NOVEL CHLORINE *E6* CONJUGATE WITH DUAL TARGETING TO CANCER CELLS

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Abstract. Photodynamic therapy (PDT) has been successfully used to treat many types of tumors. However, the widespread use of PDT is limited by a number of factors, including low selectivity of photosensitizer (PS) accumulation in tumor tissue. We have synthesized the novel third-generation photosensitizer, conjugate of zinc complex of chlorine *e6* with maltose and biotin (Chl-Mal-B_7). The introduction of maltose and biotin is intended to provide high selectivity to tumor cells often characterized by high-level expression of receptors for these molecules. It was shown that Chl-Mal-B_7 intensively absorbs light and fluoresces in a far-red spectral region with a quantum yield of about 10%. Chl-Mal-B_7 demonstrated photoinduced toxicity in submicromolar concentrations against cancer cells that is several times more effective compared to nonmalignant cells.

Keywords: photodynamic therapy, photosensitizer, chlorin *e6*, glycoconjugate, biotin, targeting to tumor.

List of Abbreviations

PDT – photodynamic therapy
PS – photosensitizers
ROS – reactive oxygen species
IC50 – half-maximal inhibitory concentration

Introduction

Photodynamic therapy (PDT) is an actively developing therapeutic approach for treatment of oncological diseases. The main components of PDT are a photosensitizer (PS), light in the range of 600–800 nm ("tissue transparency window") and molecular oxygen (O_2) (Agostinis *et al*., 2011). Depending on the tumor localization, the PS is administered intravenously or topically. The driving factor of the treatment is the absorbed light energy, so, the local irradiation ensures the locality of the therapeutic effect and protects other organs and tissues. Light irradiation is performed after a certain drug-to-light interval established taking into account the pharmacokinetic characteristics of the PS (Zhu & Finlay, 2008). As a result of the absorption of photon, the excited PS molecule can enter two types of photochemical reactions. The type I reactions involve PS interaction with organic molecules or O_2 with electron transfer. These reactions produce various reactive oxygen species (ROS). The type II reactions are most common and are characterized by the triplet-triplet interaction of PS with molecular oxygen with direct energy transfer and formation of singlet $^1O_2$. Radical ROS and $^1O_2$ can directly react with many biological molecules, initiate chain reactions of radical oxidation and eventually lead to death of the irradiated cell (Castano *et al*., 2004; Mishchenko *et al*., 2022; Otvagin *et al*., 2022).

The effectiveness of PDT is mostly determined by photochemical and photophysical characteristics of the PS. The most of PSs approved for clinical use are of tetrapyrrole structure, and are classified into three generations (Kou *et al*., 2017). Traditionally, the first generation of PSs includes hematoporphyrin derivative (HpD) and photofrin II (a purified form of HpD). Significant disadvantages of these PSs are absorption in the range of 620–640 nm, undefined chemical composition; insufficient selectivity; and high dark toxicity. The second generation of PSs includes synthetic and modified natural chlorins (Volovetsky *et al*., 2017; Alzeibak *et al*., 2021), porphyrazines (Lermontova *et al*., 2019), bacteriochlorins (Takamitsua *et al*., 2004), phthalocyanines (Brilkina *et al*.,

2019) and similar compounds. These compounds absorb in the longer spectral region and are characterized by a higher quantum yield of $^1O_2$ (Plekhova *et al*., 2022).

Despite significant progress, insufficient selectivity of PS accumulation in tumors remains a factor limiting the wider application of PDT in clinical practice. In order to solve this problem, the development of third-generation PSs with targeted delivery to cancer cells is currently underway (Mehraban & Freeman, 2015; Kutova *et al*., 2019). One of the promising approaches is the conjugation of a second-generation PS with a targeting moiety (Mfouo-Tynga *et al*., 2021). As a latter, different agents can be used, including antibodies and their fragments, lectins, peptides, lipoproteins, hormones, mono- and polysaccharides, vitamins and low molecular weight ligands for receptors overexpressed on cancer cells (Torchilin, 2010).

In this work we have synthesized a novel targeted photosensitizer, namely a conjugate of zinc complex of chlorine *e6* with maltose and biotin. Simultaneous administration of two types of targeting molecules is intended to provide selective interaction with cancer cells. We have studied the optical properties of the obtained conjugate and its photodynamic activity against human cancer cells.

Materials and Methods

Synthesis of chlorine e6 derivative Chl-Mal-B$_7$

A Schlenk flask was filled with argon, and charged with EDC·HCl (0.058 g, 0.302 mmol) and biotin, or vitamin B_7 (0.074 g, 0.302 mmol). Anhydrous dimethylformamide (DMF; 3 mL) was added, and the mixture was stirred for 30 min at 0°C. Another Schlenk flask filled with argon was charged with chlorin derivative synthesized previously in our group (Otvagin *et al*., 2021) (0.130 g, 0.151 mmol), 4-dimethylaminopyridine (DMAP; 0.009 g, 0.074 mmol) and anhydrous DMF (3 mL). The mixture from the second flask was transferred by a syringe into the first flask. The reaction mixture was stirred at 0 °C for 3h, at room temperature for 15h, and concentrated under reduced pressure. The residue was dissolved in $CHCl_3$ (100 mL), washed with H_2O (3 × 100 mL), dried (Na_2SO_4), and concentrated. Then, obtained green powder was added to maltose azide (0.028 g, 0.076 mmol) dissolved in 6 mL DMF in the first flask. In another flask, $CuSO_4·5H_2O$ (0.003 g, 0.012 mmol), tris(benzyltriazolylmethyl)amine (TBTA; 0.007 g, 0.013 mmol), sodium ascorbate (AscNa; 0.005 g, 0.026 mmol) were dissolved in 6 mL H2O, and this mixture was added immediately into the DMF solution in the first flask. The reaction was stirred for 1.5h at 50 °C, cooled, solvent was evaporated under vacuum. Then, column chromatography separation was performed using Macherey-Nagel Kieselgel 60 (70–230 mesh). After column chromatography ($CHCl_3$/MeOH 80:20 to 60:40), deep-green solid was obtained (0.099 g, 0.069 mmol, 45%).

Characterization of Chl-Mal-B$_7$

^{1}H NMR and ^{13}C NMR spectra were recorded on Agilent DD2 400 MHz spectrometer. Chemical shifts (δ) are reported in ppm for the solution of compound in DMSO-d_6, with the residual peak of solvent as an internal reference, *J* values in Hertz. ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.51 (s, 1H), 9.50 (s, 1H), 8.84 (s, 1H), 8.65 (s, 1H), 8.34 (s, 1H), 8.22 (dd, *J* = = 17.8, 11.6 Hz, 1H), 8.00 (s, 1H), 7.88 (t, *J* = = 5.5 Hz, 1H), 6.35 (d, *J* = 19.2 Hz, 3H), 6.21 (d, *J* = 17.9 Hz, 1H), 5.99 (d, *J* = 11.6 Hz, 1H), 5.72 (s, 1H), 5.62 – 5.38 (m, 4H), 5.09 (d, *J* = = 18.6 Hz, 1H), 5.06 – 5.00 (m, 1H), 4.98 – 4.90 (m, 2H), 4.61 – 4.49 (m, 2H), 4.42 (d, *J* = 7.0 Hz, 1H), 4.38 – 4.18 (m, 4H), 4.05 (s, 1H), 3.89 – 3.39 (m, 28H), 3.14 – 2.97 (m, 2H), 2.77 (dd, *J* = 12.4, 5.0 Hz, 1H), 1.66 (t, *J* = = 7.5 Hz, 3H), 1.59 (d, *J* = 7.0 Hz, 3H). 13C NMR (101 MHz, DMSO-d_6): δ 173.32, 172.16, 171.91, 170.11, 165.06, 162.74, 162.66, 151.52, 148.14, 145.96, 144.82, 143.90, 143.11, 141.39, 140.62, 138.62, 137.13, 133.90, 132.97, 132.16, 130.69, 129.50, 121.85, 119.40, 101.94, 101.24, 100.90, 99.86, 93.08, 87.05, 79.10, 77.91, 76.64, 73.52, 73.21, 72.40, 71.51, 69.84, 69.71, 69.66, 69.26, 68.95, 60.99, 60.73, 60.23, 59.15, 55.38, 52.21, 51.62, 46.34, 37.11, 35.10, 34.07, 32.12, 30.30, 28.18, 28.01, 25.26, 22.87, 18.88, 17.90, 12.31, 11.64, 10.93.

Mass spectra were recorded using the MALDI method on a time-of-flight Bruker Microflex LT mass-spectrometer. TLC analyses were carried out on Merck TLC Silica gel 60 F254. MALDI-TOF: calculated for $C_{66}H_{88}N_{12}O_{18}ZnS$ $[M+H]^+$ m/z 1435.5; found m/z 1435.4.

Photophysical Measurements

Absorption and fluorescence spectra were recorded for 5 μM Chl-Mal-B_7 solution with a Synergy MX spectrophotometer-spectrofluorometer (BioTek). The molar extinction coefficient ε was determined using the following equation:

$$\varepsilon = D/\mathrm{cl}, \quad (1)$$

where D is optical density, l is path length, and c is concentration.

The fluorescence quantum yield φ_1 was calculated using the equation:

$$\varphi_1 = \frac{\varphi_2 F_1 D_2}{F_2 D_1}, \quad (2)$$

where F_1 and D_1 are the integral fluorescence intensity and optical density of the Chl-Mal-B_7, respectively; φ_2 is the quantum yield of rhodamine B (Sigma, USA) in water (0.31); and F_2 and D_2 are the integral fluorescence intensity and optical density of rhodamine B, respectively. The fluorescence was excited at 410 nm, and the optical density was measured at the same wavelength. The fluorescence signal was detected at 550−850 nm.

Culturing of cell lines

The experiments were performed on cell line on human epidermoid carcinoma A431 and Chinese hamster ovarian cell line CHO.

Cells were cultured in Eagle MEM medium (PanEco, Russia) with 10% (v/v) fetal calf serum (HyClone, USA) and 2 mM L-glutamine in 5% CO_2 at 37 °C. At each passaging stage, the cells were treated with Trypsin-EDTA (1:1) solution (PanEco, Russia).

Analysis of Cellular Uptake of Chl-Mal-B_7

Cells were seeded in glass bottom 96-well plates (Corning, USA) at the density of 5×10^3 cells per cell followed by 24h incubation in 5% CO_2 at 37 °C. The medium was then exchanged with fresh serum-free growth medium containing 5 μM of a tested compound; and the cells were incubated for 4 hours. At the end of the incubation, the cells were washed three times from the photosensitizer with 10 mM phosphate buffered saline (PBS) and fixed with 4% formaldehyde solution for 30 minutes.

The cells were imaged using a laser scanning confocal microscope Axio Observer Z1 LSM 710 NLO/Duo (Carl Zeiss, Germany) equipped with C-Apochromat 63× water immersion objective lens with numerical aperture of 1.2. Fluorescence was excited at 405 nm and registered in the range of 600–740 nm.

Fluorescence intensity of the cytoplasmic region of the cells was measured using ZEN 2012 program (Carl Zeiss, Germany); at least 10 cells in two-three fields of view were analyzed.

Study of Photoinduced Cytotoxicity

Cells were seeded in 96-well plates at the density of 4×10^3 cells per well followed by 24h incubation. The medium was then exchanged with fresh serum-free growth medium containing tested compound in different concentrations. After 4 hours, the medium was exchanged with full fresh growth medium.

To estimate the photoinduced toxicity of the tested compound, the cells were exposed to light irradiation using LED light source providing a homogeneous light distribution in 96-well plates (Shilyagina *et al*., 2014) under temperature controlled conditions (37 °C) on TermoStat plus (Eppendorf, Germany). The irradiation dose was of 20 J/cm^2 in the spectral range of 655–675 nm with power density of 32 mW/cm^2 and the total exposure time of 10 min 25 sec.

The viability of cell cultures was assessed 24 hours after irradiation using the microculture tetrazolium assay (MTT assay). The cells were incubated with serum-free medium containing 0.5 mg/mL MTT reagent (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazole bromide; Alfa Aesar, UK) for 4 hours. Then, the formazan formed from the reduction of MTT by cells' dehydrogenases was dissolved in dimethyl sulfoxide (DMSO), and the absorbance was measured at 570 nm with a Synergy MX plate reader (BioTeck, USA).

The same procedure was performed for the estimation of the dark toxicity of the compound, except for that there was no cells exposure to LED light.

Cell viability was expressed as the ratio of the optical density of treated and untreated cells (in percentage). Data analysis and calculation of half-inhibition concentration IC_{50} was performed using the GraphPad Prism 6 software (Software Inc., San Diego CA) and a four-parameter model for the log-normal distribution.

Results

A novel third generation water-soluble PS, named Chl-Mal-B_7, was synthesized and studied. This agent is a conjugate of zinc complex of chlorine *e6* with maltose and biotin (Fig. 1). Chlorin derivative synthesized previously in our group was used as a precursor for a photosensitizing moiety (Otvagin *et al.*, 2021). Its functionalization included two steps, where conjugation with biotin was achieved under a Steglich-type amidation reaction (El-Faham & Albericio, 2011). Then, dipolar cycloaddition of resulted alkyne and maltose azide catalyzed by CuSO4/AscNa catalytic system in the presence of TBTA ligand (Otvagin *et al.*, 2018) allowed us to synthesize targeted molecule Chl-Mal-B_7 with 45% yield.

The synthesized compound Chl-Mal-B_7 has two absorption maxima in the short-wavelength (Soret band) and the long-wavelength (Q-band) spectral regions (Fig. 2). The log of molar extinction coefficient (log ε) is 4.6 and 4.1, respectively. More intense light absorption registered for Chl-Mal-B_7 in the short-wavelength region is typical for all chlorin PSs (Ferreira *et al.*, 2008); it confirms the preservation of the photophysical properties of the chlorin structure in the Chl-Mal-B_7 conjugate.

Chl-Mal-B_7 demonstrates fluorescence in far-red spectral region with maximum at 646 nm (Fig. 2, Table 1). It has been previously reported that the fluorescence of chlorin *e6* in aqueous solutions at pH 7.8 has a peak at 650 nm (Zhang & Görner, 2009). Therefore, conjugation with two targeted moieties lead to a slight effect on fluorescent properties. Chl-Mal-B_7 has a high value of fluorescence quantum yields (10.2%), which make the compound promising for application in photodiagnosis.

To study the Chl-Mal-B_7 cellular uptake, the PSs was added to the culture medium of A431 and CHO cells at concentration of 5 μM. Chl-Mal-B_7 was rapidly accumulated by cells within 4h and localized primarily in cell cytoplasm with weak penetration into the nuclei (Fig. 3). We assume that presence of a large hydrophobic macrocyclic group in the Chl-Mal-B_7 molecule is a reason for the rapid redistribution between the medium and cellular structures and can promote its accumulation in intracellular membranes. Thus, Chl-Mal-B_7 combines the properties of water-solubility due to the introduced hydrophilic substituents (maltose and biotin) and lipophilicity. The latter property is favorable for the manifestation of photodynamic properties, since membranes are commonly considered as the most sensitive primary targets of PDT.

To assess the photodynamic potential of Chl-Mal-B_7, an MTT assay was performed on a cancer cell culture (A431) and a non-malignant cell culture (CHO). The compound demonstrated no toxicity in the dark against CHO at concentrations up to 100 μM, however, it showed slight dark toxicity towards A431 with IC_{50} in the dark about 24 μM (Fig. 4, Table 2).

Irradiation of cells pre-treated with Chl-Mal-B_7 at a dose of 20 J/cm^2 resulted in pronounced photoinduced cell death. However, different degrees of sensitivity of cell cultures were found. Photodynamic activity of Chl-Mal-B_7 is more than 7 times higher against malignant cells: IC_{50} was about 0.6 μM for A431 compared to 4.5 μM for CHO.

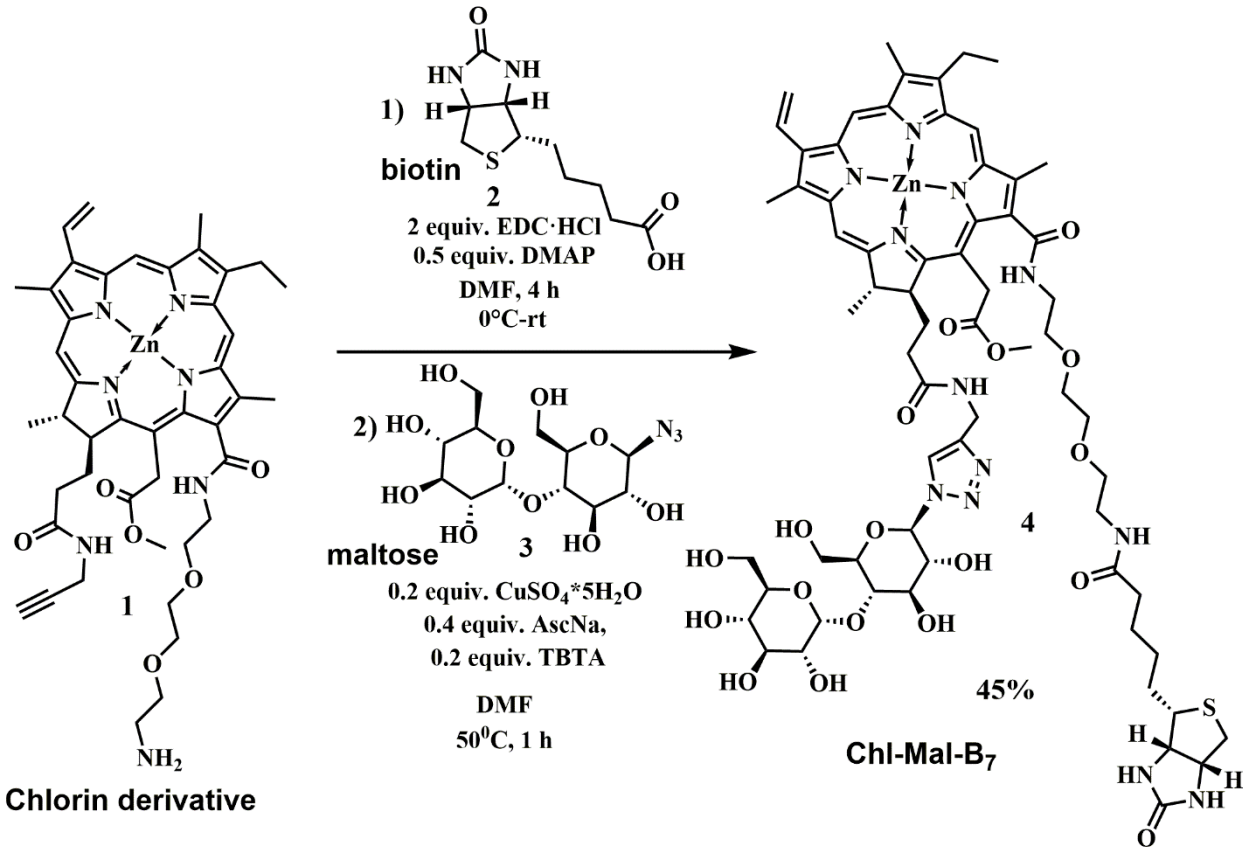

Fig. 1. Synthesis of chlorin e_6 conjugate Chl-Mal-B_7

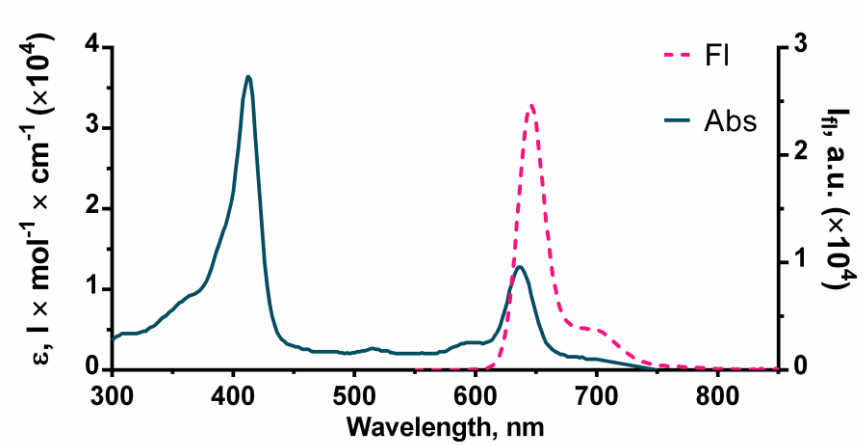

Fig. 2. Absorption and fluorescence (λ_{ex} 410 nm) spectra for 5 μM aqueous solution of Chl-Mal-B_7

Table 1

Photophysical characterization of Chl-Mal-B_7

λ_{abs} (nm)/log ε		λ_{em} (nm)	Φ_F (%)
Soret band	Q-band		
412 / 4.6	636 / 4.1	646	10.2

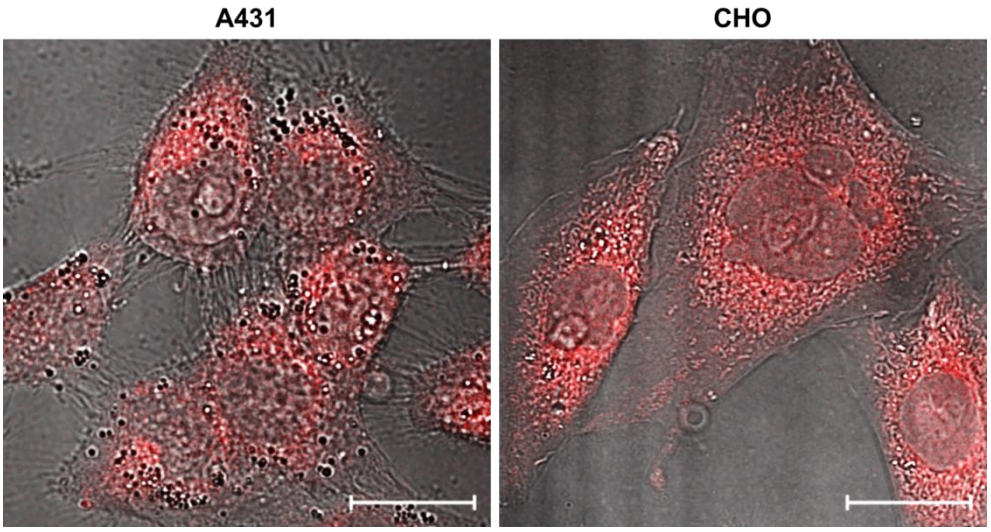

Fig. 3. Confocal images of the fixed cells after 4h incubation in serum-free growth medium containing 5 μM of Chl-Mal-B_7, the merged images in transmitted light and red fluorescence are shown, bar is 20 μm

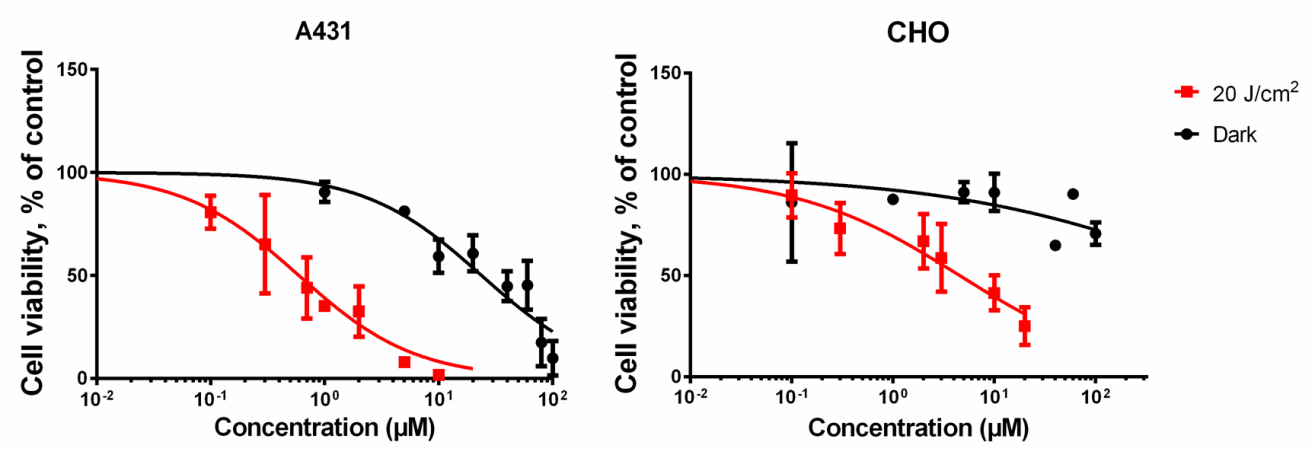

Fig. 4. Relative viability of A431 and CHO cells treated with Chl-Mal-B_7 in dark or under light exposure

Table 2

Cytotoxic activity of Chl-Mal-B_7

Cell culture	$IC_{50\ dark}$, μM	$IC_{50\ light}$, μM
A431	23.8 [16.74 to 34.05]	0.6 [0.4 to 0.9]
CHO	>100	4.5 [2.6 to 7.8]

Discussion

Development of new approaches to increase the selectivity of drugs accumulation in tumor tissue is among the main research trends in anticancer drug discovery. In PDT, the most common targeting strategy is based on PS conjugation with a ligand specifically interacting with molecules overexpressed on the surface of tumor cells. This approach suggests an increase in intracellular PS delivery through receptor-me-

diated endocytosis (Martin-Saldaña *et al*., 2022).

In our work, a third-generation PS was synthesized, which is a novel conjugate of zinc complex of chlorine *e6* with two targeting ligand, maltose and biotin.

The saccharide-PS conjugation strategy is by far the most widely used. Malignant cells are characterized by increased consumption of glucose due to increased activity of glycolysis (Warburg effect) (Liberti & Locasale, 2016). For many types of tumors, this metabolic state of cells is accompanied by overexpression of glucose transporters. In particular, overexpression of GLUT1, GLUT3, and GLUT12 is reported by many researchers; it has been shown that the expression of these transporters in cancer cells is 10-12 times higher than in normal cells (Kunkel *et al*., 2003; Young *et al*., 2011; Barron *et al*., 2016).

Another distinctive feature of transformed cells is aberrant glycosylation, which promotes increased expression of lectins, in particular galectins. These are glycan-binding proteins that can specifically bind to sugar residues, triggering a cascade of molecular events in the cell (Liu & Rabinovich, 2005). There are a number of reports on an increase in the selectivity of glycosylated PSs towards cancer cells. The conjugation of PSs with glucose (Kuzmina *et al*., 2020), galactose (Dukh *et al*., 2022), maltose (Narumi *et al*., 2021) and others improves the hydrophilic properties of the molecules, and increases the selectivity towards tumor cells in *in vitro* and *in vivo* studies (Otvagin *et al*., 2018; Otvagin *et al*., 2019;).

Due to the heterogeneous nature of the tumor, single targeting strategy may have limited success in clinical practice. The concept of simultaneous targeting multiple tumor markers is proposed to improve therapeutic efficacy by targeting multiple tumor cell subtypes including tumor-associated stromal cells. This approach demonstrated a certain success, for example, in the development of dual-targeting liposomes (Huang *et al*., 2020; Lu *et al*., 2019).

The second targeting moiety used in our work is biotin, which is also a promising ligand for active drug targeting of cancer cells (Russell-Jones *et al*., 2004; Ren *et al*., 2015). Biotin plays an important role in cell growth and proliferation as well as in cell signaling. Actively proliferating cancer cells are characterized by increased consumption of biotin and a high level of expression of biotin-specific transporters (Sodium Dependent Multivitamin Transporter) (Vadlapudi *et al*., 2012). Recently published works have experimentally evidenced an increase in the uptake of biotin conjugates of organic molecules (Maiti & Paira, 2018) and in particular PS (Liu *et al*., 2021) by cancer cells. Biotin has also been shown to increase selectivity and water solubility in various nanosize drug delivery systems (Park *et al*., 2015).

To conclude, the synthesized novel conjugate of zinc complex of chlorine *e6* with maltose and biotin, Chl-Mal-B_7, possesses a combination of properties that make it a promising candidate for PDT of tumors. The absorption of far-red light by Chl-Mal-B_7 and the fluorescence with quantum yield of about 10% allow the use of the compound for photodiagnosis to clarify the localization and size of the tumor focus. High photoinduced toxicity against cancer cells confirms its potency as a photosensitizer. Moreover, the presence of dual targeting to cancer cells is intended to ensure high selectivity of tissue distribution and minimization of negative side-effects in normal tissue as well as high therapeutic efficacy at low administered doses. Verification of the latter assumption is planned as part of a subsequent study of Chl-Mal-B_7 therapeutic potency on tumor-bearing animals.

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