APPLICATION OF PHOTODYNAMIC THERAPY IN THE STERILISATION OF TUMOUR CELLS TRANSPLANTED INTO LABORATORY ANIMALS

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Abstract

In this study, we tried to estimate the effectiveness of the antimicrobial activity of photodynamic therapy (PDT) without the destruction of tumour cells. Chlorin e6 in concentrations from 0.05 to 1.0 µg/mL and total dose of light 100 J/sq.cm were used. Samples from different tumours were tested for a presence of bacteria. Next, cells from the same tumours were treated with chlorin e6-PDT, chlorin e6, and light alone, or not treated. The survival rate was counted, and the cells were injected back into the animals. PDT caused the eradication of bacteria such as Proteus sp., Streptococcus α−haemolyticus, and others: the specimen became aseptic. After Ce6-PDT treatment and in control groups, tumour growth was observed in all rats 7-10 d after transplantation. The tumours were palpable and macroscopically visible. Our studies showed that PDT kills bacterial flora without the destruction of tumour cells. Thus, tumour cells free of bacteria could be used for other experiments.

Key words: tumours, photodynamic therapy, bacteria, chlorin e6.

Photodynamic therapy is a well-known method for the treatment of various malignant tumours and non-tumour lesions. For more than a decade, photodynamic therapy is used as antimicrobial modality. This photodynamic antimicrobial chemotherapy (PACT) is emerging as a promising therapy for bacterial infections (2). The photosensitiser is taken up by a pathogen, bacteria, or virus, and then illuminated by light at an appropriate wavelength. This can lead to the inactivation of the pathogen through the production of highly reactive free radical species, which induce oxidative damage to lipids, proteins and DNA/RNA, and/or adduct formation between the photosensitiser and microbial biomolecules (14). Recently, much attention was paid to photoinactivation by the application of a novel, positively charged meso-substituted porphyrin, e.g. 5-[4- (1-dodecanoylpyridinium)]-10,15,20-triphenyl-porphyrin incorporated into a poly-cationic liposome. This porphyrin was successfully tested against a typically antibiotic-resistant pathogen, such as methicillin-resistant Staphylococcus aureus (5). The other modification of antimicrobial PDT comprises conjugates between photosensitisers and proteins. This was achieved by the binding of IgG with tin (IV) chlorin e6. The conjugate showed (itself to be effective against methicillin-resistant Staphylococcus aureus. IgG-Sn Ce6 and Sn Ce6 killed bacteria in the light- and photosensitiser-dose-dependent manner (3).

Among the many different microorganisms, fungal pathogens were also subjected to PDT. A fungal pathogen Cryptococcus neoformans was found to be susceptible to photodynamic inactivation by the use of a polycationic conjugate of polyethyleneimine and chlorin e6 (7).

There are a number of possible initial targets of PACT applications, including periodontal diseases, impetigo, atopic dermatitis, acne vulgaris, infected wounds, and superinfected psoriatic plaques (11). In another study, methylene blue and visible light were applied to evaluate the killing of microorganisms encountered on healthy and affected skin (Staphylococcus aureus, S. epidermidis, Streptococcus pyogenes, Corynebacterium minutissimum, Propionibacterium acnes, and Candida albicans). The results indicated that the PDT of the skin may represent a useful alternative to conventional antimicrobial therapy (16). The antimicrobial PDT was also used for the inactivation of vesicle stomatitis virus (VSV) in red blood cell concentrates (RBC) with limited damage to the cells (15). In this study, another cationic porphyrin, mono-phenyl-tri(N-methyl-4-pyridyl)-porphyrin chloride [Tri-P(4)] was shown to be effective. Next, the series of viruses and bacteria was spiked into 60% RBC
concentrates and the inactivation of pathogens was determined after PDT with 25 µM Tri-P(4) treatment and light up to 360 kJ/sq.m. It was found that PDT with Tri-P(4) inactivated a wide range of pathogens, but not cell-associated HIV and a non-enveloped virus, and compromised RBC quality. Previous studies (17, 18) have revealed that PDT could be used in the sterilisation of RBC concentrates from VSV, herpes simplex virus-1 (HSV-1), encephalomyocarditis virus (EMCV), and bovine viral diarrhoea virus (BVDV) at little, if any, harm to RBC. Similar results with respect to VSV were achieved by Trannoy et al. (15); however, they observed that PDT may compromise the RBC quality (15). Photodynamic therapy can also be used in the inactivation of pathogens in platelet concentrates. In that case, the photodynamic treatment effect with thionine and light was significantly enhanced when it was followed by irradiation with UV-B (12). Depending on the functional groups introduced into the molecule, fullerenes can effectively photoinactivate either or both pathogenic microbial cells and malignant cancer cells. The mechanism appears to involve superoxide anion, as well as singlet oxygen (13).

In all the studies briefly mentioned above, the antimicrobial role of PDT was more or less satisfactory confirmed.

Our aim in the present study was to confirm whether photodynamic therapy can be applied in the preventive sterilisation of tissue/tumour samples transplanted into laboratory animals, i.e. rats. Failures in the successful transplantation, or inoculation, of experimental tumours into rats are often related to an existing contamination by bacteria of the transferred tissues. The risk of contamination by animal fur, plastic cage, food or sawdust, or infection caused by the operator is always possible, especially when the cells are transferred from one individual to another.

**Material and Methods**

**Photosensitiser.** Chlorin e6 crystals (Porphyrin Products, Inc., USA) were dissolved in 9.8 ml of 0.9% NaCl. The total dissolution of chlorin e6 was achieved thanks to the addition of 0.2 ml of 0.05 M potassium hydroxide to the main solution. Next, the solution was diluted to achieve concentrations from 0.05 to 1.0 µg/mL (0.05; 0.1; 0.2; 0.5, and 1.0).

**Light source.** This was a halogen lamp (Penta Lamps Teclas). The wavelength was 630 nm, and the total dose of light was 100 J/sq.cm. Irradiations were performed on Petri dishes after a 24 h incubation of the tumour cells with the photosensitiser in the dark.

**Animals.** Inbred female Buffalo and Wistar rats from own breeding, 3-4 months of age and 200-240 g of weight, were used. They were kept in plastic cages in a humid atmosphere at room temperature. The Buffalo rats were transplanted with Morris hepatoma (MH); the Wistar rats with mammary carcinoma (MC) and sarcoma Yoshida (SY) - all tumours obtained from Institute of Oncology, Poland. Approval from the Local Bioethics Committee for Animal Research was obtained.

**Tumour cell lines.** After 10 d from the transplantation of above-mentioned tumours, i.e. MH, MC, and SY, they were excised, minced in case of MH and MC, and then kept in medium RPMI-1640 supplemented with L-glutamine, 10% FBS (foetal bovine serum), 100 U/mL of penicillin, and 100 µg/mL of streptomycin (Sigma). They were kept in plastic flasks at 37°C. Before irradiation, the cells were centrifuged at 1,000 rpm for 10 min and resuspended back in the medium. The tumour cells, after being treated with PDT (Ce6 – 0.1 or 0.2 µg/mL, light 100 J/sq.cm), were injected back into the rats. Similarly, the tumour cells subjected to Ce6 alone and light alone, were injected back into the animals. We used these two doses of Ce6 because they were found to be the lowest and highest range of the tested doses where the application of light was required to obtain an aseptic specimen. Tumour growth was then observed in all the rats: 7-10 d after the transplantation. The tumours were palpable and macroscopically visible. They were excised after that time, and microscopical examination (formalin fixed, paraffin embedded, and haematoxylin-eosin stained slides) confirmed the histological structure of the lesions.

**Bacteriological studies.** The cells obtained from above three tumours were tested for the presence of pathogenic bacteria. The tumour cells had been previously treated with chlorin e6-PDT, with chlorin e6 alone, light alone, or not treated. The incubation with chlorin e6 (doses from 0.05 to 1.0 µg/mL: 0.05; 0.1; 0.2; 0.5, and 1.0) lasted 24 h and then the cells were irradiated with light at 630 nm for 20 min to achieve 100 J/sq.cm. All the tumour cells were seeded on agar, blood agar, and McConkey medium. They were incubated under aerobic conditions at 37°C for 18-24 h. The colonies growing on the McConkey medium characteristic for *Enterobacteriaceae* sp. were identified by use of the API20E test (bioMérieux, France). The colonies suspected of belonging to *Streptococcus* sp. were seeded on Bile Esculin Azide–LAB-AGARTM medium (Biomed, Kraków, Poland), and incubated under aerobic conditions at 37°C for 18-24 h. The colonies changing the colour of the medium to black (decomposing esculin) were classified as *Enterococcus* sp. The other colonies were seeded onto blood agar, which was incubated under aerobic conditions at 37°C for 18-24 h. After that time, the colonies were stained by use of the Gram-method and identified using API Strep test (bioMérieux, France). The colonies suspected of belonging to *Staphylococcus* sp. were seeded on Bile Esculin Azide–LAB-AGARTM medium (Biomed, Kraków, Poland), and incubated under aerobic conditions at 37°C for 18-24 h. The colonies confirmed the histological structure of the lesions.

**Preparation and evaluation of cytological smears.** The cytological smears from the tumours were

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The text is a continuation of the previous discussion on the effectiveness of photodynamic therapy (PDT) in inactivating pathogens and malignant cancer cells. The study aimed to confirm whether PDT can be applied as a preventive sterilisation method for tissue/tumour samples transplanted into laboratory animals, specifically rats.

The authors used chlorin e6 crystals as the photosensitiser and a halogen lamp as the light source. Inbred female Buffalo and Wistar rats were used as the experimental subjects. The tumours tested included MH, MC, and SY. The cells were treated with different doses of chlorin e6 and light, and their effectiveness was assessed through various methods such as histological examination and bacterial culture.

The bacteriological studies were performed to identify any pathogenic bacteria that might compromise RBC quality. The colonies suspected of belonging to *Enterobacteriaceae* and *Streptococcus* were identified using specific tests and methods.

The cytological smears from the tumours were prepared and evaluated to confirm the histological structure of the lesions.
done in the routine way, air-dried at room temperature, fixed in 70% ethanol, and stained with haematoxylin-eosin. They were then evaluated by two independent pathologists using a light microscope at a magnification of 200x. The number of surviving (living) tumour cells was counted from five high-power microscope fields on one glass slide from one specimen, e.g. treated with light only, or with chlorin e6 only: the mean value and standard deviation of the data from the fields were next calculated. We confirmed the obtained results by using the MTT test (data not shown).

Results

The studies carried out showed that the application of photodynamic therapy allows the destruction of microorganisms in the tumours used for the transplantation in rats. It was confirmed that chlorin e6 (Ce6) in low doses, i.e. from 0.05 to 0.1 µg/mL after irradiation with light in a dose of 100 J/sq.cm, caused the eradication of bacteria such as Proteus sp., Streptococcus α-haemolyticus, Corynebacterium sp. and Staphylococcus aureus. It is worth emphasising that Ce6 in doses of 0.2, 0.5, and 1.0 µg/mL induced a similar effect in the absence of light. The results are shown in Table 1.

### Table 1

Results of microbiological analysis of tumours subjected to photodynamic therapy and in control group

<table>
<thead>
<tr>
<th>Chlorin e6 (µg/mL)</th>
<th>Bacteria (before irradiation)</th>
<th>Bacteria (after irradiation 100 J/sq.cm)</th>
</tr>
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<tbody>
<tr>
<td>0.05</td>
<td><em>Proteus</em> sp. 1.5x10³&lt;br&gt;<em>Streptococcus</em> α-haemolyticus 6x10²&lt;br&gt;<em>Corynebacterium</em> sp. 8x10²&lt;br&gt;<em>Staphylococcus</em> sp. 4x10²</td>
<td>Sterile</td>
</tr>
<tr>
<td>0.1</td>
<td><em>Streptococcus</em> α-haemolyticus 3.2x10³&lt;br&gt;<em>Corynebacterium</em> sp. 1x10²&lt;br&gt;<em>Staphylococcus</em> sp. 5x10²</td>
<td>Sterile</td>
</tr>
<tr>
<td>0.2</td>
<td>Sterile</td>
<td>Sterile</td>
</tr>
<tr>
<td>0.5</td>
<td>Sterile</td>
<td>Sterile</td>
</tr>
<tr>
<td>1.0</td>
<td>Sterile</td>
<td>Sterile</td>
</tr>
<tr>
<td>0</td>
<td><em>Proteus</em> sp. 1.5x10³&lt;br&gt;<em>Streptococcus</em> α-haemolyticus 6x10²&lt;br&gt;<em>Corynebacterium</em> sp. 8x10²&lt;br&gt;<em>Staphylococcus</em> sp. 5x10²</td>
<td>Sterile</td>
</tr>
</tbody>
</table>

(Control group)  

![Fig. 1. The number of surviving cells of mammary carcinoma treated with PDT and in control groups. Std. – standard; CI_01_light - 0.1 µg/mL of chlorin e6 + light; CI_02_light - 0.2 µg/mL of chlorin e6 + light; control groups: CI_01 - 0.1 µg/mL of chlorin e6; CI_02 - 0.2 µg/mL of chlorin e6; light – only light without chlorin e6; (-) – no chlorin, no light.](image-url)
Fig. 2. The number of surviving cells of sarcoma Yoshida treated with PDT and in control groups. Symbols are explained in the footnote to Fig. 1.

Fig. 3. The number of surviving cells of Morris hepatoma treated with PDT and in control groups. Symbols are explained in the footnote to Fig. 1.
Photodynamic therapy was repeated on the three aforementioned tumour cell lines. The results showed that in all three cases, a significant but not total in vitro decrease in the amount of tumour cells was observed. For example, in mammary carcinoma, the mean value of the number of well-preserved surviving cells from the five fields in the control group was found to be 149.2. This amount was reduced to 61.8 and 109.2 in groups of cells treated with Ce6-PDT at Ce6 doses of 0.1 and 0.2 µg/mL, respectively. Ce6 alone also caused a decrease in the number of tumour cells to 138.6 and 86 after incubation with Ce6 (0.1 and 0.2 µg/mL, respectively) (Fig. 1).

In the Yoshida sarcoma control group, the mean value of the number of cells from the five fields was found to be 142.6. In all other groups, except for (those treated with) light treated, a strong decrease in the number of cells was observed; however, this was never zero (Fig. 2).

The results were only slightly different in Morris hepatoma. In the control group, the number of cells was 199.4 on average, whereas after Ce6-PDT with 0.1 µg/mL, a slight increase was found, i.e. 212 cells. In contrast, the Ce6-PDT with 0.2 µg/mL Ce6, resulted in a decrease to 142, as was expected (Fig. 3).

Discussion

Although various tumours are successfully treated with PDT, its application on microorganisms (PACT) has not yet been found to have specific biological applications. With the present study, we would like to emphasise the possible application of PACT as a method of preventive sterilisation of tumours transplanted into laboratory animals. In previous studies, Gad et al. (8) reported the use of PDT to treat localised infections caused by Staphylococcus epidermidis and Staphylococcus aureus. They have used two cationic photosensitisers including poly-lysine-chlorin e6 conjugate and methylene blue and anionic free chlorin e6 (Ce6). They have found that free Ce6 killed more mutant strains than the wild type (8). PACT has also been successfully used in the treatment of burn wounds in mice with established Staphylococcus aureus infections (10) or Escherichia coli (9).

Photosensitised inactivation of microorganisms is typically a multi-target process, thus the selection of photosensitive microbial strains is very unlikely.

In a number of studies, different porphyrins have been used in PACT, e.g. Banfi et al. (1) assessed the effect of a panel of seven tetraaryl-porphyrins in vitro against Escherichia coli, Pseudomonas aeruginosa, and Staphylococcus aureus. From that experiment, it has been concluded that the presence of positively charged groups on a porphyrin frame is fundamental for photosensitiser antibacterial activity; however, a certain degree of lipophilicity may improve this efficiency (1). The chlorin e6 used in the present study is an anionic compound. The possibility of using a bacteriophage to deliver tin(IV) chlorin e6 to Staphylococcus aureus has also been considered (4). In that study, the substantial killing of methicillin- and vancomycin-intermediate strains of Staphylococcus aureus were achieved using low concentrations of the conjugate, 1.5 µg/mL and low light doses, 21 J/cm², under which the viability of human epithelial cells in the absence of bacteria was unaffected (4). In our study, the PACT conditions were also rather mild with regard to the low photosensitiser dose; however, the dose of light was higher than in other related studies. This has also been observed in other studies, where the extensive eradication of pathogens could be achieved under mild irradiation conditions, such as short incubation times and low fluence-rates, which guarantee a high degree of selectivity in comparison with the main constituents of host tissue, such as keratinocytes and fibroblasts (11).

For optimising the antibacterial activity of the photosensitiser meta-tetrahydroxyphenylchlorin, it has been encapsulated in mixed cationic liposomes composed of different ratios of dimyrystol-sn-glycero-phosphatidylethanolamine and any of four cationic surfactants derived from 1-prolinol (2). The other porphyrin incorporated into the polycationic liposomes, i.e. 5-[4-(1-dodecanoylpyridinium)]-10,15,20-triphenylporphyrin, was tested against methicillin-resistant Staphylococcus aureus, a known antibiotic-resistant pathogen (5). The use of such liposomes as carriers dramatically potentiates the antibacterial activity of the photosensitiser. The carrier disorganises the native three-dimensional architecture of the bacterial wall, thereby enhancing its permeability to the photosensitiser (5).

The mechanism of the PACT is connected with the interaction of the photosensitiser with the outer wall on the surface of several types of bacterial and yeast cells, and with the above-mentioned increase in permeability and accumulation of the compound at the cytoplasmic membrane level (11). These photosensitisers are highly active toward both wild strain and antibiotic-resistant Gram-positive and Gram-negative bacteria and yeasts (11). Some failures in the PACT of Gram-positive bacteria can be related to slime production and the stationary phase: these problems can be overcome by using cationic photosensitisers (8). The other method reported by Embleton et al. (3), comprised of the application of IgG-tin(IV) chlorin e6 conjugate. They have found that the conjugate killed methicillin-resistant Staphylococcus aureus in a light-dose- and photosensitiser-dependent manner, and in the selected way.

In some studies, the efficacy of photodynamic therapy against microbial flora achieved up to 80% in the reduction of colony-forming unit counts (6). The important parameter is always the photodynamic dose, i.e. a photosensitiser x light dose that can be elevated by the increase of chemical or/and light dose. The antibacterial PDT does not actually require high photodynamic doses, and this enables the sterilisation, without damage of transplanted tumour cells.

In our study we have checked and confirmed the susceptibility of some bacteria, like Staphylococcus sp. to PDT; in other studies, the fungal pathogen
Cryptococcus neoformans rom2 mutant was hypersusceptible to photodynamic inactivation (7). That experiment proved the significance of cell wall integrity in microbial susceptibility to PACT.

References