The Effect of Some Amphiphilic Porphyrins on the Transmembrane Potential of Cultured L929 Cells

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This study include in the same frame promising structures tailored as photoactive agents for photodynamic therapy of cancer versus one of the most versatile cell lines from experimental point of view, fibroblasts type, subcutaneous connective tissues- L929. Transmembrane potential was studied establishing important features in the behavior of chemical vectors against cultured cells via membranar media. Two amphiphilic porphyrins, 5,15-bis-(4-hydroxy-3-methoxyphenyl)-10,20-bis-(4-carboxymethylphenyl)porphyrin and Zn(II)-5,15-bis-(4-hydroxy-3-methoxyphenyl)-10,20-bis-(4-carboxymethylphenyl)porphyrin were assessed in terms of the effect on membrane potential. The comparative effect of free base porphyrin and metalloporphyrin with amphiphilic structure, reveal hyperpolarization phenomena and bring informations about consequences of active compound concentration against cell membrane.

Keywords: amphiphilic porphyrins, transmembrane potential, mouse L929 fibroblasts

Tetrapyrroles are among the most studied agents to be used in biomedical domain, especially in photodynamic therapy (PDT), due to their multivalent profile including photochemical, redox, coordination and molecular flexibility special properties [1, 2].

Thus, it is known that PDT agents selectively accumulate in malignant tissue against the healthy one [3] and, when activated with the proper wavelength, they can lead to the production of singlet oxygen, a highly reactive species that triggers the development of oxidative chain reactions within the cell, resulting in its destruction [4-7].

While the exact mechanism by which the PDT agents accumulate in the diseased tissue is still unclear, several studies tried to explain the interaction of the cell membrane with tetrapyrrole compounds. Among these studies, few investigate the effect of the porphyrinic structures on the transmembrane (plasmatic membrane) potential.

The cell membrane, a bi-dimensional oriented, complex lipoprotein system, separates the cell from the outer aqueous medium, and also ensures the fluxes of the molecules relevant for the cellular metabolism from an outside the cell. One of the most important features implicated in the entry and exit of the substances from the cell is the transmembrane potential, defined as the voltage difference between the inside and outside surfaces of the lipid bilayer; the membrane potential arises mainly from the interaction of the ionic channels with the ionic pumps of the membrane, which maintain different ions concentrations at the inner and outer side of the membrane. The membrane potential in case of non-excitable cells has a rather constant value, being called resting potential. The opening or closing of an ionic channel can induce a decrease of the absolute value of the transmembrane potential (i.e., a depolarization of the membrane), or an increase of that value (a hyperpolarization of the cell membrane). Both changes can result in the normal metabolism of the cell; for example, it is considered that membrane depolarization can lead to apoptosis [8-10].

The selectivity, penetration and distribution of PDT agents, mainly porphyrinic type structures, in different types of tissue and cells obviously depends on the chemical structure of each compound (the number, size, position and length of the substituents, the presence of a metallic ion in the core of the tetrapyrrole ring, the distribution charge on the molecule and its hydrophobic/hydrophilic balance). Also, their efficiency in PDT depends on the mechanism by which the destruction of the malignant cell is activated.

Photosensitizers can enter the cell membrane by diffusion both across and along the membranes, by non-specific endocytosis, or even by pinocytosis. Large aggregates can also be internalized via phagocytosis [11]. Literature data suggest the need for the PDT agents to be in the proximity of the target molecule for effective singlet oxygen quenching [12, 13]. It is believed that one mechanism through which the PDT functions is the lipid peroxidation induced by the singlet oxygen generated in the activation-deactivation of the fluorescent agent. Another mechanism is that of modulating the gramicidin A channels [14].

Tetraphenylporphyrin derivatives have been developed as a new class of synthetic ligands for potassium channels [15], but they also can act as PDT agents.

The membrane potential can be measured by different techniques. In the past decades, voltage-dependent fluorescent probes were synthesized, allowing an indirect method of assessing the ionic transfer through the cell membrane. The fluorescent voltage-sensitive probe *bis*-(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4(3)$) is a fluorescent probe that binds to intracellular or inner cell membrane proteins, the process having as a result an increase of the fluorescent signal. The depolarization of the membrane generates an influx of the probe to the cell, therefore an increase of the emission intensity; equally, the hyperpolarization of the membrane diminishes the flow of the probe into the cell, therefore the fluorescent signal is reduced as compared with normal (control) cell [16]. In this way, if a certain compound generates a change in the resting potential of the cell, this can be notified by using $DiBAC_4(3)$ as a voltage sensitive molecular reporter.

Continuing our researches regarding amphiphilic porphyrins with biomedical applications, [17-29], in this study we synthesized a new A_2B_2 type porphyrinic complex, Zn(II)-5,15-*bis*-(4-hydroxy-3-methoxyphenyl)-10,20-*bis*-(4-carboxymethylphenyl)porphyrin (Zn(II)P) (fig. 1), and

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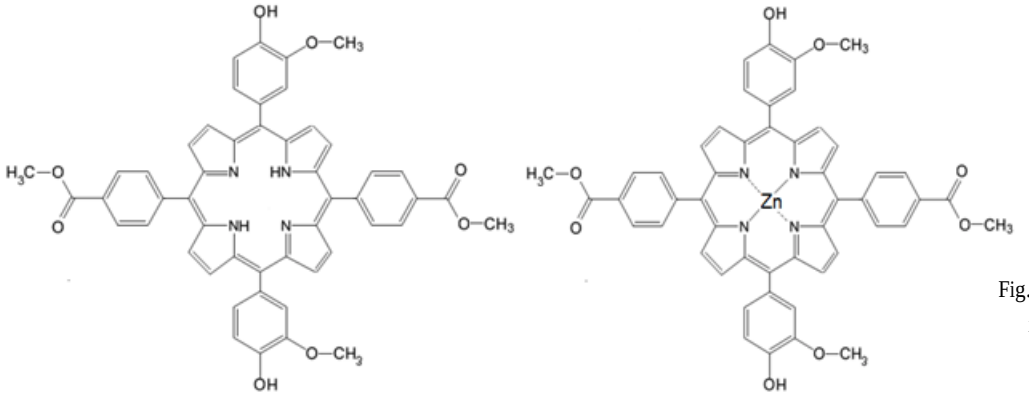

Fig. 1. Molecular structures of investigated porphyrins

a: 5,15-*bis*-(4-hydroxy-3-methoxyphenyl)-10,20-*bis*-(4-carboxymethylphenyl)porphyrin
b: Zn(II)-5,15-*bis*-(4-hydroxy-3-methoxyphenyl)-10,20-*bis*-(4-carboxymethylphenyl)porphyrin

assessed its effect on the transmembrane potential of L929 cultured cells.

In order to highlight the influence of the metallic ion on transmembrane potential, we included in experiments free base porphyrin, 5,15-*bis*-(4-hydroxy-3-methoxyphenyl)-10,20-*bis*-(4-carboxymethylphenyl)porphyrin (P) (fig. 1), compound reported in a previous study [30].

Experimental part

Materials and methods

Commercially available chemicals and solvents were used as received from Sigma-Aldrich and Merk. The free base porphyrin, 5,15-*bis*-(4-hydroxy-3-methoxyphenyl)-10,20-*bis*-(4-carboxymethylphenyl)porphyrin, subjected to the biological study was obtained according to the procedure described previously [30]. Zn(II)-5,15-*bis*-(4-hydroxy-3-methoxyphenyl)-10,20-*bis*-(4-carboxymethylphenyl)porphyrin was synthesized in this study and its structural characterization was performed by the elemental analysis of C, H and N with an automatic Carlo Erba L-1108 analyzer, FT-IR spectrometry with a FT-IR Tensor 27 spectrophotometer and UV-Vis spectroscopy with a Lambda 35 Perkin-Elmer spectrophotometer. The NMR spectra were recorded with a 400 MHz Bruker NMR spectrometer. The porphyrin solutions were freshly prepared in the spectrally pure solvents and kept in dark until the measurement to prevent photodegradation.

Synthesis of Zn(II)-5,15-bis-(4-hydroxy-3-methoxyphenyl)-10,20-bis-(4-carboxymethylphenyl) porphyrin

The synthesis of Zn(II)P complex was performed by refluxing a dichloromethane solution containing a mixture of 5,15-*bis*-(4-hydroxy-3-methoxyphenyl)-10,20-*bis*-(4-carboxymethylphenyl)porphyrin (0.25 mmol) and anhydrous zinc acetate (0.25 mmol) in the presence of 2,6-dimethylpyridine, at 60°C for 30 min. The presence of porphyrinic complex in the reaction mixture was evaluated by UV–Vis spectroscopy while TLC tests of the reaction product allowed us to establish the conditions for purification of the Zn(II)P. The crude product from the synthesis reaction, was purified through column chromatography (Al_2O_3 90, Merck, 63–200 μm 70-230 mesh, CH_2Cl_2/diethyl ether 50:1 (*v/v*)) and finaly by TLC.

Zn(II)P was obtained with a yield of 92%, as violet crystals insoluble in water, soluble in ethanol, dimethylsulfoxide, dichloromethane, chloroform and polyethylene glycol 200.

Transmembrane potential study

Polyethylene glycol 200 (PEG 200, Sigma-Aldrich) was used as solvent to prepare the test porphyrinic solutions, because is a pharmacologically accepted solvent which limit the uptake of nanostructures by blood phagocytes, increasing their bioavailability [31]. In addition, PEG 200 is commonly used in pharmaceutical formulation in order to overcoming the molecular aggregation of the porphyrinic photosensitizers. For each porphyrin above, stock solutions of 0.25 mM were prepared, which were used to test the effect of the porphyrins upon cells.

For the transmembrane potential study, the mouse L929 fibroblasts from subcutaneous connective tissue (ATCC CCL-1) purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). L929 is a fibroblast adherent cell line from subcutaneous connective, areolar and adipose tissue originating from *Mus musculus* (mouse). Cell line bought from ATCC was kept frozen between experiments. Cells were counted and adjusted to 10^5cells/mL with DMEM culture medium (Biochrom, Berlin, Germany). Fluorescent voltage-sensitive probe *bis*-(1,3-dibutylbarbituric acid)trimethine oxonol ($DiBAC_4$(3)) bought from Molecular Probes Fisher Scientific, Inc., was used as a reporter of the changes in the resting potential of the cells; the probe was dissolved to a concentration of 2 M (stock solution) in DMSO and was kept to -20 °C between experiments. Aliquots of 2 mL from each kind of cells were incubated at room temperature for 20 min with different concentrations of porphyrins in the range 0.5–2.5μM, then the influence of the porphyrins on the transmembrane potential of the cells was evaluated using the increase/decrease of the fluorescence emission intensity of the probe $DiBAC_4$(3) measured at 513 nm, with excitation at 495 nm (relative units).

The probe concentration on the cells was of 2 μM. Sets of cells incubated only with corresponding amounts of PEG 200 were used as controls.

The results were expressed as relative percent change of the intensity signal of the treated cells *vs.* controls:

$$I_e = 100 \times (1 - I_{sample}/I_{control}) \quad (1)$$

The measurements were performed whith Jasco FP 6500 spectrofluorimeter in standard 1 cm pathlength cuvettes from polymethyl metacrylate.

Results and discussions

Mesoporphyrinic complex, Zn(II)P was synthesized by the classical methods [18] that involves refluxing of equimolar ratios of the porphyrinic ligand [30] and metal salt in the presence of a basic catalyst. The structural evaluation of Zn(II)P was performed by elemental analysis, NMR, FT-IR and UV-Vis spectrometry. Analytical and spectral data of the amphiphilic porphyrins used in study are presented in table 1. The formation of Zn(II)P in the synthesis reaction, was spectrally confirmed by the absence of FT-IR and ^{1}H-NMR spectral signals associated the proton of the N-H group which belongs to the porphyrinic core of the ligand (table 1). Thus, the comparative analysis of the ^{1}H-NMR data of the porphyrinic ligand (P) and its zinc complex (Zn(II)P), reveals the absence of the signal at -2.79 (s, 2H) [30], confirming the involvement of both nitrogen atoms of porphyrinic ring in the coordination of the metallic ion. Moreover, the UV-Vis spectral analysis has clearly highlighted a typical spectrum for Zn(II)P complex, described by a Soret band with a maximum at ~403 nm, accompanied by two Q bands situated in the 528 -567 nm spectral range (fig. 2 and table 1).

The FT-IR spectrum of the Zn(II)P includes typical vibration modes of both porphyrin macrocycle and phenyl substituents. Thus, the spectral band at 3418 cm^{-1} is attributed to the stretching vibration of the -OH functional group in the porphyrin complex. In the FT-IR spectrum of the Zn(II)P, the spectral band at ~2924 cm^{-1} is attributed to the C-H stretching of the phenyl groups and the spectral signal at about 1720 cm^{-1} can be attributed to the C-O groups. Also, for C=O stretching vibration, a medium absorption band was indentified at ~1624 cm^{-1}. Another IR band was identified in the higher wave number region, at ~2860 cm^{-1} and is assigned to the stretching vibration motion of C-H bond in the -O-CH$_3$ group.

Both compounds (P and Zn(II)P) were evaluated from the point of view of their action on the transmembrane potential of L929 cultured cells.

The time of action of the porphyrins upon the cells was set to 20 min due to the fact that in experiments for which the cells were subjected to the action of the porphyrins for 24 h, the transmembrane potential increased by about 10% (i.e. the porphyrins exerted a hyperpolarizing effect). However, in the PDT process, the cells undergo the action of the porphyrins in a lesser time, being eliminated by excretion in a few hours [32]. Meanwhile, the irradiation procedure in PDT implies also tenths of minutes, so it could be important whether the hyperpolarization process influences or not the fluxes of the ions in or out the cells [32].

Due to the fact that free base and the Zn(II) complex of the porphyrin present fluorescence in the proximity of the characteristic wavelengths for the probe DiBAC$_4$(3), we first tested if any spectral interference of the stimuli and the probe could affect the results. Thus, we excited the samples also at 405 nm, and monitored the emission signal

Table 1

ANALYTICAL AND SPECTRAL DATA OF THE AMPHIPHILIC PORPHYRINS USED IN STUDY

Porphyrinic compoun	Elemental analysis% (calc/exp) C H N	IR (ν, cm^{-1})	^{1}H-NMR (δ ppm) (400 MHz, $CDCl_3$)	UV-Vis** λ_{max} (nm)
*P $C_{50}H_{38}N_4O_8$	72.99 (72.87) 4.62 (4.53) 6.81 (6.67)	3414, 3314, 2922, 2862, 1717, 1624, 1606, 1511, 1436, 1261, 1099, 1020, 867, 798, 734	-2.79 (s, 2H), 4,01 (s, 6H); 4,11 (s, 6H), 5,34 (s, 2H); 7,26 (s, 2H); 7,30 (d, 2H); 7,71 (d, 2H); 7,98 (d, 4H); 8,20 (d, 4H); 8,30(d, 4H); 8,44 (d, 4H)	402, 498, 530, 572, 628
Zn(II)P $C_{50}H_{36}N_4O_8Zn$	70.17 (70.02) 4.21 (4.11) 6.54 (6.38)	3418, 2924, 2860, 1720, 1624, 1608, 1515, 1446, 1264, 1080, 1025, 860, 797	3,80 (s, 6H); 4,11 (s, 6H), 5,20 (s, 2H); 7,12 (s, 2H); 7,22 (d, 2H); 7,70 (d, 2H); 7,82 (d, 4H); 8,20 (d, 4H); 8,38 (d, 4H); 8,64 (d, 4H)	403, 528, 567

*ref [30], **solvent CH_2Cl_2

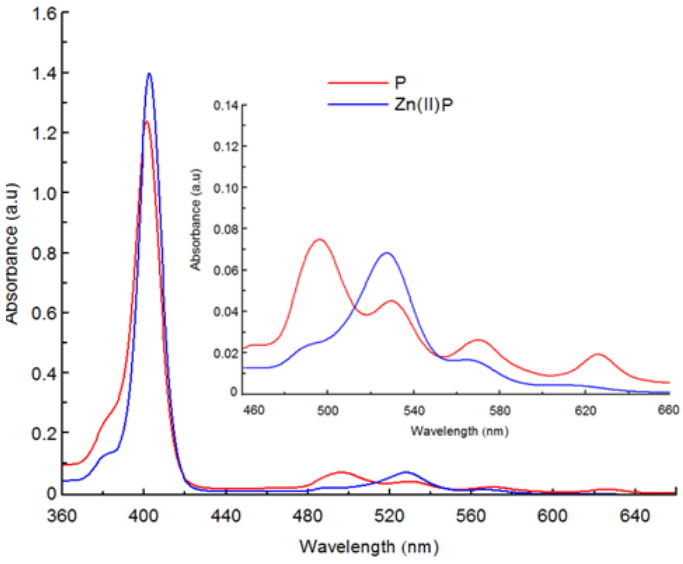

Fig. 2. Absorption spectra of the porphyrins under study in dichloromethane, c=2.5×10^{-6} M

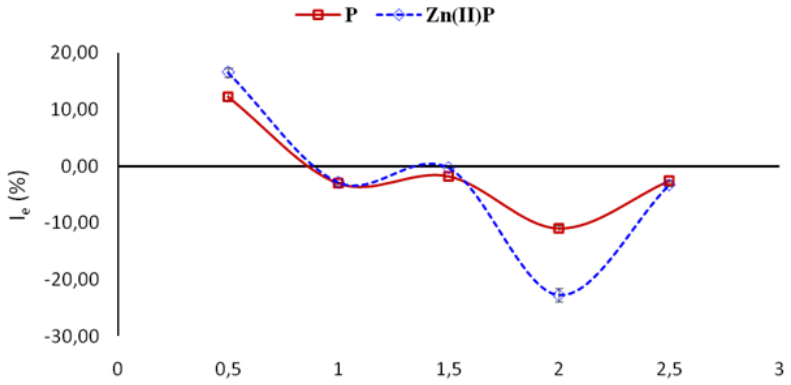

Fig. 3. The effect of 5,15-*bis*-(4-hydroxy-3-methoxyphenyl)-10,20-*bis*-(4-carboxymethylphenyl) porphyrin and Zn(II)-5,15-*bis*-(4-hydroxy-3-methoxyphenyl)-10,20-*bis*-(4-carboxymethylphenyl) porphyrin (c=0.5-2.5 μM solvent PEG 200) on the transmembrane potential of L929 cells

within the range 500-600 nm. We could note that the emission of the porphyrin in this range is very weak, so there is no spectral interference appears between the probe and the porphyrins, i.e. the signal of the voltage-sensitive probe is not affected. Under the action of the porphyrins dissolved in PEG 200, generally the cell membrane was depolarized (i.e., the absolute value of the transmembrane potential decreased in samples incubated for 20 min with the porphyrins as compared to control cells). The depolarization, while low, was dose-dependent (fig. 3). Interestingly, for the lowest dose of porphyrin tested (0.5 μM), a hyperpolarization of the membrane effect appeared for both compounds. This could be due to the low dose of porphyrin on the cells, thus the overall effect on the channel is low. In case of 2μM, a pronounced depolarization was noticed for both compounds, suggesting a bimodal behavior (and thus different action mechanisms) on the voltage-gated channels of the cell membrane. Also, for the Zn(II) complex, a higher depolarization effect was observed than in the case of the porphyrinic ligand.

Our results are in accordance with those obtained by Horning et al. [15], who showed that tetraphenylporphyrin derivatives can bind to potassium-voltage channel, having binding sites: one modulating the ion flow through the channel pore, the other binding targeting the voltage-sensor protein movement.

As studies showed also that one of the mechanisms through which porphyrins permeate the cell membrane is passive diffusion, somehow inhibited at lower temperatures [33], the fact that the experiments were carried out at 25 Celsius degrees can be considered a limitation of our study.

Anionic *meso*-tetraphenylporphyrin initially accumulates in lysosomes, but change their localization upon cell irradiation [34]. The irradiation-induced redistribution of these compounds seems to depend on the cell-cycle state of the cells [35]. This can also account for our results, and should be a controlled parameter in further studies.

Conclusions

The study revealed a depolarizing effect, dose-dependent, of the studied porphyrins on L929 cultured cells. For both compounds, the behavior at lower concentration is similar in terms of hyperpolarization effect. With the increasing of concentration, the polarizing effect of porphyrins proves to be lower. At the same value of 2μM a pronounced depolarization was noticed for ligand and its Zn(II) complexes. The pattern including in the same frame the membrane polarization and the concentration of the active compound linked by a direct connection is an effective tool in future protocols targeting one suitable photosensitizer.

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