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PHOTODYNAMIC THERAPY TARGETED TO PATHOGENS

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Abstract

Photodynamic therapy (PDT) employs a non-toxic dye termed a photosensitizer (PS) together with low intensity visible light, which, in the presence of oxygen, produce cytotoxic species. PS can be targeted to its destination cell or tissue and, in addition, the irradiation can be spatially confined to the lesion giving PDT the advantage of dual selectivity. This promising approach can be used for various applications including microbial inactivation and the treatment of infections. Resistance to PDT has not been shown and multiantibiotic-resistant strains are as easily killed as naïve strains. It is known that Gram (+) bacteria are more sensitive to PDT as compared to Gram (−) species. However, the use of cationic PS or agents that increase the permeability of the outer membrane allows for the effective killing of Gram (−) organisms. Some PS have an innate positive charge, but our approach is to link PS to a cationic molecular vehicle such as poly-L-lysine. This modification dramatically increases PS binding to and penetrating through the negatively charged bacterial permeability barrier. Due to focused light delivery the use of PDT is possible only for localized infections. Nonetheless numerous diseases can be treated. Selectivity of the PS for microbes over host cells, accurate delivery of the PS into the infected area, and PDT dose adjustment help minimize side effects and give PDT an advantage over conventional therapy. There are only a few reports about the use of antimicrobial PDT in animal models and clinical trials. We have used genetically modified bioluminescent bacteria to follow the effect of PDT in infected wounds, burns, and soft tissue infections in mice. Not only were bacteria infecting wounds, burns, and abscesses killed, but mice were saved from death due to sepsis and wound healing was improved.

Keywords

bacteria; photosensitizer conjugate; fungus; localized infection

Photodynamic therapy (PDT) is a therapy for cancer and other diseases that has received regulatory approvals for several indications in many countries (1). The basic principles of PDT have been summarized in numerous reviews (2–4). Briefly, relatively harmless substances named photosensitizers (PS) after irradiation with low intensity light in presence of oxygen produce cytotoxic species that lead to cell destruction. The cytotoxic species can arise via two mechanisms known as Type I and Type II photoprocesses. The first leads to production of radical ions and reactive oxygen species (5) and the second to formation of the excited state reactive singlet oxygen (6) (Fig. 1). Compared to other cytotoxic therapies PDT has the advantage of dual selectivity: PS can be targeted to diseased tissue or cells, and light can be focused in the site of the lesion.

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Although the photochemistry is the same for cancer and antimicrobial PDT, there are major differences in the PS structures and cellular targets. Most of the PS that are under investigation for the treatment of cancer and other diseases are based on the tetrapyrrole nucleus including porphyrins, chlorins, bacteriochlorins, phthalocyanines and texaphyrins. These molecules have been chosen for their low toxicity in the absence of light to mammalian cells and to animals, and for their tumor-localizing properties. PS that have been studied for their ability to kill microorganisms have distinct molecular frameworks. Examples are halogenated xanthenes such as Rose Bengal (RB) poly-L-lysine chlorin (e6) coryngated (pL-ce6) (7), phenothiazines such as toluidine blue O (TBO), and methylene blue (8) and poly-L-lysine-chlorin(e6) conjugates (9) (Fig. 2.). The main targets for photodynamic inactivation (PDI) of mammalian cells are lysosomes, mitochondria and the plasma membrane (2) while in microbial cells damage to the outer membrane plays a major role (9) with a possible role for DNA damage.

PDT can be used against various microorganisms including viruses, bacteria, and fungi (10). Due to spatially confined light delivery PDT can be used only for localized infections. Advantages of PDT compared to standard therapies include the following. The development of resistance to PDT has not been shown to occur (11) and is thought to be very unlikely. Multiantibiotic-resistant strains are as easily killed as naïve strains (12). Topical PDT can be used for infections in non-perfused tissue such as burns. PDT may have less, toxicity as compared to other topical antimicrobial. In addition side effects can be minimized by the use of PS targeted towards microorganisms rather than host cells (13). In this review we will discuss photoinactivation of members of various microbial groups and initial approaches to treating animal models of infections.

Targeted antimicrobial PDT

Antimicrobial PDT has been known for about a century and many basic factors that govern the susceptibility of various bacteria to photodynamic inactivation mechanisms are known; however much remains to be explored.

It has been shown that Gram (+) bacteria are relatively easy to kill by PDT, while Gram (−) bacteria show significant resistance (14). This is due to differences in their cell wall structures. Since the Gram stain acts like many PS used in PDT, it can be imagined that Gram (+) species that readily take up dye are also easily killed while the reverse is true of Gram (−) bacteria. Gram (−) bacteria have a complex many layered outer barrier structure consisting of a glycocalyx, lipolysaccharide, outer membrane lipid bilayer, periplasm, peptidoglycan cell wall, and plasma membrane lipid bilayer (15–16). This barrier keeps out most PS therefore specific methods have to be adopted to ensure that the PS can penetrate the bacterium (14). These methods consist of the use of polymyxin B nonapetide (17) or ethylenediamine tetracetic acid (18) to disturb the structure of the outer membrane or choosing the PS molecule to bear a cationic charge (19).

Differences in susceptibility to PDI between species from the same Gram classification are harder to explain. Factors that may be important include membrane permeability barriers (*Pseudomonas aeruginosa* for instance is harder to kill than *Escherichia coli*), differences in antioxidant enzymes or DNA repair mechanisms, and simple factors such as the size of the microbial cell.

Most bacteria do not absorb light, rather they scatter light. Light scattering may have a slight dependence on Gram classification inasmuch as it affects their size and shape. In a few cases bacteria do absorb light because they are colored (i.e. they have a natural pigment). This pigment (especially if it is composed of porphyrins) may act as a natural photosensitizer that is naturally synthesized or accumulated inside the bacterial cell. This is the case for some

bacteria such as *Propionibacterium acnes* (20), *Helicobacter pylori* (21), and some others that accumulate porphyrins that allow their photokilling without an the need for adding an exogenous PS.

Positively charged PS can be bound to the negatively charged surface of bacteria from both groups (Gram + and Gram −). Polycationic conjugates between poly-L-lysine chains (pL) and ce6 developed in our laboratory (9) are particularly suited to fight both Gram (+), Gram (−) bacteria, and fungi (Fig. 2). There is the evidence that the conjugates are not only bound to the outer surface of microorganisms, but also penetrate the permeability barrier. Other PS may be only loosely bound to microorganisms, or indeed incapable of binding or penetrating the cells. In our experiments we compared the process of carrying out the irradiation in the presence of the PS in the solution in which the bacteria are suspended, with the process whereby the bacteria are centrifuged and resuspended (washed) after incubation with the dye, and therefore only the dye actually bound to the cells remains. We compared the PDI activity of three PS (pL-ce6 prepared from a 100-lysine chain, TBO and RB) against the Gram (−) *E. Coli* (Fig.3a–c). It can be seen that the pL-ce6 conjugates are highly effective against *E. coli* (1 μM and up to 1 J/cm2) and this is independent of whether the dye is washed out or not (Fig. 3a). TBO is much less effective (35 μM and up to 2 J/cm2) and in addition is much more active if left in solution with the bacteria than when washed out (Fig. 3b). RB is comparably effective to TBO (35 μM and up to 8 J/cm2), but is only active in mediating the photokilling of *E. coli* when it remains in solution, and is completely inactive after washing (Fig. 3c). These observations imply that the conjugate strongly binds to the cells (and presumably penetrates the Gram (−) permeability barrier), TBO does bind to some extent to the bacteria but that the dye in solution is more active, and in the case of RB, extracellular singlet oxygen plays the major role.

PDT is known to be effective against various fungi, however, higher doses compared to bacteria have to be used. Polycationic pL-ce6 conjugates are highly effective in conjunction with relatively small amounts of light in killing pathogens from different classes. In addition to the Gram (−) *E. coli*, we used *Staphylococcus aureus* (a Gram + pathogen), and *Candida albicans* (a dimorphic pathogenic fungus/yeast). In Fig. 4a we compare the dose effect on the survival fraction of increasing the conjugate concentration combined with a single fluence (12 J/cm2) of 665-nm light. Even though the light-mediated killing of the fungus (*C. albicans)* needed a significantly higher concentration of pL-ce6 than did the bacterial species, we still achieved five logs of killing with 25 μM. In Fig. 4b we compare the relative effectiveness of the three PS against *C. albicans.* The conjugate was dramatically more potent than the alternative PS, TBO and RB. We achieved 3–4 more logs of kill with one eighth of the concentration suggesting that polycationic conjugates may be the most promising PS for fungal disease.

Another approach for targeting PS towards microorganisms includes linking of PS to an antibody against the organism. Using TBO-antibody conjugates, 100% elimination of *Porphyromonas gingivalis* was achieved (22). Monoclonal antibodies and PS conjugates were also used against *P. aeruginosa.* Effective and specific killing was achieved after illumination (23). Non-specific IgG conjugates with bacteriochlorophyll-serine derivative were toxic against *S. aureus* after irradiation, however in this case, the uptake of the conjugate by the bacteria was less as compared to free PS (24). This finding once again shows that some PS have to penetrate the bacteria in order to be effective, while others work extracellularly.

PDT for localized infection

For a long time infections were always successfully treated with topical and systemic antibiotics, however recent and rapid emergence of multi-antibiotic resistant strains of

bacteria is of considerable concern. Both traumatic wounds and burns may contain significant amounts of non-perfused tissue due to compromise of the capillary circulation. These factors seriously limit the use of systemic antibiotics. Topically applied antimicrobials may also face a problem in penetrating to bacteria that have colonized the damaged tissue. Therefore there is significant need for new therapies. PDI of bacteria in wounds may be an effective means of killing bacteria while simultaneously stimulating the host immune system and enhancing wound healing.

Although PDT is generally effective in killing mammalian cells as well as microbial cells, certain factors can be taken into account to enhance selectivity for microbial cells over host cells. Firstly the PS should be applied topically into the infected area rather than injected systemically. This allows the PS to come directly into contact with the microbes instead of being delivered via the capillaries and coming into contact with host cells first. Secondly the structure of the PS (cationic charge and macromolecular structure) means that the PS binds rapidly to microbes but only slowly is taken up by host cells, thus giving temporal selectivity for the microorganisms. Hence if light is delivered relatively soon after PS administration, collateral damage to host tissue will be minimized.

Despite a considerable amount of work in the literature on PDI of bacteria in vitro, its use to treat animal models of localized infection is rare (25–26). This may be partly due to inefficient PS targeting and killing of bacteria compared to eukaryotic cells, but another reason is likely to be the difficulty in following the progress of localized infections in rodents. The use of genetically engineered luminescent bacteria and a sensitive low-light imaging camera to follow the extent and amount of infection in real-time in living animals is a solution to this problem (27). We have demonstrated the use of this approach in several murine models.

Our initial experiments were done with excisional wounds infected with a relatively non-pathogenic *E. coli* (28). Topical application of pL-ce6-conjugates in combination with 660-nm light led to light dose dependent reduction in luminescence in treated wounds not seen in control wounds or wounds treated with conjugate alone or light alone. The treatment did not damage the host tissue, treated wounds healed equally well as control uninfected wounds. An invasive strain of the Gram (−) pathogen *P. aeruginosa* was used for further studies (29). Dose dependent reduction in luminescence was observed after topical application of pL-ce6 conjugates followed by red light (Fig. 5a–g). Wounds were totally clear 24 hours later (Fig. 5h). The dark-conjugate treated wounds still had an appreciable bacterial infection as judged by the residual luminescence (Fig. 5i–m). We were able to quantify the reduction in luminescence present in the infected wounds using the ARGUS software. The resulting curves are plotted in Fig. 5n. The PDT treated group shows a semi-logarithmic relationship between bacterial luminescence and delivered fluency, until 99% of the luminescence has disappeared after 240 J/cm2. There is a significant difference between the luminescence found from the conjugate in the dark group, compared with that in the light alone and absolute control groups. This is due to two factors: firstly to a degree of dark toxicity of the conjugate towards *P. aeruginosa,* and secondly to the ability of the bacteria in the absolute and light alone control wounds to continue to multiply. All mice bearing untreated infected wounds or those treated with light alone or conjugate alone, developed septicemia when the bacteria reached the bloodstream, and died between 1 and 4 days after infection. By contrast 90% of the mice whose wounds were treated with PDT survived (Fig. 5o), surrounding host tissue was spared, and the wounds healed well.

Reports using PDT as a cancer therapy in patients have suggested that the healing after PDT is surprisingly effective. In infected wounds in mice we have shown not only the strong antimicrobial effect of PDT and its ability to destroy microbial toxins, but also a stimulation

of wound healing. If the PDT regimen is appropriate this stimulation of wound healing can be demonstrated in non-infected wounds. A novel area of PDT research is its use to treat chronic wounds such as non-healing leg ulcers.

A similar technique was used for monitoring of soft tissue infections (30). Stably transformed bioluminescent mouse pathogenic *S. aureus* cells were injected into the thigh muscles of cyclophosphamide treated mice. Twenty-four hours later a pL-ce6 conjugate was injected into the area of infected muscle; light was delivered 30 minutes later as a surface spot or by interstitial fiber. Light dose dependent loss of luminescence was seen. However in some cases the infection recurred.

The only fungus where photoinactivation was tested in vivo in an infection model is *C. albicans* (31). An immunodeficient murine model (SCID mice) was used. Methylene blue was introduced into the mouse oral cavity at a concentration 450–500 μg/mL followed by 275 J of light from a diffusing tip fiber. This totally eliminated yeasts as determined by swab culture. These data suggest that antimicrobial PDT might have diverse possible clinical applications in humans that will be discussed below.

Clinical applications

Data on clinical applications of PDI are limited, and most of the studies were devoted to antiviral PDT, but a few antibacterial clinical trials were also undertaken. In 1970s there were numerous clinical trials and clinical practice for treatment of recurrent herpes simplex lesions (32–33). Topical application of 1% aqueous solution of Neutral Red followed by a 15 minute exposure to a 40 W bulb filtered to transmit 440 – 550 nm was used. Treatments were discontinued due to weak effectiveness and possible carcinogenicity. However recently several groups showed that 5-aminolevulinic acid (ALA) based PDT utilizing red light can be used against molluscum contagiosum (caused by poxviruses), HPV, vesicular stomatitis virus (34–35). Abdel-Hady *et al.* (36) used topical ALA-PDT to treat high-grade vulval intraepithelial neoplasia lesions but observed a short-term response in only one third of cases.

Antiviral PDT is also widely used for blood and blood product decontamination. Psoralens, methylene blue, merocyanine 540, porphyrins/chlorins, and phthalocyanines are usually used as PS (37). It has been established that multiple sites, such as the envelope and core proteins, the inner core structures, RNA, and reverse transcriptase are targets for photodynamic inactivation of viruses (38–39); however further studies on antiviral PDT are needed.

Studies about the use of clinical PDT for treating bacterial infections are even less common. There is one report of PDT being used to treat abscesses by topical administration of PS in patients by Lombard et al. (26). Five patients with brain infection after craniotomy and surgical drainage were treated by instilling hematoporphyrin into the abscess bed and irradiating 5 minutes afterwards. Positive clinical responses were observed.

Other clinical trials have been devoted to treatment of *H. pylori* infections. *H. pylori* is an endemic pathogenic bacterium causing gastroduodenal ulceration in humans and is linked to the development of stomach cancer. Increasing reports mention the emergence of antibiotic resistance to conventional triple drug therapy (40) prompting the search for alternative treatments (41). A clinical trial was carried out in 13 patients using oral 5-ALA (20 mg/kg) and, 45 minutes later, a zone of gastric antrum was illuminated through an endoscope with a blue laser (410 nm, 50 J/cm2) (42). A greater eradication of *H. pylori* in biopsies from illuminated areas compared to control zones was demonstrated. However, we have recently discovered that *H. pylori* accumulates natural endogenous PS - porphyrins and therefore the

use of an additional PS in the stomach can be avoided (43). We enrolled ten symptomatic patients positive for *H. pylori.* Blue light (405±2 nm) was delivered through a flexible optical fiber into gastric antrum. The mean reduction in *H. pylori* colonies was 91%, however some patients had reduction of 99% (44).

P. acnes (the cause of acne) was also successfully treated with a combination of topical ALA and red light 22 in patients (45). PDT caused a transient acne-like folliculitis, but then sebum excretion was eliminated for several weeks. There was histological evidence of sebaceous gland damage and bacterial porphyrin fluorescence was also suppressed. Clinical clearance of inflammatory acne was achieved for at least 20 weeks after multiple treatments and 10 weeks after a single treatment. Transient hyperpigmentation, superficial exfoliation, and crusting were observed, which cleared without scarring. Similar results were also reported by Japanese workers (46). However *P. acnes* as well as *H. pylori* can be killed in the absence of dye (47). High-intensity 405–420 nm blue light was used in this study and the response was more than 80%. Significant reduction of 59–67% of inflammatory acne lesions after only eight treatments of 8–15 minutes was also achieved. No adverse effects or patient discomfort were noted. Although PDT is know to be effective against eukariotic pathogens, just one clinical application has so far been reported. Cutaneous leishmaniasis (caused by an intracellular parasite) was treated in 10 patients, the combination of topical Metvix (an aminolevulanic acid ester) and red light was employed. There was no recurrence ten months after therapy (48).

CONCLUSION AND POSSIBLE FUTURE APPLICATIONS

Future clinical applications of antimicrobial PDT may involve two broad groups of diseases in which PS and light may be relatively easily delivered to the site of infections. The first group includes trauma-associated infections. Its clinical manifestations vary from superficial infections caused by various Gram (+) and Gram (−) bacteria, to necrotizing fasciitis (*S. aureus, Streptococcus spp,* or polymicrobial species), gas gangrene (*Clostridium spp),* necrotizing cellulitis, and Fournier's gangrene (synergistic mixtures of aerobes and anaerobes).

The second group is even more diverse. It includes stubborn chronic infections in body cavities and surfaces that generally respond poorly to systemic antibiotics. Examples of these infections include bacterial keratitis, keratomycoses, dermatophytoses, abscesses, sinusitis, otitis media, periodontal diseases, and urinary tract infections. Microbial species involved in these conditions include various bacteria, filamentous fungi, eukaryotic parasites and (to a lesser extent) viruses all of which have been shown to be susceptible to in vitro PDI.

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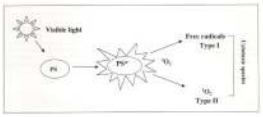

Fig.1.

Schematic illustration of photophysical/photochemical mechanisms of PDT. After irradiation with visible light the ground state PS moves to the excited state. Excited state PS transfers its energy to the ground state of molecular oxygen (a triplet). This results in the PS returning to the ground singlet state, and the oxygen rising to the excited singlet state (Type II). Alternatively the PS may undergo reactions with substrates leading to free radicals (Type I). Both singlet oxygen and free radicals are highly cytotoxic species that can react with proteins, nucleic acids and lipids in cells.

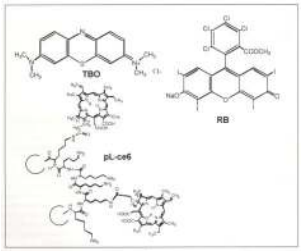

Fig.2.
Structural chemical formulae of PS used in antimicrobial PDT. TBO is toluidine blue O, RB is Rose Bengal, pL-ce6 is poly-L-lysine chlorin(e6) conjugate.

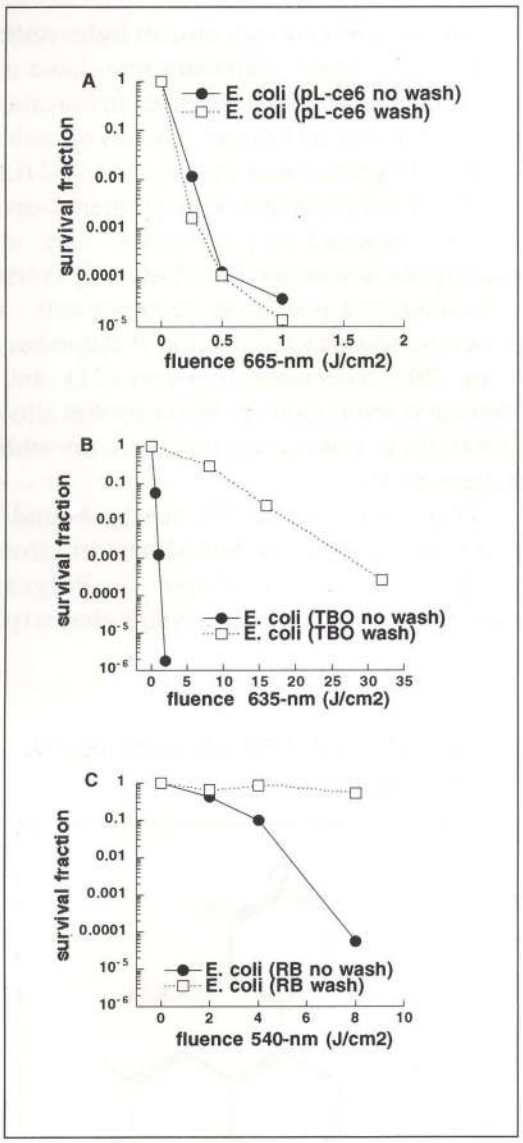

Fig.3.

PDI of *E. coli* mediated by various PS with and without removal of the PS from the cell suspension (washing). A: pL-ce6 at 0.75 μM with 665-nm light; B: TBO at 35 μM with 635-nm light, C: RB at 35 μM with 540-nm light.

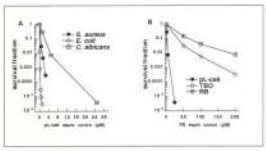

Fig.4.

A: PDI mediated by pL-ce6 against representative members of three microbial classes. The concentration of pL-ce6 was increased and a constant fluence (12 J/cm2) of 665-nm light was delivered and survival fractions measured. B: PDI mediated by three PS against *C. albicans*. Concentrations of PS were varied and a constant fluence (12 J/cm2) of the appropriate wavelength light was administered.

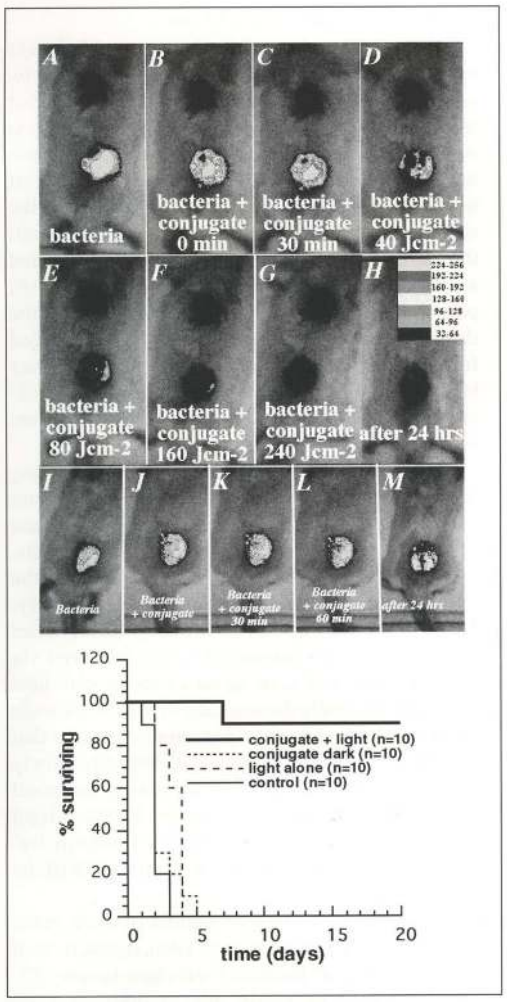

Fig.5.

(Panels A–H): Successive overlaid luminescence (gray scale) images and monochrome LED images of a representative mouse bearing an excisional wound infected with 5×10^6 luminescent *P*. aeruginosa and treated with pL-ce6 conjugate and increasing doses of light. (Panels I–M): A representative mouse treated with pL-ce6 and kept in the dark. Panel N: Mean pixel values of luminescence signals from defined areas measuring 1200 pixels covering infected wounds determined by image analysis. The four groups comprise absolute control, light alone control, dark conjugate control, and PDT treated. Data points are means of values from the wounds on ten mice per group and bars are SD. Panel O: Kaplan-Meier survival plot for the four groups of mice described in panel N.