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Novel Hydrophilic Conjugates of Chlorin e_6 with Fructose: Synthesis and Estimation of Photodynamic Activity

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The conjugates of natural and synthetic porphyrins with carbohydrates are known to be promising antitumor photosensitizers (PS). The presence of monosaccharide fragments at the periphery of the porphyrin macrocycle leads to an increase in bioavailability and increases their uptake by cancer cells. We synthesized new hydrophilic conjugates of chlorin e_6 with fructose by alkylation of the amino groups of chlorin e_6 amides with one and two ethylenediamine or hexamethylenediamine fragments on the macrocycle periphery with triflate of 2,3:4,5-di-O-isopropylidene-β-D-fructopyranose, followed by removal of diisopropylidene protection by 70% aqueous trifluoroacetic acid. In all cases, monoalkylation occurs, which allows the chemoselective insertion of one fructose fragment for each amino group present in the initial chlorin. For most of the studied compounds, it is possible to obtain a solution containing at least 0.2 mg/mL without the use of auxiliary substances, which indicates the possibility of obtaining water-soluble forms. The synthesized conjugates were shown to exhibit pronounced photodynamic activity at concentrations at which dark cytotoxic effect is not observed (HeLa, A549, HT-29 cancer cell cultures were used as test objects).

Keywords: Methylpheophorbide *a*, chlorin e_6, fructose, cytotoxicity, alkylation.

Новые гидрофильные конъюгаты хлорина e_6 с фруктозой: синтез и оценка фотодинамической активности

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Литературные данные свидетельствуют о том, что конъюгаты природных и синтетических порфиринов с углеводами являются перспективными противоопухолевыми фотосенсибилизаторами (ФС). Наличие моносахаридных фрагментов на периферии порфиринового макроцикла приводит к увеличению биодоступности и увеличивает их поглощение раковыми клетками. Нами синтезированы новые гидрофильные конъюгаты хлорина e_6 с фруктозой путем алкилирования аминогрупп амидов хлорина e_6 трифлатом 2,3:4,5-ди-О-изопропилиден-β-D-фруктопиранозы с последующим удалением диизопропилиденовой защиты 70% водным раствором трифторуксусной кислоты. Во всех случаях происходит моноалкилирование, что позволяет хемоселективно вводить один фрагмент фруктозы для каждой аминогруппы, присутствующей в исходном хлорине. Для большинства исследованных соединений возможно получение раствора, содержащего не менее 0.2 мг/мл, без использования вспомогательных веществ, что указывает на возможность получения водорастворимых форм. Показано, что синтезированные конъюгаты проявляют выраженную фотодинамическую активность в концентрациях, при которых темновой цитотоксический эффект не наблюдается (в качестве тест-объектов использовали культуры раковых клеток HeLa, A549, HT-29).

Ключевые слова: Метилфеофорбид *a*, хлорин e_6, фруктоза, цитотоксичность, алкилирование.

Introduction

The literature data suggest that the conjugates of natural and synthetic porphyrins with carbohydrates are the promising antitumor photosensitizers (PS).[1–21] The dark and photoinduced cytotoxicity of such conjugates may depend on the nature and amount of carbohydrate fragments, the mode of their binding to the macrocycle, and the structure of the macrocycle itself. It is possible to improve the therapeutic ratio and increase of the PS effectiveness by varying of these parameters.[2,3,5-10] The presence of monosaccharide fragments at the periphery of the porphyrin macrocycle leads to an increase in bioavailability and increases their uptake by cancer cells compared to photosensitizers already used in medical practice, such as chlorin e_6 and thalaporfin, due to active transport through the cell membrane.[9,22] Chlorophyll *a* derivatives are a good basis for obtaining new antitumor and PS.[2] For the introduction of carbohydrate fragments to the periphery of the chlorophyll *a* derivatives macrocycle, click chemistry,[2,13] metathesis reactions,[2] the formation of ester[23,24] and amide bonds,[2] as well as alkylation of amino groups.[25] In the case of alkylation of amino groups, a stable C-N-C bond is formed between the fragments of chlorin e_6 and galactose.[25] Insertion of fructose moieties may promote active transport because fructose is selectively taken up by cells via the carbohydrate transporter GLUT5 and GLUT5 is abundantly expressed in many cancers.[26] In the present work, chlorin e_6 derivatives with one and two fructose fragments on the periphery of the macrocycle were synthesized by alkylation of the amino groups of chlorin e_6 derivatives with diisopropylidene fructose triflate followed by removing isopropylidene protection in acidic conditions (Scheme), and the photodynamic activity of the compounds obtained was evaluated using cultures of cancer cells of the HeLa (cervical cancer cells), A549 (lung adenocarcinoma) and HT-29 (human colon adenocarcinoma) lines as a test object.

Experimental

General

Mass spectra of synthesized compounds were recorded on the "Thermo-Finnigan LCQ Fleet" device. Infrared spectra were measured in KBr tablets on the "IR Prestige 21" device (Shimadzu). ^{1}H (working frequency 300 MHz) and ^{13}C (working frequency 75 MHz) NMR spectra of the synthesized compounds were recorded on Bruker AVANCE-II-300 (standard impulse Bruker software for one and two-dimensional experiments). UV-vis spectra were recorded on a spectrometer UV-1700 "Shimadzu" (wavelength range of 200-1100 nm). The samples were analyzed in quartz cuvettes (10 mm thick). The reaction was controlled using TLC method on Sorbfil slides. Extraction of the reaction products was done using column chromatography on silica gel Alfa Aesar 70–230 mesh. Methylpheophorbide *a* **1**[27] (from pheophytin *a*[28]) aminochlorin **2**,[29] diaminochlorin **4**,[29] aminochlorin **3**[30,31] and 2,3:4,5-Di-O-isopropylidene-1-O-(trifluoromethanesulfonyl)-β-D-fructopyranose **6**[32] were obtained according to literature methods.

Synthesis

Chlorin e_6 13,17-N,N′-(6-aminohexyl)-diamide-15-methyl ester ***5***. To the solution of 500 mg (0.82 mmol) methylpheophorbide *a* **1** in 30 mL of $CHCl_3$, hexamethylendiamine (1.5 g, 12.9 mol) was added. The mixture was stirred at room temperature (~23 ºC) for 5 h (TLC, $CHCl_3$–C_2H_5OH (95% aq.), 3:1). Next, the solvent ($CHCl_3$) was evaporated in vacuo, and the reaction mixture was heated in a glycerine bath for 8 h at 42 ºC. Then, the reaction mixture was diluted with chloroform (100 mL), washed with water until neutral p*H*, the organic layer was dried over anhydrous Na_2SO_4, and solvent was evaporated in vacuo. A residue after the evaporation was purified by chromatography on silica gel (eluent: $CHCl_3$-C_2H_5OH (95% aq.)-ammonia (25% aq.), 5:5:1). Yield: 270 mg (41 %) as a dark green crystalline powder. UV-Vis ($CHCl_3$-C_2H_5OH (95% aq.), 1:1) λ_{max} nm ($I_{relative}$ (%)): 662 (23), 606 (3), 527 (3), 500 (8), 401 (100). IR (KBr) ν cm^{-1}: 3080 (C–H, vinyl group), 1739 (C=O, ester), 1680 ("amide-I"), 1602 ("chlorin band"), 1540 ("amide-II"). MS (ESI) *m/z*: 807.6 (calcd. for $[M+H]^+$ 807.5). ^{1}H NMR (300 MHz, DMSO-d_6, Me_4Si) δ_H ppm: -2.11 (1H, s, III-NH); -1.81 (1H, s, I-NH); 1.23 - 1.64 (16H, m), 2.93-3.37 (12H, m) (17(4)-CH_2, 17(5)-CH_2, 17(6)-CH_2, 17(7)-CH_2, 17(8)-CH_2, 17(9)-CH_2, 13(2)-CH_2, 13(3)-CH_2, 13(4)-CH_2, 13(5)-CH_2, 13(6)-CH_2, 13(7)-CH_2, 17(9)-NH_2, 13(7)-NH_2); 1.68 (3H, t, 8(2)-CH_3, J = 5.6 Hz); 1.83 (3H, d, 18(1)-CH_3, J = 3.0 Hz); 2.02-2.26 (3H, m), 2.61-2.76 (1H, m) (17(1)-CH_2, 17(2)-CH_2); 3.34 (3H, s, 7(1)-CH_3); 3.53 (3H, s, 2(1)-CH_3); 3.55 (3H, s, 12(1)-CH_3); 3.70 (3H, s, 15(3)-CH_3); 3.84 (2H, q, 8(1)-CH_2, J = 7.3 Hz); 4.34 (1H, d, 17-H, J = 9.6 Hz); 4.62 (1H, q, 18-H, J = 6.9 Hz); 15(1)-CH_2: 5.31 (1H, d, J = 18.3 Hz), 5.57 (1H, d, J = 18.3 Hz); 6.19 (1H, d, 3(2)-$H_{(cis)}$, J = 11.7 Hz); 6.47 (1H, d, 3(2)-$H_{(trans)}$, J = 17.4 Hz); 7.93 (1H, m, 17(3)-NH); 8.34 (1H, dd, 3(1)-H, J = 11.7, 17.4 Hz); 9.14 (1H, s, 20-H); 9.17 (1H, m, 13(1)-NH); 9.77 (1H, s, H-5); 9.79 (1H, s, H-10).

Conjugate 7. To the solution of aminochlorin **2** (300 mg, 0.45 mmol) in 30 mL of THF, 2,3:4,5-Di-O-isopropylidene-1-O-(trifluoromethanesulfonyl)-β-D-fructopyranose **6** (353 mg, 0.90 mmol) and 74 mg (0.90 mmol) of anhydrous CH_3COONa were added. The mixture was refluxed for 6 h (TLC, CCl_4-acetone, 4:1). The mixture was cooled, filtered from excess of sodium acetate and the solvent (THF) was evaporated in vacuo. A residue after the evaporation was diluted with chloroform (100 mL), washed with water (twice in 250 mL), the organic layer was dried over anhydrous Na_2SO_4, and solvent was evaporated in vacuo. A residue was purified by chromatography on silica gel (eluent: CCl_4–acetone, 5:1). Yield: 205 mg (50 %) as a dark green crystalline powder. UV-Vis ($CHCl_3$) λ_{max} nm ($I_{relative}$ (%)): 663 (16), 608 (4), 529 (5), 502 (7), 406 (100). IR (KBr) ν cm^{-1}: 3086 (C–H, vinyl group), 1740 (C=O, ester), 1680 ("amide-I"), 1605 ("chlorin band"), 1537 ("amide-II"). MS (ESI) *m/z*: 909.3 (calcd. for $[M+H]^+$ 909.5). ^{1}H NMR (300 MHz, $CDCl_3$, Me_4Si) δ_H ppm; -1.77 (1H, s, III-NH); -1.56 (1H, s, I-NH); 1.09 (3H, s), 1.42 (3H, s), 1.43 (3H, s), 1.48 (3H, s) (8^a-CH_3, 9^a-CH_3, 11^a-CH_3, 12^a-CH_3); 1.76 (3H, d, 18(1)-CH_3, J = 6.6 Hz); 1.77 (3H, t, 8(2)-CH_3, J = 6.6 Hz); 1.81-1.96 (1H, m), 2.13-2.34 (2H, m), 2.51-2.70 (1H, m) (17(1)-CH_2, 17(2)-CH_2); 3.15-3.32 (3H, m), 3.92-4.04 (2H, m) (13(2)-CH_2, 13(3)-CH_2, 13(3)-NH); 5^a-CH_2: 2.98 (1H, d, J = 12.6 Hz), 3.13 (1H, d, J = 12.3 Hz); 3.37 (3H, s, 7(1)-CH_3); 3.54 (3H, s, 2(1)-CH_3); 3.61 (3H, s, 12(1)-CH_3); 3.66 (3H, s, 17(4)-CH_3); 3.81 (3H, s, 15(3)-CH_3); 6a-CH_2: 3.72 (1H, d, J = 13.2 Hz), 3.86 (1H, d, J = 12.0 Hz); 3.78-3.90 (2H, m, 8(1)-CH_2); 4.15 (1H, d, 17-H, J = 8.1 Hz); 4.31 (1H, d, J = 2.1 Hz), 4.38-4.58 (3H, m) (18-H, 2^a-H, 3^a-H, 4^a-H); 15(1)-CH_2: 5.31 (1H, d, J = 19.2 Hz), 5.63 (1H, d, J = 18.9 Hz); 6.18 (1H, d, 3(2)-$H_{(cis)}$, J = 11.1 Hz); 6.39 (1H, d, 3(2)-$H_{(trans)}$, J = 18.3 Hz); 7.00 (1H, t, 13(1)-NH, J = 4.7 Hz); 8.13 (1H, dd, 3(1)-H, J = 11.7, 17.7 Hz); 8.85 (1H, s, 20-H); 9.69 (1H, s, H-5); 9.73 (1H, s, H-10).

Conjugate ***8***. To the solution of aminochlorin **3** (300 mg, 0.41 mmol) in 30 mL of THF, diisopropylidenefructose triflate **6** (322 mg, 0.82 mmol) and anhydrous CH_3COONa (67 mg, 0.82 mmol) were added. The mixture was refluxed for 6 h (TLC: $CHCl_3$–CH_3OH, 10:1). The reaction mixture was treated similarly to the procedure used for the synthesis of the compound **7**. A residue after evaporation was purified by chromatography on silica gel (eluent: $CHCl_3$-CH_3OH, 5:1). Yield: 217 mg (55 %) as a

dark green crystalline powder. UV-Vis ($CHCl_3$) λ_{max} nm ($I_{relative}$ (%)): 663 (12), 592 (4), 528 (7), 503 (7), 407 (100). IR (KBr) ν cm^{-1}: 3080 (C–H, vinyl group), 1739 (C=O, ester), 1686 ("amide-I"), 1604 ("chlorin band"), 1541 ("amide-II"). MS (ESI) *m/z*: 965.5 (calcd. for $[M+H]^+$ 965.6). ^{1}H NMR (300 MHz, $CDCl_3$, Me_4Si) δ_H ppm: -1.90 (1H, s, III-NH); -1.80 (1H, s, I-NH); 1.32 (3H, s), 1.38 (3H, s), 1.49 (3H, s), 1.52 (3H, s) (8^a-CH_3, 9^a-CH_3, 11^a-CH_3, 12^a-CH_3); 1.67-1.83 (6H, m, 18(1)-CH_3, 8(2)-CH_3); 2.11-2.29 (3H, m), 2.52-2.66 (1H, m) (17(1)-CH_2, 17(2)-CH_2); 1.55-1.61 (4H, m), 2.99-3.17 (2H, m), 3.25-3.36 (2H, m), 3.45-3.70 (7H, m), 3.71-3.91 (4H, m) (13(2)-CH_2, 13(3)-CH_2, 13(4)-CH_2, 13(5)-CH_2, 13(6)-CH_2, 13(7)-CH_2, 13(7)-NH, 5^a-CH_2, 6^a-CH_2, 8(1)-CH_2); 3.31 (3H, s, 7(1)-CH_3); 3.50 (3H, s, 2(1)-CH_3); 3.54 (3H, s, 12(1)-CH_3); 3.64 (3H, s, 17(4)-CH_3); 3.81 (3H, s, 15(3)-CH_3); 4.20 (1H, d, *J* = 7.2 Hz), 4.27 (1H, d, *J* = 2.1 Hz), 4.58 (1H, dd, *J* = 2.1, 8.1 Hz) (2^a-H, 3^a-H, 4^a-H); 4.38 (1H, d, 17-H, *J* = 8.7 Hz); 4.50 (1H, q, 18-H, *J* = 7.1 Hz); 15(1)-CH_2: 5.28 (1H, d, *J* = 19.2 Hz), 5.54 (1H, d, *J* = 19.2 Hz); 6.11 (1H, d, 3(2)-$H_{(cis)}$, *J* = 11.7 Hz); 6.33 (1H, d, 3(2)-$H_{(trans)}$, *J* = 17.4 Hz); 6.66 (1H, t, 13(1)-NH, *J* = 5.3 Hz); 8.07 (1H, dd, 3(1)-H, *J* = 11.4, 18.0 Hz); 8.84 (1H, s, 20-H); 9.64 (1H, s, H-5); 9.69 (1H, s, H-10).

*Conjugate **11***. To the solution of diaminochlorin **4** (350 mg, 0.50 mmol) in 30 mL of THF diisopropylidenefructose triflate **6** (785 mg, 2.00 mmol) and anhydrous CH_3COONa (164 mg, 2.00 mmol) were added. The mixture was refluxed for 12 h (TLC: $CHCl_3$–CH_3OH, 12:1). The reaction mixture was treated similarly to the procedure used for the synthesis of the compound **7**. A residue after the evaporation was purified by chromatography on silica gel (eluent: $CHCl_3$–CH_3OH, 5:1). Yield: 197 mg (33 %) as a dark green crystalline powder. UV-Vis ($CHCl_3$) λ_{max} nm ($I_{relative}$ (%)): 663 (15), 608 (3), 529 (5), 502 (6), 408 (100). IR (KBr) ν cm^{-1}: 3080 (C–H, vinyl group), 1739 (C=O, ester), 1686 ("amide-I"), 1605 ("chlorin band"), 1544 ("amide-II"). MS (ESI) *m/z*: 1179.7 (calcd. for $[M+H]^+$ 1179.6). ^{1}H NMR (300 MHz, $CDCl_3$, Me_4Si) δ_H ppm: -1.80 (1H, s, III-NH); -1.77 (1H, s, I-NH); 0.98 (3H, s), 1.03 (3H, s), 1.15 (3H, s), 1.22 (3H, s), 1.26 (3H, s), 1.41 (3H, s), 1.45 (3H, s), 1.49 (3H, s) (8^a-CH_3, 9^a-CH_3, 11^a-CH_3, 12^a-CH_3, 8^b-CH_3, 9^b-CH_3, 11^b-CH_3, 12^b-CH_3); 1.67-1.81 (6 H, m, 8(2)-CH_3, 18(1)-CH_3); 1.96-2.15 (2H, m), 2.25-2.42 (2H, m) (17(1)-CH_2, 17(2)-CH_2); 2.48-2.75 (4 H, m), 2.90-3.22 (6H, m), 3.41-3.65 (4H, m), 3.70-3.96 (6H, m), 4.07-4.34 (4H, m), 4.42-4.67 (4H, m) (2^a-H, 3^a-H, 4^a-H, 5^a-CH_2, 6^a-CH_2, 2^b-H, 3^b-H, 4^b-H, 5^b-CH_2, 6^b-CH_2, 8(1)-CH_2, 13(2)-CH_2, 13(3)-CH_2, 17(4)-CH_2, 17(5)-CH_2, 13(3)-NH, 17(5)-NH, 17-H, 18-H); 3.35 (3H, s, 7(1)-CH_3); 3.53 (3H, s, 2(1)-CH_3); 3.60 (3H, s, 12(1)-CH_3); 3.79 (3H, s, 15(3)-CH_3); 15(1)-CH_2: 5.36 (1H, d, *J* = 18.3 Hz), 5.53 (1H, d, *J* = 18.3 Hz); 5.90 (1H, m, 17(3)-NH); 6.17 (1H, d, 3(2)-$H_{(cis)}$, *J* = 10.8 Hz); 6.39 (1H, d, 3(2)-$H_{(trans)}$, *J* = 18.3 Hz); 7.26 (1H, m, 13(1)-NH); 8.12 (1H, dd, 3(1)-H, *J* = 11.3, 17.9 Hz); 8.83 (1H, s, 20-H); 9.66 (1H, s, H-5); 9.72 (1H, s, H-10).

*Conjugate **12***. To the solution of diaminochlorin **5** (350 mg, 0.43 mmol) in 30 mL of THF diisopropylidenefructose triflate **6** (506 mg, 1.29 mmol) and anhydrous CH_3COONa (106 mg, 1.29 mmol) were added. The mixture was reflux for 12 h (TLC: $CHCl_3$–CH_3OH, 9:1). The reaction mixture was treated similarly to the procedure used for the synthesis of the compound **7**. A residue after the evaporation was purified by chromatography on silica gel (eluent: $CHCl_3$–CH_3OH, 1:1). Yield: 257 mg (46 %) compound **12** as a dark green crystalline powder. UV-Vis ($CHCl_3$) λ_{max} nm ($I_{relative}$ (%)): 662 (12), 530 (6), 503 (6), 408 (100). IR (KBr) ν cm^{-1}: 3080 (C–H, vinyl group), 1740 (C=O, ester), 1682 ("amide-I"), 1601 ("chlorin band"), 1545 ("amide-II"). MS (ESI) *m/z*: 1291.8 (calcd. for $[M+H]^+$ 1291.8). ^{1}H NMR (300 MHz, $CDCl_3$, Me_4Si) δ_H ppm: -1.77 (1H, s, III-NH); -1.58 (1H, s, I-NH); 1.23-1.57 (6 H, m), 1.59-1.85 (12H, m) (17(4)-CH_2, 17(5)-CH_2, 17(6)-CH_2, 17(7)-CH_2, 17(8)-CH_2, 17(9)-CH_2, 8(2)-CH_3, 18(1)-CH_3); 1.28 (3H, s), 1.30 (3H, s), 1.35 (3H, s), 1.41 (3H, s), 1.44 (3H, s), 1.48 (3H, s), 1.52 (3H, s), 1.55 (3H, s) (8^a-CH_3, 9^a-CH_3, 11^a-CH_3, 12^a-CH_3, 8^b-CH_3, 9^b-CH_3, 11^b-CH_3, 12^b-CH_3); 1.88-2.13 (3H, m), 2.26-2.41 (1H, m) (17(1)-CH_2, 17(2)-CH_2); 2.48-2.96 (12H, m), 3.08 (1H, d, *J* = 12.3 Hz), 3.46-3.60 (1H, m), 3.62-3.98 (8H, m), 4.12-4.21 (4H, m), 4.24 (1H, d, *J* = 8.1 Hz), 4.34 (1H, d, *J* = 1.5 Hz), 4.45-4.56 (3H, m), 4.61 (1H, dd, *J* = 1.5, 8.1 Hz) (2^a-H, 3^a-H, 4^a-H, 5^a-CH_2, 6^a-CH_2, 2^b-H, 3^b-H, 4^b-H, 5^b-CH_2, 6^b-CH_2, 8(1)-CH_2, 13(2)-CH_2, 13(3)-CH_2, 13(4)-CH_2, 13(5)-CH_2, 13(6)-CH_2, 13(7)-CH_2, 13(3)-NH, 17(9)-NH, 17-H, 18-H); 3.34 (3H, s, 7(1)-CH_3); 3.51 (3H, s, 2(1)-CH_3); 3.54 (3H, s, 12(1)-CH_3); 3.80 (3H, s, 15(3)-CH_3); 5.03 (1H, m, 17(3)-NH); 5.41 (2H, s, 15(1)-CH_2); 6.16 (1H, d, 3(2)-$H_{(cis)}$, *J* = 12.0 Hz); 6.37 (1H, d, 3(2)-$H_{(trans)}$, *J* = 17.4 Hz); 6.61 (1H, m, 13(1)-NH); 8.10 (1H, dd, 3(1)-H, *J* = 11.7, 17.4 Hz); 8.34 (1H, s, 20-H); 9.66 (1H, s, H-5); 9.71 (1H, s, H-10).

*Synthesis of compounds **9**, **10**, **13**, **14** (general procedure).* To the 150-170 mg of compounds **7**, **8**, **11**, **12** 5 mL of 90 % aq. trifluoroacetic acid (TFA) was added. The reaction mixture was stirred at room temperature (~23 ºC) for 4 h. Next, excess of the trifluoroacetic acid was evaporated at low pressure. A residue after evaporation was diluted by 5 mL of $CHCl_3$–CH_3OH mixture (20:1), the resulting mixture was purified by chromatography on silica gel (eluent: $CHCl_3$–CH_3OH, 5:1 for compound **9**; $CHCl_3$–CH_3OH, 1:1 for compound **10**; $CHCl_3$–C_2H_5OH (95% aq.), 5:1 for compound **13**, $CHCl_3$–C_2H_5OH (95% aq.), 1:2 for compound **14**).

*Conjugate **9*** was obtained from compound **7** (150 mg, 0.17 mmol) as a dark green crystalline powder (yield: 130 mg, 92 %). UV-Vis (C_2H_5OH 95% aq.) λ_{max} nm ($I_{relative}$ (%)): 662 (31), 606 (3), 527 (3), 500 (9), 399 (100). UV-Vis (H_2O) λ_{max} nm ($I_{relative}$ (%)): 663 (24), 613 (4), 502 (10), 399 (100). IR (KBr) ν cm^{-1}: 3084 (C–H, vinyl group), 1740 (C=O, ester), 1680 ("amide-I"), 1603 ("chlorin band"), 1542 ("amide-II"). MS (ESI) *m/z*: 829.5 (calcd. for $[M+H]^+$ 829.4). ^{1}H NMR (300 MHz, DMSO-d_6, Me_4Si) δ_H ppm: -2.00 (1H, s, III-NH); -1.75 (1H, s, I-NH); 1.67 (3H, t, 8(2)-CH_3, *J* = 6.2 Hz); 1.70 (3H, d, 18(1)-CH_3, *J* = 7.5 Hz); 2.08-2.43 (3H, m), 2.65-2.80 (1H, m) (17(1)-CH_2, 17(2)-CH_2); 3.32 (3H, s, 7(1)-CH_3); 3.53 (3H, s, 2(1)-CH_3); 3.54 (3H, s, 12(1)-CH_3); 3.59 (3H, s, 17(4)-CH_3); 3.72 (3H, s, 15(3)-CH_3); 3.39-3.50 (5H, m), 3.62-3.69 (4H, m), 3.75-4.17 (9H, m) (2^a-H, 3^a-H, 4^a-H, 5^a-CH_2, 6^a-CH_2, 8(1)-CH_2, 13(2)-CH_2, 13(3)-CH_2, 13(3)-NH, 1^a-OH, 2^a-OH, 3^a-OH, 4^a-OH); 4.45 (1H, d, 17-H, *J* = 10.2 Hz); 4.64 (1H, q, 18-H, *J* = 6.9 Hz); 15(1)-CH_2: 5.33 (1H, d, *J* = 19.2 Hz), 5.34 (1H, d, *J* = 18.3 Hz); 6.19 (1H, d, 3(2)-$H_{(cis)}$, *J* = 11.7 Hz); 6.46 (1H, d, 3(2)-$H_{(trans)}$, *J* = 17.4 Hz); 8.32 (1H, dd, 3(1)-H, *J* = = 11.7, 17.7 Hz); 9.13 (1H, s, 20-H); 9.40 (1H, m, 13(1)-NH); 9.75 (1H, s, H-5); 9.81 (1H, s, H-10). ^{13}C NMR (75 MHz, DMSO-d_6, Me_4Si) δ_C ppm: 11.47 (7(1)); 12.20 (2(1)); 12.52 (12(1)); 18.27 (8(2)); 19.03 (8(1)); 23.39 (18(1)); 29.87, 31.01 (17(1), 17(2)); 37.49 (15(1)); 48.58 (18); 51.80 (17(4)); 52.46 (15(3)); 53.06 (17); 64.47, 69.16, 69.57, 70.36, 101.52, 102.09, 115.63, 119.60 (13(2), 13(3), 1a, 2a, 3a, 4a, 5a, 6a); 94.68 (20); 96.24 (5); 98.98 (10); 103.04 (15); 122.56 (3(2)); 129.38 (13); 129.70 (3(1)); 129.79 (12); 130.76 (2); 134.41 (7); 134.47 (4); 135.27 (11); 135.37 (3); 136.71 (1); 138.64 (8); 144.97 (14); 158.21 (9); 158.62 (6); 168.20 (16); 169.02 (19); 170.15 (13(1)); 173.29 (17(3)); 173.68 (15(2)).

*Conjugate **10*** was obtained from compound **8** (150 mg, 0.16 mmol) as a dark green crystalline powder (yield: 136 mg, 95 %). UV-Vis ($CHCl_3$-C_2H_5OH (95% aq.), 1:1) λ_{max} nm ($I_{relative}$ (%)): 662 (30), 606 (3), 526 (3), 500 (9), 399 (100). UV-Vis (H_2O) λ_{max} nm ($I_{relative}$ (%)): 663 (24), 613 (4), 502 (10), 399 (100). IR (KBr) ν cm^{-1}: 3080 (C–H, vinyl group), 1739 (C=O, ester), 1680 ("amide-I"), 1605 ("chlorin band"), 1538 ("amide-II"). MS (ESI) *m/z*: 885.7 (calcd. for $[M+H]^+$ 885.5). ^{1}H NMR (300 MHz, DMSO-d_6, Me_4Si) δ_H ppm: -2.09 (1H, s, III-NH); -1.82 (1H, s, I-NH); 1.40-1.94 (6H, m), 2.89-3.25 (4H, m), 3.25-3.78 (10H, m), 3.79-3.97 (6H, m) (2^a-H, 3^a-H, 4^a-H, 5^a-CH2, 6^a-CH_2, 8(1)-CH_2, 13(2)-CH_2, 13(3)-CH_2, 13(4)-CH_2, 13(5)-CH_2, 13(6)-CH_2, 13(7)-CH_2, 13(3)-NH, 1^a-OH, 2^a-OH, 3^a-OH, 4^a-OH); 1.60-1.74 (6H, m, 8(2)-CH_3, 18(1)-CH_3); 2.07-2.42 (3H, m), 2.63-2.79 (1H, m) (17(1)-CH_2, 17(2)-CH_2); 3.33 (3H, s, 7(1)-CH_3); 3.54 (6H, s, 2(1)-CH_3, 12(1)-CH_3); 3.59 (3H, s, 17(4)-CH_3); 3.70 (3H, s, 15(3)-CH_3); 4.43 (1H, d, 17-H, *J* = 10.2 Hz); 4.65 (1H, m, 18-H); 15(1)-CH_2: 5.36 (1H, d, *J* = 18.3 Hz), 5.56 (1H, d, *J* = 17.7

Hz); 6.18 (1H, d, 3(2)-$H_{(cis)}$, J = 11.1 Hz); 6.45 (1H, d, 3(2)-$H_{(trans)}$, J = 18.3 Hz); 8.32 (1H, dd, 3(1)-H, J = 11.7, 17.7 Hz); 9.15 (1H, s, 20-H); 9.20 (1H, m, 13(1)-NH); 9.77 (1H, s, H-5); 9.81 (1H, s, H-10). ^{13}C NMR (75 MHz, DMSO-d_6, Me_4Si) δ_C ppm: 11.48 (7(1)); 12.19 (2(1)); 12.54 (12(1)); 18.27 (8(2)); 19.42 (8(1)); 23.45 (18(1)); 25.46, 26.35, 26.82, 29.33, 64.37, 69.19, 69.55, 70.29, 101.20, 102.09, 115.51, 119.46 (13(2), 13(3), 13(4), 13(5), 13(6), 13(7), 1a, 2a, 3a, 4a, 5a, 6a); 29.91, 31.05 (17(1), 17(2)); 37.24 (15(1)); 48.55 (18); 51.81 (17(4)); 52.31 (15(3)); 53.15 (17); 94.66 (20); 96.20 (5); 99.00 (10); 103.26 (15); 122.47 (3(2)); 129.84 (13); 129.98 (3(1)); 130.54 (12); 130.70 (2); 134.20 (7); 134.27 (4); 135.21 (11); 135.62 (3); 136.66 (1); 138.41 (8); 144.76 (14); 158.30 (9); 158.72 (6); 168.20 (16); 168.31 (19); 169.83 (13(1)); 173.32 (17(3)); 173.67 (15(2)).

Conjugate ***13*** was obtained from compound **11** (150 mg, 0.13 mmol) as a dark green crystalline powder (yold: 118 mg, 89 %). UV-Vis (H_2O) λ_{max} nm ($I_{relative}$ (%)): 656 (22), 601 (4), 501 (9), 399 (100). IR (KBr) ν cm^{-1}: 3079 (C–H, vinyl group), 1739 (C=O, ester), 1686 ("amide-I"), 1603 ("chlorin band"), 1541 ("amide-II"). MS (ESI) *m/z*: 1019.6 (calcd. for $[M+H]^+$ 1019.5). ^{1}H NMR (300 MHz, DMSO-d_6, Me_4Si) δ_H ppm: -2.02 (1H, s, III-NH); -1.76 (1H, s, I-NH); 1.60-1.76 (6H, m, 8(2)-CH_3, 18(1)-CH_3); 1.94-2.25 (4H, m, 17(1)-CH_2, 17(2)-CH_2); 3.34 (3H, s, 7(1)-CH_3); 3.54 (3H, s, 2(1)-CH_3); 3.55 (3H, s, 12(1)-CH_3); 3.72 (3H, s, 15(3)-CH_3); 2.16-2.90 (6H, m), 3.24-3.80 (23H, m), 3.81-3.96 (8H, m), 3.97-4.10 (2H, m), 4.15 (1H, d, J = 4.5 Hz) (2^a-H, 3^a-H, 4^a-H, 5^a-CH_2, 6^a-CH_2, 8(1)-CH_2, 13(2)-CH_2, 13(3)-CH_2, 13(3)-NH, 1^a-OH, 2^a-OH, 3^a-OH, 4^a-OH, 2^b-H, 3^b-H, 4^b-H, 5^b-CH_2, 6^b-CH_2, 17(4)-CH_2, 17(5)-CH_2, 17(5)-NH, 1^b-OH, 2^b-OH, 3^b-OH, 4^b-OH); 4.42 (1H, d, 17-H, J = 8.7 Hz); 4.62 (1H, q, 18-H, J = 7.5 Hz); 15(1)-CH_2: 5.30 (1H, d, J = 19.2 Hz), 5.51 (1H, d, J = 18.3 Hz); 6.21 (1H, d, 3(2)-$H_{(cis)}$, J = 11.7 Hz); 6.28 (1H, m, 17(3)-NH); 6.48 (1H, d, 3(2)-$H_{(trans)}$, J = 18.3 Hz); 8.35 (1H, dd, 3(1)-H, J = 11.9, 17.6 Hz); 9.15 (1H, s, 20-H); 9.40 (1H, m, 13(1)-NH); 9.77 (1H, s, H-5); 9.81 (1H, s, H-10). ^{13}C NMR (75 MHz, DMSO-d_6; Me_4Si) δ_C ppm: 11.50 (7(1)); 12.22 (2(1)); 12.58 (12(1)); 18.29 (8(2)); 19.41 (8(1)); 23.51 (18(1)); 28.84, 30.72 (17(1), 17(2)); 38.57 (15(1)); 48.82 (18); 52.50 (15(3)); 53.20 (17); 53.76, 56.50, 61.00, 61.14, 64.32, 64.48, 69.08, 69.19, 69.44, 69.55, 70.30, 70.39, 75.84, 75.96, 101.90, 102.90 (13(2), 13(3), 17(4), 17(5), 1a, 2a, 3a, 4a, 5a, 6a, 1b, 2b, 3b, 4b, 5b, 6b); 96.07 (20); 99.01 (5); 101.45 (10); 103.06 (15); 122.58 (3(2)); 129.15 (13); 129.73 (3(1)); 129.84 (12); 130.70 (2); 132.08 (7); 134.38 (4); 135.30 (11); 135.43 (3); 136.75 (1); 138.60 (8); 144.97 (14); 158.29 (9); 158.71 (6); 168.61 (16); 169.18 (19); 170.22 (13(1)); 173.17 (17(3)); 173.27 (15(2)).

Conjugate ***14*** was obtained from compound **12** (170 mg, 0.13 mmol) as a dark green crystalline powder (yield: 133 mg, 90 %). UV-Vis (H_2O) λ_{max} nm ($I_{relative}$ (%)): 657 (22), 602 (5), 501 (10), 400 (100). IR (KBr) ν cm^{-1}: 3083 (C–H, vinyl group), 1739 (C=O, ester), 1686 ("amide-I"), 1601 ("chlorin band"), 1542 ("amide-II"). MS (ESI) *m/z*: 1131.7 (calcd. for $[M+H]^+$ 1131.6). ^{1}H NMR (300 MHz, DMSO-d_6, Me_4Si) δ_H ppm: -2.11 (1H, s, III-NH); -1.81 (1H, s, I-NH); 1.34-1.62 (6 H, m), 1.62-1.74 (12H, m) (17(4)-CH_2, 17(5)-CH_2, 17(6)-CH_2, 17(7)-CH_2, 17(8)-CH_2, 17(9)-CH_2, 8(2)-CH_3, 18(1)-CH_3); 1.75-1.90 (2H, m), 2.02-2.22 (2H, m) (17(1)-CH_2, 17(2)-CH_2); 2.82-3.21 (10H, m), 3.27-3.75 (12H, m), 3.76-4.05 (8H, m), 4.15 (1H, m, J = 3.6 Hz), 4.54-5.01 (3H, m), 5.39-5.67 (2H, m), 5.90-6.10 (1H, m), 6.64-6.81 (1H, m) (2^a-H, 3^a-H, 4^a-H, 5^a-CH_2, 6^a-CH_2, 2^b-H, 3^b-H, 4^b-H, 5^b-CH_2, 6^b-CH_2, 8(1)-CH_2, 13(2)-CH_2, 13(3)-CH_2, 13(4)-CH_2, 13(5)-CH_2, 13(6)-CH_2, 13(7)-CH_2, 13(3)-NH, 17(9)-NH, 2^a-OH, 3^a-OH, 4^a-OH, 5^a-OH, 2^b-OH, 3^b-OH, 4^b-OH, 5^b-OH); 3.33 (3H, s, 7(1)-CH_3); 3.53 (3H, s, 2(1)-CH_3); 3.55 (3H, s, 12(1)-CH_3); 3.70 (3H, s, 15(3)-CH_3); 4.34 (1H, d, 17-H, J = 8.7 Hz); 4.63 (1H, m, 18-H); 15(1)-CH_2: 5.31 (1H, d, J = 18.3 Hz), 5.57 (1H, d, J = 16.8 Hz); 6.19 (1H, d, 3(2)-$H_{(cis)}$, J = 10.8 Hz); 6.46 (1H, d, 3(2)-$H_{(trans)}$, J = 17.7 Hz); 7.80 (1H, m, 17(3)-NH); 8.33 (1H, dd, 3(1)-H, J = 11.7, 17.7 Hz); 9.14 (1H, s, 20-H); 9.20 (1H, m, 13(1)-NH); 9.77 (1H, s, H-5); 9.80 (1H, s, H-10). ^{13}C NMR (75 MHz, DMSO-d_6, Me_4Si) δ_C ppm: 11.26 (7(1)); 11.48 (2(1)); 12.16 (12(1)); 18.29 (8(2)); 19.44 (8(1)); 22.85 (18(1)); 23.52, 23.73, 25.28, 25.47, 26.18, 26.37, 26.45, 26.84, 28.83, 29.36 (2C), 30.28, 61.01, 67.91, 70.27, 76.00, 79.68, 82.27, 83.31, 96.23, 100.04, 102.13, 115.79, 119.76 (13(2), 13(3), 13(4), 13(5), 13(6), 13(7), 17(4), 17(5), 17(6), 17(7), 17(8), 17(9), 1a, 2a, 3a, 4a, 5a, 6a, 1b, 2 b, 3 b, 4 b, 5 b, 6 b); 31.01, 32.92 (17(1), 17(2)); 37.18 (15(1)); 48.74 (18); 52.27 (15(3)); 53.31 (17); 94.62 (20); 98.99 (5); 101.06 (10); 103.20 (15); 122.41 (3(2)); 129.13 (13); 129.87 (3(1)); 129.99 (12); 130.39 (2); 134.05 (7); 134.17 (4); 135.17 (11); 135.62 (3); 136.71 (1); 138.27 (8); 144.76 (14); 148.94 (9); 153.30 (6); 164.47 (16); 168.96 (19); 169.84 (13(1)); 172.16 (17(3)); 173.27 (15(2)).

Dark and photoinduced cytotoxicity

In this work, we used human cervical cancer cell line (HeLa) (BioloT, Russia), pulmonary adenocarcinoma (A549) (BioloT, Russia) and human colon adenocarcinoma (HT-29) (BioloT, Russia). The cells were cultured in a growth medium DMEM/F12 (PanEco, Russia) containing 10% of fetal bovine serum (FBS) (HyClone, USA), and 1% L-glutamine (PanEco, Russia) without antibiotics at 37 °C and 5% CO_2. Investigation of dark and photoinduced cytotoxic activity was carried out according to the procedure described by Pylina *et al*.[33] The experiment was performed in six biological replicates. Survival index was assessed by the FMCA method as described by Lindhagen *et al*.[34] The statistical differences were estimated by Student t-test.

Results and Discussion

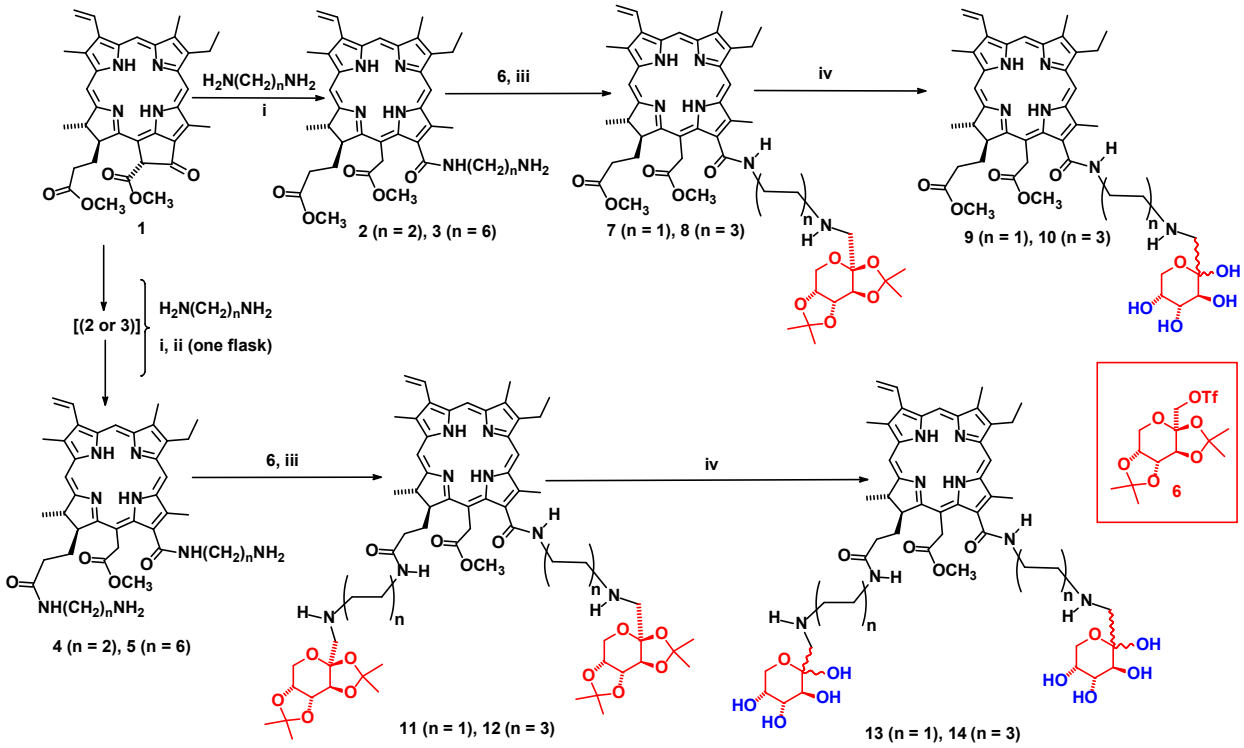
The alkylation of the amino groups of chlorin e_6 derivatives **2-5** with diisopropylidene fructose triflate **6** (Scheme) was used in this work for the insertion of fructose fragments to the periphery of the chlorin macrocycle. The presence of one or two amino groups in the molecules of chlorophyll derivatives makes it possible to vary the amount of introduced carbohydrate fragments. The compounds **2-5** were synthesized on the basis of methylpheophorbide *a* **1**. The mono-derivatives of chlorin e_6 **2** and **3** with an amino group removed from the macrocycle by spacers of various lengths, were synthesized by the action of ethylenediamine ore hexamethylenediamine on methylpheophorbide *a* **1** according to the methods developed by us earlier.[27-31] The insertion of the second amino group was carried out by amidation of the ester group at position 17 of the 13-amide derivative **2** or **3**, which is formed as a result of the methylpheophorbide *a* **1** exocycle recovering. This reaction was described earlier for ethylenediamine, opening and amidation can be carried out "in one flask".[29] A similar reaction for hexamethylene-diamine (HMDA) has not been described in the literature and was carried out for the first time in the present work. The amidation with hexamethylenediamine has some features associated with the fact that HMDA is a solid under normal conditions. While the reaction with ethylene-diamine occurs at room temperature, amidation with hexamethylenediamine requires heating to 42 °C and the formation of a melt in which the reaction occurs. Alkylation of amino chlorins **2-5** with a 2,3:4,5-di-O-isopropylidene-1-O-(trifluoromethanesulfonyl)-β-D-fructo-pyranose **6** allows to insert one diisopropylidene-fructose fragments into each amino group and thus to obtain chlorins with one (compounds **7** and **8**) and two (compounds **11** and **12**) carbohydrate fragments (Scheme 1). After removing the

isopropylidene protection by the common method (hydrolysis in 90% aq. TFA), hydrophilic chlorin derivatives containing one (compounds **9** and **10**) or two (compounds **13** and **14**) fructose fragments connected to the macrocycle by a spacer of two and six methylene groups were obtained (Scheme 1).

The structure of the synthesized new compounds was established on the basis of data of NMR, IR and UV-vis spectroscopy, as well as mass spectrometry. Mass spectra (ESI) of the aminochlorins **2-5** alkylation products of with triflate derivatives **6** (compounds **7**, **8**, **11** and **12**), as well as conjugates with fructose **9**, **10**, **13** and **14**, peaks show peaks, the *m/z* values of which correspond to protonated molecular ions with structures of compounds **7-14**. In the UV-vis spectra of all *de novo* synthesized compounds, absorption bands characteristic of the chlorin chromophore are observed. In the ^{1}H and ^{13}C NMR spectra of conjugates **7**, **8**, **11**, **12**, the signals of the chlorin macrocycle and fragments of diisopropylidene-fructose are observed. The ratio of the intensities of the signals of protons of the chlorin macrocycle and carbohydrate fragments in the ^{1}H NMR spectra corresponding to the presence of one (for compounds **7** and **8**) and two (compounds **11** and **12**) carbohydrate fragments in the molecule, which correlates with the data of mass spectrometry. The presence in the spectra of ^{1}H NMR of chlorins **7**, **8**, **11** and **12** singlet signals that correspond, judging by the integral intensity, to the methyl groups of the diisopropylidene protection of one (in compound **7** and **8**) and two (in compound **11** and **12**) fragments indicates that the reaction is selective and the isopropylidene protection at the conditions of its carrying out is persistent. In the ^{1}H and ^{13}C NMR spectra of compounds **9**, **10**, **13** and **14**, there are no signals corresponding to protons and carbon atoms of the diisopropylidene protection, whereas the signals of protons of hydroxyl groups formed after removal of diisopropylidene fragments are observed, as well as the signals of chlorin macrocycle and fragments of fructose, which confirms the complete removal of the protection.

As already noted, the presence of fructose fragments increases the solubility of porphyrin compounds in water. We obtained preliminary data on the solubility of compounds **9**, **10**, **13** and **14** in water. When dissolved in water, compounds **9**, **10**, **13** and **14** generate fluorescent in UV light solutions, which indicates the presence of single molecules in the aqueous phase, that is, the formation of a true solution. For compounds **9**, **13** and **14**, it is possible to obtain a solution containing at least 0.2 mg/mL without the use of auxiliary substances, which indicates the possibility of obtaining water-soluble forms.

To evaluate the dark and photoinduced (LED-irradiated at a wavelength of 660 nm and light exposure value of 12 J/cm^2) cytotoxicities of compounds **9**, **10**, **13** and **14** towards HeLa, A549 and HT-29 cells, the survival values were determined at three different concentrations of 0.1, 1.0, and 10.0 μM as in our previous studies (Figure).[33] It should be noted that a significant decrease in survival occurs with photoinduced exposure of tumor cells to the studied compounds at concentrations of 1 and 10 μM, with the exception of compound **14**, as a result of photoinduced exposure of which at a concentration of 1 μM, more than 50% of cells survive. When cells are treated with compounds **9**, **10**, **13** and **14** at concentrations of 1 and 10 μM for 72 h without exposure to light, more than 60% of tumor cells survive. More than 80% of cells survive under dark and photodynamic exposure of compounds **9**, **10**, **13** and **14** at a concentration of 0.1 μmol/L. Thus, all conjugates synthesized exhibit a pronounced photodynamic effect at those concentrations at which they do not have a dark cytotoxic effect.



Scheme 1. (i) $CHCl_3$, 23 ºC, 3-5 h; (ii) 23 ºC, 20 h for **4**, 42ºC, 8 h for **5**; (iii) THF, NaOAc, reflux, 6-12 h; (iv) TFA (90%-H_2O), 23 ºC, 4 h.

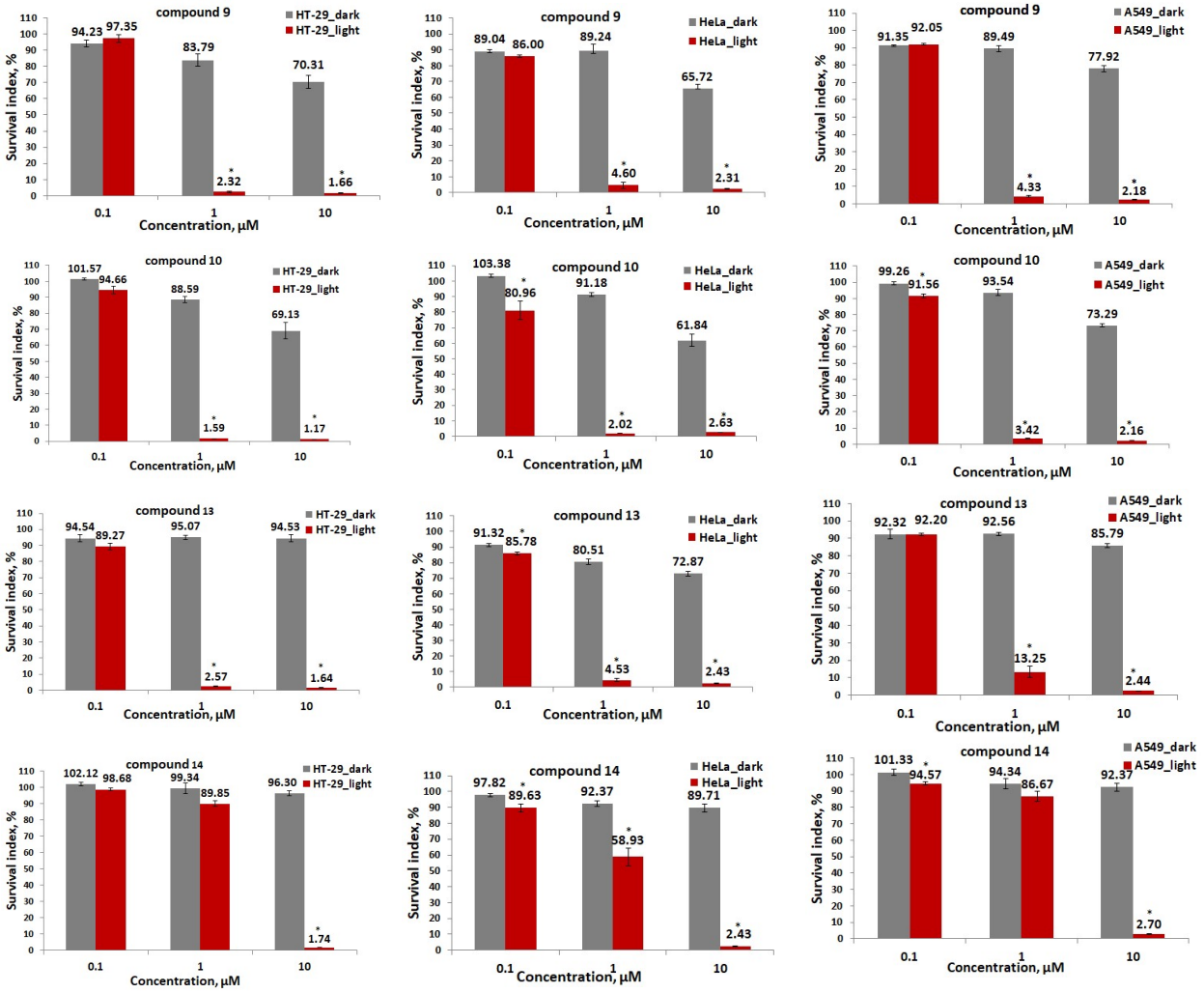


Figure 1. Survival rate of HeLa, A549 and HT-29 cell lines estimated with FMCA after photoinduced and dark exposure to test compounds (average value obtained using 6 separately treated microcultures are presented with SEM). *– difference from control (dark exposure) is significant at $p < 0.05$ (Student's t-test).

Conclusions

Thus, here we synthesized new hydrophilic conjugates of chlorin e_6 with fructose by alkylation of the amino groups of chlorin e_6 amides with one and two ethylene diamine or hexamethylene diamine fragments on the macrocycle periphery with triflate of 2,3:4,5-di-O-isopropylidene-β-D-fructopyranose, followed by removal of diisopropylidene protection by 70% aqueous trifluoroacetic acid. In all cases, monoalkylation occurs, which allows the chemoselective insertion of one fructose fragment for each amino group present in the start chlorin. For most compounds studied, it is possible to obtain a solution containing at least 0.2 mg/mL without the use of auxiliary substances, which indicates the possibility of obtaining water-soluble forms. The synthesized conjugates were shown to exhibit pronounced photodynamic activity at concentrations at which dark cytotoxic effect does not observe (HeLa, A549, HT-29 cancer cell cultures were used as test objects).

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