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Photodynamic treatment for multidrug-resistant Gram-negative bacteria: Perspectives for the treatment of *Klebsiella pneumoniae* infections

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ABSTRACT

The emergence of multi-drug resistance for pathogenic bacteria is one of the most pressing global threats to human health in the 21st century. Hence, the availability of new treatment becomes indispensable to prevent morbidity and mortality caused by infectious agents. This article reviews the antimicrobial properties of photodynamic therapy (PDT), which is based on the use of photosensitizers compounds (PSs). The PSs are non-toxic small molecules, which induce oxidative stress only under excitation with light. Then, the PDT has the advantage to be locally activated using phototherapy devices. We focus on PDT for the *Klebsiella pneumoniae*, as an example of Gram-negative bacteria, due to its relevance as an agent of health-associated infections (HAI) and a multi-drug resistant bacteria. *K. pneumoniae* is a fermentative bacillus, member of the Enterobacteriaceae family, which is most commonly associated with producing infection of the urinary tract (UTI) and pneumonia. *K. pneumoniae* infections may occur in deep organs such as bladder or lungs tissues; therefore, activating light must get access or penetrate tissues with sufficient power to produce effective PDT. Consequently, the rationale for selecting the most appropriate PSs, as well as photodynamic devices and photon fluence doses, were reviewed. Also, the mechanisms by which PDT activates the immune system and its importance to eradicate the infection successfully, are discussed.

1. Introduction

1.1. Multi-drugs resistance in Klebsiella pneumoniae

The emergence of multi-drug resistance in pathogenic bacteria is one of the most pressing global threats to human health in the 21st century. The WHO and US CDC described the situation as a global crisis and an impending catastrophe of a return to the pre-antibiotic era [1]. Thus, in the US, almost 23,000 persons die annually as a direct consequence of antibiotic-resistant infections [2]. Because bacteria have been accumulating progressively more resistance factors, it results in, progressively, less antimicrobial alternatives for effective treatment [3]. Taking this into account, the WHO has updated a list for global priority of antibiotic-resistant bacteria whom desperate require R&D to produce new antimicrobial options [4]. As shown in Table 1, the list considers as critic, at first priority, the multi-drug resistant Gram-negative bacteria; *Acinetobacter baumannii, Pseudomonas aeruginosa,* and Enterobacteriaceae producing extended-spectrum β-lactamase (ESBL) plus carbapenemase [4,5]. Those are members of the bacterial pathogens collectively named ESKAPE group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) [6]. Among them, *Klebsiella pneumoniae* stand out as a major health-associated infections (HAIs) producer (30%) [7,8]. *Klebsiella pneumoniae* is a fermentative Gram-negative bacillus, member of the family of Enterobacteriaceae, which is most commonly associated with producing infection of the urinary tract (UTI) and pneumonia [6,9]. Despite *K. pneumoniae* produce primary infections, most of these are HAIs presented in medically compromised patients [8,10]. The HAIs producers *K. pneumoniae* strains have increasingly acquired high levels of antimicrobial drug resistance, reaching 61.4% for multidrug-resistant (MDR), 22% for extensively drug-resistant (XDR) and 1.8% for pan-drug-resistant (PDR) [11]. *Klebsiella pneumoniae* is naturally resistant to ampicillin through plasmid-encoded ampicillin-hydrolyzing β-lactamases, such as SHV-1

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Table 1
The priority list of WHO of bacteria for which new antibiotics are urgently needed.

Priority 1: CRITICAL	
1	*Acinetobacter baumannii*, carbapenem-resistant
2	*Pseudomonas aeruginosa*, carbapenem-resistant
3	Enterobacteriaceae, carbapenem-resistant, ESBL-producing
Priority 2: HIGH	
4	*Enterococcus faecium*, vancomycin-resistant
5	*Staphylococcus aureus*, methicillin-resistant, vancomycin-intermediate and resistant
6	*Helicobacter pylori*, clarithromycin-resistant
7	*Campylobacter* spp., fluoroquinolone-resistant
8	*Salmonellae*, fluoroquinolone-resistant
9	*Neisseria gonorrhoeae*, cephalosporin-resistant, fluoroquinolone-resistant
Priority 3: MEDIUM	
10	*Streptococcus pneumoniae*, penicillin-non-susceptible
11	*11Haemophilus influenzae*, ampicillin-resistant
12	*12Shigella* spp., fluoroquinolone-resistant

gene (and to a lesser extent TEM-1 and TEM-2 genes), but mostly remain sensitive for third-generation cephalosporins [3]. For strains of *K. pneumoniae* producing HAIs, a single point mutation of the blaSHV-1 gene generates a β-lactamase able to hydrolyze most penicillins and extended-spectrum cephalosporins (cefuroxime, third- and fourth-generation cephalosporins, and aztreonam) [12]. Those, extended-spectrum β-lactamase (ESBL) producer bacteria are MDR strains and belong to the Ambler class A of β-lactamases inhibited by β-lactamase inhibitors like clavulanic acid, sulbactam, or tazobactam [12]. An increasing number of *K. pneumoniae* strains are producers of newer β-lactamases, including the carbapenem-hydrolyzing enzymes that are found mostly in the hospital environment [11,13,14]. Carbapenemases are β-lactamases that hydrolyze penicillins, cephalosporins, carbapenems, and monobactams [12,15]. In many geographic areas, the carbapenemase-producing *Klebsiella pneumoniae* (KPC) has been established as an important nosocomial pathogen, mainly causing severe infections in critically ill patients like bloodstream infections of HAIs [14,16]. The most relevant carbapenemase enzyme carried by *K. pneumoniae* are carbapenemase-2 (bla_{KPC-2}) and New Delhi Metallo-β-lactamase-1 (NDM-1) [17].

In Latin America, carbapenemase-producing Enterobacteriaceae are widespread since 2005, when the first cases were isolated in Colombia, Brazil, and Argentina [18]. The first cases of *K. pneumoniae* bla_{KPC-2} isolated in Latin America were in two Colombian patients who have not traveled abroad [19]. In Argentina, all of the 63 *K. pneumoniae* isolates of a surveillance study were of ST258 strain that belongs to the K1 serogroup, harboring the bla_{KPC-2} gene [20]. In Chile, the first case of a *Klebsiella pneumoniae* bla_{KPC} isolates was from a Chilean patient who brought the strain from Italy in 2012. The patient acquired this strain as a nosocomial disease during his hospitalization in an Italian hospital [21]. After this finding, active surveillance started in Chile, and between 2012 and 2013, were isolated from public hospitals more than 30 *K. pneumoniae* bla_{KPC} strains [22]. Seventeen of them were characterized by MLST and PFGE, resulting in 13 strains of the dominant MDR HAIs producer genotype of ST258 strain [22]. Then, carbapenemase-producing enterobacteria are a potential threat because these are difficult to find in routine cultures of patients from public hospitals [23]. Many of KPC also produce resistance to other antibiotics such as quinolone and aminoglycoside, mainly acquired in hospital environments [24]. Those strains that are XDR are a serious concern by the rapid increase of carbapenemase-producing isolates, expressing enzymes such as bla_{KPC-2} and NDM-1 [17]. For XDR bacteria, when causing serious infection, the treatment options are reduced to the use of tigecycline or Colistin [25]. In Chile, the isolation of Enterobacteriaceae PDR strains has been increasing in hospitals since 2004 particularly for the Gram-negative HAIs producing, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* isolated from ICU inpatients [25]. In Chilean public hospitals, the treatment for patients infected with PDR strains is efficient and based on the use of intravenous colistin, which costs is about U$ 120 a day for 10 or more days [25]. The overall prevalence of colistin-resistant Enterobacteriaceae is 0.67%, which is 0.4% for *K. pneumoniae* [26]. Thus, the global increase of pan-resistant Enterobacteriaceae has resulted in increased use of colistin even with agricultural purpose, with the risk of accelerating the inevitable emerging resistance [1,27]. Until late 2015, all described resistance mechanisms to polymyxins rest on chromosomally encoded genes, then resistance to colistin was not known to spread from cell to cell [28]. However, from intensive farming animal facilities where polymyxins are massively used, a resistance gene to colistin, the *mcr*-1 has emerged. The *mcr*-1 gene is encoded in a plasmid that spread laterally as it can conjugate and easily transfer the resistance to other bacteria [1]. Although conventional wastewater treatment reduces bacterial load, including strains that carry the *mcr*-1 gene, it has been reported that an increase of up to 2 logs in the abundance of bacteria carrying *mcr*-1 gene has occurred over a period of 5 years [29]. Although in Chile have not yet been reported, to date, the *mcr*-1 gene has been identified in several strains of Enterobacteriaceae producing HAI, in numerous countries across five continent, including Peru and Brazil [30–32]. Even in the absence of colistin use, the *mcr-1* gene may spread within hospital environments [33], hence raises the possibility that the spread of *mcr*-1 gene will not be contained [30]. Thus, the epidemiological evidence indicates that Chile is not free to the emergence of MDR HAIs producer strains and particularly the expansion of dominant strains of MDR *K. pneumoniae* such as the ST258. Then, this article is a revision of state of the art for the importance of *K. pneumoniae* as a HAIs producer, their MDR acquisition and we will review a new strategy for treatment based on photodynamic therapy (PDT). We also include a revision of the immune system activation by PDT and its importance for the success of the therapy.

1.2. Importance of K. pneumoniae in Healthcare-associated infection

Klebsiella pneumoniae can be present in the intestinal tract and the nasopharynx as a saprophyte at different carrying rates from 5 to 38%, or 1–6% respectively. These colonization rates increase in direct proportion to the length of stay into the hospital environment up to 77% and 19%, in the stool and nasopharynx, respectively [6]. The global rate of HAIs in the United States is approximately 5% and results in 88,000 death annually with an overall annual direct medical costs of $35.7–45 billion dollars during 2009 [34]. In Chile, similar global prevalence is observed, 4.8 HAIs per 100 patients, with 3% mortality were registered during 2015. In public hospitals in Chile, hospitalizations by UTI increase on average four times bed days when a HAIs complicate it and in the case of pneumonia increases up to 5.5 times [35,36]. The increase in bed days represents a general cost to the Chilean health service of US $ 70,000,000 calculated to the year 1993 [37]. In Chile the more prevalent HAIs are; UTI 1.03%, pneumonia without ventilation 0.6%, and bacteremia 0.34% [38]. Upon overall UTI-HAIs, the main etiological agents are *K. pneumonia* (24.66%) and *E. coli* (23.05%). For ventilator-associated pneumonia, Gram-negative bacteria are the most prevalent, where, although *K. pneumoniae* is ranked fourth, its prevalence is significantly high, 15% [38]. Urinary tract infection is the most common of the IAAS with a worldwide prevalence of 35% [39], being in Chile, up to 19.8% [38]. For inpatient at hospitals, 80% of all UTIs are related to the use of a urethral catheter. Finally, close to 15%–25% of hospitalized patients have an urethral catheter at some point during their stay, most of them for 2–4 days [40]. By the other hand, the main route of hospital pneumonia is by micro-aspirations due to operative procedures, such as sedation, intubation, and also vomiting or alteration of swallowing [41]. The mortality rates for hospital pneumonia are estimated at 10% and increase when pneumonia is associated with ventilation that can rise from

20% to 60%, depending on the patient and the severity of the underlying diseases [42]. The main HAIs, urinary tract infection and pneumonia lead to produce a significant increase in the time of hospitalization of patients, and in both *K. pneumoniae*, an MDR producer bacteria represents a high proportion. The control of *K. pneumoniae* infection is based in the innate and acquired immune response. It starts with physical barriers such as the mucociliary lining into the respiratory tract or the urine flow in the genitourinary tract. After overcoming the physical barrier, *K. pneumoniae* face humoral defenses like the complement that produce the bacterial lysis by forming a pore or guide the opsonophagocytosis [43]. The complement activation by either of three ways; classical, alternative or lectin, induce the release of pro-inflammatory mediators that attract innate immune cells. Cellular damage of infected tissues also induces the recruitment of immune cells into the infection area. The use of antibacterial therapies that activate the immune system to increase the elimination of bacteria would be a great contribution to solve infections that are difficult to eradicate. In this context, monoclonal anti-capsular antibodies have recently been developed that lead the death of *K. pneumoniae* due to opsonophagocytosis [44]. Although these therapies proved to be efficient in eradicating bacteria, this and other antibody-based therapies are strain specific for a limited number of serotypes. To treat the large number of capsular variants of *K. pneumoniae*, a less specific treatment should be developed. Photodynamic therapy, for example, has the quality of producing interspecific oxidative stress in bacterial cells, while stimulating the immune response [45].

2. Photodynamic therapy

In a scenario like this, therapeutic alternatives that have antimicrobial properties but different from antibiotics should be explored. The photodynamic therapy (PDT) and the use of small photosensitizer complex have been developed to produce locally activated cytotoxic action against cancer cells [45] and microorganisms [46], like *K. pneumoniae* [47]. The photosensitizers are non-toxic molecules, able to absorb the energy of specific wavelength and transfer it to oxygen molecules present in biological solutions to produce the activated forms of superoxide radicals ($OH^{\bullet}$) or singlet oxygen ($^1O_2$) [48]. Both, the $OH^{\bullet}$ and the $^1O_2$ may produce reactive species of oxygen (ROS) that have the ability to promoting bacterial cell death through oxidation of closer organic macromolecules. The oxidation of macromolecules, such as those contained in the plasma membrane; Proteins and lipids, or nucleic acids, resulting in non-specific bacterial death [49,50]. The photosensitizer molecules are exceptional in terms of its capacity to swipe to a triplet state the activated electron that jumped to a more energetic orbital in a singlet state when earning energy from light. As shown in Fig. 1, the triplet state is achieved by a spin-flip through an intersystem crossing [51]. The PS in triplet state recover its basal state by one of two types of photodynamic effects; donating an electron (Type I), to soluble oxygen producing the $OH^{\bullet}$, or transferring the energy (Type II) to a triplet molecular oxygen activating it to the unstable $^1O_2$ state [48].

2.1. Photosensitizer used for inactivation of K. pneumoniae

Although the PDT has been used to treat bacterial infection for a long time, most of them have a focus on periodontal health as treatment of halitosis and bacterial infection of the oral cavity [49,52]. Very few initiatives have explored the antimicrobial activity of these molecules to treat microorganisms producing non-superficial infections. Several works describe the use of PDT to treat infections with Gram-negative bacteria, for example, oral infections by *P. aeruginosa* [53], or also to treat urinary tract infections by *P. aeruginosa* or *E. coli* [54,55]. In Table 2, we summarized some of the most recent efforts to eradicate *K. pneumoniae* by PDT. For example, organic no-metallic molecules such as 5-aminolevulinic acids activated using white light inactivated *in vitro* planktonic growing cells and biofilm of *K. pneumoniae* in a concentration-dependent manner [47]. On the other hand, surfaces covered by polydimethylsiloxane nanocomposites in a quantum dot array were able to inactivate *K. pneumoniae* after 15 min of irradiation [56]. The use of phenothiazinium has shown an *in vitro* reduction of 3 $\log_{10}$ in biofilm formation of *K. pneumoniae* [57]. Moreover, conjugates of gold nanoparticles with monomeric methylene blue killed 97% of clinical isolates of MDR *K. pneumoniae* [58]. The Type II photodynamic effect was produced using iodine added to improve the photofrin activity over Gram-negative bacteria, including *K. pneumoniae* [59]. The use of a different photosensitizer was reported, as natural deep eutectic solvents were able to reduce the *K. pneumoniae* load *in vitro* when conjugated with citric acid [60]. The eutectic solvents have been suggested to be a third liquid phase in organisms, apart from water and lipids [61]. More interesting, there is a report in the literature of one effort to eradicate *K. pneumoniae* infections *in vivo*. In which, tincture of methylene blue was used to paint petechiae produced by infection of MDR *K. pneumoniae* in the mouth of domestic snakes. The inoculation site was subsequently irradiated with laser light of 660 nm for 80 s per point, achieving complete recovery of the patients [62]. One of the mechanisms of action of PDT when used in combination therapies with antibiotics, is that it decreases the MIC of the latter. This effect has been observed in both Gram-positive cocci and Gram-negative bacilli [63].

2.2. Selection of photosensitive molecules for in vivo use

The design and synthesis of photosensitizers are restricted because they must accomplish specific characteristics such as; be a good light absorber at low energy (near-infrared, with high molar absorption coefficient > 20,000 $M^{-1}\,cm^{-1}$) to minimize the dose of photosensitizer and to penetrate better the tissues. At these, absorption bands must have a triplet excited state of easy access to promote energy transfer from a photoexcited sensitizer molecule to generate highly cytotoxic singlet oxygen and destroy bacterial cells [64]. The photosensitizer molecule must have low levels of dark toxicity, and low probability of adverse pharmacological effects for both, humans and experimental animals. The preference for absorption at a red light wavelength or near-infrared is due to these wavelengths penetrate deeper into the tissues. Also, in tissues, both absorption and light scattering are minimized with longer wavelengths. Absorption bands at shorter wavelengths have less penetration and are more energetic, therefore are more likely to produce photosensitivity on the skin (the power of sunlight falls to $\lambda >$ 600-nm). Absorption bands at higher wavelengths (> 800 nm), would not produce photons with enough energy to raise the photosensitizers into a triplet state to transfer the energy to the molecular oxygen although a strategy of interstitial photodynamic therapy can be used [65]. Photosensitizers should have a long lifetime of the excited state (in the order of μs) to have efficient energy transfer with the oxygen molecules, and they must be ideally soluble in water or soluble in pharmacologically compatible vehicles. They should not be aggregated indissolubly in biological environments as this reduces their photochemical efficiency. In term of pharmacokinetics, patients clearance should be rapid, as well as < 24 h to avoid the need for light protection after treatment or photosensitivity [45,66]. As infections of the urinary tract or pneumonia caused by *K. pneumoniae* occur in internal organs like lung and bladder, the photosensitizer molecule of choice should be excited and have antimicrobial activity between wavelengths of 600 to 800 nm in order to penetrate the tissues [67].

To minimize side effects, and maximize the antimicrobial capacities, the photosensitizers must act preferentially over microorganism's cells rather than over eukaryotic animal cells. Some authors suggest the use of cationic PS that should electrostatically better interact with the anionic bacterial envelope rather than the almost neutral, mammalian cell membrane [68]. Besides, the chemical nature of photosensitizer will determine how much the PS will bind the bacterial envelope [69]. Many dyes intended to mark specifically bacterial structures can be

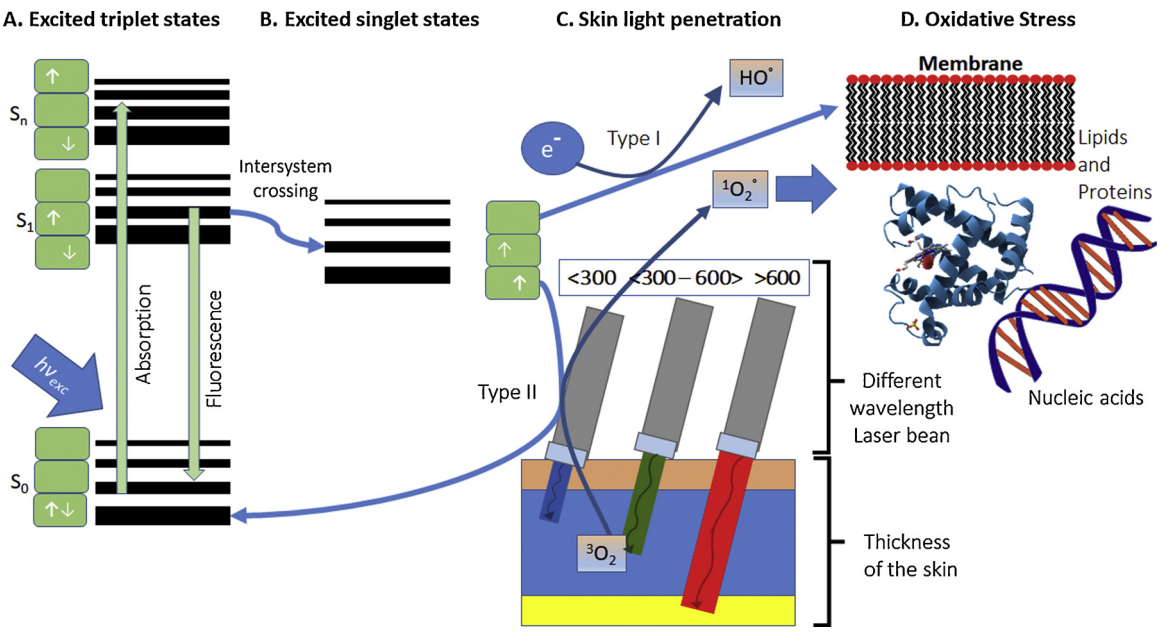

Fig. 1. Mechanism of operation of photodynamic therapy. A, Jablonski's diagram shows how an electron with a singlet ground state moves to a higher energy level when is excited by light-absorbing the quantized energy from photons. The electron can access from S_0 to singlet-excited state (such as S_1). **B**, From the S_1 by intersystem crossing process, the electron change their spin, stabilizing its state from the order of n-seconds to μ-seconds. **C**, the photosensitizer molecule, needs to be excited with enough energy; therefore, light must penetrate deep enough into the tissues or skin to reach it. From the excited state, the electron can return to its baseline state S_0, releasing the quantized energy transferring it to molecular oxygen. The molecular oxygen, which is in its basal triplet state ($^3O_2$), is in turn energized into an unstable excited state of singlet oxygen ($^1O_2^*$). **D**, Singlet oxygen is not a ROS, but it can interact with nearby macromolecules, producing the alkylation of proteins and lipids from cell membranes, as well as the disintegration of DNA.

used to direct the fate of the PS on the bacterial surface [70]. Some non-cationic porphyrins such as photofrin show good PDT activity over Gram-negative bacteria, but only when they are associated with an active radical [59]. One structure predominantly found in Gram-negative bacterial envelopes such as lipopolysaccharides and polysaccharides can be used to direct specifically the binding of PS molecules. For example, concanavalin-A help dextran coupled PS particles to bind the envelope rich in polysaccharides and kill multidrug-resistant Gram-negative bacteria [58]. It is essential to keep in mind that most of pathogenic *Klebsiella pneumoniae* have a bacterial envelope decorated with a thick polysaccharide capsule [6]. Hence, it is reasonable to design PS with the ability to selectively bind motifs found on the surface of *K. pneumoniae,* which could significantly increase its effectiveness and decrease adverse effects on host cells.

2.3. Light delivery strategies

The choice for the light delivery strategy should consider the PS nature (absorption, emission or cost), quality of the infection in terms of size of lesions, location, accessibility, and kind of surrounding tissue. The therapeutic effects will be obtained when a properly calculated dosimetry is applied, taking into account the aforementioned factors. For superficial lesions, irradiation of skin surface with a light-emitting lamp with enough power at the right wavelength should be enough. For example, an unplugged diode laser device emitting 3.5 W/cm^2 of light fluence was successfully used to treat *K. pneumoniae* infection in a stomatitis infection of domestic snakes [62]. However, if the localization of infection is in internal organs, devices that are more specific should be used. For infections located deep in the body or in bulky organs, optical fibers can be inserted directly into the affected tissue to deliver the light dose. This technique is denominated interstitial photodynamic therapy (iPDT) [65]. Certain kind of fiber arrays are considered better than others to ensure a homogenous delivery of light power, as cylindrical diffusers allow a shorter treatment duration than flat cleaved fibers [71]. The bladder is an organ that by its architecture and form, is an excellent target for phototherapy due to the ability to

Table 2
List for research and development of PDT for *K. pneumoniae*.

Photosensitizer	Technique	State	Application	Author
Methylene blue	Red light	In vivo	Oral mucosa	Eduardo, et al. 2019
Indocyanine green, EDTA*	Laser beam	In vitro	Planktonic bacteria and biofilms	Li, et al. 2019
Polydimethylsiloxane	Quantum dots	In vitro	Bacteria cultures	Marković, et al. 2019
Methylene blue	Fiber optic	In vivo	Intravesicular light delivery	Huang, et al. 2018
Monomeric methylene blue	Laser beam	In vitro	Bacteria cultures	Khan, et al. 2017
Methylene blue	Laser beam	In vivo	Snakes infection	Grego, et al. 2017
Eutectic solvents	Blue light	In vitro	Bacteria cultures	Wikene, et al. 2017
Zinc(II) phthalocyanines	Visible light	In vitro	MDR Bacteria cultures	Miretti, et al. 2017
Phenothiazinium	Laser beam	In vitro	Biofilms and planktonic cells	Misba, et al. 2017
Potassium Iodide	Blue light	In vitro	Bacteria cultures	Huang, et al. 2017
5-aminolevulinic acid	white light	In vitro	Biofilms and planktonic cells	Liu, et al. 2016
Fluorescent cephalosporins	white light	In vitro	Bacteria cultures	Xiao, et al. 2013
Methylene blue, TBO* and ALA*	white light	In vitro	Bacteria cultures	Yow, et al. 2011

*ALA = delta-aminolevulinic acid, TBO = toluidine blue O, EDTA = ethylenediamine tetraacetate.

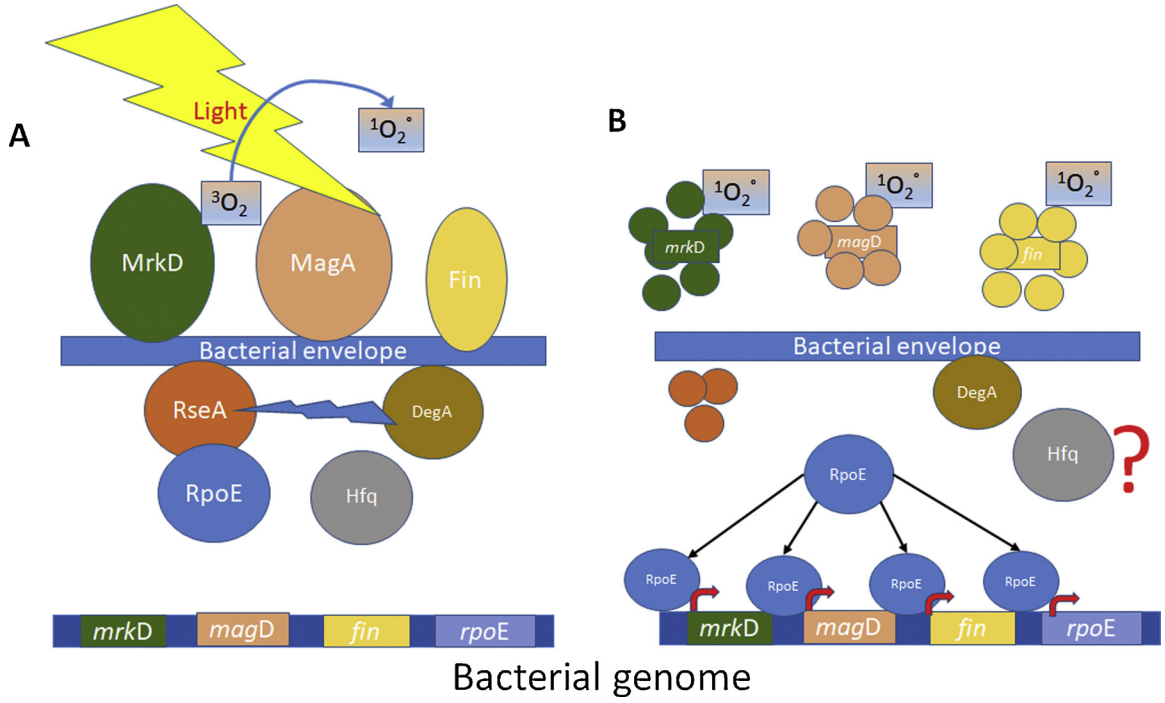

Fig. 2. Proposed model for the control of genes related to the bacterial envelope affected by photodynamic therapy. A. In the bacterial envelope, there are several extracytoplasmic factors, such as the pili protein (MrkD, Fin) or the capsule proteins (MagA). On the cytoplasmic side of the plasma membrane, is the extracytoplasmic regulatory protein, RpoE. The RpoE is inactive when it is bound to the repressor protein RseA. The photo-oxidative stress produced by PDT converts molecular oxygen into singlet oxygen, which causes damage to cell envelope proteins and lipids. Another cytoplasmic protein, DegA, senses damage to the envelope structures and becomes active. B, Activated DegA degrades the repressor protein DegA, releasing the RpoE transcriptional factor. The RpoE is a master gene that regulates the expression of 77–104 genes, many of them encode for extracytoplasmic structures, and possibly some of them are genes that encode capsule and fimbriae structures such as *mag*A, *mrk*D, and *fin* among others. The mechanism of action of the Hfq protein during photooxidative stress in Gram-negative bacteria is not yet fully understood.

disperse uniformly the light supplied. In clinical trials, 73% success rates have been achieved in the treatment of superficial bladder cancer, indicating a good penetration of photodynamic therapy using an argon laser on haematoporphyrin derivative [45]. More recently, the PDT was successfully applied even for treatment of muscle-invasive tumors in a model of rat bladder cancer. A glass fiber was used to irradiate transurethrally the rat´s bladder with a light dose of 10 J/cm^2 [72]. In the market, there are inflatable balloons able to adapt the shape of hollow organs like the bladder, with a strongly scattering material [73]. In conclusion, PDT can be applied for the treatment of infections located in almost all types of tissues or organs. Therefore, the best combination of PS, light delivery system, and photon flow dosimetry should be carefully chosen.

3. Oxidative stress in prokaryotes and *Klebsiella pneumoniae*

Oxidative stress is a well-known strategy that host cells use to eliminate pathogenic bacteria. Bacteria are exposed to reactive oxygen species (ROS). Two classes of ROS are generated, by electron transfer (type I) and by energy transfer (type II) [74]. Monocytes, neutrophils, and macrophages are cells of the innate immune system that, using NADPH oxidase, pump electrons into the phagocytic vacuole, producing mainly the type I ROS superoxide ($O_2^{\bullet -}$) from molecular oxygen. The superoxide generates hydrogen peroxide (H_2O_2) and other ROS like $HO^{\bullet}$ that kills or inhibit the growth of bacteria engulfed into the phagocytic vacuole [75]. Pathogenic bacteria can also be killed by exposition to oxidative stress out of cells into luminal space of gut by H_2O_2 released by resident microbiota [76] or by intestinal cells [77]. On the other hand, Type II ROS are produced by photooxidative stress and by macrophages respiratory burst that generates highly reactive $^1O_2$ [78]. ROS compounds affect multiple prokaryotic targets at once, for example, the H_2O_2 diffuse through the prokaryotic envelope and induce oxidation of amino acids, and transcriptional stress [79]. Consequently, bacteria produce several direct oxidantive detoxification enzymes. In *Escherichia coli* the stress by H_2O_2 is sensed by the two components regulator *oxy*R/S that controls the expression of several genes such as; *kat*G (hydroperoxidase) *ahp*CF (alkyl hydroperoxide reductase), *oxy*S (a regulatory protein), *dps* (a non-specific DNA-binding protein), *fur* (ferric uptake regulation), *gor*A (glutathione reductase), and *grx*A (glutaredoxin) [80]. Stress by superoxide and nitric oxide is sensed in *E. coli* by a different signal cascade, controlled by the *sox*R regulator that activates the expression of only one gene, *sox*S. The activated *sox*S regulates the expression of superoxide dismutase *sod*A, *sod*B, and *sod*C homologs [81]. A blast search revealed an 89% identity of *E. coli sod*A to an orthologous in *Klebsiella pneumoniae* in the position 75569–76184 of the genome of the strain HS11286.

There are not many studies explaining the bacterial response to $^1O_2$ induced oxidative stress, and most of the knowledge is in the photosynthetic α –proteobacteria, *Rhodobacter sphaeroides*. The response to photooxidative stress in *R. sphaeroides* is initiated by small noncoding RNAs (sRNA) and the RNA chaperone Hfq that activate the alternative sigma factor RpoE [82]. The sRNAs such as; RSP_1090 and RSP_1091 are activated by exposure to $^1O_2$, or by sunlight itself, and promote the release of the RpoE protein from a chaperone that keeps it inactive, the anti-sigma factor ChrR [82]. The RpoE protein is the extra-cytoplasmic stress-response sigma factor-24 (σ^{24}). In *E. coli* controls the expression of about 77–106 genes such as *rpo*E itself, periplasmic folding catalysts, biosynthesis genes for lipid A and membrane-derived oligosaccharides [83,84]. A BLAST search revealed that neither the anti RpoE *chr*R gene nor the sRNAs; RSP_1090 or RSP_1091 have their orthologs in any sequence of *K. pneumoniae* available on GenBank. On the other hand, in *E. coli*, *rpo*E is negatively regulated by binding to the RseA membrane

protein and is released by the proteolytic activity of DegA protein on RseA [84]. The DegA activity initiates after sensing damage on the envelope by the accumulation of misfolded membrane proteins or periplasmic proteins [84]. Both *rse*A and *deg*A genes are present in *K. pneumoniae* genome (Blast search). In addition, a blast search found that RNA-binding Hfq gene is present in all available *K. pneumoniae* sequences strains (Fig. 2A). In *E. coli*, the Hfq protein modulates the envelope stress response mediated by the *rpo*E regulator [85,86]. The Hfq protein is involved in post-transcriptional regulation of gene expression in environmental adaptation and virulence by binding sRNAs [87–89]. Also, the Hfq protein modulates motility and promotes resistance to cellular stresses such as oxidative stress or low pH [90]. Although it is unclear whether there is specificity in response to each type of ROS, whether there is a cross-protective ability of proteins in each stress response or whether cellular damage caused by $^1O_2$ can increase the production of other ROS [74], envelope damage seems to be sensed by *rpo*E activating mechanisms. The *rpo*E regulon gene composition suggests a significant role in manteinng the integrity of the bacterial envelope [84]. Besides, the sRNAs specific response showed by *R. sphaeroides* bacteria, is essential during the *rpo*E regulon activation by the $^1O_2$ stress [91]. Additionally, multiple omic data of *K. pneumoniae* transcriptome found 15 sRNA and 185 antisense RNA [92]. Nine of the sRNAs, found in the *K. pneumoniae genome* bind Hfq protein; RyhB, SraL, SroB, SraC/RyeA, MicF, SraD, GcvB, SraH, and IsrN [92].

3.1. Modulation of Virulence factors of Klebsiella pneumoniae during photooxidative stress

Klebsiella pneumoniae has evolved into two distinct epidemiologically and clinically defined pathotypes; the classical strains (cKP) and the hypervirulent strains (hvKP) [93]. The cKP strains are responsible for most of the infections mainly found in hospitals and are prone to acquire antimicrobial resistance [15]. On the other hand, the hvKP strains are not associated with HAIs, do not produce MDR strains and although are a serious life threat for young, healthy people from the community, its dissemination are circumscribed to Asia [93]. As a Gram-negative bacteria, *K. pneumoniae* shows several virulence factors common to Enterobacteria [6]. Factors such as fimbriae, capsule, enterobactin, and biofilm formation are found in almost all *K. pneumoniae* strains and represent the classical virulence factors [6,94]. Among those, in *K. pneumoniae,* the pili factors are encoded in the genome, by the Type 1 pili (T1P), Type 3 pili (T3P) and the *E. coli* common pilus (ECP) genes [95]. The pili factors are required for initial colonization of epithelial cells from the trachea, renal tubule, lung tissues, and biofilm formation [96]. The T1P participation in the pathogenesis of lower UTI as well as for pyelonephritis is amply documented [96]. The T3P is also associated with UTI [96], and its biosynthesis is encoded on the *mrk*ABCDF operon [10]. On the other hand, one of the most important virulence determinants of *K. pneumoniae* is the polysaccharide capsule, which is involved, among others, in resistance to opsonophagocytic complement-mediated killing [6] and inhibition of differentiation and functional capacity of macrophages [9]. The capsule biosynthesis is encoded by an operon of approximately nine open reading frames (ORFs) located in, the capsular polysaccharide synthesis (CPS) region of 25 kb in length and contain a total of 20 ORFs [97]. One characteristic for virulence of *K. pneumoniae* associated to capsule is the production of a colony phenotype known as hypermucoviscous, an extreme “stickiness” of these colonies on agar plates [9]. The hypermucoviscous phenotype has been associated with invasive nature that correlates with invasive liver abscess syndrome, metastatic infections and bacteremia [9]. The production of the hypermucoviscous phenotype had associated with the expression of mucoviscosity-associated gene A (*mag*A) and the regulator of mucoid phenotype A gene (*rmp*A) [98]. These genes are related to the biosynthesis of polysaccharide capsules where mutants for *mag*A lose their network of exopolysaccharides and become susceptible to serum and phagocytosis [99]. The *mag*A gene was renamed as wzy_k1 (AB355924) [100], is part of the capsule biosynthesis operon into the CPS region and is restricted to the K1 serotype [97]. The *rmp*A gene is a plasmid-encoded regulator, of the extracapsular polysaccharide synthesis and was first related to producing the mucoid phenotype virulence factor, distinct from capsule synthesis [97]. It is also more prevalent in hypermucoviscous strains than regular capsule producing strains, and in ESBL producing strains of *K. pneumoniae* [101]. In *K. pneumoniae,* the orthologous of *oxy*R was linked to resistance to H_2O_2 exposure and for intestinal colonization through fimbrial synthesis and biofilm formation [102]. The OxyR regulates the expression of genes encoding the fimbrial virulence factors types 1 (*fin*) and 3 (*mrk*D) during *K. pneumoniae* colonization of gut [102] and resistance-modulation-cell division *acr*B gene [103]. Since the sigma factor *rpo*E is activated upon damage occurs in the cell envelope, it is very likely that it promotes the expression of genes involved in the biosynthesis of the polysaccharide capsule such as *mag*A and *rmp*A or the fimbria like *mrk*D (Fig. 2B).

4. Photodynamic and the immune response over microbial infection

Photodynamic therapy induces a strong inflammatory reaction that occurs at the site on which the illumination is applied [104]. Most of the knowledge of the inflammatory processes caused by PDT is based on the experienced for cancer treatment [45]. For the PDT treatment of cancer cells, innate and acquired immune response are both indispensable to produce the therapeutic outcome. In an example, the occlusion of blood vessels that irrigate treated tissues, eliminate any therapeutic activity of the PDT [105]. The PDT not only influence the local tissues but also influence systemically modulating the population profile of immune cells and the cytokines levels in peripheral blood. For example, in patients for neck and head cancer, modulate the absolute numbers and the frequency of $CD4^+$ regulatory T lymphocyte (T_{reg}) and NK cells [106]. In the same work, the levels of circulating HMGB1, IL-6, and IL-10 cytokines were also modulated by PDT [106]. In addition, in immunocompromised mice the bone marrow transplant results in the reconstruction of anti-tumoral immune response and restoration of the lost effectiveness of PDT [107]. However, the photodynamic effect to overcome the microbial infection is dependent on the innate immune response and is less dependent on the acquired immune response [108]. Hamblin and co-workers have contributed most of the knowledge for the effect of PDT in the immune response to bacteria [46]. This response is based on the recruitment of innate immune cells as the main source of therapeutic effectors promoted by the host. For example, in a model of bacterial arthritis, the recruitment and accumulation of polymorphonuclear neutrophil cells (PMN) are essential for the therapeutic effect of PDT [109,110]. While there is evidence that MDR *K. pneumoniae* have developed strategies to overcome the normal killing action of PMNs, they remain the main effector of innate immunity [111,112]. The cell recruitment is an induced response of innate immunity promoted by signaling released by damaged epithelial cells as well as innate immune cells. The traumatic injury over local cells induced by phototherapy promotes the release of immunogenic damage-associated molecular patterns (DAMPs), which are host self-derived danger signals [113]. Those molecules are normally hidden into cellular structures, released and exposed by dying cells and are sensed by innate cells receptors such as TLRs [113]. Korbelik et al. 2006 suggest differentiating the DAMPs molecules derived from PDT action in three major groups: cell-derived molecules, extracellular matrix degradation, and extravasated plasma proteins [114]. Some cell-derived molecules like heat shock proteins (HSPs) [115], phospholipase and arachidonic acid [116,117] elicit an effectively innate-immune response. This immune response initiates by signaling through transcriptional regulators such as NF-κB or AP1 that induce the expression of several cytokines and cell mediators [107]. For example, the Hspa1b molecule produced by fibroblast exposed to PDT, ligate the TLR-2 and TLR-4 receptors on the

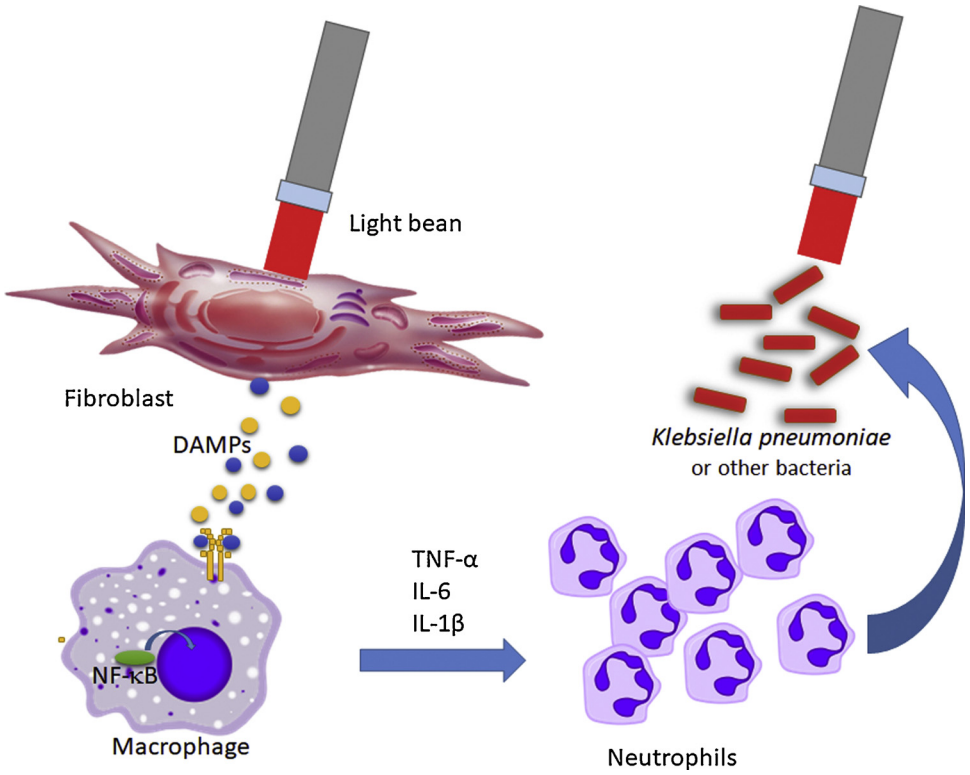

Fig. 3. Mechanism of action of immune system stimulation. The light that affects the area where the photodynamic treatment was applied causes damage to skin epithelial cells. Among them, for example, fibroblasts cells release DAMPs that are generally hidden from the immune system. DAMPs serve as markers of damage and bind pattern recognition receptors found on the surface of macrophages, such as TLR-2 and TLR-4. These, when bound to their ligand, activate a signaling cascade that culminates with the activation of the transcriptional factor NF-kB. The activation of NF-kB activates the expression and release of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β. The pro-inflammatory cytokines produce the recruitment and activation of leukocytes of innate immunity, such as polymorphonuclear cells (PMN). PMNs activated by pro-inflammatory cytokines have high activity against bacteria found in the area. Meanwhile, photodynamic treatment exerts an effect on the cellular structures of bacteria, weakening and killing them, facilitating the action of effector cells of the innate immune system.

macrophage cell membrane that via NF-κB pathway induces the expression of the inflammatory cytokines TNF-α, IL-6 and IL-1β [118]. The release of innate-immunity mediators such as HSPs, and arachidonic acid, and also complement proteins, and all those attract immune cells into the injured tissue [119,120]. Macrophages and dendritic cells are recruited with the primary function to eliminate the DAMPs, and cellular debrides originated by photoreactivity induced cytotoxic damage, to facilitate the local healing of the affected tissues [113]. The immunomodulatory effects result in an increased number of macrophages, monocytes, mast cells, and dendritic cells that induce PMN activation [109]. Also, the activation of the complement components like production of C3a accompanied to the overexpression of its receptor C3aR participate in PMNs and macrophages activation [121]. The magnitud of circulating PMNs activation and their onsite accumulation after PDT treatment is directly proportional to the efficacy of the therapy [122]. Hence, the participation of PMNs activated by PDT treatment should be the most important effector mechanisms deploy by host immunity to solve the tissue infections [123,124]. Indeed, the TDP induced damage over PMNs must be minimized, to affect not greatly its participation as effector agents [125]. Furthermore, the best antibacterial effect can be obtained when optimal photon fluxes are achieved where they are high enough to generate a therapeutic effect, but low enough not to affect neutrophil survival [125]. As shown in Fig. 3, the single photooxidative effect on the bacterial cell structure is not sufficient to eliminate the infection in vivo, but requires the activation of the innate immune response by the PDT.

5. Conclusions

In conclusion, the low generation of new antibiotics effective against bacteria resistant to multiple drugs, and their rapid dissemination makes essential to find different therapeutic alternatives. Photodynamic therapy can help where there is an empty therapeutic space, facilitating the elimination of infection when antibiotic options are exhausted and avoiding the progression of resistance. Many efforts are being made to produce new photosensitizing molecules in cancer therapy. At the same time, safety and efficacy tests are being carried out for these new molecules. These same platforms can be used for development, proof of concept, and clinical studies that would facilitate the availability of photodynamic products against infections for clinical use in humans.

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