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All you need is light

Antimicrobial photoinactivation as an evolving and emerging discovery strategy against infectious disease

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The story of prevention and control of infectious diseases remains open and a series of highly virulent pathogens are emerging both in and beyond the hospital setting. Antibiotics were an absolute success story for a previous era. The academic and industrial biomedical communities have now come together to formulate consensus beliefs regarding the pursuit of novel and effective alternative anti-infective countermeasures. Photodynamic therapy was established and remains a successful modality for malignancies but photodynamic inactivation has been transformed recently to an antimicrobial discovery and development platform. The concept of photodynamic inactivation is quite straightforward and requires microbial exposure to light energy, typically wavelengths in the visible region, that causes the excitation of photosensitizer molecules (either exogenous or endogenous), which results in the production of singlet oxygen and other reactive oxygen species that react with intracellular components, and consequently produce cell inactivation. It is an area of increasing interest, as research is advancing (1) to identify the photochemical and photophysical mechanisms involved in inactivation, (2) to develop potent and clinically compatible photosensitizer, (3) to understand how photoinactivation is affected by key microbial phenotypic elements (multidrug resistance and efflux, virulence and pathogenesis determinants, biofilms), (4) to explore novel delivery platforms inspired by current trends in pharmacology and nanotechnology and (5) to identify photoinactivation applications beyond the clinical setting such as environmental disinfectants.

The Reality of Infection and Antimicrobial Resistance

One of the scientific highlights of the 20th century was, without doubt, the development of successful prevention and control efforts for infectious diseases worldwide. Since the development of penicillin and subsequent development and synthesis of other antibiotics, vaccines and antiseptics, victory against pathogens has been repeatedly declared.[1] By the 1980s, pharmaceutical companies were convinced that there were already enough antibiotics. It was time to "close the book on infectious diseases" and the emphasis was shifted to more threatening clinical problems such as cancer, diabetes and heart disease.

Microorganisms, however, had a different opinion. The extensive and inappropriate use of antibiotics gradually led to the development of pervasive antimicrobial resistance. Penicillin was first put into widespread use in the early 1940s and by 1944 half of all clinical *Staphylococci* spp isolates were resistant to this proclaimed "miracle drug."[2] Today infectious disease is the second most important killer in the world, number three in developed nations and fourth in the USA.[3] It is the third leading cause of death in Europe, mostly in elderly and debilitated populations, and despite existing antibiotic therapies and vaccines, infectious diseases remain the leading cause of mortality and morbidity.[4] Worldwide, 17 million people die each year from bacterial infections.[5]

Five classes of antibiotic-resistant pathogens are emerging as major threats to public health: methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus faecalis* (VRE), multidrug-resistant mycobacteria, Gram-negative pathogens and fungi.[6] In addition to these established threats we are confronting even more challenging clinical scenarios including carbapenem-resistant *Klebsiella pneumonia,* New Delhi metallo-β-lactamase containing enterobactericae, as well as the German *Escherichia coli* outbreak caused by a previously unknown strain, all of which are responsible for significant morbidity and mortality.[7-9]

Photodynamic Therapy (PDT) as an Antimicrobial Approach

As the efficacy of antibiotics decreases and the end of the "antibiotic era" gets closer, major international research efforts to

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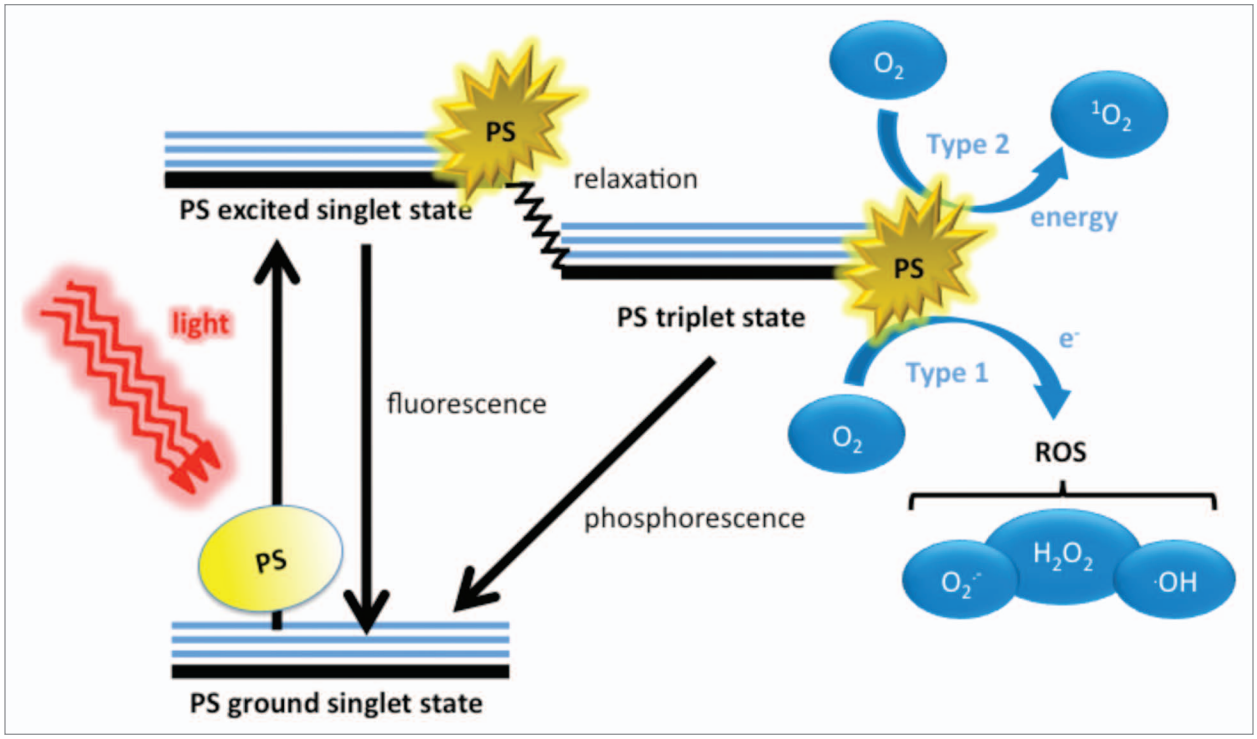

Figure 1. Schematic illustration of photodynamic therapy including the Jablonski diagram. The PS initially absorbs a photon that excites it to the first excited singlet state and this can relax to the more long-lived triplet state. This triplet PS can interact with molecular oxygen in two pathways, type I and type II, leading to the formation of reactive oxygen species (ROS) and singlet oxygen respectively.

discover new ways to eradicate bacteria are evolving. The emphasis is now on how to comprehend, prevent and if possible eliminate multidrug resistance in concert with exploring new ways to kill microbial pathogens. In addition to target-based conventional discovery there is an array of promising novel approaches currently under investigation. A prominent member of this list is the light-based platform of photodynamic therapy (PDT). PDT was accidentally discovered over 100 years ago by Oskar Raab and Hermann von Tappiener when they noticed that *Paramecium* spp protozoans stained with acridine orange died upon exposure to bright light.[10] In the 1970s, PDT began to be explored for the selective destruction of cancer.[11] Since then, PDT has emerged as a tool for the treatment of various malignancies and is the principle tool for the treatment of age-related macular degeneration.[12] Recently, it has been transformed to a discovery and treatment alternative for localized infections.[13]

PDT involves the use of harmless visible light combined with a light-sensitive dye—the photosensitizer (PS)—and oxygen present in and around cells. After illumination with the light of the appropriate visible wavelength, the PS is energized to an excited state that can undergo molecular collisions with oxygen, resulting in the formation of reactive oxygen species (ROS) and singlet oxygen. PDT is a highly selective modality as (1) hyperproliferating cells selectively uptake PS[14] and (2) cell death is spatially limited to regions where light of the appropriate wavelength is applied. As microbial cells possess very fast growth rates, much like that of malignant cells, it was suggested that PDT could be used for microbial cell destruction—this became a reality in the mid-1990s.[15]

Since then, antimicrobial photodynamic-inactivation (PDI) and therapy has been developed as a prolific discovery and development platform, exploring many aspects of the microbial phenotype related to multidrug resistance such as efflux systems, biofilms, bacterial spores and virulence determinants. This trend, in concert with rationalized synthesis and delivery efforts for new PS, has populated the literature with a variety of preclinical and clinical antimicrobial PDT applications.

The Photophysical Processes of PDT

The three principle elements of PDT are the PS, visible light and oxygen. These elements, when combined, yield potent oxidizing species (**Fig. 1**). In the ground state, a PS is said to be in the singlet state, whereby all of its electrons are spin paired in low energy orbitals. Upon application of light corresponding to the absorption peak of the PS, the electron in the highest occupied molecular orbital (HOMO) of the PS is excited to the lowest unoccupied molecular orbital (LUMO), causing the PS to reach the unstable and short-lived excited singlet state. In this state, several processes may rapidly occur.[16] The most critical of these to PDT is the reversal of the excited electron's spin, known as intersystem crossing to the triplet state of the PS. This triplet state is less energetic than the excited singlet state, but has a considerably longer lifetime, as the excited electron, now with a spin parallel to its former paired electron, may not immediately fall back down (as it would then have identical quantum numbers to that of its paired electron, thus violating the Pauli Exclusion Principle). Accordingly,

the excited electron in the PS triplet state may first obtain correct spin orientation (a relatively slow process) and then fall to ground levels (phosphorescence) or the PS may interact with molecules abundant in its immediate environment. Because of the Selection Rules that specify that triplet-triplet interactions are spin-allowed while triplet-singlet interactions are spin-forbidden, the PS triplet can react readily with molecular oxygen that is a triplet in its ground state (**Fig. 1**).

The Photochemical Generation of Oxidizing Species

The discussion of PDT's oxidizing characteristics is centered on molecular oxygen (O_2). As mentioned above, the ground state of oxygen is a triplet state, whereby the two outermost orbitals are unpaired but spin parallel. When the PS is in the long-lived triplet state, it may interact with O_2 in two distinctly different ways.[17,18] The Type I process occurs when the PS directly transfers an electron, sometimes in concert with proton donation, to O_2, yielding superoxide anion ($O_2^{\bullet-}$), which can then go on to form other ROS including the hydroxyl radical ($^{\bullet}OH$), and hydrogen peroxide (H_2O_2). Alternatively, the Type II process occurs when the energy used to excited the PS to the triplet state is transferred to O_2, thus "flipping the spin" of an outermost O_2 electron and shifting it into the orbital containing the other electron, which in turn leaves one orbital entirely unoccupied (a violation of Hund's rule). Termed singlet oxygen ($^1O_2$), this form of oxygen (not considered a radical as its electrons are spin-paired) is extremely short-lived and reactive, owing to its electron configuration instability.

The formation of ROS through the Type 1 process results in the stripping of electrons from biological macromolecules, including lipids, proteins and nucleic acids.[16] $^{\bullet}OH$, arguably the most reactive of the three ROS formed, will abstract electrons to become a hydroxide ion, which then may easily form water via obtaining a proton. $O_2^{\bullet-}$ too, may abstract electrons, forming a peroxide ion that immediately abstracts protons to form H_2O_2; however, in biological systems it is not particularly reactive. Nonetheless, $O_2^{\bullet-}$ may be converted to H_2O_2 and O_2 by superoxide dismutase. H_2O_2 is only considered truly reactive when it reacts with ferrous iron in what is known as the Fenton reaction:

$$H_2O_2 + Fe^{2+} \rightarrow OH^- + {}^{\bullet}OH + Fe^{3+}$$

which results in the homolytic fission of the oxygen-oxygen bond in H_2O_2 to yield a hydroxide ion and $^{\bullet}OH$ via the oxidation of ferrous iron to ferric iron.[19] H_2O_2 is removed through catalase, forming water and oxygen gas. Although $^{\bullet}OH$ is not broken down by enzymatic reactions, it may be quenched by antioxidants, including antioxidant peptides (e.g., glutathione) or by antioxidant sugars (e.g., ascorbic acid).[20]

Because $^1O_2$ is not a radical, it reacts with biological molecules through quite different mechanisms, making the Type 2 pathway responsible for different macromolecular modifications. $^1O_2$ tends to favor reacting with double bonds and sulfur moieties (both of which have high electron densities) and may interact with aromatic components of macromolecules in Diels-Alder cycloadditions.[21,22] Unlike ROS, $^1O_2$ cannot be broken down by enzymes but can be quenched by antioxidants.

Properties of Photosensitizers

PSs are usually organic delocalized aromatic molecules consisting of a central chromophore with auxiliary branches (auxochromes) that are responsible for further electron delocalization of the PS, thus altering the absorption spectra of the PS.[23] Due to extensive electron delocalization, PSs tend to be deeply colored. This means that the energy required to excite the electrons in the HOMO to the LUMO is low compared with less delocalized molecules and therefore the absorption bands are in the longer wavelength (red) spectral region and are large, reflecting the high probability of excitation. Acridine orange was the first photodynamic agent used.[24] Most of the PSs that have been employed for the treatment of cancer and other tissue diseases are based on the tetrapyrrole nucleus, with emphasis in the use of porphyrins. Chlorins, bacteriochlorins phthalocyanines as well as a plethora of dyes with different molecular frameworks have been frequently proposed as antimicrobial PSs (**Fig. 2**).[25,26] These include halogenated xanthenes, such as Rose Bengal (RB),[27] perylenequinones, such as hypericin[28] phenothiazinium salts, such as toluidine blue O (TBO) and methylene blue (MB),[29] cationic buckminsterfullerenes (e.g., C_{60}),[30,31] psoralens (furanocoumarins).[32]

In contrast, fairly recently a genetically encoded PS was developed from the hydrozoan chromoprotein anm2CP, a homolog of green fluorescent protein (GFP). The PS was named KillerRed: It generates ROS upon irradiation with green light and has been proven potent against *E. coli* and malignant cells in vitro.[33] As microorganisms produce and accumulate porphyrins the appealing hypothesis of endogenous photosensitization is also an alternative pathway of photoirradication.[34,35]

PDI for Microbial Pathogens: The Permeability Barrier

The permeability barrier in Gram-negative bacteria is accountable for the observed susceptibility differences between Gram-positive and Gram-negative species when treated with neutral or anionic PS.[36] These observations then prompted the use of molecules such as polymyxin nonapeptide to enhance permeabilization of Gram-negative bacterial outer membranes in combination with PDT.[13] Moreover, investigators took cell envelope chemical properties of Gram-negative and Gram-positive bacteria into account (namely that both possess inherently anionic structures) and developed cationic PS effective against both bacterial groups.[37] This critical discovery guided the efforts in exploring PDT as a potential modality for the eradication of resistant pathogens.

Yeasts and fungal pathogens are variable in their cell envelopes, possessing outer wall mixtures of glucans, mannan and chitin polymers. This feature makes them inherently more permeable to external substances than Gram-negative bacteria. The hypothesis that cationic PS would more efficient in PDI has been tested in *Candida albicans*, arguably the most common fungal pathogen.

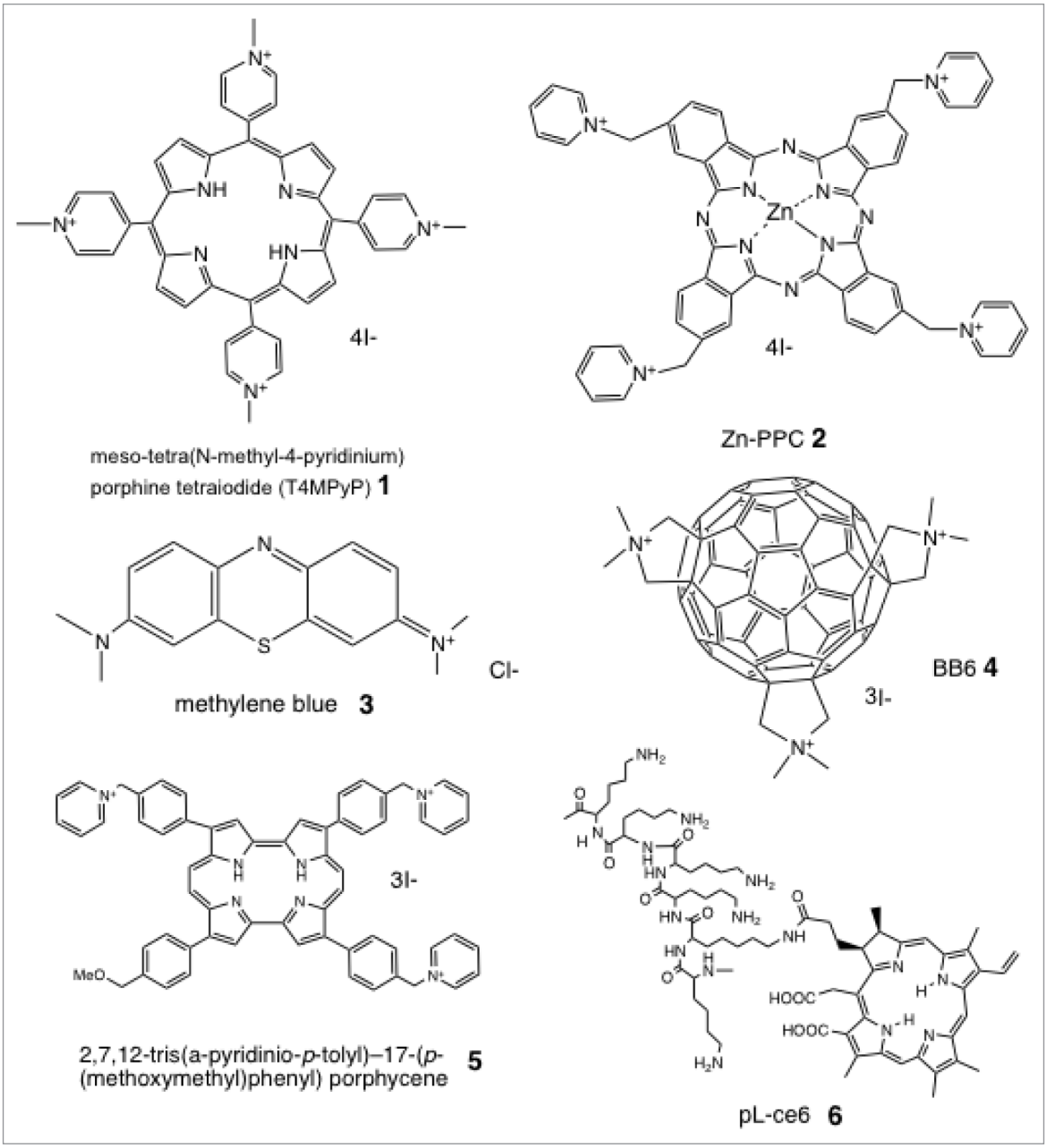

Figure 2. Chemical structures of some representative antimicrobial PS. (1) Cationic porphyrin, meso-tetra(N-methyl-4-pyridinium) porphine tetraiodide (T4MPyP); (2) cationic phthalocyanine, ZnPPC; (3) phenothiazinium salt, methylene blue; (4) cationic functionalized fullerene, BB6; (5) cationic porphycene, 2,7,12-tris(a-pyridinio-p-tolyl)-17-(p-(methoxymethyl)phenyl) porphycene; (6) poly-l-lysine chlorin(e6) conjugate, pL-ce6.

C. albicans has been inactivated by the PSs Photofrin (Porfimer sodium), RB and Al(III)-tetrasulphonated phthalocyanine.[38-40]

Overcoming the permeability barrier and subsequently enhancing the PDI efficacy has been addressed recently both in bacteria using the polycationic biopolymer chitosan[41] and fungi employing saponins.[42]

Antibiotic-Resistant Pathogens

Methicillin-resistant *Staphylococcus aureus* (MRSA) is estimated to cause ~19,000 deaths per year in the United States.[43] Apart from their high mortality rate, MRSA infections lead to an estimated $3–4 billion of additional health care costs per year. Furthermore, the rising prevalence of MRSA increases the likelihood that vancomycin-resistant *S. aureus* (VRSA)[44]—just as deadly as MRSA but more challenging to treat—will become a new scourge in hospitals. Vancomycin-resistant *Enterococcus faecalis* (VRE) has been also a common threat in hospital settings for at least 15 years.[45] An important feature of antimicrobial PDT is that antimicrobial-resistant isolates are just as susceptible to PDT as their naïve counterparts are, as best demonstrated by a reduction in survival fractions between MRSA and wild-type

S. aureus.[46] The use of the phenothiazinium dye TBO with a 632.8 nm He laser can completely eradicate MRSA.[46] A number of PS, including cationic substituted Zn(II)-phthalocyanines,[47] poly-*S*-lysine-porphyrin conjugates,[48] meso-tetrahydroporphyrin, tetrahydroporphyrin-tetratosylat (THPTS),[49,50] and cationic water-soluble gallium(III) phthalocyanines (GaPcs),[51] can substantially reduce MRSA populations (4–5 $\log_{10}$). Moreover, it has been shown that a PS conjugate of polyethylenimine and chlorin(e6) (pEI-ce6) in concert with red light is capable of reducing MRSA colony viable counts by 2.7 $\log_{10}$ in a murine skin abrasion model.[52] MB was active against VRE[53] as well as vancomycin-porphyrin conjugates are able to eliminate in vitro vancomycin-sensitive and vancomycin-resistant Enterococci.[54]

Multidrug-resistant (MDR) and pandrug-resistant (PDR) Gram-negative bacteria are less prevalent than MRSA but pose an equally grave threat of truly untreatable infections.[55,56] In a model study, it was demonstrated that 60 MDR *Pseudomonas aeruginosa* isolates could be killed (up to 6–7 $\log_{10}$ reduction in viable cell counts) using TBO-PDT.[57] This study also demonstrates that antibiotic-resistant *P. aeruginosa* was just as susceptible to PDI as antibiotic-susceptible strains. Another report demonstrated that PDI with cationic phthalocyanines has a substantial phototoxic effect in MDR strains of *Aeromonas hydrophila*.[58] Using the PS Tri-P(4), other investigators have obtained PDI reduction of *Yersinia enterolitica* viable counts by 5 $\log_{10}$.[59]

MDR and extensively drug-resistant (XDR) strains of *Mycobacterium tuberculosis* (MDR-TB and XDR-TB), are a rising threat in the developing world.[60] MDR-TB treatment requires a 2 y course of antibiotics with serious side effects; XDR-TB is even more difficult to cure and often fatal.[61] PDT for TB studies have been focused on the homologous system *M. bovis* Bacille de Calmette et Guerin (BCG) both in vitro employing phenothiaziniums and in murine models of localized mycobacterial induced granulomatous infection.[62,63] In a similar fashion the effects of PDT have been assessed in infections caused by *M. marinum*[64] and on rapidly growing nontuberculous mycobacteria keratitis.[65]

Candida spp are the third leading cause of catheter-related infections, and are associated with the highest mortality of all catheter-related infections.[66] Although prevention of invasive candidiasis using azole prophylaxis can be effective in selected high-risk patient populations, selection for invasive infection by resistant non-*albicans Candida* species or molds is a potentially devastating consequence. Despite improvements in antifungal therapy, the high attributable mortality rate due to Candida infections has improved little from two decades ago. Even with appropriate therapy, attributable mortality remains 15–49%.[67] Moreover, an episode of candidemia significantly increases length of hospital stay and cost of care. In one analysis, the estimated cost of an episode of care for candidemia was $34,123 per Medicare patient and $44,536 per private insurance patient (1997 US$), with an overall economic impact of $2 billion annually in the US.[68]

It has also been demonstrated that *C. albicans* biofilms are sensitive to Photofrin PDT and that *C. albicans* germ tubes, which are able to survive H_2O_2 stress, have been eliminated by PDT.[69] A recent study employing Photogem® as a PS with a light emitting diode (LED) had a significant effect in PDI against fluconazole-resistant *C. albicans* and *C. glabrata*.[70]

PDI for Fungal Pathogens

Cryptococcus neoformans is an encapsulated yeast that may cause cryptococcosis, a potentially fatal disease affecting immunocompromised patients, which occurs with the inhalation of the infectious inoculum. In an attempt to explore the role of the cell wall integrity pathway in PDI, Fuchs et al. has shown that employing pEI-ce6 in combination with 665 nm red light leads to reduction in *C. neoformans* KN99α (wild-type serotype with intact cell wall) viability by 2 $\log_{10}$ whereas the PDI-effect in the isogenic mutant *rom2* (cell wall defective) was substantially higher (4 $\log_{10}$). PDI employing pEIce6 in concert with the cell wall specific antifungal capsofungin, potentiates cell killing.[71]

PDT also has promising potential in the treatment of superficial fungal skin infections caused by dermatophytes. *Trichophyton rubrum* is responsible for *Tinea pedis* (athelete's foot), fungal folliculitis, onychomycosis and dermatophytosis (ringworm). Employing an ex vivo infection model of human stratum corneum of *T. rubrum*, Smijs et al. incubated samples with the PS 5,10,15-tris(4-methylpyridinium)-20-phenyl-(21H,23H)-porphine trichloride (Sylsens B) and deuteroporphyrin monomethylester.[72] Upon light application, both PS were shown to be active antifungals. Moreover, 5-aminolevulinic acid (5-ALA) and red light has an effect in the treatment of onychomycosis.[73]

The list of PDT-inactivated fungi includes *Trychophyton mentagrophytes*, *Trychophyton tonsurans*, *Microsporum cookei*, *Microscporum gypseum*, *Microsporum canis*, *Epidermophyton floccosum*, *Nannizia cajetani*, *Metarhizium anisopliae*, *Aspergillus nidulans*, *A. fumigatus* and *Fusarium* sp, employing a variety of PS, offering a new avenue for antifungal therapies.[74,75]

PDI and Multidrug Efflux

The role of multidrug efflux in antimicrobial PDT is a fairly recent trend under investigation. Efflux mechanisms are broadly recognized as major components of resistance to many classes of antimicrobials.[76] Efflux occurs due to the activity of membrane transporter proteins widely known as multidrug efflux systems (MES).[77,78] MES are implicated in a variety of physiological roles other than efflux and identifying natural substrates and inhibitors is an active and expanding research topic.[79] There is an apparent structural similarity between designated efflux substrates and a number of PS especially in their amphilicity. Therefore, it is important to understand how the interaction of PS with efflux systems will affect their PDI potential. This has been explored for phenothiazinium PS and both bacterial and fungal MES.[80,81] This interaction seems to be less obvious for different PS chemotypes. Porphyrin uptake and efflux seems to be regulated by the TolC system in *E. coli*.[82] In *Streptococcus agalactiae* two coregulated efflux transporters modulate intracellular heme and protoporphyrin IX availability.[83] The participation of MES in porphyrin-mediated PDI has been implied in ATP

Binding Cassette (ABC) mammalian systems.[84] In contrast, the PDI pattern of amphipilic protoporphyrin diarginate PPArg(2) in a variety of efflux-related *S. aureus* strains showed no correlation for the PS with MES.[85]

The use of small molecules that block MES, known as multidrug efflux pump inhibitors (EPIs), in combination with conventional antibiotics has been proposed as a plausible antimicrobial alternative. An array of biochemical approaches have yielded a number of promising EPIs in a series of pathogenic systems.[86] This synergistic discovery platform has been exploited in PDT for the potentiation of the phototoxic action of PS that are designated substrates of efflux systems.[87] It has been shown that near-infrared light can cause selective photodamage of multidrug-resistant pathogens.[88] In a recent study, it has been demonstrated that photodamage of multidrug-resistant Gram-positive and Gram-negative bacteria by near-infrared (870 nm/930 nm) light potentiates erythromycin, tetracycline and ciprofloxacin.[89] Although the antibiotics used in this study are MES substrates and therefore it is reasonable to assume that near infrared light may play role in efflux inhibition the experimental evidence is rather weak and this possibility requires further exploration. The mechanism is hypothetical at this stage and not clearly distinct from PDI as it potentially involves an optically mediated mechano-transduction of cellular redox pathways, decreasing DeltaPsi and increasing ROS.

Biofilm Eradication

Microorganisms in nature thrive through adherence to both living and inanimate surfaces, doing so via forming biofilms.[90] Biofilms have been found to be involved in approximately 80% of all infections. The dense and protected environment of the film as well as the significantly different properties from free-floating bacteria of the same species have been implicated to as much as 1,000-fold resistance to detergents, antiseptics and antibiotics.[91] There is an expanding body of literature regarding PDT-based biofilm eradication strategies, with emphasis in the use of different PS for biofilm related phenotypes and microbial species.[92] By using isogenic pairs of wild-type and transposon mutants deficient in capsular polysaccharide and slime production in *S. epidermidis* and *S. aureus,* it has been established that the cationic PSs pL-ce6 and MB can overcome the protective effect of extracellular slime and stationary bacterial growth to PDI susceptibility.[93] TBO has a substantial impact on PDI of staphylococcal biofilms decreasing cell numbers (5 $\log_{10}$ after irradiation with red light) disrupting biofilm architecture and suggests damage to bacterial cell membranes.[94] PDI with merocyanine 540 has a comparable effect in viability of biofilms from both Gram-positive pathogens when 400 Jcm^{-2} green light is used.[95,96] PDI with the cationic porphyrin, tetra-substituted N-methyl-pyridyl-porphine (TMP) was effective in both biofilm models when combined with antibiotics or host defense mechanisms.[97,98]

Tri-meso (N-methyl-pyridyl), meso (N-tetradecyl-pyridyl) porphine (C14) has significantly better PDI effect in the eradication of *S. epidermidis* biofilms when compared with the parental tetra-substituted N-methyl-pyridyl-porphine (C1).[99] TBO-mediated PDI has an impact on *Streptococcus mutans* biofilms in different maturity stages (4 $\log_{10}$ with red light for mature biofilms),[100] as well as mature *S. sobrinus* and *S. sanguinis* biofilms.[101] Erythrosine was found to inactivate *S. mutans* biofilms better than MB and protoporphyrin and this effect was enhanced at 2 $\log_{10}$ by light fractionation.[102,103] Erythrosine is also more potent than MB against *Aggregatibacter actinomycetemcomitans* biofilms, a system where the anionic PS RB and TBO have a considerable impact.[104,105] PDI mediated by both 5-ALA and TMP at different concentrations can eliminate *P. aeruginosa* biofilms.[106,107]

PDI eradication of microbial biofilms would address a variety of challenging clinical conditions. This list includes urinary tract infections, catheter infections, middle-ear infections, sinusitis, formation of dental plaque[108] periodontitis,[109] gingivitis, endodontics,[110] osteomyelitis[111] infected contact lenses, endocarditis, infections in cystic fibrosis[112] and infections of permanent indwelling devices such as joint prostheses and heart valves and implants.[113] In the head and neck area, biofilms are a major etiologic factor in periodontitis, wound infections, oral candidiasis,[114] and sinus and ear infections. Peri-implantitis involves the bacterial colonization, typically in the form of biofilms, of implant surfaces and may lead to patient infection and damage to the implant surface. Dörtbudak et al. used TBO PDT to successfully decontaminate implants with bacterial colonization of 15 patients, leading to the reduction in bacterial counts by approximately 2 $\log_{10}$.[115]

One of the major security and bioterrorism threats of the 21st century is that of *Bacillus anthracis*, which cannot be inactivated by heat, antibiotics or other antimicrobial agents. Demidova and Hamblin demonstrated that *Bacillus* spp spores, some of which are more robust than that of *B. anthracis*, may be destroyed by the phenothiazinium dyes dimethylmethylene blue, MB, new methylene blue, and TBO with the application of red light.[116] Oliveira et al. demonstrated that *Bacillus cereus* endospores could be inactivated by porphyrin PS and light.[117] This suggests that PDT may be actively applied in military and national security applications for the decontamination of anthrax spores.

PS Delivery and Nanomedicine

The unique physical and chemical properties of nanoparticles, particularly their small size and high surface-to-volume ratio, allow this technology to surpass barriers and gain access to biologic molecules and systems. Since modern science permits the manipulation of nanosized materials, the size, shape and chemical characteristics may be altered in order to facilitate molecular interactions. As such, they can be engineered as vehicles to carry various therapeutic or diagnostic agents and are potentially useful for medical applications including targeted drug delivery, gene therapy and cell labeling.[118]

PDT has also attracted the interest of nanotechnology as the effectiveness of the treatment can be greatly enhanced by the use of nanoparticles. In the last decade, different approaches to the combination of nanoparticles and PDT have been investigated in relation to the antimicrobial applications of the technique. One

use of the nanoparticles is to improve the delivery of PS to the bacteria; others use the nanoparticles to improve the inactivation kinetics.[119] Many of the PSs being studied for PDI of bacteria are based on the tetrapyrrole nucleus, such as porphyrins, chlorins and phthalocyanines, are lipophilic and easily form aggregates in aqueous solution, resulting in the loss of photosensitizing activity.[120,121] To overcome this problem, suitable PS carriers were designed to deliver PSs, e.g., liposomes,[120,122,123] micelles[124] and nanoparticles.[125,126] Among these systems, liposomes are most commonly employed to incorporate lipophilic PSs and have been proved to enhance the antimicrobial PDI of various PSs, not only because liposomes increase the solubility and stability of PSs, but also because they can facilitate the penetration of PSs into bacteria by means of fusion processes or disturbing the cell walls.[127] However, these reported liposomal formulations mainly aimed to deliver PSs passively, while little research was done to apply liposomes actively targeted to bacteria for PDI of bacteria.

The Question of Resistance Development of Pathogenic Microorganisms to PDI

The studies and reports discussing the potential of microbes to develop resistance to PDT are scattered and quite controversial. The non-selective nature of antimicrobial PDI appears as a competitive advantage in the activation of a specific microbial resistance pathway. In a conventional biological study of routine stress followed by re-growth, 5,10,15-tris(1-methylpyridinium-4-yl)-20-(pentafluorophenyl)-porphyrin triiodide [Tri-Py(+)-Me-PF] was employed as PS and *V. fischeri* and *E. coli* were used as model cells. After ten cycles of partial inactivation followed by re growth, neither of the bacteria developed resistance to the photodynamic process.[128]

Superoxide dismutase is upregulated following protoporphyrin-mediated PDI in *S. aureus* and RB-mediated PDI in *S. mutans* induces the bacterial heat shock protein—responsible for refolding denatured proteins to native conformations and stabilizing lipid membranes during stress—GroEL expression.[129] These observations are in accordance with those of St. Denis et al. who demonstrated that sub-lethal PDI stress increased the expression of the two major bacterial heat shock proteins GroEL and Dnak and that exposing *E. coli* and *E. faecalis* to heat pretreatment prior to PDI (a positive upregulator) conferred stress tolerance, increasing *E. coli* cell viability by 2 $\log_{10}$ and *E. faecalis* cell viability by 4 $\log_{10}$. PDI with RB in the yeast *S. cerevisae* demonstrated a role of Yap1p and Skn7p in the defense against singlet oxygen.[130]

From a Conventional Platform to the Alignment with the "Microbial Phenotype"

Advances in microbial physiology shed light on a series of pathways, components and phenotypes that may serve as potential alternative and attractive targets for antimicrobial drug discovery. Recent studies have dissected social (intercellular) interaction at the molecular level through analysis of both synthetic and natural microbial populations.[131] These approaches have revealed novel molecular mechanisms that stabilize cooperation among cells and define new roles of population structure for the evolution of cooperative interactions. This knowledge of interaction parameters is changing the view of microbial processes, with emphasis on pathogenesis and antibiotic resistance, and suggests new ways to fight infection by exploiting social interaction.[131] Evidently, bacteria have the ability to enter into a dormant (non-dividing) state. The molecular mechanisms that underlie the formation of dormant persister cells are now being unraveled.[132] Accumulating evidence suggests that seemingly disparate phenomena as latent bacterial infections, un-culturable microorganisms and biofilm multidrug tolerance are defined by persisters.[132] Targeting bacterial virulence factors is also a novel approach under investigation for the development of new antimicrobials that can be used to disarm pathogens in the host.[133]

The broad-spectrum activity and the non-specific action of antimicrobial PDI should be explored deeper to address these biological phenomena. There is no documented evidence on whether PDT can disrupt these sophisticated microbial defensive lines. We have to take into account that photoinactivation is able to eradicate microorganisms without discriminating resistant isolates, both planktonic and biofilm species. This is in concert with the potential of localized photooxidative stress to inactivate virulence factors[134,135] and virulence determinants[136-138] in the absence of any documented conventional resistance mechanism. The possibility of active efflux seems to be related with some but not all the molecular classes of PSs although improved delivery methods may overcome this barrier. A single antimicrobial PDT treatment in vitro potently inactivated protease activity and resulted in a 4 $\log_{10}$ reduction in the viability of *P. gingivalis*.[139] Dose and time-of-exposure experiments revealed that protease inactivation occurred at lower concentrations of PS and less time of light exposure. Also, antimicrobial PDT treatment has been shown to be potently and functionally inactivated IL-1β and TNFα.[139]

Antimicrobial PDT: From Bench Top to Bed Side

With the results from in vitro and animal studies being promising, a number of clinical applications for antimicrobial PDT have been tested and performed in vivo. PDT has been proposed for many dental applications due to the accessibility to the oral cavity. In contrast the complexity of oral microflora makes this microenvironment quite challenging for the deployment of novel antimicrobials. It was demonstrated that phenothiazinium-mediated PDT and 660 nm light in root canals infected by the predominant endodontic pathogen *E. faecalis* leads to 99.9% reduction in viability (TBO) in an approximate 97% reduction in viable enterococci (MB).[140,141] Oral biofilms often exacerbate healing of root canals, which led Garcez et al. to demonstrate that *P. aeruginosa* and *Proteus mirabilis* biofilms could be reduced in root canals of extracted teeth. Using the pEI-ce6 PS conjugate in concert with 660 nm diode laser, PDT reduced detected cell viability by 95%.[142]

Helicobacter pylori infections are responsible for stomach ulcers, associated with severe morbidity and contribute to the development of adenocarcinoma of the stomach lining.[143]

Milson et al. demonstrated that TBO was capable of reducing *H. mustelae* viability by 90% in infected ferret stomachs.[144] A clinical trial with 13 *H. pylori*-infected patients exposed to oral 5-aminolevulinic acid and 410 nm endoscopic light resulted in the significant viability reduction.[145] An important observation is that *H. pylori* naturally accumulates porphyrins, which may then act as endogenous PS.[146] This is actually also true in *H. pylori* infected patients, whom the application of 405 nm endoscopic light alone was capable of reducing Colony Forming Unit (CFU) counts by about 90%.[147]

PDT's very nature makes it ideal for the treatment of skin, wound and burn infections, all of which are easily accessible for light therapies.[148-150] XF73, a cationic porphyrin PS, is able to reduce MRSA growth by >3 $\log_{10}$ in a porcine skin infection model.[151] PDT with polycationic PS conjugates and 665 nm light in murine excisional wounds led to a reduction in infectious organisms, permitting mouse survival to reach 90%, and reduce substantially pathogen viability.[152] TBO and light cured mice of otherwise fatal *Vibrio vulnificus* wound infections in murine models.[153] Burns often become infected due to impaired immune responses, destruction of skin vasculature and the cutaneous barrier; accordingly, burn patients typically die of resistant nosocomial infections. PDT was effective in treating *Acinetobacter baumannii* burn infections in mice, reducing viability of the pathogens at 3 $\log_{10}$.[154]

Finally, PDT may have prospective applications in the treatment of soft tissue infections. An anti-*P. aeruginosa* monoclonal antibody-conjugated tin(IV) chlorin(e6) PS and 630 nm light leads to a drop of a >75% in the number of viable *P. aeruginosa* in a subcutaneous pseudomonad infection.[155]

Several new PDT clinical applications have been developed in recent years. Ondine Biomedical has a large clinical trial in progress using MB-PDT for nasal decontamination of MRSA before surgery (www.ondinebio.com/wp-content/uploads/2011/04/OBP-NR-041511-Final.pdf). The same company is planning a second clinical trial of photodisinfection for the in situ microbial disinfection of endotracheal tubes as a means to prevent ventilator associated pneumonia (www.ondinebio.com/wp-content/uploads/2011/05/OBP-NR-051011-Final.pdf). A related company called Sinuwave is exploring the use of MB-PDT to combat chronic sinusitis (www.sinuwave.com).

Conclusion

PDT is not a conventional drug discovery platform since three elements (PS, visible light and oxygen) are essential for successful deployment. Though successful methodology to treat infectious disease with PDT will be evolved in due course of time, it is important to realize that photoinactivation of microbes is an exclusively localized process and many other infectious diseases may continue to need systemic therapy unless PDT therapy is developed which can stimulate the host immune system. It is well established in that PDT in anticancer therapy induces host immune responses that have components of innate and adaptive immune systems. In principle the same process should operate when infections are treated with PDT. The effect of PDT on the host immune system is an important implication of PDT that is an open avenue that requires investigation in the area of infection. Researchers have bypassed some of the difficulties associated with new antimicrobial development by developing facile whole-animal screens that utilize the well-studied nematode *Caenorhabditis elegans,* the great wax moth *Galleria mellonella* and the fruit fly *Drosophila melanogaster* as model hosts to identify and develop new classes of antimicrobial agents with anti-virulence or immunomodulatory efficacy and evaluate toxicity or efficacy. The amenability of these non-vertebrate hosts to large screens has made these model hosts useful to identify or develop active compounds against either bacterial or fungal pathogens.[156-158] Therefore, the design of host-pathogen studies exploring the ability of PDT to interfere with virulence determinants requires sophisticated tools and approaches. The recent example of a host-parasite model to assess intracellular targeting specificity of novel phthalocyanines[159] will inspire similar explorations.

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