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Evaluation of the interplay among the charge of porphyrinic photosensitizers, lipid oxidation and photoinactivation efficiency in *Escherichia coli*

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ABSTRACT

Photodynamic inactivation (PDI) is a simple and controllable method to destroy microorganisms based on the production of reactive oxygen species (ROS) (*e.g.*, free radicals and singlet oxygen), which irreversibly oxidize microorganism's vital constituents resulting in lethal damage. This process requires the combined action of oxygen, light and a photosensitizer (PS), which absorbs and uses the energy from light to produce ROS. For a better understanding of the photoinactivation process, the knowledge on how some molecular targets are affected by PDI assumes great importance. The aim of this work was to study the relation between the number and position of positive charges on porphyrinic macrocycles and the changes observed on bacterial lipids. For that, five porphyrin derivatives, bearing one to four positive charges, already evaluated as PS on *Escherichia coli* inactivation, have been tested on lipid extracts from this bacterium, and also on a simple liposome model. The effects were evaluated by the quantification of lipid hydroperoxides and by analysis of the variation of fatty acyl profiles. *E. coli* suspensions and liposomes were irradiated with white light in the presence of each PS (5.0 μM). Afterwards, total *E. coli* lipids were extracted and quantified by phosphorus assay. Lipid oxidation on bacteria and on liposomes was quantified by ferrous oxidation in xylenol orange (FOX2 assay) and the analysis of the fatty acid profile was done by gas chromatography (GC). As previously observed for *E. coli* viability, an overall increase in the lipid hydroperoxides content, depending on the PS charge and on its distribution on the macrocycle, was observed. Analysis of the fatty acid profile has shown a decrease of the unsaturated fatty acids, corroborating the relation between lipid oxidation and PDI efficiency. Bacterial membrane phospholipids are important molecular targets of photoinactivation and the number of charges of the PS molecule, as well as their distribution, have a clear effect on the lipid oxidation and on the efficiency of PDI. The distinct extent of the formation of lipid hydroperoxy derivatives, depending on the PS used, is a good indicator of this process. The FOX2 assay allowed the detection of lipid peroxidation of *E. coli* membrane after PDI with all the five porphyrins, however, it was not the most appropriated method to quantify the relative lipid oxidation caused by PS with different efficiencies. The fatty acid analysis used to quantify the extent of lipid oxidation by the different PS provided better results. The same results were observed for the liposome model. Consequently, the model system based on liposomes is a fast and simple method that can be used for the screening of the efficiency of new PS, before proceeding with the more complex studies on bacterial models.

1. Introduction

The emergence of antibiotic resistance of microbial pathogens is a severe problem worldwide [1]. Infections caused by these antibiotic-resistant microorganisms concern clinicians practicing in every field of medicine. Consequently, the search for alternative

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strategies is needed. The photodynamic inactivation (PDI) has been emerging as one of the most promising alternatives for the control of pathogenic microorganisms.

In the last decade, it has been used in the treatment of localized oncologic or skin diseases. This process relies on the combination of light, a chemical compound known as photosensitizer (PS), and molecular oxygen. Phenothiazine derivatives, xanthene dyes or tetrapyrrolic macrocycles, particularly porphyrins and phthalocyanines, have been used as PS [2]. When activated by light, in the presence of oxygen, these molecules lead to the formation of reactive oxygen species (ROS) capable of oxidizing many biological molecules such as enzymes, proteins, lipids and nucleic acids, achieving a cytotoxic effect [3,4].

Recent studies point out that membrane lipids might be particularly important targets in the photosensitization process [5]. ROS cause direct oxidative modification on bacterial lipids, namely on unsaturated lipids, and indirect modifications through reactive products of lipid peroxidation [3,6]. Lipid peroxidation could potentially alter surrounding components like proteins, nucleic acids and other molecules, in addition to lipids themselves, and represent a deleterious event associated with photo-oxidative stress [3].

Escherichia coli is commonly chosen as a representative biological model of Gram-negative bacteria which are not as susceptible as Gram-positive bacteria to photosensitization with a variety of porphyrins [5,7]. The presence of lipopolysaccharides negatively charges the bacterial outer membrane, making it impermeable to neutral or anionic compounds [8]. Besides, the well balanced architecture of the different constituents of the outer membrane hampers the interaction of the PS with the cytoplasmic membrane and intercepts the photogenerated ROS [9]. However, it is well documented that many of neutral and anionic PS can become effective against Gram-negative bacteria if they are co-administrated with outer membrane disrupting agents such as $CaCl_2$, EDTA or polymyxin B nonapeptide, which are able to promote electrostatic repulsion with destabilization of the cell wall organization [10,11]. The co-administration of these agents allows that a significant concentration of PS penetrates into the cytoplasmic membrane, which can be photosensitized after light activation of the PS. The same effect can be achieved by the use of cationic PS. The electrostatic forces generated between the positively charged PS and the constituents of the Gram-negative cell wall, as mentioned above, allow the binding and eventual penetration of the PS into the cell.

A study concerning the photoinactivation of *E. coli* in the presence of five porphyrin derivatives bearing one to four positive charges but with the same *meso*-substituent group (Fig. 1) has shown the following order of efficiency: Mono-Py^+-Me-PF < Di-Py^+-Me-PF_{opp} < Tetra-Py^+-Me < Di-Py^+-Me-PF_{adj} < Tri-Py^+-Me-PF after 90 min of treatment and Mono-Py^+-Me-PF < Di-Py^+-Me-PF_{opp} < Tetra-Py^+-Me ≈ Di-Py^+-Me-PF_{adj} ≈ Tri-Py^+-Me-PF after 270 min of treatment [unpublished data]. The results have demonstrated that the efficiency of PDI is strongly affected by the charge number and its distribution on the PS.

The aim of this work was to evaluate if the bacterial phospholipids are similarly affected by the number and position of the positive charges on the porphyrin core after PDI. For this analysis, *E. coli* was used as a model of Gram-negative bacteria and the PDI was performed under the same conditions of the previous work and in the presence of the same cationic porphyrin derivatives, presented in Fig. 1. The porphyrins with different charge number, but bearing the same pentafluorophenyl *meso*-substituent group, were used to evaluate exclusively the effect of the number and charge distribution on the oxidation of bacterial lipids. In addition, the possibility of using the liposome-based system 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) as a simple model of Gram-negative bacterial phospholipids to verify if the PS efficiency is reflected on phospholipid oxidation was also considered.

2. Material and methods

2.1. Photosensitizers

The porphyrin derivatives 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetra-iodide (Tetra-Py^+-Me), 5,10,15-tris

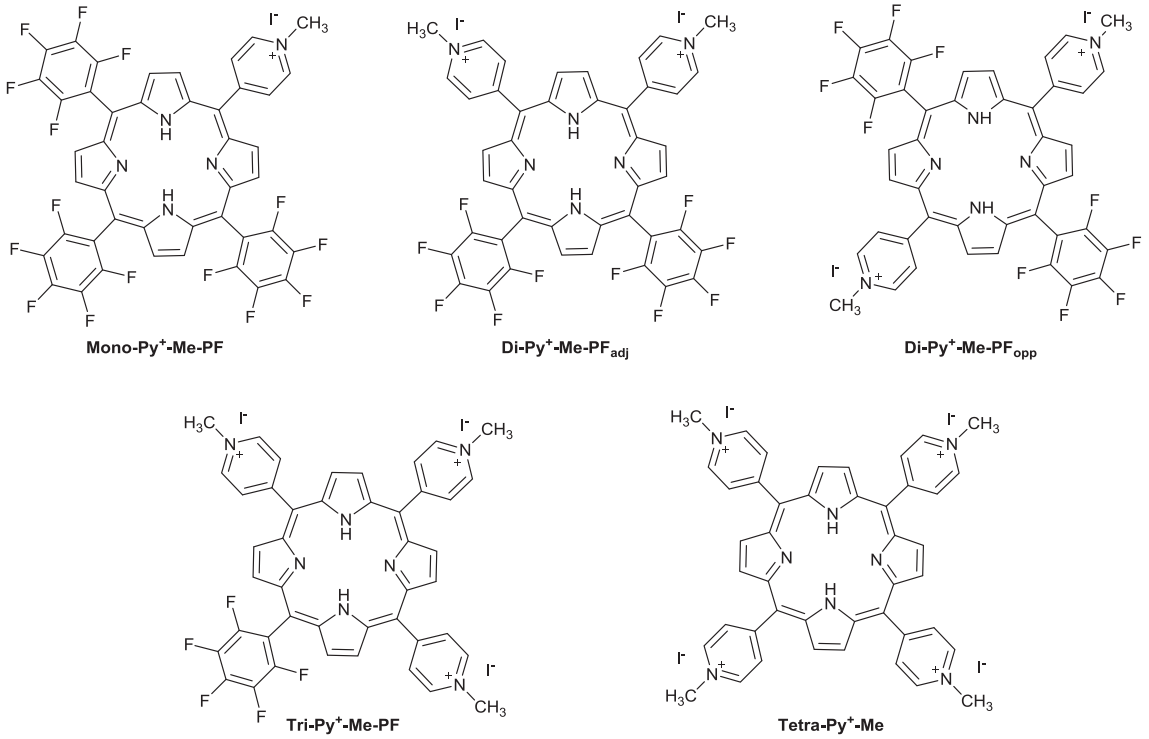

Fig. 1. Chemical structures of the cationic porphyrins used in this study.