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Photodynamic effect of lanthanide derivatives of *meso*-tetra(*N*-methyl-4-pyridyl)porphine against *Staphylococcus aureus*

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Photodynamic therapy (PDT), used for cancer treatment, is also an alternative method for eradication of drug-resistant bacteria. This method utilizes a nontoxic light-activated dye, called a photosensitizer, and visible light to produce reactive oxygen species that lead to bacterial cell death. The purpose of this study was to investigate the bactericidal effect of PDT using lanthanide derivatives of *meso*-tetra(N-methyl-4-pyridyl)porphine against *Staphylococcus aureus* strains. The new photosensitizers appeared to be photodynamically ineffective. No enhancement of antistaphylococcal activity of TMPyP was observed after the conjugation of the porphyrin with lanthanide ions. Additionally, a significant difference in the susceptibility of two bacterial strains to unmodified TMPyP was observed.

Keywords: MRSA, photodynamic therapy, photosensitizer, porphyrin

INTRODUCTION

The growing resistance of bacteria to routinely used antibiotics has prompted a search for alternative antimicrobial approaches (Taylor *et al.*, 2002). One such approach is photodynamic therapy (PDT), a commonly known method of cancer treatment (Jori & Brown, 2004; Kato *et al.*, 2004). It is based on the activation of a photosensitizer by light of appropriate wavelength which leads to bacterial cell death via oxidative damage (Pervaiz, 2001). The highly reactive oxygen species formed initiate oxidative reactions which 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). Among bacteria, *Staphylococcus aureus* is the major cause of health-care associated infections (Dzierzanowska *et al.*, 2004). The most difficult to treat, using classical methods such as antibiotic therapy, are methicillin-resistant strains (Lopaciuk & Dzierzanowska, 2002). In recent years, several studies of successful photoinactivation of bacteria with *meso*-substituted cationic porphyrins have been described especially in the case of Gram-positive bacteria that are the area of our interest (Merchat *et al.*, 1996; Kubat *et al.*, 2000; Lambrechts *et al.*, 2004). However, the ability of these compounds to penetrate the complicated structure of LPS in the bacterial cell envelope facilitates fighting not only Gram-positive but also Gram-negative bacteria which, until recently, was possible only upon treating bacteria with membrane permeabilizers (Malik *et al.*, 1992; Hamblin *et al.*, 2002).

In this work we investigated the photodynamic effect of new photosensitizers, lanthanide derivatives of *meso*-tetra(*N*-methyl-4-pyridyl)porphine on two *S. aureus* strains. Experiments involving a comparison of the bactericidal effects of various porphy-

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Abbreviations: DMSO, dimethylsulfoxide; MRSA, methicillin-resistant *Staphylococcus aureus*; LPS, lipopolysaccharide; PDT, photodynamic therapy; TMPyP, *meso*-tetra(*N*-methyl-4-pyridyl)porphine tetratosylate salt.

rin photosensitizers have already been performed in our laboratory (Grinholc *et al*., 2007; 2008a; 2008b). We have shown that using exogenous sensitizers like protoporphyrin IX and protoporphyrin diarginate, an effective reduction in viable count could be obtained (Grinholc *et al*., 2007; 2008a). However, with the use of endogenous, aminolevulinic acid-induced porphyrins, the obtained bactericidal effect was much lower (Grinholc *et al*., 2007).

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bactericidal effect of PDT was tested on clinical, methicillin-resistant, and reference, methicillin-sensitive Newman ATCC 25904 *S. aureus* strains. MRSA (methicillin-resistant *S. aureus*) was isolated from a patient suffering of local infection (wound) and hospitalized in the Provincial Hospital in Gdańsk (Poland). For experimental purposes, the strains were cultivated in Trypcase-soy broth (bioMerieux) and grown aerobically at 37°C for 24 h.

Photosensitizer. The photosensitizers, *meso*-tetra(*N*-methyl-4-pyridyl)porphine tetratosylate salt (TMPyP) (Aldrich) and its lanthanide derivatives (LnTMPyP) were used. Europium and praseodymium chlorides were synthesized at the Faculty of Chemistry, University of Wrocław (Poland). Ytterbium chloride was from Aldrich. Stock solutions of 1 mg/ml of photosensitizer and of lanthanide chlorides were prepared in 100% DMSO and stored at 4°C until use.

Light source. The illumination was carried out using a BioStimul Lamp (Biotherapy, Czech Republic) which emits polarized (96% level of polarization) and monochromatic light (624 nm ± 18 nm) that is coincident with the photosensitizer's absorbance. 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. The power density of light was 3.3 mW/cm^2.

Photosensitization and irradiation procedure. The photodynamic effect was studied after photosensitization with lanthanide derivatives of TMPyP: europium, praseodymium and ytterbium. Overnight bacterial cultures were diluted with fresh Trypcase-soy broth to OD_{600}=0.1–0.11 and then incubated with 10 μM photosensitizer for 15 min at 37°C. After photosensitization, 0.1 ml aliquots of each cell suspension were transferred to wells in a 96-well plate and illuminated with a light dose of 12 J/cm^2 at room temperature. The light source was placed 1 cm over the bacterial suspensions. The plastic cover was removed from the microtiter plate to ensure the highest light energy delivery to the studied probes. The light energy was measured with the same experimental conditions and revealed the light dose of approx. 0.2 J/cm^2 per minute. These illumination conditions were used in our previous reports (Grinholc *et al*., 2007; 2008a). Next, the cell suspensions were serially 10-fold diluted with phosphate-buffered saline (PBS). Ten microliters of each dilution was plated onto Trpcase-soy agar plates and incubated at 37°C for 24 h. After the incubation, the number of colony forming units (CFUs) was counted. Control samples consisted of bacterial cell suspensions irradiated and non-irradiated in the absence of photosensitiser or incubated with the photosensitiser in the dark. Additional controls consisted of bacteria treated with lanthanide chlorides with and without illumination.

In a second set of experiments photosensitization was carried out with various TMPyP concentrations (5, 10, 20 and 40 μM) and light doses of 6, 12 and 18 J/cm^2. When different TMPyP concentrations were analyzed, the applied light dose was 12 J/cm^2. When studying different light doses, the photosensitizer's concentration was 10 μM. The procedure and control samples were as described above. All experiments were performed at least in triplicate.

RESULTS

Photodynamic effect of lanthanide derivatives of TMPyP

The obtained results revealed that under the conditions used, conjugation of TMPyP with lanthanide ions did not enhance the photodynamic effect of the porphyrin against *S. aureus.* In the Newman strain the use of EuTMPyP led to a similar reduction in the viable count as the use of the porphyrin alone (Fig. 2A). Conjugation of TMPyP with praseodymium or ytterbium reduced its photodynamic effect (Fig. 3A, 4A). Similar results were obtained for the MRSA strain (Fig. 2B, 3B, 4B). Interestingly, the clinical MRSA strain turned out to be more susceptible to the photodynamic effect than the reference Newman strain.

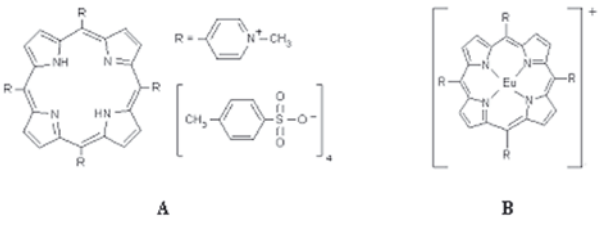

Figure 1. Chemical structure of photosensitizers TMPyP and EuTMPyP.

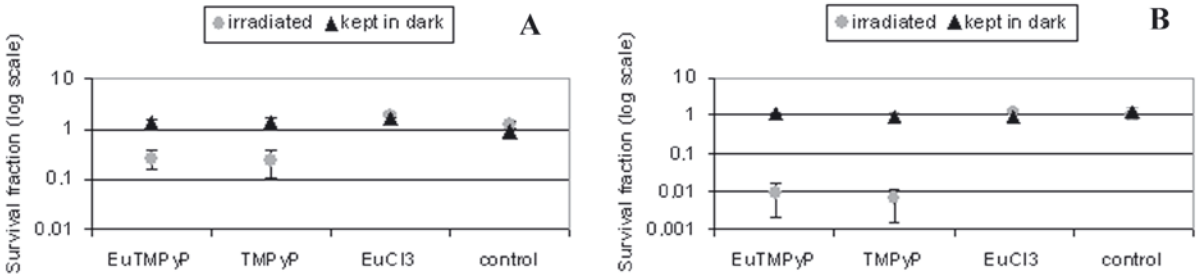

Figure 2. Bactericidal effect of 10 μM europium derivative of TMPyP (EuTMPyP) on *S. aureus* Newman (A) and MRSA (B).
Delivered light energy was 12 J/cm^2. Controls consisted of cell suspensions irradiated or non-irradiated in the absence of photosensitizers.

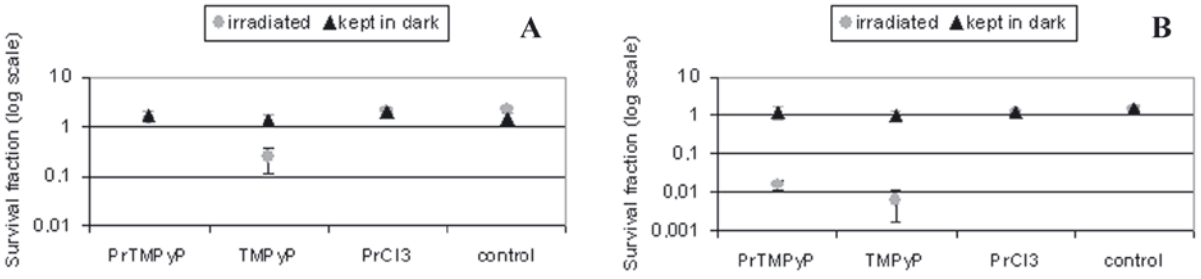

Figure 3. Bactericidal effect of 10 μM praseodymium derivative of TMPyP (PrTMPyP) on *S. aureus* Newman (A) and MRSA (B).
Delivered light energy was 12 J/cm^2. Controls consisted of cell suspensions irradiated or non-irradiated in the absence of photosensitizers.

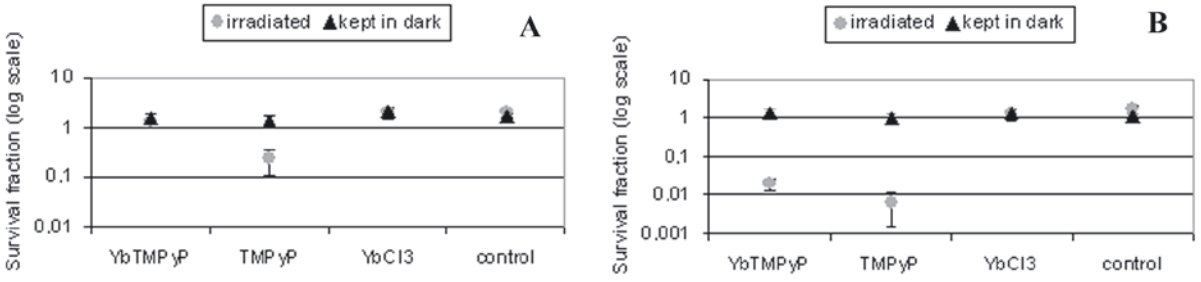

Figure 4. Bactericidal effect of 10 μM ytterbium derivative of TMPyP (YbTMPyP) on *S. aureus* Newman (A) and MRSA (B).
Delivered light energy was 12 J/cm^2. Controls consisted of cell suspensions irradiated or non-irradiated in the absence of photosensitizers.

Photodynamic effect of various concentrations of TMPyP and light doses

With the use of different TMPyP concentrations, a concentration-dependent decrease of bacterial growth was observed for both strains tested. In addition, the methicillin-resistant *S. aureus* was observed to be more sensitive to photodynamic treatment than the reference Newman strain. A difference of approx. 1–2 $\log_{10}$-unit reduction was observed between these two strains (Fig. 5A and B).

The bactericidal effect was also light-dose dependent (Fig. 6). The light dose of 18 J/cm^2 resulted in a more efficient bacterial eradication than 6 or 12 J/cm^2. Again, the MRSA strain was more susceptible to PDT than the reference strain (Fig. 6B).

DISCUSSION

The aim of this work was to investigate the photodynamic effect of lanthanide derivatives of the cationic *meso*-porphyrin TMPyP. While successful photoinactivation of various bacteria with *meso*-substituted cationic porphyrins has already been described, lanthanide derivatives of this porphyrin have not been examined (Merchat *et al.*, 1996; Kubat *et al.*, 2000; Lambrechts *et al.*, 2004). The idea

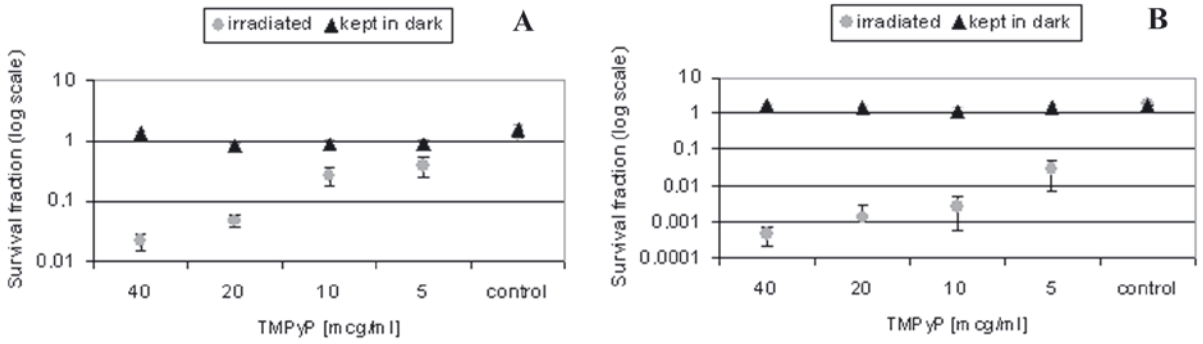

Figure 5. Bactericidal effect of various concentrations of TMPyP on *S. aureus* Newman (A) and MRSA (B).
Delivered light energy was 12 J/cm^2. Controls consisted of cell suspensions irradiated or kept in darkness in the absence of photosensitizer.

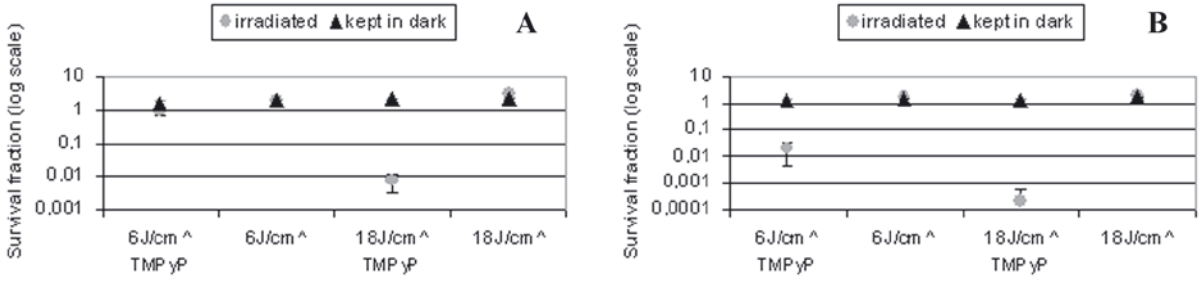

Figure 6. Bactericidal effect of 10 μM TMPyP and various light doses on *S. aureus* Newman (A) and MRSA (B).

of conjugation of TMPyP with lanthanide ions came from chemists from the Faculty of Chemistry, University of Wrocław (Poland). According to the literature data, lanthanides are used for detection and identification of microorganisms. The method is based on the interaction of fluorescent probes, consisting of lanthanide ions, with intracellular bacterial components (Ivanovskaia *et al.*, 1999). The results of the present studies indicated that conjugation of TMPyP with lanthanides did not enhance the antistaphylococcal effect of the porphyrin. It is worth noting that the efficiency of fluorescence emission of porphyrins and their lanthanide derivatives is pH-dependent and the lowest efficiency was observed at pH about 7 (Wiglusz *et al.*, 2004). It means that in therapy other mechanism should be considered beside the role of the oxygen singlet state. The photodynamic effect of TMPyP was dependent on the concentration of the photosensitizer as well as the light dose at the experimental conditions used. We can not draw such a general conclusion for higher sensitizer concentrations that were not tested. Not surprisingly, the use of TMPyP in a higher concentration or an increased light dose resulted in a more efficient eradication of bacteria. The illumination time ranged from 0.5 h to 1.5 h. Such long exposition to light may cause inconvenience, especially during clinical trials. However, the illumination time is strongly dependent on the light source that is used. In this work a BioStimul lamp, delivering the light dose of 0.2 J/cm^2 per minute, was used. Replacing the device with a helium-neon laser that delivers the energy of 4.2 J/cm^2 per minute, reduces the illumination time of the sample even to a few minutes (Embleton *et al.*, 2002). In all experiments illumination in the absence of the photosensitizer, or incubation with the photosensitizer in the dark did not influence the viability of the studied *S. aureus* strains. On this basis one may conclude that the studied photosensitizer did not express toxicity in darkness.

In the present studies two strains of *S. aureus* were used, characterized by different susceptibility to antibiotics. The antibiotic resistance patterns were determined by disc-diffusion method (not shown). Interestingly, the two strains also presented different susceptibility to the photodynamic treatment with TMPyP. The methicillin-resistant *S. aureus* was more sensitive to PDT than the reference Newman strain. The molecular basis for this different susceptibility requires further investigation. The possible mechanisms underlying this different susceptibility to PDT could be biofilm production, which may block the sensitizer's penetration into the bacterial cell; differences in the level of the sensitizer's accumulation; emergence and overexpression of some efflux pumps that could potentially reduce the uptake level of the sensitizer; etc.

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