Հայաստանի Գիտությունների Ազգային Ակադեմիա
Национальная Академия Наук Армении
National Academy of Sciences of Armenia

Հայաստանի Կենսաբանական Հանդես
Биологический Журнал Армении
Biological Journal of Armenia

• Փորձարարական և տեսական հոդվածներ • Экспериментальные и теоретические статьи •
• Experimental and theoretical articles •

**Biolog. Journal of Armenia, 4 (64), 2012**

# ACTION OF FATTY ACIDS ON THE BINDING CAPACITY OF PORPHYRINS TO BLOOD PROTEINS: I. SPECTRAL INVESTIGATIONS

**A.G. GYULKHANDANYAN[1], R.K. GHAZARYAN[2], V.N. KNYUKSHTO[3], A.S. STASHEUSKI[3], B.M. DZHAGAROV[3], G.V. GYULKHANDANYAN[1]**

*[1]Institute of Biochemistry, NAS of Armenia,*
*[2]Department of Chemistry, Pharmaceutical Faculty, Yerevan State Medical University*
*[3]Institute of Physics, NAS of Belarus, Minsk, Belarus*
*gvg536898@yahoo.com*

It is shown that long-chain fatty acids (palmitate, stearate) can compete with the porphyrins for binding sites on molecules of serum albumin and hemoglobin, significantly reducing the level of binding of protein-porphyrin pairs. It is also shown that the main carrier protein of porphyrins in blood is the serum albumin.

*Proteins – fatty acids – porphyrins – serum albumin – hemoglobin – spectroscopy*

Ցույց է տրված, որ երկարաշղթա ճարպաթթուները (պալմիտինաթթուն, ստեարինաթթուն) կարող են մրցակցել պորֆիրինների կապման տեղերի համար շիճուկային ալբումինի և հեմոգլոբինի մոլեկուլներում, ինչը զգալիորեն իջեցնում է սպիտակուց-պորֆիրին զույգերի կապման աստիճանը: Ցույց է տրված նաև, որ արյան մեջ պորֆիրինների հիմնական սպիտակուց տեղափոխիչը շիճուկային ալբումինն է:

*Սպիտակուցներ – ճարպաթթուներ – պորֆիրիններ – շիճուկային ալբումին – հեմոգլոբին – սպեկտրոսկոպիա*

Показано, что длинноцепочечные жирные кислоты (пальминовая, стеариновая) могут конкурировать с порфиринами за места связывания на молекулах сывороточного альбумина и гемоглобина, что значительно снижает уровень связывания пары белок-порфирин. Показано также, что основным белком-носителем порфиринов в крови является сыворо-точный альбумин.

*Белки – жирные кислоты – порфирины - сывороточный альбумин – гемоглобин – спектроскопия*

Blood proteome is a medium, which rapidly respond to a variety of changes in an organism, including diseases. In photodynamic therapy of tumors (PDT) binding and delivery of photosensitizers (PS) to the tumor is one of the important tasks of this method [7]. Delivery of PS was implemented through the blood and such proteins as serum albumin, hemoglobin, and lipoproteins play a key role in this problem [1, 9, 10, 12]. Presence of ligands, including fatty acids, greatly complicates the solution of tasks in relation to the possible competition of the two ligands (porphyrin and fatty acid) for binding sites on carrier proteins. Among photosensitizers for photodynamic therapy of the most actively being explored cationic porphyrins [7, 13]. Previously methods were developed and a series of cationic porphyrins with different peripheral groups and

central metals (more than 80 compounds) were synthesized [11, 3]. The aim of this study was to investigate the influence of long-chain fatty acids (FA) on the binding of porphyrins to macromolecule of carrier proteins and determination of the most probable carrier protein of porphyrins in the blood.

Presented problems we solved in two steps: 1) study the photophysical properties of porphyrins and selection of suitable porphyrins by criterion of generation of singlet oxygen and 2) study of the binding of selected porphyrins to carrier proteins. To solve the problems of the first stage the studies and detailed analysis of the photophysical properties of new porphyrins and metalloporphyrins (total 21 compounds) were conducted. The problems of the second stage were resolved via methods of optical spectroscopy and molecular docking.

***Materials and methods***. Bovine serum albumin (BSA), bovine serum albumin free fatty acid (БСА, FFA), human serum albumin-free fatty acid (HSA), human hemoglobin (Hb), sodium salts of fatty acids (palmitic and stearic) were purchased from Sigma-Aldrich Chemical Co. (USA). Water-soluble cationic porphyrins and metalloporphyrins with different central metal atoms (Zn, Ag, Co, Fe, Mn, Cu, etc.) and various peripheral functional groups (hydroxyethyl-, butyl-, allyl-, methallyl-) were synthesized at the Yerevan State Medical University in accordance with the procedures described in [11, 3]. The structure of synthesized compounds was investigated and confirmed by NMR, IR, and absorption spectroscopy. In fig. 1 shows the basic structure of the investigated porphyrins:

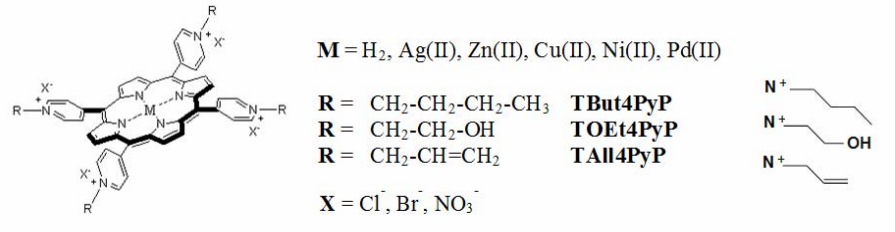


**Fig.1**. Basic structure of cationic porphyrins.

All other reagents were analytical clean grade.

For studies of photophysical properties of porphyrin solutions were prepared in distilled water and studied at room temperature in 10 mm quartz cuvette. To determine the fluorescence quantum yield we used a relative method. As a standard solution we used of Zn-tetraphenylporphin in toluene with the fluorescence quantum yield 3.0%. The quantum yields of singlet oxygen ($\gamma_\Delta$) we were determined by a relative method. As a standard we used meso-tetra [4-N-metilpiridil] porphyrin (TM4PyP) with $\gamma_\Delta$ = 77% [2].

Luminescence spectra were recorded on the laser fluorometer, created in the laboratory of Photonics of molecules of the Institute of Physics, NAS of Belarus. In standard configuration, excitation of samples was carrying out by laser pulses with energies of 4 uJ and duration 0.7 ns, with the frequency of 1 kHz at a wavelength of 531 nm.

Absorption spectra and binding constants of porphyrins with proteins was determined by the method described in [5]. Fatty acids were injected with the proteins in the appropriate ratio (the ratio of protein-fatty acid ranged from 1:1 to 1:5). The binding constant (K) of protein-porphyrin pairs (as well as protein-porphyrin-fatty acid) was determined by analyzing the decrease in the absorption of the Soret band when increasing concentrations of the corresponding protein (or protein-fatty acid) were added to the porphyrin solution, according to the modified equation of Kapp et al. [8]:

$$A_o/A_o\text{-}A = 1/fK[C_{prot}] + 1/f \qquad (1)$$

where $A_o$ and A are the porphyrin solution absorbance in the absence and presence of the protein, respectively. The value of K was defined from an adsorption isotherm. On the adsorption isotherm $A_o/A_o$-A (ordinate) vs. $1/[C_{prot}]$ (abscissa), the value 1/f is a segment on the axis $A_o/A_o$-A corresponding to a point $1/[C_{prot}] = 0$.

The experimental results were statistically processed using a Student test.

***Results and Discussion.*** *1. Luminescent properties of new cationic porphyrins.* For all 21 compounds absorption and fluorescence spectra were measured. Moreover, for all complexes of metalloporphyrins containing atoms of Co, Ag, Fe and Cu, were not found own glow of complex or the impurity luminescence of free-base porphyrin. For other compounds - free porphyrins and their complexes with Zn atoms characteristically a significant fluorescence. All the studied compounds were photostable.

*2. The quantum yields of singlet oxygen and triplet characteristics of water-soluble cationic porphyrins.* From the set of all new porphyrins maximum close of spectral absorption characteristics to the known porphyrin TM4PyP had TBut4PyP. The quantum yields of singlet oxygen, and kinetic characteristics of the glow of singlet oxygen for TM4PyP were investigated in tris-buffer and for TBut4PyP in various aqueous solutions (tris-buffer, deionized, distilled and redistilled water): for TM4PyP $\gamma_\Delta$=77%, for TBut4PyP $\gamma_\Delta$=78-79%. Table 1 shows the quantum yields of singlet oxygen, and kinetic characteristics of the glow of singlet oxygen of solution of zinc porphyrin complexes which investigated in distilled water at room temperature.

We note that there are the differences in rise time ($\tau_T$) of signal of singlet oxygen for 3-N-pyridyl and 4-N-pyridyl of derivatives and of free bases. In solutions of metalloporphyrins similar were only durations of luminescence quenching of singlet oxygen ($\tau_\Delta$). Perhaps the introduction of the metal ion has significantly changed the triplet characteristics of porphyrins, which reflected in the differing for all metallocomplexes values $\gamma_\Delta$ and $\tau_T$.

**Table 1***. The quantum yields of singlet oxygen production of porphyrins

| Compound | $\gamma_\Delta$, % | $\tau_T$, mks | $\tau_\Delta$, mks |
|---|---|---|---|
| $H_2$TByt4PyP | 78 | 1.9 | 3.7 |
| ZnTByt4PyP | 90 | 2.7 | 3.9 |
| ZnTByt3PyP | 84 | 3.0 | 3.9 |
| ZnTOEt4PyP | 87 | 2.8 | 3.7 |
| ZnTOEt3PyP | 87 | 2.5 | 3.6 |
| ZnTAll4PyP | 91 | 2.6 | 3.7 |

*The sta

ndard deviation does not exceed 5%

*3. The influence of fatty acids on the binding of porphyrins with carrier proteins.* Previously [4-6], we investigated the binding of cationic porphyrins with carrier-proteins of ligands in the blood (serum albumin and hemoglobin) and it was shown that the binding is non-specific and reversible, and also depends on the pH. So for ZnTByt4PyP porphyrin the binding constants with BSA changed from $6.0\times10^4$ $M^{-1}$ to $2.0\times10^6$ $M^{-1}$ at pH 4.0 and 9.5, respectively [5]. Relatively low binding constants of porphyrins and metalloporphyrins ($0.95\text{-}3.5\times10^5$ $M^{-1}$) indicate the electrostatic character of interactions of cationic porphyrins with negatively charged molecule of BSA [4]. At the same time it is well known that a major function of serum albumin in blood is the binding and transport of fatty acids. In this connection we investigated the effect of long chain fatty acids (palmitic and stearic) on binding of porphyrins with carrier-proteins. Using the received binding constants for the two porphyrins (TBut4PyP and Zn-TBut4PyP) and two proteins (HSA Fatty Acid free and Hb) in the absence of fatty acids [5, 6], we assessed changes in the binding constant in the presence of fatty acids (palmitic and stearic). The results for two proteins and two porphyrins indicate a clear tendency to reduce the binding constant with increasing ratio fatty acid/protein in a solution of porphyrin (tab. 2 and 3). In this case, the binding constant of pair porphyrin TBut4PyP-HAS with

competition of fatty acid (at a ratio of fatty acid/protein = 1:1) according spectral data is reduced in the 1.3-1.6 times, whereas for Hb the decrease is more significant: in the 2.2-2.4 times. From these data an important conclusion follows that the fatty acids (palmitic and stearic) play an essential role in the binding of ligands for SA and Hb. Tables testifies also that binding with porphyrins in the presence of fatty acids compared with the BSA in the case of Hb in solution occurs the more significant decrease of binding constants. This testifies that the main carrier protein of porphyrins in blood is serum albumin.

**Table 2***. Evaluation of binding constants for HSA/porphyrin pairs in the presence of fatty acids.

| Ratio FA: protein | HSA (Zn-TBut4PyP) K ($\times 10^5$ $M^{-1}$) | | HSA (TBut4PyP) K ($\times 10^5$ $M^{-1}$) | |
|---|---|---|---|---|
| | Na palmitate | Na stearate | Na palmitate | Na stearate |
| 1 : 1 | 2.07 | 1.68 | 2.43 | 1.87 |
| 2 : 1 | 1.65 | 1.47 | 0.77 | 1.02 |
| 4 : 1 | 1.11 | 1.22 | 0.70 | 0.64 |
| 5 : 1 | 0.74 | 1.0 | 0.31 | 0.57 |

* Each value is the average of 4 experiments. The standard deviation does not exceed 5%.

**Table 3***. Evaluation of binding constants for human Hb/porphyrin pairs in the presence of fatty acids.

| Ratio FA: protein | Hb (Zn-TBut4PyP) K ($\times 10^5$ $M^{-1}$) | | Hb (TBut4PyP) K ($\times 10^5$ $M^{-1}$) | |
|---|---|---|---|---|
| | Na palmitate | Na stearate | Na palmitate | Na stearate |
| 1 : 1 | 1.35 | 1.28 | 1.36 | 1.24 |
| 2 : 1 | 1.08 | 1.26 | 1.23 | 1.18 |
| 4 : 1 | 0.81 | 1.19 | 1.14 | 1.15 |
| 5 : 1 | 0.69 | 1.05 | 0.92 | 0.86 |

* Each value is the average of 4 experiments. The standard deviation does not exceed 5%.

Based on studies of photophysical and spectral properties of porphyrins have made the following conclusions:

1. Free base porphyrins and their zinc complexes exhibits high photosensitizing activity.

2. The nature of the aqueous solution has no effect on the high efficiency of singled oxygen of studied porphyrins.

3. The most promising porphyrins for photodynamic investigations by criterion of generating singlet oxygen are metalloporphyrins ZnTByt4PyP and ZnTAll4PyP.

4. It is shown that long-chain fatty acids (palmitate, stearate) can compete with the porphyrins for binding sites on molecules of serum albumin and hemoglobin, significantly reducing the level of binding of protein-porphyrin pair. This fact should be considered when applied the method of PDT of tumor and photosensitizer dose calculations must consider the level of fatty acids in blood.

5. Results testify that binding with porphyrins in the presence of fatty acids compared with the BSA in the case of Hb in solution occur the more significant decrease of binding constants. This testifies that the main carrier protein of porphyrins in blood is serum albumin.

This work was supported by grants from the State Committee of Science of the Ministry of Education and Science of Armenia (Project 11RB-016) and the Foundation for Fundamental Research of the Republic of Belarus (Project F11ARM-017).

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*Received 15.11.2012*