

Inhibition of Multidrug-Resistant Gram-Positive and Gram-Negative Bacteria by a Photoactivated Porphyrin

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Submitted 21 November 2016, revised and accepted 12 January 2017

Abstract

The authors studied the *in vitro* antibacterial activity of the photo-activated porphyrin meso-tri(N-methyl-pyridyl), mono(N-tetradecyl-pyridyl)porphine (C14) against four multidrug-resistant bacteria: *Staphylococcus aureus*, *Enterococcus faecalis* (Gram-positive), *Escherichia coli*, *Pseudomonas aeruginosa* (Gram-negative). Using 10 µg/ml of porphyrin and 60 sec irradiation we observed the remarkable susceptibility of *S. aureus* and *E. faecalis* to treatment while, under the same conditions, *E. coli* and *P. aeruginosa* showed very low susceptibility. In a later stage, suspensions of Gram-negative bacteria were processed with EDTA before photo-activation, obtaining a significant decrease in viable counts. In view of the results, if the combination of low porphyrin concentrations and short irradiation times will be effective *in vivo* also, this approach could be a possible alternative to antibiotics, in particular against localized infections due to multidrug-resistant microorganisms.

Key words: antibiotic-resistant bacteria; laser light irradiation; porphyrin; photo-activation treatment

Antibiotic resistance is a very current topic health concern and represents one of the most important challenges of the 21st century to human health because, due to extensive use over the last decades, antibiotics are gradually losing their effectiveness. For example, methicillin resistant *Staphylococcus aureus* (MRSA) isolated since the early 1960s, still represents more than 60% of all the *S. aureus* isolates in US hospitals (NNIS 2004), despite recent data demonstrating that the incidence of serious infections due to MRSA has decreased since 2005 in numerous settings (Dantes *et al.*, 2013). At the same time the appearance of a further resistance against vancomycin (vancomycin resistance *Staphylococcus aureus*, VRSA) (Sievert *et al.*, 2002) and its possible transfer by conjugation (de Niederhäusern *et al.*, 2011), could aggravate the situation. Also *Enterococcus* spp., isolated from hospital and food-animal samples, has developed resistance against many antibiotics, including vancomycin (de Niederhäusern *et al.*, 2007). Finally, considering the increase of multiresistant Gram-negative bacilli (MRGNB) such as *Klebsiella* spp., *Escherichia coli* and *Pseudomonas aeruginosa* to beta-lactam antibiotics (penicillins, cephalosporins, monobactams, carbapenems) (Shaikh *et al.*, 2015), it

becomes increasingly urgent to experiment different antimicrobial treatments. A possible alternative is represented by photodynamic therapy (PDT), a treatment that achieves cytotoxic activity using a combination of visible light, a chemical compound photosensitizer and oxygen. The antimicrobial PDT was overtaken by the discovery of antibiotics, but today could offer new therapeutic opportunities for localized infections and those that don't require systemic therapies, especially if caused by multidrug-resistant bacteria. Among the several advantages of antimicrobial PDT, the most important are non-target specificity and the few side effects. Furthermore, bacterial inactivation is obtained with an action not related to the antibiotic-resistance mechanisms (Jori *et al.*, 2006; Tavares *et al.*, 2010). A remarkable variety of photosensitizing compounds (porphyrins, metallo-porphyrins and derivatives), when activated, have shown efficacy in the photo-killing of pathogenic bacteria regardless of their sensitivity or resistance to antibiotics (Lazzeri *et al.*, 2004; Merchat *et al.*, 1996b), but their efficacy can significantly change, relatively to the microorganism target (Huang *et al.*, 2010). Generally neutral and anionic photosensitizers exhibit considerable phototoxic activity against Gram-positive and

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no significant activity against Gram-negative bacteria, unless the outer membrane permeability is enhanced, prior to irradiation, by treatment with chelating agents (Reddi *et al.*, 2002). In contrast, cationic porphyrins, under appropriate conditions, promote efficient inactivation of Gram-negative bacteria also, without the need for modifying the permeability of the outer membrane (Merchat *et al.*, 1996a). During the planning of the PDT it must therefore take into account this particular feature, relative to the photosensitizers employed, as well as the operating conditions. It is therefore extremely crucial to define a protocol that would allow to obtain a reduction of the microbial cells at the same time preventing damage to the host tissues. In view of these premises, the present work investigated the antibacterial activity of a cationic porphyrin against two Gram-positive and two Gram-negative multidrug-resistant bacteria employing low concentrations of the photosensitizer, short incubation in the dark and short times of exposure to a monochromatic laser light. In a second step the Gram-negative bacteria were subjected to the same treatment after exposure to EDTA.

The following microorganisms, all from the American Type Culture Collection (Manassas, VA, USA), were used: methicillin resistant *S. aureus* ATCC BAA-2094 and vancomycin resistant *E. faecalis* ATCC BAA-2128 (Gram-positive), multidrug-resistant *E. coli* ATCC BAA-2452 and *P. aeruginosa* ATCC BAA-2109 (Gram-negative). The strains were grown at 37°C for 24 h in Tryptic Soy broth or Tryptic Soy agar (TSB or TSA, Difco Laboratories, Detroit, MI) and were maintained at 80°C in the appropriate cultivation broth containing 20% (v/v) glycerol (Merck, Darmstadt, Germany).

We used a meso substituted tetracationic porphyrine (meso-tri(N-methyl-pyridyl), mono(N-tetradecylpyridyl)porphine (C14) (kindly provided by Prof. Jori, European Patent Application EP 1 457 113 A1 2004) a synthetic compound of the tetrapyrrole series, having three positive charges situated in peripheral substituents of the tetrapyrrolic macrocycle core (alkyl chain), and one hydrophobic hydrocarbon chain of 14 carbon atoms as a peripheral substituent tail. Cell irradiation experiments were performed by using a diode laser with 635 nm wavelength, output power 50 mW, 300 nm diameter optical fibers (LAMBDA Scientifica, Vicenza, Italy).

Overnight cultures in TSB of *S. aureus* ATCC BAA-2094, *E. faecalis* ATCC BAA-2128, *E. coli* ATCC BAA-2452 and *P. aeruginosa* ATCC BAA-2109, were centrifuged at 10,000 × g for 10 min at 4°C. The supernatant was discarded and the cells were harvested, washed twice, and resuspended in Phosphate Buffer Saline (PBS, pH 6) to give of about 10⁷ CFU/ml. The resultant bacterial suspensions were aseptically distributed in eppendorf tubes and added of suitable volume of porphyrin

C14 to a final concentration of 5 and 10 µg/ml. The bacterial cells were mixed carefully, incubated in the dark and room temperature for 60", to allow the binding and/or uptake of the porphyrin, and subjected to irradiation for 30" and 60" using a monochromatic light laser. The laser fibre was placed in the bottom of eppendorf tube and a spiral movement was manually performed to ensure uniform diffusion of the light. Afterward tenfold dilutions of each suspension were seeded on TSA and, following a 24 h incubation at 37°C, viable counts (CFU/ml) were determined. Two controls were performed (only laser light irradiation and porphyrin without any irradiation).

In a second step of the study, in consideration of the low susceptibility of Gram-negative bacteria to the PD treatment, suspensions of *E. coli* and *P. aeruginosa* were processed with EDTA (3 mM) for 60" before the treatment with 10 µg/ml of porphyrin and 60" of laser light irradiation.

The experiments were repeated three times, the data (log bacterial count) were averaged and standard deviation was calculated. Bacterial reduction in percentage was determined using the following formula:

$$R\% = [(B-A) / B] \times 100$$

R: percentage reduction of the microbial cells;

A: sample microbial suspension (CFU/ml);

B: control microbial suspension (CFU/ml)

The decline rates of *S. aureus*, *E. faecalis*, *E. coli* and *P. aeruginosa* were analyzed using a t-test for paired data. A statistical probability equal to or less than 0.05 was considered significant.

The antibacterial activity of the photo-activated porphyrin against *S. aureus* ATCC BAA-2094, *E. faecalis* ATCC BAA-2128, *E. coli* ATCC BAA-2452 and *P. aeruginosa* ATCC BAA-2109 is summarized in Table I. The Gram-positive bacteria showed a remarkable susceptibility to photodynamic treatment. With the highest parameters employed (10 µg/ml of porphyrin, 60" of dark incubation time and 60" of irradiation) we obtained a bactericidal activity with viable counts decrease of 3.7 and 4.4 log CFU/ml for *S. aureus* and *E. faecalis* (99.98% and 99.996% reduction, respectively) ($p < 0.01$ compared to controls). Even using the lower parameters (5 µg/ml of porphyrin, 60" of dark incubation time and 30" of irradiation) we obtained a significant reduction (99.29% and 99.52%, respectively). In the same experimental conditions, and also employing the highest treatment parameters, the Gram-negative bacteria *E. coli* and *P. aeruginosa* showed a lower susceptibility (decrease respectively of 40.8% and 73%, $p > 0.05$). This outcome is probably due to the low outer membrane permeability that affects the uptake of the porphyrin (Reddi *et al.*, 2002). Our results against the Gram-negative bacteria, are partially in disagreement with the studies of Merchat *et al.* (1996a) who found

Table I
Antibacterial activity of photo-activated porphyrin against *S. aureus*, *E. faecalis*, *E. coli* and *P. aeruginosa* after 1 min dark incubation

Start	laser 30"	laser 60"	Por 5 µg	Por 10 µg	Por 5 µg laser 30"	Por 5 µg laser 60"	Por 10 µg laser 30"	Por 10 µg laser 60"
<i>S. aureus</i>	6.96* ± 0.07	6.95 ± 0.08	5.98 ± 0.04	5.53 ± 0.04	4.87 ± 0.01	4.28 ± 0.03	3.87 ± 0.01	3.25 ± 0.02
	7.02 ± 0.04*	12.5%**	13.46%	90.76%	96.73%	99.29%	99.82%	99.93%
<i>E. faecalis</i>	7.02 ± 0.08	7.11 ± 0.07	5.14 ± 0.02	4.95 ± 0.07	4.90 ± 0.03	4.56 ± 0.09	3.17 ± 0.05	2.83 ± 0.06
	7.22 ± 0.05	37.12%	22.15%	91.79%	99.46%	99.52%	99.78%	99.991%
<i>E. coli</i>	7.18 ± 0.04	7.06 ± 0.06	7.12 ± 0.04	7.11 ± 0.06	7.10 ± 0.08	7.08 ± 0.01	7.02 ± 0.03	6.99 ± 0.05
	7.21 ± 0.02	8.7%	8.7%	20.12%	20.7%	21.95%	26.2%	35.5%
<i>P. aeruginosa</i>	5.73 ± 0.09	5.74 ± 0.03	5.63 ± 0.03	5.56 ± 0.08	5.61 ± 0.05	5.60 ± 0.02	5.54 ± 0.07	5.51 ± 0.02
	6.08 ± 0.06	55%	54%	64.4%	69.7%	65.67%	66.6%	70.83%
								73%

* Log CFU/ml, ** % reduction, Por = porphyrin

that cationic porphyrins like C14 show bactericidal activity without the addition of chelating agents. This discrepancy could be due to the feature of our experimental design, carried out in mild operating conditions: low dark incubation time with photosensitizer (60") and low laser light irradiation time (30"–60"). In the second step of the study, the addition of EDTA to the cell suspensions before PDT treatment has produced a decrease of about 2,7 and 3,5 log CFU/ml in *E. coli* and *P. aeruginosa* viable counts (Table II), with a reduction of 99.83 and 99.97 %, respectively ($p < 0,01$). Relatively to controls, the only exposure to laser light does not caused significant effects on bacterial viability, while the not photo-activated porphyrin at the highest concentration (10 µg/ml) produced a reduction of 1.5 and 2.3 log CFU/ml for *S. aureus* and *E. faecalis*, and lower than 1 log for *E. coli* and *P. aeruginosa*. This antibacterial activity observed without laser light irradiation could be ascribed to the presence of the long hydrocarbon tail, which can interact with hydrophobic areas in the cell membrane, inducing a marked alteration of the native three-dimensional architecture and impairing specific metabolic processes (Maraggia, 2006).

According to our results if the experimental design employed in this study is proved effective in bacterial photo-inactivation also *in vivo*, the antimicrobial PDT

could be suitably used for less invasive treatments that do not require systemic antibiotic therapies. Porphyrin C14 or a similar photosensitizer could be employed for the treatment of localized infections, chronic wounds, oral candidiasis and in the dental field for cariogenic and periodontal diseases (Jori *et al.*, 2006) in particular when caused by multidrug-resistant Gram-positive and Gram-negative bacteria. The low sensitivity of Gram-negative bacteria observed in our study can be overcome by employing a combination of porphyrin and EDTA. Using this association we achieved a reduction in viable counts such as those observed for the Gram-positive bacteria. The advantage of PDT, as appears from our data, is that the bactericidal activity is obtained employing low dark incubation time with photosensitizer, low dosages of porphyrin and very short irradiation times; as previously referred by Dai *et al.* (2009), if irradiation is performed at short intervals after photosensitizer application (minutes), the PDT damage to host tissue will be minimized. Membranes and cell wall components are the main targets of PDT and the photosensitizers do not need to enter the cell, but the adhesion to these structures is sufficient for bacterial inactivation. In this way the microorganisms don't have the possibility to develop resistance through the known mechanisms. Unlike antibiotics, repeated

Table II
Antibacterial activity of photo-activated porphyrin against *E. coli* and *P. aeruginosa* (60" dark incubation with 10 µg/ml of porphyrin) after EDTA addition

Start	Laser 60"	Por 10 µg	EDTA 60"	EDTA 60" Por 10 µg	Laser 60" Por 10 µg	EDTA 60" Laser 60" Por 10 µg
<i>E. coli</i>	7.6* ± 0.07	7.58 ± 0.04	7.54 ± 0.08	7.28 ± 0.02	7.34 ± 0.05	4.92 ± 0.02
	7.70* ± 0.04	18%**	24%	30%	62%	56%
<i>P. aeruginosa</i>	7.40 ± 0.03	7.34 ± 0.08	7.34 ± 0.09	7.32 ± 0.05	5.34 ± 0.04	4.38 ± 0.07
	7.89 ± 0.04	67.53%	71.81%	71.81%	72.98%	99.72%
						99.97%

* log CFU/ml, ** % reduction, Por = porphyrin

photodynamic treatments do not seem to induce the selection of resistant bacteria, as singlet oxygen and free radicals interact with numerous cell structures and different metabolic pathways of the microorganisms (Wainwright and Crossley, 2004). Consequently, this therapeutic approach may be a viable alternative to the use of antibiotics in particular against infections due to multidrug-resistant bacteria, opening new prospects for the use of photosensitized processes in the medical field.

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