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Research paper

The application of antimicrobial photodynamic therapy on *S. aureus* and *E. coli* using porphyrin photosensitizers bound to cyclodextrin

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ABSTRACT

Photodynamic therapy is usually used against malignant and non-malignant tumors. Nowadays, due to resistance of bacterial strains, we are looking for a new antimicrobial strategy to destroy bacteria with minimal invasive consequences. The worldwide increase in antibiotic resistance among different classes of gram-positive and gram-negative bacteria has led to the search for alternative anti-microbial therapies such as antimicrobial PDT (aPDT). Development antimicrobial technology combines a non-toxic compound, called photosensitizer, visible light of the appropriate wavelength, and the generation of reactive oxygen species. In this work, the photosensitizers TMPyP and ZnTPPS4 are investigated for photodynamic and antimicrobial photodynamic therapy. We tested these two porphyrins on two cell lines and two bacterial strains to compare effectiveness. In addition, we applied photosensitizers bound in the complex created with hp-β-cyclodextrin. The light-emitting diodes were used at the doses 0, 1, 5, 10 J/cm^2 for cells and 0, 150 J/cm^2 for bacteria. Tested concentrations for cells and microbes were from 0.5 to 50 μM and from 0.78 to 100 μM, respectively. From this work it can be concluded that TMPyP is a promising compound both in aPDT and in PDT, particularly in contrast to ZnTPPS4, which was efficient only in PDT. Furthermore, the eradication of gram-positive bacteria is possible only with higher concentrations of ZnTPPS4.

Introduction

The widespread problem of antibiotic resistance in pathogens such as *Staphylococcus aureus* has prompted the search for new antimicrobial approaches in recent times. Antimicrobial photodynamic therapy is being actively studied as a possible alternative to antibiotic treatment for localized infections (Wainwright 2009; Dai et al. 2009). The ability of *S. aureus* to develop multidrug resistance is well documented, and antibiotic resistance in an increasing number of bacteria has shown the need for alternative therapies to treat infections, with antimicrobial photodynamic therapy (aPDT) being a potential candidate (Gois et al. 2010). Antimicrobial photodynamic therapy follows principles similar to those of photodynamic therapy (PDT), which is more widely known for its application to the treatment of non-cancerous diseases and cancerous lesions. Antimicrobial photodynamic therapy can be defined as the eradication of target cells by a reactive oxygen species produced through the interaction of a chemical photosensitizer (PS) and light of the appropriate wavelength (ideally at the maximum absorption of the PS) (Gois et al. 2010). It is a non-invasive approach based on the following principles: non-toxic photoactive drugs (usually called photosensitizers – PSs) can be activated by doses of visible light, and subsequently, forms of reactive oxygen species (ROS) can be created through either electron transfer (type I) or energy transfer (type II) reactions. In PDT, reactive oxygen species (ROS) – mainly singlet oxygen generated by the illuminated photosensitizer that was introduced into the bacteria – were found to cause a lethal effect (Lipovsky et al. 2009). These ROS will react with many cellular components that will induce oxidative processes leading to cell death (Michaeli and Feitelson 1994; Stark 2005; Ravanat et al. 2000). There are two basic mechanisms that have been proposed to account for the lethal damage caused to bacteria by PDI: DNA damage and damage to the cytoplasmic membrane, allowing leakage of cellular contents or the inactivation of membrane

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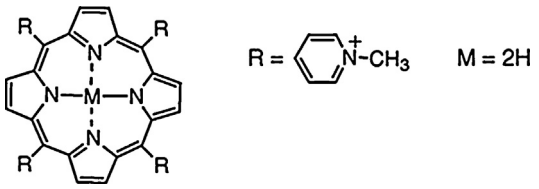

Fig. 1. Molecular structure of photosensitizer TMPyP (Mosinger et al., 2006).

transport systems and enzymes (Hamblin and Hasan 2004). Therefore, antimicrobial PDT could become a therapeutic modality of general acceptance and widespread application, at least in those infections that become chronic after prolonged antibiotic treatment. Unlike antibiotics, repeated photosensitizations of bacteria do not induce the selection of resistant strains as singlet oxygen and free radicals interact with several cell structures and different metabolic pathways in microbial cells (Schastak et al. 2010). Antimicrobial photodynamic therapy, as a treatment for localized infection, will only be useful if it can be tolerated by the patient. It has been shown that human cells (keratinocytes and fibroblasts) can survive aPDT conditions that are lethal to microorganisms. The differences in susceptibility are likely to be due to differences in cell size and structure (Cassidy et al. 2009).

Materials and methods

Photosensitisers and cyclodextrin

We used two photosensitizers $ZnTPPS_4$ and TMPyP. $ZnTPPS_4$ was prepared by Jiri Mosinger at the Department of Inorganic Chemistry, Faculty of Sciences, Charles University in Prague, Prague, Czech Republic. TMPyP (Fig. 1) is commercially available and was purchased from Sigma–Aldrich. These porphyrins were diluted in saline solution (PBS) before their use. The investigated concentrations were 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50 and 100 μmol/l for *S. aureus*; 12.5, 25, 50 and 100 μmol/l for *Escherichia coli*. For tested cell lines (NIH3T3 and HeLa) were used concentrations from 0.5 to 50 μmol/l. For higher effectiveness of photosensitisers in PDT we prepared a complex solution photosensitizer: hp-β-cyclodextrin (Sigma–Aldrich) in three volume ratios – 1:4, 1:1 and 2:1 for the concentrations mentioned above. These complexes were also prepared in saline solution (PBS).

Cell lines and culture conditions

The NIH 3T3 (mouse embryonic fibroblast cell line) and HeLa (cervical cancer cell line) cells were grown in 96-well microplates (10^4 cells/well), using the cultivation medium DMEM (Dulbecco's modified Eagle's medium) with additions of 0.5, 1, 5, 10 and 50 μmol/l photosensitizers at 37 °C, 5% CO_2 for 24 h in darkness. The viability of the cell lines was then investigated using methods for evaluating cell damage (Kolarova et al. 2009).

Bacterial strains and culture conditions

For our PDT study we used the gram-positive bacteria strain *Staphylococcus aureus CCM 4223* (ATCC 29213) and the gram-negative strain *E. coli CCM 4225* (ATCC 35218). The bacteria were used at a density of 1–2 colonies per plate and grown aerobically in 2 ml Brain Heart Infusion (Himedia) aerobically at 37 °C, 5% CO_2 for 45 min in darkness. Subsequently, an amount of 50 μl, approximately 1×10^6 CFU/ml was placed in each well for experiment.

Light source and exposure

A homemade LED-based device like a light source (LEDs 414 nm) for the irradiation of experimental microplates was used. It was especially designed to illuminate the culture plate. Light source with uniform energy density was assembled from a field of neighboring superficially emitting LED diodes arranged into hexagonal form, where triplet of neighboring LED diodes forms equilateral triangle. LED diodes are fixed to a board opposite sample place arranged on distance elements. The light source is protected by National Patent CZ 302829 B6.

Samples of bacteria with photosensitizers or complex PS:CD were exposed under aerobic conditions for an irradiation of 150 J/cm^2 (for 56 min) in 96-well microplates at 37 °C. Samples of cell lines were irradiated by light in a total dose 1, 5 and 10 J/cm^2 (for 20, 100 and 200 s).

Cytotoxicity of porphyrins on cell lines

The toxic effects of TMPyP (5,10,15,20-tetrakis(N-methylpyridinium-4-yl)porphyrin) and $ZnTPPS_4$ (Zinc-5,10,15,20-tetrakis(4-sulphonatophenyl)porphyrin) on NIH3T3 and HeLa cell lines were evaluated by measuring the cytocidal effect after treatment with the porphyrins in the dark without light exposure. In all cases, the cell lines were incubated for 24 h in the dark at 37 °C and 5% CO_2 atmosphere with the selected photosensitizers at concentrations ranging between 0.5 and 50 μmol/l. After dark incubation, the amounts of H_2O_2 and cell viability were measured without any irradiation.

Photodynamic therapy applied on cell lines

Determination of ROS

The production of ROS in cells was investigated by molecular probe CM-H_2DCFDA (5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester) (Invitrogen). After incubation with photosensitizers, the cells were treated with 10 μmol/l CM-H_2DCFDA for 30 min at 37 °C in darkness. Fluorescence of CM-DCF (excitation/emission: 495/530 nm) was recorded by the 96-well microplate reader Synergy HT (BioTek, Winooski, Vermont, USA) (Kolarova et al. 2007). Two columns of wells were used as a negative control (a photodynamically treated cell line in the absence of a photosensitizer). The general procedure was mentioned in a previous article (Hanakova et al. 2012).

Cell viability test

Phototoxicity was evaluated by the MTT test. After photodynamic reaction, the cells were incubated at 37 °C, 5% CO_2 for 24 h in fresh DMEM. Before the start of the viability measurement, DMEM was replaced by 0.5 mg ml^{-1} MTT (Sigma–Aldrich) dissolved in PBS and incubated for 3.5 h at 37 °C and 5% CO_2 atmosphere. The MTT solution was then carefully removed and replaced with 100 μl of DMSO to dissolve formazan crystals. The absorbance of the prepared solution was measured in the 96-well microplate reader Synergy HT at 570 and 690 nm (Krestyn et al. 2009). The general procedure was mentioned in a previous article (Hanakova et al. 2012).

Determination of PDT on cell lines via microscopy

The effectiveness of PDT and morphological changes of cells were determined via microscope Olympus IX81 with DSU unit (Olympus Corporation, Japan) before and after the therapy. The first part of microscopy was carried out immediately after preparation of the cell lines. Before the second part of microscopy, the cell samples were incubated for 24 h with photosensitizers ± CD and after

the treatment incubated for another 5 h in the dark at 37 °C and 5% CO_2.

Cytotoxicity of porphyrins on bacterial cells

The toxic effects of TMPyP and $ZnTPPS_4$ on *S. aureus* and *E. coli* strains were evaluated by measuring the extent of the growth inhibition (bacteriostatic/bactericidal effect) and reduction of survival (cytocidal effect) after treatment with the porphyrins in the dark without light exposure. In all cases, 50 μl of the bacterial cell suspension (10^6 cells/ml) were incubated for 45 min in the dark at 37 °C with the selected photosensitizer at concentrations ranging from 0.78 (for *S. aureus*) or 12.5 μmol/l (for *E. coli*) to 100 μmol/l. After dark incubation absorbances were measured for 24 h without any irradiation.

Photodynamic inactivation of bacterial strains

The samples were distributed to four test groups:

1. Only light – bacterial samples were irradiated with appropriate wavelengths of light in the absence of any photosensitisers.
2. Only photosensitisers (±CD) – bacterial samples with photosensitisers added to the final concentrations of each sample and not irradiated.
3. Photosensitisers (±CD) and light – bacterial samples had photosensitizers added to the final concentrations as in group 2, but were then subsequently treated with light of an appropriate wavelength as in group 1.
4. Controls using bacterial samples not treated by either light or photosensitisers (±CD) (Chan and Lai 2003).

The suspensions of bacteria (10^6 CFU/ml) were tested in 96-well microplates (GAMA GROUP a.s., Czech Republic), where photosensitizers were diluted by a geometric series from 0.78 to 100 μmol/l. After 45 min in darkness, the bacterial culture appeared to be in the exponential growth phase. The microplates were irradiated from above by a light source (LEDs 414 nm) of 150 J/cm^2 at 37 °C; the absorbance was measured on a spectrophotometer (BioTek, Winooski, Vermont, USA) using the program Gen5 at a wavelength of 630 nm every 1 h for a total of 24 h. The efficacy of photosensitizers to produce photoinactivation on bacterial strains was determined spectrophotometrically. The experiments were repeated at least three times. The control samples were bacteria not treated with photosensitizers or light and bacteria exposed to light in the absence of photosensitizers (groups 3 and 4).

Determination of bacterial survival

The survival of bacterial cells following illumination was monitored by counting their viable number after the exposure of the suspended bacteria to light. Bacterial cultures grown under the same conditions but without light exposure served as controls. The absorbance of the suspension at 630 nm was monitored at predetermined time periods for determining growth curves. The percent of growth inhibition and cell survival in the treated and untreated cells was calculated by the following equation:

$(A_X - A_0)/(A_C - A_0) \times 100$ – percentage of viable counts of bacteria, where A_X and A_C are the absorbances measured after 45 min of incubation in the darkness, and measuring a timeframe of 0, 6, 12, 18 and 24 h of absorbance for the treated and control cell suspensions, respectively, with A_0 representing the initial absorbance. All experiments were repeated at least three times.

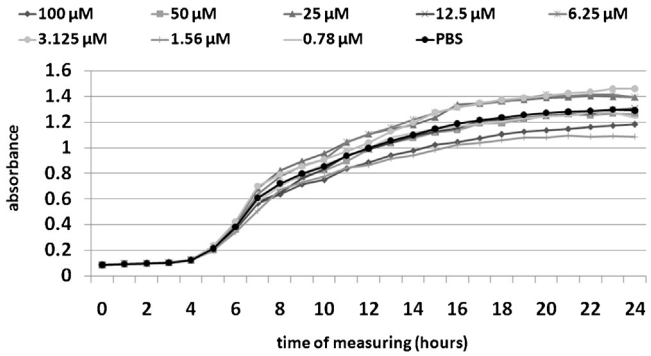

Fig. 2. Control growth curves of *S. aureus* for photosensitizer $ZnTPPS_4$ in ratio 1:4 with CD without irradiation.

Statistical analysis

The Kruskal–Wallis test and consequently Mann–Whitney test were used to compare cell viability between cell lines, ratios and concentrations.

We used the regression coefficients to detect a statistically significant absorbance trend in time. A 95% confidence interval (CI) is a type of interval estimate of a population parameter and is used to indicate the reliability of an estimate. We used it to compare the regression coefficients. If two regression coefficients have non-overlapping intervals, they are significantly different.

The analyses were performed with the SPSS software vision 15 (SPSS, Ing. Chicago, USA) and statistical significance was set at $p < 0.05$.

Results

Cytotoxicity of porphyrins on cell lines and bacterial strains

We used non-irradiated plates as a control with photosensitizers (all concentrations) and PBS for all cell lines and bacterial strains. Use of photosensitizers without irradiation did not markedly influence the viability of cell lines (viability maximally decreased only to 90%, data not presented). The growth curves of the photosensitizing compounds aligned with the curves of PBS (Fig. 2). Their progression was almost identical in all cases.

Determination of PDT effect on cell lines by means of microscopy

Microscopy was used for a rough viability analysis of melanoma and non-melanoma cell lines. Methylene blue is primarily used for staining of bacteria. In the case of cell lines it is used only as a photosensitive agent. Methylene blue has been also applied to various microbiological estimations such as viable cell count, drop plate count and membrane filtration methods (Bapat et al. 2006).

In Fig. 3 the cell lines NIH3T3 and HeLa before treatment are displayed. Both cell lines maintained shape, but after treatment NIH3T3 cells were transformed and HeLa cells were more spherical in comparison to their former elongated state. On all figures, the influence of treatment on cells while using light dose of 5 J/cm^2 for concentration 1 μM of TMPyP in ratio 1:1 with CD is shown.

In this manner, the microscopic images of NIH3T3 and HeLa cell lines in the black and white light mode appear at an original image magnification of 400 (Figs. 3 and 4). In the first case (Fig. 3A and B) we immediately took a photo before PDT. In the second case (Fig. 4A and B), before acquiring the images, the cells were exposed to 1 μM TMPyP at 5 J/cm^2 for 24 h after which we applied the treatment.

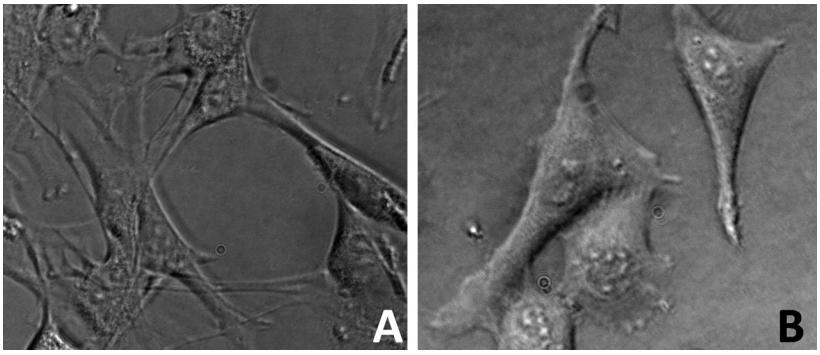

Fig. 3. NIH3T3 cells (A) and HeLa cells (B) before treatment of PDT (original image magnification of 400 times).

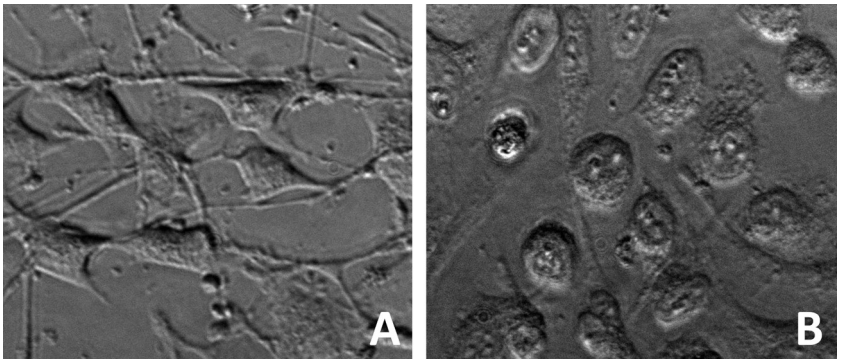

Fig. 4. NIH3T3 cells (A) and HeLa cells (B) after treatment of PDT (original image magnification of 400 times).

Determination of ROS and cell viability test

In a previous article (Hanakova et al. 2012), we discussed some of the effects of porphyrin photosensitizers on the NIH3T3 cell line. Firstly, the cell cytotoxicity of all photosensitizers with cyclodextrin was tested on the NIH3T3 and HeLa cell lines. The cells were incubated with the tested photosensitizers and cyclodextrin for 24 h in darkness. Then ROS production determination and MTT assay were performed. According to our results, the most efficient light dose to the NIH 3T3 and HeLa cell lines was 5 J/cm^2. The lowest concentrations of TMPyP (±CD; from 0.5 μmol/l) were highly efficient (Fig. 5). In this regard, the viability of the cell lines HeLa and NIH3T3 decreased to 12% and 10% for 0.5 μM concentration of TMPyP in ratio 1:1, respectively (Fig. 5). The photosensitizer ZnTPPS$_4$ was not more efficient. The viability did not decrease at values which were obtained by TMPyP. Fig. 6 shown that the production of relative fluorescence units (ROS production) for photosensitizer TMPyP is generally high (for NIH3T3 is higher than for HeLa cell line).

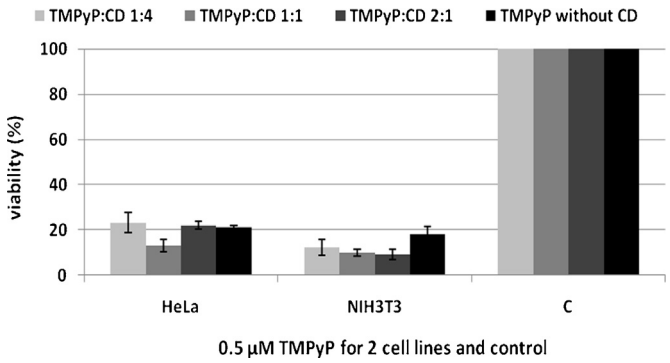

Fig. 5. Dependence of cell line survival on the photosensitizer 0.5 μM TMPyP utilizing a light dose of 5 J/cm^2. Cell viability was determined by measuring an enzyme activity of living cells using the MTT test. The total yield of the MTT product by the control (only irradiated cells without PS and CD) was set at 100% (column C).

Photodynamic inactivation of bacterial strains

Fig. 7A shows the potent effect of aPDT on the eradicated bacterial strain *S. aureus* for TMPyP in all concentrations and at a ratio of 1:4 with CD. The same results were obtained at a ratio of 1:1. Higher concentrations are effective for photosensitizer ZnTPPS$_4$ in ratio 1:4 (Fig. 7B) and samples without CD were not induced, we observed a trend only at a concentration of 100 μM for this compound (Fig. 7C).

E. coli as representative for gram-positive strain was eradicated only for the highest concentration of 100 μM TMPyP for ratio 2:1. For the photosensitizer ZnTPPS$_4$ at a ratio of 2:1, aPDT was not successful (Fig. 8A and B).

From the results shown in Figs. 7 and 8, it is clear that PDT applied to a strain of gram-positive bacteria showed greater efficiency than to gram-negative bacteria.

Determination of bacterial survival

After 24 h using the photosensitiser TMPyP at a ratio of 2:1 with CD, the viability of *S. aureus* decreased by 8% at a concentration of 100 μM, and by 14% for concentrations of 50 and 25 μM (Fig. 7D). Bacterial strains of *S. aureus* were eradicated for TMPyP without CD after 24 h with values of 12%, 16% and 23% viability for concentrations of 100, 50 and 25 μM, respectively (data not shown).

Fig. 8C shows the viability of *E. coli* using TMPyP without CD, which was decreased to 14%, 14% and 84% for 100 μM, 50 μM and 25 μM, respectively. For the photosensitizer ZnTPPS$_4$, the values of viability did not decrease below 90%.

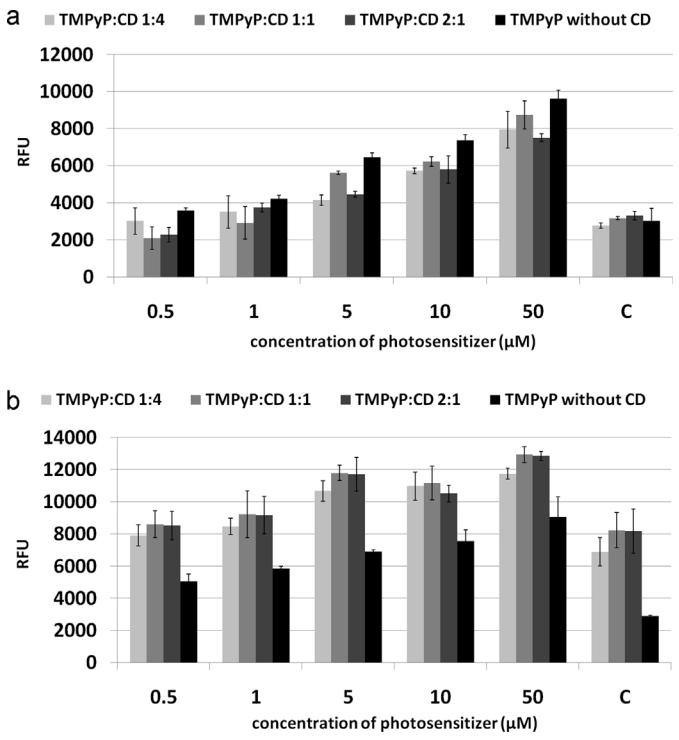

Fig. 6. (A) Dependence of RFU on the concentration of thephotosensitizer TMPyP, tested on HeLa cell line with light dose of 5 J/cm^2. (B) Dependence of RFU on the concentration of the photosensitizer TMPyP, tested on the NIH3T3 cell line with a light dose of 5 J/cm^2. The total RFU product by the control (only irradiated cells without PS and CD) represents column C.

Statistical analysis

The Kruskal–Wallis test with multiple post hoc comparisons by Mann–Whitney U-tests with Bonferroni correction showed that the values of cell viability in 50 μM concentration at a ratio of 1:4 and 1:1 were lower than without CD ($p = 0.005$ or $p = 0.007$).

Regression coefficients were positive and statistically significant, $p < 0.05$. It indicates the increasing absorbance during time.

Generally in the case of the confidence intervals of the regression coefficients for the control curves without photosensitiser do not overlap with the CI of growth curves for bacterial strains for all concentrations. It means that the absorbance of control samples was increasing and the absorbance of bacterial strains was not increasing.

The statistically important regression coefficients and related concentrations for *S. aureus* are shown in Tables 1 and 2. These data are from experiments with microplates irradiated with a light of 150 J/cm^2. For the gram-positive bacterial strains, all data are statistically important.

In the case of *S. aureus* (TMPyP:CD – 1:4) in Table 1 the confidence intervals are not overlapped for concentrations of 25 μM to 50, 12.5, 6.25 and 3.125 μM. In the ratio 1:1 and 2:1 all concentrations are not overlapped with PBS growth curve of *S. aureus*.

PBS-chosen wells containing only PBS, without the photosensitizer TMPyP, but irradiated with a light dose of 150 J/cm^2.

In the case of *S. aureus* (ZnTPPS4:CD – 1:4) the growth curves of PBS and 0.78 μM are not overlapped with other concentrations. In the case without cyclodextrin the PBS, 100 and 25 μM are not overlapped with other concentrations.

Discussion

In this study, we compared the photosensitivity of cell lines NIH3T3 and HeLa and the antimicrobial effectiveness of two

Table 1
Statistical analysis – regression coefficient and calculated 95% CIs (left and right limit) for bacterial strain *S. aureus*, photosensitizer TMPyP and irradiation with a light dose of 150 J/cm^2.

Concentration (μmol/l)	Regression coefficient	95% CI		*p*
		Lower limit	Upper limit	
Photosensitizer TMPyP bacterial strain S. aureus ratio 1:4				
100	0.00061	0.0004	0.0008	<0.0001
50	0.00053	0.0003	0.0007	<0.0001
25	0.00107	0.0008	0.0013	<0.0001
12.5	0.00042	0.0002	0.0006	<0.0001
6.25	0.00024	0.0001	0.0004	0.011
3.125	0.00025	0.0001	0.0004	0.007
1.56	0.00069	0.0005	0.0009	<0.0001
0.78	0.00061	0.0004	0.0008	<0.0001
PBS	0.02525	0.0214	0.0291	<0.0001
Photosensitizer TMPyP bacterial strain S. aureus ratio 1:1				
100	0.00072	0.0005	0.0010	<0.0001
50	0.00067	0.0004	0.0009	<0.0001
25	0.00046	0.0004	0.0005	<0.0001
12.5	0.00044	0.0003	0.0006	<0.0001
6.25	0.00039	0.0002	0.0006	0.0003
3.125	0.00026	0.0001	0.0005	0.007
1.56	0.00043	0.0003	0.0006	<0.0001
0.78	0.00036	0.0002	0.0005	0.0005
PBS	0.02525	0.0214	0.0291	<0.0001
Photosensitizer TMPyP bacterial strain S. aureus ratio 2:1				
100	0.00072	0.0005	0.0010	<0.0001
50	0.00063	0.0004	0.0009	<0.0001
25	0.00045	0.0002	0.0006	0.0004
12.5	0.00049	0.0003	0.0007	<0.0001
6.25	0.00033	0.0001	0.0005	0.0010
3.125	0.00031	0.0001	0.0005	0.0015
1.56	0.00033	0.0001	0.0005	0.0013
0.78	0.00031	0.0001	0.0005	0.0021
PBS	0.02525	0.0214	0.0291	<0.0001
Photosensitizer TMPyP bacterial strain S. aureus without CD				
100	0.00091	0.0006	0.0012	<0.0001
50	0.00076	0.0005	0.0010	<0.0001
25	0.00064	0.0004	0.0009	<0.0001
12.5	0.00054	0.0003	0.0007	<0.0001
6.25	0.00030	0.0002	0.0004	<0.0001
3.125	0.00044	0.0003	0.0006	<0.0001
1.56	0.00027	0.0001	0.0004	0.0041
0.78	0.00027	0.0001	0.0005	0.0081
PBS	0.02525	0.0214	0.0291	<0.0001

Table 2
Statistical analysis – regression coefficient and calculated 95% CIs (left and right limit) for bacterial strain *S. aureus*, photosensitizer $ZnTPPS_4$ and irradiation by 150 J/cm^2 PBS – chosen wells containing only PBS, without photosensitizer $ZnTPPS_4$, but irradiated with a light dose of 150 J/cm^2.

Concentration (μmol/l)	Regression coefficient	95% CI		*p*
		Lower limit	Upper limit	
Photosensitizer $ZnTPPS_4$ bacterial strain S. aureus ratio 1:4				
100	0.00029	0.0001	0.0005	0.0041
50	0.00158	0.0013	0.0018	<0.0001
25	0.00032	0.0001	0.0005	0.0007
12.5	0.00028	0.0001	0.0005	0.0032
6.25	0.00036	0.0002	0.0005	<0.0001
3.125	0.00025	0.0001	0.0004	0.0010
1.56	0.00553	0.0025	0.0085	0.0009
0.78	0.02005	0.0150	0.0252	<0.0001
PBS	0.02525	0.0214	0.0291	<0.0001
Photosensitizer $ZnTPPS_4$ bacterial strain S. aureus without CD				
100	0.00026	0.0001	0.0005	0.0164
50	0.00856	0.0068	0.0103	<0.0001
25	0.01058	0.0089	0.123	<0.0001
12.5	0.00493	0.0029	0.0069	<0.0001
PBS	0.02525	0.0214	0.0291	<0.0001

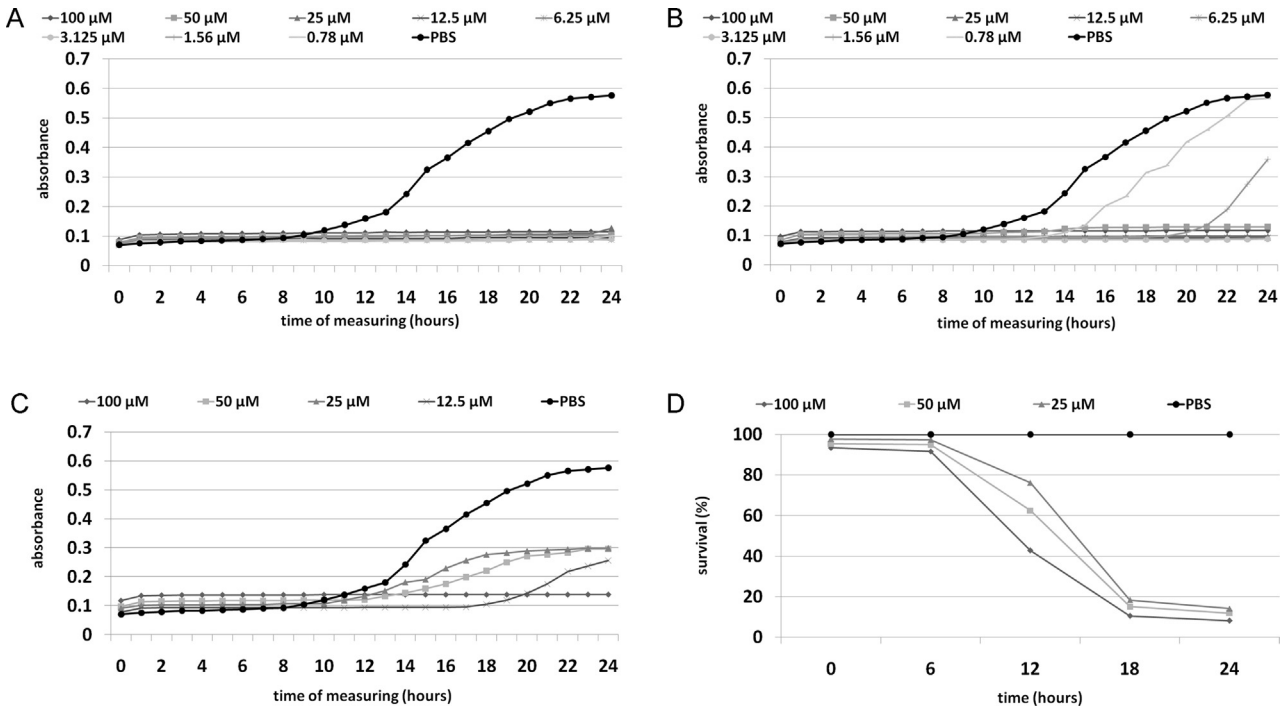

Fig. 7. (A) Growth curves of *S. aureus* measured during 24 h for photosensitizer TMPyP at a ratio of 1:4 with CD irradiated with a light dose of 150 J/cm^2. (B) Growth curves of *S. aureus* measured during 24 h using the photosensitizer $ZnTPPS_4$ at a ratio of 1:4 with CD irradiated with a light dose of 150 J/cm^2. (C) Growth curves of *S. aureus* measured during 24 h using the photosensitizer $ZnTPPS_4$ without CD irradiated with a light dose of 150 J/cm^2. (D) Survival of the bacterial strain of *S. aureus* (%) using the photosensitizer TMPyP at a ratio of 2:1 with CD irradiated with a light dose of 150 J/cm^2.

pathogen microbial cells. We focused on investigating the cytotoxic effect of photosensitizes and the decrease in viability of the two bacterial strains *S. aureus* and *E. coli*.

It has been found that low visible light modulates cytokine levels and growth factors, increases tissue oxygenation, and leads to increased mammalian cell proliferation and migration (particularly fibroblasts). The results of cellular changes *in vitro* and *in vivo* are used to explain increased light-induced healing of chronic wounds (Lipovsky et al. 2009). In evaluating the potential of PDT for the clinical treatment and prevention of burn wound infections, it is important not only to assess its antimicrobial efficacy, but also the cytotoxic effects on healthy cells that are involved in the wound healing process, such as keratinocytes, fibroblasts and leukocytes (Lambrechts et al. 2005). Because of these reasons, we studied the

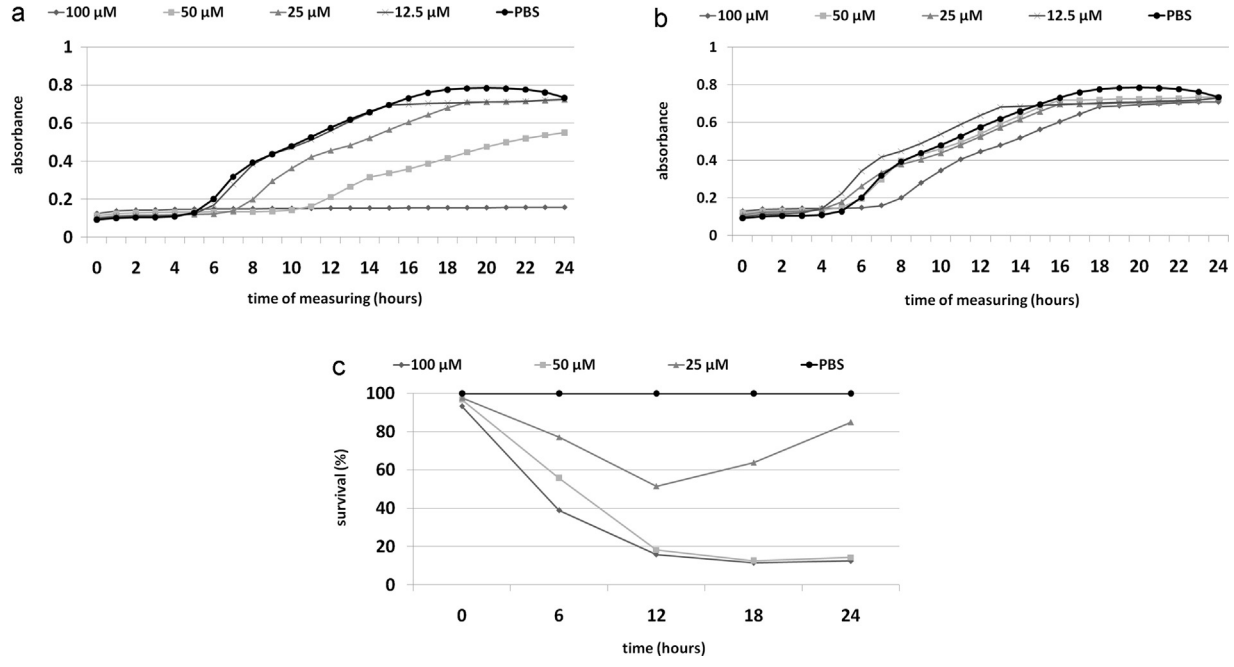

Fig. 8. (A) Growth curves of *E. coli* measured during 24 h using the photosensitizer TMPyP at a ratio of 2:1 with CD irradiated with a light dose of 150 J/cm^2. (B) Growth curves of *E. coli* measured during 24 h using a photosensitizer $ZnTPPS_4$ at a ratio of 2:1 wih CD irradiated with a light dose of 150 J/cm^2. (C) Survival of the bacterial strain of *E. coli* (%) using the photosensitizer TMPyP without CD irradiated with a light dose of 150 J/cm^2.

effect on the cancer cell lines and fibroblasts, since these cells play a crucial role in the healing of wounds.

TMPyP was the most efficient photosensitizer for destroying gram-positive bacteria *S. aureus* at all concentrations, photosensitizer $ZnTPPS_4$ only in the highest concentrations from 100 to 25 μmol/l. Survival bacteria were under 20% during successful aPDT. The gram-negative bacteria strain *E. coli* was not strongly sensitive to aPDT with TMPyP photosensitizer. The photosensitizer $ZnTPPS_4$ was not sufficiently efficient in any type of experiment. O'Riordan et al. (2005) illustrated the antimicrobial mechanism of action of PDT for gram-positive as well as gram-negative bacterial strains. Photoinactivation of gram-positive and gram-negative bacteria is based on the concept that certain photosensitizers (PS) can accumulate in significant amounts in or at the cytoplasmic membrane, the critical target to induce irreversible damage in bacteria. The positive charge of the PS appears to promote a tight electrostatic interaction with negatively charged sites at the outer surface of the bacterial cell (Maisch et al. 2004). Cationic porphyrins have attracted considerable attention as effective photodynamic sensitizers. Due to their binding affinity toward nucleic acids, these porphyrins can selectively photocleave DNA and inhibit telomerases. One of the most studied cationic porphyrin is 5,10,15,20-tetrakis (*N*-methylpyridinium-4-yl) porphyrin (TMPyP). External binding and ion-pairing was observed for interaction with cyclodextrins (Mosinger et al. 2006). Cyclodextrins are shaped like truncated cones. The cavity is composed of glucopyranose units linked by glycosidic oxygens in the interior. This linkage and contribution of the hydrogen bonding create a rigid hydrophobic cavity that can accommodate a variety of molecules of appropriate size. The cyclodextrin size increases with the number of glucose units ranging from 6 to 8, designated as α-, β- and γ-cyclodextrins (Lang et al. 2001). Jori et al. (2006) demonstrated the effect of visible light irradiation in the presence of TMPyP on gram-positive and gram-negative bacteria. They pointed out that the anionic derivative is active only against gram-positive bacteria, while it has no toxic effect on gram-negative bacteria even after prolonged exposure to light, contrary to what was observed for the positively charged porphyrin (Jori et al. 2006). Thus, although the binding of the photosensitizer with the cytoplasmic membrane (whose integrity represents a critical parameter for cell survival) of gram-positive bacterial cells is relatively fast and efficient, gram-negative bacteria shows a low permeability to photosensitizers, such as porphyrins or their analogs, having a polycyclic chemical structure (Jori 2006). It has been repeatedly reported in recent studies that cationic porphyrins are more active than anionic or non-ionic ones against both gram-positive and gram-negative bacteria (Banfi et al. 2006).

Porphyrins, because of their electronic structure, are predetermined to noncovalent interactions, to the formation of molecular assemblies and, finally, to an important role in molecular recognition. As most porphyrins are also used as photosensitizers for photodynamic therapy, assembling with other molecules may dramatically affect their photophysical properties and, hence, the final therapeutical effect (Mosinger et al. 2009).

Therefore, the rapidly increasing emergence of antibiotic resistance among pathogenic bacteria may be bringing to an end a period extending over the past 50 years, termed "the antibiotics era". Bacteria replicate very rapidly and a mutation that helps a microbe survive in the presence of an antibiotic drug will quickly become predominant throughout the microbial population. The inappropriate prescription of antibiotics and the failure of some patients to complete their treatment regimen also exacerbate the problem (Hamblin and Hasan 2004).

As the incidence of antimicrobial resistant bacterial strains rises, the development of new, alternative and adjuvant antimicrobial wound treatments is becoming increasingly important (Lambrechts et al. 2005).

In this paper we wish to report on the application of porphyrins and their effectiveness in photodynamic and antimicrobial photodynamic therapy on cell lines and bacterial strains. Our results are important and very successful for future development in aPDT area, especially because of the still growing need to use antibiotics for such infections.

Conclusion

The growing resistance to antibiotics and other chemotherapeutics has led to efforts to find antimicrobial treatment to which bacteria will not easily develop resistance. A potential alternative may be antimicrobial photodynamic inactivation (PDI) (Grinholc et al. 2008).

Due to many positive results, PDT seems to be one possible option to the medical sphere. The target of the present study was to test the efficiency of photosensitizers on the cell lines and bacterial strains and to eradicate gram-positive and gram-negative bacteria or at least decrease microbial growth. This work describes experiments targeted on PDT and aPDT with respect to further tests.

Cationic porphyrins have attracted considerable attention as effective photodynamic photosensitizers. One of the most studied cationic porphyrin is 5,10,15,20-tetrakis(*N*-methylpyridinium-4-yl)porphyrin (TMPyP). Our work showed that TMPyP is more effective than zinc porphyrin ZnTPPS4 considering the reduction of the bacterial growth (Mosinger et al. 2006).

Ideal photosensitisers should meet several criteria: chemical stability and constant composition, water-solubility, high quantum yield of $^1O_2$ generation and no cytotoxicity in the dark (Yano et al. 2011). It should be enriched in the target tissue, such as a tumor as selectively as possible, and be eliminated from the body sufficiently quickly to avoid generalized skin photosensitization. It should not self-aggregate much in the body. Synthesis of the photosensitizer should be relatively easy and the starting materials readily available. Its large-scale production should also be feasible to make it cost-effective and widely applicable. The photosensitizer should be stable enough to avoid degradation processes, e.g. photobleaching. However, the photobleaching tendency can be an advantage as well, because it can shorten the durative of generalized photosensitivity after PDT, and also because it can increase the treatment selectivity (Nyman and Hynninen, 2004).

In this study we used cyclodextrin that served as carrier for increasing effects. In the future we will primarily focus on different bacterial strains and new alternative conditions for aPDT.

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References

Banfi S, Caruso E, Buccafurni L, Battini V, Zazzaron S, Barbieri P, et al. J Photochem Photobiol B: Biol 2006;85:28–38.

Bapat P, Nandy SK, Wangikar P, Venkatesh KV. Quantification of metabolically active biomass using methylene blue dye reduction test (MBRT): measurement of CFU in about 200 s. J Microbiol Methods 2006;65(1):107–16.

Cassidy CM, Tunney MM, McCarron PA, Donnelly RF. Drug delivery strategies for photodynamic antimicrobial chemotherapy: from benchtop to clinical practice. J Photochem Photobiol B: Biol 2009;95:71–80.

Chan Y, Lai CHH. Bactericidal effects of different laser wavelengths on periodontopathic germs in photodynamic therapy. Lasers Med Sci 2003;18:51–5.

Dai T, Huang YY, Hamblin MR. Photodynamic therapy for localized infections – state of the art. Photodiagn Photodyn Ther 2009;6:170–88.

Gois MM, Kurachi CH, Santana EJB, Mima EGO, Spolidório DMP, Pelino JEP, et al. Susceptibility of *Staphylococcus aureus* to porphyrin-mediated photodynamic antimicrobial chemotherapy: an in vitro study. Lasers Med Sci 2010;25:391–5.

Grinholc M, Szramka B, Kurlenda J, Graczyk A, Bielawski KP. Bactericidal effect of photodynamic inactivation against methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* is strain-dependent. J Photochem Photobiol B: Biol 2008;90:57–63.

Hamblin MR, Hasan T. Photodynamic therapy: a new antimicrobial approach to infectious disease? Photochem Photobiol Sci 2004;3(5):436–50.

Hanakova A, Bogdanova K, Tomankova K, Binder S, Bajgar R, Langova K, et al. Study of photodynamic effects on NIH 3T3 cell line and bacteria. Biomed Pap (Med Faculty, University of Palacky Olomouc, Czech Republic) 2012;156 [Available online] http://biomed.papers.upol.cz/corproof.php?tartkey=bio-000000-0363#.UXYz5kp_TAg

Jori G. Photodynamic therapy of microbial infections: state of the art and perspectives. J Environ Pathol Toxicol Oncol 2006;25(1–2):505–19.

Jori G, Fabris C, Soncin M, Ferro S, Coppellotti O, Dei D, et al. Photodynamic therapy in the treatment of microbial infections: basic principles and perspective applications. Lasers Surg Med 2006;38:468–81.

Kolarova H, Bajgar R, Tomankova K, Kolar P, Kubinek R. Photodynamic and sonodynamic treatment by phthalocyanine on cancer cell lines. Ultrasound Med Biol 2009;35:1397–404.

Kolarova H, Bajgar R, Tomankova K, Krestyn E, Dolezal L, Halek J. In vitro study of reactive oxygen species production during photodynamic therapy in ultrasound-pretreated cancer cells. Physiol Res 2007;56(1):27–32.

Krestyn E, Bajgar R, Kolarova H, Tomankova K. Photodynamic properties of $ZnTPPS_4$, $ClAlPcS_2$ and ALA in human melanoma G361 cells. Toxicol In Vitro 2009;34:286–91.

Lambrechts SAG, Schwartz KR, Aalders MCG, Dankert JB. Photodynamic inactivation of fibroblasts by a cationic porphyrin. Lasers Med Sci 2005;20:62–7.

Lang K, Kubat P, Lhotak P, Mosinger J, Wagnerova DM. Photophysical properties and photoinduced electron transfer within host–guest complexes of 5,10,15,20-tetrakis(4-N-methylpyridyl)porphyrin with water-soluble calixarenes and cyclodextrins. Photochem Photobiol 2001;74(4):558–65.

Lipovsky A, Nitzan Y, Friedmann H, Lubart R. Sensitivity of *Staphylococcus aureus* strains to broadband visible light. Photochem Photobiol 2009;85:255–60.

Maisch T, Szeimies RM, Jori G, Abels CH. Antibacterial photodynamic therapy in dermatology. Photochem Photobiol Sci 2004;3:907–17.

Michaeli A, Feitelson J. Reactivity of singlet oxygen toward amino acids and peptides. Photochem Photobiol 1994;59(3):284–9.

Mosinger J, Janošková M, Lang K, Kubát P. Light-induced aggregation of cationic porphyrins. J Photochem Photobiol A: Chem 2006;181:283–9.

Mosinger J, Slavětínská L, Lang K, Coufal K, Kubát P. Cyclodextrin carriers of positively charged porphyrin sensitizers. Org Biomol Chem 2009;7:3797–804.

Nyman ES, Hynninen PH. Research advances in the use of tetrapyrrolic photosensitizers for photodynamic therapy. J Photochem Photobiol B: Biol 2004;73:1–28.

O'Riordan K, Akilov OE, Hasan T. The potential for photodynamic therapy in the treatment of localized infections. Photodiagn Photodyn Ther 2005;2:247–62.

Ravanat JL, Di Mascio P, Martinez GR, Medeiros MHG, Cadet J. Singlet oxygen induces oxidation of cellular DNA. J Biol Chem 2000;275:40601–4.

Schastak S, Ziganshyna S, Gitter B, Wiedemann P, Claudepierre T. Efficient Photodynamic therapy against gram-positive and gram-negative bacteria using THPTS, a cationic photosensitizer excited by infrared wavelength. PLoS ONE 2010;5(7):e11674.

Stark G. Functional consequences of oxidative membrane damage. J Membr Biol 2005;205:1–16.

Yano S, Hirohara S, Obata M, Hagiya Y, Ogura S, Ikeda A, et al. Current states and future views in photodynamic therapy. J Photochem Photobiol C: Photochem Rev 2011;12:46–67.

Wainwright M. Photoantimicrobials – so what's stopping us? Photodiagn Photodyn Ther 2009;6:167–9.