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The interaction of chlorin photosensitizers for photodynamic therapy with blood transport proteins

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ABSTRACT

Photodynamic therapy (PDT) is still considered to be a promising antitumor modality whose potential is not fully disclosed. This unique combination of visible light, molecular oxygen and a light-sensitive molecule, *i.e.* a photosensitizer (PS), leads to a generation of reactive oxygen species inducing direct tumor cell death, damage to tumor microvasculature and induction of a local inflammatory reaction. A much higher selectivity of tumor targeting during PDT can be achieved by the PS binding to appropriate delivery vehicles with pronounced affinity to tumor tissue. Blood lipoproteins are often considered to be such agents enhancing PS tumor accumulation. Here, we focus on the interaction between a series of charged chlorin PSs synthesized on a chlorophyll *a* platform with blood transport proteins. The ability of PS molecules to form molecular complexes with potential carries – biocompatible polymers or surfactants is also considered and briefly discussed. Our experimental studies do indicate that a charge sign, number and relative position of charged groups in the macrocyclic molecule strongly influence the PS-protein interaction. The monocationic chlorin PS with a pronounced hydrophobic surface is found to be delivered by lipoproteins, while trianionic chlorin e_6 is preferentially associated with serum albumin. The addition of biocompatible micellar or polymeric carriers widely used to improve biocompatibility of many drugs remains an elution profile almost unchanged despite strong PS-carrier binding. It is important that both di- and tricationic chlorins are not associated with any transport proteins and the mechanism of their accumulation in tumors must be different from other PSs. Taking into account that lipoproteins are highly important carriers for PS molecules in antitumor photodynamic therapy, we can make an important conclusion that chlorins bearing one cationic group at a certain position of a macrocycle are more efficient photosensitizing agents compared to anionic or polycationic macrocycles.

1. Introduction

Photodynamic therapy (PDT) is a minimally invasive modality with promising results in treating various cancers and many non-malignant diseases [1–3]. PDT consists of the unique combination of a photosensitizer (PS) which is a light-sensitive molecule, molecular oxygen and light with a specific wavelength. Light activates PS molecules, which leads to generation of reactive oxygen species (ROS) being toxic to target cells. The appearance of ROS in intracellular space or tumor vascular is capable of destroying cancer lesions [4–7]. The most popular photosensitizers in clinical practice are appropriate derivatives of macroheterocycles, such as porphyrins, chlorins or phthalocyanines [8–10].

The attachment of charged groups to hydrophobic chlorophyll is known to be a good strategy to improve aqueous solubility of potential agents [6,9,11]. The water-soluble second-generation chlorin PSs such as “Fotoditazin” and “Fotoran e_6” have gained a good reputation in

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clinical practice due to their high efficacy in treating many tumors, low dark toxicity, rapid clearance from the body, high purity and low cost [3,11–14]. Meantime, chlorin macrocycles with appropriate cationic groups can be considered as perspective multi-targeted photosensitizers [1,9] demonstrating pronounced affinity towards both malignant mammalian and microbial cells. Unlike anionic PSs, these compounds are able to photoinactivate Gram-negative pathogens penetrating their outer lipopolysaccharide membrane [1,9].

The efficacy of antitumor PDT is strongly dependent of a specific PS ability to be selectively accumulated in malignant tissue compared to normal cells [15–17]. The important factor responsible for targeted PS accumulation in tumors is a binding to an appropriate carrier. Furthermore, it is worthy of note that many chlorin PSs have a tendency to hydrophobic association in water [18] and form large aggregates even at low solute concentrations [10,19]. This phenomenon poses a challenge in clinical practice since generation of singlet oxygen in aggregates is significantly suppressed. The most common way to solve this problem is to use appropriate delivery agents such as biocompatible polymers or micellar surfactants [9,16,17] which are able to form molecular complexes with macrocycles.

Being intravenously administrated a PS interacts immediately with blood proteins and cell elements and the PS distribution in the body depends on its affinity to these entities. The major transport proteins in human blood are albumins, low-density lipoproteins (LDL) and high-density lipoproteins (HDL) [20]. These species play an important role in the delivery of drugs to targeted places in the body and are responsible for the subsequent therapeutic effect [20]. Low-density lipoproteins (LDL) are primarily involved in the transport of cholesterol and other lipids from the liver to peripheral tissues. They consist of a hydrophobic core surrounded by a monolayer of phospholipids, cholesterol and apolipoproteins [21]. It is considered [22–25] that hydrophobic PS molecules slightly soluble in water preferentially bind to LDL and better penetrate cancer cell membranes. Tumor cells often take up many LDL molecules due to upregulation of LDL receptors, which can be exploited for targeted delivery of PSs [26].

High-density lipoproteins (HDL) are another class of lipoproteins responsible for the reverse transport of cholesterol and other lipids from peripheral tissue to the liver [27]. The transport of some amphiphilic PSs is associated with HDL [28], but compared to LDL, HDL species are mainly accumulated by macrophages surrounding tumor cells, which may negatively affect the efficacy of PDT [20,24,29].

Albumins are the most abundant plasma proteins and play an important role in maintaining osmotic pressure. Many biomolecules, including fatty acids, hormones and drugs are transported by appropriate albumins [30]. Some PS molecules can also bind to specific sites in albumin molecules to be transported to tumors. Although albumin is also accumulated in tumor tissue, this way is considered to have lower efficacy due to prevailing vascular damage and not to cancer cells [20].

The PS-blood protein interaction is strongly dependent of the chemical structure and polarity of the structure of a PS molecule. We have mentioned above that hydrophobic PSs tend to bind to lipoproteins, whereas well-soluble ionic PSs are bound to albumins [20]. The elucidation of the molecular mechanisms governing the PS-protein interaction may shed additional light on PS biodistribution, which is an important step of developing more efficient PDT strategies.

This study focuses on distribution of charged chlorin photosensitizers (see Fig. 1) between human blood transport proteins and the effect of a PS functional group and a passive carrier on the PS-protein interaction using gel filtration chromatography.

2. Experimental section

2.1. Photosensitizer synthesis and identification

A detailed description of PS synthesis can be found in the original papers [31–33], and the identification of the PS studied is given in the

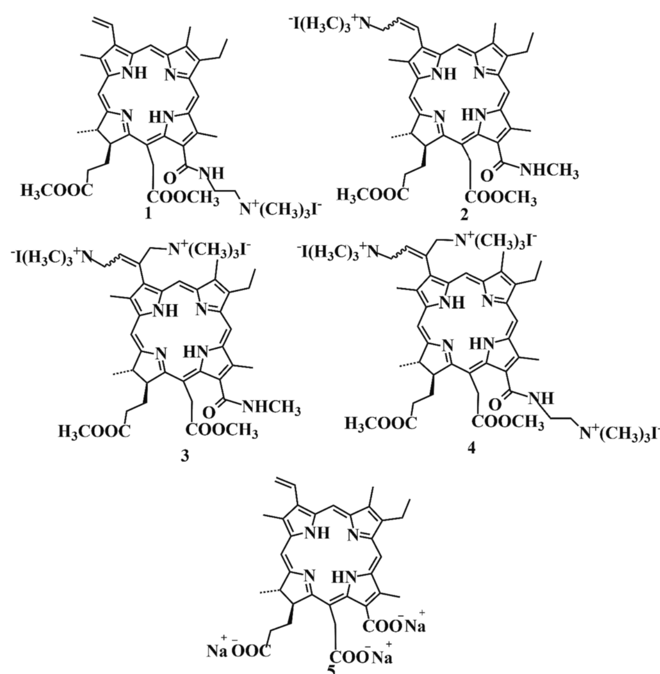

Fig. 1. Molecular structures of the PSs studied: 13(1)-N-(2-N'N'N'-trimethylammonioethyl iodide)amide chlorin e_6 15(2),17(3)-dimethyl ester **1**; 3(2)-(N,N,N-trimethylaminomethyl iodide) chlorin e_6 13(1)-N-methylamide 15(2),17(3)-dimethyl ester **2**; 3(1),3(2)-*bis*-(N,N,N-trimethylaminomethyl iodide) chlorin e_6 13(1)-N-methylamide 15(2),17(3)-dimethyl ester **3**; 3(1),3(2)-*bis*-(N,N,N-trimethylaminomethyl iodide) chlorin e_6 13(1)-N'-(2-N'',N'',N''-trimethylammonioethyl iodide)amide 15(2),17(3)-dimethyl ester **4**; chlorin e_6 trisodium salt **5**.

Suppl. Mat. File.

2.2. Chemicals

Water was distilled twice. Phosphate saline buffer (PSB, Agat-med, for biochemical laboratories) was prepared by dissolving a solid formulation in a liter of purified water to reach the pH value of 7.4. Nonionic surfactant polyoxyethylene (20) sorbitan monooleate (Tween 80, Panreac, pharma grade), polyvinylpirrolidone (PVP, Merck, pharma grade, $M = 10000$ g mol^{-1}), Thrombin (Renam, pure lyophilisate), calcium chloride (Panreac, >99 %), an “Acrilex P-200” gel (Vekton, pure) and human plasma (Ivanovo Regional Transfusion Station, frozen sterile product) were used as supplied.

2.3. Interaction with potential passive carriers

The PS-Tween 80 interaction was studied with the spectrophotometric titration technique described several times before [9,33,34]. The experimental data for comp. **2** are compiled in the Suppl. Mat. File.

2.4. Binding to serum proteins

The interaction of chlorin PSs with serum proteins was studied by the gel filtration method [35] with a self-built 1.5 × 70 cm “Acrilex P-200” column. Fibrinogen was removed from defrosted human plasma with pure thrombin and $CaCl_2$. After 3–4 h incubation, pure serum was accurately separated from the precipitate and stored frozen. Before to start gel filtration an appropriate amount of serum was defrosted and dissolved in PSB to obtain a 70% serum PSB solution. Then, an appropriate PS was dissolved in as serum solution to reach the solute molality of 80 μmol·kg^{-1}. One milliliter of this solution was packed into the “Acrilex P-200” column with a special syringe to form a packed bed.

Then, the packed bed was equilibrated with PSB as a mobile phase for separating blood proteins. The PSB flow allowed low-density lipoproteins to pass almost unhindered through the column, while smaller high-density lipoproteins and albumin were retarded according to its partial penetration into a gel matrix.

Solution fractions of 2.5 ml were collected for a spectrophotometric analysis. The absorption of serum proteins in PSB was also determined, and the appropriate separation curves (elution profiles) were obtained (see Fig. 2). Additionally, serum fractions without any PS were analyzed with a "COBAS 6000″ platform (Switzerland) to determine the concentration of lipoproteins and albumins after gel filtration. Other details are given in the Suppl. Mat. File and the results are summarized in Table 1.

3. Results and discussion

Fig. 1 compares the structures of two monocationic (comps. **1, 2**), di- (comp. **3**) and tricationic (comp. **4**) chlorin PSs. Additionally, trisodium salt of chlorin e_6 (comp. **5**) was added as a reference PS. We see from Fig. 2 *a* that three protein fractions consisting of low-density lipoproteins (fast-eluting peak), high-density lipoproteins (intermediate peak) and albumin (slow-eluting peak) are clearly detected and independently confirmed by the biochemical analysis performed (see Fig. 2 *b*). Similar elution profiles were obtained with "Acrilex P-300" or "Sephacryl S-300" columns [35,36]. The quantity of PS molecules bound to each type of blood proteins was estimated to be proportional to the area under each absorption peak. All the experiments were repeated three times and the mean value was used to obtain the separate curves mentioned above.

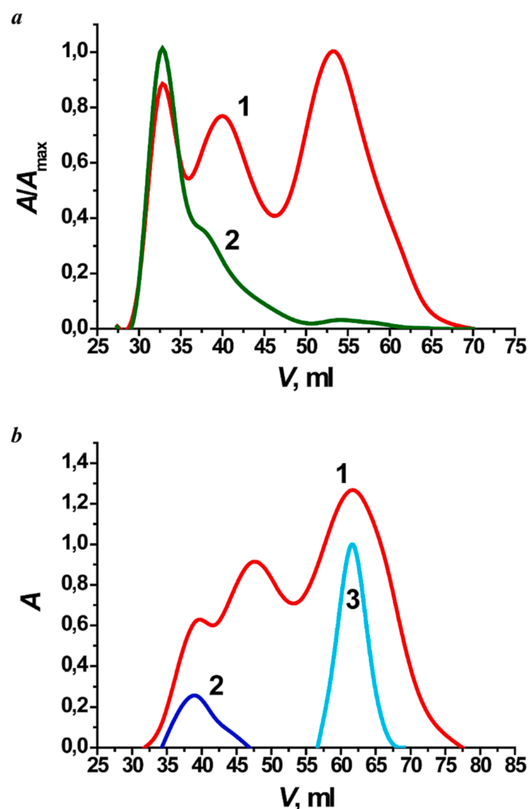

Fig. 2. (a) B-splined elution profiles from a "Acrilex P-200" column for comp. **1**: red line **1** gives the protein absorption at 280 nm and olive line **2** refers to the PS absorption profile at 665 nm; (b) the detection of serum transport proteins by a "COBAS 6000" platform: red line **1** gives the elution profile of pure blood protein sample, while blue and cyan lines **2**, **3** refer to LDL and albumins, respectively. Note that the level of HDL in diluted serum was lower than the detection limit of the devise. Here, ***A*** is absorption of an appropriate PS and serum proteins and ***V*** is a volume of an eluent exiting a gel-chromatography column.

Table 1
PS interaction with various carries [a.]

PS	Carrier *Serum transport proteins (%), gel filtration*		
	LDL	HDL	Albumin
comp.1 [9]	52 ± 2.7	40 ± 2.3	8 ± 0.5
comp. **1** + Tween 80	51 ± 2.1	39 ± 1.8	10 ± 1.1
comp. **1** + PVP	49 ± 1.4	43 ± 2.7	8 ± 2.4
comp. **2**	~ 1[b]	0	0
comp. **2** + Tween 80	~ 1[b]	0	0
comp. **3**	0	0	0
comp. **4**	0	0	0
comp. **5** [9]	2 ± 1.7	6 ± 1.5	92 ± 3.2
comp. **5** + PVP	1 ± 0.1	5 ± 1.8	94 ± 1.8
	Tween 80 or PVP, spectrophotometric titration		
comp. **1** [9]	$\lg K_{b1} = 3.73 \pm 0.31$, $n_1 = 0.71 \pm 0.07$	$\lg K_{b2} = 9.36 \pm 1.04$, $n_2 = 2.18 \pm 0.30$	
	$\lg K_{b1} = 5.66 \pm 0.08$, $N_0 = 0.39 \pm 0.11$ (PVP)		
comp. **2**	$\lg K_{b1} = 5.22 \pm 0.53$, $n_1 = 1.33 \pm 0.13$	$\lg K_{b2} = 7.78 \pm 2.52$, $n_1 = 2.09 \pm 0.75$	
comp. **3** [37]	$\lg K_{b1} = 5.66 \pm 0.29$, $n_1 = 1.76 \pm 0.09$	$\lg K_{b2} = 9.09 \pm 0.67$, $n_2 = 3.26 \pm 0.28$	
comp. **4** [37]	$\lg K_{b1} = 1.44 \pm 0.13$, $n_1 = 0.33 \pm 0.03$	$\lg K_{b2} = 14.1 \pm 2.9$, $n_2 = 3.19 \pm 0.70$	
comp. **5** [9]	$\lg K_{b1} = 3.79 \pm 0.03$, $n_1 = 0.86 \pm 0.01$	$\lg K_{b2} = 8.39 \pm 0.35$, $n_2 = 1.99 \pm 0.10$	
	$\lg K_{b1} = 4.56 \pm 0.09$, $N_0 = 1.62 \pm 0.2$ (PVP)		

[a] - K_b is a binding constant to Tween 80 micelles or PVP, n is the mean number of Tween 80 molecules in close contact with a PS molecule in a micelle and N_0 is the number of PS binding sites per a PVP molecule; [b] a little amount of the PS was found to be associated with LDL.

Fig. 2 *a* illustrates the example of gel filtration of comp. **1** with the column mentioned above. The first peak corresponds to low-density lipoproteins, while the second and third peaks refer to high-density lipoproteins and albumins, respectively. The curves obtained with a "COBAS 6000″ platform (Fig. 2 *b*) support this finding.

Recently, we have shown [9] that comp. **5** is almost entirely transported by the albumin fraction of serum proteins. This is in qualitative agreement with the previous studies exploited an "Acrilex P-300" column [35] and the finding that charged polar molecules are often delivered by albumins [20]. In contrast, Table 1 shows that comp. **1** with one cationic fragment occupying a position near two carboxylic ester groups is transported by lipoproteins. This difference can simply arise from the fact that positively charged groups surrounding a hydrophobic pocket of albumin attract a negatively charged chlorin e_6 molecule (comp. **5**), while the interaction with monocationic chlorin (comp. **1**) is repulsive. The high affinity of comp. **1** towards a lipid-like compartment found elsewhere [9] induces its preferential accumulation in a lipid core of LDL and HDL.

The increase in the number of cationic groups in a chlorin molecule leads to a total loss of the PS affinity to be transported by serum proteins. Hence, their accumulation in tumor tissue is not associated with a serum protein pathway, which can limit their potential as efficient photosensitizers in antitumor photodynamic therapy.

Comparing the structures of cationic chlorin photosensitizers shown in Fig. 1, we see that the presence of an extended apolar moiety in the PS molecule is crucial for its binding to serum lipoproteins. Indeed, both comps. **1** and **2** are isomeric monocationic derivatives of chlorin e_6. However, comp. **2** with the cationic fragment occupying the opposite

position to the carboxylic ester groups is not transported by serum proteins. Hence, the attachment of a charged fragment to the apolar vinyl group results in a loss of the PS ability to penetrate a lipid core of lipoproteins. The interaction with albumins is mainly repulsive due to the positive charge of the PS molecule and according to the absorption spectrum, comp. **2** seems to be in an associated state in serum.

We have mentioned above that many chlorin photosensitizers have a tendency to hydrophobic association in aqueous solutions [18,19] leading to the formation of nanoaggregates even at millimolar concentrations. Biocompatible polymers, micellar surfactants, dendrimers *etc.* as nanoscale passive delivery vehicles are widely used in pharmaceuticals [9,16,17] to increase PS solubility and weaken aggregation. Our recent spectrophotometric studies do indicate that both non-ionic surfactant Tween 80 and PVP form stable complexes with various chlorins [9,33,34]. The results for charged chlorins compiled in Table 1 shows that both carriers efficiently bind PS molecules, Tween 80 revealing two modes of binding. The corresponding binding constants are large, especially, with the large excess of the micellar carrier.

In the light of these results, we have performed further experiments to investigate the effect of Tween 80 or polyvinylpyrrolidone on the distribution of photosensitizers in serum. Table 1 shows that a 100-fold molar excess of Tween 80 does not change the elution profile of comps. **1**, **2**. A similar phenomenon is observed when an equimolar amount of PVP is added to comps. **1** and **5**. Thus, the distribution pattern of chlorin PSs remains unaltered both in surfactant and polymer solutions. Comp. **1** binds to LDL, HDL and albumin fractions of serum in the ratio of 50:40:10 for all the three cases. The behavior of comps. **2** and **5** is very similar (see Table 1). This fact clearly indicates that the PS-protein binding is much stronger compared to complexation with passive synthetic carriers. Importantly, the carriers themselves have no significant affinity to serum proteins and the addition of Tween 80 to a solution of comp. **2** does not change the PS behaviour.

4. Conclusion

In conclusion, we can state the following as the result of this and several earlier studies using the light-sensitive semi-synthetic compounds for antitumor and antimicrobial photodynamic therapy. First, serum proteins can efficiently deliver various chlorin PSs. However, the details of the PS-protein interaction depend on the photosensitizer structure. Polyanionic PSs are transported by albumins, while presumably hydrophobic PSs even containing one cationic group can be efficiently bound by lipoproteins. Second, both the number of cationic groups and their relative position in the macrocycle influence the PS-protein interaction. Hence, by manipulating the polarity and charge distribution of photosensitizer molecules, it is possible to optimize their binding to transport proteins and improve targeted drug delivery to tumor cells. This knowledge could ultimately contribute to the development of more efficient and selective PDT treatments for cancer patients. Third, synthetic passive carriers have their place in drug delivery, but it is evident that the affinity and binding capacity of transport proteins contribute much stronger to the distribution of photosensitizers in the body and the carriers above have little effect on their pharmacodynamics. Fourth and the most important, our results suggest that a water-soluble semi-synthetic chlorin macrocycle with a pronounced hydrophobic fragment and a high affinity towards a lipid-like compartment would be a quite efficient photosensitizer for PDT. Lipoprotein-transported comp. **1** seems to meet these criteria and should be the object for further studies.

CRediT authorship contribution statement

Philipp K. Morshnev: Methodology, Validation, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Andrey V. Kustov:** Conceptualization, Validation, Writing – original draft, Writing – review & editing, Supervision, Project administration. **Eduard A. Drondel:** Investigation, Visualization. **Ivan I. Khlydeev:** Resources, Methodology. **Olga B. Abramova:** Methodology, Investigation. **Elena B. Yaroslavtseva-Isaeva:** Methodology, Investigation. **Elena V. Lyalyakina:** Investigation, Resources. **Mikhail O. Koifman:** Supervision, Resources, Writing – review & editing. **Dmitry B. Berezin:** Conceptualization, Validation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

These data can be found in the Suppl. Mat. File

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molliq.2023.123116.

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