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Photodynamic therapy: a new antimicrobial approach to infectious disease?

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Abstract

Photodynamic therapy (PDT) employs a non-toxic dye, termed a photosensitizer (PS), and low intensity visible light which, in the presence of oxygen, combine to produce cytotoxic species. PDT has the advantage of dual selectivity, in that the PS can be targeted to its destination cell or tissue and, in addition, the illumination can be spatially directed to the lesion. PDT has previously been used to kill pathogenic microorganisms *in vitro*, but its use to treat infections in animal models or patients has not, as yet, been much developed. It is known that Gram-(−) bacteria are resistant to PDT with many commonly used PS that will readily lead to phototoxicity in Gram-(+) species, and that PS bearing a cationic charge or the use of agents that increase the permeability of the outer membrane will increase the efficacy of killing Gram-(−) organisms. All the available evidence suggests that multi-antibiotic resistant strains are as easily killed by PDT as naïve strains, and that bacteria will not readily develop resistance to PDT. Treatment of localized infections with PDT requires selectivity of the PS for microbes over host cells, delivery of the PS into the infected area and the ability to effectively illuminate the lesion. Recently, there have been reports of PDT used to treat infections in selected animal models and some clinical trials: mainly for viral lesions, but also for acne, gastric infection by *Helicobacter pylori* and brain abscesses. Possible future clinical applications include infections in wounds and burns, rapidly spreading and intractable soft-tissue infections and abscesses, infections in body cavities such as the mouth, ear, nasal sinus, bladder and stomach, and surface infections of the cornea and skin.

1 Introduction

Photodynamic therapy (PDT) is a therapy for cancer and other diseases that has received regulatory approval for several indications in many countries.¹ Its use as a cancer treatment is based on the observation that certain non-toxic dyes—known as photosensitizers (PS)—of which hematoporphyrin derivative (HPD, also known as Photofrin) is the best-known example, accumulate preferentially in malignant tissues.² Therapy involves delivering visible light of the appropriate wavelength to excite the PS molecule to the excited singlet state. This excited state may then undergo intersystem crossing to the slightly lower energy, but longer lived, triplet state, which may then react further by one or both of two pathways known as the Type I and Type II photoprocesses, both of which require oxygen.³ The Type I pathway involves electron-transfer reactions from the PS triplet state with the participation of a substrate to produce radical ions that can then react with oxygen to produce cytotoxic

species, such as superoxide, hydroxyl and lipid-derived radicals.⁴ The Type II pathway involves energy transfer from the PS triplet state to ground-state molecular oxygen (triplet) to produce excited-state singlet oxygen, which can oxidize many biological molecules, such as proteins, nucleic acids and lipids, and lead to cytotoxicity.⁵ PDT has the advantage over other therapies of dual selectivity: not only is the PS targeted to the tumor or other lesion, but the light can also be accurately delivered to the affected tissue. Although originally developed as a cancer treatment, the most successful PDT application to date (which recently received FDA approval) has been in ophthalmology, as a treatment for age-related macular degeneration.⁶ Other non-oncological applications of PDT at a less developed stage include treatments for psoriasis,⁷ arthritis,⁸ Barretts's esophagus,⁹ atherosclerosis¹⁰ and restenosis¹¹ in both veins and arteries.

Most of the PS that are under investigation for the treatment of cancer and other tissue diseases are based on the tetrapyrrole nucleus, examples are porphyrins (HPD), chlorins (BPD, SnEt2, *m*-THPC), bacteriochlorins (TOOKAD), phthalocyanines (Pc4) and texaphyrins (Lutex) Fig. 1. These molecules have been chosen for their low dark toxicity to mammalian cells and to animals, and for their tumor-targeting properties. However many other PS (especially those that have been studied for their ability to kill microorganisms) have different molecular frameworks. These include halogenated xanthenes such as Rose Bengal,¹² phenothiazines such as Toluidine Blue O and Methylene Blue,¹³ acridines¹⁴ and perylenequinones such as hypericin¹⁵ (Fig. 2).

2 PDT for infectious disease

The rapidly increasing emergence of antibiotic resistance amongst pathogenic bacteria may be bringing to an end a period extending over the past 50 years, termed "the antibiotic era".¹⁶ Bacteria replicate very rapidly and a mutation that helps a microbe survive in the presence of an antibiotic drug will quickly become predominant throughout the microbial population. The inappropriate prescription of antibiotics and the failure of some patients to complete their treatment regimen also exacerbate the problem. Methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci are two resistant species that are causing much concern at present.¹⁷ The emergence of antibiotic resistance amongst pathogenic bacteria has led to a major research effort to find alternative antibacterial therapeutics¹⁸ to which, it is hypothesized, bacteria will not be easily able to develop resistance. Examples of these relatively novel therapies are bacteriophages,¹⁹ naturally occurring or synthetic antimicrobial peptides²⁰ and PDT.²¹ All studies that have examined the killing of antibiotic-resistant bacteria by the combination of PS and light, termed photodynamic inactivation (PDI), have found them to be equally as susceptible as their naïve counterparts.^{22,23}

Because the delivery of visible light is almost by definition a localized process, PDT for infections is likely to be applied exclusively to localized disease, as opposed to systemic infections such as bacteremia. In contrast to PDT for cancer, where the PS is usually injected into the bloodstream and accumulates in the tumor, we believe that PDT for localized infections will be carried out by local delivery of the PS into the infected area by methods such as topical application, instillation, interstitial injection or aerosol delivery. The key issues to be addressed, therefore, will be the effectiveness of the treatment in destroying sufficient numbers of the disease-causing pathogens, effective selectivity of the PS for the microbes, thus avoiding an unacceptable degree of PDT damage to host tissue in the area of infection, and the avoidance of regrowth of the pathogens from a few survivors following the treatment.

3 Photodynamic inactivation of bacteria

It has been known since the first days of PDT, early in the last century, that certain microorganisms can be killed by the combination of dyes and light *in vitro*.^{24–26} Throughout the years since those times there have been additional reports of bacteria being killed or inactivated by various combinations of PS and light (see Table 1 for a representative selection). In the 1990s, it was observed that there was a fundamental difference in susceptibility to PDT between Gram-(+) and Gram-(−) bacteria. It was found that, in general, neutral or anionic PS molecules are efficiently bound to and photodynamically inactivate Gram-(+) bacteria, whereas they are bound, to a greater or lesser extent, only to the outer membrane of Gram-(−) bacterial cells, but do not inactivate them after illumination.⁴⁴ The high susceptibility of Gram-(+) species is explained by their physiology, as their cytoplasmic membrane is surrounded by a relatively porous layer of peptidoglycan and lipoteichoic acid that allows PS to cross.⁴⁴ The cell envelope of Gram-(−) bacteria consists of an inner cytoplasmic membrane and an outer membrane that are separated by the peptidoglycan-containing periplasm (Fig. 3). The outer membrane forms a physical and functional barrier between the cell and its environment. In the outer membrane, several different proteins are present, some of them function as pores to allow passage of nutrients, whereas others have an enzymatic function or are involved in maintaining the structural integrity of the outer membrane and the shape of the bacteria.

Several groups of workers then devised approaches that would allow PDI of Gram-(−) species. The Israeli group of Nitzan and co-workers used the polycationic peptide polymyxin B nonapeptide (PMBN), which increased the permeability of the Gram-(−) outer membrane and allowed PS that are normally excluded from the cell to penetrate to a location where the reactive oxygen species generated upon illumination can cause fatal damage.⁴⁵ PMBN does not release lipopolysaccharide (LPS) from the cells, but ‘expands’ the outer leaflet of the membrane, allowing PS such as deuteroporphyrin (DP) to penetrate and permitting PDI of *E. coli* and *P. aeruginosa*.⁴⁵ Nitzan *et al.* demonstrated an interaction between PMBN and DP in solution, and speculated that this binding assisted the penetration. They used this method to kill a multi-antibiotic resistant strain of *A. baumannii* and found that DP seemed to work much better in concert with PMBN than many other PS, including porphyrins, phthalocyanines and merocyanine 540.⁴⁶ They also found that the growth medium of the bacteria made a big difference to their susceptibility to PDI, with the high protein brain–heart infusion leading to less killing than the low protein nutrient broth. Furthermore, these workers noted that the type of protein present in the medium, as well as its concentration, made a difference to the susceptibility of the bacteria. Interestingly, they found that a polylysine chain of 20 lysine residues did not allow PDI with DP.⁴⁴ Malik *et al.* have also studied a mixture of hemin and DP as a PDI agent against bacteria.⁴⁷ It has a dark cytotoxic activity on *S. aureus* and other Gram-(+) bacteria; the effect of the combined mixture was stronger than that of the separate constituents and was as strong in the dark as under illumination.⁴⁴ The total inability of the Gram-(+) cultures to recover from the combined treatment by hemin–DP in the dark suggests the possibility of the formation of an oxidizing porphyrin complex. A similar approach was taken by Bertoloni *et al.*, who found that the use of ethylenediaminetetraacetic acid (EDTA) to release LPS or the induction of competence with calcium chloride sensitized *E. coli* and *Klebsiella pneumoniae* to PDI by Hematoporphyrin or zinc phthalocyanine.⁴⁸

A second approach adopted by other groups is to use a PS molecule with an intrinsic positive charge. Wilson and co-workers have used the phenothiazine Toluidine Blue O to carry out PDI of a large range of both Gram-(+) and Gram-(−) bacteria.⁴⁹ These papers have been frequently concerned with oral bacteria,⁵⁰ but they have also studied *S. aureus*²³ and the Gram-(−) *H. pylori*.⁵¹ The growth phase of the bacteria did not make a big difference to

their susceptibility to PDI, and the presence of serum in the medium decreased the killing.⁵² A group in Italy led by Jori have used cationic porphyrins [*meso*-tetra(*N*-methyl)-4-pyridylporphine tetraiodide and tetra-{4-(*N,N,N*-trimethylanilinium)}porphine to photoinactivate Gram-(−) species such as *Vibrio anguillarum* and *E. coli*.⁵³ They found that washing the loosely bound PS from the cells before illumination decreased the killing and explained this finding by supposing that the first dose of light on PS bound to the outside of the outer membrane causes initial limited photodamage that then allows further penetration of the PS.⁵³ The group led by Brown in the UK has used cationic phthalocyanines for PDI of Gram-(−) bacteria.³⁷ They investigated *E. coli* DH5α and, in particular, the mechanism of uptake. They found that incubation with zinc pyridiniumphthalocyanine (PPc) in the dark led to increased sensitivity of the bacteria to hydrophobic, but not hydrophilic, antibiotics. Incubation with PPc also led to increased uptake of radiolabeled protoporphyrin, a process that was reversed in the presence of up to 50 mM Mg²⁺ ions. These observations are consistent with the uptake of PPc proceeding through the self-promoted uptake pathway (see next section).⁵⁴

There are reports of PDI of Gram-(−) bacteria in which it is clear that the PS does not have to penetrate the bacterium to be effective, or, indeed, even come into contact with the cells. According to these papers, if singlet oxygen can be generated in sufficient quantities near to the bacterial outer membrane, it will be able to diffuse into the cell to inflict damage on vital structures.⁵⁵ In one set of studies, the bacteria were separated from the PS by a layer of moist air and singlet oxygen in the gas phase was generated and allowed to diffuse across the gap before contacting the bacteria.⁵⁶ Gram-(−) species were harder to kill than Gram-(+) and the intracellular content of carotenoids was found to protect the bacteria from photoinactivation. In another study, the PS Rose Bengal was covalently bound to small polystyrene beads that were allowed to mix with the bacteria in suspension.⁵⁷ Some targeting systems for PDI of bacteria presumably also rely on this ability of PS bound at the outer membrane to generate reactive oxygen species that then diffuse into the cells. Yarmush and his group have covalently bound PS to a monoclonal antibody (Mab) that binds to cell surface antigens expressed on *P. aeruginosa* and demonstrated specific killing of target bacteria after illumination not produced by a non-specific Mab conjugate.^{58,59} Other workers have used a non-specific IgG that was recognized by protein A expressed on *S. aureus*.⁶⁰ They conjugated a bacteriochlorophyll–serine derivative to rabbit IgG and compared the PDI effect with that of unconjugated dye. They found that although the free dye was considerably more phototoxic than the selective targeted IgG conjugate at equal incubation concentrations, each molecule of conjugate was actually more phototoxic than each molecule of free dye because the uptake of the conjugate was 30 times less than the free dye. They attributed this difference to the conjugate only binding to “exclusive positions on the cell wall”. It is very unlikely that covalent antibody bound PS could penetrate the outer membrane and, therefore, diffusion of reactive oxygen species inwards to the interior of the cell must be occurring.

How can these two conflicting sets of findings on the necessity for PS penetration into Gram-(−) bacteria be reconciled? One possibility is that singlet oxygen can indeed diffuse into bacteria, producing fatal damage if it is generated at the outer surface or in close proximity in solution. The diffusion distance of singlet oxygen in solution has been estimated to be approximately 20 nm.³ The failure of some PS that bind to Gram-(−) species to produce any killing, however, must mean that the reactive species produced on illumination were unable to diffuse inward to sensitive sites. The fact that these PS can lead to efficient PDI of Gram-(+) species shows that the molecules have not undergone any conformational change that has rendered the PS no longer photoactive. Hence, we hypothesize that PS that operate chiefly *via* Type I mechanisms need to penetrate the Gram-

(–) outer membrane, while those that act mainly by Type II PS may not, and that the former may be more efficient.

4 Photophysics

Some mechanistic studies have implicated Type I (electron-transfer and radical) mechanisms in PDI of bacteria. Martin and Logsdon investigated a set of thiazine, xanthene, acridine and phenazine dyes, and their phototoxicity towards *E. coli*.⁴² Hydroxyl radical scavengers conferred dose-dependent protection against the photodynamic action of all of the representative dyes. Exogenous superoxide dismutase and catalase partially protected the cells against the dye-mediated phototoxicity, and prior induction of intracellular superoxide dismutase and catalase by growth in glucose minimal medium containing manganese and paraquat gave substantial protection. They concluded oxygen radicals were primarily responsible for the oxygen-dependent toxicity of the dyes examined. Strong *et al.* compared two structurally different PS immunoconjugates targeted against *P. aeruginosa* that had different photophysical properties.⁵⁹ One had a lower singlet oxygen quantum yield, yet was more efficient in PDI; this was explained by hydroxyl radical generation due to the chemical structure of the conjugate.

5 Mechanisms of damage

There are two basic mechanisms that have been proposed to account for the lethal damage caused to bacteria by PDI: (i) DNA damage and (ii) damage to the cytoplasmic membrane, allowing leakage of cellular contents or inactivation of membrane transport systems and enzymes. There is a good deal of evidence that treatment of bacteria with various PS and light leads to DNA damage. Breaks in both single- and double-stranded DNA, and the disappearance of the plasmid super-coiled fraction have been detected in both Gram-(+) and Gram-(–) species after PDI with a wide range of PS structural types.^{33,61–63} There is some evidence that PS that can more easily intercalate into double-stranded DNA can more easily cause damage.^{14,64} Guanine residues have been shown to be the most easily oxidized.⁶⁴ The damage may be able to be repaired by various DNA repairing systems.⁶⁵ However, various authors have concluded that although DNA damage occurs, it may not be the prime cause of bacterial cell death. One argument that has been used in favor of this hypothesis is that *D. radiodurans*, which is known to have a very efficient DNA repair mechanism, is easily killed by PDI.⁶⁶ The alteration of cytoplasmic membrane proteins has been shown by Valduga *et al.*⁶⁷ and Bertoloni *et al.*⁴⁸ Disturbance of cell-wall synthesis and the appearance of a multilamellar structure near the septum of dividing cells, along with loss of potassium ions from the cells was reported by Nitzan *et al.*⁴⁵

6 Endogenous porphyrins in microorganisms

It has long been known that some bacteria accumulate porphyrins under some circumstances and, consequently, exhibit red fluorescence under UVA or blue light illumination and are susceptible to killing upon illumination.⁶⁸ This was reported to be common among anaerobic species⁶⁹ and red-fluorescent pus was found in infections.⁷⁰ It is now realized that there are two mechanisms by which bacteria can naturally accumulate sufficient porphyrins to allow them to be photo-inactivated upon illumination. Firstly, there is a group of anaerobic species that are mainly oral pathogens and were previously known as black-pigmented Bacteroides species, but have now been reclassified as Porphyromonas and Prevotella species.⁷¹ These bacteria depend largely on external heme (either as hemoglobin or hemopexin) to satisfy their demand for iron⁷² and, to a greater or lesser extent, intracellularly accumulate a black pigment that consists of an iron-containing heme aggregate (*e.g.* hemozoin), together with varying amounts of iron-free protoporphyrin IX (PPIX).^{73,74} If they accumulate PPIX, they will be photosensitive;⁷⁵ however, if they mainly

acquire heme, they will not be photosensitive, as iron-containing tetrapyrroles do not carry out the appropriate photochemistry. It was shown that these bacteria did not incorporate ^{14}C -labeled 5-aminolevulinic acid (ALA) into these porphyrins, thus demonstrating that the pigment came from external heme, rather than endogenous heme biosynthesis.

In a similar fashion to mammalian cells, most bacteria use the heme biosynthetic pathway to produce porphyrins from the precursor ALA. Differences between the bacterial and mammalian pathways include the existence of a widely used alternative route to ALA from glutamate and offshoots from the pathway that lead to the heme variants and coenzyme B $_{12}$ ⁷⁶ (Fig. 4). The bacterium responsible for acne (*P. acnes*) has long been known to accumulate red-fluorescent porphyrins;⁷⁷ this property has been used to follow the response of patients to therapy by fluorescence photography of the face⁷⁸ and as the basis for phototherapy for acne treatment.⁷⁹ These porphyrins mainly consist of coproporphyrin and PPIX (with a minor contribution from uroporphyrin).⁸⁰ The fact that a tetracarboxylic porphyrin is the main constituent shows that the porphyrins must arise from endogenous heme biosynthesis, as there is no known mechanism for producing a tetracarboxylic porphyrin from a dicarboxylic porphyrin that was obtained from exogenous heme. Our laboratory has recently shown that *H. pylori* naturally accumulates enough porphyrins to allow photoinactivation with blue light and that these porphyrins contain coproporphyrin,⁸¹ suggesting that *H. pylori* behaves in a similar fashion to *P. acnes* in accumulating photoactive porphyrins as a by-product of heme biosynthesis.

It is well established that with many mammalian cell types, the provision of exogenous ALA bypasses the physiological feedback control of ALA synthetase and leads to intracellular accumulation of photoactive porphyrins, chiefly PPIX.⁸² A similar approach has been taken with some bacteria *in vitro*. Gabor *et al.* demonstrated photoinactivation of *E. coli* B, but not *E. hirae*, after ALA incubation and white light illumination.³² In another study,⁸³ they showed that non-photoactive metalloporphyrins could be formed, and a similar discovery was made by other workers for the yeast *Candida guilliermondii*.^{84–86}

van der Meulen *et al.* showed that *H. parainfluenzae*, after incubation with ALA, was killed after red light illumination.³⁶ *H. influenzae*, however, has several mutations in heme biosynthetic enzymes and obtains both tetrapyrroles and iron from exogenous heme. Consequently, it was unaffected by ALA and light.

7 *In vitro* selectivity compared to mammalian cells

Although many workers in the field of bacterial PDI *in vitro* have proposed its use as a treatment for localized infections, there have been relatively few *in vitro* studies demonstrating selective killing of microbes under conditions in which mammalian cells were spared. Soukos *et al.* showed that *Streptococcus sanguis* was killed by Toluidine Blue and red light using parameters that spared human gingival keratinocytes and fibroblasts.⁸⁷ Zeina *et al.* used a human keratinocyte cell line (H103) that resisted killing by Methylene Blue-mediated PDT under conditions that killed several cutaneous microbial species.²⁹ Recently, Soncin *et al.* reported that certain cationic ZnPc, combined with a short incubation time (5 min) and relatively low light fluences, killed both wild-type and methicillin-resistant *S. aureus*, while human fibroblasts and keratinocytes were unharmed.⁸⁸

8 Susceptibility and resistance

Bacteria are able to persist during feast and famine in many different environments, including soil, water, plants, animals and humans. They have evolved a wide array of protective mechanisms to defend themselves against starvation, extremes of heat, cold, pH, osmolarity and UV radiation, and oxidative stress. Regulation of gene expression in bacteria

occurs *via* RNA polymerases (RNAP) that are routinely isolated in two distinct forms: core RNAP, which catalyzes the polymerization of ribonucleotides into the RNA complement of a DNA template, and RNAP holoenzyme, which contains the subunits of the core molecule (B, B', and A2) plus an additional protein (sigma factor) that permits the holoenzyme to recognize promoter elements and initiate transcription at these sites.⁸⁹ Bacteria can contain a multiplicity of sigma factors (usually at least 10). Two of these factors, sigma-70 (gene *rpoD*) and sigma-54 (gene *rpoN*), direct the transcription of a wide variety of genes.⁹⁰ The other sigma factors, known as alternative sigma factors, are required for the transcription of specific subsets of genes. The alternative sigma factor S encoded by the *rpoS* gene is known to be important for survival under starvation and other stressful conditions in many bacterial species.^{91,92} Bacteria can communicate to each other when the population rises at a rate that outpaces the supply of nutrients *via* diffusible small molecules (acylhomoserine lactones)⁹³ and this quorum-sensing mechanism controls the expression of many genes.

Treatment of *E. coli* with low doses of hydrogen peroxide results in the increased expression of 30 proteins and resistance to killing by higher doses of hydrogen peroxide is induced. This observation led to the discovery that prokaryotic cells employ redox-sensing transcription factors to detect elevated levels of reactive oxygen species and regulate expression of antioxidant enzymes.⁹⁴ H₂O₂ reacts with iron to form the extremely reactive and damaging hydroxyl radical *via* the Fenton reaction. Superoxide anion (O₂⁻) accelerates this reaction because its dismutation leads to increased levels of hydrogen peroxide and also because O₂⁻ elevates the intracellular concentration of iron by attacking iron-sulfur proteins. In *E. coli*, two such transcription factors, OxyR and SoxRS, have been well characterized.⁹⁵ Both proteins are turned on and off with very fast kinetics (of the order of a few minutes), allowing rapid cellular responses to oxidative stress. It is presently unknown to what extent these antioxidant defenses will influence the susceptibility of bacteria to the reactive oxygen species generated during PDI.

A great deal of work has been carried out on the permeability barriers of varying bacteria to molecules with different structures, because one of the factors governing bacterial susceptibility to antibiotics is thought to be decreased permeability. The substantial body of work by Leive and co-workers^{96,97} in the 1960s and '70s elucidated the effects of divalent cations and the role of LPS in forming the outer membrane barrier of Gram-(−) bacteria. The ability of chelators such as EDTA to destabilize the LPS coating by removing the Mg²⁺ and Ca²⁺ ions that act as bridges between neighboring LPS molecules is now well accepted⁹⁸ (Fig. 3). Two classes of polycations, which although having much in common, also have important differences, have been studied for their ability to increase the permeability of Gram-(−) bacteria. These are the large class of naturally occurring antimicrobial cationic peptides and a much smaller group of synthetic polycations. Cationic peptides are important components of the natural defenses of most living organisms against microbial infection and, in mammals, are found in high concentrations in neutrophils, while similar peptides are inducible by injury in amphibians (magainins) and in insects (cecropins) to combat bacterial invasion.⁹⁸ The term “self-promoted uptake” was coined by Hancock and Bell to describe the uptake of cationic peptides across outer membranes of Gram-(−) bacteria.⁹⁹ The first step is the interaction of polycations with divalent cation binding sites on cell surface LPS, where they displace these ions and, being so bulky, disrupt the normal barrier property of the outer membrane causing transient “cracks” which permit passage of hydrophobic compounds, small proteins and/or antimicrobial compounds, and promote the uptake of the perturbing peptide itself. The specificity for bacteria is due to their large transmembrane potentials, high content of negatively charged lipids, and lack of cationic lipids and cholesterol, none of which apply to eukaryotic cells.¹⁰⁰

The second group of synthetic polycations, typified by poly-L-lysine (pL), has been investigated as permeabilizing agents. Vaara and Vaara showed that treatment of *Salmonella* strains and *E. coli* with pL (20 lysines) in concentrations up to $100 \mu\text{g mL}^{-1}$ ($50 \mu\text{M}$) released about 20–30% of the LPS into the medium and caused rapid increased permeability to SDS (1000-fold) and a slower increase in permeability to hydrophobic antibiotics such as novobiocin and fusidic acid (100-fold) without affecting their viability.¹⁰¹ However pL chains longer than 20 lysines (50, 115 and 300 lysine residues) had a significant cytotoxic effect on several Gram-(−) species.¹⁰² Polycations such as polyethyleneimine¹⁰³ and polyornithine¹⁰⁴ have also been shown to permeabilize Gram-(−) species.

9 Polycationic PS conjugates

In 1997, a hypothesis was formed in our laboratory that by covalently conjugating a suitable PS to a pL chain, a bacteria-targeted PS delivery vehicle could be constructed that would efficiently inactivate both Gram-(+) and Gram-(−) species. Because the resulting polycationic entity is a macromolecule, it would be taken up by mammalian cells by the time-dependent process of endocytosis, thus giving temporal selectivity for bacteria, into which the polycation could penetrate rapidly. This was demonstrated¹⁰⁵ by the preparation of a conjugate between one molecule of chlorin e6 (ce6) and a pL chain of 20 lysine residues that, after 1 min incubation and illumination with red light, killed >99% of the Gram-(+) (*Actinomyces viscosus*) and Gram-(−) (*Porphyromonas gingivalis*) oral pathogens while sparing an oral epithelial cell line (HCPC-1). A similar construct was subsequently used by another group (composed of one ce6 molecule and a 5 amino-acid lysine chain) to kill several oral pathogens in the presence of 25% whole blood.¹⁰⁶ Polo *et al.* used conjugates between pL and porphycenes with a significant phototoxic activity against Gram-(−) bacteria.¹⁰⁷ In a subsequent report,¹⁰⁸ this group showed that several strains responsible for periodontal disease were efficiently inactivated by visible light irradiation in the presence of porphycene–polylysine conjugates. Repeated photosensitization of surviving cells did not induce the selection of resistant bacterial strains nor modify their sensitivity to antibiotic treatment.

We recently compared¹⁰⁹ the effectiveness of pL–ce6 conjugates with chain lengths of either 8 or 37 lysines attached to precisely one ce6 molecule (see Fig. 2) for bacterial PDT and found the 37-lysine conjugate was able to efficiently mediate the photodestruction of both Gram-(+) and Gram-(−) species, while the 8-lysine conjugate and free ce6 were only effective against Gram-(+) bacteria. We have used pL–ce6 conjugates and red light to successfully carry out PDT of wound infections in mice (see below).

10 Bacterial virulence factors

Since some pathogenic species and strains of bacteria are more likely to cause disease than others, much effort has been devoted to determining the various molecules responsible for the propensity to cause disease, both in humans and animals. Many of these virulence factors are properties of the bacteria themselves such as motility, adhesiveness to various tissue components and resistance to host defense mechanism in serum or by phagocytes. However, other important virulence factors are secreted molecules such as enzymes, toxins and small molecules. One very attractive feature peculiar to PDT as an antibacterial treatment is the possibility that the reactive oxygen species generated from the excited state of the dye may chemically destroy many of these secreted virulence factors (especially those that are proteins). That this may be feasible has been demonstrated by Komerik *et al.*, who showed that LPS from *E. coli* and culture supernatants containing proteases of *P. aeruginosa* were inactivated after exposure to red light in the presence of Toluidine Blue O.¹¹⁰ Thus, PDT, in addition to being antimicrobial, may also be an “anti-virulence factor therapy”.

11 Photoinactivation of viruses, fungi, yeasts and parasites

Many reports have emerged over the years of the use of PS and light to kill other pathogenic microorganisms *in vitro*. Most of the *in vitro* work on viruses has been oriented to the use of the methodology for sterilization of blood or blood products^{111,112} (reviewed in ref. 113). Several reports have concluded that lipid-enveloped viruses are more susceptible to PDI than non-enveloped strains (see Table 2). Although this is a promising field, due to constraints on space, it will not be covered in detail here. Many of the clinical applications of PDT for localized infections have been concerned with lesions of viral etiology (see below).

In a similar fashion to viruses, there have been several reports on the use of PDT to kill yeasts and fungi *in vitro* (some reports are summarized in Table 3). There has been much less systematic study (compared to that carried out with bacteria) on the types of molecular features necessary in a PS in order to make it effective in mediating PDI of various species of yeast and fungi. One report concluded that membrane damage and the consequent increased permeability was the proximate cause of cell death after Methylene Blue-mediated PDT on yeast.¹³⁴

Human pathogenic parasites have also been killed by combinations of PS and light. *Plasmodium falciparum*, responsible for malaria, has been killed by PDT mediated by an *N*-(4-butanol)pheophorbide derivative (which also killed *Babesia divergens*)¹³⁵ and by silicon phthalocyanines such as Pc4¹³⁶ (see Fig. 1). *Trypanosoma cruzi* (Chagas' disease) has been killed by the combination of light and PS such as hematoporphyrin and aluminium sulfonated phthalocyanine.¹³⁷ Human helminth eggs in wastewater were inactivated by a cationic *meso*-substituted porphyrin and light.¹³⁸

12 Photoinactivation of microbes in the presence of biological material

Millson *et al.* carried out PDI of *Helicobacter mustelae* (which naturally infects the gastric mucosa of ferrets) on explanted segments of ferret stomach (*ex vivo*).¹³⁹ They found that Methylene Blue and red light produced significant killing of this Gram-(−) organism, but that much higher concentrations of PS were necessary than when the PDI was performed *in vitro*. Several workers have used various PS and light *in vitro* to kill oral bacteria growing on samples of dental plaque obtained *in vivo*.^{140–142} Lasocki *et al.*¹⁴³ studied the antibacterial photodynamic effect of arginine hematoporphyrin derivative (HpD-Arg2) using two methods. The PS and light were employed against *P. aeruginosa* and *S. aureus* either as suspension cultures in nutrient broth or as colonies growing on isolated mouse muscles. The PDI effect was 1000 times greater in the case of suspension cultures and the concentration of dye necessary for optimal effects was much lower.

13 PDT of infections *in vivo* (animal models)

There have been (rather surprisingly) few attempts to demonstrate PDI of bacteria in the tissue of living animals or to carry out PDT in animal models of infection. Berthiaume *et al.*¹⁴⁴ evaluated the efficacy of antibody-targeted photolysis to kill bacteria *in vivo* using PS immunoconjugates. After infecting the dorsal skin in mice with *P. aeruginosa*, both specific and non-specific tin(IV) chlorin e6–monoclonal antibody conjugates were injected at the infection site. After a 15 min incubation period, the site was exposed to 630 nm light with a power density of 100 mW cm^{−2} for 1600 s. Illumination resulted in a greater than 75% decrease in the number of viable bacteria at sites treated with a specific conjugate, whereas normal bacterial growth was observed in animals that were untreated or treated with a non-specific conjugate. Orenstein *et al.*¹⁴⁵ studied a mixture of deuteroporphyrin and hemin for PDI of *S. aureus*. They found that the mixture was strongly bactericidal in the dark *in vitro*,

while deuteroporphyrin alone required light to produce killing. They only found bacterial killing in a guinea pig model of an infected burn (*S. aureus*) with the mixture in the dark.

Teichart *et al.*¹⁴⁶ evaluated the efficacy of using Methylene Blue-mediated PDT to treat oral candidiasis in an immunosuppressed murine model, mimicking what is found in human patients. SCID mice were inoculated orally with *C. albicans* by swab 3 times a week for a 4 week period. Mice received a topical oral cavity administration of 0.05 mL MB solution at 250–500 $\mu\text{g mL}^{-1}$; 10 min later, the mice were irradiated with 664 nm light from a diode laser with a cylindrical diffuser. Swabs were taken before and after treatment and cultured for determination of the colony-forming unit (CFU). The highest MB concentrations and light totally eradicated *C. albicans* from the oral cavity, a reduction from 2.74 $\log_{10}$ to 0 CFU.

Our laboratory has demonstrated the use of PDT with polycationic PS conjugates to treat infections in excisional wounds in mice.¹⁴⁷ We used genetically engineered bacteria that emit luminescence, which we detected using a sensitive low light imaging camera.^{148–150} These bacteria have been transfected with a plasmid containing the *Photorhabdus luminescens* lux operon (luxABCDE) that encodes for not only the luciferase enzyme, but also the biosynthetic enzymes necessary for biosynthesis of the luciferase substrate. Hence, in the presence of ATP from the bacterium and external oxygen, these bacteria will glow in the dark. The rate of luciferase enzyme turnover in the presence of substrate allows for real-time measurements, and the enzyme is active at the body temperature of mammals. An image captured by the camera of a living mouse gives information about the intensity and spatial spread of the infection, and each animal can be followed longitudinally, dramatically reducing the numbers of animals needed to study treatment of infections. This method is an improvement on the traditional use of survival or body fluid sampling and subsequent plating and colony counting.

We used a non-pathogenic strain of *E. coli* that allowed four wounds on the back of each mouse to be infected with 1 million bacteria and, 30 min later, pL-ce6 conjugate was added to wounds 1 and 4.¹⁴⁷ After a further 30 min, wounds 3 and 4 were illuminated with red light and the mouse was imaged in the camera at each stage and after consecutive incremental fluences of light had been delivered. There was a light dose dependent reduction of bacterial luminescence from the wound treated with conjugate and light that was not seen in any of the control wounds (see Fig. 5). Wound healing was similar in PDT-treated wounds compared to untreated wounds, showing that no unacceptable harm was done to the host tissue by PDT. We then used a highly pathogenic strain of *P. aeruginosa* in a single excisional wound on the mouse back.¹⁵¹ In this case, PDT-treated mice were saved from death caused by the bacteria invading the bloodstream, which was the fate of mice whose wounds were untreated or received light or conjugate alone. PDT-treated wounds healed faster than those of mice that were treated with an alternative topical antimicrobial, silver nitrate solution. This result may be due to the possible PDT-mediated inactivation of extracellular virulence factors secreted by the bacteria that are responsible both for bacteria invading the tissue and causing damage to the wound bed, delaying wound healing, as is seen in mice where the bacteria have been killed by an alternative means.

14 Clinical applications

Most of the clinical applications of PDT for treatment of infections so far have been directed towards viral lesions. In the 1970s, there was a burst of popularity in treating herpes simplex lesions by topical PDT^{152,153} (reviewed in ref. 154). Several dyes (of which the most popular choice was Neutral Red) were topically applied to oral or genital herpes lesions, followed by illumination, generally with white light. However, this practice diminished after

Myers *et al.*¹⁵⁵ carried out a controlled clinical trial showing no therapeutic effects in 96 patients and a possible adverse effect on orolabial lesions. In addition to this, concern was raised about the possible carcinogenic effects of the treatment.¹⁵⁶

Papillomatosis, caused by human papillomatosis virus (HPV), has been treated by systemic and topical PDT in several anatomic locations. In the respiratory tract, papillomatosis is a potentially life-threatening disease that affects both children and adults, and can result in complete respiratory obstruction. Conventional therapies cannot prevent multiple recurrences. Systemic PDT with dihematoporphyrin ether (4.25 mg kg^{-1}) was tested in 48 patients, who received 50 J of 630 nm laser light 48 h after application of the drug.¹⁵⁷ There was notable improvement with a significant decrease in papilloma growth rate compared to control patients. Three year follow-up of a subset of patients confirmed that the improvement was maintained. Similar results were reported by Abramson *et al.*¹⁵⁸ and by Bujia *et al.*¹⁵⁹ Karrer *et al.*¹⁶⁰ treated a 65 year-old woman who had suffered from wart-like lesions on the hands, lower arms and forehead for about 45 years, diagnosed as epidermodysplasia verruciformis. PDT was performed using a 20% ALA ointment applied for 6 h to the lesions and irradiating with 580–740 nm light (160 mW cm^{-2} , 160 J cm^{-2}). Following PDT, blistering and crusting of the lesions occurred, but these healed completely within 2–3 weeks without scarring and the cosmetic result was excellent. Twelve months after PDT, a few lesions had recurred on the hands. Although permanent cure of epidermodysplasia verruciformis cannot be achieved, topical PDT might result in better control of HPV-induced lesions.

Abdel-Hady *et al.*¹⁶¹ used topical ALA–PDT to treat high grade vulval intraepithelial neoplasia (VIN 2–3) lesions, but observed a short-term response in only one third of cases. Unifocal lesions were found to be more responsive than multifocal and pigmented lesions. They measured HPV infection, HLA expression and immune infiltrating cells in VIN biopsies from responders and non-responders. There was a greater likelihood of HPV positivity associated with a lack of response of VIN to PDT, and VIN non-responders were more likely to show HLA class I loss compared with responders. There was a significant increase in CD8 infiltration (cytotoxic T-cells) in post-treatment VIN responders compared with non-responders. High risk HPV infection and lack of cell-mediated immunity may play a role in the observed poor response of lower genital lesions to topical PDT.

There is one report of PDT being used to treat localized bacterial infections by topical administration of PS: Lombard *et al.* treated 5 patients with brain abscesses after craniotomy and surgical drainage by introducing hematoporphyrin into the abscess bed and illuminating 5 min afterwards, producing a positive clinical response.¹⁶²

H. pylori is an endemic pathogenic bacterium that causes 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,¹⁶³ prompting the search for alternative treatments.¹⁶⁴ A preliminary clinical trial was carried out in 13 patients using oral 5-ALA (20 mg kg^{-1}); 45 min later, a zone of gastric antrum was illuminated through an endoscope with a blue laser (410 nm, 50 J cm^{-2}).¹⁶⁵ Greater eradication of *H. pylori* in biopsies from illuminated areas compared to control zones was demonstrated.

Acne is caused by the growth of *P. acnes* in the sebaceous glands. PDT with topical ALA was tested in 22 subjects with acne vulgaris on the back, each of whom was treated in four sites with ALA plus red light, ALA alone, light alone and neither ALA nor light.¹⁶⁶ Twenty percent topical ALA was applied with 3 h occlusion and 150 J cm^{-2} broad-band light (550–700 nm) was used. 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. There was clinical clearance of inflammatory acne for at least 20 weeks after multiple treatments and 10 weeks after a single treatment. Transient hyperpigmentation, superficial exfoliation and crusting were observed, all of which cleared without scarring. Similar results were also reported by Japanese workers.^{167,168}

15 Conclusion and possible future applications

While the use of PDT to treat infections is clearly in its infancy, there may be significant future applications. Multi-antibiotic resistance of pathogens, especially bacteria, is a rapidly growing and alarming phenomenon, and alternative methods of treating localized infections are being urgently sought. In addition, in many localized infections, oral or systemically delivered antibiotics are not particularly effective, either because the bacteria are infecting a tissue that is not well perfused, or because the bacteria are present in the tissue as a biofilm (a state that is known to dramatically reduce antibiotic susceptibility). In addition, the rise in the number of immunosuppressed organ transplant, cancer and AIDS patients has led to the increased occurrence of intractable infections.

We can envisage PDT being used in the future to combat otherwise hard to treat localized infections. The basic premise is that the PS should be capable of local, topical or intracavitary administration into the infected area and, after a suitable time, the appropriate dose (fluence and fluence rate) of the optimum wavelength of light should be delivered into the infected area *via* a fiber optic, diffusing tip, fiber bundle or implantable light-emitting diode, or by direct illumination of a surgically exposed area.

Surgical wound infections account for 25% of nosocomial infections and frequently display some degree of antibiotic resistance. Species involved include *S. aureus*, *Enterococcus* spp. and Gram-(−) enteric bacilli. Patients who have intestinal surgery, who are neutropenic due to cancer chemotherapy or other medication, or who have diabetes or other vascular disease are at increased risk of post-surgical wound infection. It may be possible when these infected wounds need surgical intervention to apply topical PDT, especially for drug-resistant strains.

Acute soft-tissue infections are relatively rare, but they can have devastating consequences to patients. The spread can be rapid, the mortality rate is high (up to 50%) and mutilating surgery is frequently the only means of arresting the unrelenting course of the disease. The group includes such manifestations such as 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). In these infections, repeated excisions of affected tissue are frequently necessary and topical PDT could have a role to play in rapidly reducing the bacterial burden and, hence, reducing the extent of surgical debridement.

An abscess is the common result of an infection in soft tissue and many internal organs, such as liver, lungs, brain and peritoneum. It is the result of a massive influx of neutrophils that kill bacteria but also produce enzymes that lead to local tissue destruction and accumulation of pus (a mixture of living and dead bacteria, dead neutrophils and liquified tissue). An abscess that is successful in limiting the spread of the infection becomes surrounded by a collagen membrane and the contents may be under considerable pressure. The bacteria responsible include *S. aureus*, *Klebsiella* spp., enteric bacilli and anaerobes such as *Bacteroides* spp. Surgical drainage is the normal treatment for abscesses and it could advantageously be followed by topical application of PS followed by illumination to remove residual infection from the abscess bed.

The effect of burns in destroying the cutaneous barrier, rendering the affected tissue non-perfused, and depressing immune defenses, means that they very commonly become infected. In the past, the majority of patients with serious burns died from infections. The introduction of topical antimicrobial treatments and early excision and skin grafting has reduced the death rate significantly. The ubiquitous pathogen *P. aeruginosa*, together with *S. aureus*, *P. mirabilis*, *Candida* spp. and filamentous fungi are frequently responsible. Burn infections frequently lead to failure of skin grafts. Topical antimicrobials employed include mafenide acetate, mupirocin, silver nitrate and silver sulfadiazine, and topical PDT may also have a role to play.

Chronic and acute sinusitis can present a significant clinical problem as they involve an infected cavity that is sometimes poorly responsive to systemic antibiotics and surgery. The bacteriology can vary among cases, but coagulase-negative Staphylococci, *S. pneumoniae*, *H. influenzae*, *Moraxella catarrhalis* and *S. aureus* have been shown to be the commonest organisms implicated in chronic sinusitis, and antibiotic resistance is a major problem. Because the sinus is a bony cavity coated by a mucosal layer and is accessible by a catheter or fiber optic for PS and light delivery, it is a possible candidate for antimicrobial PDT. Ear infections such as bacterial otitis media (*H. influenzae*, *Pneumococcus* spp., *S. pneumoniae* and *Chlamydia pneumoniae*) could also be susceptible.

Periodontal disease is caused by a set of pathogenic bacterial species which, along with a wide range of host-compatible species, form complexes in subgingival biofilms (plaques) and are responsible for clinical inflammation and periodontal destruction. A PS may be injected into the periodontal pocket, followed by illumination with fiber optics inserted into the infected area. This technique keeps the bactericidal effect confined to the disease lesion so that beneficial microflora at other sites in the mouth would remain intact. In a similar manner, tooth surface infections by oral or mutans Streptococci (*Fusobacterium nucleatum*, *Actinomyces viscosus*) responsible for dental caries could also be susceptible.

Urinary tract infections (UTI) comprise one of the commonest classes of localized bacterial infections. The majority are caused by *E. coli* strains (uropathogenic), but *Proteus*, *Staphylococcus*, and *Klebsiella* spp. can also be involved. The bacteria initially adhere to the urothelium of the bladder or urethra, causing cystitis, but can then ascend the urinary tract, leading to pyelonephritis. For anatomical reasons, women are much more likely to contract these infections than men. Although UTIs are usually effectively treated using oral antibiotics that build up to effective concentrations in the urine, recurrence is common and bacterial resistance is becoming increasingly problematic. PDT has been clinically used to treat superficial bladder cancer, wherein the PS is injected systemically and light is delivered intravesically. We hypothesize that intravesical delivery of both PS and light should avoid bladder damage and show utility as a therapy for bacterial cystitis.

As mentioned previously, *H. pylori* infection has been shown to be strongly associated with the presence of inflammation in the gastric mucosa (chronic superficial gastritis), and especially with polymorphonuclear cell infiltration (chronic active gastritis). Once acquired, *H. pylori* persists, usually for life, unless eradicated by antimicrobial therapy.¹⁶⁹ *H. pylori* is a major cause of peptic ulcer disease, a human carcinogen and is implicated in the development of gastric cancer (the most important gastrointestinal malignancy in the world). *H. pylori* organisms are spiral, microaerophilic, Gram-(−) bacteria that colonize the gastric mucosa and secrete urease and other virulence factors that increase their pathogenicity. Combination antibiotic therapy leads to eradication rates of about 80%, but at the expense of side effects and possible poor patient compliance. Increasing antibiotic resistance and the existence of non-responsive patients suggest that alternative strategies for *H. pylori* eradication need to be sought. The clinical trial using ALA referred to previously¹⁶⁵ and the

discovery in our laboratory that *H. pylori* is killed by blue light due to accumulation of endogenous porphyrins⁸¹ suggest that light-based therapies may play a role in combating this infection. The relative ease of PS and/or light delivery into the stomach may mean that this is one of the first antibacterial applications of PDT to be used clinically.

Corneal infections such as bacterial keratitis (*P. aeruginosa*, *Capnocytophaga canimorsus*, *Serratia marcescens*, *Chlamydia trachomatis* or *S. aureus*) and keratomycosis (fungal infection) could be treated with topical PDT. Dermatophytoses or fungal infections of the skin and nails are a common problem affecting millions of people worldwide, but especially in countries with hot and humid climates. These infections may take the form of ringworm (tinea corporis), athlete's foot (tinea pedis), onychomycosis (toenail fungus), tinea capitis (scalp), tinea cruris (groin) and tinea barbae (beard). The causative organisms are frequently *Trichophyton* spp., *Epidermophyton floccosum* or *Microsporum canis*. Dermatophytoses are most commonly treated with topical antifungal preparations, although therapeutic success is limited because of the lengthy duration of treatment required, poor patient compliance and high relapse rates at specific body sites. Although at present there are only sporadic reports of dermatophytes developing resistance to antifungals, by analogy with antibiotic resistance in bacteria, it is likely only a matter of time before widespread resistance emerges for dermatophytes. The superficial nature of fungal infections encourages the testing of topical PDT as a therapy.

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Fig. 1.
Chemical structures of PS used for PDT in clinical trials.

Fig. 2.
Chemical structures of non-tetrapyrrole PS used for PDI of microbial species.

Fig. 3. Diagrams illustrating differences in membrane structure between Gram-(+) and Gram-(-) bacteria.

Fig. 4.
Heme biosynthetic pathways of bacteria.

Fig. 5.
PDT of excisional wounds on the backs of mice infected with bioluminescent *E. coli* and treated by topical application of pL-ce6 conjugate, followed by irradiation with red light.

Table 1

Reports of photoinactivation of Gram-(+) and Gram-(−) bacteria *in vitro*

Species (Gram status)	Photosensitizer	Author	Year
<i>Escherichia coli</i> (−)	<i>N</i> -Alkylpyridylporphyrins	Benov <i>et al.</i> ²⁷	2002
<i>Acinetobacter baumannii</i> (−), <i>E. coli</i>	Cationic hydrophilic porphyrin	Nitzan and Ashkenazi ²⁸	2001
<i>S. aureus</i> (+), <i>Staphylococcus epidermidis</i> (+), <i>Streptococcus pyogenes</i> (+),	Methylene Blue	Zeina <i>et al.</i> ²⁹	2001
<i>Corynebacterium minutissimum</i> (−), <i>Propionibacterium acnes</i> (−)			
<i>S. aureus</i> , <i>S. epidermidis</i> , <i>Pseudomonas aeruginosa</i> (−), <i>E. coli</i> , <i>Proteus mirabilis</i> (−)	“Photosens”	Tolstykh <i>et al.</i> ³⁰	2001
<i>S. aureus</i> , <i>Streptococcus pneumoniae</i> (+), <i>Enterococcus faecalis</i> (+), <i>Haemophilus influenzae</i> (−), <i>E. coli</i> , <i>P. aeruginosa</i>	Methylene Blue, Toluidine Blue	Usacheva <i>et al.</i> ³¹	2001
<i>E. coli</i> , <i>Enterococcus hirae</i> (+),	Zinc phthalocyanine tetrasulfonate, tetraphenylporphyrin derivatives, ALA	Gabor <i>et al.</i> ³²	2001
<i>Deinococcus radiodurans</i> (+)	Rose Bengal	Schafer <i>et al.</i> ¹²	2000
<i>S. aureus</i>	Hematoporphyrin	Bertoloni <i>et al.</i> ³³	2000
<i>E. coli</i>	ALA	Szocs <i>et al.</i> ³⁴	1999
<i>S. aureus</i>	Photofrin, <i>m</i> -THPC, hypericin	Kubin <i>et al.</i> ¹⁵	1999
<i>S. aureus</i> (methicillin resistant)	Methylene Blue and derivatives	Wainwright <i>et al.</i> ²²	1998
<i>S. aureus</i>	Malachite Green isothiocyanate	Golding <i>et al.</i> ³⁵	1998
<i>Haemophilus parainfluenzae</i> (−)	ALA	van der Meulen <i>et al.</i> ³⁶	1997
<i>E. coli</i> , <i>P. aeruginosa</i> , <i>Enterococcus seriolicida</i> (+)	Zinc pyridiniumphthalocyanine	Minnock <i>et al.</i> ³⁷	1996
<i>E. coli</i> , <i>S. aureus</i>	Cationic, neutral and anionic tetraphenylporphyrins	Nitzan <i>et al.</i> ³⁸	1995
<i>Bacillus subtilis</i> (+), <i>Streptococcus faecalis</i> (+)	Hematoporphyrin derivative	Shawer and Cooper ³⁹	1990
<i>Helicobacter pylori</i> (−)	Hematoporphyrin derivative	Bedwell <i>et al.</i> ⁴⁰	1990
<i>Salmonella typhimurium</i> (−)	Rose Bengal	Dahl <i>et al.</i> ⁴¹	1989
<i>E. coli</i>	Thiazines, xanthenes, acridines and phenazines	Martin and Logsdon ⁴²	1987
<i>Brochothrix thermosphacta</i> , <i>D. radiodurans</i> , <i>Streptococcus</i> , <i>Micrococcus</i> , <i>Staphylococcus</i> , <i>Bacillus</i> , <i>Arthrobacter</i> <i>kurthia</i> , <i>Pseudomonas</i> spp., <i>Enterobacteriaceae</i>	Rose Bengal	Banks <i>et al.</i> ⁴³	1985

Table 2Reports of photoinactivation of viruses *in vitro*

Species ^a	Photosensitizer	Author	Year
SFV, VSV	Buckminsterfullerene (C ₆₀)	Kasermann and Kempf ¹¹⁴	1997
VSV	AlPcS4, Pc4	Moor <i>et al.</i> ¹¹⁵	1997
Vaccinia virus	Sulfonated phthalocyanines	Allen <i>et al.</i> ¹¹¹	1995
HIV-1	Methylene Blue	Bachman <i>et al.</i> ¹¹⁶	1995
HIV-1	Hypocrellin	Hudson ¹¹⁷	1994
HIV-1, VSV, influenza virus	Hypericin, Rose Bengal	Lenard <i>et al.</i> ¹¹⁸	1993
SFV, SV40, calicivirus	Methylene Blue	Mohr <i>et al.</i> ¹¹⁹	1993
HSV-1, cytomegalovirus, HTLV, HIV-1, Sindbis virus, FEV	Merocyanine 540	Sieber <i>et al.</i> ¹²⁰	1992
EIAV	Hypericin	Carpenter and Kraus ¹²¹	1991
HSV	Sapphyrins	Judy <i>et al.</i> ¹²²	1991
VSV, FLV	Benzoporphyrin derivative	Neyndorff <i>et al.</i> ¹²³	1990
SIV	Dihematoporphyrin ether	Chanh <i>et al.</i> ¹²⁴	1989
HSV-1	Hematoporphyrin derivative	Matthews <i>et al.</i> ¹²⁵	1988

^a SFV = Semliki forest virus; VSV = vesicular stomatitis virus; HIV-1 = human immunodeficiency virus-1; HSV-1 = herpes simplex virus-1; HTLV = human T cell leukemia/lymphoma virus; FEV = Friend erythroleukemia virus; EIAV = equine infectious anemia virus; FLV = feline leukemia virus; SIV = simian immunodeficiency virus.

Table 3Reports of photoinactivation of fungi and yeasts *in vitro*

Species	Photosensitizer	Author	Year
<i>Aspergillus fumigatus</i>	Green 2W	Friedberg <i>et al.</i> ¹²⁶	2001
<i>Saccharomyces cerevisiae</i>	Glucosyl porphyrins	Carre <i>et al.</i> ¹²⁷	1999
<i>Kluyveromyces fragilis</i>	Rose Bengal, Methylene Blue, Toluidine Blue	Lazarova and Tashiro ¹²⁸	1995
<i>Kluyveromyces marxianus</i>	Toluidine Blue, CIAIPc	Paardekooper <i>et al.</i> ¹²⁹	1995
<i>S. cerevisiae</i>	Hematoporphyrin	Sharma and Jain ¹³⁰	1994
<i>Candida albicans</i>	Rose Bengal	Lazarova ¹³¹	1993
<i>C. albicans</i>	Zinc phthalocyanines	Bertoloni <i>et al.</i> ¹³²	1992
<i>S. cerevisiae</i>	Eosin Y	Cohn and Tseng ¹³³	1977