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Strategies to potentiate antimicrobial photoinactivation by overcoming resistant phenotypes†

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Abstract

Conventional antimicrobial strategies have become increasingly ineffective due to the emergence of multidrug resistance among pathogenic microorganisms. The need to overcome these deficiencies has triggered the exploration of alternative treatments and unconventional approaches towards controlling microbial infections. Photodynamic therapy was originally established as an anti-cancer modality and is currently used in the treatment of age related macular degeneration. The concept of photodynamic inactivation requires cell 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 reactive oxygen species. ROS produce cell inactivation and death through modification of intracellular components. The versatile characteristics of PDT prompted its investigation as an anti-infective discovery platform. Advances in understanding of microbial physiology have shed light on a series of pathways, and phenotypes that serve as putative targets for antimicrobial drug discovery. Investigations of these phenotypic elements in concert with PDT have been reported focused on multidrug efflux systems, biofilms, virulence and pathogenesis determinants. In many instances the results are promising but only preliminary and require further investigation. This review discusses the different antimicrobial PDT strategies and highlights the need for highly informative and comprehensive discovery approaches.

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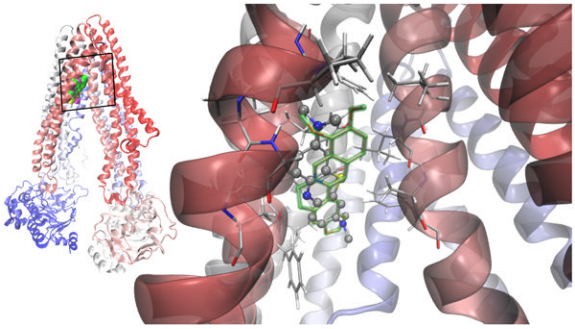

Introduction

The 20th century saw the discovery of antibiotics and led to a wide array of successful methods for preventing and controlling infectious diseases. This fostered a mindset that the war against infectious microbes had been won and research efforts were shifted to more pressing matters such as cancer, diabetes, and heart disease. In the 1980s the consensus among pharmaceutical companies was that there were enough antibiotics already on the pharmacy shelf and they began to redirect their research efforts accordingly [1]. Optimism transformed into skepticism, however as outbreaks and epidemics of new, re-emerging, and drug--resistant infections arose. The microorganisms responsible for these infections possessed effective and dynamic pathogenic capabilities and gave rise to the term “superbugs”. Infectious diseases in the twenty-first century continue to be a dangerous threat. Each year over 13 million deaths worldwide are attributed to the emergence of new infectious diseases or to the re-emergence of diseases previously thought to be under control.

Addressing this challenge requires rational as well as unconventional antimicrobial discovery efforts [1]. A prominent player in these efforts is likely to be the light-based technology known as antimicrobial photodynamic inactivation or photodynamic therapy [2], which uses harmless visible light in combination with non-toxic photosensitizers to control infections. Antimicrobial PDT was accidentally discovered over 100 years ago with the observation that *Paramecium spp.* protozoans stained with acridine orange died upon exposure to bright light[3]Historically PDT has been more prominent in the cancer setting and is currently used for the treatment of age-related macular degeneration.[4] Recent years have seen the migration of PDT research efforts and ophthalmology settings towards being used as a discovery and treatment alternative for localized infections [5].

PDT involves the use of harmless visible light combined with a light-sensitive dye – the photosensitizer – and oxygen present in and around cells. After illumination with the light of the appropriate 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), including singlet oxygen by energy transfer or hydroxyl radicals by electron transfer. The high selectivity of PDT for rapidly growing and thus hyperproliferating malignant cells [6] suggested it should be useful for microbial cell destruction [7]. Studies of antimicrobial PDT have focused on: (i) exploring the photophysical and photochemical properties of the approach (ii) exploring chemical properties to develop more effective and clinically compatible PSs (iii) bypassing the microbial permeability barrier and investing in novel delivery methodologies (iv) preclinical and clinical investigations of PDT applications.

PSs are usually organic aromatic molecules with a high degree of electron delocalization. They contain a central chromophore with auxiliary branches (auxochromes) which add

further electron delocalization to the PS and thus alter the absorption spectra [8]. Porphyrins, chlorins, bacteriochlorins, phthalocyanines as well as a plethora of dyes with different molecular frameworks have been proposed as antimicrobial PSs [9,10]. These dyes include halogenated xanthenes (e.g. Rose Bengal (RB)), [11] perylenequinones (e.g. hypericin) [12], phenothiazinium salts, (e.g. toluidine blue O (TBO) and methylene blue (MB)) [13], cationic fullerenes (e.g. derivatives of C_{60}), [14, 15] and psoralens (e.g. furanocoumarins) [16].

In just 20 years antimicrobial PDT has emerged as a discovery and development platform inspiring a proliferation of light-based antimicrobial explorations worldwide. However, the potential for microbial resistance development using PDT remains under-investigated. Studies of resistance have been sporadic but they are rapidly increasing, with recent reports examining key elements of the microbial phenotype. These include multidrug efflux systems, biofilm, spore formation, virulence and pathogenicity determinants. The emerging consensus is that the effectiveness of PDT may be profoundly impacted by all these systems, but the exact mechanisms of these effects remain elusive. This review aims to summarize and provide critical commentary around these aspects of antimicrobial PDT. It also aims to highlight the mechanistic similarities and differences between PDI and conventional antimicrobials. Collecting this diverse information may transform PDI from an alternative discovery platform to a dynamic anti-infective countermeasure.

Efflux and Antimicrobial PDT

Efflux mechanisms are major components of resistance to many classes of antimicrobials as well as chemotherapeutic agents [17]. Efflux results from the activity of membrane transporter proteins, widely known as multidrug efflux systems (MES) [18, 19]. These systems perform essential roles in cellular metabolism and they differ in membrane topology, energy coupling mechanisms, and, most importantly, in substrate specificities [20]. Identifying natural substrates and inhibitors of efflux systems is an active and expanding research topic [21].

Based on their sequence similarity, efflux systems were classified into the following six super-families: ATP-binding cassettes (ABC), major facilitators (MFS), resistance-nodulation cell division (RND), small multidrug resistance family (SMR), multi-antimicrobial extrusion protein family and multidrug endosomal transporters (MET). The first five families were found in microorganisms while the MET family appears to be restricted to higher eukaryotes. Representatives of all groups are expressed in mammalian cells [22]. ABC transporters are the largest super family, containing seven subfamilies designated A to G based on sequence and structural homology [23]. The best-studied families of fungal MES are from *Saccharomyces cerevisiae*, especially those responsible for pleiotropic drug resistance (PDR). Members of this family are highly conserved and are often responsible for drug resistance among pathogenic fungal species [24, 25].

A challenging clinical scenario involves MES in *Pseudomonas aeruginosa* [26]. Sequence analysis of the *P. aeruginosa* genome has revealed the presence of MES from all five super families, with the largest number of predicted pumps, a total of 12, belonging to the RND family [27]. X-ray crystal structures of most transporter families were reported in a variety of organisms [28–36],

Studies into the effects of efflux in antimicrobial PDT have only recently commenced. Structural similarities exist between efflux substrates and a number of PSs, most notably their amphipathic nature. The participation of MES in PS mediated PDT has been observed with ABC mammalian systems. Primary evidence came initially from investigations with porphyrins and the system ABCG2 (or Breast Cancer Resistance Protein BCRP). Transport of phytoporphyrin (phylloerythrin) was blocked by the ABCG2-specific inhibitor

fumitremorgin C (FTC) in human embryonic kidney cells transfected with full length human ABCG2 [37, 38]. Serum-dependent export of protoporphyrin IX by ABCG2 in T24 cells was also demonstrated [39]. In a more comprehensive study a series of conjugates of substrate PSs with varying groups attached to different positions on the tetrapyrrole macrocycle were designed. Pyropheophorbides and purpurinimides were found to be substrates for ABCG2 which affected the phototoxic response of a side population of stem cell-like cancer cells to PDT [40]. This was also the case for hypericin and ABG2 and ABCC1 (or multidrug resistance-associated protein 1, MRP1) where both systems affected the outcome of hypericin-mediated PDT in HT-29 adenocarcinoma cells [41]. In these two systems it is clear that MES affect PDT for a variety of PS chemotypes. In contrast, for ABCB1 (P-glycoprotein, P-gp) the evidence for PS substrates is sporadic and contradictory. For example, it has been shown that the multidrug resistance modulator and Cyclosporine A Analogue SDZ-PSC 833 potentiates the photodynamic activity of chlorin e6 independently of P-gp in multidrug resistant human breast adenocarcinoma cells [42]. Furthermore, psoralen inhibits the function of the transporter in the dark [43]. A hypericin-mitoxantrone (MTZ, chemotherapeutic) cocktail plus illumination with blue light potentiates cytotoxicity in bladder and breast cancer cells that -overexpress P-gp [44].

Phenothiazinium dyes MB and TBO are amphipathic cations and physicochemically similar to the antibacterial alkaloid berberine, a well-characterized substrate of MFS efflux systems in Gram-positive bacteria [45, 46]. This raised the possibility that phenothiazinium PSs could also be substrates of microbial efflux systems. Recent experimental evidence indicated that phenothiaziniums were NorA (MFS) substrates in *Staphylococcus aureus* and possibly MexAB (RND) substrates in *P. aeruginosa* [47]. This evidence was not supported by a model study using 60 *P. aeruginosa* clinical isolates overexpressing efflux systems it was demonstrated that antibiotic-resistant *P. aeruginosa* cells are as susceptible to TBO-mediated PDI as susceptible strains [48].

The observation that ABC transporters and not MFS affect MB-mediated PDI in the pathogenic yeast *Candida albicans* is perplexing [47, 49]. Furthermore, the structurally related phenothiazines thioridazine and chromazine have been characterized as inhibitors as opposed to substrates of a variety of pathogen efflux systems [50–52]. A recent study identified a sigma factor network responsive to cell-envelope damage by thioridazine in *Mycobacterium tuberculosis* suggesting roles other than efflux inhibition or anti-mycobacterial activity for the compound [53]. One plausible explanation for this complex behavior comes from the promiscuous substrate specificities of efflux systems. For example, clinically important PDR transporters include *C. albicans* Cdr1p (CaCdr1p) and CaCdr2p, which are orthologs of *S. cerevisiae* Pdr5p (ScPdr5p) and mammalian ABCG2 and ABCC1 transporters. Fungal PDR efflux pumps have relatively promiscuous substrate specificities that are thought to be defined primarily by their transmembrane domains. These specificities often partially overlap among family members in a particular organism and thus provide broad-spectrum protection against xenobiotic threat, including that posed by the widely-used and well-tolerated azole and triazole drugs. Both RND and ABC systems expel a wealth of potential substrates. This overlap in substrate specificity highlights the obstacles to blocking pumps efficiently.

The well documented promiscuity may explain the additional scattered reports for the interaction of porphyrins with microbial efflux systems. Porphyrin uptake and efflux seem to be regulated by the TolC system in *E coli* [54]. In *Streptococcus agalactiae*, two coregulated efflux transporters modulate intracellular heme and protoporphyrin IX availability [55]. In contrast the PDI pattern of amphipilic protoporphyrin diarginate PPArg in a variety of efflux related *S. aureus* strains showed no correlation for the PS with MES [56].

This wealth of contradicting information prompt us to apply a combination of first principles Quantum Mechanics calculations (QM) together with a docking protocol to understand whether the two phenothiazinium dyes MB and TBO are *a priori* candidates of being substrates of an ABC transporter. We used the ABCB1 mammalIan P-gp as a paradigmatic structural model. Despite its homology with other yeast and bacterial ABC transporters, the mouse P-gp is the only eukaryotic ABC experimental structure [30], crystallized in a drug-binding competent state [57]. We compared the docking properties of phenothiaznium dyes with berberine. The methodology and results are summarized on Figure 2 and Table 2. It was found that MB and berberine have a strong binding affinity with ABCB1 where as TBO showed less affinity. TBO had also a second binding site with less than 1 kcal/mol of difference in energy with respect to the main site. Both dyes share the site with berberine (Figure 2), in their lowest and most populated cluster of conformations. This pocket involves hydrophobic and aromatic residues mainly from TM 4, 5 and 6.

The use of small molecules known as multidrug efflux pump inhibitors (EPIs) that block MES in combination with conventional antibiotics has been proposed as an approach for circumventing efflux-mediated antimicrobial resistance. Biochemical approaches have yielded a number of promising EPIs in several pathogenic systems [58]. This concept of synergistic action has been exploited in PDT to potentiate the phototoxic action of phenothiazinium PSs [59]. The PDT effect of MB or TBO was substantially enhanced by small molecule EPIs in *S. aureus* that affect NorA as assessed by both reduction of viable cells and fluorescent dye accumulation. The potentiation is less pronounced against *P. aeruginosa* with MexAB.

It has been shown that near-infrared light can cause selective photodamage to multidrug resistant pathogens [60]. 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 potentiated the action of erythromycin, tetracycline and ciprofloxacin [61]. Although the antibiotics used in this study are MES substrates, and it is therefore reasonable to assume that near infrared light may play role in efflux inhibition, the experimental evidence is weak and this requires further exploration. The potentiation mechanism is hypothetical at this stage and not clearly distinct from PDI, as it may involve an optically mediated mechano-transduction of cellular redox pathways, decreasing DeltaPsi and increasing ROS.

Development of Microbial Resistance to PDT and analogies with bacteriocidal antibiotics

The first step towards addressing microbial resistance development to PDT is understanding the mechanisms and their implications. PDT leads to the production of singlet oxygen and other ROS through a variety of photochemical mechanisms resulting in cell death. This mechanism is by default analogous to bacteriocidal antibiotics but recent reports suggested that the analogy may go much deeper. It has now been demonstrated that the three major classes of bactericidal antibiotics (quinolones, b-lactams and aminoglycosides) regardless of drug-target interaction, stimulate the production of hydroxyl radicals in Gram-negative and Gram-positive bacteria, which ultimately contribute to cell death [62]. In the same report it was observed that bacteriostatic drugs do not produce hydroxyl radicals. The mechanism of hydroxyl radical formation induced by bactericidal antibiotics is the end product of an oxidative damage cellular death pathway involving the tricarboxylic acid cycle, a transient depletion of NADH, destabilization of iron-sulfur clusters, and stimulation of Fenton chemistry. The same group at Boston University employed systems-level approaches and phenotypic analyses to elucidate the pathway by which the aminoglycosides kanamycin and gentamycin ultimately trigger hydroxyl radical formation. In brief, the pathway involves

mistranslation and misfolding of membrane proteins and signaling through the envelope stress-response two-component system, with the redox-responsive two-component system playing an associated role [63].

As three of the main resistant components to antimicrobials (permeability barrier, multidrug efflux systems and drug availability) have been extensively explored, the focus had naturally shifted to the remaining two, target and pathway modification. The non-selective nature of PDT and the lack of specific molecular targets for the ROS produced during PDT means that it is unlikely that a specific microbial resistance pathway could develop. Reports discussing the potential for microbes to develop resistance to PDT are scattered and provided no clear conclusions. In a study of routine stress exposure followed by re growth, the photosensitizer 5,10,15-tris(1-methylpyridinium-4-yl)-20-(pentafluorophenyl)-porphyrin triiodide (Tri-Py(+)-Me-PF) was employed against *Vibrio fischeri* and *E. coli* model cells. After ten cycles of partial inactivation followed by regrowth, neither of the bacteria developed resistance to the photodynamic process [64]. Giulianai et al subjected *P. aeruginosa, S. aureus* and *C. albicans* to 20 consecutive PDI treatments with cationic Zn(II) phthalocyanine RLP068/Cl, but did not find any resistant mutants [65]. Only *S. aureus* showed increased MIC in dark conditions, but even in this case, the susceptibility of the mutated bacteria to PDI was not affected by their MIC increase.

Up regulation of the key oxidative stress enzyme superoxide dismutase has been observed following protoporphyrin-mediated PDT in *S. aureus* and RB-mediated PDT in *S. mutans.* This correlated with induction of GroEL, the bacterial heat shock protein responsible for refolding denatured proteins and stabilizing lipid membranes during stress [66]. Expression of the bacterial heat shock protein Dnak was also increased after sub-lethal PDI stress [67]. In the same study, heat pre-treatment (a positive up regulator) prior to PDI for *E. coli* and *Enterococcus faecalis* 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 for Yap1p and Skn7p in defense against singlet oxygen insult [68].

Efflux Pump Inhibitor-Photosensitizer Hybrids and their Potential use in PDT

The interaction between efflux systems and some but not all the molecular classes of PS was documented. The potentiation of PS mediated PDI by EPIs is emphatically demonstrated at least for Gram-positive bacteria and fungi. As MES also play a role in invasion, adherence and colonization by microbial cells, a PDT-EPI based combination approach may in some cases reduce bacterial virulence *in vivo*. A major obstacle, however, may arise from the fact that efflux systems manipulation could cause unexpected toxicities due to the multitude of physiological roles MES may play in human cells. In this context, efforts directed at specifically inhibiting efflux pumps operating only in prokaryotes may offer a greater chance of therapeutic success. Interestingly, it has been shown that target bacteria respond to clinical challenge with EPIs by developing resistance mutations that decrease the efficacy of the EPI [69, 70]. Recently it was demonstrated that reserpine can select multidrug resistant *Strepotococcus pneumoniae* strains [71].

The threat of cross-resistance to various different antibiotics elevates the complexity of EPI discovery ventures. Addressing this requires that efflux substrates and inhibitors be clearly differentiated, particularly with respect to PS for use in antimicrobial PDT. It also demands rational approaches that simultaneously address both the photochemical mechanistic aspects of PDT and efflux phenotypic variations. A reported strategy of “dual antimicrobial action” targeting NorA in Gram-positives may serve as a useful precedent for addressing these issues. A hybrid compound (SS14) created by fusing the plant antimicrobial berberine to the

synthetic NorA EPI INF55 was found to be an effective antimicrobial against *S. aureus*, including mutant strains that over express NorA [72]. MIC's for SS14 against *S. aureus* were 2–16 times lower than berberine and combinations of berberine with INF55. The hybrid rapidly accumulated in bacterial cells and showed higher efficacy than vancomycin in a *Caenorhabditis elegans* model of enterococcal infection [72]. Analogs of SS14 exhibited similar antimicrobial activities [73, 74] suggesting that significant structural changes can be made to these hybrids without adversely affecting their ability to block MES or their antibacterial activity. Such hybrids are predicted to have an advantage over separate compound administration in terms of synchronous or near synchronous delivery of both agents to the appropriate bacterial target sites.

The physicochemical similarites between berberine and the phenothiazinium photosensitizers MB and TBO (i.e. both amphipatic cations) and evidence that they are overlapping substrates for various efflux pumps (e.g. NorA [47,59]) suggested that an analogous strategy might be successfully applied in antimicrobial PDT. For example, hybrids which link EPIs to phenothiaziniums may decrease efflux of the PS leading to increased microbial cell killing upon illumination Hybrids of this type are currently under investigation in our laboratories (Figure 3).

Biofilm Inactivation

Chronic infections are most often associated with the formation of biofilms, [75, 76]. The dense protective environment of biofilms along with the significant differences in properties compared to free-floating or planktonic bacteria of the same species have been implicated to confer biofilm bacteria with as much as 1000-fold higher resistance to detergents, antiseptics and antibiotics [77]. The eradication of microbial biofilms remains a key challenge in the antimicrobial discovery arena and new discoveries are required to address a number of clinical conditions. PDT studies have been explored to some degree as an alternative treatment for several recalcitrant infections. For example, PDT has been used to target dental plaques, [78] periodontitis, [79] gingivitis, endodontics, [80] osteomyelitis [81], infections in cystic fibrosis, [82] infections of permanent indwelling devices such as joint prostheses and heart valves and implants [83] and oral candidiasis [84]. Peri-implantitis involves the biofilm colonization, 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 in 15 patients, leading to the reduction in bacterial counts by approximately 2 $\log_{10,}$[85].

It is important to compare PDI with conventional antibiotics both in terms of mechanism and efficacy when targeting biofilms. Biofilms generally do not restrict penetration of antibiotics [86], but they do form a barrier to the larger components of the immune system [87–89]. There is a wealth of literature describing PDT-based anti-biofilm strategies which focuses mostly on the use of different PSs against a variety of microbial species [90]. In contrast there are only a limited number of studies exploring the effects of PDT on phenotypic biofilm elements (e.g. adhesins). Moreover, there is no consensus as to which is the most reliable model for evaluating PDT efficacy against biofilms. The majority of published reports use methodologies where biofilms are grown in/on plastic or silicon microtiter plates and surfaces. These bioassays have been repetitively criticized for lack of robustness and occasionally yield inconsistent results. A very recent report [91] discussed the impact of PDT on the viability of *Streptococcus mutans* cells in an artificial biofilm model that used sterile chambered cover glasses, to form a salivary pellicle layer. PDT using phenothiazine chloride and red laser gave a significant reduction of biofilm bacterial viability as measured by Live-Dead assay. PDT studies with clinically relevant multi-species

biofilms are virtually non-existent with the notable exception being studies of dental plaque-derived biofilms and endodontic polymicrobial infections *in vitro* [92, 93].

By using isogenic pairs of wild-type and transposon mutants of *Staphylococcus epidermidis* and *S. aureus* deficient in capsular polysaccharide and slime production it has been shown that the cationic PSs pL-c(e6) and MB can overcome the protective effect of extracellular slime and stationary bacterial growth to PDI [94]. TBO has a substantial impact on PDI of *staphylococcal* biofilms which it decreases cell numbers ($5\log_{10}$ after irradiation with red light), disrupts biofilm architecture and damages bacterial cell membranes [95]. PDI with merocyanine 540 has a comparable effect on the viability of biofilms from Gram-positive pathogens when 400 J/cm^2 green light is used [96, 97]. Tri-meso (N-methyl-pyridyl), meso (N-tetradecyl-pyridyl) porphine (C14) has a significantly greater PDI effect in eradicating *S. epidermidis* biofilms compared with the parent tetra-substituted N-methyl-pyridyl-porphine (C1) [98]. TBO mediated PDI affects *Streptococcus mutans* biofilms in different stages of maturity (4 $\log_{10}$ with red light for mature biofilms) [99] as well as mature *S. sobrinus* and *S. sanguinis* biofilms [100]. Erythrosine was found to inactivate *S, mutans* biofilms better than MB and protoporphyrin with the effect enhanced to 2 $\log_{10}$ by light fractionation [101, 102]. Erythrosine is also more potent than MB against *Aggregatibacter actinomycetemcomitans* biofilms as are the anionic PSs RB and TBO [103, 104]. PDT with 5-ALA and TMP at different concentrations can inactivate *P. aeruginosa* biofilms [105, 106].

Although the PDT results in many systems seem promising they don't address the central problem. The paradox of chronic biofilm infections is that they are often unresponsive to antimicrobial therapy even when caused by a pathogen that is not resistant to the antimicrobial agent. The simple explanation is that the agent fails to effectively reach at least some cells *in vivo*, resulting in a relapsing infection [107]. As light delivery is location specific and dependent this has been implied in a few PDT explorations. For example *Helicobacter pylori,* a Gram-negative spiral bacterium that forms biofilms on the gastric mucosae, naturally accumulates porphyrins, which may then act as endogenous PSs [108]. In *H. pylori* infected patients the application of 405 nm endoscopic light alone is capable of reducing CFU counts by about 90% [109].

The bulk of cells in biofilms are actually highly susceptible to killing by antimicrobials and it is indeed only a small fraction of cells known as persisters that remain alive following antimicrobial treatments [110]. Persisters represent a subpopulation of cells that spontaneously go into a dormant, non-dividing state. When a population is treated with a bactericidal antibiotic, regular cells die but the persisters survive. According to the persister cell model of chronic relapsing infections, antimicrobial agents working in concert with the immune system are able to eliminate all regular and persister cells from the bloodstream, along with regular cells from the biofilm [111]. The only remaining live cells are then persisters present in biofilms and it is these persisters that repopulate causing the infection to relapse once the level of the bactericidal agent drops. This model seems to be more realistic and is partially supported by PDT explorations. For example, optimal light dosimetry was required to simultaneously maximize bacterial killing and allowed neutrophil accumulation into the infected site when for Photofrin-mediated PDT with red light was tested in a murine MRSA bacterial arthritis model [112]. Additionally, PDT employed a three dimethylpyrrolidinium functionalized C60-fullerene in a mouse wound *P. aeruginosa* biofilm infection did not enhance survival but when the PDT was combined with a suboptimal dose of tobramycin a synergistic therapeutic effect was observed, with 60% of mice surviving compared to 20% with tobramycin alone [113]. PDT with the cationic porphyrin, tetra-substituted N-methyl-pyridyl-porphine (TMP) resulted in almost complete

eradication of *staphylococcal* biofilms when they were exposed to vancomycin or subjected to the phagocytic action of whole blood [114, 115].

PDI inter-relationship with the "microbial phenotype"

The broad-spectrum activity and what appears to be non-specific action of antimicrobial PDI should be explored deeper in alignment with the complexity of the microbial phenotype. There is no documented evidence whether or not PDI can disrupt sophisticated microbial defensive lines. We have to take into account that PDI is able to eradicate microorganisms without producing resistant isolates, both in planktonic and biofilm forms. This is in concert with the potential of localized photooxidative stress to inactivate virulence factors in the absence of any documented conventional resistance mechanism. PDI with the phenothiazinium MB inhibits in a dose -dependent manner the biological activities of the proteinaceous virulence factors V8 protease, alpha-haemolysin and sphingomyelinase *in S. aureus* TBO has a similar effect in the two key bacterial virulence factors in both *E. coli* and *P. aeruginosa*, lipopolysaccharide (LPS) and proteases [116, 117]. A single antimicrobial PDT treatment *in vitro* inactivated protease activity and resulted in a 4-$\log_{10}$ reduction in the viability of *P. gingivalis*. 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 functionally inactivate IL-1 beta and TNF-alpha [118].

A series of pathways, components and phenotypes that may serve as potential alternative and attractive targets for antimicrobial drug discovery are under investigation. One alternative approach is targeting the bacterial communication system (quorum sensing, QS) with emphasis the signal molecules that bacteria produce and detect and thereby coordinate their behavior in a cell-density dependent manner (quorum sensing inhibitors, QSI [119]. There is no evidence for the effect of PDI to QS although the action of PDT with a QSI seems like an attractive has been a plausible combinatorial alternative. In some bacteria QS and RND efflux pump expression are linked. For example, the extracellular autoinducer concentration was significantly reduced when BpeAB-OprM in *Burkholderia pseudomallei* and MexAB-OprM in *P. aeruginosa* were knocked out [120] suggesting that inhibition of these efflux pumps could be useful therapeutically. Recent studies have dissected intercellular interaction at the molecular level through analysis of both synthetic and natural microbial populations. 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 [121].

Conclusion

PDI is not a conventional drug discovery platform as it is usually understood, since three elements (PS, visible light and oxygen) are essential for successful deployment. Many of the elements of the bacterial phenotype may come to play an important role as PDI evolves. A set of technical challenges will have to be met using sophisticated tools and approaches to address complex biological questions regarding resistance mechanisms, biofilm inactivation and persister cell formation. There are no validated examples of biofilm photoinactivation or PDT being used in reliable polymicrobial infection models. Minimal information exists for the design of host-pathogen studies exploring the ability of PDT to interfere with virulence determinants. One example is a report of a host-parasite model to assess intracellular targeting specificity of novel phthalocyanines against Leishmania parasites infecting macrophages and dendritic cells [122].

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Mark Haynes PhD Dr Haynes earned his bachelor's degree from McGill University and his PhD from The Department of Microbiology and Immunology at The University of Miami's School of Medicine. His work has focused on the biology of autoimmune diseases and the role particular immune cells play in disease development or resolution. His current work involves studying lymphocyte signaling, protein-protein interactions, and the involvement of antibody recognition in autoimmune disease. He is currently the Immunology Project Leader in the University of New Mexico Center for Molecular Discovery (UNMCMD)

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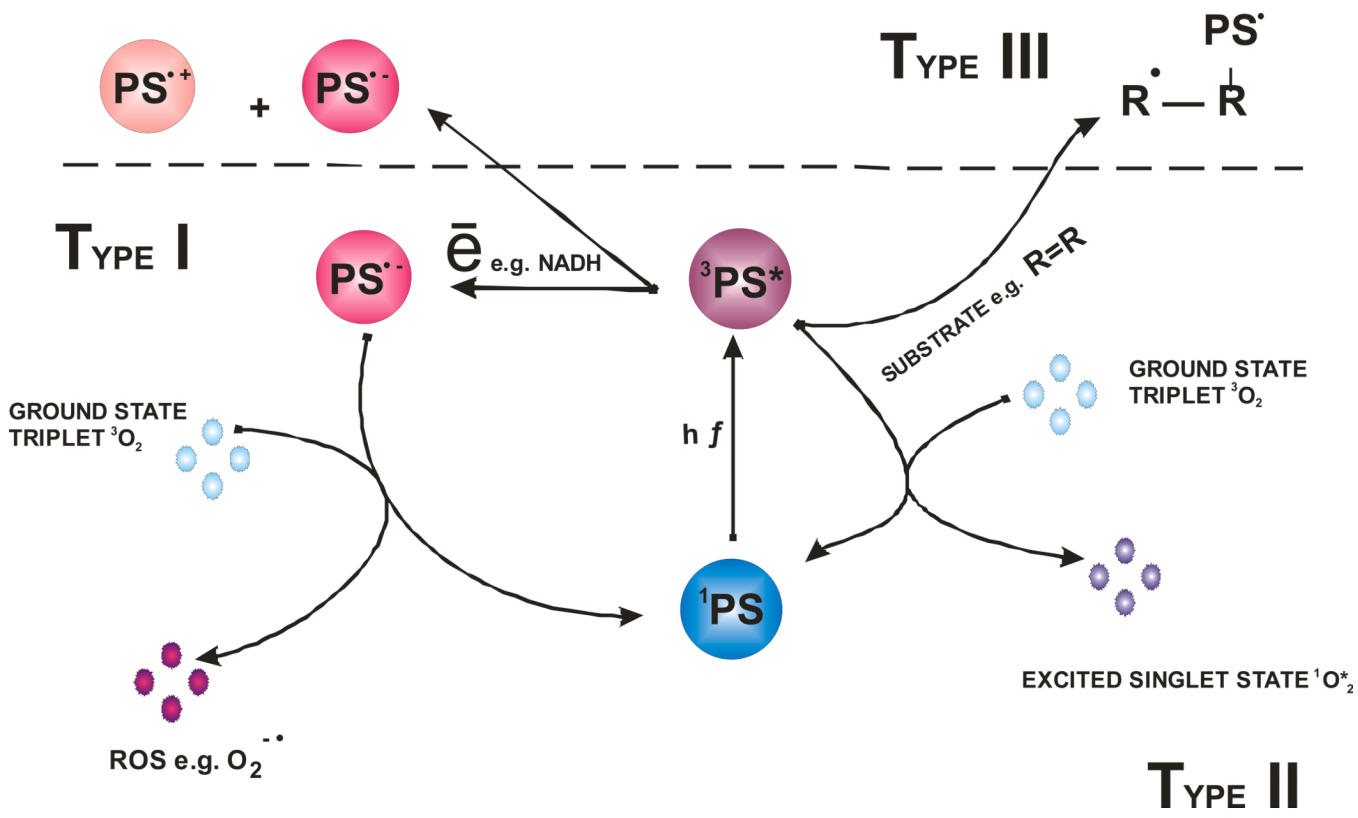

Figure 1.

Schematic illustration of photodynamic acion. 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. In the absence of oxygen the PS may interact with a substrate (R) in a pathway known as Type III.

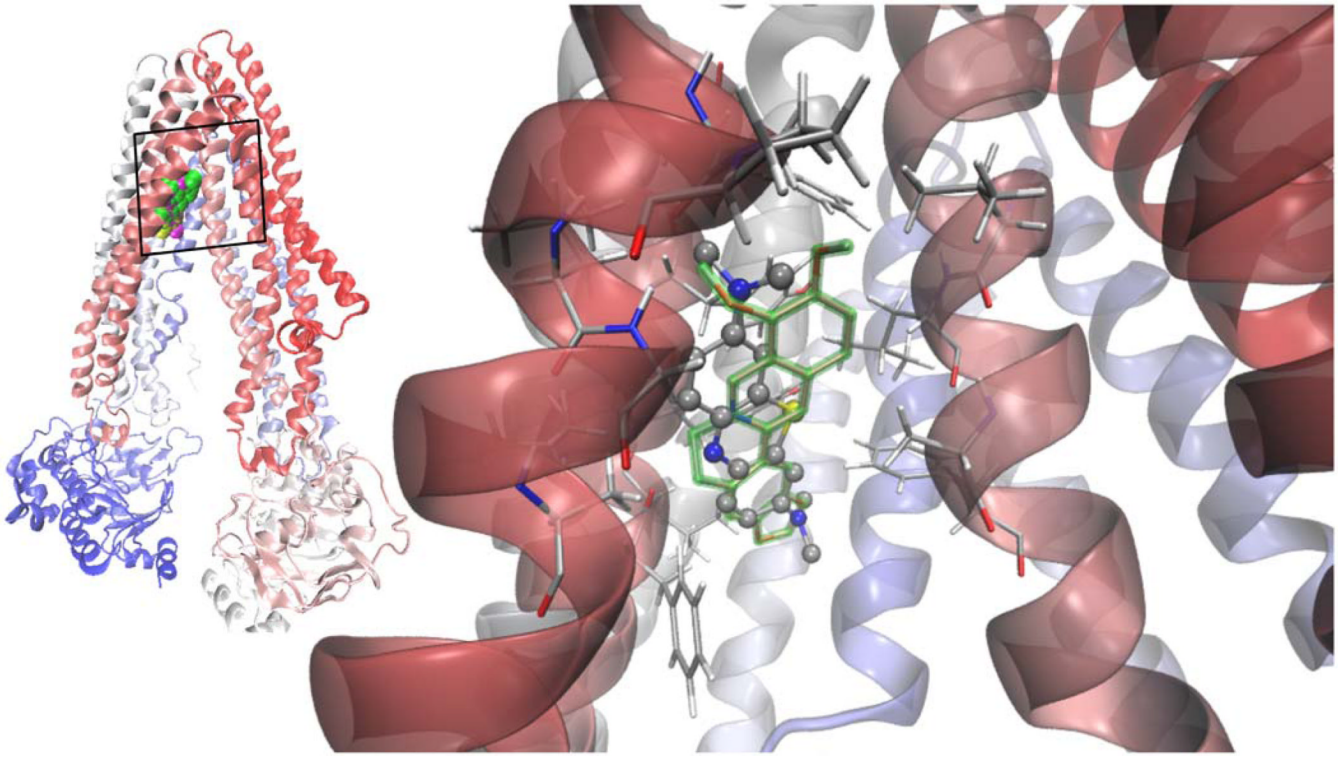

Figure 2.

Left: superimposed Van der Waals (VdW) models of the lowest energy docked structures of berberine [75] TBO (yellow) and MB (magenta) in apex of the inverted "V" depicted by the transmembrane α-helices 4 and 6 as they separate from top to bottom (in this orientation the extracellular side at the top; cartoon representation of the protein colored according to the sequence, from red to blue). *Right*: MB showed a clear specificity for the site overlapping with berberine, the calculated binding energies being practically indistinguishable. The hydrophobic pocket of negative electrostatic potential is rich in hydrophobic and aromatic residues, mainly form helices 4, 5 and 6.

The structure of the dyes were optimized with the method for fundamental vibrational frequencies B3LYP/6-311+G(d,p level of theory using the gaussian03 [129] package in a model polar solvent (PCM acetonitrile) [130]. The structures were docked onto the whole chamber formed by the twelve Transmembrane Domains (TMs) of the protein at the height to which they span the cell membrane in order to locate their binding sites, using a Lamarckian genetic algorithm implemented in Autodock 4.2.3. Afterwards, the whole protocol was applied in a smaller region comprising their main binding site. Their highly delocalized charge distribution was modeled from the QM results instead of the default program charges [131].

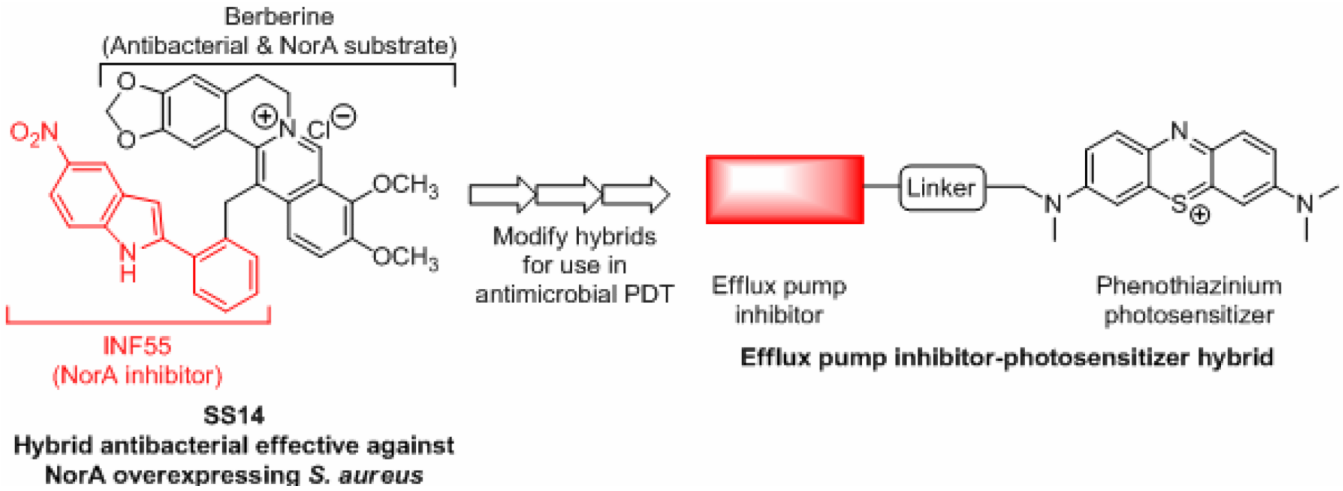

Figure 3.
Extension of the hybrid antimicrobial approach [72, 74] to compounds for use in antimicrobial PDT

Table 1

Combinatorial antimicrobials based on PDT and representatives of the dual antimicrobial platform

PDT-combinations	Synergist	Microorganism(s)	Target(s)	Reference
Polycationic conjugates of chlorin	visible light	*S. aureus, E. coli, P. aeruginosa, C. albicans*	non specific reactive oxygen species (ROS)	[123]
Methylene Blue (MB), Tolouidine Blue (TBO)	visible light, EPIs	*S. aureus, P. aeruginosa, C. albicans*	ROS	[49, 59]
TBO	visible light, EPIs	*E. faecalis biofilms*	ROS	[124]
functionalized C60-fullerene-tombramycin	visible light	*P. aeruginosa biofilms*	ROS	[113]
Dual Action Antimicrobials				
Chitosan-silver nanoparticle	-	*P aeruginosa, P. mirabilis, A. baumannii*	membrane	[125]
Berberine-Indole Derivatives	-	*S. aureus, E. faecalis, E. faecium, B. anthracis, B. cereus*	DNA and membrane	[72, 126, 127]
Oxazolidinone-Quinolone	-	*S. aureus, E. faecalis, E. faecium E. coli, H. pylori*	DNA gyrase and topoisomerase IV	[128]

Table 2

Summary of molecular docking results for MB, TBO compared to berberine.

Compound	Binding Energy (kcal/mol)
Berberine	−5.38
MB	−5.29
TBO	−4.40