$) δ_H ppm: –2.01 (br.s, 1H, $N^{I}H$); –1.78 (br.s, 1H, $N^{III}H$); 1.70 (d, 3H, *J*=6.6 Hz, $C^{18(1)}H_3$); 1.71 (t, 3H, *J*=7.3 Hz, $C^{8(2)}H_3$); 1.75–1.99 (m, 2H, $C^{17(2)}H_2$); $C^{17(1)}H_2$: 2.06–2.28 (m, 1H), 2.29–2.47 (m, 1H); 3.26 (d, 3H, $C^{13(2)}H_3$, *J*=4.4 Hz); 3.27 (s, 3H, $C^{7(1)}H_3$); 3.47 (s, 3H, $C^{2(1)}H_3$); 3.51 (s, 3H, $C^{12(1)}H_3$); 3.73 (m, 2H, $C^{8(1)}H_2$, *J*=7.3 Hz); 3.75 (s, 3H, $C^{15(3)}H_3$); 4.26 (br.s, 1H, dioxCH$_2$O*H*); 4.03–4.59 (m, 4H, dioxC*H*$_2$OH, $C^{17}H$, $C^{18}H$); 4.87 and 4.98 (all d 1H, *J*=12.1 Hz, dioxC*H*$_2$OOChl); 5.26, 5.44 (all d 1H, $C^{15(1)}H_2$, *J*=19.1 Hz); 6.14 (d, 1H, $C3^{(2)\text{-}cis}H$, *J*=11.7 Hz); 6.34 (d, 1H, $C^{3(2)\text{-}trans}H$, *J*=18.3 Hz); 6.66–6.81 (br.m, 1H, $C^{13(1)}NH$); 7.22–7.39 (m, 2H, Ar-diox); 7.95–8.05 (m, 2H, Ar-diox); 8.05 (dd 1H, $C^{3(1)}H$, *J*=17.6 Hz, *J* =1.7 Hz); 8.77 (s, 1H, $C^{20}H$); 9.56 (s, 1H, C^5H); 9.59 (s, 1H, $C^{10}H$). ^{13}C NMR (75 Hz, $CDCl_3$) δ_C ppm: 11.28 ($C^{7(1)}$); 11.88 ($C^{2(1)}$); 12.13 ($C^{12(1)}$); 17.65 ($C^{8(2)}$); 19.64 ($C^{8(1)}$); 23.04 ($C^{18(1)}$); 27.25 ($C^{13(2)}$); 29.43 ($C^{17(2)}$); 30.25 ($C^{17(1)}$); 37.75 ($C^{15(1)}$); 49.31 (C^{18}); 52.09 ($C^{15(3)}$); 52.78 (C^{17}); 56.10 (*C*H$_2$OH(diox)); 57.28 (diox-*C*H$_2$OOCCH$_2$CH$_2$-chl); 93.53 (C^{20}); 98.73 (C^5); 101.28 (C^{10}); 102.27 (C^{15}); 119.19 (CH-Ar-diox); 119.95 (CH-Ar-diox); 121.65 ($C^{3(2)}$); 128.28 (C^{13}); 129.41 ($C^{3(1)}$); 129.72 (C^{12}); 130.25 (C^2); 131.29, 131.78 (CH-Ar-diox); 134.54 (C^7); 134.77 (C^4); 134.85, 134.92 (C^3; C^{11}); 136.03 (C^1); 136.74, 136.78, 137.23 (C-Ar-diox); 138.86 (C^8); 142.57 (C-Ar-diox); 144.70 (C^{14}); 148.95 (C^9); 154.14 (C^6); 166.06 (C^{16}); 168.77 (C^{19}); 170.06 ($C^{13(1)}$); 172.49 ($C^{17(3)}$); 174.16 ($C^{15(2)}$).

*Chlorin e_6 13(1)-N-(4'-N'N'-dimethylpiperazinio iodide) amide, 15(2),17(3)-dimethyl ester (**2**)* was obtained according to the reference [31]. UV-Vis (EtOH) λ_{max} nm (lgε): 399 (5.13), 499 (4.10), 526 (3.64), 606 (3.71), 662 (4.65). Mass spectrum (ESI) *m/z*: for M^+ ($C_{42}H_{53}N_6O_5$) found 721.4, calculated 721.4; for MH^{2+} ($C_{42}H_{54}N_6O_5$) found 361.4, calculated 361.2. ^{1}H NMR (300 MHz, $CDCl_3$) δ_H ppm: (mixture of atropisomers 2.4 to 1 (according to the intensity of one of the CH protons at the position 15(2)), differing in the mutual arrangement of the chlorin macrocycle and the amide group at position 13, sign "/" separates the signals of atropisomers differing in chemical shift): –1.63/–1.47 (br.s, 1H, $N^{I}H$); –1.51/–1.40 (br.s, 1H, $N^{III}H$); 1.60–1.74 (br.m, 3H, $C^{8(2)}H_3$); $C^{18(1)}H_3$: 1.77 (d, 3H, *J*=7.2 Hz)/1.68–1.74 (br.m, 3H); 1.82–2.67 (m, 4H, $C^{17(1)}H_2$, $C^{17(2)}H_2$); $N^+(CH_3)_2$: 3.19/3.26 (s, 3H), 3.26/3.12 (s, 3H); 3.37/3.41 (s, 3H, $C^{2(1)}H_3$); 3.47/3.49 (s, 3H, $C^{7(1)}H_3$); 3.51/3.56 (s, 3H, $C^{12(1)}H_3$); 3.63 (s, 3H, $C^{17(4)}H_3$); 3.73/3.69 (s, 3H, $C^{15(3)}H_3$); 3.04–3.86 (m, 6H, CH_2 piperazine fragment); 3.66–3.84 (m, 2H, $C^{8(1)}H_2$); 4.17–4.56 (m, 4H, $C^{17}H$, $C^{18}H$, CH_2 piperazine fragment); $C^{15(1)}H_2$: 4.99 (d, 1H, *J*=17.7 Hz)/4.97 (d, 1H, *J*=18.5 Hz), 5.80 (d, 1H, *J*=17.7 Hz)/5.54 (d, 1H, *J*=18.5 Hz); 6.08–6.17 (m, 1H, $C3^{(2)\text{-}cis}H$); 6.34 (dd, 1H, *J*=17.9 and 1.0 Hz, $C^{3(2)\text{-}trans}H$); 8.03 (dd, 1H, *J*=17.9 Hz and 11.7 Hz, $C^{3(1)}H$); 8.82/8.77 (s, 1H, $C^{20}H$); 9.59/9.54 (s, 1H, C^5H); 9.67/9.61 (s, 1H, $C^{10}H$). ^{13}C NMR (75 Hz, $CDCl_3$) δ_C ppm: 11.26/11.19 ($C^{7(1)}$); 12.11 ($C^{2(1)}$); 12.99/13.15 ($C^{12(1)}$); 17.65 ($C^{8(2)}$); 19.58/19.51 ($C^{8(1)}$); 22.97/22.91 ($C^{18(1)}$); 29.64/29.96 ($C^{17(2)}$); 31.18/31.01 ($C^{17(1)}$); 35.83 (CH_2 piperazine fragment); 37.47 ($C^{15(1)}$); 40.49 (CH_2 piperazine fragment); 49.13/49.46 (C^{18}); 51.63/51.34 ($C^{17(4)}$); 52.26 ($C^{15(3)}$); 52.37/52.44 ($N^+(CH_3)_2$); 52.62 ($N^+(CH_3)_2$); 53.33 (C^{17}); 60.57 (CH_2 piperazine fragment); 61.05 (CH_2 piperazine fragment); 93.93/93.74 (C^{20}); 98.87/98.81 (C^5); 101.83 (C^{10}); 101.99 (C^{15}); 122.02 ($C^{3(2)}$); 122.87/123.06 128.12 (C^{13}); 129.21 ($C^{3(1)}$); 129.41, 130.73/130.83 (C^{12}, C^2); 134.21/134.03 (C^7); 134.41 (C^4); 134.96/135.03 (C^3; C^{11}); 136.29/136.20 (C^1); 139.39/139.51 (C^8); 145.33/145.27 (C^{14}); 149.25/149.07 (C^9); 155.11/155.04 (C^6); 167.15/166.71 (C^{16}); 169.38/168.65 (C^{19}); 169.43/169.75 ($C^{13(1)}$); 173.43/173.51 ($C^{17(3)}$); 174.24/173.64 ($C^{15(2)}$).

*Pheophorbide a 17(3)-dioxidine ester (**6**)*.[19] To a solution of 193.0 mg (0.33 mmol) of pheophorbide *a* (**5**) in 50 mL of methylene chloride, 98.2 mg (0.80 mmol) of 4-dimethyl-aminopyridine (DMAP), 171.8 mg (0.67 mmol) of 2-chloro-N-methylpyridinium iodide (CMPI), and 152.6 mg (0.68 mmol) of dioxidine were added. The mixture was refluxed in a flask for 30 min under TLC control (eluent CCl_4 : acetone = 2:1). The reaction mixture was then transferred to a separatory funnel and washed with 5–10% hydrochloric acid to remove an excess of DMAP and by-products of 2-chloro-N-methylpyridinium iodide conversion. Subsequently, the acid was washed with distilled water until pH became neutral. The resulting solution was dried over anhydrous sodium sulfate and evaporated under reduced pressure at the temperature of 40–50 °C. The residue obtained after evaporation

was chromatographed on silica gel using a mixture of CCl_4 : acetone (ratios ranging from 40:1 to 1:1) as the eluent. The eluate containing the main substance was then evaporated under reduced pressure. The yield of the desired product (**6**) obtained as a dark blue-black crystalline powder was 131.9 mg (51%). Mass spectrum (ESI) *m/z*: MH^+ ($C_{45}H_{45}N_6O_8$) 797.3 (calculated) 797.2 (found); MNa^+ ($C_{45}H_{44}N_6NaO_8$) 819.3 (calculated), 819.4 (found). IR (KBr) ν_{max} cm^{-1}: 3393, 3094, 2959, 2924, 2868, 2733, 1740, 1696, 1616. ^{1}H NMR (300 MHz, $CDCl_3$) δ_H ppm (mixture of atropisomers *a* to *a'* with the ratio 2.4 to 1 according to the integral of C^5; the signals of isomer *a'*, which differs in chemical shifts from the signals of similar protons of the minor isomer, are separated with the sign "/"): –1.66/–1.49 (1H, br.s, $N^{I}H$), 0.50/0.61 (1H, br.s, $N^{III}H$), 1.71/1.70 (3H, t, *J* = 7.4 Hz, $C^{8(2)}H_3$), 1.82 (3H, d, *J* = 7.3 Hz, $C^{18(1)}H_3$), 17–CH_2CH_2COODiox: 2.13–2.28 m (1H), 2.32–2.46 m (1H), 2.47–2.74 m (2H); 3.24/3.22 s (3H, 7-CH_3), 3.42/3.40 s (3H, 2-CH_3), 3.68/3.67 s (3H, 12-CH_3), 8-C$H_2$$CH_3$: 3.69 m (2H, *J* = 7.4 Hz)/3.64–3.74 m (2H); 3.87/3.81 s (3H, 13(2)-CO_2CH_3), 17-H: 4.24 br.d (1H, *J* = 7.9 Hz)/4.34 br.d (1H, *J* = 7.4 Hz), 4.45 m (1H, *J* = 7.2 Hz, 18-H), 4.44–4.55 br. m (1H CH_2O*H*(diox)), CH_2OH(diox): 4.83 d (2H, *J* = 4.1 Hz)/ 4.88 d (2H, *J* = 6.3 Hz), diox-CH_2OOCCH_2CH_2-pheo: 5.40 d (1H, *J* = 12.8 Hz), 5.45 d (1H, *J* = 12.8 Hz), 6.20 dd (1H, *J* = 11.6 and 1.2 Hz, 3-CH=CH*H*cis), 6.26/6.15 s (1H, 13(2)CH), 6.30 d (1H, *J* = 18.1 and 1.2 Hz, 3-CH=CH*H*trans), 7.65–7.83 m (2H, Ar-diox), 7.99/7.97 dd (1H, *J* = 18.1 and 11.6 Hz, 3-C*H*=CH_2), 8.39–8.49 m (2H, Ar-diox), 8.57/8.50 (1H, s, H^{20}), 9.36/9.32 s (1H, H^5), 9.50/9.48 s (1H, H^{10}). ^{13}C NMR ($CDCl_3$), δ_C ppm: 11.22 (C(7(1))), 12.07 (C(2(1), C(12(1))), 17.38 (C(8(2))), 19.44 (C(8(1))), 23.00/22.53 (C(18(1))), 29.56 (C(17(2))), 30.60 (C(17(1))), 50.11 (C(18)), 50.91/50.77 (C(17)), 52.83/53.05 (C(13(4))), 56.68/56.52 (CH_2OH(diox)), 57.89 (diox-CH_2OOCCH_2CH_2-pheo), 64.69/65.76 (C(13(2)), 93.12 (C(20)), 97.57 (C(5)), 104.44 (C(10)), 105.31 (C(15)), 119.72 (Ar-diox), 120.47 (CH-Ar-diox), 122.79 (C(3(2))), 129.08 (C(13)), 131.79 (C(3(1))), 131.91 (C(12), C(2)), 132.34 (CH-Ar-diox), 136.22 и 136.29 C (C(7), C(4)), 136.50 (C(3)), 137.29 (C-Ar-diox), 137.37 (C-Ar-diox), 137.60 (C-Ar-diox), 137.93 C(11), 142.08 (C(1)), 142.98 (C-Ar-diox), 145.25 (C(8)), 149.57 (C(14)), 151.00 (C(9)), 155.66 (C(6)), 160.78 (C(16)), 169.61 (C(19)), 172.04 (C(13(3)), 172.41 (C(17(3))), 189.54 (C(13(1)).

Chlorin e_6 13(1)-N-(4'-N'-methylpiperazinyl)amide, 15(2)-methyl-17(3)-dioxidine ester (7). 17(3)-Dioxidine ester of pheophorbide *a* (**6**) in the amount of 23.3 mg (0.03 mmol) was dissolved in 5 mL of chloroform and mixed with 0.1 mL (1 mmol) of N-methylpiperazine. The resulting mixture was stirred for 2.5 h at 40 °C. Then the reaction mixture was washed with distilled water until a neutral pH was reached. The resulting solution was dried over anhydrous sodium sulfate and evaporated under reduced pressure at a temperature of 40–50 °C. The residue after evaporation was chromatographed on silica gel (eluted with a mixture of CCl_4 : acetone in ratios from 20:1 to 1:1, then with a mixture of chloroform:methanol in ratios from 30:1 to 6:1). 17.9 mg (67%) of the target product (**7**) was obtained as a dark green crystalline powder. Mass spectrum (ESI) *m/z*: $(M+2H)^+$ ($C_{50}H_{58}N_8O_8$) 898.4 (calculated), 898.1 (found); MNa^+ ($C_{50}H_{56}N_8NaO_8$), 919.4 (calculated), 919.5 (found). IR (KBr) ν_{max} cm^{-1}: 3100, 2957, 2924, 2862, 2743, 1736, 1607. ^{1}H NMR (300 MHz, $CDCl_3$) δ_H ppm: (mixture of atropisomers as 2 to 1 ratio according to the intensity of H^{20} signals, differing in the mutual arrangement of the chlorin macrocycle and the amide group in position 13, the sign "/" separates the signals of atropisomers differing in chemical shifts): –1.97/–1.80 (br.s, 1H, $N^{I}H$); –1.74 (br.s, 1H, $N^{III}H$); 1.76 (t, 3H, *J* =7.6 Hz, $C^{8(2)}H_3$); $C^{18(1)}H_3$: 1.77 (d, 3H, *J*=7.4 Hz)/1.69 (d, 3H, *J*=7.2 Hz); 1.83–2.65 (m, 8H, $C^{17(1)}H_2$, $C^{17(2)}H_2$, CH_2 piperazine fragment); 2.36/2.48 (s, 3H, $N(CH_3)$; 2.65–2.99 (m, 2H, CH_2 piperazine fragment); 3.03–3.26 (m, 2H, CH_2 piperazine fragment); 3.34/3.33 (s, 3H, $C^{7(1)}H_3$); 3.51/3.49 (c, 3H, $C^{2(1)}H_3$); 3.55 (s, 3H, $C^{12(1)}H_3$); 3.88/3.81 (s, 3H, $C^{15(3)}H_3$); 3.77–3.85 (m, 2H, $C^{8(1)}H_2$); 3.97–4.14 (m, 1H) and 4.31–4.60 (m, 3H) 4.03–4.59 (m, 4H, $C^{17}H$, $C^{18}H$, CH_2 piperazine fragment); 4.75 br.s (1H, dioxCH_2O*H*); 4.68 and 4.73 (all d 1H, *J*=13.8 Hz, dioxCH_2OOChl); 5.18–5.49 (m, 2H, dioxCH_2OH); $C^{15(1)}H_2$: 5.06 (d, 1H, *J*=19.0 Hz)/ 5.18–5.49 (m, 2H), 5.77 (d, 1H, *J*=19.0 Hz)/ 5.18–5.49 (m, 2H); $C3^{(2)\text{-}cis}$H: 6.15 (dd, 1H, *J* =11.6 and 1.0 Hz)/6.14 (dd, 1H, *J*=11.6 and 1.0 Hz); $C^{3(2)\text{-}trans}$H: 6.37 (dd, 1H, *J*=18.0 and 1.0 Hz)/6.35 (dd 1H, *J*=18.0 and 1.0 Hz); 7.39–7.58 (m, 2H, Ar-diox); 7.97–8.32 (m, 3H, Ar-diox, $C^{3(1)}H$); 8.85/8.79 (s, 1H, $C^{20}H$); 9.64/9.60 (s, 1H, C^5H); 9.68 (s, 1H, $C^{10}H$). ^{13}C NMR (75 Hz, $CDCl_3$) δ_C ppm: 11.34 ($C^{7(1)}$); 12.17/12.13 ($C^{2(1)}$); 12.10/12.32 ($C^{12(1)}$); 17.69 ($C^{8(2)}$); 19.68 ($C^{8(1)}$); 23.06/22.95 ($C^{18(1)}$); 29.50/29.60 ($C^{17(2)}$); 30.62/30.46 ($C^{17(1)}$); 37.23/37.85 ($C^{15(1)}$); 42.01/41.89 (CH_2 piperazine fragment); 46.05/46.12 (NCH_3); 47.24/47.16 (CH_2 piperazine fragment); 49.24/49.46 (C^{18}); 52.23/52.32 ($C^{15(3)}$); 52.62/53.10 (C^{17}); 55.01/54.93 (CH_2 piperazine fragment); 55.23/55.27 (CH_2 piperazine fragment); 56.52/56.46 (CH_2OH(diox)); 57.64/57.58 (diox-CH_2OOCCH_2CH_2-chl); 93.84/93.62 (C^{20}); 98.75 (C^5); 100.92/101.14 (C^{10}); 102.43/102.50 (C^{15}); 119.39/119.44 (CH-Ar-diox); 120.13/120.16 (CH-Ar-diox); 121.59 ($C^{3(2)}$); 126.12/126.41 (C^{13}); 129.46 ($C^{3(1)}$); 130.12/129.99 (C^{12}); 130.21 (C^2); 131.41/131.57, 131.95/132.07 (CH-Ar-diox); 133.67/133.64 (C^7); 134.45/134.48 (C^4); 134.74/ 134.78, 135.20 (C^3; C^{11}); 136.20/136.13 (C^1); 136.99/137.03, 137.04/137.10, 137.54 (C-Ar-diox); 138.76/138.79 (C^8); 142.92/142.88 (C-Ar-diox); 144.70/144.64 (C^{14}); 149.16/149.00 (C^9); 154.21/154.15 (C^6); 166.78/166.24 (C^{16}); 168.59/168.40 (C^{19}); 168.90/168.76 ($C^{13(1)}$); 172.54/172.64 ($C^{17(3)}$); 173.15/173.03 ($C^{15(2)}$).

Chlorin e_6 13(1)-N-(4'-N'N'-dimethylpiperazinio iodide) amide, 15(2)-methyl-17(3)-dioxidine ester (3). The solution containing 17.9 mg (0.02 mmol) of 13(1)-N-(4'-N'-methylpiperazinyl)amide-15(2)-methyl-17(3)-dioxidine ester of chlorin e_6 (**7**) in 1.5 mL of pre-dried chloroform was mixed with 0.5 mL (0.008 mol) of methyl iodide. The reaction mixture was stirred at room temperature for 1 hour, then the solvent was evaporated to dryness. The resulting product (**3**) was obtained as a dark green crystalline powder with the yield of 21.9 mg (97%). MS(ESI) *m/z* M^+I^-: $(M)^+$ ($C_{51}H_{59}N_8O_8$) 911.4 (calculated), 911.6 (found); $(MH)^{2+}$ ($C_{51}H_{60}N_8O_8$) 456.2 (calculated), 456.5 (found). IR (KBr) ν_{max} cm^{-1}: 3094, 2966, 2926, 2868, 2731, 1734, 1638, 1603. ^{1}H NMR (300 MHz, $CDCl_3$) δ_H ppm: (mixture of atropisomers as 2 to 1 ratio according to the intensity of H^{20} signals, differing in the mutual arrangement of the chlorin macrocycle and the amide group in position 13, the sign "/" separates the signals of atropisomers differing in chemical shifts): –2.25 (br.s, 1H, $N^{I}H$); –2.10 (br.s, 1H, $N^{III}H$); 1.41–1.72 (m, 6H, $C^{8(2)}H_3$, $C^{18(1)}H_3$); 1.73–2.68 (m, 8H, $C^{17(1)}H_2$, $C^{17(2)}H_2$, CH_2 piperazine fragment); $N^+(CH_3)_2$: 3.06/2.97 (s, 3H), 3.12 (s, 3H); 2.88–3.80 (m, 6H, CH_2 piperazine fragment, $C^{8(1)}H_2$); 3.32/3.25 (s, 3H, $C^{7(1)}H_3$); 3.36 (s, 3H, $C^{2(1)}H_3$); 3.39/3.36 (s, 3H, $C^{12(1)}H_3$); 3.60 (s, 3H, $C^{15(3)}H_3$); 3.80–5.56 (m, 11H, $C^{17}H$, $C^{18}H$, CH_2 piperazine fragment, dioxCH_2O*H*, dioxCH_2OH, dioxCH_2OChl, $C^{15(1)}H_2$); $C3^{(2)\text{-}cis}$H: 6.07 (d, 1H, *J*=11.6 Hz)/6.02 (d, 1H, *J*=12.0 Hz); $C^{3(2)\text{-}trans}$H: 6.24 (d, 1H, *J*=18.3 Hz)/6.16 (d, 1H, *J*=18.7 Hz); Ar-diox: 6.87–7.04 (m, 1H), 7.17–7.30 (m, 1H), 7.39–7.68 (m, 1H), 7.83–7.91 (m, 1H); 7.70–8.01 (m, 1H, $C^{3(1)}H$); 8.80 (s, 1H, $C^{20}H$); 9.38/9.30 (s, 1H, C^5H); 9.54 (s, 1H, $C^{10}H$). ^{13}C NMR (75 Hz, $CDCl_3$) δ_C ppm: 11.01/11.06 ($C^{7(1)}$); 12.05/11.96 ($C^{2(1)}$); 12.05/12.14 ($C^{12(1)}$); 17.19/17.37 ($C^{8(2)}$); 19.39/19.55 ($C^{8(1)}$); 23.02/22.90 ($C^{18(1)}$); 29.54/29.71 ($C^{17(2)}$); 30.77 ($C^{17(1)}$); 37.38/38.26 ($C^{15(1)}$); 40.71 (CH_2 piperazine fragment); 49.05/49.41 (C^{18}); 51.11/50.52 ($C^{15(3)}$); 52.62/53.01 (C^{17}); 51.37/52.34, 54.10 (NCH_3); 56.11 (CH_2 piperazine fragment); 56.51 (CH_2 piperazine fragment); 56.87/58.56 (CH_2OH(diox) HSQC); 60.81/60.60 (diox-CH_2OOCCH_2CH_2-chl HSQC); 94.54 (C^{20}); 98.26/98.45 (C^5); 101.09/100.93 (C^{10}); 102.58/103.15 (C^{15}); 118.79/119.22 (CH-Ar-diox); 119.33/119.70 (CH-Ar-diox); 122.33 ($C^{3(2)}$); 123.84/124.72 (C^{13}); 129.77 ($C^{3(1)}$); 130.06 (C^{12}); 130.92/131.02 (C^2); 131.16/131.25, 131.72 (CH-Ar-diox); 134.74, 134.99/135.14 (C^7, C^4); 135.52/135.40, 135.70 (C^3, C^{11}); 136.14 (C^1); 136.26,

136.66/136.72 137.47/137.61 (C-Ar-diox); 139.51/139.76 (C^{8}); 142.40/142.78 (C-Ar-diox); 143.97/144.46 (C^{14}); 149.16/149.00 (C^{9}); 154.21/154.15 (C^{6}); 167.86 (C^{16}); (C^{19}); 168.61 ($C^{13(1)}$); 172.49/172.41 ($C^{17(3)}$); 172.92/173.50 ($C^{15(2)}$).

Biological tests

Biological experiments are described in details in [33] and just briefly given here.

Cell culture. HeLa cervical cancer cells, *A549* lung cancer cells and human colon adenocarcinoma *HT29* cells were used for experiments. Cell cultures for all experiments were maintained in a DMEM/F12 (Paneco, Russia) supplemented with 10% fetal calf blood serum (FBS) (Thermo Scientific HyClone, UK), at 37 °C and 5% CO_2, without antibiotics. Cells were reseeded using a 0.05% solution of trypsin-EDTA with Hank's salts (PanEco, Russia) 2 times a week.

Stock solution of compounds studied preparation. Correspondent amounts of porphyrin derivatives were dissolved in DMSO (Amresco, USA) to get 100 μM stock solution. Working concentrations (0.01–100 μM) were prepared by diluting stock with DMSO. 1 μM of the compound solution or the same amount of solvent was added to each well of 96-well plate to get 0.5% (v/v) DMSO in cell suspension.

Cytotoxicity assessment. Cells were seeded in 96-well plates and incubated under standard conditions for 72 hours. Suspension density was equal to 5000 cells per well for *HeLa* cells and 20000 for *A549* and *HT29* cells. For the phototoxicity determination the plates were irradiated with a LED panel (IBC, Belarus) described elsewhere [29] during 20 min and then incubated for 70 hours under standard conditions. The intensity of the light spot (power density) was measured by an "Argus 03" power meter. The total light dose was equal to 12 $J \cdot cm^{-2}$. Cell survival was assessed with fluorometric microculture cytotoxicity assay (FMCA) according to the protocol.[35] After the medium was removed, 100 μL of Fluorescein Diacetate solution was added and cells were incubated at 37 °C and 5% CO_2 for 40 min. Fluorescence intensity measurements were made at 485 nm (excitation) / 520 nm (emission) on a microplate reader CLARIOstar (BMG LABTECH, Germany). Cell viability was calculated as a percent of control (1 μM of pure DMSO). Experiments were done in six repeats.

Conclusions

In conclusion, new chlorin photosensitizer **3** combining the cationic N,N-dimethylpiperazinio and heterocyclic "Dioxidine" fragments in one molecule was synthesized from pheophorbide *a* using the sequence of chemical reactions including esterification of the activated carboxylic group, nucleophilic cleavage of cyclopentanone ring and quaternization of the tertiary amino substituent. Surprisingly, the synthesized compound containing several charged centers in a molecule was found to be sparingly soluble in water. The dark and photoinduced activity of the target compound and previously synthesized similar macroheterocycles carrying only a heterocyclic (**1**) or cationic (**2**) fragment was studied in relation to three tumor cell cultures like cervical cancer (*HeLa*), lung cancer (*A549*), and human colon adenocarcinoma (*HT29*). Different photokilling behavior of chlorins studied towards to the certain strains of the malignized cells was specified. Unlike bifunctional PS **3**, chlorins containing N,N-dimethylpiperazinio or "Dioxidine" fragments separately demonstrate higher photoinduced cytotoxicity. Monocationic chlorin **2** has proven to show the best photosensitizing efficacy in the series of the compounds studied.

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