Acta Biochimica Polonica

Vol. 55 No. 1/2008, 85–90

on-line at: www.actabp.pl

Regular paper

Photodynamic effect of protoporphyrin diarginate ($PPArg_2$) on methicillin-resistant *Staphylococcus aureus* and human dermal fibroblasts

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Received: 20 September, 2007; revised: 13 December, 2007; accepted: 16 January, 2008
available on-line: 23 January, 2008

The worldwide rise in the antibiotic resistance of bacteria forces the development of alternative antimicrobial treatments. A potential approach is photodynamic inactivation (PDI). The aim of the present study was to determine the phototoxicity of protoporphyrin diarginate ($PPArg_2$) against methicillin-resistant *Staphylococcus aureus* and human dermal fibroblasts. Different concentrations (0 to 20 μM) of $PPArg_2$ and light dose of 6 J cm^{-2} were tested. Cell viability was evaluated using the methylthiazoletetrazolium (MTT) assay. Incubation with 10 μM followed by illumination yielded a 3.6 log_{10}-unit reduction in the viable count for *Staphylococcus aureus*. At the same experimental conditions, only 22.5% of the fibroblasts were photoinactivated. Protoporphyrin diarginate at concentrations up to 20 μM demonstrated no toxicity towards *S. aureus* or fibroblasts when not irradiated. These results suggest that the protoporphyrin diarginate exerts a high bactericidal effect against methicillin-resistant *S. aureus* strain without harming eukaryotic cells.

Keywords: antimicrobial photodynamic inactivation, cytotoxicity, fibroblasts, MRSA, phototoxicity

INTRODUCTION

As the incidence of bacterial strains resistant to antimicrobial agents rises, the development of new antibacterial strategies becomes increasingly important. In the past decade, the first clinical isolate of a methicillin resistant *Staphylococcus aureus* (MRSA) strain with reduced susceptibility to vancomycin was reported in Japan (Hiramatsu *et al.*, 1997) and later also in the United States and France (Ploy *et al.*, 1998; Smith *et al.*, 1999). In 2002 the first documented case of an infection caused by vancomycin-resistant *S. aureus* was reported (Sievert, 2002). Additionally, MRSA are important etiological factors responsible for health care-associated life-threatening infections (Grinholc *et al.*, 2007a; Kurlenda *et al.*, 2007). Particularly burn wound infections remain a potentially serious problem and are an important cause of death. In some countries, e.g. Portugal, MRSA strains can constitute up to 65% of all *S. aureus* isolates in hospitals and produce many therapeutic problems including MRSA outbreaks (Aires de Sousa *et al.*, 1998; Leski *et al.*, 1998; Kurlenda *et al.*, 2007). In Poland the average prevalence of MRSA in hospitals varies from 2.3% to 59.9% (Hryniewicz *et al.*, 1993; Piechowicz *et al.*, 1993). Many human pathogens are now multiresistant to antimicrobial drugs and skin infections with such organisms may be particularly difficult to treat.

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Abbreviations: ALA, delta-aminolevulinic acid; cfu, colony forming units; DMSO, dimethylsulfoxide; EC_{50}, 50% effective concentration; MRSA, methicillin-resistant *Staphylococcus aureus*; MTT, methylthiazoletetrazolium; PDI, photodynamic inactivation; PPArg2, protoporphyrin diarginate.

An alternative therapeutic approach may be to use photodynamic inactivation (PDI), which employs visible light in the presence of a photosensitizing agent. An activated sensitizer may react with molecules from its direct environment by electron or hydrogen transfer. The sensitizer, in its photoactive triple state, can react with a local substrate to form cytotoxic radicals (type 1 reaction) or transfer its energy directly to oxygen generating the reactive singlet oxygen (type 2 reaction) (Wainwright, 1998). Both pathways can lead to or induce cell death. These highly reactive oxygen species initiate further oxidative reactions in the direct environment. Free radicals and peroxides, which are produced as a result of PDI photooxidation reactions, may damage a number of cellular structures, like the bacterial cell wall, lipid membranes, enzymes, or nucleic acids (Halliwell & Gutteridge, 1984; Baumler *et al.*, 1999).

Different chemical compounds with photoactive properties have already been tested against Gram-positive bacteria. Various photosensitizers such as haematoporphyrin (Bertoloni *et al.*, 2000), porphyrin derivatives (Lasocki *et al.*, 1999; Lambrechts *et al.*, 2005a; 2005b), phenothiazinium salts (Bisland *et al.*, 2006; Tegos & Hamblin, 2006), chlorin (Embleton *et al.*, 2002; Gad *et al.*, 2004) and 5-aminolaevulinic acid-induced porphyrin sensitizers (Nitzan *et al.*, 2004; Bisland *et al.*, 2006) have been studied and found to demonstrate high bactericidal effect against *S. aureus* strains after illumination with visible light. However, in evaluating the potential of PDI for the clinical treatment of, particularly, skin infections, it is important to assess not only the bactericidal efficacy, but also the cytotoxic effects against healthy dermal cells. The cytotoxicity of many sensitizers described above toward keratinocytes and fibroblasts has been studied (Haddad *et al.*, 1999; Ramaiah *et al.*, 2002; Zeina *et al.*, 2002; 2003; Chiu *et al.*, 2005; Lambrechts *et al.*, 2005a; 2005b; Maisch *et al.*, 2005). Zeina *et al.* (2002) reported that antimicrobial photodynamic therapy sufficient to reduce microbes by seven log cycles would have little cytotoxic and no genotoxic effect on keratinocytes. Haylett *et al.* (2003) showed that following photoinactivation, some DNA damage was detected in fibroblasts, but it was fully repaired within 24 h of treatment. Soukos *et al.* (1996) claimed that photosensitization of keratinocytes and fibroblasts did not reduce cell viability in given experimental conditions, whereas an effective bactericidal activity was obtained. Previously published data confirm the high bactericidal activity of protoporphyrin diarginate ($PPArg_2$) against an *S. aureus* strain (Grinholc *et al.*, 2007b). However, its toxicity towards fibroblasts has not been investigated yet.

The use of visible light in conjunction with an appropriate photosensitizer may be a useful alternative to antibiotics for microbial skin infections. However, it is important to determine the effect of PDI on skin cells using the same conditions that are known to be effective against microbes. Therefore, the aim of the present *in vitro* studies was to investigate the photodynamic inactivation of a multiresistant *S. aureus* strain and healthy human dermal fibroblasts, to determine if microbes could be effectively killed without damaging adjacent fibroblasts.

MATERIALS AND METHODS

Chemicals. All cell culture material was purchased from Gibco-Invitrogen (Paisley, UK). All other chemicals were purchased from Sigma-Aldrich (Germany).

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

Cell line. Human skin fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units ml^{-1} penicillin and 100 $\mu g\ ml^{-1}$ streptomycin. Cultures were maintained in a humidified atmosphere containing 5% CO_2 at 37°C.

Photosensitizer. The stock solution of the photosensitizer protoporphyrin diarginate ($PPArg_2$) (Institute of Optoelectronics, Military University of Technology, Warszawa, Poland) was prepared in distilled water at a concentration of 10 mM and stored at –20°C in darkness until use.

Light source. The illumination was performed with a BioStimul Lamp (Biotherapy, Czech Republic). Delivered light energy was determined with the use of a light power meter (model LM1, CARL Zeiss, Germany) and was approx. 0.2 J cm^{-2} per minute. The BioStimul Lamp emits polarized (96% level of polarization) monochromatic light (624 nm ± 18 nm).

Phototoxicity assay of bacteria. The bacterial culture was grown overnight at 37°C in nutrient trypcase soy broth (BioMerieux, France) and then diluted with fresh broth to an appropriate density (10^7 ml^{-1} bacterial cells). Such *S. aureus* culture was incubated with different concentrations of the protoporphyrin diarginate sensitizer (0 to 25 μM) for 30 min in the dark. After the incubation, the cells were transferred into a 96-well microtiter plate (100 μl per well) and illuminated for 30 min (6 J cm^{-2}). Control wells were neither sensitized with the photosensi-

tizer nor exposed to the light source or were incubated with the photosensitizer only. After illumination the survival of the bacteria was determined by counting the numbers of colony forming units (cfu). Serially diluted aliquots of treated and untreated (no sensitizer, no light) cells were plated on trypcase soy agar (BioMerieux, France) and the number of cfu ml^{-1} was counted after 18 to 24 h of incubation at 37°C. Each experiment was done three times. Survival fractions were expressed as ratios of cfu of bacteria treated with light and photosensitizer to cfu of untreated bacteria.

Phototoxicity assay with eukaryotic cells. Cells (5×10^4) were seeded into 96-well plates and allowed to adhere overnight. Protoporphyrin diarginate was then added to the medium in the concentration range of 0 to 20 μM and cells were incubated for 30 min at 37°C in the dark prior to irradiation. As the EC_{50} value was found within the studied $PPArg_2$ concentration range, no further concentrations were analyzed. Irradiation was performed with red light using a BioStimul lamp for 30 min (6 J cm^{-2}). After illumination, cell survival was determined 24 h later by a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT is used as an indicator of metabolically active cells, in which a color reaction dependent on enzyme activity takes place in mitochondria, and this activity can be measured. Following treatment, MTT (0.5 mg ml^{-1}) was added and cells were incubated for 2 h at 37°C. Cells were lysed with DMSO and the absorbance of the formazan solution was measured at 550 nm with a plate reader (Victor, 1420 multilabel counter).

Data analysis and statistics. Each experiment was performed in triplicate. All primary data are presented as means with standard deviations of the mean. Statistical analysis was performed with two-way analysis of variance (ANOVA) with Bonferroni post-test. A P value of < 0.05 was considered as statistically significant in each experiment.

RESULTS

Phototoxicity against methicillin resistant *Staphylococcus aureus*

A clinical *S. aureus* strain was used to determine the antibacterial toxicities of protoporphyrin diarginate. Illumination of MRSA following incubation with different concentrations (0 to 25 μM) of $PPArg_2$ caused a significant decrease in viability, as determined by the survival percentage (Fig. 1). $PPArg_2$ at 10 μM exhibited significant antibacterial activity after illumination for 30 min (6 J cm^{-2}) (killing efficacy, 3.6 $\log_{10}$-unit reduction) (Table 1). No toxicity of

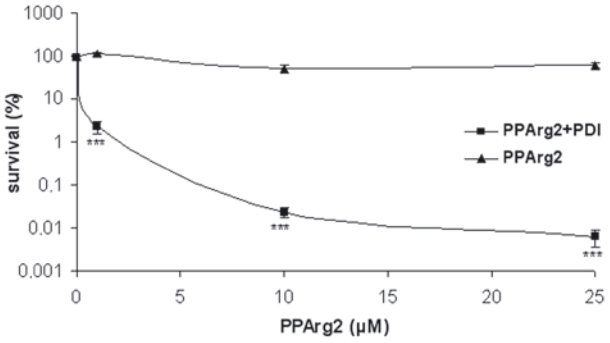

Figure 1. Survival of *S. aureus* exposed to different concentrations of $PPArg_2$ and a light dose of 6 J cm^{-2}. Samples exposed to $PPArg_2$ and light (filled squares); samples exposed to sensitizer without illumination (filled triangles). Each point is the mean of three experiments ± S.D. Statistical analysis was performed with two-way ANOVA with Bonferroni post-test to compare the two corresponding data points at each concentration of the two curves. ***$P < 0.001$.

$PPArg_2$ at concentrations up to 25 μM was observed without illumination (Fig. 1) (Table 1).

Phototoxicity to eukaryotic cells

To determine whether protoporphyrin diarginate induces cytotoxic and phototoxic activity in eukaryotic cells, the viability of cells treated with the sensitizer was assessed with the MTT assay. The toxicity of different concentrations of protoporphyrin diarginate to human skin cells was tested by using healthy dermal fibroblasts. As shown in Fig. 2, incubation of fibroblasts with $PPArg_2$ yielded reduced cell viability only upon illumination. The corresponding 50% effective concentration (EC_{50}) after 30 min of incubation and a light dose of 6 J cm^{-2} was evaluated at 17 μM. Incubation of fibroblasts with protoporphyrin diarginate at concentrations up to 20 μM without illumination did not influence cell viability (Fig. 2).

The concentrations of $PPArg_2$ used in the bacterial and eukaryotic cell toxicity experiments showed concentration-dependent differences upon illumination. Protoporphyrin diarginate at a concentration of 10 μM exerted 3.6 $\log_{10}$-unit reduction in viable count after illumination, whereas at this con-

Table 1. Photodynamic inactivation of fibroblasts and *S. aureus* with different concentrations of $PPArg_2$.

Illumination parameters: 30 min, 6 J cm^{-2}

	Reduction in viability count [%(S.D.)]		
	10 μM $PPArg_2$	17 μM $PPArg_2$	20 μM $PPArg_2$
Fibroblasts	22.5 (6.1)	50 (5.5)	66.5 (5.1)
S. aureus	99.97 (0.01)	99.988 (0.005)	99.99 (0.002)

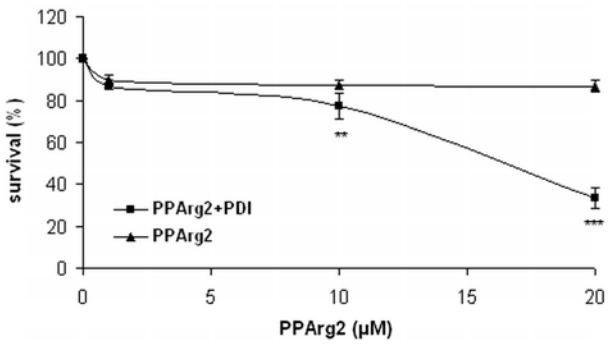

Figure 2. $PPArg_2$ dose-dependent PDI of fibroblasts. Illumination parameters: 30 min, 6 J cm^{-2} (filled squares). Toxicity of $PPArg_2$ after 30 min incubation at 37°C in the dark (filled triangles). Each point is the mean of three experiments ± S.D. Statistical analysis was performed with two-way ANOVA with Bonferroni post-test to compare the two corresponding data points at each concentration of the two curves. $^{**}P < 0.01$; $^{***}P < 0.001$.

centration fibroblasts were still viable with or without illumination (Figs. 1 and 2) (Table 1).

DISCUSSION

The challenge in antimicrobial photoinactivation (PDI) is to find a therapeutic window in which bacteria are effectively eradicated without harming the surrounding tissue. It is important not only to assess the antimicrobial activity, but also the cytotoxic effects towards healthy dermal cells. In this study, we analyzed the *in vitro* toxicity of PDI towards human dermal fibroblasts using protoporphyrin diarginate as the photosensitizer. The results of the present study show that the photosensitization with $PPArg_2$ at the low concentration of 10 μM was effective in killing a methicillin-resistant *Staphylococcus aureus* strain, against which it achieved 3.6 log_{10}-unit reduction. At the same concentration and with the same incubation time, and a dose of light of only 6 J cm^{-2}, $PPArg_2$ had little effect on skin cells, with 77.5% of fibroblasts still viable *in vitro*. Interestingly, previously published data (Grinholc *et al.*, 2007b) reveal that at the same $PPArg_2$ concentration but different incubation time (15 min), 2.1 log_{10} reduction could be achieved even though a 12 J cm^{-2} light dose was applied. In the present study, with a prolonged incubation time (30 min) and reduced light dose (6 J cm^{-2}), 3.1 log_{10} unit reduction was obtained. It is in accordance with bacterial uptake studies of $PPArg_2$ showing that after 30 min of incubation, the highest amount of photosensitizer is accumulated inside the bacterial cell (not shown). Lambrechts *et al.* (2005b) claim that a 90% reduction in cell viability is very substantial for fibroblasts, and, for bacteria, a 4 log (99.99%) reduction in viability is often used as an acceptable indicator. Comparing our data, we conclude that fibroblasts are substantially less sensitive to PDI with the use of $PPArg_2$ than are *S. aureus*.

In regard to the previously published data (Lambrechts *et al.*, 2005b), the cell viability assay (MTT), in our study, was performed 24 h after photodynamic treatment. Lambrechts and coworkers indicated that an increased survival value was observed when the MTT assay was performed immediately following PDI. The authors claim that this can be explained by the fact that the MTT assay is unable to distinguish between early apoptotic and living, healthy cells (Lambrechts *et al.*, 2005b). In consequence, it means that still-alive early apoptotic cells are recognized in MTT assay as healthy, even though apoptosis has already been induced by the photodynamic action. To avoid underestimation of cytotoxicity, we decided to perform the viability test after 24 h of post-treatment incubation. The studies of Lambrechts *et al.* (2005b) involved photoinactivation with the use of another porphyrin-based sensitizer, 5-phenyl-10,15,20-tris(*N*-methyl-4-pyridyl)porphyrin chloride (TriP[4]). In those studies, a significant reduction in viability of fibroblasts was obtained with 0.78 μM photosensitizer, and no survival was observed with concentrations of 12.5 μM and greater. EC_{50} for TriP[4] was estimated at 2 μM. When the bactericidal effect of TriP[4] was analyzed against *S. aureus*, a 5 log_{10}-unit reduction could be obtained at the concentration of 3.1 μM. Other porphyrin-based sensitizers were analyzed by Maisch *et al.* (2005), who studied novel XF porphyrin derivatives. The EC_{50}s of those novel sensitizers ranged from 0.047 to 0.47 μM. With those sensitizers Maisch *et al.* (2005) reached an approx. 3 log_{10}-unit reduction at the very low concentration of 0.005 μM. In the case of $PPArg_2$, the EC_{50} value for fibroblasts was evaluated at 17 μM and a 3.6 log_{10}-unit reduction could be reached at the concentration of 10 μM. Moreover, the applied light dose was only 6 J cm^{-2}, and not 27 J cm^{-2} or 13.7 J cm^{-2} as in the studies mentioned above, respectively.

Not only exogenous but also endogenous, naturally occurring porphyrins within target cells are used as photosensitizing agents. The production of these endogenous porphyrins is stimulated by the administration of 5-amino levulinic acid (ALA). Previously published studies demonstrate that *S. aureus* is able to produce high amounts of porphyrins upon induction by ALA, which may be used as an ideal stimulator of production of endogenous sensitizers in photodynamic therapy (Nitzan *et al.*, 2004; Bisland *et al.*, 2006). 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. Chiu *et al.* (2005) analyzed the phototoxicity of ALA-induced porphyrins against normal adult and neonatal fibroblasts. The cells were incubated for 3 h with ALA at the concentration of 1 mg ml^{-1}. At 10 or 20 J cm^{-2}, near total cell death was observed, while at 5 J cm^{-2} the cell viability was comparable to controls. Also in the studies of Haddad *et al.* (1999), the effect of ALA-based photodynamic therapy on the viability of normal fibroblasts was evaluated. Fibroblasts were incubated with ALA at the low concentration of 2.5 μg ml^{-1} for 48 h. Cells were then illuminated with light doses of 50, 100 and 200 J cm^{-2}. It was reported that the photodynamic therapy caused no significant change in fibroblast viability at all light doses. In the previous report, Grinholc *et al.* (2007b) present a more detailed comparison of the light source used with the recently published data obtained using other light sources.

To ensure that photoinactivation (PDI) has the potential for clinical use in antimicrobial treatment, it is necessary to assess the cytotoxicity of the studied sensitizers towards healthy dermal cells, in particular the cells that are involved in the wound healing process, such as fibroblasts and keratinocytes. Such research is still being performed (Zeina *et al*., 2002; 2003; Lambrechts *et al*., 2005b; Maisch *et al*., 2005). Our results are in accordance with the above studies and are very promising. On the basis of the presented results we can conclude that antibacterial PDI could be an alternative to standard topical antibiotic treatment. However, despite these promising results, only assessment of the technique against a much wider number of clinical *S. aureus* isolates can prove the efficacy of PDI for the inactivation of bacteria *in vivo*. Observations against one strain take no account of the clonal/genotypic variance that undoubtedly is present in the population, and deserves further investigation.

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