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Photosensitization of Bacteria to Visible Light by Meso-Substituted Porphyrins

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Derivados porfínicos com 2 ou 4 substituentes catiônicos nas posições meso do macrociclo tetrapirrolico são ligados predominantemente pela membrana citoplasmática das bactérias Gram(+) e Gram(-). Por irradiação com luz visível fotossensibilizam uma inativação eficiente de ambos os tipos de bactérias (mais de 90% de mortes por irradiação com 4 lâmpadas de tungstênio de 250 W na presença de 8.4 μM do fotossensibilizador). Pelo contrário, porfinas meso-substituidas com substituentes aniônicos, assim como uma porfirina com substituição aniônica periférica (hematoporfirina), somente são fotoativas frente a bactérias Gram(+), apesar da ligação eficiente à membrana das espécies Gram(-) e da similaridade com os análogos catiônicos na fotogeração de espécies intermediárias citotóxicas.

Porphine derivatives bearing 2 or 4 cationic substituents in the meso positions of the tetrapyrrolic macrocycle are predominantly bound by the cytoplasmic membrane of Gram(+) and Gram(-) bacteria and, upon visible light irradiation, photosensitize an efficient inactivation of both bacterial types (over 99% killing upon 10 min irradiation with four 250 W tungsten lamps in the presence of 8.4 μM photosensitizer). On the contrary, anionic meso-substituted porphines as well as one anionic peripherally substituted porphyrin (haematoporphyrin) are photoactive only toward Gram(+) bacteria in spite of efficient binding to the membrane of Gram(-) species and close similarity with the cationic analogues in the photogeneration of cytotoxic intermediate species.

Keywords: *meso-porphines, photosensitization, bacteria, photosterilization*

Introduction

The dye-sensitized photoinactivation of bacteria is a very active area of research[1,2]. While Gram(+) bacteria are very sensitive to the action of a variety of visible light-absorbing photosensitizers, Gram(-) bacteria usually show low levels of photosensitivity, possibly due to a protective effect exerted by the highly organized outer wall[3], which includes murein, a peptidoglycan imparting mechanical rigidity, as well as a trilamellar structure outside the peptidoglycan layer. In fact, Gram(-) bacteria can be readily

photosensitized if the permeability of such wall is increased by treatment with chemical or biological agents, such as $CaCl_2$, Tris-EDTA or the polymixin nonapeptide[4,5].

Previous studies from our laboratory showed that the above picture is obtained also in the case of typical photodynamic sensitizers, such as haematoporphyrin and water- or lipid-soluble derivatives of Zn(II)-phthalocyanine[5,6]. We now extend our investigations to meso-substituted porphines, since the presence of selected functional groups on the methine carbon atoms was shown to impart peculiar photobiological properties to porphyrins[7,8], at least in the case of mammalian cells.

Experimental

Three porphyrin photosensitizers were used in the present investigation, namely haematoporphyrin (Hp, Porphyrin Products), meso-tetra (4-sulphonatophenyl) porphine ($TPPS_4$, Porphyrin Products) and meso-tetra(4N-methyl-pyridinium)porphine (T_4MPyP, Alfa Inorganics). Selected spectroscopic and photophysical properties of these porphyrins are summarized in Table 1. For photosensitization studies the porphyrins were dissolved in phosphate-buffered aqueous solution, at pH 7.4, and at 8.4 μM concentration.

Enterococcus seriolicida, a Gram(+) bacterium, and *Escherichia coli*, a Gram(-) bacterium, were grown in brain heart broth (Difco) and cultivated at 37 °C under aerobic conditions. The cells from broth cultures in the logarithmic phase of growth were harvested as previously described[12] and finally resuspended in 5 mM phosphate buffer, pH 7.4, in order to obtain an absorbance of 0.87 at 650 nm.

Typically, cells were incubated with 8.4 μM porphyrin solutions (1 mL) for 5 min at 37 °C; cell pellets were washed once with 5 mM phosphate-buffer, pH 7.4, and treated with 2% aqueous sodium dodecyl sulphate (SDS) in order to disrupt the cells and obtain the incorporation of the porphyrin in a monomeric state into the surfactant micelles. The porphyrin concentration was then estimated by a spectrophotofluorimetric procedure[12]. In a parallel set of experiments, porphyrin-loaded cells (10^6 cells/mL, 12 mL in Pyrex test tube) were exposed to the light of four 250 W tungsten bulbs using the same experimental arrangement as previously described[5]. The cell survival at different irradiation times was determined by the colony forming units per mL after serial 10-fold dilution of control and irradiated cells.

Table 1. Selected spectroscopic and photophysical properties of porphyrin photosensitizers (data in neutral aqueous solution, monomeric samples).

Property	Hp	$TPPS_4$	T_4MPyP
Typical functional groups	2-carboxylate (peripheral)	4-sulphonate (meso)	4 N-methyl-pyridinium (meso)
Abs max (nm)	398	410	424
Extinction coefficient (M^{-1} cm^{-1})	157,000	163,000	194,000
Triplet quantum yield[9,10]	0.63	0.84	0.92
Singlet oxygen quantum yield[10,11]	0.32	0.75	0.74

Results

The amount of porphyrin recovered from the *E. seriolicida* and *E. coli* cells after one washing with the phosphate buffer is given in Table 2. No significant variations in the amount of cell-bound porphyrin were observed if the incubations were prolonged for 60 min at 37 °C. Under these conditions, the porphyrins exhibited no appreciable intrinsic cytotoxic activity toward the two bacterial strains, since no significant difference in cell survival was observed between dark incubated and control cells.

On the other hand, upon visible light irradiation all three porphyrins caused a rapid inactivation of *E. seriolicida* (Table 3), while *E. coli* cells appeared to be photosensitive only to the action of T_4MPyP (Table 4). In order to induce some degree of photosensitivity of *E. coli* when irradiated in the presence of Hp and $TPPS_4$, it was necessary to add either $CaCl_2$ or Tris-EDTA, *i.e.* chemical agents which are known to alter the permeability of the outer membrane of

Table 2. Amount of porphyrin recovered from bacterial cells after 5 min incubation with 8.4 μM photosensitizer and one washing step with PBS recoveries expressed as nmoles of porphyrin/10^7 cells.

Porphyrin	*E. seriolicida*	*E. coli*
Hp	13.51	2.99
$TPPS_4$	0.29	1.04
T_4MPyP	1.00	0.97

The porphyrin concentration was determined by spectrophotofluorimetric analysis after extraction of the photosensitizer from the cells with 2% aqueous SDS.

Table 3. Decrease in the survival of *Enterococcus seriolicida* cells irradiated in the presence of 8.4 μM porphyrins. The figures indicate the logarithmic decrease in the overall survival.

Photosensitizer	Irradiation time (min)		
	1	5	10
Hp	4.5	6.0	7.5
$TPPS_4$	0.0	4.2	4.5
T_4MPyP	4.0	4.9	6.1

The survival of photosensitized cells (full visible light-irradiation with four 250 W tungsten lamps) was estimated by plating on solid growth medium and counting the number of colony forming units after 1 day incubation of the plates at 37 °C.

Table 4. Decrease in the survival of *Escherichia coli* cells irradiated in the presence of 8.4 μM porphyrins. The figures indicate the logarithmic decrease in the overall survival.

Photosensitizer	Irradiation time (min)		
	1	5	10
Hp	0.0	0.0	0.0
Hp + $CaCl_2$	0.5	2.5	4.5
TPPS4	0.0	0.2	0.2
T_4MP_yP	0.0	3.1	4.5

The survival of photosensitized cells (full visible light-irradiation with four 250 W tungsten lamps) was estimated by plating on solid growth medium and counting the number of colony forming units after 1 day incubation of the plates at 37 °C. Survival studies irradiation of Hp +Tris/EDTA give values identical with those obtained for Hp + $CaCl_2$.

Gram(-) bacteria. Thus, 10 min-irradiation of *E. coli* with Hp in the presence of both permeabilizing agents decreased its survival by about 4 logarithmic units (8), whereas when $TPPS_4$ was used as the photosensitizer, cell survival decreased by about 5 logarithmic units. In all cases, irradiation of the *E. seriolicida* or *E. coli* cells under identical experimental conditions, but in the absence of photosensitizers, had no effect on cell survival.

Preliminary cell fractionation studies, to be reported in a greater detail elsewhere, show that for all the porphyrins tested the cytoplasmic membrane represents the main binding site in the case of both Gram(+) and Gram(-) bacteria, since at least 80% of each photosensitizer was recovered from this subcellular compartment.

Discussion

The results reported in the present paper partly confirm previous findings indicating that Gram(-) bacteria are not affected by irradiation with full spectrum visible light or selected visible wavelengths in the presence of neutral or anionic porphyrins and phthalocyanines (see the introductory paragraph). Since photosensitivity of such bacterial cells is generated by the addition of permeabilizing chemicals, the hypothesis that the structural organization of the outer wall, which is typical of Gram(-) bacteria, probably acts as a scavenger for the photogenerated potentially cytotoxic transient species is further reinforced. Actually, the same result was obtained when singlet oxygen was produced externally to *E. coli* cells by photoexcitation of a photosensitizing dye (rose Bengal) which was immobilized on glass beads[12].

A novel observation, however, is represented by the possibility to photosensitize the inactivation of *E. coli* even in the absence of additives when a positively charged meso-substituted porphine is used. Preliminary findings from our laboratory, as well as from the group of Prof. S. Brown (University of Leeds, UK; personal communication) appear to indicate that this observation is of general validity, since it is obtained for a variety of Gram(-) bacterial strains and several types of cationic porphyrins with different chemical structures and 1 to 4 positive charges. It is unlikely that such a peculiar behaviour of cationic porphyrins reflects specific photophysical features, since the spectroscopic properties of T_4MPyP in the ground and excited triplet state, as well as its quantum yield for singlet oxygen generation, are essentially similar with those found for Hp and $TPPS_4$ (see Table 1). Moreover, all three porphyrins appear to bind predominantly to the plasma membrane, as suggested by the preliminary experiments on their subcellular distribution and by previous observations on the early involvement of membrane proteins in the photoinduced cellular damage[5].

Thus, while the reasons underlying the higher photoefficiency of T_4MPyP toward Gram(-) bacteria are not apparent at the present stage of our investigations, we hypothesize that the presence of positive charges has an orientating effect of the porphyrin toward specific sites in the membrane compartments whose integrity is critical for the functional activities of cells. This item is being presently addressed in our laboratory through a concerted application of biochemical, physiological and photobiological techniques.

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