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Bactericidal Effects of the Fotolon (Chlorin e6) on Gram-Negative and Gram-Positive Strains Isolated from Wound Infections

Bakteriobójczy wpływ Fotolonu (chlorinu e6) na Gram-ujemne i Gram-dodatnie szczepy izolowane z zakażonych ran

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Abstract

Background. Surgical site infections and diabetes foot infections are the very serious therapeutic problem. Because of the alarming bacterial drug resistant level and difficulty in wound infections treatment, an alternative or supportive bactericidal cure have to be developed. One of them is Photo Dynamic Inactivation (PDI) of bacterial strains.
Objectives. Test and discussion of the PDI efficiency using the Fotolon (chlorin e6) against Gram-positive and Gram-negative standard and wild type strains.
Material and Methods. Eight clinical isolates of *Pseudomonas* sp. and one clinical strain of *Acinetobacter baumanii* were tested. As references the authors used control strains from American Type Culture Collection: Gram-positive: *Staphylococcus aureus* ATCC 29213, and Gram-negative: *Klebsiella pneumoniae* ATCC 700601, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853. PDI procedure were carried out using photosensitive compound – the Fotolon (chlorin e6) and laser light.
Results. For *S. aureus* strain fivefold decrease of colony forming units was obtained. For other control bacteria only twice decrease level was observed. The *P. putida* and *P. stutzeri* isolates were as sensitive as Gram-positive *S. aureus*, what is very interesting phenomenon. The remaining Gram-negative strains showed 0.5–2 log of cfu decrease. The authors found that high concentration of the Fotolon in short incubation time gives smaller bactericidal effect than lower concentration in long incubation period.
Conclusions. Both Gram-positive and Gram-negative types of bacteria were sensitive to PDI with Fotolon, however different PDI susceptibility of clinical isolates were observed (**Adv Clin Exp Med 2006, 15, 2, 279–283**).

Key words: photodynamic inactivation, Fotolon (chlorin e6), Gram-positive bacteria, Gram-negative bacteria.

Streszczenie

Wprowadzenie. Zakażenia ran pooperacyjnych oraz infekcje związane ze „stopą cukrzycową" są bardzo poważnym problemem terapeutycznym. Ze względu na alarmujący wzrost oporności drobnoustrojów na antybiotyki oraz trudności w leczeniu zakażeń ran istnieje konieczność rozwoju nowych alternatywnych lub wspomagających metod leczniczych. Jedną z nich może być fotodynamiczna inaktywacja (PDI) szczepów bakteryjnych.
Cel. Przebadanie skuteczności PDI w stosunku do Gram-dodatnich i Gram-ujemnych szczepów bakteryjnych, zarówno standardowych, jak i dzikich.
Materiały i metody. Przetestowano sześć klinicznych szczepów *Pseudomonas* sp. i jeden kliniczny izolat *Acinetobacter baumanii*. Jako kontrole użyto szczepy z kolekcji ATCC (American Type Culture Collection): Gram-dodatni *Staphylococcus aureus* ATCC 29213 oraz Gram-ujemne: *Klebsiella pneumoniae* ATCC 700601, *Escherichia coli* ATCC 25922 i *Pseudomonas aeruginosa* ATCC 27853. Procedurę fotodynamicznej inaktywacji przeprowadzono za pomocą Fotolonu (chlorinu e6) i światła laserowego.
Wyniki. W przypadku szczepu *S. aureus* zaobserwowano 5-krotny spadek liczby jednostek tworzących kolonie (cfu). Gram-ujemne szczepy kontrolne wykazywały tylko dwukrotny spadek cfu. Izolaty *P. putida* i *P. stutzeri* by-

ły tak samo wrażliwe na PDI jak Gram-dodatni *S. aureus,* co jest bardzo interesującym zjawiskiem. Pozostałe szczepy Gram-ujemne wykazywały spadek cfu w granicach 0,5–2 log. Stwierdzono, że wysokie stężenie Fotolonu zastosowane przy krótkim czasie inkubacji charakteryzowało się mniejszym efektem bakteriobójczym niż niskie stężenie aplikowane przez długi czas.
Wnioski. Zarówno bakterie Gram-dodatnie, jak i Gram-ujemne były wrażliwe na PDI z zastosowaniem Fotolonu, szczepy kliniczne jednak wykazywały zróżnicowaną wrażliwość (**Adv Clin Exp Med 2006, 15, 2, 279–283**).

Słowa kluczowe: fotodynamiczna inaktywacja, Fotolon (chlorin e6), bakterie Gram-dodatnie, bakterie Gram-ujemne.

The wound infections, especially surgical site infections and diabetes foot infections are the very serious therapeutic problem. They account for 14–16% of the estimated 2 million nosocomial infections affecting hospitalized patients in the United States [1]. The World Health Organization demonstrates a prevalence of these infections varying from 5 to 34% of total nosocomial infections [2].

The most common bacteria isolated from the wound infections belong to [3–5]: Gram-positive: *Staphylococcus aureus, Staphylococcus coagulase-negative*, *Streptococcus* sp., *Enterococcus* sp.; Gram-negative: *Enterobacteriaceae* (*Escherichia coli*, *Enterobacter* sp., *Klebsiella* sp., *Proteus* sp.) and *Pseudomonadales* (*Pseudomonas aeruginosa*, *Acinetobacter* sp.).

The bacteria causing the hospital-acquired infections are in most cases multidrug resistant [4–6]. From the Gram-positive bacteria there are isolated methicillin resistant *Staphylococcus aureus* (MRSA) [5–7] and vancomycin resistant enterococci (VRE) [5, 6, 8]. The *Pseudomonadales* are natural resistant to many antibiotics and chemotherapeutics and nosocomial isolates could be unsusceptible to almost all active antibiotics [9–12]. Because of this alarming resistance level and difficulty in wound infections treatment, an alternative or supportive bactericidal cure have to be developed. One of them is Photo Dynamic Inactivation (PDI) of bacterial strains.

The photodynamic therapy of bacterial infections starts with topical administration of PS. Owing to the fact, the time needed to bind large PS molecules (molecular weight ~18 500) to the bacteria cells is relatively short (up to 30 minutes) in comparison with eukaryotic cells, the PDI show good selectivity for bacteria [13, 14].

Next the PS is excited with light of proper wavelength and proper fluency, to initiate cytotoxic damage to the surrounding living structures by highly reactive species generation (e.g. single oxygen, hydroxyl radicals, etc.).

The PDI itself is regarded as efficient and non-recovering antimicrobial therapeutic procedure against Gram-positive bacteria. Moreover, its activity is independent of the antibiotic sensitivity spectrum of the treated pathogen [15, 16].

The photodynamic activity of photosensitizer (PS) used in PDT (photodynamic therapy) is dependent on their hydrophobic or hydrophilic properties accountable to their side chains on the tetra-pyrrole ring, as well as to the structure and chemical composition of the cellular targets like proteins or lipids. Whereas traditional photosensitizes (non-charged) have shown to be effective in Gram-positive bacteria inactivation, structural features (outer membrane) occurring in the Gram-negative bacteria creates chemical and physical barrier which does not allow to photo-inactivate these bacteria.

There are three approaches to extend antimicrobial PDI effectiveness towards Gram-negative bacteria, e.g. 1) destabilization of the outer membrane structure making it permeable to photosensitizes molecules [17–20], 2) application of the positively charged phthalocyanine photosensitizers [21–24], which electro-statically bind to negatively charged outer membrane of the bacteria, 3) the use of specific antibody-linked PS to increase the binding selectivity to Gram-negative bacteria covers [25, 26].

The first approach combines PDI treatment with small peptide polymyxin-B application. Also other compounds like Tris/EDTA together with non-cationic photosensitiser were used to increase effectiveness. The second approach utilizes poly-L-lysine (non-bactericidial polymer) and chlorin e6 conjugates with different length of the lysine chain. The PS modified towards polycationic character binds to anionic sites of lipopolysaccharide (LPS) – the component of the outer membrane. This binding is regarded to weaken the intermolecular interaction of the LPS, disorganize its structure leading to increased permeability for drugs to reach the cytoplasmic membrane.

In the study the authors have tested and discussed the Photo Dynamic Inactivation (PDI) efficiency of the Fotolon against Gram-positive and Gram-negative standard and wild bacteria strains.

Material and Methods

Microorganisms

In the study the authors have used the clinical strains isolated from wound infections of the

patients hospitalized in surgical wards in Wroclaw. The bacteria belonging to *Pseudomonadales* were used as the appropriate experimental strains. These were: *Pseudomonas aeruginosa* – 6 strains, *Pseudomonas stutzeri* – 1 strain, *Pseudomonas putida* – 1 strain, *Acinetobacter baumanii* – 1 strain. As references the authors used control strains from American Type Culture Collection: Gram-positive: *Staphylococcus aureus* ATCC 29213, and Gram-negative: *Klebsiella pneumoniae* ATCC 700601, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853.

Photosensitizer

The authors used the Fotolon containing chlorin e6 (Ce6) and PVP polyvinylpyrrolidone dissolved in 0.9% NaCl solution (PF). The ratio of mass fractions of chlorin e6 and PVP is 1 : 1. The PVP polymer does not influence spectral properties of Fotolon in comparison to pure chlorin e6, however Ce6 builds into the hydrophobic part of the PVP, leading to lack of photosensitizer agglomeration [27]. Photo bleaching of the Fotolon was experimentally examined and it was determined, that in PF solution it looses 80% of its activity with 30 J/cm^2 light dose delivered. Moreover it was determined from absorption spectra that due to polymerization of e6 molecules, PF solution of Fotolon looses its activity after several days from the day of preparation, leading to decrease in effective photo activity of PS concentration. Therefore the authors used PS solution prepared just before the experiments.

Light Source

As an excitation source, pigtailed laser diode was used (LaserSecura, Poland) emitting at 650 nm matching exactly Q absorption band's maximum of chlorin e6 (Fotolon) in phosphate buffer (0.9% NaCl). The plate was fixed about 4 cm above the laser fiber tip and power meter (Coherent, Field Master) equipped with measuring head (Coherent, LM-10) shielded with 0.5 cm^2 diaphragm was used to obtain 200 mW/cm^2 light flux. The surface of the plastic plate reflected around 12% of light, however the 96-well plates were covered with the same kind of plastic reflecting 12% of the laser light back to the well. Thus 200 mW/cm^2 light flux is assumed to be preserved during all experiments. The total light dose delivered was then 120 J/cm^2, which is in excess enough to photo activating all e6 molecules. In fact, 2.5-minute lasting treatment procedure could have been used to obtain the same results.

Experimental procedure

Two kinds of experiments were carried out: 1) 50 μg/ml chlorin e6 dose with long (120 min) incubation time with bacterial culture before illumination, and 2) 500 μg/ml chlorin e6 dose with shorter (30 min) incubation time with bacterial culture before illumination. Bacteria were incubated in the dark at room temperature. The volume of 100 μl of bacterial culture in concentration 10^6 cells/ml diluted in sterile phosphate buffer (PBS) was placed into the wells of a flat bottom 96-well microtitration plate in triplicate and illuminated. To determine colony forming units (cfu) the aliquots 10 μL were taken from each well, serially diluted, placed on Mueller Hinton Agar (MHA) and incubated for 24 h at 37°C in the dark. The controls were bacteria untreated with PS or light, bacteria exposed to the light in the absence of PS and bacteria treated with PS but not illuminated. The cfu of the last two control groups, exhibited no changes of cfu in comparison to no PS – no light control group.

Results

The reduction of colony forming units of the reference and clinical strains is presented on Figure 1. The presented data are normalized to the respective (no PS, no light) control group cfu, to easily compare different strains.

In control group the authors have obtained fivefold decrease of colony forming units of *S. aureus*. It is well known that chlorin e6 has a good activity on Gram-positive bacteria. The next three control strains were more susceptible to the higher concentration of photosensitizer, and only about twice decrease level was observed.

The clinical strains behavior could not be predicted from Gram-positive or Gram-negative character of the bacteria. The *Pseudomonas putida* and *Pseudomonas stutzeri* isolates were as sensitive as Gram-positive *S. aureus,* what is very interesting. The other strains showed various susceptibility to PDI of the Fotolon. It held within 0.5–2 orders decrease of cfu.

But if one looks closer on strains No 4 and No 5 one could see unexpected dependencies. High concentration of Fotolon gives smaller decrease of cfu than lower concentration does. It turned out that these strains are the mutants resistant to imipenem (one of the carbapenem antibiotics).

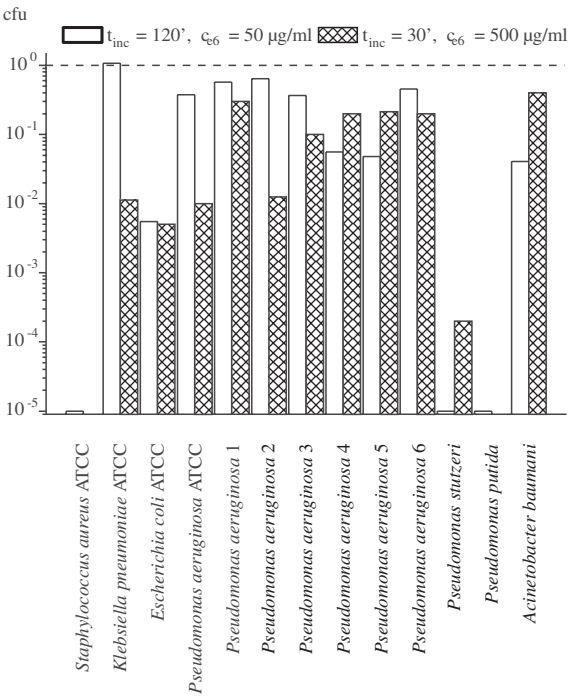

Fig. 1. Cfu parameter for all strains examined within the two kinds of experiments. The data are normalized to the cfu of respective control group: t_{inc} – incubation time; c_{e6} – chlorin e6; cfu – colony forming units

Ryc. 1. Wskaźnik cfu dla wszystkich badanych szczepów w dwóch rodzajach eksperymentów. Dane zostaly znormalizowane w stosunku do cfu odpowiedniej grupy kontrolnej: t_{inc} – czas inkubacji; c_{e6} – chlorin e6; cfu – jednostki tworzące kolonie

The mechanisms of *Pseudomonas aeruginosa* resistance to carbapenems are well known [9–12]. These are 1) drug inactivation through out of enzymes activity (carbapenemases), 2) target alternation – is not detected, 3) prevention of drug influx correlated with loss of membrane protein called OprD porin and 4) active extrusion of drug from the cell by efflux pump system.

Studied *Pseudomonas aeruginosa* strains No 4 and No 5 exhibited the reduced number of porin OprD. The MIC (minimum inhibitory concentration) of imipenem for these strains makes 16–32 μg/ml (low level resistance). This means that the entrance of the drug into the cell is limited.

In studied mutant isolates the authors have small number of this channel, so when the chlorin e6 use this porin for influx, one will see that lower concentration of photosensitizes but longer incubation time will be more effective than higher concentration acting during short time as this was the result of conducted experiment. That allows the authors to suppose that OprD is most probably responsible for influx of chlorin e6 into the *Pseudomonas aeruginosa* cell.

Both types of bacteria Gram-positive and Gram-negative were sensitive to PDI with Fotolon, however different PDI susceptibility of clinical isolates was observed. Larger clinical strain collections should then be verified for therapeutical application of Fotolon in PDI. Moreover, due to non-specific PDI action against bacterial and patient cells, possibly short times of incubation the bacteria with the PS compound should be applied. Thus modifications to the chlorin e6 compound or other photosensitiser are expected to increase the antimicrobial efficiency. Nevertheless it was postulated that OprD was probably responsible for influx of chlorin e6 into the *Pseudomonas aeruginosa* cell. Since the photodynamic antimicrobial inactivation mechanism is not precisely known, the last conclusion seems to be extremely important for further PDI efficiency enhancement.

References

[1] Emori TG, Gaynes RP: An overview of nosocomial infections, including the role of microbiology laboratory. Clin Microb Rev 1993, 4, 428–442.

[2] Mayon-White RT, Ducel G, Kereselidze T: An international survey of the prevalence of hospital-aquired infection. J Hosp Infect 1988, 2, 11 Suppl A: 43–48.

[3] NNIS System: National Nosocomial Infections Surveillance (NNIS) report, data summary from October 1986–April 1996, issued May 1996. A report from the National Nosocomial Infections Surveillance (NNIS) System. Am J Infect Control 1996, 5, 380–388.

[4] Revathi G, Puri J, Jain BK: Bacteriology of burns. Burns 1998, 4, 347–359.

[5] Siami G, Christou N, Eiseman I, Tack KJ and the Clinafloxacin Severe Skin And Soft Tissue Infections Study Group: Clinafloxacin versus Piperacillin-Tazobactam in Treatment of Patients with Severe Skin and Soft Tissue Infections. Antimicrob. Agents Chemother 2001, 45, 525–531.

[6] Carmeli Y, Castro J, Eliopoulos GM, Samore MH: Clinical Isolation and Resistance Patterns of and Superinfection with 10 Nosocomial Pathogens after Treatment with Ceftriaxone versus Ampicillin-Sulbactam. Antimicrob. Agents Chemother 2001, 45, 275–279.

[7] Bell JM, Turnidge JD and SENTRY APAC Participants: High Prevalence of Oxacillin-Resistant *Staphylococcus aureus* Isolates from Hospitalized Patients in Asia-Pacific and South Africa: Results from SENTRY Antimicrobial Surveillance Program, 1998–1999. Antimicrob. Agents Chemother 2002, 46, 879–881.

[8] Harbarth S, Cosgrove S, Carmeli Y: Effects of Antibiotics on Nosocomial Epidemiology of Vancomycin-Resistant Enterococci. Antimicrob. Agents Chemother 2002, 46, 1619–1628.

[9] Livermore DM, Woodford N: Carbapenemases: a problem in waiting? Curr Opin Microbiol 2000, 3, 489–495.
[10] Livermore DM: Of Pseudomonas, porins, pumps and carbapenems. J Antimicrob Chemother 2001, 47, 247–250.
[11] Quinn JP, Dudek EJ, DiVincenzo CA, Lucks DA, Lerner SA: Emergence of resistance to imipenem during therapy for *Pseudomonas aeruginosa* infections. J Infect Dis 1986, 154, 289–294.
[12] Senda K, Arakawa Y, Nakashima K, Ito H, Ichiyama S, Shimokata K, Kato N, Ohta M: Multifocal outbreaks of metallo-—lactamase-producing *Pseudomonas aeruginosa* resistant to broad-spectrum β-lactams, including carbapenems. Antimicrob Agents Chemother 1996, 40, 349–353.
[13] Hamblin MR, Zahra T, Contag CH, McManus AT, Hasan T: Optical monitoring and treatment of potentially lethal wound infections *in vivo.* J Infect Dis 2003, 187, 1717–1725.
[14] Soukos NS, Wilson M, Burns T, Speight PM: Photodynamic effects of toluidine blue on human oral keratinocytes and fibroblasts and *Streptococcus sanguis* evaluated *in vitro*. Lasers Surg Med 1996, 18, 253–259.
[15] Embleton ML, Sean PN, Cookson BD, Wilson M: Selective lethal photosensitization of methicillin-resistant *Staphylococcus aureus* using an IgG-tin (IV) chlorin e6 conjugate. J Antimicrob Chemother 2002, 50, 857–864.
[16] Soncin M, Fabris C, Busetti A, Dei D, Nistri D, Roncucci G, Jori G: Approaches to selectivity in the Zn(II)-phthalocyanine-photosensitized inactivation of wild-type and antibiotic-resistant *Staphylococcus aureus.* Photochem Photobiol Sci 2002, 10, 815–919.
[17] Ehrenberg B, Malik Z, Nitzan Y: Fluorescence spectral changes of hematoporphyrin derivative upon binding to lipid vesicles *Staphylococcus aureus* and *Escherichia coli* cells. Photochem Photobiol 1985, 41, 429–435.
[18] Malik Z, Ladan H, Nitzan Y: Photodynamic inactivation of Gram-negative bacteria Problems and possible solutions. J Photobiol Photochem B: Biol 1992, 1, 262–266.
[19] Bertoloni G, Rossi F, Valduga G, Jori G, van Lier J: Photosensitiseing activity of water- and lipid-soluable phthalocyanines on *Escherichia coli.* FEMS Microbiol Letters 1990, 59, 149–55.
[20] Nitzan Y, Malik Z, Ehrenberg B: Photosensitization of microbial cells. In Photobiology: the Science and its Applications. Eds.: Riklis E. Plenum Press, New York, 815–820.
[21] Merchat M, Bertoloni G, Giacomoni P, Villanueva A, Jori G: Meso-substituted cationic porphyrins as efficient photosensitizers of Gram-positive and Gram-negative bacteria. J Photochem Photobiol 1996, 32, 153–157.
[22] Hamblin MR, O'Donnell DA, Murthy N, Rajagopalan K, Michaud N, Sherwood ME, HasanT: Polycationic photosensitiser conjugates: effects of chain length and Gram classification on the photodynamic inactivation of bacteria. J Antimicrob Chemother 2002, 49, 941–951.
[23] Minnock A, Vernon DI, Schofield J, Griffiths J, Parish JH, Brown ST: Photoinactivation of bacteria. Use of a cationic water-soluble zinc phthalocyanine to photoinactivate both Gram-negative and Gram-positive bacteria. J Photochem Photobiol B: Biol 1996, 32, 159–164.
[24] Vaara M: Agents that increase the permeability of the outer membrane. Microbiol Rev 1992, 56, 395–411.
[25] Berthiaume F, Reiken SR, Toner M, Tompkins RG, Yarmush ML: Antibody-targeted photolysis of bacteria *in vivo.* Bio-Technology 1994, 12, 703–706.
[26] Friedberg JS, Tompkins RG, Rakestraw SL, Warren SW, Fischman AJ, Yarmush ML: Antibody-targeted photolysis. Bacteriocidal effects of Sn (IV) chlorin e6-dextran-monoclonal antibody conjugates. Ann NY Acad Sci 1991, 618, 383–393.
[27] Parkhots MV, Knyukshto VN, Isakov GA, Petrov PT, Lepeshkevich SV, Khairullina AY, Dzhagarov BA: Spectral-luminescent studies of the "photolon" photosensitizer in model media and in blood of oncological patients. J App Spectr 2003, 70, 921–926.

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