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Communication

Bactericidal effect of photodynamic therapy against methicillin-resistant *Staphylococcus aureus* strain with the use of various porphyrin photosensitizers

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Photodynamic therapy (PDT) is based on photosensitizers activated by light of appropriate wavelength. Their activation leads to generation of singlet oxygen and free radicals responsible for the cytotoxic effect. The aim of this project was to compare the bactericidal effect of PDT using different porphyrin photosensitizers against a methicillin-resistant *Staphylococcus aureus* strain. Exogenous sensitizers (protoporphyrin IX and newly synthesized derivative, protoporphyrin diarginate) induced a 3 $\log_{10}$-unit reduction in bacterial viable counts. With the use of endogenous, ALA-induced porphyrins, a 1.6 $\log_{10}$-unit reduction was obtained. The sensitizers tested executed their antibacterial activity with no essential change in the antibiotic resistance pattern of the studied strain.

Keywords: MRSA, photodynamic therapy, photosensitizer, porphyrin

INTRODUCTION

Photodynamic therapy (PDT) is a potential therapy against cancerous tumours (Szurko *et al.*, 2003; Chwilkowska *et al.*, 2003) and has recently been studied against a wide range of bacteria, fungi, yeasts, and viruses that cause serious problems of contemporary medicine (Jori & Brown, 2004). Photodynamic therapy is based on chemicals called photosensitizers that are activated by light of adequate wavelength. Its activation leads to the generation of singlet oxygen and free radicals responsible for the cytotoxic effect against specific cells (Romanova *et al.*, 2003). The aim of this study was to analyze the bactericidal effect of PDT against methicillin-resistant *Staphylococcus aureus* (MRSA) which is the most important etiological agent responsible for hospital-acquired infections (Kurlenda *et al.*, 2007). Some MRSA infections may be life-threatening, especially in the case of immuno-compromised patients, causing bacteremia, endocarditis, sepsis or toxic-shock syndrome (Jarraud *et al.*, 2002). Their multiresistance to antibiotics leads to many therapeutic problems, and for this reason an alternative method to antibiotic therapy needs to be developed. It seems that photodynamic therapy may be an effective and alternative therapeutic option against *S. aureus* strains (Embleton *et al.*, 2004; Gad *et al.*, 2004) and potentially against other bacterial pathogens like *S. epidermidis* (Zeina *et al.*, 2001; Gad *et al.*, 2004), *Propionibacterium acnes* (Ashkenazi *et al.*, 2003) *Listeria monocytogenes* (Romanova *et al.*, 2003), *Streptococcus pyogenes*, *Corynebacterium minutissimum*, *Bacillus subtilis*, and *Enterococcus faecalis* (Shawar & Cooper, 1990; Zeina *et al.*, 2001). Different chemical compounds with photoactive properties have already

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Abbreviations: ALA, δ-aminolevulinic acid; c.f.u., colony forming units; MRSA, methicillin-resistant *Staphylococcus aureus*; PDT, photodynamic therapy; $PPArg_2$, protoporphyrin diarginate; PPIX, protoporphyrin IX.

been tested against Gram-positive bacteria. Various photosensitizers such as haematoporphyrin (Bertoloni *et al.*, 2000), porphyrin derivatives (Lasocki *et al.*, 1999; Lambrechts *et al.*, 2005), phenothiazinium salts (Bisland *et al.*, 2006; Tegos & Hamblin, 2006), chlorin (i.e. SnCe6) (Gad *et al.*, 2004; Embleton *et al.*, 2005) and δ-aminolevulinic acid-induced porphyrin sensitizers (Nitzan *et al.*, 2004; Bisland *et al.*, 2006) were studied and found to demonstrate a high bactericidal effect after illumination with visible light against *S. aureus* strains.

Photoactivated sensitizers such as protoporphyrin IX (PPIX) and arginine haematoporphyrin derivatives (HpD-Arg_2) reveal bactericidal activity against several bacterial species, including MRSA (Orenstein *et al.*, 1997; Lasocki *et al.*, 1999). Protoporphyrin diarginate ($PPArg_2$), which was recently synthesized and has not been described yet, was used in this study. Additionally, previously published studies demonstrate that *S. aureus* is able to produce high amounts of porphyrins upon induction by δ-aminolevulinic acid (ALA) which may be used as an ideal stimulator of endogenous sensitizer production in photodynamic therapy (Nitzan *et al.*, 2004; Bisland *et al.*, 2006). The aim of this study was to compare the bactericidal effect achieved by the action of the above-mentioned photosensitizers.

As damage of genomic and plasmid DNA is one of the results of photodynamic activity (Romanova *et al.*, 2003) and some antibiotic resistance mechanisms are DNA-dependent, a change in the resistance pattern has been suggested to be observed. The influence of photodynamic therapy on the antibiotic resistance pattern of the studied strain was therefore analyzed.

MATERIALS AND METHODS

Protoporphyrin diarginate. $PPArg_2$ is a newly synthesized chemical compound and its bactericidal effect as a photosensitizer has not been studied so far. Its synthesis, purification and molecular characterization is described in published Polish, European and United States patents (Konarski, 1990; Padzik-Graczyk *et al.*, 1993; Graczyk & Konarski, 1994; 1995; Graczyk & Konarski, 1997). The molecular data concerning $PPArg_2$, such as molecular formula, molecular weight, composition and nominal mass are presented in Fig. 1. The molecular data concerning unmodified protoporphyrin IX are presented in Fig. 2.

MRSA isolate. The investigated clinical strain of MRSA (methicillin-resistant *Staphylococcus aureus*), of known resistance level (Table 1), was isolated from the Provincial Hospital in Gdańsk. The isolate was characterized by Gram-staining and its ability to produce coagulase and clumping factor using Slidex Staph Plus (BioMerieux). Additionally, the species was identified using the biochemical identification system ID 32 Staph (BioMerieux).

Photodynamic therapy. Photodynamic therapy with the use of exogenous porphyrin photosensitizers was conducted as follows: stock solutions of photosensitizers at a concentration of 10 mM were prepared (PPIX (MP Biomedicals) in 100% DMSO (Sigma) and $PPArg_2$ in distilled water) and stored at –20°C in the darkness until use. The bacterial culture was grown overnight at 37°C in nutrient trypcase soy broth (BioMerieux) and then diluted with fresh broth to the density OD_{600} 0.05. An appropriate volume of stock photosensitizer solution (0.8 to 4 μl) was added to 0.8 ml of the MRSA culture to achieve the desired final concentration, from 10 to 50 μM. The culture was incubated at 37°C for 15 min in the darkness and then loaded into a 96-well plate and treated with an appropriate light dose. The total volume of the culture in each well was 0.1 ml. An identical microplate was incubated in the darkness in the same conditions and served as a control. Thus, there were three types of controls: *S. aureus* treated solely with light, kept with photosensitizer in the darkness and *S. aureus* kept without sensitiz-

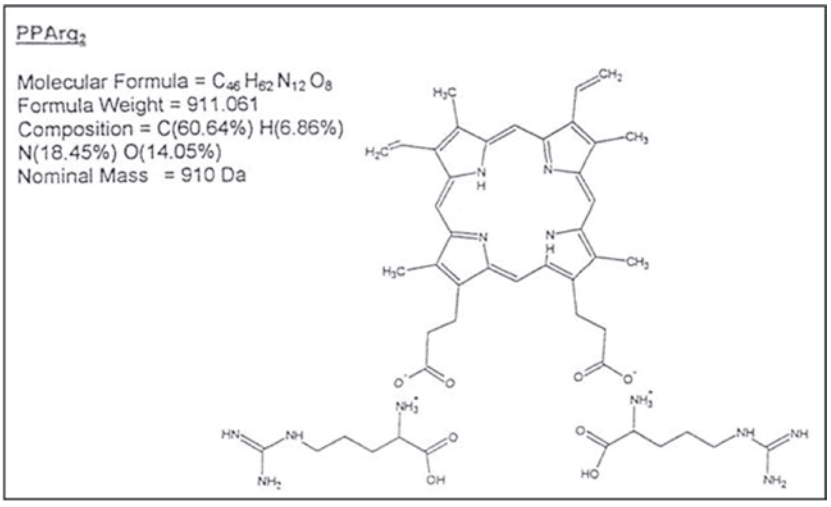

Figure 1. Molecular characteristics of protoporphyrin diarginate ($PPArg_2$).

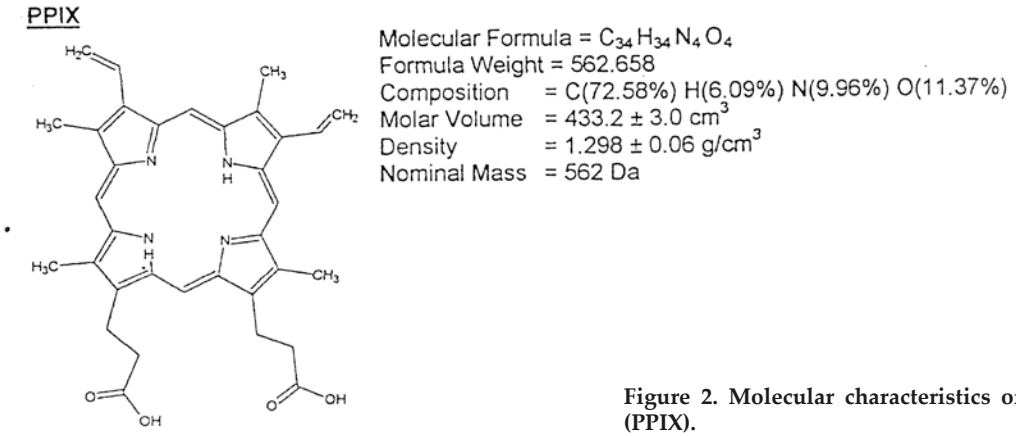

Figure 2. Molecular characteristics of protoporphyrin IX (PPIX).

er and light. After the illumination, aliquots (10 μl) were taken from each well to determine the number of colony-forming units (c.f.u.). The aliquots were serially diluted 10-fold in PBS (0.13 mol/l NaCl, 8.1 mmol/l Na_2HPO_4, 1.47 mmol/l KH_2PO_4, 2.68 mmol/l KCl) to give dilutions of 10^{-3} (for PDT with PPIX and $PPArg_2$) and 10^{-4} (for PDT with ALA). Aliquots (10 μl) of each of the dilutions were plated on trypcase soy agar (BioMerieux). After 18 h of incubation at 37°C in the darkness the colonies formed were counted and the results were analyzed statistically. In the case of ALA-induced production of endogenous porphyrins analogous conditions were used, with the δ-aminolevulinic (MP Biomedicals) stock concentration of 1 M prepared in distilled water and the time of incubation of the bacterial culture with ALA ranging from 1 to 24 hours. The final concentration of ALA was 10 mM. Each experiment was done three times.

Antibiotic susceptibility. The antibiotic susceptibility of PDT-treated and untreated strain was determined by the disc-diffusion method according to the criteria of the recommendations for susceptibility testing (Hryniewicz *et al.*, 2005). The influence of the PDT treatment on the antibiotic susceptibility was tested under experimental conditions showing the highest level of the bactericidal effect. In the case of exogenous sensitizers, it was done at the concentration of 50 μM and the light dose of 12 J/cm^2. When the endogenous sensitizers were analyzed, the antibiotic susceptibility was determined after the PDT treatment following 24 h incubation with ALA and the light dose of 50 J/cm^2. After the PDT treatment, 10-μl aliquots of each of the dilutions were plated as above, incubated for 18 h at 37°C in the darkness, then five colonies were picked at random, diluted in PBS and used to determine the antibiotic susceptibility (Hryniewicz *et al.*, 2005). The following antibiotic discs were used: oxacillin (OX, 1 μg), erythromycin (E, 15 μg), lincomycin (L, 15 μg), gentamycin (GM, 10 μg), ciprofloxacin (CIP, 5 μg), sulphamethoxazole/Trimethoprim (SXT, 23.75/1.25 μg) and vancomycin (VA, 30 μg) for a standard antibiogram, and tetracycline (TE, 30 μg), rifampicin (RA, 5 μg), fusidic acid (FA, 10 μg), penicillin G (PEN, 10 units), ampicillin (AMP, 10 μg), mupirocin (MUP, 200 μg), nitrofurantoin (NF, 300 μg), ofloxacin (OFX, 5 μg), trimethoprim (TMP, 5 μg), teicoplanin (TEC, 30 μg), chloramphenicol (CHL, 30 μg), clindamycin (DA, 2 μg), netilmicin (NET, 30 μg), tobramycin (TOB, 10 μg), amikacin (AK, 30 μg), kanamycin (K, 30 μg), linezolid (LZD, 30 μg), telithromycin (TEL, 15 μg) and quinupristin/dalfopristin (QD, 15 μg) for an extended antibiogram.

Light source. The illumination was carried out using a BioStimul Lamp which emits polarized (96% level of polarization) monochromatic light (624 nm ± 18 nm) (BIOTHERAPY, Czech Republic). A light power meter (model LM1, CARL ZEISS, Jena, Germany) served to determine the delivered light energy, which was approx. 0.2 J/cm^2 per minute.

RESULTS AND DISCUSSION

Protoporphyrin IX as well as $PPArg_2$ exert an effective antibacterial activity even in the concentration of 10 μM, illumination lasting for 60 min and the light dose of only 12 J/cm^2. In these experimental conditions the phototoxic effect resulted in 2.4 and 2.1 $\log_{10}$-unit reduction in viable counts (for PPIX and $PPArg_2$, respectively). A cytotoxic effect of 0.2 and 0.22 $\log_{10}$-unit reduction was observed (for PDT with PPIX and $PPArg_2$, respectively) (Figs. 3 and 4). As DMSO was used to dissolve the non-polar PPIX, the cytotoxicity of DMSO as a control solution was analyzed. To give the final concentration of PPIX ranging from 10 to 50 μM, the concentrations of DMSO were 0.1 to 0.5%, respectively. No cytotoxic or phototoxic effect of DMSO was observed at these conditions (not shown). When the ALA-induced endogenous porphyrins were studied, the bactericidal

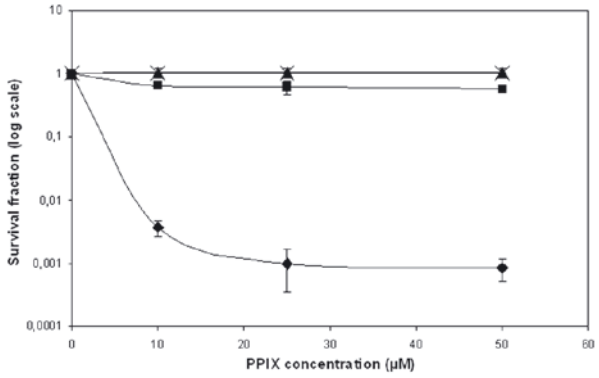

Figure 3. Photo- and cytotoxic effect of protoporphyrin IX (PPIX) with the light dose of 12 J/cm^2.
The survival rate was calculated from the number of c.f.u. in the PDT-treated sample divided by the number of c.f.u. in the sample kept in the darkness without photosensitizer. Designations are as follows: PDT-treated *S. aureus* (◆), *S. aureus* treated solely with light (▲), kept with photosensitizer in the darkness (■), and without sensitizer and light (×). Each experiment was done three times, and error bars show S.D.

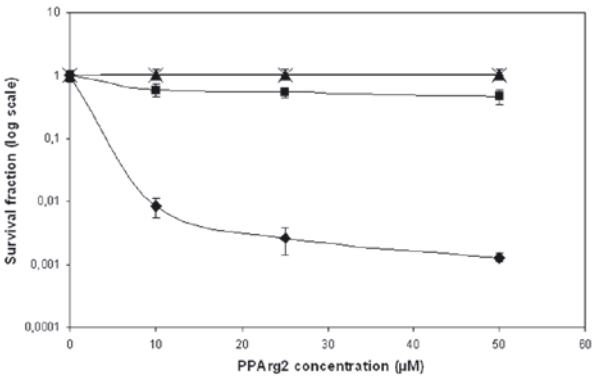

Figure 4. Photo- and cytotoxic effect of $PPArg_2$ with a light dose of 12 J/cm^2.
Survival fraction determined as in Fig. 3. Designations are the same as in Fig. 3.

effect of 1.6 $\log_{10}$-unit reduction was obtained after 24 h of incubation and the light dose of 50 J/cm^2 (Fig. 5). The cytotoxic effect after this incubation time was approx. 0.3 $\log_{10}$-unit reduction.

Lasocki *et al*. (1999) reported that photodynamic therapy with the use of another water-soluble porphyrin sensitizer, haematoporphyrin diarginate derivative (HpD-Arg_2), at the concentration of 25 μg/ml revealed a high bactericidal effect against *S. aureus*. In their studies 99.99% eradication was reached with the illumination carried out using a home-made visible light source (the light intensity was 80 klx and the illumination time was 30 min). Similar results were obtained by Szpakowska *et al*. (2001). In those studies, regarding MRSA strains, the minimal bactericidal concentration (MBC) of HpD-Arg_2 ranged from 1.6 to 50 μg/ml. Our studies show that concentration of 9 μg/ml of protoporphyrin diarginate gives a (99.4%) 2.2 $\log_{10}$-unit reduction in viable counts. A (99.9%) 3 $\log_{10}$-unit reduction was achieved using the concentration of 45 μg/ml (50 μM) of $PPArg_2$. However, in our studies the illumination of the bacterial cultures was performed with the use of a 630 nm wavelength light source. Banfi *et al.* (2006) studied the antibacterial activity of different tetraaryl-porphyrin photosensitizers and showed that even in the concentration of 1–3 μM an almost 7 $\log_{10}$-unit decrease could be obtained. However, their studies were performed with the use of broadband white light (380–780 nm) of a 500 W tungsten-halogen lamp and the delivered light energy was approx. 133 J/cm^2. In our studies the delivered light energy was only 12 J/cm^2. Porphyrin-based photosensitizers were also studied by Maisch *et al.* (2005). In their studies incubation with only 0.005 μM porphyrin-derivative sensitizer followed by illumination yielded a 3 $\log_{10}$-decrease in the viable cells. We

Table 1. Antibiotic susceptibility of PDT-treated and untreated *S. aureus* strain.

S. aureus	Antibiotics[a]:									
	Standard antibiogram									
	OX	**E**	**L**	**GM**	**CIP**	**SXT**	**VA**			
Untreated	0	0	18	0	0	27	20			
PDT-treated[b]	0	0	17	0	0	26	19			
	Extended antibiogram									
	TE	**RA**	**FA**	**PEN**	**AMP**	**MUP**	**NF**	**OFX**	**TMP**	**TEC**
Untreated	10	32	36	10	14	38	30	15	35	21
PDT-treated[b]	10	30	34	10	13	36	30	14	33	20
	CHL	**DA**	**NET**	**TOB**	**AK**	**K**	**LZD**	**TEL**	**QD**	
Untreated	34	28	35	36	33	32	30	21	25	
PDT-treated[b]	34	26	34	34	32	32	30	20	25	

[a]For abbreviations used see Materials and Methods in Antibiotic susceptibility paragraph. [b]$PPArg_2$–based photodynamic therapy (sensitizer concentration 50 μM and the light dose of 12 J/cm^2). The resistance pattern was determined with the disc-diffusion method (numbers present the diameters of zones of growth inhibition in mm).

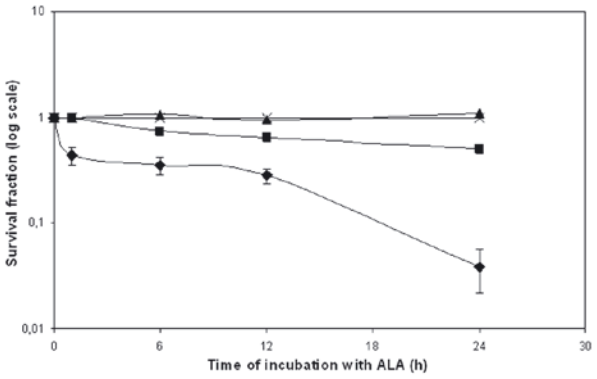

Figure 5. Photo- and cytotoxic effect of 10 mM ALA with a light dose of 50 J/cm^2.
Survival fraction determined as in Fig. 3. Designations are the same as in Fig. 3.

suppose that such a high bactericidal effect resulted from the illumination performed with an incoherent light source with the emission λ of 380 to 480 nm. It must be considered that the absorption spectra of porphyrins show an intense Soret band with a maximum centered at about 422 nm and a series of weak bands, between 500 and 650 nm, whose intensities are below 5% of the Soret band intensity. Thus, illumination carried out with a 380–480 nm light source results in a much more efficient excitation of the porphyrin sensitizers.

In the case of ALA-induced production of endogenous porphyrin sensitizers, Nitzan *et al*. (2004) reported that staphylococcal strains produced high amounts of porphyrins when incubated with 0.38 mM ALA for 4 h. Upon illumination of the ALA-induced strains with 407–420 nm blue light, a decrease of five orders of magnitude was demonstrated with a light dose of 50 J/cm^2. Total eradication could be achieved with a 100 J/cm^2 dose. In our studies a 1.6 $\log_{10}$-unit reduction in viable counts was reached after 24 h of incubation with 10 mM ALA and the light dose of 50 J/cm^2. Probably, such ambiguous bactericidal effects result from illumination performed with the use of a different light source. Although δ-aminolevulinic acid is known to be phototoxic, it was important to evaluate its bactericidal effect using the same experimental conditions as for $PPArg_2$ and PPIX. This allowed the conclusion to be drawn that exogenous sensitizers exert a higher bactericidal effect than the endogenous sensitizers produced upon administration of ALA. When the light dose of 12 J/cm^2 was delivered to the studied sample, no bactericidal effect could be observed (not shown). Moreover, the 24-hour incubation with ALA is not suitable as a treatment scheme from the clinical point of view. This incubation time could probably be shortened even to 4 h according to Nitzan *et al.* (2004) when a light source emitting 407–420 nm blue light is used.

On the basis of these preliminary studies we may conclude that all the photosensitizers tested, including ALA-induced endogenous porphyrins, may exert a high bactericidal effect against methicillin-resistant *S. aureus* strains. Moreover, protoporphyrin diarginate ($PPArg_2$) was as effective as unmodified protoporphyrin IX. Being water-soluble, $PPArg_2$ may penetrate the infected tissues deeper and hence exert its bactericidal effect more effectively, which is significant from the clinical point of view. Moreover, the fact that $PPArg_2$ is water-soluble eliminates the use of DMSO or other cytotoxic solvent to prepare the solution of the photosensitizer. This is clinically important, as such toxic compounds may decrease the viability of eukaryotic cells surrounding the site of bacterial infection. The obtained results suggest that protoporphyrin diarginate may exert a high antibacterial effect. However, more efficient, although also more complicated, photoinactivation systems have been found, i.e. those described by Embleton *et al.* (2005) using phage-mediated delivery of the sensitizer into the target cell. Another effective photoinactivation system for methicillin-resistant *S. aureus* strains is proposed by Ferro *et al.* (2006) who suggest the use of liposome-mediated delivery of the photosensitizing agents.

Additionally, we asked whether the resistance pattern of the studied MRSA strain changes in the course of PDT action using both endogenous and exogenous sensitizers. Malik *et al.* (1994) revealed that bacteria surviving porphyrin treatment had an impaired resistance to penicillin caused by the damage of plasmids responsible for 13-lactamases synthesis. In our study no essential change in the resistance pattern was observed. The resistance to antibiotics was the same before and after the PDT treatment based on exogenous as well as endogenous photosensitizers. The obtained results suggest that photodynamic therapy with the use of protoporphyrin diarginate may be an effective bactericidal method against multiresistant *Staphylococcus aureus* strains and potentially against other bacterial pathogens.

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