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Antimicrobial activity of photodynamic therapy in combination with colistin against a pan-drug resistant *Acinetobacter baumannii* isolated from burn patient

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ABSTRACT

Nosocomially-acquired multi-, extensively-, and pandrug resistant (MDR, XDR, and PDR) strains of microorganisms such as *Acinetobacter baumannii* remain a serious cause of infection and septic mortality in burn patients. Treatment of patients with nosocomial burn wound infections is often complicated by drug-resistant strains of *A. baumannii*. Today, many researchers are focusing on the investigation of novel non-antibiotic strategies such as photodynamic therapy (PDT). We report a new PDT strategy that suppresses colistin resistance in PDR *A. baumannii* by interfering with the expression of a pmrA/pmrB two-component system. In the current study, *A. baumannii* with a PDR feature isolated from a burn patient was used as a test strain. PDT was carried out using toluidine blue O (TBO) and light-emitting diode (LED) as a photosensitizer and radiation source, respectively. The antimicrobial susceptibility profiles were assessed for cells surviving PDT. The effects of sub-lethal PDT (sPDT) on the expression of the pmrA/pmrB two-component signal transduction system were evaluated by real-time quantitative reverse transcription PCR. Results of drug susceptibly testing (DST) in LED and TBO groups separately showed that the bacteria were resistant to all tested antibiotics, while the DST result of the LED + TBO group showed highly declining bacterial growth when compared with the control group. Reduction in the expression of *pmrA* and *pmrB* was observed in the treated strains after sPDT. This represents the first conclusive example of a direct role for the PDT in breaking antibiotic resistance by directly modulating two-component system activity.

1. Introduction

Burn wounds are commonly infected with bacterial pathogens and have a high risk of infection by nosocomially-acquired multidrug-resistant (MDR) bacteria. These patients are also immunosuppressed, which makes them susceptible to many infection agents [1]. In deep partial-thickness and full-thickness burns, removing devitalized tissue, early excision, grafting, and topical antimicrobial therapy is the current standard of care and the primary method for reducing infection risk and length of hospital stay [2,3]. Insufficient initial antimicrobial therapy to treat burn wound infections results in higher mortality rates [2]. If a Gram-negative MDR pathogen is isolated from burn wound infections, colistin should be considered [2,3]. However, not only does colistin have a narrow therapeutic window, but worldwide reports indicate that the extensive use of colistin as a last resort to control gram-negative MDR pathogen infections has led to a worrying growing trend of colistin resistance among this bacteria [4]. This presents serious challenges to clinicians facing lack of affordable and effective treat-

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ment of critically ill patients with MDR pathogen infections and has prompted photodynamic therapy (PDT) as adjunctive therapy in the treatment of antibiotic-resistant localized infections [5].

Acinetobacter baumannii is one of the most prevalent Gram-negative MDR nosocomial agents [6–8], which cause severe infection in burn patients and leads to a high mortality rate [9,10]. Treatment of patients who are infected with these bacteria is very complicated [4,11]. New strains of *A. baumannii* have emerged which are resistant to the colistin that was re-introduced as the drug of "last resort" [12]. The major mechanism of colistin resistance in *A. baumannii* is the modification of the lipid A moiety of lipopolysaccharide (LPS) with phosphoethanolamine as a result of mutations in a two-component signal transduction system, pmrA/pmrB, which leads to the up-regulated expression of the pmr operon [13]. Today many researchers pointed out that the investigation of novel non-antibiotic strategies, which can combat against infectious diseases, should be introduced, and research projects should be developed in this area [14–18].

One of these approaches is light-based antimicrobial therapy [15–23]. This strategy relies on the ability to eradicate microbes regardless of antibiotic resistance, and the rates and mechanisms of resistance to such methods are very rare among microbial populations [24]. Briefly, the basis of the photodynamic therapy (PDT) mechanism is a combination of nontoxic photosensitizers (PSs) and harmless visible light that produce reactive oxygen species (ROS). The ROS's are able to oxidize biomolecules and thereby kill cells. Commonly, PDT is used for treatment of localized infections because the tissues should be accessible for exposure to PSs. Killing bacteria without interfering with wound healing is one of the best advantages of this method [24]. Therefore, the aim of our present study was to evaluate the antimicrobial activity of PDT in combination with antibiotics against colistin-resistant PDR *A. baumannii* (CR-PDR-AB) clinical isolate.

2. Materials and methods

In the current study, CR-PDR-AB isolated from a burn patient was used as a test strain [25]. The CR-PDR-AB strain is resistant to ampicillin-sulbactam, amikacin, cefepime, ceftazidime, colistin, ciprofloxacin, gentamicin, imipenem, levofloxacin, minocycline, meropenem, piperacillin-tazobactam, piperacillin, rifampicin, tobramicin, tetracycline, and trimethoprim-sulfamethoxazole, but is susceptible to tigecycline. Minimum inhibitory concentration (MIC) results for the CR-PDR-AB strain challenged with colistin were found to be 32 mg/mL as previously described [25]. The CR-PDR-AB strain where characterized as PDR phenotype according to the international expert proposal for Interim standards guidelines [26]. Fresh bacterial colonies were inoculated with 10 mL of brain heart infusion (BHI) broth. Then, the colonies were incubated at 37 °C in a shaker bath at 100 rpm for 15 h. Log phase of bacterial growth was achieved by adding 400 μL of the suspension to 10 mL fresh BHI broth and incubated for 5 h. For confirmation of log phase growth, the optical density (OD) of suspension was checked by spectrophotometry according to a previous study [23].

2.1. Photosensitizer and light sources

TBO (Merck, Frankfurter, Germany) was dissolved in distilled H_2O to obtain a final concentration of 100 mg/L. Then, the solution was decontaminated by filtration and subsequently kept in dark room [23]. The light-emitting diode (LED) (FotoSan 630 nm LAD, CMS dental, Denmark) with 630 nm of emission was used as a light source.

Table 1
Groups description and their treatments.

Groups	Type of treatment
C	A control group without any treatment
TBO	The bacterial solution was exposed only to photosensitizer for 60s
LED	The bacterial solution was exposed only to LED for 60s
LED + TBO_1	The bacterial solution was exposed photosensitizer plus LED for 60s
LED + TBO_2	The bacterial solution was exposed photosensitizer plus LED for 90s

2.2. PDT experiments

The bacterial solution was divided into 5 micro test tubes (group) for each assay (Table 1). Aliquots of 100 μL of each bacterial suspension were placed in 96-well microtiter plates which have zero and ten percent light absorption and reflection, respectively, at wavelength between 400- 1000 nm, then incubated at 37 °C with TBO at a final concentration of 50 mg/L in the dark and at room temperature (23–26 °C) for 10 min and exposed to LED (2.000–4.000 mW/cm^2). The LED device was fixed 1 mm above the top surface of microtiter plate by a microphone stand.

2.3. Drug susceptibly testing

The PDT drug susceptibly testing (DST) was performed for all of the 5 groups by disk diffusion method according to Clinical Laboratory Standards Institute (CLSI) guidelines [27]. Antibiotic discs such piperacillin, ceftazidime, doripenem, and colistin (MAST, UK) were used in this study. Finally, the drug resistance patterns of each group were compared to each other.

2.4. Analysis of the expression of pmrA and pmrB genes following PDT by relative quantitative real-time (q) RT-PCR

To determine whether the integrity of LPS was still present in the sPDT-treated *A. baumannii* strains, the qRT-PCR assay was used to assess the expression of *pmrA* and *pmrB* genes according to a previous study [28]. The sPDT against *A. baumannii* was in a combination of 0.37 mg/mL TBO and an irradiation time of the LED for 60 s, with an estimated average output light energy 180 J/cm2 as described previously [23]. The fold changes of the *pmrA* and *pmrB* genes' expression levels were calculated by the $2^{-D\Delta Ct}$ method using the Relative Expression Software Tool (REST) 2009 software (QIAGEN) [29]. The mRNA expression levels were revealed as n-fold differences relative to the calibrator.

2.5. Statistical analysis

STBOS 21.0 (STBOS Inc., Chicago, IL) was employed for statistical analysis. ANOVA and Tukey's test were used to assess the difference between the relative quantities of the *pmrA* and *pmrB* gene expressions in CR-PDR-AB under the treatments. All experiments were performed in triplicate, and results were reported as mean ± standard deviation (SD). *P*-values < 0.05 in all experiments were considered statistically significant.

3. Result

Drug susceptibly testing of the control group showed that the bacterium was resistant to all tested antibiotics, according to CLSI guidelines (Fig. 1A). In addition, the results of DST in the LED and TBO groups separately showed that the bacteria were resistant to all tested antibiotics (Fig1B and C). The DST result of both the LED + TBO_1 and LED + TBO_2 groups showed a high decrease in the bacterial growth compared with the control group (Fig. 1D and E). In addition, in the LED + TBO_2 group around the antibiotic discs, rare

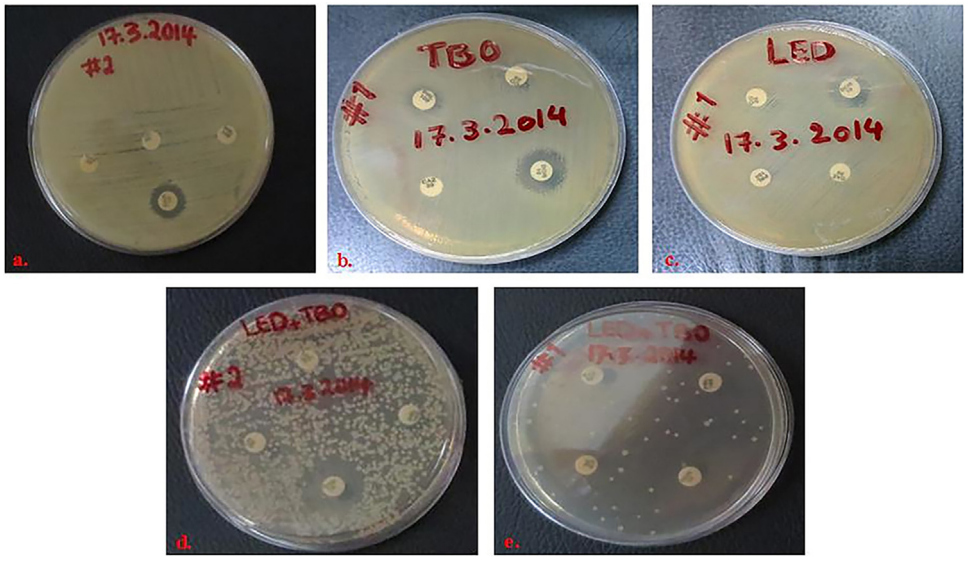

Fig. 1. (a) Result of Drug susceptibly test (DST) for control group; (b) Result of DST for TBO group; (c) Result of DST for LED group; (d) Result of DST for LED + TBO1 group; (e) Result of Drug susceptibly testing for LED + TBO2 group; Abbreviation, CAZ: Ceftazidime, Co: Colistin, PRL: Piperacillin, DO: Doripenem.

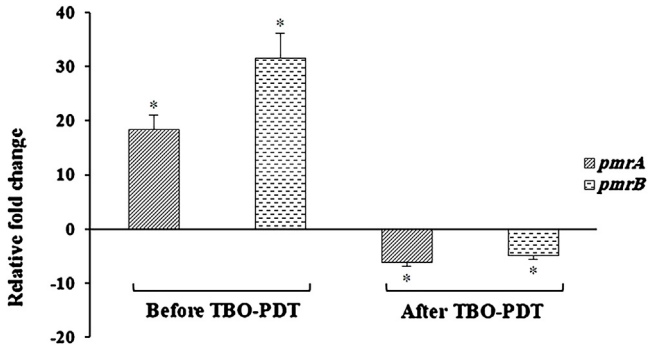

Fig. 2. Effect of sPDT on the *pmrA* and *pmrB* genes expression by CR-PDR-AB. Real-time qRT-PCR data for each gene were normalized against those obtained for the *16S rRNA* control. The mRNA concentration was calculated as $2^{-\Delta\Delta Ct}$ for each virulence gene, where ΔCT represents the threshold cycle (CT) value of the gene subtracted from the CT value of the *16S rRNA* control. Values are mean ± standard error of the mean of cDNA concentrations for each gene transcript from three replicate experiments. *, $P < 0.05$ is significantly different from the control (strains grown without treatment).

numbers of bacterial colonies were seen in comparison to the control group. In addition, inhibition growth zones of antibiotic discs were increased extensively, and even the bacterial colonies were countable in the last group.

Our data showed that before sPDT, the expression levels of *pmrA* and *pmrB* increased 18.4- and 31.7-fold (both $p < 0.05$) in colistin-treated CR-PDR-AB compared with control (no exposure to colistin), respectively (Fig. 2). In sPDT-treated CR-PDR-AB, 6.1- and 4.9-fold reduction in the expression of *pmrA* and *pmrB* genes in CR-PDR-AB was seen, respectively (all $p < 0.05$).

4. Discussion

Burn wounds are a global public health problem, as they induce metabolic and inflammatory alterations that predispose the patient to various severe complications, such as burn wound infections. The survival rate is currently a favorable 97% for patients admitted to burn centers. This can be largely attributed to advancements in burn wound care and treatment [2].

Burn patients are at high risk for drug-resistant infection, which often results in significantly delayed wound healing, longer hospitalization, and higher mortality. Infection can lead to sepsis or septic shock, which results in impaired perfusion of burn tissue that delays wound healing. Furthermore, the leading mortality following a severe burn are septic shock and multiorgan failure, so prevention and treatment of burn wound infection is a primary concern in the management of burn patients [2,3]. The emergence of MDR *A. baumannii* as a problematic nosocomial pathogen among patients with burn wound infection has drawn clinical attention to colistin as an old antimicrobial agent that is active against MDR *A. baumannii* [30]. Unfortunately, worldwide reports indicate that the widespread administration of colistin as a last treatment option to control MDR *A. baumannii* infections has led to an alarming growing trend of colistin-resistance among MDR *A. baumannii* [31]. Recurrently, the management of burn wound infections by MDR *A. baumannii* proves very challenging because *A. baumannii* isolates often develop antimicrobial resistance and no new drugs are currently in the pipeline to eliminate PDR *A. baumannii* infections. Thus, surveillance of the activities of PDT as adjunctive therapy are urgently needed to guide physicians to make decisions regarding the treatment of antibiotic-resistant localized infections [32].

In the present study, we evaluated the antimicrobial effects of PDT against *A. baumannii* as combination therapy with colistin. According to our results, PDT had a positive effect against pan drug resistant strain of *A. baumannii* isolated from burn patients. The patient who was infected with this strain did not respond to antibiotic therapy. However, PDT could almost eliminate the bacterial population *in vitro*. Therefore, this method is a useful approach that can eradicate the bacterial infection regardless of resistance. In addition, the important parameters of PDT against *A. baumannii* are the TBO concentration and time of radiation. In the present study, TBO showed favorable antimicrobial effects. In addition, when the time of radiation changed from 60 to 90 s, the antimicrobial effect was enhanced. Ragàs et al. in 2012 surveyed the *in vivo* effect of PDT against *A. baumannii* from a burn wound. They used methylene blue as antimicrobial PS for treating an *A. baumannii* burn infection and concluded that this PS is highly effective against *A. baumannii* [33].

Today, colistin is used as the last line of drugs for the treatment of *A. baumannii* infections [34]. It causes bacterial cell death directly through membrane lysis [33]. Unfortunately, infections caused by strains with resistance to this antibiotic have increased worldwide [34]. Sampson et al. showed the killing effect of colistin was increased in the presence of reactive oxygen spices (ROS) even

in colistin resistant strains [35]. On another hand as described PDT works by ROS producing [23]. Another effect of PDT on bacterial cells is mediated by membrane disruption and permeability [23]. In this situation, antibiotic molecules can be directed to bacterial cells easily and interact and inactive its targets. Therefore, we can conclude that PDT can be used in combination with antibiotics such as colistin even in drug resistant strains. The electrostatic interaction between cationic PSs, such as TBO, and negatively charged lipid A phosphates in the OM of Gram-negative bacteria may displace Mg^{2+} and Ca^{2+} on the LPS network. This displacement can disrupt outer membrane (OM) permeability which facilitates the transit of a variety of compounds, including various antibiotics, into the cells. In addition, the TBO-facilitated transfer also enhances its own transfer across OM through a self-promoted uptake [34–39].

In *A. baumannii*, the current studies reported an association of the *pmrA* and *pmrB* genes with colistin resistance [13]. In fact, mutations in *pmrA* and *pmrB* genes may participate in colistin resistance due to induced modification of the lipid A of outer membrane LPS [13]. The expression of the *pmrA* and *pmrB* genes was downregulated (6.1- and 4.9-fold in CR-PDR-AB) when exposed to sPDT treatments. This observation indicated that the PDT might reverse phosphoethanolamine modification of lipid A responsible for colistin resistance.

In conclusion, emergence and dissemination of highly drug resistant organisms in the world have shown that antibiotic therapy alone cannot control of this situation. New methods and approaches are needed for prevention and treatment of infections due to resistant organisms. PDT is one of the best approaches for treatment of local infection such as burn infections that can be used as combination therapy with antibiotics. Our data represents the first decisive example of a procedure that breaks antibiotic resistance by directly modulating two-component regulatory system activity.

Disclosure statement

There are no competing financial interests.

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