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Antimicrobial blue light and photodynamic therapy inhibit clinically relevant β-lactamases with extended-spectrum (ESBL) and carbapenemase activity

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ABSTRACT

Introduction: The production of β-lactamases by Gram-negative bacteria is among the most important factors of resistance to antibiotics, which has contributed to therapeutic failures that currently threaten human and veterinary medicine worldwide. Antimicrobial photodynamic therapy and antimicrobial blue light have a broad-spectrum antibacterial activity against multidrug-resistant and hypervirulent pathogens.
Objective: To investigate the ability of antimicrobial blue light to inhibit the hydrolytic activity of clinically relevant β-lactamase enzymes (*i.e.,* KPC, IMP, OXA, CTX-M, and SHV), with further comparison of the inhibitory effects of antimicrobial blue light with methylene blue-mediated antimicrobial photodynamic therapy.
Methods: Blue LED light (λ = 410 ± 10 nm) alone or red LED light (λ = 660 ± 10 nm) in combination with methylene blue were used to inactivate, *in vitro*, suspensions of *Klebsiella pneumoniae* strains producing clinically important β-lactamase enzymes assigned to the A, B and D Ambler molecular classes. Furthermore, β-lactamase activity inhibition mediated by antimicrobial blue light and methylene blue-mediated antimicrobial photodynamic therapy was measured by using the chromogenic β-lactam substrate nitrocefin.
Results: β-lactamase activities were effectively inactivated by both visible light-based approaches. In this regard, antimicrobial blue light and methylene blue-antimicrobial photodynamic therapy led to a significant reduction in the hydrolysis of nitrocefin (81–98 %).
Conclusion: Sublethal doses of antimicrobial blue light and methylene blue-mediated antimicrobial photodynamic therapy are equally effective to inhibit clinically significant β-lactamases, including extended-spectrum β-lactamases and carbapenemases.

1. Introduction

The epidemiological success of pandemic β-lactamases has been linked to therapeutic failures that currently threaten human and veterinary medicine worldwide [1]. Currently, the World Health Organization has published a global priority list of antibiotic-resistant bacteria, in which carbapenemases- and/or extended-spectrum β-lactamases (ESBLs)-producing Enterobacterales were ranked in the critical priority group [2]. In this regard, these bacteria are in the spotlight because they have been widely identified in community- and hospital-associated infections, in human and veterinary medicine, being frequently associated with high mortality rates [1,3–5].

Klebsiella pneumoniae is a major nosocomial pathogen that is currently challenging global health systems [6,7]. In this regard, the rapid dissemination of international high-risk clones of *K. pneumoniae* (*e.g.,* sequence types ST11, ST258, ST307, ST340 and ST442) in hospital settings is a serious epidemiological concern because these lineages have been associated with resistance to medically relevant β-lactams

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antibiotics (*i.e.,* third-generation cephalosporins and carbapenems) [8].

Methylene blue-mediated antimicrobial photodynamic therapy (MB-aPDT) and antimicrobial blue light (aBL) at 400−470 nm have been extensively investigated against pathogenic bacteria, including those resistant to clinically important antimicrobials [9,10]. More recently, it has been demonstrated that MB-aPDT could impair the enzymatic activity of some bacterial carbapenemases avoiding degradation of carbapenem antibiotics [11]. To gain a deeper understanding of the inhibitory effects of visible light against β-lactam enzymes activity, we have investigated the potential of aBL in inhibiting β-lactamases belonging to the A, B and D Ambler molecular classes [12], produced by international clones of *K. pneumoniae*. Additionally, inhibitory effects of aBL compared to MB-aPDT were evaluated.

2. Materials and methods

2.1. Bacterial strains

Two previously characterized *K. pneumoniae* strains were used. *K. pneumoniae* strain KPBr-1 (GenBank accession number: NZ_PDMV00000000.1) belongs to ST442 and produces IMP-1 metallo-β-lactamase, and SHV-110 and CTX-M-9 ESBL enzymes; whereas *K. pneumoniae* strain ICBKPS3.2 (GenBank accession number: JABEPG000000000.1) belongs to ST11 and produces KPC-2 carbapenemase, CTX-M-2 and SHV-182 ESBL enzymes, and OXA-2 class D β-lactamase. Additionally, *K. pneumoniae* ATCC strain 700603, which produces SHV-18 ESBL, was used as control. β-lactamases produced by *K. pneumoniae* strains used in this study cover relevant classes of the molecular classification proposed by Ambler [12].

2.2. aBL and aPDT experiments

Fresh colonies of *K. pneumoniae* were inoculated into 10 mL of Mueller-Hinton broth (Difco, USA). The inocula were prepared from overnight broth cultures under shaking regimen incubated at 37 °C. The bacteria were then centrifuged at 10,000*g* for 10 min, washed twice and suspended in PBS (phosphate buffer solution, pH 7.4). The inocula were standardized to approximately 10^9 colony forming units (CFU)/mL by turbidity of the suspension with a spectrophotometer (λ = 625 nm; optical path =1 cm; optical density = 0.667). One milliliter of each sample was added individually in a 12-well plate for irradiation procedure.

Two different irradiation protocols were used. For MB-aPDT, we used a red LED probe (660 ± 10 nm, Prototype 1, BioLambda, Brazil). The irradiance was kept constant at 100 mW/cm^2. MB (> 95 % purity, Sigma-Aldrich, USA) was used at 100 μM in PBS. The aBL was performed using a LED irradiator (410 ± 10 nm, LEDbox, BioLambda, Brazil). No exogenous photosensitizer (PS) was added to the system. Blue light irradiance was kept constant at 38.2 mW/cm^2. Both LED devices were positioned above the samples. Radiant exposure levels for aBL and MB-aPDT are presented at Supplementary Table 1. Three types of controls were used for MB-aPDT experiments: non-treated cells (PBS only), light alone (highest light dose without MB exposure), and MB alone (MB exposure without light during the entire experimental period). For aBL was considered only non-irradiated group for negative control. After the irradiation procedure, samples were serially diluted (1:10) in PBS, seeded on Mueller Hinton agar plates and incubated at 37 °C for 24 h for CFU quantification [13]. All microbiological experiments were conducted in technical triplicates, and replicated in 3 different experimental days, which totalized 9 samples for each strain evaluated.

Survival fraction values were determined as averages of $\log_{10}$ reduction of CFU and normalized in relation to each respective non-irradiated control group. Inactivation kinetics analysis and the calculated lethal dose for inactivation of $3\log_{10}$ ($LD_{99.9}$) values were obtained as previously described [14].

2.3. β-lactamase activity determination

The bacterial β-lactamase activity and its inhibition by visible light platforms were measured by using an assay based on the chromogenic substrate, nitrocefin (Sigma Chemical Co., St. Louis, MO), a chromogenic cephalosporin that changes from yellow to red when the amide bond in β-lactam ring is hydrolyzed by β-lactamases [15].

In brief, a volume of 1 mL bacterial cell solution at final concentration of 10^9 CFU/mL was sampled before and after aBL and MB-aPDT. The evaluated samples were irradiated with calculated doses for inactivation of $LD_{99.9}$ with aBL and MB-aPDT, and compared to negative control (untreated sample), red light-only and MB-only treatments. These cells were lysed in sodium phosphate buffer containing 1% Triton X-100, and the cell extracts were centrifuged (16,000*g*, 20 min, 4 °C). Supernatant samples (5 μL) were then placed in technical duplicates into a 96-well microplate, and the β-lactamase activity represented by hydrolysis rates of nitrocefin was determined every minute for 1 h, at 25 °C, by absorbance measurements at 490 nm (SpectraMax M4; Molecular Devices, USA), where the nitrocefin hydrolyzed product is uniquely absorbed. All results were averaged over duplicates and replicated in 3 different experimental days. Data were presented as means ± standard deviation.

2.4. Statistical analyses

Statistical analysis of $LD_{99.9}$ and β-lactamase inhibition was performed by one-way ANOVA with Tukey as post-test, using Prism 8.0 software (GraphPad, USA). All inference analyses adopted a significance level of $p < 0.05$.

3. Results and discussion

Antibacterial activity of visible light-based approaches was confirmed (Fig. 1). In this regard, β-lactamase producers showed more sensitivity to MB-aPDT when compared to aBL. For MB-aPDT treatment, all *K. pneumoniae* strains presented similar inactivation kinetics with more than $3\log_{10}$ of inactivation after 16 J/cm^2 (2 min and 40 s) (Fig. 1, Table 1 and Supplementary Table 2). On the other hand, in aBL groups we observed different inactivation kinetics amongst *K. pneumoniae* strains. In fact, the ATCC strain achieves a microbial load reduction of $3\log_{10}$ after 637.40 J/cm^2 (106 min), whereas strains KPBr-1 and ICBKP3.2 require 836.87 J/cm^2 and 892.28 J/cm^2 (139 and 148 min), respectively (Fig. 1, Table 1 and Supplementary Table 2). Previous studies have demonstrated that *K. pneumoniae* is more tolerant to antimicrobial blue light and photodynamic therapy, when compared to other clinically important bacteria such as *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* [14]. Recently, we have further demonstrated that even hypervirulent and hypermucoviscous strains of *K. pneumoniae*, considered a clinical challenge, can also be effectively inactivated by MB-aPDT and aBL [16].

Regarding enzymatic inactivation, initially β-lactamases from *K. pneumoniae* strains exhibited rapid hydrolysis of the nitrocefin substrate, whereas sublethal doses of MB-aPDT and aBL led to a significant reduction of nitrocefin hydrolysis ($p < 0.0001$) (Fig. 2), supporting that both aBL and MB-aPDT strategies are equally effective to inhibit β-lactamases, including carbapenemases and ESBLs.

Due to its broad-spectrum bactericidal activity, broad-spectrum cephalosporins are often the primary therapeutic choice for life-threatening infections, whereas carbapenems are considered last resort options [17]. Worryingly, the production of ESBLs and carbapenemases has limited the use of this antibiotics [17–19]. Furthermore, β-lactamases-producing Enterobacterales frequently display resistance to other antimicrobial classes [20].

A previous study demonstrated that MB-aPDT impair the activity of class A (SME-3), B (VIM-1) and D (OXA-48) carbapenemases from *Serratia marcescens*, *K. pneumoniae*, and *Enterobacter aerogenes* strains,

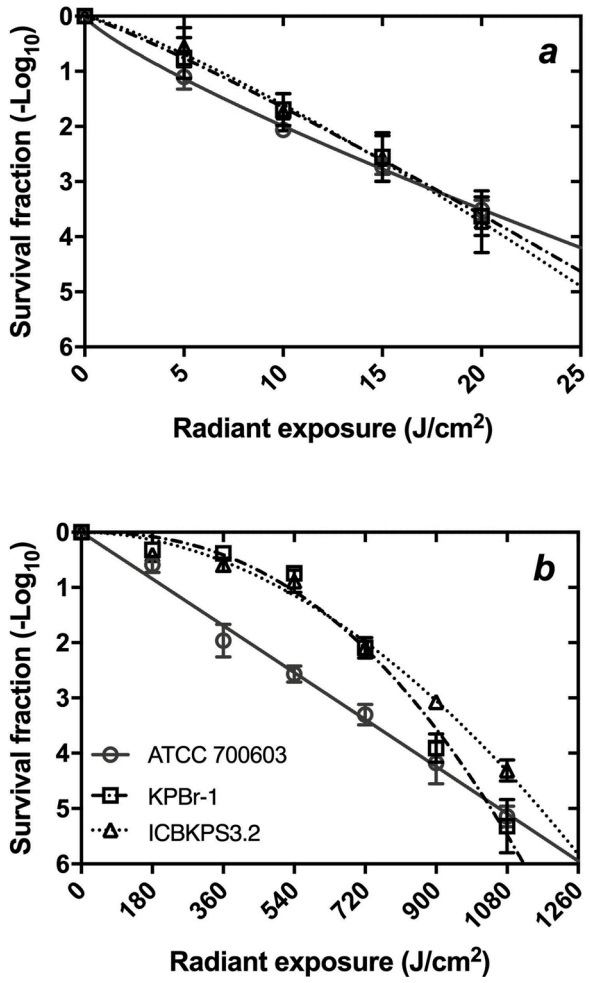

Fig. 1. Kinetics of *Klebsiella pneumoniae* strains induced by MB-aPDT (660 ± 10 nm) irradiation (a), and aBL (λ = 410 ± 10 nm) (b). Data are presented as survival fraction (log10 reduction) in function of radiant exposure (J/cm^2). The bars indicate SD of the mean at each data point and are not shown where their height is shorter than symbols.

Table 1
Lethal dose ($LD_{99.9}$) and tolerance factor (T) average values estimated using the Weibull model for *Klebsiella pneumoniae* strains inactivated by aBL and MB-aPDT*.

Strain	MB-APDT		Blue light	
	$LD_{99.9}$ (J/cm^2)	T	$LD_{99.9}$ (J/cm^2)	T
KPBr-1	16.99	1.12	836.87	2.36
ATCC 700603	16.53	0.81	637.40	1.00
ICBKPS3.2	16.71	1.22	892.28	1.92

* The values are showed in radiant exposure (J/cm^2) and minutes.

respectively [11]. In this study, we report the further inhibition of KPC-2 and IMP-1 metallo-β-lactamases, SHV-18, SHV-110, SHV-182, CTX-M-2 and CTX-M-9 ESBLs, and OXA-2 class D β-lactamases, by aBL and MB-aPDT. In this way, it has been demonstrated that MB-aPDT destroys the bacterial genome leading to dysfunction in the DNA coding for carbapenemases. Such observation is in line with the fact that cationic PSs, such as MB, tend to bind negatively charged moieties of the cells, including the DNAs. Specifically, the presence of MB in bacterial cytoplasm has indicates that MB-aPDT has the potential to damage chromosomal and plasmid genes, including those encoding for carbapenemases, following oxidative damage on varied targets [11].

In summary, our results confirm inhibition of clinically relevant

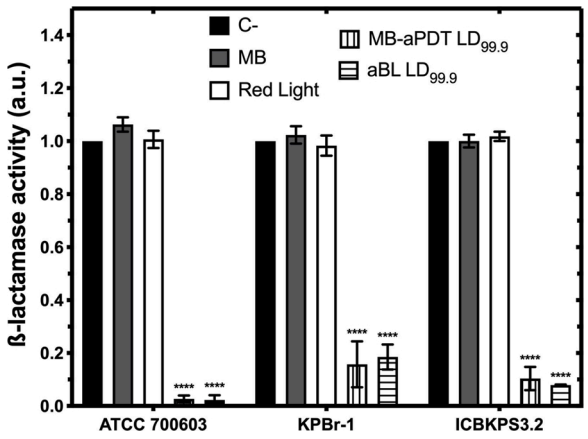

Fig. 2. β-lactamase activity and inhibition evaluated by chromogenic hydrolysis of nitrocefin substrate. The negative control (C-), methylene blue (MB), red light, and samples exposed to irradiance calculated for lethal dose of 99.9 % ($LD_{99.9}$) by aBL and MB-aPDT are presented as means ± standard deviation.

β-lactamases by MB-aPDT, and demonstrate, for the first time, the further inhibition of β-lactamases by aBL. Although, aBL is highly effective against nosocomial clones of *K. pneumoniae*, MB-aPDT is faster than aBL to inactivate *in vitro* suspensions of critical priority *K. pneumoniae* strains, as well as to inhibit their β-lactamases. This is particularly important for clinical applications. However, even though ultraviolet lamps are being used for disinfection of healthcare environments and material surfaces, blue light could be a new potential tool since it is could be used in inhabited hospital settings [21]. In this case, long exposure times seem to be not an important issue. Additionally, combining MB-aPDT /aBL with clinically relevant β-lactams antibiotics would be useful to evaluate other clinically important aspects. Testing aBL with the β-lactamase-producing pathogens that are more sensitive to aBL would be also interesting. We hope our findings encourage further researches in these areas.

4. Conclusion

Our findings ratify the use of aBL and MB-aPDT against drug-resistant pathogens and also open a new avenue in the fight against β-lactamases. Subsequent studies should encompass the combination of light-based antimicrobial techniques with other antimicrobial classes. In a prospective scenario, light-based therapies could extend the use of broad-spectrum cephalosporin and carbapenem antibiotics, initially for the treatment of topical infections caused by β-lactamase producers.

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Ethical approval

Not required/not applicable.

Declaration of Competing Interest

C. P. Sabino is an associate at BioLambda but declares to only have scientific interest on this study. There are no further conflicts of interest to be declared.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.pdpdt.2020.102086.

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