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New Sensitizers Developed on a Methylpheophorbide a Platform for Photodynamic Therapy: Synthesis, Singlet Oxygen Generation and Modeling of Passive Membrane Transport

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Abstract

This study focuses on the behavior of new potential sensitizers for photodynamic therapy of cancer developed on a chlorophyll a platform. Pheophorbide a 17-(3) methylester and its two glycol derivatives have been synthesized from chlorophyll and identified via visible, UV-, NMR- and MS-spectra. The behavior of photosensitizers in solutions has been studied with various experimental techniques. They are found to generate singlet oxygen with a sufficient quantum yield and reveal a tendency to effectively penetrate into cell membranes due to high lipophilicity. Thermodynamic analysis indicates that the sensitizer transfer from a water-like to a lipid-like medium is controlled by a large and negative enthalpic term excepting the case of the most polar solute, where for phosphate saline buffer the favorable entropic term dominates. Our study highlights the important feature dealing with the temperature dependence of partition coefficients between saline buffer and 1-octanol which is found to be surprisingly strong for hydrophobic solutes and temperature independent for the species containing both H-donor and H-acceptor groups.

Keywords: Photodynamic therapy; Reactive oxygen species; Photosensitizers; Partition coefficients; Hydrophobicity; Thermodynamics of solvation

Introduction

Earlier diagnostics and effective treatment of tumors are very important for increasing the survival rate and quality of life of cancer patients worldwide [1]. Fluorescence diagnostics and photodynamic therapy (PDT) with the appropriate photosensitizer (PS) of the first or second generations are very promising options for visualization and local little-invasive treatment of not deep-seated malignancies [1]. PDT consists of three non-toxic essential components: PS, visible light, and oxygen [1-3]. Most of PSs which are currently in use are based on a tetrapyrrole structure, similar to that observed in hemoglobin. These species are able to absorb irradiated photon energy, transfer it to nearby oxygen molecules producing reactive oxygen species such as singlet oxygen $^1O_2$, different radical forms or superoxide anion [1-3]. The most important form $^1O_2$ ($^1\Delta_g$) reveals a very short lifetime and, therefore, small diffusion into a tissue that limits photodynamic damage of PS to its nearest surrounding [1]. The latter strongly depends on the solute structure, therefore, different PSs reveal variable localizations in cancer cells [1,4]. For instance, porfimer sodium localizes mostly in lipid membranes; chlorin e_6 derivatives target lysosomes, mitochondria membranes or endoplasmic reticulum; phthalocyanines reveal a broad spectrum of affinity to cell organelles [1]. Another important point is that the sensitizer localization in cancer cells is likely to influence mechanisms of PDT [3]. In particular, Pd-bacteriopheophorbide is found to form appreciable amounts of hydroxyl and superoxide radicals in an aqueous medium (cytozol) but not in apolar organic solvents (lipid membrane), where only $^1O_2$ is detected [3].

Thus, the PS interaction with surrounding molecules, its ability for membrane penetration, light and temperature induced instability play a crucial role for PDT efficacy [3,5]. These processes for many sensitizers are still poorly studied. However, this information is necessary both to improve the available therapeutic options making them more effective, efficient and affordable for patients and suggest new approaches to make further progress in visualizing and treating cancer.

Here, we provide much deeper insight into the problems mentioned above and focus attention on the behaviour of new potential PSs created on a chlorophyll a platform in a liquid state. This study covers not only synthesis of macrocycle species with the pH-independent glycol residues, their spectral identification and ability to generate singlet oxygen, but also gives important information about thermodynamics of transfer from an aqueous to a lipid-like environment in the physiological temperature range. Being taken into account together, these quantities are believed to provide further progress in understanding the PS behaviour in vivo.

Materials and Methods

Synthesis

The description of PSs synthesis, their identification, purification of solutes and solvents are given as a supporting material. The solute structures are given in Figure 1.

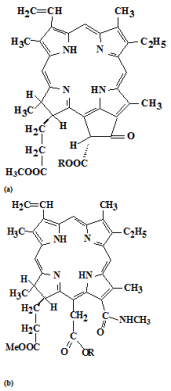

Figure 1: Structures of pheophorbide 17(3)-dimethyl ester a (R = CH_3, 1), pheophorbide a 13(2)-diethylene glycol ester 17(3)-methyl ester (R = -CH_2-CH_2-O-CH_2-CH_2-OH, 2) (a) and chlorin e_6 13(1)-N-methylamide, 15(2)-diethylene glycol, 17(3)-methyl ester (R = -CH_2-CH_2-O-CH_2-CH_2-OH, 3) (b).

Physico-chemical techniques

Singlet oxygen quantum yield estimation: The time-resolved photoluminescence of $^1O_2$ at 1270 nm was studied with the LIF-200 pulsed laser fluorimeter using a nitrogen laser (λ = 337 nm) with the pulse frequency of 30 Hz, the pulse energy of 20 μJ and duration of 2 ns [6]. The FD-10 GA germanium photodetector equipped with a IKS glass band pass filer with the short-wave transmission cut at 980 nm was used [6].

PS	Solvent	
	Benzene	1-octanol
1	0.44[a]	0.47
3	0.62	0.50
Chlorine e_6	0.61[b] [12]	0.67 [12], 0.55 [13][c]

Table 1: Quantum yield of singlet oxygen $^1O_2$ for pheophorbide a 17(3)-dimethyl ester (1), chlorine e_6 13(1)-N-methylamide, 15(2)-diethylene glycol, 17(3)-methyl ester (3) and chlorine e_6 sodium salt in non-aqueous solvents. [a] – Uncertainties are estimated to be within 10% in benzene and 15% in 1-octanol; [b,c] – values in toluene and ethanol, respectively.

The quantum yield of 1O2 (γ_Δ) in 1-octanol (OctOH) and benzene was estimated with a comparative method using Pd mesoporphyrin-IX dimethyl ester or mesotetraphenylporphine as standards. This choice was dictated by their sufficient solubility and reliable quantum yields for both PSs [7-9]. For benzene solutions of mesotetraphenylporphine the $\gamma_{\Delta\ st}$ value was chosen equal to 0.67 [7] For Pd mesoporphyrin-IX dimehyl ester in OctOH $\gamma_{\Delta\ st}$ = 1 was used [8,9]. The γ_Δ values given in Table 1 were computed via the following equation:

$$\gamma_\Delta/\gamma_{\Delta st} = I_0/I_0st \quad (1)$$

where γ_Δ, I0 and $\gamma_{\Delta\ st}$, I0st were the quantum yield and the initial intensity of luminescence obtained from decay kinetics at τ=0 for PS and the standard, respectively.

Partition coefficients determination

Partition coefficients (P) between OctOH and water or phosphate saline buffer were determined with the method of isothermal saturation which, in principal, was identical to that applied earlier for solubility measurements [10]. Here we have, however, exploited thermostated hermetic 50 ml glass cells with effective stirring which were similar to the cell reported elsewhere [11]. Weighed amounts of a sensitizer solution in OctOH with the concentration of 50 μmol/kg and standard phosphate buffer (pH = 7.4) or bidistilled water with the volume ratio of 35:65 were placed into a cell and intensively stirred with a magnetic stirrer usually for two days. The temperature instability in the cell during the experiment was within ± 0.05 K.

When equilibrium was reached, the stirrer was switched off to achieve phase separation. Then two milliliters of a lipid-like fraction were quickly taken up with a stainless steel needle fixed in the lid into a syringe and weighed with analytical balances. The equilibrium concentration of methyl pheophorbide a or its derivatives were analyzed spectrophotometrically using previously obtained calibration plots.

These were found to be linear for the sensitizer concentration range of 10 μmol/kg-800 μmol/kg. This procedure was repeated from eight to ten times for each temperature studied and the mean value of the absorbance coefficient was selected to compute the equilibrium PS content in OctOH. The PS concentration in an aqueous phase was computed as the difference between the equilibrium and initial concentrations in OctOH. The P values given in Table 2 were computed via the well-known equation:

$$P = C_{m\ OctOH}/C_{m\ aq} \quad (2)$$

where $C_{m\ OctOH}$ and $C_{m\ aq}$ were equilibrium molalities of PS in 1-octanol and aqueous phases, respectively.

T / K	*P*			
	1	2	3	3 1-octanol/water
298.15	210.1 ± 6[a]	77.7 ± 4	32.6 ± 1.6	186.1 ± 16
308.15	151.1 ± 12	49.2 ± 4	33.7 ± 1	67.9 ± 2
318.15	78.4 ± 2	34.6 ± 1.1	33.1 ± 0.4	33.9 ± 0.6

Table 2: Partition coefficients of pheophorbide a 17(3)-dimethyl ester (1), pheophorbide a 13(2)-diethylene glycol ester 17(3)-methyl ester (2) and chlorine e_6 13(1)-N-methylamide, 15(2)-diethylene glycol, 17(3)-methyl ester (3) in the 1-octanol/phosphate buffer and 1-octanol/ water biphasic systems at different temperatures. [a]-Values are in molality scale, errors represent the twice standard deviation.

Results and Discussion

Generation of singlet oxygen by PSs

The most important property for any PS which is considered as a certain agent for PDT is generation of singlet oxygen. For estimating

the quantum yield and lifetime of $^1O_2$ in a lipid-like phase we have determined these quantities in benzene and 1-octanol. Luminescence of singlet oxygen is found to decay according to a biexponential law with a slow component due to $^1O_2$ quenching. We have computed constants of luminescence decay and a lifetime of singlet oxygen using the *I-f*(τ) curves. The lifetime quantities being 31 and 19 microseconds for benzene and OctOH, respectively, are found to be nearly independent of the sensitizer structure.

They are in a good agreement with values reported elsewhere [12,13]. The γ_Δ quantities are given in Table 1 where they are compared to those for chlorine e6 sodium salt in non-aqueous solvents [12,13]. We see from Table 1 that all macrocycles generate 1O2 with a moderate yield and can be used in a PDT treatment. For the given PS this quantity is, in general, slightly larger in apolar solvents than in polar alcohols. It is obvious that the quantum yield and lifetime should be yet smaller in an aqueous medium.3 From this point of view it would be preferable that our PSs target mitochondrial or cellar membranes rather than, say, lysosomes or cytozol. Thus, the information about the PSs membrane affinity is of some use to make assumptions of their localization in human cells.

PSs partition between water-like and lipid-like phases

One of the most important goals of the present work is to study the interaction of PSs with their environment in a liquid phase because these interactions greatly contribute to their activity in vivo [3]. It is known [14] that the human body may be viewed as a series of lipid-like membrane barriers dividing water-like filled compartments. Hence, it is reasonable to estimate lipophilicity of macrocycles using their distribution between aqueous and lipid phases. The inner hydrophobic core of a membrane is usually modeled by 1-octanol (more precisely by OctOH containing about 4 vol% of water). Similarly, water or aqueous phosphate buffer is used to mimic the aqueous filled compartment [14].

Table 2 shows that the partition coefficients between phosphate buffer and 1-octanol are very large indicating that all macrocycles are hydrophobic. These values, however, reveal some important features which are noteworthy. First, for the most hydrophilic sensitizer containing both H-acceptor and H-donor groups (compound 3) the P values are the smallest and independent of the temperature. Hence, for such PSs the partition coefficients are identical at the physiological and standard temperatures. In contrast, more hydrophobic sensitizers reveal the significant temperature dependence of P values (Table 2).

Second, saline buffer is a much friendlier medium for PSs than cold water. The situation, however, changes at higher temperatures where partition coefficients are almost equal. Third, the appearance of active -NH-proton in the solute molecule strongly encourages the PSs transfer to an aqueous phase, but again only at low temperatures. Being more than twice as different at 298 K, the P coefficients for 2 and 3 become nearly identical at 318 K.

Thermodynamics of PSs solvation

The partition coefficients presented in Table 2 allow providing simple thermodynamic analysis of the PS transfer from an aqueous to a non-aqueous medium. Since we consider dilute solutions, for the free energy of the solute transfer from water to OctOH one can write:

$$\Delta_t G^0 = \Delta_{sol} G^0{}_{OctOH} - \Delta_{sol} G^0{}_{aq} \approx -RT\ln\frac{C_{mOctOH}}{C_{maq}} = -RT\ln P \quad (3)$$

Hence, the partition coefficients given in Table 2 are proportional to the free energy of PS transfer from a water-like to a lipid-like environment. The temperature dependence of the Δ_tG^0 values can be expressed in terms of the original approach of Clark and Glue [15]. Assuming the temperature independent enthalpy change for the partition coefficient one can write:

$$R\ln P = -\frac{\Delta_t G^0}{298.15} + \Delta_t H^0\left[\frac{1}{298.15} - \frac{1}{T}\right] \quad (4),$$

where 298.15 is the reference temperature and Δ_tH^0 is the standard enthalpy of transfer at the reference temperature, respectively. The coefficients of eq (4) are listed in Table 3. Figure 2, comparing experimental and computed RlnP values, illustrates that there is rather a good agreement between experimental and calculated quantities for all cases. Thus, the simplifications made (see eqs (3, 4)) seem to be accurate for this restricted temperature range.

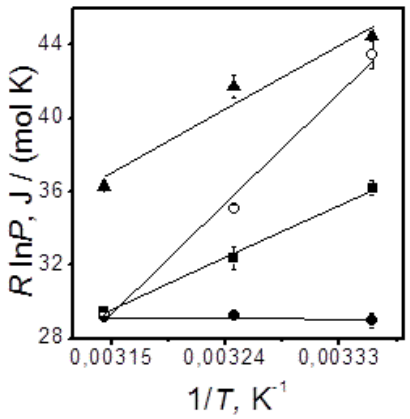

Figure 2: The temperature dependence of the *R*ln*P* values for pheophorbide a 17(3)-dimethyl ester (▲), pheophorbide a 13(2)-diethylene glycol ester 17(3)-methyl ester (■) and chlorin e_6 13(1)-N-methylamide, 15(2)-diethylene glycol, 17(3)-methyl ester (●) between phosphate saline buffer and 1-octanol; (○) gives values for chlorin e_6 13(1)-N-methylamide, 15(2)-diethylene glycol, 17(3)-methyl ester for partition between bidistilled water and 1-octanol. Lines represent a calculation according to eq (4).

Free energy values are negative for all cases indicating strong PS affinity to a lipid-like environment. The enthalpic term, in general, favors the PS transfer. Solvation of hydrophobic PS in OctOH is significantly more exothermic due to mainly a stronger solute-solvent attraction in a non-aqueous phase.

In fact, for the most hydrophobic compound 1 the enthalpy of transfer is large and negative, whereas for the more hydrophilic compound 3 enthalpies of solvation in OctOH and saline buffer are almost identical (Table 3).

Thermodynamic quantity	Solute				
	1	2	3	3 1-octanol/water	CH_2[b]
Δ_tG^0, kJ/ mol	-13.4 ± 0.3[a]	-10.8 ± 0.08	-8.7 ± 0.05	-12.9 ± 0.24	-3.50 (-2.6)
Δ_tH^0, kJ/ mol	-38.7 ± 8	-31.9 ± 2	-0.6 ± 1	-67.2 ± 6	-1.78 (-5.7)
$(-T\Delta_tS^0)$, kJ/mol	25.3 ± 8	21.1 ± 2	-8.1 ± 1	54.3 ± 6	-1.72 (3.1)
r_f, J /(mol K)	0.14	0.11	0.03	0.02	-

Table 3: Thermodynamics of transfer for pheophorbide a 17(3)-dimethyl ester (1), pheophorbide a 13(2)-diethylene glycol ester 17(3)-methyl ester (2) and chlorine e_6 13(1)-N-methylamide, 15(2)-diethylene glycol, 17(3)-methyl ester (3) in the 1-octanol/phosphate buffer and 1-octanol/ water biphasic systems at 298.15 K. a Errors represent 95 % confidence limit and rf is the standard deviation of the fit, b – computed from ref. [16-18] as the difference between 1-heptanol and 1-hexanol. Thermodynamic quantities of methylene group solvation in OctOH are given in brackets.

However, there is another important point dealing with the solute size which should be taken into account. It is known that accommodation of hydrophobic units of moderate size creates excluded volume without disrupting water structure, since water-water hydrogen bonding simply goes around a solute [16,17]. Enthalpies and entropies of solvation for such species are negative and change rather linearly with the solute size (see, for instance, refs. [18,19]). However, large hydrophobic objects strongly disrupt the H-bond network of water inducing depletion of solvent density near an extended apolar surface [16,17]. This collective enthalpic effect caused by the loss of some H-bonding near the surface leads to positive entropies of solvation for species larger than 1 nm [16]. The size of chlorophyll macrocycle is of the same order of magnitude. Moreover, being almost planar these species can additionally strengthen the collective drying effects mentioned above. Our thermodynamic analysis appears to provide further grist to this mill.

Table 3 compares thermodynamic transfer functions for PSs and hydrophobic methylene group [19]. If enthalpies and free energies of transfer are both negative for methyl pheophorbide a and -CH_2-group, entropic terms -$T\Delta_{solv}S^0$ reveal different signs. For small methylene group it is negative, whereas for large hydrophobic PS molecules the entropic term is positive. Table 3 shows that solvation of -CH_2-group in OctOH is accompanied by negative enthalpic and entropic changes. The same effect is observed in water [18]. However, the -$T\Delta_{solv}S^0$ there term is more positive, which leads to the positive free energy of hydration. There is no reason to expect that the entropy of solvation of methyl pheophorbide a in OctOH reveals any abnormalities in comparison with hydrophobes of a smaller size (see, for example, values for normal alcohols [19]), i.e. the -$T\Delta_{solv}S^0$ term should be rather large and positive. If the similar result were valid for water we would have the large and positive -$T\Delta_{hydr}S^0$ term for methyl pheophorbide a leading to the nearly identical the -$T\Delta_tS^0$ values for -CH_2-group and PS. However, Table 3 shows that for methyl pheophorbide a the -$T\Delta_tS^0$ value is large and positive. Hence, the hydration term for this large hydrophobic molecule is significantly smaller than it would be expected. Thus, the exothermic transfer of hydrophobic PSs from water to OctOH may result not only from stronger solute-OctOH attractions but also from the large enthalpic cost for PS accomodation in water. The introduction of hydrophilic residues into the macrocycle allows to avoid this hydrophobic inhibition due to H-bond formation and corresponding water reorganization effects [16,17]. We believe that hydration of such PSs reveals many common features with the behavior of planar mosaic structures modelled by Zangi and Berne [20], where the number and position of hydrophilic centers strongly influence the water structure near the surface.

Conclusion

In this paper, we have focused attention on the behaviour of recently synthesized macrocyclic compounds which are believed to be of some importance for photodynamic therapy. The macrocycles are able to generate singlet oxygen in a lipid-like phase with a moderate yield. This ability is found to be nearly independent of the solute structure. Another important fact illuminated here for the first time is the strong temperature dependence of the partition coefficients between lipid-like and water-like phases. For hydrophobic PSs the P values are very large at 298 K and decrease significantly with the temperature. Even for the most hydrophilic compound 3 the l_g P value is of 1.5 in a physiological temperature range, which is quite enough for effective transfer across the lipid barrier [14] and target cellular or mitochondrial membranes. Thus, the PS_s studied can be, in principal, applied as certain agents for inactivating pathogenic bacteria or tumors. However, they are only slightly soluble in an aqueous phase, which requires developing delivery systems for their administration. Polyethylene glycol [21] or polyvinyl pyrrolidone [5] can be used here as a simple potential drug delivery vehicle to enhance PSs solubility and prevent their self-association in an aqueous phase.

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