Antimicrobial photodynamic therapy – what we know and what we don’t

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ABSTRACT

Considering increasing number of pathogens resistant towards commonly used antibiotics as well as antiseptics, there is a pressing need for antimicrobial approaches that are capable of inactivating pathogens efficiently without the risk of inducing resistances. In this regard, an alternative approach is the antimicrobial photodynamic therapy (aPDT). The antimicrobial effect of aPDT is based on the principle that visible light activates a per se non-toxic molecule, the so-called photosensitizer (PS), resulting in generation of reactive oxygen species that kill bacteria unselectively via an oxidative burst. During the last 10–20 years, there has been extensive in vitro research on novel PS as well as light sources, which is now to be translated into clinics. In this review, we aim to provide an overview about the history of aPDT, its fundamental photochemical and photophysical mechanisms as well as photosensitizers and light sources that are currently applied for aPDT in vitro. Furthermore, the potential of resistances towards aPDT is extensively discussed and implications for proper comparison of in vitro studies regarding aPDT as well as for potential application fields in clinical practice are given. Overall, this review shall provide an outlook on future research directions needed for successful translation of promising in vitro results in aPDT towards clinical practice.

Biofilm infections and antimicrobial resistance as a major clinical challenge

Bacteria persist in structured biofilm ecosystems and rarely in cultures of single species that are traditionally used by microbiologists to study the behavior of microorganisms in vitro (Davey and O’Toole 2000). In biofilms, microorganisms are remarkably less susceptible towards antimicrobials as compared to their planktonic counterparts (Mah and O’Toole 2001; Stewart and Costerton 2001). The mechanisms of tolerance and resistance in biofilms include slow penetration of antimicrobials through the biofilm matrix, altered microenvironment within the biofilm, different stress response of bacterial cells, and the formation of a sub-population of so-called persister cells (Chambless et al. 2006). Considering that biofilm infections are caused by a diversity of microbial species, potential resistances within the biofilm can easily be transferred among different species by horizontal gene transfer (Lewis 2001; Burmølle et al. 2006; Madsen et al. 2012; Wolcott et al. 2013).

It has been estimated that more than 60% of all microbial infections are caused by biofilms, also with reference to hospital-acquired infections, where sterile conditions are often compromised (Lewis 2001; Busscher et al. 2012). Frequent biofilm infections include dental infections (caused by dental plaque), dermal infections, urinary tract infections, middle-ear infections, endocarditis and implant- or catheter-associated infections, whereby at least the latter potentially can lead to serious, i.e. lethal, outcomes for patients (Lewis 2001; Zimmerli et al. 2004; Choy et al. 2008; Marsh et al. 2011).

Nowadays, several pathogens (especially Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Enterococcus faecalis, and Acinetobacter baumannii) have been reported to be increasingly resistant to a wide range of antibiotics (Laxminarayan et al. 2016; Renwick et al. 2016).
Some of them, e.g. methicillin-resistant *S. aureus* (MRSA) strains and the multi-resistant extended spectrum beta-lactamase (ESBL)-producing bacteria have become established pathogens in hospitals (McCarthy et al. 2010; Holmes et al. 2016; Laxminarayan et al. 2016).

After the discovery of penicillin in 1928, the golden era of antibiotics began in the 1940s and lasted until the end of 1960s with the development of different classes of antibiotics including aminoglycosides, tetracyclines, chloramphenicol, macrolides, glycopeptides, oxazolidinones, ansamycins, quinolones, and streptogramins (Aminov 2010; Renwick et al. 2016). However, as mentioned above, pathogens became resistant against most of these antibiotics and from the 1970s until the present, a lack of new antibiotics threatens to result in a stand-off with human pathogens and a return to the pre-antibiotic era (Aminov 2010; Lewis 2012; Laxminarayan et al. 2016; Renwick et al. 2016). This also relates to antifungal drug resistance, which can occur with all drug classes, and involves acquired resistance in susceptible strains and selection of inherently less susceptible species (Perlin et al. 2017). *Candida* and *Aspergillus* species such as *Candida glabrata, Candida auris, and Aspergillus fumigatus* have been reported to increasingly acquire resistance against major antifungal drugs, such as azoles and echinocandins (Perlin et al. 2017). It should also be kept in mind that resistance against disinfectants like chlorhexidine digluconate may correlate with antibiotic resistance as previously reported for *Staphylococci* (Meyer and Cookson 2010; Horner et al. 2012; Kampf 2016). Such a correlation between disinfectant resistance and antibiotic resistance makes the widespread use of disinfectants questionable (Kampf 2016).

A recent review on antimicrobial resistance, which was sponsored by the UK Department of Health and commissioned in 2014 by the UK Prime Minister reported that the number of deaths attributable to global antibiotic resistance will increase to 10 million per year until 2050 compared to the current 700,000 annual deaths (Renwick et al. 2016). Furthermore, this report stressed the reluctance of pharmaceutical companies to invest into the development of new antibiotics.

Therefore, there is an urgent need for alternative treatment methods against resistant microorganisms. These methods should also be designed to prevent the emergence of antibiotic resistance after use, e.g. acting according to a multi-target mode of action instead of the key-lock principle known from conventional antibiotics.

Consequently, in a recent review in *Lancet Infectious Diseases*, several alternatives for antibiotics were summarized and discussed from a medical and economical point of view (Czaplewski et al. 2016). The alternatives mentioned there included antibodies, probiotics, phage lysins, different classes of bacteriophages, immune stimulation (e.g. phenyl butyrate and vitamin D), vaccines, antimicrobial, anti-biofilm, and innate host defense peptides, etc. to name a few. The authors concluded that without sufficient funding, these novel treatment modalities to replace or supplement antibiotics will not become available in clinical practice, as funding currently is the key limiting factor that is stalling a global response to antibiotic resistance (Czaplewski et al. 2016).

Interestingly, in this review, the use of antimicrobial photodynamic therapy (aPDT) as an alternative approach instead of antibiotics and antiseptics was not mentioned (Czaplewski et al. 2016), although this term reveals 2287 citations in PubMed (National Centre for Biotechnology Information; status as of November 8 2017). This fact emphasizes that the potential for aPDT unfortunately has been underestimated or even been neglected. In addition, leading scientists in the field of aPDT have addressed this topic in a personal view article in *Lancet Infectious Diseases*, where the authors promoted to give funding not only for conventional antimicrobials but also for light-based approaches highlighting the potential of aPDT as an alternative antimicrobial approach in this upcoming era of resistance (Wainwright, Maisch, et al. 2017). The present review aims to provide a concise and critical overview about the current status quo of research on aPDT as well as on knowledge gaps and to offer an outlook on future research directions for translation of aPDT towards clinical practice.

**Antimicrobial photodynamic therapy – what we know**

**History**

The principle of photodynamic therapy (PDT) was accidentally discovered in 1900 by the medical student, Oscar Raab in Munich, who was involved in a study on the toxicity of the dye acridine red on *Paramecium* spp. as part of his doctoral thesis (Raab 1900). The results of his experiments using acridine red in small concentrations were inconsistent and not reproducible, despite many replicates. However, he and his supervisor Hermann von Tappeiner recognized that the observed toxicity was dependent on the time of the day and the amount of daylight, since this had been the only changing parameter (Szeimies 2005). Later on, von Tappeiner applied this approach with Albert Jesionek clinically for treatment of skin carcinomas (Jesionek and Tappeiner 1905) and coined the term “photodynamic phenomenon” (Tappeiner 1909).
Jodlbauer and von Tappeiner in 1904 and Huber in 1905 were the first to describe the successful photodynamic inactivation of bacteria (Jodlbauer and Tappeiner 1904; Huber 1905).

While anti-cancer PDT is clinical reality now for 25 years at least, e.g. in the treatment of actinic keratosis or basal cell carcinoma, antimicrobial applications of the photodynamic process have only been rediscovered in response to the emergence of the first drug-resistant infections in the healthcare sector during the early 1990s (Wainwright, Maisch, et al. 2017; Wainwright 2017a).

**Fundamental mechanisms**

The principle of aPDT is based on the combination of three compounds, a *per se* non-toxic molecule, the so-called photosensitizer (PS), light of a spectral range appropriate for excitation of the PS (typically from the visible to near infrared spectrum) and molecular oxygen (Wainwright 1998; Wainwright, Maisch, et al. 2017). Figure 1 shows the Jablonski diagram depicting the mechanism of aPDT: after absorption of a photon (A), the PS transfers from its ground singlet state ($S_0$) to an excited singlet state ($S_n$). Subsequently, the PS can lose energy by emitting fluorescence (F) or heat (H) via internal conversion, thereby returning to $S_0$, or it can be converted to a longer-living excited triplet state PS ($T_1$) by so-called inter-system crossing (ISC). From this state, it can return to its ground state ($S_0$) either by phosphorescence emission (P) or by two mechanisms generating reactive oxygen species (ROS), as follows: in type I mechanism, charge (i.e. electrons) is transferred to surrounding substrates leading to formation of superoxide radicals ($O_2^-$) that can undergo dismutation into hydrogen peroxide ($H_2O_2$), the precursor of the highly reactive free hydroxyl radicals (HO•), which are formed via Fenton-like reactions. In contrast, in type II mechanism, energy (but no charge) is transferred directly to the ground state molecular oxygen ($^1O_2$), leading to emergence of singlet oxygen ($^1O_2$), which is nothing else than energized molecular oxygen (+0.98 eV = +1.57 x 10⁻¹⁹ J). After such a cycle of absorption and generation of ROS, the PS is in its ground state ($S_0$) again and therefore ready for absorption of a new photon to generate more ROS. Accordingly, one PS molecule can generate thousands of molecules of $^1O_2$ before being destroyed. The ratio of both photodynamic mechanisms is unique for each PS and depending on the respective chemical structure, whereby the singlet oxygen quantum yield $\Phi_A$ describes the amount of type II mechanism (Maisch et al. 2007; Alves et al. 2014; Baptista et al. 2017; Wainwright, Maisch, et al. 2017).

**Photosensitizers**

Since the discovery of the photodynamic process in the beginning of the 20th century, the development of appropriate “light-activatable dyes” was initiated for use as PS. Even before, several dyes had already been synthesized in Germany by the end of the 19th century.
(from the 1860s) in the context of a booming German textile industry (López-Muñoz et al. 2005). Within the last 20 years, several approaches were done to optimize the photodynamic properties of PS by modifying the chemical structure or by developing new classes of PS (Wainwright, Maisch, et al. 2017). In the following, main classes of PS used for aPDT are described shortly (chemical structures of the most used compounds are shown in Figure 2). For a more detailed description of all the distinct PS classes, the reader may be referred to the excellent review of Heidi Abrahamse and Michael R. Hamblin (Abrahamse and Hamblin 2016).

**Phenothiazinium derivatives**

The first phenothiazinium dye (Methylene Blue) was developed by Heinrich Caro in the 1870s (Caro 1878). These dyes are composed of a three-ring π-system with auxochromic side groups, carry a single positive charge and exhibit a singlet oxygen quantum yield below 0.5.
therefore acting mainly according to type I mechanism (Wilkinson et al. 1993). They show strong absorption in the red spectrum (≈600–680 nm), which is advantageous for their application as PS due to the better tissue penetration of light from longer wavelengths (Felgenträger et al. 2013). Examples for classic pheno-thiazinium-based PS employed in aPDT are Methylene Blue [3,7-bis(dimethylamino)-phenothiazin-5-ium chloride] or Toluidine Blue [3-amino-7-(dimethylamino)-2-methyl-phenothiazin-5-ium chloride], which have been continuously refined during the last 20 years by several chemical modifications (e.g. New Methylene Blue, Di-Methyl-Methylene Blue) (Wainwright et al. 1998; Wainwright 2000; Gollmer et al. 2015).

Impact in the field: first clinically approved PS (dentistry), standard PS in vitro

**Porphyrin, chlorin, and phthalocyanine derivatives**

Porphyrins, chlorins, and phthalocyanines are structurally comparable heterocyclic-macrocyclic compounds consisting of four pyrrole cycles (porphyrins, phthalocyanines) or three pyrrole and one pyrroline subunits (chlorins). Porphyrins and chlorins carry up to eight positive charges and act predominantly according to type II mechanism with singlet oxygen quantum yields between 0.5 and 0.8 (Fernandez et al. 1997). Furthermore, they exhibit an intense absorption maximum at ≈405 nm (Soret band) and smaller peaks at wavelengths longer than 500 nm (Q bands) (Gouterman 1961).

Many of these compounds are occurring in nature; for instance, it is well known that several oral bacteria form endogenous porphyrins in their metabolism, that is why they can be successfully inactivated by irradiation with visible light only in vitro (Cieplik, Späh, et al. 2014; Pummer et al. 2017). Examples for chemical modifications of porphyrins that are commonly used as PS are TMPyP [5,10,15,20-tetrakis(1-methyl-4-pyridinium)-porphyrin tetra-p-toluenesulfonate], a four-fold positively charged derivative (Collins et al. 2010), and XF-73 [5,15-bis-[3-trimethylammoniopropoxy]-phenyl]-porphyrin chloride], a porphyrin derivative with two positive charges (Maisch et al. 2005). With regard to chlorins, mainly cationic derivatives of chlorin-e6 are used for aPDT, e.g. Photodithazine®, which is a commercially available chlorin-e6 derivative with two positive charges (Dovigo et al. 2013). In contrast to porphyrins and chlorins, phthalocyanines are hydrophobic and uncharged, wherefore they have to be combined with methods for solubilisation, e.g. encapsulation in nano-emulsions, to be used as effective PS for aPDT (Ribeiro et al. 2015).

Impact in the field: TMPyP widely used as standard PS in vitro

**Xanthene derivatives**

Anionic xanthene dyes like Eosin Y, Erythrosine and Rose Bengal are derived from fluorescein and show intense absorption in the green spectrum (≈480–550 nm) (DeRosa 2002; Goulart et al. 2010). Although they act mainly according to type II mechanism (singlet oxygen quantum yields ranging between 0.6 and 0.8) (Wilkinson et al. 1993), their negative charge impedes with successful use as PS for aPDT (Cieplik, Tabenski, et al. 2014).

Impact in the field: sparse studies in more recent years

**Fullerene derivatives**

Fullerenes are soccer-ball shaped cage molecules made exclusively from carbon atoms, with fullerene C60 being the most studied compound, which has been functionalized in multiple ways by adding positively charged moieties (Mizuno et al. 2011; Sharma et al. 2011). They show strong absorption in the UV and visible range due to their extended π-conjugated system of molecular orbitals and react predominantly according to both type I and type II mechanism depending on chemical modifications and solvents (Mroz et al. 2007).

Impact in the field: unique class of PS, sparse studies on effect on biofilms yet.

**Phenalenone derivatives**

Phenalenone chromophors are biosynthesized by plants to defend against pathogens using sun to generate singlet oxygen (Flors and Nonell 2006). Santi Nonell et al. were the first to report about PNS [1H-Phenalen-1-one-2-sulfonic acid] as an “extremely efficient singlet molecular oxygen sensitizer for aqueous media” reacting almost quantitatively according to type II mechanism (Nonell et al. 1993). From then, PNS was used as a standard-PS for photophysical measurements but was not applied for aPDT due to its negative charge. Later, several positively charged and water-soluble phenalen-1-one derivatives were introduced with SAPYR as their lead compound (carrying a positively charged pyridinium-methyl moiety) (Cieplik et al. 2013). These phenalen-1-one derivatives exhibit singlet oxygen quantum yield above 0.9 and absorb in the UV/blue spectrum (≈320–430 nm) (Späh et al. 2014; Tabenski et al. 2016).

Impact in the field: SAPYR as first water-soluble exclusive type-II PS.
Riboflavin derivatives

Riboflavin, i.e. vitamin B2, has already been known to generate singlet oxygen upon UVA irradiation (Regensburger et al. 2012). However, hydrophilicity was very poor due to the lack of positive charges leading to unexceptional antimicrobial efficacy (Makdoumi and Bäckman 2016). Recently, riboflavin derivatives substituted with up to eight positive charges showed considerable improvement in photodynamic efficacy (Maisch et al. 2014). These novel riboflavin derivatives show absorption in the UVA/blue spectrum (≈350–470 nm) and singlet oxygen quantum yields between 0.7 and 0.8 (Maisch et al. 2014).

Impact in the field: safe in humans, as decomposition of flavin molecules is ubiquitous

Curcumin derivatives

Curcumin [(1E,6E-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is a naturally occurring intense yellow dye isolated from the rootstocks of Curcuma longa and approved as food additive E100. Due to its poor hydrophilicity, several chemical modifications of curcumin carrying positive charges have been developed recently (Tortik et al. 2014; Spaeth et al. 2017). Curcumin and its derivatives act nearly quantitative according to type I mechanism and exhibit absorption in the UV/blue spectrum (≈300–500 nm) (Araújo et al. 2014).

Impact in the field: curcumin approved as food additive, novel positively charged derivatives with enhanced water solubility

Light sources

For light-activation of a PS, three main light sources have been described for the field of aPDT in the literature so far: lasers (e.g. argon, diode, or neodymium doped: yttrium, aluminum, and garnet [Nd:YAG] lasers), light-emitting diodes (LEDs) and gas-discharge lamps (e.g. quartz-tungsten-halogen or xenon-discharge lamps) (Wilson and Patterson 2008). In contrast to lasers and LEDs, halogen lamps have the advantage that they can be spectrally filtered to match any PS; however, they cannot be efficiently coupled into optical fiber bundles or liquid light guides, and they cause more heating as compared to lasers and LEDs (Nagata et al. 2012). Heating caused by a given light source must be considered for any clinical application of aPDT in the human body. Depending on the irradiation period and the respective light source used, the energy dose applied can cause a temperature increase, potentially leading to tissue damages (Spranley et al. 2012). For example, irradiation of teeth with a halogen lamp for 45–60 s increased the temperature of dentin by around 5 °C (Weerakoon et al. 2002), while an increase in temperature of 5.5 °C is known to have adverse effects on the dental pulp (Nammour et al. 2010; Nagata et al. 2012).

All in all, for irradiation of a given PS, the spectral emission and the intensity of the respective light source as well as the mode of light-delivery (via optical fiber or directly) are more important than the type of the light source itself, e.g. laser, LED, or gas-discharge lamp.

Antimicrobial efficacy of aPDT in vitro

The antimicrobial efficacy of aPDT employing many distinct PS had been investigated in a very large number of in vitro studies since the 1990s. Thereby, many of these studies showed promising results, often achieving inactivation rates of more than 5 log10 steps of CFU (colony forming units), which is defined as a disinfecting effect according to infection control guidelines (Boyce and Pittet 2002).

In this instance, it must be considered that many of these studies have been conducted for inactivation of planktonic cultures, i.e. free-floating bacterial cells, which is a convenient method for high-throughput antimicrobial screening of many compounds, e.g. in order to evaluate whether slight chemical modifications of a given PS can enhance antimicrobial efficacy (Kiesslich et al. 2013).

However, for evaluating the efficacy of a given antimicrobial approach it is mandatory to study biofilms, as it is well known that bacteria embedded in biofilms exhibit very different properties as compared to their planktonic counterparts like an up to 1000-fold higher tolerance towards antimicrobials (Marsh 2004; Marsh et al. 2011). Besides all the other factors responsible for this higher tolerance of bacteria in biofilms, especially the biofilm matrix (extracellular polymeric substance; EPS) may protect the bacteria against aPDT-mediated
killing in two-fold way by preventing penetration of the PS throughout the biofilm (interactions of positively charged PS molecules with negatively charged EPS residues, redox processes, and π–π-interactions of aromatic surfaces) and by hampering light propagation in deeper layers of the biofilm (Mah and O’Toole 2001; Stewart and Costerton 2001).

For an overview of the antimicrobial efficacy of aPDT for inactivation of bacterial biofilms in vitro, the reader may be kindly referred to a recent review of our group, where antimicrobial efficacy rates of distinct PS are elaborated according to the respective classes of PS in detail (Cieplik, Tabenski, et al. 2014).

With regard to fungi, it is also well known that fungal biofilms are much more tolerant than planktonic fungal cultures (Lyon et al. 2011). For aPDT, the majority of studies has focused on Candida biofilms (Baltazar et al. 2015). Since the antifungal action of aPDT seems to be strain-dependent (Lyon et al. 2011), there is need to investigate the efficacy of aPDT also for biofilms of other major fungal pathogens (Baltazar et al. 2015). For more detailed insights into the effects of aPDT on fungal cells, it may also be referred to recent reviews in the literature (Lyon et al. 2011; Pinton et al. 2012; Baltazar et al. 2015). Interestingly, mixed bacterial-fungal biofilms differ from purely bacterial or fungal biofilms because fungi represent biotic surfaces to which bacteria can adhere (Seneviratne et al. 2007). Such mixed biofilms may be more resistant than biofilms formed solely from bacteria or fungi, e.g. due to a more complex EPS composition (Wargo and Hogan 2006).

**Appropriate comparison of distinct aPDT systems in vitro**

When the antimicrobial efficacy of distinct PS is to be compared, it is crucial to adjust the respective irradiation parameters. Hereby, the distinct absorption and emission characteristics of given PS and their corresponding light sources must be considered. As according to the photodynamic principle one PS molecule is excited by the absorption of one photon, adjusting the number of photons absorbed by a PS molecule upon irradiation by its corresponding light source seems appropriate to compare the antimicrobial photodynamic efficacy rates of distinct PS per excited molecule (Cieplik et al. 2015). The number of absorbed photons per second is given by the following equation:

\[
\text{absorbed photons per second} = \sum_{\lambda} \left(1 - 10^{-\varepsilon(\lambda) \times c \times d} \right) \times P_{\text{em}}(\lambda) \times \frac{\lambda}{c_0 h}
\]

Adjusting numbers of absorbed photons is the only way to take characteristics of both, the PS and the light source, into account, while other ways for adjustment of irradiation parameters (e.g. energy doses) only consider the characteristics of the light source, but not the PS. Therefore, applying this formula ensures that the biological efficacy of distinct PS can be compared independent of their photo-physical absorption characteristics (Cieplik et al. 2015, 2016).

**Putative main target structures of aPDT**

A particular advantage of aPDT is its non-selective action, focusing on multiple molecular targets like proteins, lipids, and nucleic acids (Almeida et al. 2015). Usually, oxidative damage of two crucial cellular components, i.e. cytoplasmic membranes and DNA, is proposed to be responsible for aPDT-mediated killing of bacteria (Hamblin and Hasan 2004; Alves et al. 2014). These oxidative targets of aPDT mostly depend on the respective cellular localization of the given PS as well as on the maximal time-limited diffusion length of the emerging ROS. For instance, due to its short lifetime of 3–4 µs, the diffusion length of 1O2 is not longer than 0.3 µm and depending on the surrounding medium (Baier et al. 2005; Maisch et al. 2007). Therefore, oxidative damage usually takes place in close vicinity to the respective PS molecule, for which there are three options in general (Alves et al. 2014):

- A PS just stays in close proximity to a bacterial cell without any effective binding, which may limit the oxidative damage to outer structures like the cell wall or the cytoplasmic membrane, or
- A PS actively binds to the bacterial cell via electrostatic attraction, hydrogen bonds, or van-der-Waals interactions, potentially leading to a similar damage pattern limited to outer structures, or
- A PS enters a bacterial cell by binding and subsequent diffusion or active transport into the cytoplasm, resulting in damage of intracellular components like cytoplasmic proteins or DNA.

Localization of a PS may be dependent on the chemical structure of the respective PS (e.g. molecular size, extinction coefficient, spectral radiant power, concentration of PS, thickness of solution, wavelength, velocity of light, Planck constant).
charge, lipophilicity), its concentration, solvent as well as the cell wall structure of the targeted bacteria (e.g. Gram-staining characteristics) (Alves et al. 2014). However, these binding mechanisms and the cellular localizations of given PS are not fully understood yet and reports are contradictory (Ragás et al. 2010; Preuß et al. 2013; Alves et al. 2014; Mang et al. 2016; Gollmer et al. 2017). For instance, while Ragas et al. reported a dual localization of the porphyrin-PS TMPyP inside (bound to DNA) and outside (attached to the cell wall) in *Escherichia coli* based on spectroscopic and photophysical measurements (Ragás et al. 2010), Preuß et al. found no uptake of TMPyP in *E. coli* as measured by fluorescence lifetime imaging technique (Preuß et al. 2013). In accordance to the latter study, Gollmer et al. revealed by highly-sensitive fluorescence microscopy that accumulation of TMPyP in the cytoplasm and the cell wall is minor in *S. aureus* and that this PS may only enter the cell after preceding cell wall damage (Gollmer et al. 2017). On the contrary, the phenothiazinium dye Methylene Blue seems to locate intracellularly because its photodynamic efficacy can be increased by addition of efflux pump inhibitors (Tegos et al. 2008).

Nevertheless, for damage of DNA, a PS has to build up high-intracellular concentrations (Cadet et al. 2010). Accordingly, it seems reasonable that main targets are the external structures like cell walls and cytoplasmic membranes rather than DNA (Almeida et al. 2015). Accordingly, from classic studies on the extremophile bacterium *Deinococcus radiodurans*, it is known that this species can be easily inactivated by aPDT, although it is known for its very efficient DNA repair mechanisms allowing to withstand even very high amounts of ionizing radiation (Schäfer et al. 1998; Nitzan and Ashkenazi 1999). In contrast, nucleic acids may only be affected when bacterial cell walls and cytoplasmic membranes have already been destroyed by the oxidative burst generated by aPDT treatment (Almeida et al. 2015). Attachment of the positively charged PS to the negatively charged cell wall and membrane structures of bacteria seems more probable than actual uptake of the PS. However, this is dependent on the chemical structure of the respective PS, its predominant mechanism of action and probably also on the targeted bacterial species. For further insights on cellular targets of aPDT in bacteria, the reader may be referred to the excellent review of Alves et al. (2014).

**Potential for development of resistances towards aPDT**

In general, development of resistances in pathogens is more likely for approaches that aim at one specific target structure according to the so-called key-lock principle like antibiotics or most antiseptics (McDonnell and Russell 1999; Maillard 2002). In this instance, it is easy for pathogens to overcome the antimicrobial challenge by punctual mutations, expression of efflux pumps, or up-regulation of defense-associated enzymes. As stated above, aPDT is a non-selective approach with multiple targets in focus. Therefore, it has been assessed to be very improbable that bacteria could develop resistance towards aPDT (Maisch 2015a; Wainwright, Maisch, et al. 2017).

**Bacterial response towards oxidative stress generated by aPDT**

Oxidative stress is somehow ubiquitous in bacterial life, either formed intracellularly in bacterial metabolism or due to interaction with host cells or cells from other bacterial species. While molecular oxygen (O\(_2\)) is rather unreactive, it can cross typical biological membranes very quickly (Imlay 2013). Its reactivity derives from the metabolic formation of ROS due to a four-electron reduction series with the addition of consecutive electrons generating superoxide anions (O\(_2^-\)), hydrogen peroxide (H\(_2\)O\(_2\)) and free hydroxyl radicals (•OH) (Gerschman et al. 1954; Imlay 2013). These ROS are known to be able to oxidize various biomolecules and cause substantial damage. The condition of accumulation of ROS inside a cell is called oxidative stress (Imlay 2013). Bacteria have developed defense mechanisms for detoxification of these ROS by highly specific and effective enzymatic pathways like those catalyzed by superoxide dismutase (Sod), catalase, peroxidase as well as enzymes of the glutathione system (Miller and Britigan 1997; Pomposiello and Demple 2002; Staerck et al. 2017).

Theoretically, bacteria may be able to adapt towards ROS generated from type I mechanism in aPDT by up-regulating expression of these enzymes (Maisch 2015a; Kashef and Hamblin 2017). Nakonieczna et al. observed that Sod activity as well as transcript levels of sodA and sodM increased in clinical isolates of *S. aureus* after aPDT with protoporphyrin IX, but only in strains that were found susceptible to aPDT. This emphasizes that Sod activity may not directly affect the vulnerability of *S. aureus* towards aPDT (Nakonieczna et al. 2010). As oxidative damage only occurs at the site of the localization of a PS, the distance between the oxidative burst (most probably occurring at the cell envelope as described above) and these intracellularly located defense enzymes may be too long, wherefore these enzymes may not be able to help bacteria to a significant extent to survive aPDT challenge (Maisch 2015a).
In contrast, against singlet oxygen (\(^1\)O\(_2\)) generated from type II mechanism of aPDT, there is no specific enzyme-mediated defense system known in bacteria. Furthermore, it is very improbable that bacteria could develop such a system as \(^1\)O\(_2\) is no oxygen radical, but just energized molecular oxygen \((+0.98 \text{ eV})\). However, it is known from plants, that chemical quenchers of \(^1\)O\(_2\) (mainly carotenoids, particularly \(\beta\)-carotene, and \(\alpha\)-tocopherol) are formed during photosynthesis when more light energy is absorbed than can be used (Krieger-Liszkay 2005; Ramel et al. 2012). Further research on this phenomenon in bacteria may be worthwhile.

**Expression of efflux pumps**

Another major mechanism of bacterial resistance is given by transmembrane efflux systems, which lead to a decrease of the intracellular concentrations of noxious compounds by extrusion in the extracellular environment (Alekshun and Levy 2007). This may also be an issue for resistance towards aPDT (Tegos and Hamblin 2006; Tegos et al. 2008) as it has been shown that efflux pumps reduce killing efficacy of aPDT with Methylene Blue by decreasing intracellular concentrations of Methylene Blue and associated intracellular ROS (Tegos et al. 2008; Rineh et al. 2017). However, although many classes of PS have been tested so far (Wainwright, Maisch, et al. 2017), only phenothiazinium compounds like Methylene Blue have been found to be potential substrates for efflux pumps in bacteria (Tegos and Hamblin 2006), but also in *Candida albicans* (Prates et al. 2011). This reduced antimicrobial photodynamic efficacy due to efflux and can nonetheless be overcome by adjunctive application of specific efflux pump inhibitors, as it has been shown for planktonic cultures (Tegos et al. 2008; Kishen et al. 2010) and biofilms (de Aguiar Coletti et al. 2017) *in vitro* as well as in an *in vivo* murine wound infection model (Rineh et al. 2017, 2018).

**Studies on induction of resistance towards aPDT**

Development of resistance is generally studied by applying sublethal doses of given treatment modalities and subsequent culture of the surviving bacteria in repeated cycles (Alves et al. 2014). There have been very few studies so far that tried to induce resistances in pathogens towards aPDT by such repeated passages of treatment and re-growth (Lauro et al. 2002; Pedigo et al. 2009; Giuliani et al. 2010; Tavares et al. 2010). Lauro et al. showed that *Aggregatibacter actinomycetemcomitans* and *Peptostreptococcus micros* were not able to develop detectable resistance towards aPDT with porphycene-polylysine conjugates as PS after 10 repeated cycles of partial inactivation followed by subsequent culture of outliving bacteria (Lauro et al. 2002). Further, this aPDT treatment did also not modify the susceptibility towards various classes of antibiotics (Lauro et al. 2002). Pedigo et al. investigated the potential of *E. coli* and antibiotic-sensitive and resistant strains of *S. aureus* (MSSA and MRSA) to develop resistance towards repeated aPDT treatment with MB as a PS. For both species, no differences were observed when tested through 11 (*E. coli*) or even 25 passages (MSSA, MRSA), respectively (Pedigo et al. 2009). Tavares et al. came to the same result with strains of *Vibrio fischeri* and *E. coli* after 10 cycles of aPDT with the tricationic porphyrin derivative Tri-Py\(^+\)-Me-PF (Tavares et al. 2010). Likewise, Giuliani et al. found no development of resistance even after 20 daily passages of aPDT using the tetraationic Zn(II)-phthalocyanine derivative RLP068/Ci as PS on *S. aureus*, *Pseudomonas aeruginosa* and *Candida albicans* (Giuliani et al. 2010).

However, it must be considered that there are two fundamental limitations of these studies that should be kept in mind: First, these studies have been conducted on bacteria (or yeasts) in planktonic cultures (Lauro et al. 2002; Pedigo et al. 2009; Giuliani et al. 2010; Tavares et al. 2010). In contrast, when bacteria are in a sessile biofilm-mode, as it is their preferred way of life *in vivo*, the risk of development of resistances generally is higher than when growing as planktonic cultures, e.g. via horizontal gene transfer (Boles et al. 2004; Marsh et al. 2011). Besides that, it is known that bacteria acquire resistance towards a given antimicrobial when they are continuously exposed to low, i.e. sub-antimicrobial, doses for a prolonged period while aPDT is designed to be a relatively short procedure. Consequently, Kashef and Hamblin suggested that a more realistic way to investigate the potential of resistance towards aPDT would be to expose bacteria to continuous defined sub-antimicrobial levels of aPDT (Kashef and Hamblin 2017).

Having these limitations in mind, aPDT treatment seems not to induce resistance towards antibiotics: Cassidy et al. exposed clinical isolates of *S. aureus* and *P. aeruginosa* to sublethal aPDT with TMPyP and Methylene Blue as PS for a total of 72 h (irradiation at \(t=0, 24, \text{ and } 48 \text{ h}\)) for habituation to aPDT and afterwards tested susceptibility towards various antibiotics as well as aPDT. They found no decreased susceptibility towards antibiotics nor aPDT (Cassidy et al. 2010).

**Potential fields for clinical application of aPDT**

In a very recent review, Mark Wainwright drew the example of tonsillitis, a very common disease of
bacterial and/or viral origin, which is usually treated by systemic prescription of amoxicillin without clarifying its etiology beforehand. For this disease, application of a photoantimicrobial followed by irradiation for a given period, for instance by means of a fiber-optic device, may lead to sufficient antimicrobial effects without any risk of disturbance of the intestinal microbiota by systemic antibiotics or exposure of bacteria to sub-inhibitory concentrations that potentially may induce resistance (Wainwright 2017b). However, until now only three PS have reached clinical approval for use in humans in combination with light, i.e. phenothiazinium dyes, Methylene Blue and Toluidine Blue as well as hep-tacyanine dye Indocyanine Green (Wainwright, Maisch, et al. 2017), which rather reacts according to a photothermal than a photodynamic mechanism (Engel et al. 2008). Furthermore, these systems are only approved for use in dentistry as adjuvant approach for treatment of periodontal or endodontic infections (Wainwright, Maisch, et al. 2017).

In general, application of aPDT may work best for localized infections, preferably at easy accessible parts of the body, where light propagation is no problem (Wainwright, Maisch, et al. 2017; Wainwright 2017b). Nevertheless, with the aid of endoscopes and fiber-optic devices, most regions of the body are accessible for local application of PS and subsequent irradiation, e.g. ear, nose, oral cavity, gastrointestinal tract, urogenital tract or lungs (Wainwright, Maisch, et al. 2017). In the following paragraphs, two major fields for clinical application of aPDT are summarised:

**Oral infections**

In dentistry, there may be several conditions, where application of aPDT could be useful, either for restricting the use of antibiotics or for improving treatment outcomes (Gursoy et al. 2013): in periodontology, systemic use of antibiotics is a common treatment modality for severe cases of periodontitis in order to ensure favorable treatment outcomes (Slots 2004). As this is discussed more controversially nowadays (Preus et al. 2014), there has recently been an increasing interest in alternative approaches like aPDT (Gursoy et al. 2013). There have been some clinical studies on the effects of aPDT on the healing outcomes when applied as an adjunct to mechanical treatment of severe chronic (Tabenski et al. 2016) or aggressive periodontitis (Arweiler et al. 2014). Most of these clinical trials indicate substantially better clinical short-term healing outcomes with adjunctive aPDT in terms of reduced bleeding on probing and reduction of probing pocket depths as compared with mechanical treatment alone (Sgolastra et al. 2013; Sculean et al. 2015). Altogether, the clinical data about aPDT in periodontics is still limited and the outcomes are to some extent controversial as well (Sculean et al. 2015). Accordingly, in a recent systematic review, it was concluded that evidence supporting clinical long-term benefits of aPDT as an adjunct for treatment of periodontitis is not sufficient yet (Sgolastra et al. 2013). In contrast, it was recently concluded in a systematic review that use of aPDT in combination with mechanical debridement definitely brings significant improvements in patients suffering from peri-implantitis (Sivaramakrishnan and Sridharan 2018).

In endodontics, aPDT may be a valuable add-on for disinfection of heavily infected root canals or in view of so-called “single-visit endodontics” (Chrepa et al. 2014). Garcez et al. reported that adjunctive aPDT leads to a significant further reduction of bacterial load in root canals (Garcez et al. 2010). In addition, histopathological studies in dogs indicated more favorable healing of periapical osseous lesions and less inflammation after additional treatment with aPDT (Silva et al. 2012; Borsatto et al. 2016). Likewise, in a recent randomized clinical trial, it was found that additional application of aPDT resulted in better radiographic healing of primary endodontic infections six months after endodontic treatment as compared to conventional endodontic therapy alone (de Miranda and Colombo 2018).

There are also some clinical studies and case reports on the efficacy of aPDT for treatment of oral fungal infections, e.g. oral candidiasis and denture stomatitis (Javed et al. 2014), where aPDT was found to be as effective as standard antifungal treatment (Mima et al. 2012; Alves et al. 2017). Furthermore, aPDT was also suggested as an additional antimicrobial measure for treatment of deep carious lesions when carious dentin is only partially removed (Cieplik et al. 2017). However, huge difference in study design and quality (e.g. lack of controls) as well as the fact that almost only phenothiazinium dyes but no other classes of PS have been used as PS in clinical studies do not allow for general conclusions on the clinical efficacy of aPDT for treatment of oral infections.

**Skin infections and chronic wounds**

In dermatology, aPDT may be a valuable treatment modality for all kinds of skin infections from bacterial as well as fungal origin, but especially for treatment of chronic wounds (Maisch et al. 2004; Brown 2012; Baltazar et al. 2015). In this context, it is noteworthy that beneficial effects of aPDT on host tissues have been proposed (but not yet definitely confirmed), such
as stimulation of growth factors and immune response, potentially leading to enhanced wound healing, which may be valuable for the application of aPDT for treatment of chronic wounds or ulcers (Brown 2012).

In the first randomized, placebo-controlled clinical trial on aPDT in chronic wounds (chronic leg ulcers and diabetic foot ulcers), Morley et al. showed significant and broad-spectrum bacterial cell kill plus a promising trend towards accelerated wound healing employing the phenothiazinium derivative PPA904 as a PS (Morley et al. 2013). Likewise, when Lei et al. investigated the effects of 5-aminolevulinic acid-mediated aPDT for treatment of chronic skin ulcers in lower limbs infected with P. aeruginosa (as compared to treatment with red light alone), they found significantly better healing outcomes in the aPDT group in terms of reduction in the mean ulcer area (Lei et al. 2015). Use of aPDT was even suggested to be an option for diabetic foot ulcers for prevention of amputations (Tardivo et al. 2014). Tardivo et al. found in a randomized clinical trial that the amputation rate in the group treated with aPDT with phenothiazinium dyes Methylene Blue and Toluidine Blue was only 2.9% the amputation rate in the control group (Tardivo et al. 2014). aPDT may even prevent surgical debridement of diabetic foot ulcers and is considered as an efficient and affordable treatment method for the diabetic foot (Tardivo et al. 2017).

Future research directions (what we don’t know yet)

The “perfect” aPDT system

A “perfect” aPDT system should combine high antimicrobial efficacy without any serious side-effects for patients. Furthermore, its handling should be as easy as possible. For this instance, characteristics of both, the PS as well as the corresponding light source, must be considered. Nowadays, it is agreed that a PS should combine the following features (adopted and modified from (Cieplik, Tabenski, et al. 2014; Maisch 2015b)):

- Hydrophilicity and positive charge(s) for high-affinity to binding or attachment towards negatively charged bacterial cell walls (Alves et al. 2009)
- Low-molecular weight for facilitating penetration through biofilm structures (Cieplik et al. 2013)
- High \( ^1 \text{O}_2 \) quantum yield, reaction according to type II mechanism (Maisch et al. 2007; Cieplik et al. 2015)
- High-photostability during irradiation (Engel et al. 2008)
- No toxicity or mutagenicity in the dark towards eukaryotic cells as well as microorganisms (Soukos and Goodson 2011)
- “Therapeutic window” or effective concentration range, where microorganisms can be killed sufficiently without damage of eukaryotic cells (Muehler et al. 2017)

Recently, it was further discovered by the group of Michael R. Hamblin that the antimicrobial efficacy of given PS used for aPDT (e.g. Methylene Blue, Rose Bengal) can be potentiated by the addition of simple inorganic salts (Hamblin 2017), in particular potassium iodide (Vecchio et al. 2015; Huang et al. 2017; Wen et al. 2017). The proposed mechanism for this potentiating effect of iodide is likely due to reaction of singlet oxygen with iodide and formation of iodine radicals, hydrogen peroxide, and molecular iodine (Hamblin 2017).

For a light source to be employed in aPDT, it is crucial that its emission spectrum fits for the absorption spectrum of the respective PS used. Another vital point is the ability of the emitted light to penetrate biological tissues (e.g. skin) to such an extent that the appropriate tissue depth can be reached to be effective, which may even be more complicated in the presence of blood. Due to less scattering and absorption of biological tissues at higher wavelengths, near-infrared light (700–2500 nm) penetrates these tissues more efficiently than visible light, while at wavelengths longer than 950 nm, this effect is weakened due to increased absorption of water and lipids (Smith et al. 2009). Therefore, light from a window from 650–950 nm may be regarded as optimal (Smith et al. 2009). Furthermore, recently Piazena et al. reported local maxima of spectral transmittance for human skin at central wavelengths of 1082 nm and 1268 nm (Piazena et al. 2017). Besides that, it would be particularly favorable to use a light source with beneficial effects on eukaryotic tissues. For example, recently a light source was introduced emitting visible and water-filtered infrared-A light (Al-Ahmad et al. 2013; Karygianni et al. 2014; Al-Ahmad et al. 2015; Al-Ahmad et al. 2016), which is known for its wound healing capabilities (Hoffmann et al. 2016). This light source is designed in such a way that the whole incoherent radiation of a 3000 Kelvin halogen bulb is filtered by passing a cuvette containing water. The water filter absorbs some bands within infrared A and most parts of infrared B (1400–3000 nm) and C (3000–1000 000 nm), which leads to elimination of the thermal burden to the surface of the tissue caused by the reaction of the filtered components with the water content of biological tissues (Fuchs et al. 2004; Jung and Grune 2012). The remaining wIRA penetrates to
deeper layers of the tissue and causes therapeutically beneficial warming (+2.7 °C at a tissue depth of 2 cm), an increase of the oxygen partial pressure (+32% at a tissue depth of 2 cm) and blood circulation, and subsequently stimulates the metabolism of the cells including the immune reaction (Fuchs et al. 2004; Hartel et al. 2006; Mercer et al. 2008; Felbert von et al. 2010; Schumann et al. 2011).

Nevertheless, it must be considered in this regard that many PS with proven in vitro efficacy, such as phenalenones and porphyrins, exhibit their main absorption in the violet/blue spectrum (i.e. 400–500 nm) (Cieplik, Tabenski, et al. 2014). A potential approach to combine these PS with light from longer wavelengths with good tissue penetration capabilities may be the use of photon upconversion (UC), which refers to a process where sequential absorption of two or more photons leads to the emission of light at shorter wavelength than the excitation wavelength (Haase and Schäfer 2011). In recent years, high-quality UC-nanocrystals with controlled solubility, particle size, and optical properties have been synthesized (Haase and Schäfer 2011). Therefore, applying these UC-nanocrystals may allow for combining the benefits of effective PS with absorption in the violet/blue spectrum with the better tissue penetration properties of light from longer wavelengths (Hamblin 2018). Thus, linking aPDT and UC may merit further investigation.

Investigating the mechanism of aPDT

For evaluating the potential of development of resistances in pathogens towards aPDT, it is crucial to narrow down its mechanism and find the actual main target structures that are damaged or destroyed by the oxidative burst induced by aPDT. For this purpose, mostly biochemical methods and transmission electron microscopy have been employed so far because the use of fluorescence microscopy was limited by the small size of bacteria and the faint fluorescence properties, i.e. photo-bleaching, of many PS (Alves et al. 2014; Gollmer et al. 2017). However, as stated above, very recently Gollmer et al. were the first to apply novel highly-sensitive fluorescence microscopy to study the incorporation of PS inside bacteria (in this case: S. aureus) taking advantage of fluorescence enhancement of Methylene Blue and TMPyP upon entering the bacterial cytoplasm after aPDT-mediated oxidative deterioration of the cell wall and cytoplasmic membrane (Gollmer et al. 2017). This technique merits further investigations on distinct classes of PS, but also on more bacterial species with different Gram-staining characteristics.

Another versatile method may be the use of flow cytometry in combination with appropriate fluorescence dyes to assess distinct vital parameters of bacterial cells (Nebe-von Caron et al. 1998; Joux and Lebaron 2000). While commercially available as kits (e.g. LIVE/DEAD™ BacLight™ Bacterial Viability Kit, Molecular Probes) are often used in fluorescence microscopy, these dyes can also be employed for flow cytometry for quantitative assessment. Combinations of dyes like SYTO-9 and propidium iodide, for instance, can give information whether major damage of cytoplasmic membranes occurs due to a given treatment (Tawakoli et al. 2013; Cieplik, Steinwachs, et al. 2018). Many other fluorescent dyes are available assessing metabolic activity, membrane potential, or intracellular pH-values (Novo et al. 2000; Ambriz-Aviña et al. 2014). Flow cytometry applying many distinct fluorescent dyes, so called multi-parameter flow cytometry, therefore may be used for assessing the effects of aPDT on distinct cellular structures in a semi-quantitative approach (Novo et al. 2000).

These methods can be combined with the more traditional electron microscopic techniques, which allow for additional visualization. For instance, changes in the appearance of the cytoplasm (vacuolations, condensations, etc.) or of cytoplasmic membranes can be envisioned by means of transmission electron microscopy (Wood et al. 1999).

All in all, for deeper insights into the mechanism and damage patterns of aPDT towards bacteria distinct methods must be combined in order to resolve these complex mechanisms. Furthermore, it is mandatory to not only test one class of PS, as the sites of accumulation as well as the photo-physical properties (e.g. singlet oxygen quantum yields) of given PS can vary widely and will have huge impact on the damage patterns of aPDT.

Translation in clinical practice

While there has been a discovery void of conventional antibiotics and antiseptics during the last 20 years, lots of new classes of PS, such as phenalenones, fullerences, or derivatives of naturally occurring porphyrins, chlorins, and curcumins, have been developed (Wainwright, Maisch, et al. 2017), whereby many of them have shown promising results in in vitro studies so far (Cieplik, Tabenski, et al. 2014; Maisch 2015b). However, as stated above, many of these studies have been conducted using planktonic cultures, while it is well known that bacteria organized in biofilms exhibit an up to 1000-fold higher tolerance towards antimicrobials (Marsh 2004; Marsh et al. 2011).
For example, Methylene Blue, one of the three PS with clinical approval, has shown high efficacy in studies on planktonic bacteria (Gollmer et al. 2015; Núñez et al. 2015; Cieplik et al. 2016), but seems to be much less effective for inactivation of biofilms (Cieplik, Tabenski, et al. 2014). In a recent study from our group, we compared the antimicrobial photodynamic efficacy of Methylene Blue and the phenalenone derivative SAPYR for inactivation of monospecies biofilms of *Enterococcus faecalis* SAPYR for inactivation of monospecies biofilms of *Enterococcus faecalis* and *Actinomyces naeslundii* and found killing rates of about 5 log10 steps for SAPYR while Methylene Blue exhibited no reduction at all, when the number of absorbed photons were adjusted to equal amounts for both PS (Cieplik et al. 2015). Therefore, the clinical potential of aPDT may still be underestimated as many more effective PS, e.g. SAPYR, are not available for clinical application yet. This should be considered, when data from clinical studies, for instance in periodontology, is assessed.

For further pre-clinical evaluation of novel aPDT systems, it may be valuable to conduct studies in more complex biofilm models, which rather simulates the biofilms found in vivo in the oral cavity, on skin surfaces, in wounds, or on catheters. Therefore, instead of the currently used in vitro biofilm models comprising up to 10–15 species (Guggenheim et al. 2001; Belibasakis and Thurnheer 2014; Tabenski et al. 2014; Cieplik, Steinwachs, et al. 2018; Cieplik, Wimmer, et al. 2018), microcosm biofilms cultured from patient samples (Sissons 1997; Exterkate et al. 2010; Fernandez et al. 2017) or biofilms grown in situ (Karygianni et al. 2014; Al-Ahmad et al. 2015; Al-Ahmad et al. 2016) may represent the vast complexity of naturally occurring biofilms to a greater extent. Furthermore, animal models may be valuable as well in order to evaluate the clinical efficacy of aPDT, e.g. for treatment of periodontitis (de Oliveira et al. 2010) or infected burn wounds (Hamblin et al. 2002; Demidova et al. 2005). Finally, randomised clinical trials are mandatory. Thus, availability of funding is crucial, but still is limited for the field of aPDT, at least when compared to research on conventional antimicrobials (Czaplewski et al. 2016; Wainwright, Maisch, et al. 2017).

For later clinical application, the costs of a given new antimicrobial approach must be considered as well in relation to conventional antimicrobials on the market. An effective aPDT system, which is too expensive for under-developed countries or even emerging economies, will precede the uncontrolled over-the-counter purchase of conventional antimicrobial drugs in these countries, which in turn is known to contribute to the development of antibiotic resistance (Morgan et al. 2011).

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