Effectiveness of photodynamic therapy on Gram-negative bacteria

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The emergence of antibiotic resistance among pathogenic bacteria has lead efforts to find alternative antimicrobial therapeutics to which bacteria will not be easily able to develop resistance. Therefore, the development of alternative therapeutic modalities based on different strategies is being actively pursued. Photodynamic inactivation (PDI) can be a useful approach, particularly for the treatment of localized infections. This therapy is based on the oxidative destruction of biological molecules by active oxygen species generated by photo-excited molecules (photosensitizers). Studies have showed that there is a distinction in susceptibility to PDI between Gram-positive and Gram-negative bacteria. Gram-positive bacteria are more sensitive to photoinactivation than Gram-negative bacteria. This was attributed to the differences in structure and organization of the bacterial envelopes and cell walls. PDI is a promising alternative therapy mainly because there is no development of microbial resistance due to the existence of multiple targets. This chapter will focus on some aspects of the photodynamic inactivation of Gram-negative bacteria and some results obtained by our group with Escherichia coli and Pseudomonas aeruginosa using photosensitizers like Methylene Blue, Rose Bengal, Zn-phthalocyanine and Hypericin.

Keywords Photodynamic inactivation; resistance to antibiotics; Escherichia coli; Pseudomonas aeruginosa; photosensitizer.

1. Introduction

Since the discovery of the sulfonamides and penicillin between 1920s and 1930s by Alexander Fleming, antibiotics have been used to treat infectious diseases and have saved millions of lives. However, in the last years, their widespread and often indiscriminate use has developed antibacterial resistance, considered one of the most serious public health problems. Bacteria replicate very rapidly and a mutation that helps a microbe to survive in the presence of an antibiotic drug will quickly become predominant throughout the microbial population. Furthermore transferable genetic elements such as plasmids encoding resistance enzymes and efflux pumps can be transferred between species [1].

According to Jori et al, the evolution of antibacterial resistance is further exacerbated by several factors such as inappropriate or excessive prescription of antibiotics, the widespread addition of chemical compounds in the animal feed; increase global travel, the expansion of poverty among populations in third world countries and especially, wide range of adaptation mechanisms of microbial cells [2].

The development of new antibacterial therapy is an emergency for the treatment of localized infections. Photodynamic inactivation of microorganisms is a promising alternative to kill or eliminate pathogens that are infecting tissues [3]. Photodynamic inactivation involves synergistic combination of non-toxic dyes known as photosensitizers, molecular oxygen and low doses of visible light at an appropriate wavelength in order to produce reactive oxygen species (ROS) such as singlet oxygen and hydroxyl radical that are able to kill bacteria cells [4,5]. Unlike in conventional chemotherapy, there is no developing of cell resistance due to the unspecific oxidation of the many potential target molecules such as proteins, enzymes and unsaturated lipids [6]

There are significant differences in the effectiveness of Photodynamic Therapy (PDI) with respect to photoinactivation of Gram-positive and Gram-negative bacteria [7]. Gram-positive bacteria are rather more sensitive to neutral or anionic drugs due to their highly negatively charged surface [1,8]. This difference in susceptibility between the two bacterial classifications is explained by their physiology. Gram-positive species have a relatively simple envelope and consists of just two layers, the cytoplasmic membrane surrounded by a relatively porous cell wall composed of peptidoglycan and lipoteichoic acids that allows photosensitizer to cross [9,10]. The Gram-negative bacteria envelope is a highly complex multilayered structure, which consists of an inner cytoplasmic membrane and an outer membrane that is separated by the peptidoglycan-containing periplasm. The outer membrane forms an effective permeability barrier between the cell and its environment, tending to restrict the binding and penetration of many photosensitizer structures [11]. This document shows the importance of the choice of the photosensitizer concerning its chemical structure, charge and hidrofobicity to reach photoinactivation of Gram-negative bacteria. We present some of our results with Escherichia coli and Pseudomonas aeruginosa using the photosensitizers Methylene Blue, Rose Bengal as well as Hypericin and Zn-phthalocyanine.
2. Photodynamic Inactivation

2.1. General aspects

The increasing occurrence of multi-antibiotic resistant bacteria has caused large diffusion of infectious diseases that represents a major challenge for human health worldwide [8,12]. The development of new antibiotics should be continued as they are of primary importance to maintain the effectiveness of antimicrobial treatment. However, experience in the past decades has shown that new antibiotics may be effective for a restricted period only [13]. This way, it became urgent the need to develop new procedures to prevent the microbial growth. Photodynamic inactivation of microorganisms is a promising alternative that was discovered more than 100 years ago by Oscar Raab. This author first observed the phototoxicity of acridine orange against Paramecium caudatum [5,8,14]. Jesionek and Von Tappeiner reported that light combined with sensitizing agents and oxygen could destroy cells and coined the term photodynamic action or photodynamic effect [15]. Despite these findings, practical application of this phenomenon did not appear until the second half of the 20th century. PDI was first proposed for use in the area of tumor therapy and later the use in antimicrobial therapy was studied [8,16].

PDI is based on the oxidative destruction of biological molecules by active oxygen species generated by photo-excited molecules (photosensitizers) in the presence of oxygen [10]. The main advantages of this therapy are: 1) due to the large number of potential targets which produces cell damages to microorganisms, there is no cell resistance development using this method; 2) photosensitizer is localized preferentially in bacteria and not in tissue or human cells; 3) it is possible to reach a selective photoinactivation [6,12,17] i.e., the photocytotoxic action against microbial cells with minimal damage to the host tissues.

2.2. Mechanism of photoinactivation

Irradiation of a photosensitizer with light at an appropriate wavelength, in the presence of oxygen, produces free radicals and singlet oxygen that causes cell damages that will lead to microorganisms inactivation [1,5]. According to Salmon-Divon et al, bacterial PDI is based on three concepts: the accumulation of a photosensitizer is preferably in the microbial cell; the targeted illumination induces photochemical activation of molecular oxygen into its excited state (singlet oxygen), a highly reactive oxygen species; singlet oxygen reacts with various neighboring macromolecule targets in the bacteria and produce a lethal damage to cell [11].

Two oxidative mechanisms have been proposed to explain photodynamic microbial damage at the molecular level. In type I photo-damage reaction, the photosensitizer interacts with a biomolecule producing free radicals. This process occurs through the electron transference between the photosensitizer in the triplet excited state and components of the system, generating superoxide anion radical [10,16]. Type II reaction is generally accept as the major pathway in photo-oxidative microbial cell damage, by which singlet molecular oxygen is produced through the energy transfer of the photosensitizer in triplet state [10].

There are differences in susceptibility to PDI between Gram-positive and Gram-negative bacteria [1,2,4,5,8,10,16,18,19]. Usually, even molecules such as anionic and neutral photosensitizers can bind efficiently with Gram-positive bacteria and induce growth inhibition or death under visible light [5]. On the other side, due to the Gram-negative bacteria, a highly complex multilayered structure, several approaches have been tested in order to enable PDI of this type of bacteria [18-21]. The polycationic peptide polymyxin B nonapeptide (PMBN) can increase the permeability of the Gram-negative outer membrane allowing the penetration of the photosensitizer that is normally excluded from the cell [11,19]. Another approach is based on the use of ethylenediaminetetraacetic acid (EDTA) [19,22] or cationic photosensitizers. These dyes can increase the efficiency of the photoinactivation process in Gram-negative bacteria once the positive charges on the photosensitizer molecule appear to promote a tight electrostatic interaction with negatively charged sites at the outer surface of the bacterial cells [8,23]. The mechanism by which the cationic photosensitizer works in Gram-negative bacteria is thought to be “the self-promoted uptake pathway” in which these cationic dyes first displace divalent cations, Ca$^{2+}$ and Mg$^{2+}$ from their position on the outer membrane where they can act as an anchor for the negatively charged lipopolysaccharide (LPS) molecules [1]. This process leads to disorganization of the outer membrane and increases the permeability of the bacterial cells that became more sensitive to cationic compounds [1,23].

2.3. Antimicrobial photosensitizers agents

The effectiveness of PDI requires a photosensitive substance in adequate concentration, light in a wavelength as close as possible to the maximum absorption of the photosensitizer and an appropriate light dose.

A large number of compounds with photodynamic activity are now available: phenothiazines like methylene blue (MB); xanthenes like rose bengal (RB), cationic zinc phthalocyanines and anthraquinone-derivative like hypericin (Figure 1).
Methylene blue is widely known as a histological dye [25]. The characteristic color of MB is caused by the strong absorption band in the 550-700 nm region with maximum molar absorptivity of 85,000 M$^{-1}$ cm$^{-1}$ at 664 nm [25-26]. Many studies showed the effectiveness of MB to reduce the microorganisms survival. Wainwright et al, have shown photobactericidal activity of MB against vancomycin resistant Enterococcus spp (400-1000 μmol L$^{-1}$) and methicillin resistant strains of Staphylococcus aureus (5-50 μmol L$^{-1}$) at 6.3 J cm$^{-2}$ [27-28].

Figure 2 shows that MB induced a decrease in the survival of two the Gram-negative bacteria strains after 10 minutes of exposure to light (6 and 12 J cm$^{-2}$) at 630 nm and concentrations up to 10 μmol L$^{-1}$. Increasing the light dose from 6 to 12 J cm$^{-2}$, the reduction in the survival raised by 2 log for P. aeruginosa and by 1 log for E. coli cells. Thus, the best condition of inactivation of Gram-negative bacteria with MB is incubation for 10 minutes, concentration of 10 μmol L$^{-1}$ and irradiation with 12 J cm$^{-2}$ in order to reach a decrease of 5 log for P. aeruginosa and 4 log for E. coli.

Rose bengal is a cyclic compound that contains three aromatic rings in a linear arrangement and an oxygen atom in the center of the ring [29]. Rose Bengal has a high absorption coefficient in the 450-600 nm range and a tendency to transfer electrons from its excited triplet state, producing long-lived radicals [30-32]. Its photodynamic mechanism consists of the formation of 80% singlet oxygen and 20% superoxide anion [10,33]. Rossoni et al, studied the photoactivation of Enterobacteriaceae strains with 50 μmol L$^{-1}$ RB, irradiation with blue light (460 nm) for 180 s and 200 mW, reducing the number of CFU per milliliter by 4 log [34]. Demidova and Hamblin [35] compared the efficacy of RB and toluidine blue on S. aureus, E. coli and C. albicans and verified that toluidine blue was less effective than RB in all cases. Additionally, much higher concentrations of RB were needed to kill E. coli (35 μmol L$^{-1}$) and C. albicans (200 μmol L$^{-1}$) comparing with S. aureus (0.25 μmol L$^{-1}$) [35]. These authors suggested that the extracellular reactive oxygen species (ROS) are responsible for killing these species.
The photoinactivation of *E. coli* and *P. aeruginosa* with RB and MB obtained in our group are also presented in figure 2. It can be noted that in all the conditions, RB was more efficient to kill both microbial cells. At the concentration of 10 µmol L⁻¹ and incubation of 10 minutes followed by irradiation with 12 J cm⁻² at 450 nm RB is able to reduced 4 and 6 logs of *E. coli* and *P. aeruginosa*, respectively, whereas MB in the same conditions at 630 nm reduced 5 log of *P. aeruginosa* and 4 log of *E. coli*. This fact can be explained by the higher quantum yield of singlet oxygen production of RB compared with MB [36].

Phthalocyanines are heterocyclic adducts composed of a tetrapyrrole nucleus connected by nitrogen bridges which absorb between 660-700 nm [37]. These dyes are effective photosensitive substances which are resistant to chemical or photochemical degradation [37]. Photodynamic effect of the Zn-phthalocyanines has been found to be markedly influenced by their chemical structure [12,38]. According to Ryskova et al [8], after phthalocyanines irradiation, a large quantity of singlet oxygen species is generated and they are able to remain in the excited state for a longer period compared to methylene blue. Figure 3 presents our results with the Zn-phthalocyanine, showing that this photosensitizer at a concentration of 4.5 x 10⁻⁵ µmol L⁻¹ is able to inactivate *E. coli* cells (7 log reduction), while it is necessary 10 µmol L⁻¹ of MB to reduce just 5 log, in the same conditions (10 minutes incubation, light dose 12 J cm⁻²). The efficacy of the Zn-phthalocyanine in photoinactivation of methicillin-resistant *S. aureus* strains has been studied by Soncin et al, who observed that concentrations as low as 0.1 µmol L⁻¹ lead to 4-5 log decrease in the cell survival after 5 minutes exposure to light at a fluence rate of 50 mW cm⁻² [12]. Mantareva et al, showed that Gram-negative *P. aeruginosa* can be effectively treated with more water soluble derivative of cationic Zn-phthalocyanines but at high concentration (6 µmol L⁻¹) and dose (60 J cm⁻²) [39]. In our results (Figure 3), it can be observed that the Zn-phthalocyanine concentrations and light doses that promoted significant growth inhibitions in bacteria were lower than the ones used in previous studies.

![Fig. 3: Photosensitization of *E. coli* with Zn-phthalocyanine and Hypericin incubated by 10 minutes in two light doses: (A) 6 J cm⁻² and (B) 12 J cm⁻².](image)

Hypericin is a natural photoactive pigment that, according to reports, has antineoplastic and antidepressant properties. This dye belongs to the chemical class of the phenanthro-pyrene-quinones that are bio-synthesized by plants of the genus Hypericum, in which the most common is the *Hypericum perforatum* [6,40-41]. This natural substance has been used in traditional Chinese medicine for thousands of years [42]. Some studies have reported the use of hypericin as a potent antimicrobial agent [40-41] and this efficacy may be increased about 100-fold if the pigment is simultaneously exposed to visible light [42] when hypericin generates reactive oxygen species (ROS) [43]. Despite of hypericin being a neutral molecule, our results show (Figure 3) that it is able to photoinactivate *E. coli* after irradiation at 630 nm. The increase in light dose as well as in the photosensitizer concentration leads to increased bacteria inactivation as presented in figure 3. Hypericin at concentration of 4.5µmol L⁻¹ and a light dose of 12 J cm⁻² leads to a decrease in CFU of *E. Coli* (7.3 log reduction), almost twice when using 6 J cm⁻² (4 log reduction). Comparing the results of *E. coli* photoinactivation with hypericin with the other results, obtained using MB and RB, it is notable that MB and RB even at higher concentrations are not enough to reach the same bacteria inactivation as when hypericin is used.
3. Conclusions

The arising of a large variety of antimicrobial-resistant pathogenic microorganisms has led to increased rates of diseases and mortality caused by infections that were easily treated in the past. Many studies has evidenced that PDT could be used in the treatment of localized infections by bacteria without causing bacterial resistance.

The efficacy of PDT on the studied microorganisms depends on many factors: charge of the photosensitizer, incubation time, light dose, and the rate of drug uptake. Although cationic photosensitizers are more efficient to inactivate Gram-negative bacteria, we have showed the efficacy of neutral photosensitizer like hypericin. This may be due to higher production of singlet oxygen when using hypericin than using methylene blue [44]. All these photosensitizers present a photodynamic efficiency that is light dose and concentration dependent. The best results to photoinactivate Gram-negative bacteria obtained in our group were achieved with the Zn-phthalocyanine and hypericin, which even at very low concentration could reduce E.coli by 8 and 7 log, respectively after 10 minutes of incubation and 12 J cm$^{-2}$ light dose. The choice of the photosensitizer is very important for the appropriate administration, effectiveness and to avoid damage in the host tissues. Thus, antibacterial PDT can be an alternative to topical antibiotic therapy.

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References


