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Strategies to optimize photosensitizers for photodynamic inactivation of bacteria

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ABSTRACT

The Infectious Diseases Society of America (IDSA) highlights that over the past several years, the number of new antibacterial drugs approved continues to decrease (Boucher et al., 2009) [1]. Bacteria are very good in developing resistance against antibiotics in a short time. Therefore new approaches like antibacterial photodynamic inactivation of bacteria (aPDI) will become more important in the future as antimicrobial resistance is expected to continue to increase. This review summarises the potential of the susceptibility of bacteria to aPDI and the strategies to optimize leading photosensitizers which are useful for aPDI. The most appropriate photosensitizers belonging to the chemical classes of phenothiazinium, porphyrine, fullerene and perinaphthenone. They all share the following characteristics: positively-charged, water-soluble and photostable. Taken together the most promising clinical applications of aPDI are (i) decolonization of pathogens on skin, (ii) treatments of the oral cavity like periodontitis and root canal infection and (iii) superinfected burn wounds, because these are relatively accessible for photosensitizer application and illumination.

1. Introduction

Today the increasing resistance of bacteria against antibiotics is one of the most important clinical challenges; the so-called "ESKAPE"-pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* strains) are the superbugs of the 21th century, because they can "escape" more or less any single kind of antibiotic treatment. The actual WHO's 2014 report on global surveillance of antimicrobial resistance reveals that "antibiotic resistance is no longer a prediction for the future; it is happening right now, across the world, and is putting at risk the ability to treat common infections in the community and hospitals [2]. Without urgent and coordinated action, the world is heading towards a post-antibiotic era, in which common infections and minor injuries, which have been treatable for decades, can once again kill" [2]. Both selection and evolution of antibiotic-resistant bacteria is a non-stoppable process due to the random rate of mutation in the bacteria that can provoke antibiotics ineffective within short time [3]. Therefore new approaches are needed acting as a multi-target process to avoid the development of resistances in bacteria. The antibacterial photodynamic inactivation of bacteria (aPDI) represents such a multi-target damaging process. No specific extracellular or intracellular localization of the photosensitizer is needed and no specific targets are in the focus for the oxidative burst mediated by aPDI [4].

Therefore aPDI will become more important in the future as antimicrobial resistance is expected to continue to increase.

2. General aspects of photodynamic inactivation of bacteria

At the beginning of the 20th century *Proteus vulgaris* was inactivated by the combination of a fluorescent dye, light and oxygen for the first time [5,6]. Tappeiner termed this interaction of light, oxygen and a dye as "photodynamic reaction". Nowadays the lethal effect of aPDI is based on the principle that visible light activates a photosensitizer to lead the formation of reactive oxygen species, which induces a phototoxic damage immediately during illumination. The absorption of light (visible wavelength range 400–700 nm) by the ground state of a PS leads to a transition to its singlet state and via intersystem crossing to its excited triplet state, than two mechanisms of action take place [7]. Type I photosensitization processes can produce different kinds of reactive intermediates. In the present of oxygen, type I processes can induce the formation of species like hydrogen peroxide (H_2O_2), superoxide radical anion ($O_2^{\bullet-}$) and hydroxyl radical (·OH) via Fenton reaction. These ROS are known to effectively oxidize a wide variety of biomolecules and ultimately cause substantial biological damage.

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In type II reactions the excited PS transfers energy to molecular oxygen and generates highly reactive singlet oxygen ($^1O_2$) which photo-oxidizes biomolecules like lipids and proteins leading to lysis of cell membranes. The singlet oxygen quantum yield Φ_Δ describes the ratio of the type II mechanism of a given photosensitizer [8]. In addition both type I and type II reactions can occur simultaneously. However several photosensitizers have shown different quantum yields of singlet oxygen.

The antimicrobial photodynamic process exhibits several positive aspects for the treatment of microbial infections, including a broad spectrum of action, the efficient inactivation of antibiotic-resistant strains, the low mutagenic potential, and the lack of selection of photoresistant microbial cells [4]. Therefore an appropriate photosensitizer for aPDI should fulfill the following criteria in order to have a pronounced antimicrobial efficacy (minimum > 3$\log_{10}$ reduction of CFU) and low toxicity towards mammalian cells ("therapeutic window"):

- High $^1O_2$ quantum yield.
- Photostable.
- Broad spectrum of antimicrobial action (bacteria, fungi, parasites).
- High binding affinity for microorganisms (positively charged PS for good adherence to negatively charged bacterial cell walls).
- Low binding affinity and low toxicity for mammalian cells.
- No mutagenicity (DNA damage must be avoided).
- No dark toxicity.
- Therapeutic window (photodynamic inactivation parameters necessary where bacteria are killed efficiently without damage of eukaryotic cells).

3. Susceptibility of bacteria to aPDI

In general bacteria have developed several mechanisms to elude oxidative stress from the environment. This protective system consists of an enzyme network of proteins like catalase, peroxidase or superoxide dismutase detoxifying reactive oxygen species. Furthermore anti-oxidative molecules like carotenoids quenching the singlet oxygen as well as the triplet state of chlorophyll in photosynthetic active organisms like cyanobacteria [9]. In *Rhodobacter sphaeroides* a model bacteria to study bacteria photosynthesis singlet oxygen was found as a direct inducer of an alternative $RpoH_{II}$-type sigma factor which is required for the expression of defense factors and that deletion of $RpoH_{II}$ leads to increased sensitivity against exposure to singlet oxygen originated by methylene blue and light [10]. Upon activation of the $RpoH_{II}$ genecluster an oxidative-stress defense system is expressed where proteins are involved for quenching of ROS, detoxification of peroxides and regulate redox and iron reactions. So far in human pathogenic bacteria defense systems against singlet oxygen itself are not present. In case of type-I induced ROS by a given photosensitizer, like methylene blue, TBO or curcumin, bacteria can produce oxidative-stress defense system to avoid an oxidative damage caused by aPDI. One major oxidative stress defense system is called the two stage *soxR* and *soxS* oxidative stress regulon [11]. Here the superoxide dismutase is one of the key player enzymes which metabolize super oxide anions to hydrogen peroxide and oxygen (Eq. (1)) [12,13].

$$(2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2) \quad (1)$$

Hydrogen peroxide itself can be scavenged by both alkyl hydroperoxide reductase (*ahpCF*) and catalase (*katEG*) to water and ground state oxygen [14,15]. Thereby Hydrogen peroxide serves as a sensor molecule for the transcription factor OxyR which regulates the *oxyR* gene regulon (catalases and peroxidases) [14,15]. Furthermore H_2O_2 oxidizes Fe^{2+} via Fenton reaction whereby hydroxyl radicals are generated [16] (Eq. (2))

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^* + OH^- \quad (2)$$

The hydroxyl radical reacts with many organic compounds (e.g. fatty acids) by removal of a hydrogen atom, forming water and an alkyl radical (Eq. (3)). Than the alkyl radical reacts rapidly with oxygen forming a peroxy radical (Eq. (4))

$$OH^* + R\text{-}H \rightarrow H_2O + R^* \quad (3)$$

$$R^* + O_2 \rightarrow R\text{-}O_2 \quad (4)$$

Besides singlet oxygen, hydroxyl radicals, super oxide anions and reactive oxygen intermediates derived from singlet oxygen are the meaningful molecules that induce oxidative stress in bacteria. In aPDI the photosensitizers produce different amounts of singlet oxygen depending on the chemical structure (e.g. methylene blue: Φ_Δ 0.52 [17], TMPyP: Φ_Δ 0.77 [18] and SAPyR: Φ_Δ 0.99 [19]). Depending on the localization of the photosensitizer singlet oxygen rapidly oxidizes all double bonds of fatty acids and proteins in the direct surrounding due to its high reactivity (+0.98 eV energy), short lifetime ($<10^{-6}$ s) and limited diffusion length [18,8]. In case a photosensitizer is only attached to the bacterial surface/cell wall area, oxidation of molecules occurs only at this site leading to loss of function of proteins, enzymes and fatty acids. Therefore intracellular localized defense systems cannot help bacteria to survive the oxidative burst induced by aPDI in case the photosensitizer is attached only to the surface of bacteria. In addition aPDI is a very fast and effective approach to inactivate multi-resistant bacteria. *In vitro* studies have shown that successful inactivation up to 6-$\log_{10}$ CFU are possible within seconds (incubation time plus illumination) [20]. Therefore it is under investigation how bacteria can develop defense strategies towards a lethal dose of aPDI which can occur within seconds. Typically a timespan of minutes is necessary that bacteria can react towards stress from the environment [21]. So far photodynamic killing of antibiotic resistant bacterial strains is feasible using the same parameters (concentration of PS, incubation time and light dose) as compared to the antibiotic sensitive strain of the same bacteria specie [22,23]. However Grinholc and colleagues demonstrated a strain-dependent inactivation efficacy of aPDI treated MSSA (methicillin-sensitive) or MRSA strains [24,25]. 4 out of 80 (40 MSSA and 40 MRSA) were less susceptible to aPDI [24]. These 4 strains were MRSA strains and the overall percentage of aPDI sensitive strains was higher in the MSSA group. The difference in the bactericidal effect of aPDI could not be correlated with an enhanced biofilm formation of the "resistant" strains, even though biofilm formation is known to be a protective mechanism of bacteria to survive stress situations [24]. Furthermore Nakonieczna et al. showed that superoxide dismutase was upregulated in aPDI sensitive *S. aureus* strains but does not directly influence the antimicrobial efficacy of the aPDI treatment [26]. Transcription levels of sodA and sodM were increased after protoporphyrin IX-based photodynamic treatment but only in PDI-sensitive strains. However the oxidative stress sensitivity caused by the lack of both sod enzymes can be relieved in the presence of SOD co-factors like Mn ions and partially by Fe ions [26]. Such an upregulation of SODs is part of the survival strategy of virulent bacteria. Isogenic *sodA*, *sodM* and *sodA sodM* mutants showed a reduced virulence compared to the parental strain in a subcutaneous mouse model of infection [27]. The results of this study showed the importance of bacterial enzymic superoxide scavenging systems for the survival of pathogens within the host.