Photodynamic Therapy in the Treatment of Microbial Infections: Basic Principles and Perspective Applications

Giulio Jori, PhD,[1]* Clara Fabris, PhD,[1] Marina Soncin, PhD,[1] Stefania Ferro, MSc,[1] Olimpia Coppellotti, PhD,[1] Donata Dei, PhD,[2] Lia Fantetti, PhD,[2] Giacomo Chiti, PhD,[2] and Gabrio Roncucci, PhD[2]

[1]*Department of Biology, University of Padova, Via Ugo Bassi 58B, 35121 Padova, Italy*

[2]*Molteni Farmaceutici, 50018 Scandicci, Firenze, Italy*

Background and Objectives: Photodynamic therapy (PDT) appears to be endowed with several favorable features for the treatment of infections originated by microbial pathogens, including a broad spectrum of action, the efficient inactivation of antibiotic-resistant strains, the low mutagenic potential, and the lack of selection of photoresistant microbial cells. Therefore, intensive studies are being pursued in order to define the scope and field of application of this approach.

Results: Optimal cytocidal activity against a large variety of bacterial, fungal, and protozoan pathogens has been found to be typical of photosensitizers that are positively charged at physiological pH values (e.g., for the presence of quaternarized amino groups or the association with polylysine moieties) and are characterized by a moderate hydrophobicity (n-octanol/water partition coefficient around 10). These photosensitizers in a micromolar concentration can induce a >4–5 log decrease in the microbial population after incubation times as short as 5–10 minutes and irradiation under mild experimental conditions, such as fluence-rates around 50 mW/cm^2 and irradiation times shorter than 15 minutes.

Conclusions: PDT appears to represent an efficacious alternative modality for the treatment of localized microbial infections through the in situ application of the photosensitizer followed by irradiation of the photosensitizer-loaded infected area. Proposed clinical fields of interest of antimicrobial PDT include the treatment of chronic ulcers, infected burns, acne vulgaris, and a variety of oral infections. Lasers Surg. Med. 38:468–481, 2006.

Key words: antibiotic resistance; bacteria; microbial pathogens; phenothiazines; photosensitization; phthalocyanines; porphyrins; reactive oxygen species

INTRODUCTION

Photodynamic therapy (PDT) has now obtained regulatory approval for the treatment of selected tumors in many countries, even though the total number of clinical indications is still limited [1,2]. This development represented the end-point of several decades of intensive investigations, which provided a wealth of information as regards the correlation between the chemical structure of photosensitizing agents (especially those belonging to the porphyrin family) and their pharmacokinetic properties, as well as on their mode of action at a cellular and tissue level [3,4]. In the dermatological field, PDT based on the topical deposition of 5-amino-levulinic acid (ALA) and its methylester (MAL) has been also approved for the treatment of actinic keratosis [5,6]. As a very important consequence of the progressive building up of such database, the possibility has been opened for the extension of PDT to the treatment of various non-oncological diseases, including the prevention of arterial restenosis after balloon angioplasty, benign prostatic hyperplasia, or the therapy of autoimmune disorders and epidermal/dermal pathologies [5,7]. The use of PDT for the treatment of choroidal neovascularization secondary to age-related macular degeneration is a particularly successful example [8].

The use of PDT as a therapeutic modality for the treatment of localized microbial infections represents an emerging new field. In actual fact, the first recorded observations of photodynamic processes in medicine refer to the inactivation of microorganisms: thus, more than 100 years ago, Raab [9] reported the lethal effect of acridine and visible light on *Paramecium caudatum* and the essential involvement of light and oxygen in the process was shortly thereafter demonstrated by von Tappeiner [10], who coined the term "photodynamic." However, the potential of PDT against diseases of microbial origin was not exploited for several decades, largely for two reasons: (a) some well known pathogens, especially Gram-negative bacteria and protozoa in the cystic stage, are poorly responsive to PDT with the most traditional photosensitizing agents, including xanthene or acridine dyes and those negatively charged porphyrins that have been frequently used in tumor PDT (e.g., Photofrin, tetrasulfonated derivatives); (b) the discovery of antibiotics raised the belief that microbiologically based diseases would have been gradually reduced to a level that no longer had a serious impact on human health. However, the rapid emergence of resistance to even those antibiotics which initially appeared to be highly effective disappointed such expectations [11]: thus,

*Correspondence to: Prof. Giulio Jori, Department of Biology, University of Padova, Via Ugo Bassi 58B, 35121 Padova, Italy. E-mail: jori@bio.unipd.it

Accepted 13 April 2006
Published online 19 June 2006 in Wiley InterScience (www.interscience.wiley.com).
DOI 10.1002/lsm.20361

coagulase-positive *Staphylococcus aureus* has been shown to exhibit resistance against each new class of licensed antibiotics, and more recently the emergence of *S. aureus* strains resistant to vancomycin, a glycopeptide antibiotic which was considered as a last line of defense, has been documented [12,13]. The problem is further exacerbated by factors of social nature such as the inappropriate or excessive prescription of antibiotics, the widespread addition of antibiotics to livestock feedstuff, the more and more frequent transmission of microorganisms due to the global traveling and the expansion of poverty among populations in third world countries, as well as by the truly large variety of mechanisms adopted by microbial cells to increase their resistance to external insults. These include a thickening of their outer wall, encoding of new proteins which prevent the penetration of drugs, onset of mutants deficient in those porin channels allowing the influx of externally added chemicals, etc. [14–16]. As a consequence, it has so far proven to be very difficult to identify a comprehensive strategy for overcoming this problem.

It is thus evident that there is an urgent need for the development of innovative and efficacious approaches for combating microbial diseases. Recent findings strongly support the hypothesis that PDT can represent a viable alternative since the mode of action of photodynamic sensitizers on microbial cells is markedly different from that typical of most antibiotic drugs [17]. The main favorable features of antimicrobial PDT can be summarized as follows:

— Broad spectrum of action, since one photosensitizer can act on bacteria, fungi, yeasts, and parasitic protozoa.
— Efficacy independent of the antibiotic resistance pattern of the given microbial strain.
— Possibility to develop PDT protocols which lead to an extensive reduction in pathogen population with very limited damage to the host tissue.
— Lack of selection of photoresistant strains after multiple treatments.
— Small probability to promote the onset of mutagenicity.
— Availability of formulations allowing a ready and specific delivery of the photosensitizer to the infected area.
— Use of low cost light sources for activation of the photosensitizing agent.

Such characteristics will be discussed in the following paragraphs.

PDT OF MICROBIAL INFECTIONS: THE TARGET

Microbial cells are characterized by large differences as regards the cellular structure and organization, which has obvious effects in modulating the interaction of exogenously added photosensitizing agents with cell constituents, hence in affecting the efficiency and the mechanism of the photoinactivation processes.

As shown in Figure 1, Gram(+) and Gram(−) bacteria have profound differences in their three-dimensional architecture. Both groups of bacteria present an outer cell wall. In particular, in Gram(+) bacteria the outer wall (15–80 nm thick) contains up to 100 peptidoglycan layers, which are intimately associated with lipoteichoic and negatively charged teichuronic acids. This wall displays a relatively high degree of porosity, since various macromolecules, such as glycopeptides and polysaccharides with a molecular weight in the 30,000–60,000 range, were found to readily diffuse to the inner plasma membrane [18]. Thus, in this class of bacteria, the outer wall does not act

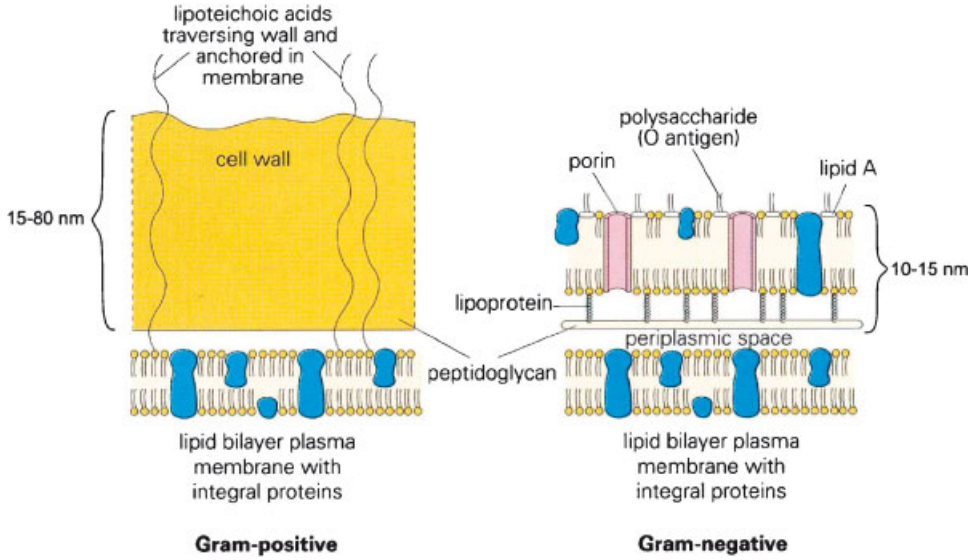


Fig. 1. Schematic representation of the outer wall and cytoplasmic membrane structure in Gram(+) and Gram(−) bacteria. [Figure can be viewed in color online via www.interscience.wiley.com.]

as a permeability barrier for the most commonly used photosensitizers, whose molecular weight does not generally exceed 1,500–1,800 Da. On the contrary, the outer wall of Gram(−) bacteria possesses an additional 10–15 nm thick structural element, which is external to the peptidoglycan network and has a very heterogeneous composition, including proteins with porin function, lipopolysaccharide trimers and lipoproteins giving the outer surface a quasi-continuum of densely packed negative charges. Such a highly organized system inhibits the penetration of host cellular and humoral defense factors and triggers mechanisms of resistance against several antibiotic drugs: only relatively hydrophilic compounds with a molecular weight lower than 600–700 Da can diffuse through the porin channels [19,20]. It is thus necessary to devise suitable strategies which enhance the permeability of the outer wall in order to make Gram(−) bacteria sensitive to the action of photodynamic processes [17,21]. Toward this end, two such approaches have been developed. Thus, the addition of either the cationic polypeptide polymixin B [22,23] or the metal chelator ethylenediaminetetraacetic acid (EDTA) [24] was found to cause the displacement and, respectively, the removal of the Mg^{2+} and the Ca^{2+} ions which neutralize the superficial negative charges: as a consequence, electrostatic repulsion is promoted with destabilization of the native organization of the wall, inducing the release of a large fraction of the lipopolysaccharides into the medium. Studies performed with representative Gram(−) bacteria, such as *Escherichia coli* and *Klebsiella pneumoniae*, demonstrated that the adoption of this pre-treatment allows significant concentrations of the photosensitizer to penetrate to the cytoplasmic membrane, which represents a critical target for bacterial cell photoinactivation [21–24], thereby overcoming the intrinsic inertness of these microbial cells toward PDT. The potentiating effect of pre-treatment with metal chelators on the efficiency of photodynamic processes was observed also for other microbial species [25].

The photosensitivity of bacteria is affected by the physiological state: in general, cells in the logarithmic phase of growth are appreciably more susceptible to photodynamic inactivation than the corresponding cells in the stationary phase [17]. Moreover, Demidova and Hamblin [26] recently demonstrated that several types of *Bacillus* spores can be inactivated by red light irradiation in the presence of phenothiazinium dyes under mild experimental conditions; this finding is particularly important since spores are usually resistant to damage by most commonly employed antibacterial agents, hence PDT could represent an innovative approach, for example, in the sterilization of wounds contaminated by bacterial spores.

PDT can be also used to inactivate yeasts and fungi. Yeasts constitute a large group of rather disparate eukaryotic organisms, which are also enveloped by the presence of a thick external wall, composed of a mixture of glucan, mannan, chitin, and lipoproteins and separated from the plasma membrane by a periplasmic space. The available evidences point out that the response of such cells to photosensitized processes is less strictly controlled by structural factors as compared with Gram(−) bacteria [27]. Thus, even a negatively charged porphyrin derivative, such as Photofrin, is accumulated by *Candida* species, and promotes an extensive inactivation of this microorganism upon visible light irradiation [28], also at the stage of biofilm [29]. However, an initial increase of the outer wall permeability is important to enhance the photodynamic effect in yeasts [30], as further confirmed by recent findings with phthalocyanine photosensitizers (Roncucci, unpublished results). The photoinhibitory role of the outer wall is further confirmed by the observation that non-walled microorganisms, such as mollicutes, are readily susceptible of photosensitized killing upon exposure to visible light and porphyrin photosensitizers, independently of their chemical structure [31].

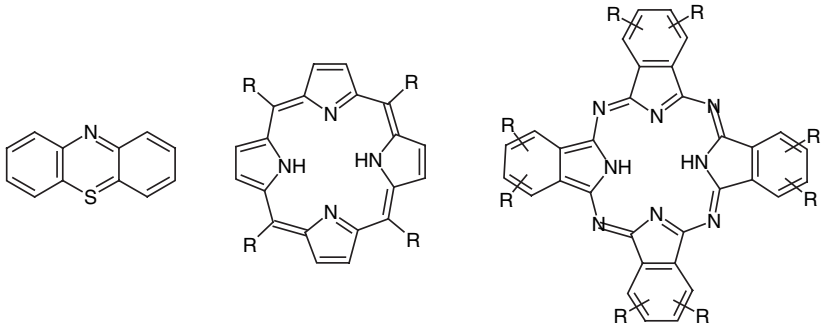
Several protozoa represent quite dangerous and even deadly human pathogens, and many out of the currently used antimicrobial therapeutic modalities are often unsuccessful. The definition of efficacious treatments is complicated since many pathogenic protozoa are so closely adapted that they are incapable of existing outside the host except as resistant stages, which are responsible for infection transmission from human to human: typical examples are represented by *Entamoeba histolytica* and *Giardia intestinalis*. Other parasitic protozoa, such as *Leishmania* spp. and *Plasmodium* spp., are also dangerous for humans and are transmitted by arthropod vectors, which makes the fight against such parasites particularly hard. A few articles demonstrated the effectiveness of PDT with Aluminum phthalocyanine against *L. amazonensis* in different cellular types, such as promastigotes and amastigotes [32]. In addition, some free-living soil and water amoebae, such as the species belonging to the genera *Acanthamoeba*, *Balamuthia*, and *Naegleria*, are recognized etiologic agents of mostly fatal amoebic encephalitis in humans and other animals with immunocompromised and immunocompetent hosts among the victims; lastly, *Acanthamoeba* spp. are agents of amoebic keratitis. Thus, the development of new therapeutic options is highly desirable. To plan a rationale approach to this problem by using PDT, it is appropriate to consider that the *Acanthamoeba* life cycle includes an active feeding trophozoite and a dormant cyst, and both stages can be infective. Because of its wall and the dormancy of the enclosed organism, the cyst allows survival during the periods which are unfavorable for growth [33]. The trophozoitic stage is delimited by a plasma membrane largely composed by phospholipids [34], which allows the penetration of several chemical compounds by phagocytosis and pinocytosis, hence it should not hinder the interaction of the photosensitizer with the cell compartments. On the other hand, the mature cysts wall consists of an outer (exocyst) and inner (endocyst) layer separated by a space. The exocyst appears to be organized in layers parallel to the cell surface, each layer resembling a fibrillar network with an interdispersed and ill-defined amorphous substance, whereas the endocyst is deposited within the exocyst wall and appears to be finely granular and mostly composed by cellulose [35,36]. Such a tightly organized structure makes the cyst highly resistant to

various chemical agents due to the osmotically inextensible endocyst wall. Hence, it is to be expected that any externally introduced photosensitizer will find major problems for advancing beyond the endocyst level.

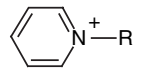
PDT OF MICROBIAL INFECTIONS: THE PHOTOSENSITIZER

A photosensitizing agent with potentially optimal properties for the treatment of microbial infections should be endowed with specific features in addition to the expected photophysical characteristics, such as a high quantum yield for the generation of both the long-lived triplet state and the cytotoxic singlet oxygen species. Such features include a large affinity for microbial cells, a broad spectrum of action in order to efficiently act on infections involving a heterogeneous flora of pathogens, a mechanism of cell inactivation minimizing the risk of inducing the selection of resistant strains or promoting the development of mutagenic processes, and the possibility to identify a therapeutic window which allows (a) the extensive killing of the disease-inducing microbial cells with minimal damage to the host tissue in the area of infection and (b) the prevention of the regrowth of the pathogens after the treatment.

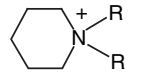
As mentioned in the previous paragraph, the use of outer wall-disrupting agents allows the extension of photodynamic inactivation to Gram(−) bacteria using a variety of photosensitizers. However, it would be desirable for clinical applications to have an effective photosensitizer without the need of additional chemicals. An important step forward in this direction was prompted by the discovery, independently made by three different laboratories, that photosensitizers that are positively charged at physiological pH values, such as phenothiazines [37], phthalocyanines [38–40], and porphyrins [41], can directly promote the photoinactivation of both Gram(+) and Gram(−) bacteria. The basic chemical structure of these polycyclic compounds is shown in Figure 2. While phenothiazine derivatives, such as methylene blue or toluidine blue, are naturally cationic owing to the involvement of one amino group in the π electron cloud resonance, porphyrins and phthalocyanines can be transformed into cationic entities through the insertion of positively charged substituents in the peripheral positions of the tetrapyrrole and, respectively, tetraazaisoindole macrocycle. Typical examples of such substituents are also shown in Figure 2: clearly, they are most frequently characterized by the presence of quaternarized nitrogen atoms, even though also the presence of amino groups with sufficiently strong basic properties to allow their protonation at neutral pH values imparts efficient antibacterial photoactivity to porphyrins and phthalocyanines [39,40,42]. The nature and number of such substituents generally have a limited



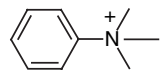
Typical Substituents (R)



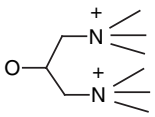
N- alkyl-pyridine



N,N – dialkyl-piperidine



N,N,N-trialkyl-aniline



1,3-di(N,N,N,-trialkyl)-propylen-diamine

Fig. 2. Basic chemical structure of phenothiazine, porphyrin, and phthalocyanine photosensitizers, and typical peripheral substituents (R) giving the photosensitizer a cationic character and enhancing the antimicrobial photosensitising efficiency.

influence on the photophysical properties of the parent compound [17,43]; however, they may appreciably affect the kinetics and extent of binding with microbial cells. In this connection, a major role is played by the degree of hydrophobicity: this parameter can be modulated by either the number of cationic moieties (up to four in meso-substituted porphyrins and to eight in α- or β-substituted phthalocyanines) or the introduction of hydrocarbon chains of different length on the amino nitrogens. Structure-activity relationship studies [44,45] suggest that amphiphilic derivatives (e.g., di- or tetracationic porphyrins or phthalocyanines) exhibit the greatest affinity for Gram(+) bacteria and yeasts [40], while more hydrophilic compounds, such octa-substituted phthalocyanines, having a n-octanol/water partition coefficient lower than about 10, bind most readily with Gram(−) bacteria. Even electrically neutral photosensitizers such as chlorin e_6 can efficiently induce the inactivation of Gram(−) bacteria provided they are combined with polycationic counterparts, such as polylysine [46,47].

It is likely that cationic photosensitizers are taken up by Gram(−) bacteria in spite of their often large molecular weight through the self-promoted uptake pathway [48]. Such pathway has been observed for selected cationic peptides, which can displace divalent cations from their binding sites on the cell surface owing to their 2–4 orders of magnitude larger affinity for such sites; moreover, owing to their bulkiness and the amphiphilic character, the polycyclic photosensitizers disrupt the normal barrier properties of the outer wall facilitating the passage of various antibiotics and other hydrophobic molecules. This action is specifically exerted on bacteria, since they differ from eukaryotic cells for the high content of negatively charged groups and lack of positively charged lipids and cholesterol [49].

Cationic phenothiazines, porphyrins, and phthalocyanines have been shown to efficiently photosensitize the inactivation of bacteria, yeasts and fungi, mycoplasmas, and pathogenic protozoa [50–52]. Therefore, at present, members of these families of compounds may represent the photosensitizers of choice for clinical PDT of microbial infections. In addition, a few bacteria, such as *Propionibacterium acnes* [53] and *Helicobacter pylori* [54], are able to produce porphyrins through a variant of the heme synthetic pathway, which makes them sensitive to visible light illumination. A controlled, prospective trial with endoscopically delivered blue light in regions of the gastric antrum was performed in ten patients and shown to cause an overall 91% reduction in *H. pylori* colonies in treated versus untreated areas [55]. A large amount of endogenous porphyrins (mainly protoporhyrin IX, even though substantial amounts of porphyrins bearing up to eight carboxylate functions are formed) is present also in black-pigmented bacteria which colonize the oral cavity, including *Prevotella intermedia* and *P. nigriscens*. As a consequence, such bacteria have been shown to be sensitive to visible light irradiation in both normal cultures and dental plaque samples [56]. Thus, PDT could be used prophylactically to stabilize the normal microbial composition of plaques by suppressing potentially pathogenic black-pigmented bacteria.

For those bacteria which do not naturally accumulate endogenous porphyrins, it is still possible to stimulate an increased synthesis of porphyrins (largely, copro- and proto-porphyrin) by the addition of ALA [57] similar to what observed for many eukaryotic cells, which is the basis for one approach to PDT of tumors [58]. In actual fact, preliminary clinical investigations [55,59] point out that the phototherapeutic effect on *H. pylori* is enhanced by utilization of topically deposited ALA Antimicrobial ALA-PDT has been described in detail in a recent article [60] and will not be discussed in this review.

PHOTODYNAMIC INACTIVATION OF MICROBIAL CELLS: IN VITRO STUDIES

A typical plot demonstrating the effect of visible light irradiation in the presence of an anionic meso(tetra-4-sulphonatophenyl)porphine ($TPPS_4$) or a cationic (tetra-N-methyl-pyridyl)porphine (TMPyP) on the survival of Gram(+) *Enteroccus seriolicida* and Gram(−) *E. coli* is shown in Figure 3A. Clearly, the anionic derivative is active only against Gram(+) bacteria, while it has no toxic effect on Gram(−) bacteria even after prolonged exposure to light, contrary to what observed for the positively charged porphyrin. This behavior is closely correlated with the mode of photosensitizer interaction with the outer cell surface as described in a previous section. As one can see, an extensive (5–6 log) drop in cell survival was obtained upon irradiation in the presence of micromolar photosensitizer concentrations using mild experimental conditions in terms of both the fluence-rate and the total light exposure time. This obtains also in the case of a methicillin-resistant *S. aureus* (MRSA) strain (data not shown). Quite interestingly, the kinetics of phthalocyanine- [39,40], $TPPS_4$- or TMPy- [42] photosensitized inactivation of a typical yeast, such as *Candida albicans*, was closely similar with that observed for Gram(+) bacteria. On the other hand, as shown in Figure 3B, in the case of protozoa such as *Acanthamoeba palestinensis*, both trophozoites and cysts undergo an extensive inactivation upon photosensitization by a tetracationic phthalocyanine; the two forms exhibit a similar dependency on the photosensitizer concentration, however cysts require a significantly longer irradiation time in order to give a similar degree of inactivation.

The efficiency of a photosensitizer as a photocytocidal antimicrobial agent can be expressed by the so-called minimal bactericidal concentration (MBC), namely the minimal photosensitizer concentration which induces a 4 log drop in survival for a given set of irradiation parameters. The MBC values for a series of porphyrins against both a wild *S. aureus* strain and MRSA, as well as against *E. coli* are shown in Table 1. The data further confirm the observation that photodynamic processes are equally effective against normal and antibiotic-resistant bacterial strains. Moreover, the highest photoactivity against Gram(+) bacteria is exhibited by amphiphilic porphyrins, for example, those having two positively

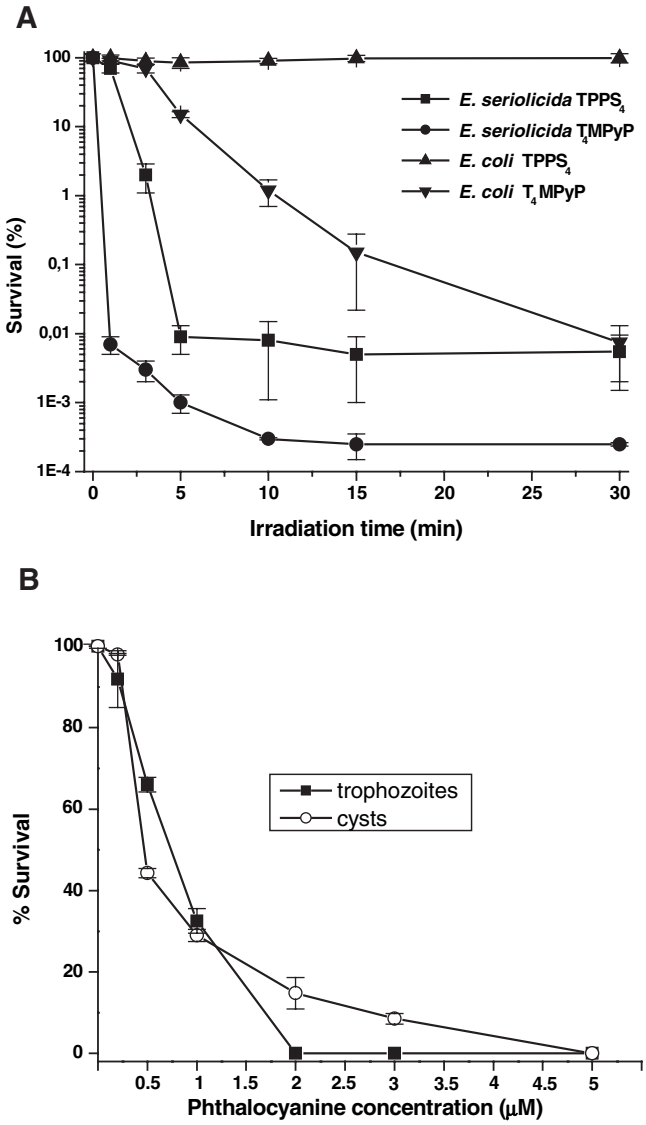


Fig. 3. **A**: Effect of the irradiation time (visible light, 50 mW/cm^2) on the inactivation of a Gram(+) bacterium (*E. seriolicida*) and Gram(−) bacterium (*E. coli*) photosensitised by a negatively charged ($TPPS_4$) and a positively charged (TMPyP) porphyrin after 5 minutes incubation with a 1 μM porphyrin solution in phosphate-buffered saline. **B**: Effect of phthalocyanine concentration on the photosensitised inactivation of *Acanthamoeba palestinensis* in the trophozoitic and cystic stage, in the presence of 1 (4), 8 (11), 15 (18), 22 (25)-tetrakis-3-(N,N,N-trimethylammonium)phenoxy-phthalocyaninato zinc (II) tetrachloride. The irradiations were performed for 10 minutes (trophozoites) and 20 minutes (cysts) using 600–700 nm light (50 mW/cm^2) after 1-hour incubation.

charged groups bound to two adjacent pyrrole rings, whereas tetracationic derivatives appear to be most active against the Gram(−) strains. A further increase in the overall photoefficiency is induced by the replacement of one N-methyl group in tetrameso(N-alkyl-pyridyl)-substituted porphyrins by longer hydrocarbon chains, which should result in a progressive increase in the hydrophobicity [45,51,52]. A maximal efficiency is observed for the porphyrin bearing one N-tetradecyl moiety, whereas a further elongation of the polymethylene chain to 18 or 22 carbon atoms is accompanied by a decrease in the antimicrobial activity. Spectroscopic studies with longer chain-substituted porphyrins showed [45] that the lower efficiency is due to a decreased lifetime of the photosensitizer excited states consequent to heavy aggregation, as it is typical of largely hydrophobic tetrapyrrole compounds in polar media [61]. Similar findings were reported by Brown et al. [62], who observed a substantial enhancement of the photoantibacterial activity of methylene blue, when the two N-methyl groups bound to the amino substituent were replaced by two butyl chains. It is likely that the hydrocarbon moiety acts as a hydrophobic arm which penetrates into lipid domains of the bacterial wall, interfering with the native three-dimensional organization and decreasing the overall stability of the system [63]. A further enhancement in the photoinactivation efficiency of porphyrins was obtained by the insertion of a spacer, such as a propoxy bridge, between the macrocycle and the cationic centers, thereby endowing the positively charged functions with a higher flexibility and facilitating their orientation for a tighter binding with anionic groups at the bacterial surface [64,65]. A disorganizing effect on the bacterial wall is also induced by pursuing a different strategy, namely by attachment of polylysine to the photosensitizer molecule: thus, Hamblin et al. [47] found that conjugation of the neutral chlorin e_6 with a polypeptide chain composed of 37 lysines led to the photoinactivation of both Gram(+) and Gram(−) bacteria, while shorter (eight lysyl residues) chains promoted no detectable phototoxic effect on Gram(−) strains. These results were fully confirmed by other laboratories using porphycene-polylysine [66] and porphyrin-polylysine [67] conjugates.

On the basis of these observations, a stepwise mechanism for the photosensitized inactivation of bacteria, yeasts, and protozoa can be schematized as shown in Fig. 4. The pathway I, which involves a direct translocation of the photosensitizer to the plasma membrane, is operative for Gram(+) bacteria and protozoa in the trophozoitic stage. Pathway II, where an initial increase in the permeability of the outer wall is required, obtains for Gram(−) bacteria, yeasts, and protozoa in the cystic stage. In all cases, the driving force for binding of the cationic photosensitizer to the negatively charged functional groups on the cell surface is of electrostatic nature, hence the binding process is completed within a very short time period: several independent reports indicate that extending the pre-irradiation incubation from 5 minutes to 1–2 hours has no effect on the amount of photosensitizer bound to the microbial cells [17,42,68]. One exception is represented by cysts of protozoa or yeasts where incubation times as long as 30 minutes are necessary in order to achieve sufficiently large endocellular concentrations of the photosensitizer [66,69]. In any case, the critical target for photosensitized killing of microbial cells appears to be represented by the

TABLE 1. Minimal Bactericidal Concentration (MBC, μM) of Porphyrins With Different Chemical Structure Upon White Light Irradiation (50 mW/cm^2; 10 minutes) of Typical Gram(+) and Gram(−) Bacteria

Porphyrin	*S. aureus* wild strain	*S. aureus methicillin resistant*	*E. coli*
meso-$TPPS_4$	1.0	1.5	>10
meso-TPyP (N-methyl)$_{2a}$ (phenyl)$_2$	0.2	0.3	2.5
meso-TPyP (N-methyl)$_4$	0.8	0.9	1.1
meso-TPyP (N-methyl)$_3$ (N-hexyl)		0.7	1.2
meso-TPyP (N-methyl)$_3$ (N-decyl)	0.8	0.7	1.5
meso-TPyP (N-methyl)$_3$ (N-tetradecyl)		0.4	1.7
meso-TPyP (N-methyl)$_3$ (N-octadecyl)	3.0	3.5	5.0
meso-TPyP (N-methyl)$_3$ (N-docosanyl)		5.0	>10

$TPPS_4$, tetra(4-sulphonatophenyl)porphine.
TPyP, tetrapyridyl-porphine; the various derivatives are substituted by alkyl chains at the level of the pyridine N atoms. (N-methyl)$_{2a}$ indicates that the two methyl groups are bound to N atoms in two adjacent pyridine rings, while the two remaining meso positions are bound with two phenyl moieties.
The MBC value is taken as the minimal photosensitizer concentration yielding a 4 log decrease in cell survival under a given set of irradiation conditions.

plasma membrane. Several lines of evidence support this conclusion:

— Fluorescence microscopy studies carried out on both bacteria and protozoa show that the photosensitizer is located at the level of the plasma membrane prior to irradiation and diffuses into the cell only after exposure to light for several minutes, corresponding with an extensive decrease in survival. Typical examples are shown by data obtained in our laboratories using a

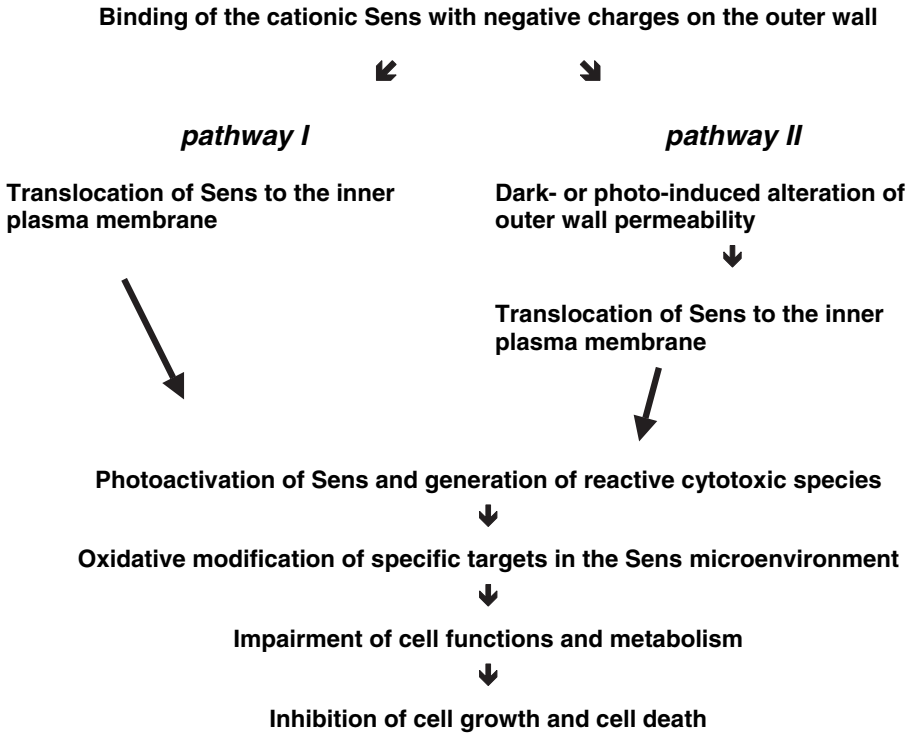


Fig. 4. Scheme illustrating the essential steps involved in the process of photosensitizer binding to microbial cells and subsequent photoinactivation. Pathway I is operative for Gram(+) bacteria and protozoa in the trophozoitic stage; pathway II is operative for Gram(−) bacteria, yeasts and protozoa in the cystic stage.

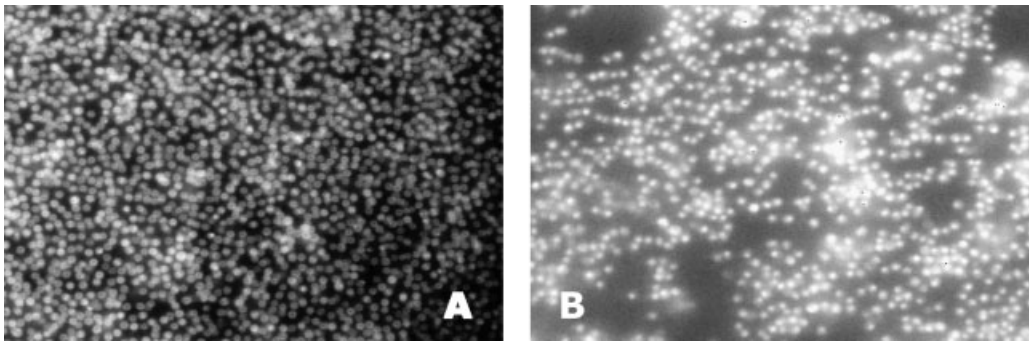


Fig. 5. Typical images obtained at the fluorescence microscope for *S. aureus* cells that had been incubated for 5 minutes in the dark with 1 μM 1 (4), 8 (11), 15 (18), 22 (25)-tetrakis-3-(N,N,N-trimethylammonium)phenoxy-phthalocyaninato zinc (II) tetrachloride (**A**) and had been subsequently irradiated for 5 minutes with 600–700 nm light at a fluence-rate of 50 mW/cm^2 (**B**). Excitation at 610 nm; emission at wavelengths above 660 nm.

tetracationic phthalocyanine as a photosensitizing agent for MRSA (Fig. 5A,B) as well as for *A. palestinensis* trophozoites (Fig. 6A–D). Even though the resolution of the fluorescence microscopy images is low owing to the small dimensions of bacterial cells, it is evident that the phthalocyanine fluorescence is largely confined in peripheral districts of unirradiated cells, whereas the whole cellular volume becomes fluorescent after 5 minutes irradiation. Similarly, in *Acanthamoeba* trophozoites the fluorescence is particularly evident in the contractile vacuole for unirradiated cells and becomes markedly more diffuse in the photosensitized samples.

— Several enzymes, which are associated with the cytoplasmic side of the *S. aureus* membrane, such as NADH, lactate or succinic dehydrogenase, are photoinactivated by phthalocyanines at a rate which closely corresponds with the rate of photoinduced cell death [70]. Similarly, several outer membrane and plasma membrane proteins undergo an extensive cross-linking

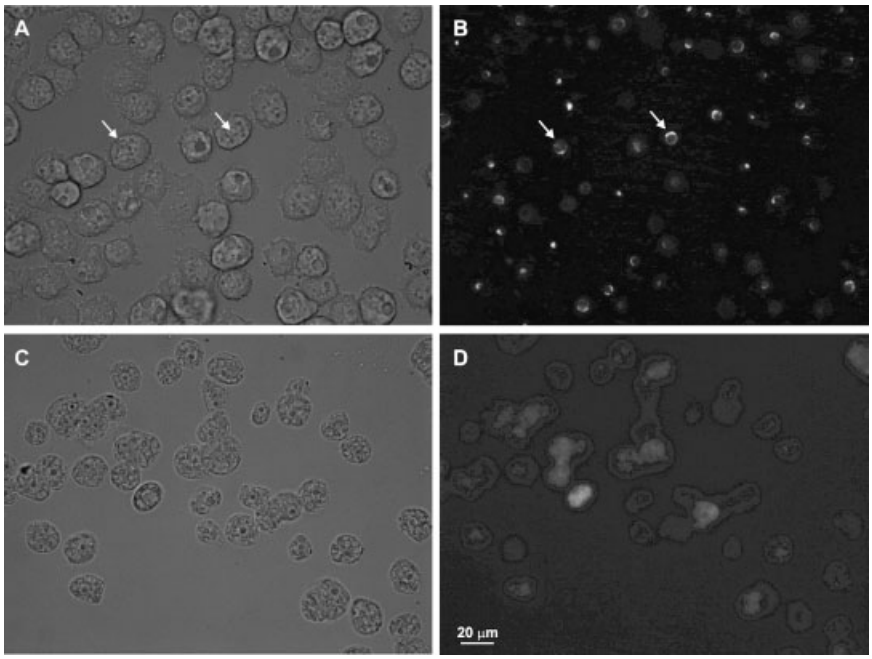


Fig. 6. Fluorescence microscopy images obtained for *A. palestinensis* trophozoites which had been incubated for 1 hour (**A**, **B**) with 5 μM 1 (4), 8 (11), 15 (18), 22 (25)-tetrakis-3-(N,N,N-trimethylammonium)phenoxy-phthalocyaninato zinc (II) tetrachloride in the dark, and subsequently irradiated for 10 minutes with 600–700 nm light (50 mW/cm^2). Pictures A and **C**: images in clear field; micrographs B and **D**: fluorescence images obtained by excitation with 620–660 nm light and collecting the emission at wavelengths in the 700–775 nm range.

in the early stages of phenothiazine photosensitization of *Porphyromonas gingivalis* [71] and cationic porphyrin photosensitization of *E. coli* [72].

— A loss of membrane barrier properties resulting in the leakage of intracellular contents, including a collapse of K^+ and ionic balance, represents an important step for loss of clonogenicity in photosensitized bacteria and yeasts, such as *S. aureus* [73] and, respectively, *Kluyveromyces marxianus* [74]. Membrane damage is also responsible for the rapid impairment of transport functions in bacteria [72] and yeasts [75]; in general, the photoprocess causes a massive reduction in the transport capacity of a wide variety of solutes, thus leading to a shortage of essential substrates for anabolic and catabolic pathways and providing an important contribution to the drop of cell viability.

— The presence of serum in the incubation medium reduces the binding of most photosensitizers to microbial cells because of competition between serum proteins (including albumin [76] and lipoproteins [77]) and plasma membrane proteins, with a consequent dramatic decrease in the efficacy of photodynamic inactivation. On the other hand, the ionic strength of the medium (e.g., salt vs. fresh water) has no appreciable effect on the efficiency of the photosensitized process [21,24]; this also obtains for changes in the pH of the medium which do not affect the chemical structure of the photosensitizer, for example, by protonation of basic functional groups [30].

— Freeze-fracture electron microscopy studies on *C. albicans* cells, which had been photosensitized by a meso-substituted cationic porphyrin, clearly indicate that the photodamage progresses from the outer leaflet of the plasma membrane to the inner leaflet [78].

One parameter which is often overlooked when assessing the degree of microbial cell susceptibility to photodynamic inactivation is represented by the cell density. As shown by Demidova and Hamblin [79], cells compete for binding with the available photosensitizer, as well as for reaction with photogenerated cytotoxic species. Similarly, under identical experimental conditions, fungal cells are usually harder to be killed by photosensitized processes as compared with bacterial cells, since their appreciably larger size requires a greater amount of singlet oxygen or oxygen radicals in order to achieve a given degree of killing.

The initial photoinduced membrane alterations are generally followed by a massive influx of the photosensitizer to endocytoplasmic districts (Fig. 5 and Ref. [78]). As a consequence, a variety of targets, including DNA, undergo photooxidative modification at later stages of the overall photoprocess, even though such damage is not directly correlated with cell death [24,69,75,78,80]. Thus, any major influence of DNA modification seems unlikely since (a) *Deinococcus radiodurans*, which possesses a very efficient DNA repair mechanism, is readily killed by photodynamic processes [81]; and (b) wild *E. coli* strains, as well as *E. coli* strains which are defective for DNA repair mechanisms, display a closely similar sensitivity to photoinactivation by a tetracationic porphyrin [72]. This pattern of cellular photomodification is in agreement with the repeatedly observed lack of mutagenic effects induced by photosensitization of microbial cells by porphyrin-type or phenothiazine derivatives [17,43,52]. However, Salmon-Divon et al. [82] recently reported that the photodynamic inactivation of *E. coli* in the presence of a tetracationic porphyrin is primarily dependent on genomic DNA photodamage rather than on protein or membrane malfunction and proposed to use the photobleaching of the green fluorescent protein chromophore in the cytoplasm as a monitor of the photoinactivation efficiency; hence, further investigations are needed before general conclusions can be drawn.

An overall examination of the results obtained in the substantial body of in vitro investigations so far carried out in the field of antimicrobial PDT suggests a possible set of optimal conditions for treatment of microbial infections, as summarized in Table 2. In particular, the combination of the short incubation time, low photosensitizer concentration and mild irradiation parameters is especially appealing since it appears to allow a selective killing of microbial pathogens under conditions in which human cells (e.g., fibroblasts or keratinocytes) are spared, as shown by a few studies with phthalocyanine [70], porphyrin [45], or phenothiazine [83,84] photosensitizers.

TABLE 2. PDT Protocol Yielding an Efficient Phototoxic Action on Gram(+)/Gram(−) Bacteria and Yeasts With Minimal Damage to Host Tissues

Parameter	Optimal values
Nature of the photosensitizer	Cationic phenothiazines, phthalocyanines, porphyrins
Cell-photosensitizer incubation time prior to irradiation	5–10 minutes
Photosensitizer dose	0.1–5 μM
Delivery system	Free or in combination with cationic polypeptides and antibodies; not effective against Gram(−) if associated with liposomes
Ionic strength	Efficiency is independent of the salt concentration
Fluence rate	Lower than 50 mW/cm^2
Total light fluence (irradiation time)	Lower than 5 J/cm^2 (15 minutes)

PHOTODYNAMIC INACTIVATION OF MICROBIAL CELLS: IN VIVO STUDIES

Relatively few reports have been published as regards the application of photodynamic processes to treat infections artificially or spontaneously developed in vivo. This is possibly due to the lack of adequate animal models. In any case, favorable results were obtained both by methylene blue-PDT of oral candidiasis induced in immunodeficient SCID mice [85] and chlorin e_6 (either free or conjugated with polylysine)-PDT of infected excisional wounds in mice [86], as well as in animal models bearing infections due to *C. albicans* and *S. aureus* using several newly synthesized phthalocyanines (Roncucci, unpublished observations). The latter experiments were recently extended to established *S. aureus* soft tissue infections [87]: apparently, the lesions responded much better to PDT performed by using the conjugated as compared with the free photosensitizer. Most importantly, the host tissue appeared to undergo minimal damage and to heal promptly upon application of PDT regimes causing a 2–3 log decrease in the bacterial population. Similar results were also obtained by using poly-lysine-chlorin e_6 conjugates to treat wound infections in mice caused by the inoculum of *Pseudomonas aeruginosa*; the PDT-treated wounds healed appreciably faster than those treated by silver nitrate [88]. In addition, a relatively mild irradiation protocol based on the local delivery of toluidine blue proved to be successful for the treatment of wound infections in mice caused by an extremely virulent Gram-negative bacillus, such as *Vibrio vulnificus* [89]. The photodynamic inactivation resulted in reduced cell motility and bacterial virulence factors.

Thus, the in vivo data so far obtained appear to support the possibility to achieve a selective or highly preferential inactivation of microbial pathogens through the choice of an adequate PDT protocol. These findings prompted Bisland et al. [90] to investigate the potential of PDT with either methylene blue or ALA for the treatment of osteomyelitis, an acute or chronic inflammation of bone and bone marrow secondary to contamination by a variety of microbial pathogens. Toward this end, a biofilm-producing *S. aureus* strain was inoculated into the tibial medullary cavity of Sprague–Dawley rats and its progression could be efficiently counteracted by illumination of the infected area with light delivered via optical fibers. This work is very important because it shows that interstitial PDT can be successfully applied also to treat deep-seated microbial infections. Interestingly, Hamblin et al. [88] developed a sensitive optical approach to monitor the state of microbial infections in wounds. The technique is based on the use of genetically engineered bacteria that emit bioluminescence, which can be detected in real time by the use of an intensified CCD camera.

An alternative approach to selectivity in antimicrobial PDT can be developed through the use of photosensitizers conjugated with antibodies directed against the target microorganism. This strategy was pioneered by Berthiaume et al. [91], who showed that a chlorin e_6 conjugate with a specific monoclonal antibody deposited on a *Pseudomonas aeruginosa*-infected dorsal skin area in mice photosensitized a much more extensive decrease in viability of the Gram(−) bacterium as compared with conjugates formed from a non-specific antibody. These findings are in agreement with previous observations [92] demonstrating that chlorin e_6 bound to an anti-*P. aeruginosa* antibody caused a selective killing of this bacterium in mixed cultures with *S. aureus*. This approach can be especially useful when PDT is applied for the elimination of a specific pathogenic organism without affecting the normal microbial flora, hence minimizing the risk of the onset of opportunistic infections, although more complicated patterns of clinical development for these conjugates can be foreseen. Recently, metal substituted non-centrosimmetrical phthalocyanines were prepared by chemical synthesis with an aim to obtain a specific targeting of either microbial or tumor cells [93].

CONCLUSIONS AND POSSIBLE CLINICAL APPLICATIONS

At present, antimicrobial PDT is still at an experimental stage. However, the rapid advances of our knowledge as regards the mode of action of photosensitizers at a subcellular, cellular and tissue level, the definition of irradiation protocols enhancing the selectivity of the photodynamic process towards microbial targets with minimal collateral damage to the hosts, and the involvement of an increasing number of research/medical centers in the pre-clinical investigations allows one to predict that PDT will be more and more frequently used for the eradication of selected localized infections caused by pathogenic microorganisms. Based on the present state-of-the-art, the following indications can be considered as most suitable for PDT treatment:

Oral Candidiasis

As shown in the previous paragraphs, *C. albicans*, which is the causative agent of oral thrush, is readily susceptible to photodynamic inactivation. Topical application of the photosensitizer would allow for a photodamage confined within the diseased lesion, thus sparing the microflora at other sites. This approach would be of particular importance for HIV-infected patients or people receiving chemo- or radio-therapy for cancer treatment [94] where *Candida* infections are quite frequent, since a local phototherapy is not expected to cause an increased burden on the immune system or undesired side effects which are associated with conventional antifungal agents [94]. Actually, *C. albicans* infections induced in the dorsum of the tongue of mice with severe immunodeficiency could be completely eradicated by red light irradiation after topical deposition of methylene blue [85].

Periodontal Diseases

These diseases involve severe inflammation of the teeth-supporting structures and are consequent to chronic infections caused by a mixture of Gram-positive and

Gram-negative bacteria growing as a biofilm to generate the so-called subgingival plaque [95]. The combined action of metalloproteases released from neutrophils in the host tissue and bacteria-derived enzymes eventually leads to destruction of the periodontal ligament and tooth loss. While current treatment protocols for chronic periodontitis involve the mechanical removal of the biofilm by laborious and often unpleasant procedures, PDT has been proposed as a viable alternative approach largely as a result of the intensive investigations carried out in this field by Wilson and coworkers [96,97]. In vitro studies have shown that several pathogens which prevail in the subgingival periodontal plaques (e.g., *P. gingivalis, Fusobacterium nucleatum*, *Staphylococcus* sp.) are efficiently eradicated by photodynamic treatment, both in aqueous suspension and as a biofilm [98]: the presence of substances typical of the oral environment, such as demineralized dentine and collagen, does not interfere with the kinetics and efficiency of the photoprocess. Moreover, in vivo experimental studies showed that toluidine blue-PDT can selectively kill *P. gingivalis* in the oral cavity and significantly decrease the level of alveolar bone loss in rats affected by periodontitis [99]. The protocol for clinical applications would involve the deposition of the photosensitizer in the dental pocket followed by irradiation with light delivered via optical fibers. The procedure can be usually completed in a few minutes. This feature would give PDT a significant advantage over treatment with antiseptics and antibiotics, which are difficult to be maintained in appreciable concentrations within the periodontal pocket for prolonged periods of time and are often poorly selective toward the target pathogen.

Healing of Infected Wounds

Indolent and chronic wounds are most frequently contaminated by bacteria, and this contamination normally causes delayed healing and prolonged hospitalization. Wound infections are commonly treated with antibiotics or various types of topical products (e.g., polymixin B, mupirocin, silver nitrate, or silver sulfadiazene); however, the emergence of antibiotic-resistant bacterial strains and the toxic effects of silver compounds call for alternative and more powerful therapeutic approaches. Once again, PDT can represent a very useful tool to treat bacterial contaminants of wounds because of the broad spectrum of action of adequately selected photosensitizing agents against both wild and antibiotic-resistant strains. Moreover, it has been demonstrated [100] that PDT, especially if applied at low light doses, can up-regulate the expression of growth factors, including cells in the perilesional districts, thereby further stimulating wound healing. The above cited pioneering work performed by Hamblin et al. [86,87] in experimental animals is providing very useful information for the definition of a successful and well-tolerated clinical protocol, which could be possibly extended to the eradication of *S. aureus* in burn wound infections [101].

Acne Vulgaris

Acne represents a pathology involving the pilosebaceous follicles. The proliferation of bacteria such as *P. acnes* and *P. granulosum* in the sebum has been invoked as one determinant of acne development [102]. These bacteria are known to produce relatively large amounts of endogenous porphyrins, hence they can be inactivated by illumination with high intensity blue light wavelengths, which are efficiently absorbed by the 400 nm-peaking Soret band of porphyrins. Preliminary clinical trials point out that direct blue light-phototherapy brings about a significant decrease in the progress of acne lesions [103]. Moreover, since ALA accumulates in sebaceous glands, PDT performed after topical application of ALA is claimed to be even more effective in the treatment of acne with reduction in the sebum production and the size of the glands; the regular skin structure is fully recovered after PDT, the only post-PDT persistent changes being represented by a decrease in the number of pilosebaceous units, as shown by studies performed on Japanese patients [104]. The beneficial action of PDT appears to include the destruction of *Propionibacterium* spp. and is of particular interest owing to the safety of this therapeutic modality for the normal host tissue.

In conclusion, the field of antimicrobial PDT is in a rapidly expanding phase, as suggested by the identification of novel photosensitizing agents, such as fullerenes [105], with apparently higher selectivity of microbial cell targeting and the proposed extension of clinical PDT to the treatment of cutaneous leishmaniasis, namely a disease which is widely diffused in Southern Europe, Near East and South America [106]. As a consequence, it is reasonable to hypothesize that antimicrobial PDT can find a specific role in the treatment of selected diseases of microbial origin, at least in the case of localized infections [52].

REFERENCES

1. Brown SB, Brown EA, Walker I. The present and future role of photodynamic therapy in cancer treatment. Lancet Oncol 2004;5:497–508.
2. Huang Z. A review of the progress in clinical photodynamic therapy. Technol Cancer Res Treatment 2005;4:283–293.
3. Detty MR, Gibson SL, Wagner SJ. Current clinical and preclinical photosensitisers for use in photodynamic therapy. J Med Chem 2004;47:3897–3915.
4. Oleinick NL, Morris RL, Belichenko I. The role of apoptosis in response to photodynamic therapy: What, where, why and how. Photochem Photobiol Sci 2002;1:1–21.
5. Calzavara-Pinton PG, Szeimies RM, Ortel B. Photodynamic therapy and fluorescence diagnosis in dermatology. Amsterdam: Elsevier, 2001.
6. Szeimies RM, Karrer S, Radakovic-Fijan S. Photodynamic therapy using topical methyl 5-amino-levulinate compared with cryotherapy for actinic keratosis: A prospective, randomized study. J Am Acad Dermatol 2002;47:259–262.
7. Peng Q. Photodynamic therapy and detection. J Environ Pathol Toxicol Oncol 2006;25:1–5.
8. Rechtman E, Ciulla T, Criswell M, Pollack A, Harris A. An update of photodynamic therapy in age-related macular degeneration. Expert Opin Pharmacother 2002;3:931–938.
9. Raab O. Uber die wirkung fluoriziender stoffe auf infusorien. Zeit Biol 1900;39:524–546.

10. von Tappeiner H. Zur kenntis der lichtwirkenden (fluoreszierenden) stoffe. Dtsch Med Wochen 1904;1:579–580.
11. Tunger O, Dinc G, Ozbakkaloglu B, Atman C, Algun U. Evaluation of rational antibiotic use. Int J Antimicrob Agents 2000;15:131–135.
12. Smith TL, Pearson ML, Wilcox KR, Cruz C, Lancaster MV, Robinson-Dunn B, Tenover FC, Zervos MJ, Band JD, White E, Jarvis WR. Emergence of vancomycin resistance in *Staphylococcus aureus*: Glycopeptide-intermediate *Staphylococcus aureus* working group. New England J Med 1999; 340:493–501.
13. Sievert D. *Staphylococcus aureus* resistant to vancomycin. Mor Mortal Wkly Rep CDC 2002;51:565–567.
14. Boyle-Vavra S, Labischinski H, Ebert CC, Ehlert K, Daum RS. A spectrum of changes occurs in peptidoglycan composition of glycopeptide-intermediate clinical*Staphylococcus aureus*isolates. Antimicrob Agents Chemother 2001;45: 280– 287.
15. Roland KL, Esther CR, Spitznagel JK. Isolation and characterization of a gene, pmrD, from *Salmonella typhimurium* that confers resistance to polymixin when expressed in multiple copies. J Bacteriol 1994;176:3589–3597.
16. Harder KJ, Nikaido H, Matsuhashi M. Mutants of *Escherichia coli* that are resistant to certain beta-lactam compounds lack the ompF porin. Antimicrob Agents Chemother 1981;20: 549–552.
17. Wainwright M. Photodynamic antimicrobial chemotherapy. J Antimicrob Chemother 1998;42:13–28.
18. Friedrich CL, Moyles D, Beveridge TJ, Hancock RE. Antibacterial action of structurally diverse cationic peptides on Gram-positive bacteria. Antimicrob Agents Chemother 2000;44:2086–2092.
19. Nikaido H. Prevention of drug access to bacterial targets: Permeability barrier and active efflux. Science 1994;264: 362–368.
20. Leive L. The barrier function of the Gram-negative envelope. Ann NY Acad Sci 1974;235:109–129.
21. Bertoloni G, Rossi F, Valduga G, Jori G, Ali H, van Lier JE. Photosensitising activity of water- and lipid-soluble phthalocyanines on prokaryotic and eukaryotic microbial cells. Microbios 1992;71:33–46.
22. Malik Z, Ladan H, Nitzan Y. Photodynamic inactivation of Gram-negative bacteria: Problems and possible solutions. J Photochem Photobiol B: Biol 1992;14:262–266.
23. Nitzan Y, Gutterman M, Malik Z, Ehrenberg B. Inactivation of Gram-negative bacteria by photosensitised porphyrins. Photochem Photobiol 1992;55:89–96.
24. Bertoloni G, Rossi F, Valduga G, Jori G, van Lier JE. Photosensitising activity of water- and lipid-soluble phthalocyanines on *Escherichia coli*. FEMS Microbiol Lett 1990;59:149–155.
25. Roncucci G, Dei D, Chiti G, Fantetti L, Giuliani F, Jori G, Rossolini GM. Antibacterial composition comprising metal phthalocyanine analogues. European Patent 1356813, 2005.
26. Demidova TN, Hamblin MR. Photodynamic inactivation of *Bacillus* spores mediated by phenothiazinium dyes. Appl Environ Microbiol 2005;71:6918–6925.
27. Paardekopper M, van Gompel AE, van Steveninck J, van de Broek J. The effect of photodynamic treatment of yeast with the sensitiser chloroalluminium phthalocyanine on various cellular parameters. J Photochem Photobiol B: Biol 1995;62: 561–567.
28. Bertoloni G, Sacchetto R, Jori G, Vernon DI, Brown SB. Protoporhyrin photosensitisation of *Enterococcus hirae* and *Candida albicans* cells. Lasers Life Sci 1993;5:267–275.
29. Bliss JM, Bigelow CE, Foster TH, Haidaris CG. Susceptibility of *Candida* species to photodynamic effects of Photofrin. Antimicrob Agents Chemother 2004;48:2000–2006.
30. Chabrier-Rosello Y, Foster TH, Perez-Nazrio N, Mitra S, Haidaris CG. Sensitivity of *Candida albicans* germ tubes and biofilms to Photofrin-mediated phototoxicity. Antimicrob Agents Chemother 2005;49:4288–4295.
31. Bertoloni G, Viel A, Grossato A, Jori G. The photosensitising activity of hematoporphyrin on mollicutes. J Gen Microbiol 1985;131:2217–2223.
32. Schuster FL, Visvesvara GS. Amoebae and ciliated protozoa as causal agents of water-borne zoonotic disease. Vet Parasitol 2004;126:91–120.
33. Dutta S, Ray D, Kolli BK, Chang KP. Photodynamic sensitization of *Leishmania amazonensis* in both extracellular and intracellular stages with aluminum phthalocyanine chloride for photolysis *in vivo*. Antimicrob Agents Chemother 2005;49:4474–4484.
34. Chlapowsky FJ, Band RN. Assembly of lipids into membranes in *Acanthamoeba palestinensis*. J Cell Biol 1971;50: 634–651.
35. Bowers B, Korn E. The fine structure of *Acanthamoeba castellanii* (Neff strain). J Cell Biol 1969;41:786–804.
36. Lloyd D, Turner NA, Khunkitti W, Hann AC, Furr JR, Russell AD. Encystation in *Acanthamoeba castellanii*: Development of biocide resistance. J Eukaryot Microbiol 2001;48:11–16.
37. Wilson M, Burns T, Pratten J, Pearson GJ. Bacteria in supragingival plaque samples can be killed by low power laser light in the presence of a photosensitiser. J Appl Bacteriol 1995;78:569–574.
38. Minnock A, Vernon DI, Schofield J, Griffith 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.
39. Roncucci G, Dei D, De Filippis MP, Fantetti L, Masini I, Cosimelli B, Jori G. Zn(II) phthalocyanines and corresponding conjugates; their preparation and use for photodynamic therapy and as diagnostic agents. US Patent 5965598, 1999.
40. Roncucci G, Fantetti L, De Filippis MP, Dei D, Jori G. Substituted metal phthalocyanines, their preparation and use thereof. European Patent 1164135, 2004.
41. Merchat M, Bertoloni G, Giacomoni P, Villanueva A, Jori G. Meso-substituted cationic porphyrins as efficient photosensitisers of Gram-positive and Gram-negative bacteria. J Photochem Photobiol B: Biol 1996;32:153–157.
42. Jori G. Photodynamische therapien in der mikrobiologie. In: Szeimies RM, Jocham D, Landthaler M, editors. Klinische fluoreszenzdiagnostik und photodynamische therapie. Berlin: Blackwell verlag, 2003, pp 360–371.
43. Jori G. Photodynamic therapy of microbial infections: State-of-the-art and perspectives. J Environ Pathol Toxicol Oncol 2006;25:505–519.
44. Giuntini F, Nistri D, Chiti G, Fantetti L, Jori G, Roncucci G. Synthesis of trimethylammonium-phenylthio-substituted phthalocyanines with different pattern of substitution. Tetrah Lett 2003;44:515–517.
45. Reddi E, Ceccon M, Valduga G, Jori G, Bommer JC, Elisei F, Latterini L, Mazzucato U. Photophysical properties and antibacterial activity of meso-substituted cationic porphyrins. Photochem Photobiol 2002;75:462–470.
46. Soukos NS, Ximenez-Fyvie LA, Hamblin MR, Socransky SS, Hasan T. Targeted antimicrobial photochemotherapy. Antimicrob Agents Chemother 1998;42:2595–2601.
47. Hamblin MR, O'Donnell DA, Murthy N, Rajagopalan K, Michaud N, Sherwood ME, Hasan T. Polycationic photosensitiser conjugates: Effects of chain length and Gram classification on the photodynamic inactivation of bacteria. J Antimicrob Chemother 2002;49:941–951.
48. Hancock RE, Bell A. Antibiotic uptake into Gram-negative bacteria. Eur J Clin Microb Infect Dis 1988;7: 713–720.
49. Falla TJ, Karunaratne DN, Hancock RE. Mode of action of the antimicrobial peptide indolicidin. J Biol Chem 1996;271: 19298–192303.
50. Jori G, Brown SB. Photosensitised inactivation of microorganisms. Photochem Photobiol Sci 2004;3:403–405.
51. Maisch T, Szeimies RM, Jori G, Abels C. Antibacterial photodynamic therapy in dermatology. Photochem Photobiol Sci 2004;3:907–917.

52. Hamblin MR, Hasan T. Photodynamic therapy: A new antimicrobial approach to infectious diseases? Photochem Photobiol Sci 2004;3:436–450.
53. Lee WL, Shalita AR, Poh-Fitzpatrick MB. Comparative studies of porphyrin production in *Propionibacterium acnes* and *Propionibacterium granulosum*. J Bacteriol 1994;133: 811–815.
54. Hamblin MR, Viveiros J, Yang C, Ahmadi A, Ganz RA, Tolkoff M. *Helicobacter pylori* accumulates photoactive porphyrins and is killed by visible light. Antimicrob Agents Chemother 2005;49:2822–2827.
55. Nitzan Y. Endogenous porphyrin production in bacteria by 5-amino-levulinic acid and subsequent bacterial photoeradication. Lasers Med Sci 1999;14:269–277.
56. Soukos NS, Som S, Abernethy AD, Ruggiero K, Dunham J, Lee C, Doukas AG, Goodson JM. Phototargeting of oral black-pigmented bacteria. Antimicrob Agents Chemother 2005;49: 1391–1396.
57. Kennedy JC, Pottier RH. Endogenous protoporphyrin IX, a clinically useful photosensitiser for photodynamic therapy. J Photochem Photobiol B: Biol 1992;14:269–277.
58. Ganz RA, Viveiros J, Ahmadi A, Khalil A, Tolkoff MJ, Nishioka NS, Hamblin MR. *Helicobacter pylori* in patients can be killed by visible light. Lasers Surg Med 2005;36:260–265.
59. Nitzan Y, Salmon-Divon M, Shporen E, Malik Z. ALA-induced photodynamic effects on Gram-positive and Gram-negative bacteria. Photochem Photobiol Sci 2004;3: 430–435.
60. Vergeldt FJ, Koehorst RBM, Vanhoek A, Shaafsma TJ. Intramolecular interactions in the ground and excited states of tetrakis(N-methyl-pyridyl)porphyrins. J Phys Chem 1995;99:4397–4405.
61. Brown SB. Antimicrobial PDT: We have the theory, what about the practice? 11th Congress of the European Society for Photobiology, Aix-les-Bains, 2005; Book of Abstracts, p 100.
62. Wilder-Smith CH, Wilder-Smith P, Grosjean P, van den Bergh H, Woodtli A, Monnier P, Dorta G, Meister F, Wagnieres G. Photoeradication of *Helicobacter pylori* using 5-aminolevulinic acid: Preliminary human studies. Lasers Surg Med 2002;31:18–22.
63. Merchat M, Spikes JD, Bertoloni G, Jori G. Studies on the mechanism of bacteria photosensitisation by meso-substituted cationic porphyrins. J Photochem Photobiol B: Biol 1996;35:149–157.
64. Caminos DA, Durantini EN. Synthesis of asymmetrically meso-substituted porphyrins bearing amino groups as potential cationic photodynamic agents. J Porphyrins Phthalocyanines 2005;9:334–342.
65. Caminos DA, Spesia MB, Durantini EN. Photodynamic inactivation of *Escherichia coli* by novel meso-substituted porphyrins by 4-(3-N,N,N-trimethylammoniumpropoxy)-phenyl and 4-(trifluoromethyl)phenyl groups. Photochem Photobiol Sci 2006;5:56–65.
66. Polo L, Segalla A, Bertoloni G, Jori G, Schaffner K, Reddi E. Polylysine-porphycene conjugates as efficient photosensitisers for the inactivation of microbial pathogens. J Photochem Photobiol B: Biol 2000;59:152–158.
67. Tomé J, Neves MGPMS, Tomé AC, Cavaleiro JAS, Soncin M, Magaraggia M, Ferro S, Jori G. Synthesis and antibacterial activity of new poly-L-lysine-porphyrin conjugates. J Med Chem 2004;47:6649–6652.
68. Jori G, Brown SB. Photosensitized inactivation of microorganisms. Photochem Photobiol Sci 2004;3:403–405.
69. Ferro S, Coppellotti O, Roncucci G, Ben Amor T, Jori G. Photosensitised inactivation of *Acanthamoeba palestinensis* in the cystic stage. J Appl Microbiol 2006; in the press.
70. Soncin M, Fabris C, Busetti A, Dei D, Nistri D, Roncucci G, Jori G. Approaches to selectivity in the Zn(II)-phthalocyanine-photosensitised inactivation of wild-type and antibiotic-resistant *Staphylococcus aureus*. Photochem Photobiol Sci 2002;1:815–819.
71. Bhatti M, Nair SP, MacRobert AJ, Henderson B, Shepherd P, Cridland J, Wilson M. Identification of photolabile outer membrane proteins of *Porphyromonas gingivalis*. Curr Microbiol 2001;43:96–99.
72. Valduga G, Breda B, Giacometti GM, Jori G, Reddi E. Photosensitisation of wild and mutant strains of *Escherichia coli* by meso-tetra(N-methyl-4-pyridyl)porphine. Biochem Biophys Res Comm 1999;256:84–88.
73. Malik Z, Babushkin T, Sher S, Hanania J, Ladan H, Nitzan Y, Salzberg S. Collapse of K^+ and ionic balance during photodynamic inactivation of leukemic cells, erythrocytes and *Staphylococcus aureus*. Int J Biochem 1993;25:1399–1406.
74. Paardekooper M, van den Broek PJA, de Bruijne AW, Elferink JGR, Dubbelman TMAR, van Steveninck J. Photodynamic treatment of yeast cells with the dye toluidine blue: All-or-none loss of plasma membrane barrier properties. Biochim Biophys Acta 1992;1108:86–90.
75. Paardekooper M, de Brujine AW, van Steveninck J, van den Broek JA. Inhibition of transport systems in yeast by photodynamic treatment with toluidine blue. Biochim Biophys Acta 1993;1151:143–148.
76. Lambrechts SAG, Aalders MCG, Verbraak FD, Lagerberg JWM, Dankert J, Schuitmaker JJ. Effect of albumin on the photoiactivation of microorganisms by a cationic porphyrin. J Photochem Photobiol B: Biol 2005;79:51–57.
77. Soncin M, Fabris C, Jori G, Roncucci G, unpublished observations.
78. Lambrechts SAG, Aalders MCG, van Marle J. Mechanistic study of the photodynamic inactivation of *Candida alibicans* by a cationic porphyrin. Antimicrob Agents Chemother 2005; 49:2026–2034.
79. Demidova TN, Hamblin MR. Effect of cell-photosensitiser binding and cell density on microbial photoinactivation. Antimicrob Agents Chemother 2005;49:2329–2335.
80. Bertoloni G, Lauro FM, Cortella G, Merchat M. Photosensitising activity of hematoporphyrin on *Staphylococcus aureus* cells. Biochim Biophys Acta 2000;1475:169–174.
81. Schafer M, Schmitz C, Horneck G. High sensitivity of *Deinococcus radiodurans* to photodynamically produced singlet oxygen. Int J Radiat Biol 1998;74:249–253.
82. Salmon-Divon M, Nitzan Y, Malik Z. Mechanistic aspects of *Escherichia coli* photodynamic inactivation by cationic tetra-meso(N-methyl-pyridyl)porphine. Photochem Photobiol Sci 2004;3:423–429.
83. Zeina B, Greenman J, Purcell WM, Das B. Killing of cutaneous microbial species by photodynamic therapy. Br J Dermatol 2001;144:274–278.
84. 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.
85. Teichert MC, Jones JW, Usacheva MN, Biel MA. Treatment of oral candidiasis with methylene blue-mediated photodynamic therapy in an immunodeficient murine model. Oral Surg Oral Med Oral Pathol Oral Radiol 2002;93:155–160.
86. Hamblin MR, O' Donnell DA, Murthy N, Contag CH, Hasan T. Rapid control of wound infections by targeted photodynamic therapy monitored by *in vivo* luminescence imaging. Photochem Photobiol 2002;75:51–57.
87. Gad F, Zahra T, Francis KP, Hasan T, Hamblin MR. Targeted photodynamic therapy of established soft-tissue interactions in mice. Photochem Photobiol Sci 2004;3:451–458.
88. 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.
89. Wong TW, Wang YY, Sheu HM, Chuang YC. Bactericidal effects of toluidine blue-mediated photodynamic action on *Vibrio vulnificus*. Antimicrob Agents Chemother 2005;49: 495–502.
90. Bisland SK, Chien C, Wilson BC, Burch S. Pre-clinical *in vitro* and *in vivo* studies to examine the potential use of photodynamic therapy in the treatment of osteomyelitis. Photochem Photobiol Sci 2006;5:31–38.
91. Berthiaume F, Reiken S, Toner M, Tompkins R, Yarmush M. Antibody-targeted photolysis of bacteria *in vivo*. Biotechnology 1994;12:703–706.

92. Embleton ML, Nair SP, Cookson BD, Wilson M. Antibody-directed photodynamic therapy of methicillin-resistant *Staphylococcus aureus*. Microb Drug Resist 2004;10:92–97.
93. Roncucci G, Dei D, De Filippis MP, Fantetti L, Nistri D. Metal substituted non-centrosimmetrical phthalocyanines analogues. Their preparation and use in photodynamic therapy and in vivo diagnostic. European Patent 1381611, 2005.
94. Berger TG. Treatment of bacterial, fungal and parasitic infections in the HIV-infected host. Semin Dermatol 1993;12:296–300.
95. Listgarten MA. Pathogenesis of periodontitis. J Clin Peridontol 1986;13:418–430.
96. Wilson M. Photolysis of oral bacteria and its potential use in the treatment of caries and periodontal disease. J Applied Bacteriol 1993;78:299–306.
97. Sarkar S, Wilson M. Lethal photosensitisation of bacteria in supragingival plaque from patients with chronic periodontitis. J Peridontal Res 1993;28:204–210.
98. Wilson M. Susceptibility of oral bacteria biofilms to antimicrobial agents. J Med Microbiol 1996;44:79–87.
99. Komerik N, Nakanishi H, Mac Robert AJ, Henderson B, Speight P, Wilson M. *In vivo*killing of *Porphyromonas gingivalis* by toluidine blue-mediated photosensitisation in an animal model. Antimicrob Agents Chemother 2003;47: 932–940.
100. Parekh SG, Trauner KB, Zarins B, Foster TE, Anderson RM. Photodynamic modulation of wound healing with BPD-MS and CASP. Lasers Surg Med 1999;24:375–381.
101. Orenstein A, Klein D, Kopolovic E, Winckler E, Malik Z, Keller N, Nitzan Y. The use of porphyrins for eradication of *Staphylococcus aureus*in burn wound infections. FEMS Immunol Med Microbiol 1998;19:307–314.
102. Lee WL, Shalita AR, Suntharalingam K, Fikrig SM. Neutrophil chemotaxis by *Propionibacterium acnes* lipase and its inhibition. Infect Immunol 1982;35:71–78.
103. Ashkenazi H, Malik Z, Harth Y, Nitzan Y. Eradication of *Propionibacterium acnes* by its endogenic porphyrins after illumination with high intensity blue light. FEMS Immunol Med Microbiol 2003;35:17–24.
104. Itoh Y, Ninomiya Y, Tajima S, Ishibashi A. Photodynamic therapy of acne vulgaris with topical delta-amino-levulinic acid and incoherent light in Japanese patients. Br J Dermatol 2001;144:575–579.
105. Tegos GP, Demidova TN, Arcila-Lopez D, Lee H, Wharton T, Gali H, Hamblin MR. Cationic fullerenes are effective and selective antimicrobial photosensisitisers. Chem Biol 2005; 12:1127–1135.
106. Gardlo K, Horska Z, Enk CD, Rauch L, Megahed M, Ruzicka T, Fritsch C. Treatment of cutaneous leishmaniasis by photodynamic tehrapy. J Am Acad Dermatol 2003;48:893–896.