Universidade de São Paulo
School of Pharmaceutical Sciences
Graduate Program in Pharmacy (Physiopathology and Toxicology)
Area of Clinical Analysis

Antimicrobial photodynamic inactivation mediated by methylene blue:
analysis of inactivation kinetics and biochemical mechanisms

Caetano Padial Sabino

Thesis to obtain the degree of DOCTOR
Advisor: Prof. Dr. Nilton Lincopan
Co-advisor: Prof. Dr. Martha Simões Ribeiro

São Paulo
2021

Universidade de São Paulo
School of Pharmaceutical Sciences
Graduate Program in Pharmacy (Physiopathology and Toxicology)
Area of Clinical Analysis

Antimicrobial photodynamic inactivation mediated by methylene blue:
analysis of inactivation kinetics and biochemical mechanisms

Caetano Padial Sabino

Original version of Thesis according to CoPGr 6018 resolution. Original is available
from the FCF/USP Postgraduate Service.

Thesis to obtain the degree of DOCTOR
Advisor: Prof. Dr. Nilton Lincopan
Co-advisor: Prof. Dr. Martha Simões Ribeiro

São Paulo
2021

Universidade de São Paulo
Faculdade de Ciências Farmacêuticas
Programa de Pós-Graduação em Farmácia (Fisiopatologia e Toxicologia)
Área de Análises Clínicas

Inativação fotodinâmica antimicrobiana mediada por azul de metileno:
análise de cinética de inativação e mecanismos bioquímicos

Caetano Padial Sabino

Tese para obtenção de título de DOUTOR

Orientador: Prof. Dr. Nilton Lincopan

Coorientadora: Martha Simões Ribeiro

São Paulo

2021

Autorizo a reprodução e divulgação total ou parcial deste trabalho, por qualquer meio convencional ou eletrônico, para fins de estudo e pesquisa, desde que citada a fonte.

Ficha Catalográfica elaborada eletronicamente pelo autor, utilizando o programa desenvolvido pela Seção Técnica de Informática do ICMC/USP e adaptado para a Divisão de Biblioteca e Documentação do Conjunto das Químicas da USP

Bibliotecária responsável pela orientação de catalogação da publicação:
Marlene Aparecida Vieira - CRB - 8/5562

S116i Sabino, Caetano Padial
Inativação fotodinâmica antimicrobiana mediada por azul de metileno: análise de cinética de inativação e mecanismos bioquímicos / Caetano Padial Sabino. - São Paulo, 2021.
81 p.

Tese (doutorado) - Faculdade de Ciências Farmacêuticas da Universidade de São Paulo. Departamento de Análises Clínicas e Toxicológicas.
Orientador: Lincopan, Nilton
Coorientador: Ribeiro, Martha

1. fotoinativação. 2. resistência bacteriana. 3. terapia fotodinâmica. 4. azul de metileno. 5. bactéria. I. T. II. Lincopan, Nilton, orientador. III. Ribeiro, Martha, coorientador.

Caetano Padial Sabino

Antimicrobial photodynamic inactivation mediated by methylene blue:
analysis of inactivation kinetics and biochemical mechanisms

Thesis referee committee to obtain the degree of DOCTOR

Prof. Dr. Nilton Lincopan

Advisor/President

Prof. Dr. Martha Simões Ribeiro

Co-advisor

Full Name

1st Referee: to be defined

Full Name

2nd Referee: to be defined

Full Name

3rd Referee: to be defined

Full Name

4th Referee: to be defined

São Paulo, 22 de janeiro de 2021

DEDICATÓRIA

Dedico este trabalho a todos, seres humanos e animais que são vítimas de infecções tratáveis por terapia fotodinâmica antimicrobiana. Que todo nosso esforço dedicado a este trabalho possa, em qualquer nível, proporcionar vidas mais longas e prósperas.

AGRADECIMENTOS

Agradeço imensamente aos meus pais, Edyr e Cecília, e familiares que me forneceram uma base sólida e sempre apoiaram para que conseguisse alcançar meus objetivos mais ousados. Também, a todos amigos que sempre estiveram ao meu lado incentivando minha dedicação aos estudos e trabalhos, mesmo nos momentos mais difíceis e distantes.

Ao meu orientador e amigo, Nilton Lincopan, que me incentivou e acreditou em meu trabalho, e investiu tamanha dedicação para alcançarmos nossos objetivos. Tenho grande admiração e me orgulho de ter a oportunidade de trabalhar em sua equipe.

À minha eterna orientadora e “mãe” científica, Martha Simões Ribeiro, que me acolheu de braços abertos desde o primeiro dia em que cheguei ao seu laboratório. Sem dúvidas, levarei para toda vida os incontáveis aprendizados que me proporciona. Ainda, abriu todos caminhos que pôde para me dar chance ao sucesso profissional pois sempre depositou em mim enorme confiança e liberdade de aprendizado.

Aos meus grandes mestres, colegas de trabalho e amigos de vida, Renato Prates, Ilka Kato, Silvia Núñez, Aguinaldo Garcez, Luis Suzuki, Alessandra Baptista, Tania Yoshimura, Fernanda Cabral, Seu Luiz, Dona Marta, João Paulo Tardivo, Fabio Sellera, Carolina dos Anjos, Milena Dropa, Miriam Fernandes, Fernanda Esposito, Helena Junqueira, Guilherme Brancini e tantos outros que no laboratório, escritório ou bar, me ajudaram a chegar até aqui. Estes além de me ensinarem a base do conhecimento que uso neste trabalho, firmaram uma forte amizade e respeito que perdurarão por toda vida.

Aos amigos e professores colaboradores, Mauro Cortez, Gilberto Braga, Raul Freitas, Victor Arana, Mark Wainwright, Michael Hamblin, Tianhong Dai e Mauricio Baptista, que contribuíram enormemente na construção deste trabalho. Também aos seus alunos e técnicos, que direta ou indiretamente me auxiliaram e apoiaram nos experimentos.

Aos meus colegas de trabalho e sócios da BioLambda, empresa da qual tenho muito orgulho de ter participado de sua criação e crescimento. Juntos, já chegamos muito longe e sei que ainda temos muito a caminhar. Muito obrigado por me aturarem no dia a dia e por acreditarem em meu sonho.

Finalmente, às agências de fomento CNPq e FAPESP pela bolsa e projetos de pesquisa financiados. Também à Faculdade de Ciências Farmacêuticas, Instituto de Ciências Biomédicas e Instituto de Química da Universidade de São Paulo e Centro de Lasers e Aplicações do Instituto de Pesquisas Energéticas e Nucleares pela infraestrutura disponibilizada.

Muito Obrigado a Todos!

***“Imagination is more important than knowledge.
For knowledge is limited,
whereas imagination embraces the entire world,
stimulating progress, giving birth to evolution.”***

Albert Einstein, 1924

ABSTRACT

SABINO, C. P. **Antimicrobial photodynamic inactivation mediated by methylene blue: Analysis of inactivation kinetics and biochemical mechanisms.** [Doctorate Thesis]. São Paulo: Universidade de São Paulo, School of Pharmaceutical Sciences; 2021.

The widespread use of antimicrobial chemotherapy in medicine and livestock production imposed an evolutive selection of drug-resistant strains worldwide. As a result, the effectiveness of our current antimicrobial armamentarium is constantly being reduced to alarming levels. Therefore, novel antimicrobial therapeutic strategies are urgently needed. Antimicrobial photodynamic therapy (APDT) comes to this scenario as a powerful tool to counteract the emergence of microbial drug-resistance. Its mechanisms of action are based on simultaneous oxidative damage of multiple targets and, therefore, it is much less likely to allow any type of microbial resistance. Therefore, the objectives of this study were focused into establishing 1) a mathematical tool to allow precise analysis of microbial photoinactivation; 2) a broad analysis of APDT effectiveness against global priority drug-resistant pathogens; 3) inhibition of β -lactamase enzymes; and 4) how the biochemical mechanisms of APDT avoid emergence of resistance. The main results obtained through the investigation led by this thesis were divided into 4 scientific articles regarding each of the above-mentioned objectives. In summary, we discovered that 1) a power-law function can precisely fit all microbial inactivation kinetics data and provide insightful information of tolerance factors and lethal doses; 2) there is no correlation between drug-resistance and APDT sensitivity, i.e., extensively drug resistant microorganisms are killed in the same kinetics as drug-sensitive controls; 3) β -lactamases are very sensitive to photodynamic inhibition; 4) biochemical mechanisms of APDT promote oxidative damages to external cell membranes, DNA and proteins whereas the main cause of microbial death seems to be directly associated with protein degradation. Thus, we conclude that APDT is effective against a broad-spectrum of pathogens and has minimum chances of promoting resistance mechanisms.

Keywords: photodynamic antimicrobial chemotherapy (PACT); antimicrobial photodynamic therapy (aPDT); photochemotherapy; photoinactivation; methylene blue; bacteria, fungi, drug-resistance.

RESUMO

SABINO, C. P. **Inativação fotodinâmica antimicrobiana mediada por azul de metileno: Análises de cinética de inativação e mecanismos bioquímicos.** [Tese de Doutorado]. São Paulo: Universidade de São Paulo, Faculdade de Ciências Farmacêuticas; 2021.

O amplo uso da quimioterapia antimicrobiana impôs uma seleção evolutiva de cepas resistentes a medicamentos. Como resultado, a eficácia dos fármacos antimicrobianos tem sido reduzida a níveis alarmantes. Portanto, novas estratégias terapêuticas antimicrobianas são urgentemente necessárias. A terapia fotodinâmica antimicrobiana (TFDA) entra neste cenário como uma ferramenta poderosa para combater a resistência microbiana. Seus mecanismos de ação são baseados no dano oxidativo sobre múltiplos alvos e, portanto, é muito menos provável que permita o surgimento de qualquer tipo de resistência. Os objetivos deste estudo foram focados ao estabelecimento de 1) modelo matemático para análise precisa da fotoinativação microbiana; 2) ampla análise da eficácia da TFDA contra patógenos resistentes a fármacos antimicrobianos de prioridade global; 3) inibição de β -lactamases por TFDA; e 4) como os mecanismos bioquímicos da TFDA evitam o surgimento de resistência. Os principais resultados obtidos através da investigação conduzida por esta tese foram divididos em 4 artigos científicos. Em resumo, descobrimos que 1) uma função de lei de potência pode ajustar com precisão todos os dados de cinética de inativação microbiana e fornecer informações detalhadas sobre fatores de tolerância e doses letais; 2) não há correlação entre resistência à quimioterapia antimicrobiana e sensibilidade à TFDA, isto é, cepas extensivamente resistentes aos antimicrobianos são inativadas sob a mesma cinética que controles sensíveis aos antimicrobianos; 3) β -lactamases são altamente sensíveis à inibição fotodinâmica; 4) os mecanismos bioquímicos da TFDA promovem danos oxidativos às membranas celulares e DNA, porém, a principal causa de morte microbiana é diretamente associada à degradação das proteínas. Assim, concluímos que a TFDA é eficaz contra um amplo espectro de patógenos e tem chances mínimas de promover mecanismos de resistência.

Palavras-Chave: Terapia fotodinâmica antimicrobiana (TFDA); fotoinativação; azul de metileno; bactéria; fungo; resistência bacteriana; fotoquimioterapia.

SUMMARY

1. General Introduction	14
1.1. Microbial Drug-Resistance	14
1.2. Antimicrobial Photodynamic Therapy	17
2. Objectives	22
3. Articles published or in process of publication	22
3.1. Main Articles	22
3.1.1. Article I	23
3.1.2. Article II	29
3.1.3. Article III	43
3.1.4. Article IV	47
3.2. Book chapters, patents and secondary articles	72
3.2.1. List of Secondary Articles	72
3.2.2. List of Patents	74
3.2.3. List of Book Chapters	75
3.2.4. List of Scientific Awards	76
4. General Conclusions	78
5. Future Perspectives	78

CHAPTER 1 – GENERAL INTRODUCTION

1.1. MICROBIAL DRUG-RESISTANCE

The overuse of antimicrobial agents over the past decades has contributed to the evolutive selection of multidrug-resistant (MDR) and extensively drug-resistant (XDR) bacterial strains against virtually all commercially available antibiotics (Figure 1). MDR bacteria are those classified as resistant to more than two classes of antimicrobials, while XDR are resistant to all but two or less classes. As a consequence, resistance of microorganisms to antimicrobials has emerged as one of the most important challenges of Medicine, emphasizing the need for novel antimicrobial strategies against drug-resistant microorganisms. Microbial resistance is expected to become a pandemic situation that requires global efforts to be avoided (1-4). The World Health Organization (WHO) published a global map highlighting the current endemic status of microbial drug-resistance, warning that a 'post-antibiotic' world could soon become a reality (3). Current treatment of many infectious human and livestock diseases already relies on a single commercially available drug (5).

Figure 1. Illustrative image showing three major causes leading to the selection of drug-resistant microorganisms. (Source: Caetano Padial Sabino)

Antimicrobial resistance introduces a profound impact on morbidity and costs associated with infections. New antibacterial drugs may be cost-effective compared to older generation drugs. However, the cost-effectiveness of developing and introducing new antimicrobials into the market before drug resistance arises is a major challenging point for independent researchers and even for pharmaceutical industries (6). In most developed and emerging countries, the highest prevalence of drug-resistant microorganisms is associated with particular carbapenem-resistant Gram-negative bacteria, glycopeptide-resistant Gram-positive bacteria and azole-resistant fungi. Infections produced by drug-resistant microorganisms are associated with increased morbidity, hospitalization rates, healthcare costs and mortality (7, 8).

The economic impact of drug-resistant infections is estimated to be \$20 billion in excess health care costs and 8 million additional hospital days in the United States and over 1.6€ billion and 2.5 million additional hospital days in the European Union (8). By 2050, the World Health Organization expects that microbial drug-resistance will reach a death toll of more than 10 million people per year and surpass cancer deaths while the World Bank estimates that it would cause an economic impact of up to US\$ 6.1 trillion, surpassing the combined GDP of Brazil and the United Kingdom.

Regarding the etiologic agents, special attention should be paid to pathogens recognized as the “ESKAPE” group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter spp.*), since they are considered prototypes of multidrug-resistant bacteria (1, 9-11). Studies have shown that the main problem in the treatment of bacterial infections caused by MDR bacteria is the resistance to carbapenems (imipenem, ertapenem and meropenem); third- (ceftazidime, cefotaxime, ceftriaxone, ceftiofur) and fourth-generation (cefepime), cephalosporins, glycopeptides and quinolones/fluoroquinolones antibiotics: the ones that were considered the last therapeutic choice (12, 13).

In the 1980s, more than 90% of all infections caused by *Enterococci* were caused by *E. faecalis* and only 5–10% by *E. faecium* (14). Currently, this ratio has dramatically changed and *E. faecium* is associated with 38–75% of all enterococcal infections, and is commonly resistant to vancomycin (15, 16). MDR *S. aureus* have become endemic in hospital settings, where the most frequent strains are methicillin-resistant (MRSA) (17). The drug resistant phenotypes have emerged as major causative pathogens in complicated skin and skin structure infections (cSSSIs) and,

unfortunately, treatment failure with vancomycin has been increasingly reported (14). Therefore, since management of cSSSIs caused by *S. aureus* is problematic, because of multifactorial transmission and limited therapeutic possibilities, studies on alternative therapies are worthy of investigation (18).

The major concern on drug resistance dissemination relies on the bacterial ability to conjugate plasmids containing virulence and resistance genes, leading to the propagation of virtually all classes of antimicrobials including broad-spectrum β -lactamases, carbapenemases, quinolones, lipopeptides and glycopeptides (3, 12, 15, 19-21). Carbapenemases are being frequently detected in plasmids of Gram-negative clinical isolates of *Enterobacterales* (e.g. KPC, IMP, GES, NDM) and non-fermenters such as *P. aeruginosa* (e.g. KPC, SPM, IMP, VIM, GES, NDM) and *A. baumannii* (e.g. OXA-23, 72, 58 and 143) (19, 21). Recently, carbapenemase-producing nosocomial strains were isolated from Brazilian urban coast, rivers and lakes used for water supply and recreational purposes, arising great public health concerns that indicates dumping of MDR-bacteria-contaminated wastewater from hospitals (22-25).

For many years, antifungal agents have greatly contributed to the improvement of public health; however, antifungal-resistant pathogens have increased, becoming a serious issue. Fungal pathogens have assumed a greater clinical significance due to the high prevalence and transmission of drug-resistant strains in healthcare units. In this regard, *Candida albicans* has been the most extensively studied pathogen because of their morbidity and mortality associated with infections, with the main incidence in immunocompromised patients (26). *Candida* species have been reported to be resistant to fluconazole, and persistent fungemia has been documented in patients under fluconazole therapy, resulting in fatal outcomes (27, 28). Yet, global surveillance studies have shown increases in fluconazole resistance among *Cryptococcus neoformans* isolates, where fluconazole failure in patients with cryptococcal disease has been attributed to heteroresistance. In brief, development of novel strategies against drug-resistant strains is critical to treat life-threatening fungal infections.

1.2. ANTIMICROBIAL PHOTODYNAMIC THERAPY

Photodynamic therapy (PDT) represents an elegant method to produce cytotoxic molecules and radicals in a space-time-controlled manner, *i.e.*, without any of the individual components (*i.e.*, light, photosensitizer and a charge or energy receptor molecule) photodynamic reactions will not initiate. This light-based therapeutic technology uses low irradiances (*i.e.* non-thermally acting) of light in combination with a non-toxic pigment, termed photosensitizer (PS), to treat localized tumors and infections (29-31). As exemplified in figure 2, a hypothetical photosensitizer excited by light ($^3\text{PS}^*$) interacts with molecular oxygen either by donation of a positive or negative charge (type I reaction) or even energy (type II reaction). Such interaction is able to produce a variety of reactive oxygen species (ROS) that can destroy bacteria, protozoa, fungi and viral particles regardless of its taxonomy (2, 30, 32-36).

So far, several molecular frameworks of potential photosensitizers have been tested against pathogenic microorganisms. Examples include halogenated xanthenes, phenothiazines, eosins, tetracyclins, quinolones, triarylmethanes, phthalocyanines, naphthalocyanines, chlorins, bacteriochlorins, porphyrins, fullerenes, and natural products such as hypericin, anthraquinones and curcumin (37). While neutral and negatively charged PS are able to effectively inactivate Gram-positive microorganisms, targeting Gram-negative bacteria (*e.g.* *Enterobacteriaceae* and non-fermenter bacilli) usually is much less efficient. On the other hand, cationic PS possess greater ability to inactivate Gram-negative bacteria, as well as Gram-positive, mycobacteria, fungi, parasites and viral particles (30-32, 35, 37-39). Current literature supports that cationic PS benefits from electrostatic interactions with the negatively charged surface and intracellular moieties of gram-negative and gram-positive bacteria, as it may repel anionic PS (31).

Figure 2. Schematic illustration of common photodynamic reaction pathways. Electron spin configurations in valence shell orbitals of radicals and singlet- and triplet-state molecules are represented as arrows inside squares. The excited singlet $^1\text{PS}^*$ decays to a triplet state ($^3\text{PS}^*$) and reacts with a receptor molecule, such as oxygen, by charge (type I) or energy (type II) donation, resulting in the formation of a variety of ROS such as free radicals, peroxides and singlet oxygen ($^1\text{O}_2$). *ISC*, *Inter System Crossing*. (Figure and legend were made by Caetano P. Sabino and published at referece 40).

Products formed from type I reactions (e.g. $\text{O}_2^{\bullet-}$, H_2O_2) may be quenched by specialized enzymes (figure 3), however, singlet oxygen produced by type II reactions faces very limited antioxidant cellular defenses based on scavengers such as carotenoids, free amino acids and glutathione (34, 41, 42).

Figure 3. General overview of type I and II photodynamic reactions in the cellular context. Ground state photosensitizer ($^1\text{PS}_0$) is excited by light ($h\nu$) to a higher energy state that decays to the long-lasting excited triplet state ($^3\text{PS}^*$) via intersystem crossing (ISC). Type I reaction initiates via charge transfer from $^3\text{PS}^*$ to $^3\text{O}_2$ forming superoxide radical anion ($\text{O}_2^{\bullet-}$). Superoxide can react with antioxidant scavengers, iron-sulfur clusters of enzyme active sites, or undergo dismutation reaction, catalyzed by superoxide dismutase (SOD), producing O_2 and hydrogen peroxide (H_2O_2). Hydrogen peroxide can be decomposed to H_2O and O_2 in reaction catalyzed by catalase (CAT) or other enzymes. Hydrogen peroxide can also be reduced by transition metals (e.g., Fenton reaction with Fe^{2+} or Cu^+) producing hydroxyl anions (HO^-) and radicals ($\text{HO}^\bullet$). Hydroxyl radicals are extremely reactive and can induce radical chain reactions in proteins, lipids, nucleic acids, and carbohydrates. Type 2 reactions produce singlet oxygen ($^1\text{O}_2^*$) via energy transfer. Singlet oxygen is also extremely reactive toward proteins, nucleic acids, and lipids. It can be quenched or scavenged by small molecular weight antioxidants, but, just like $\text{HO}^\bullet$, it is not impaired by any enzymes (figure and legend were made by Caetano P. Sabino and published at referece 40).

Antimicrobial photodynamic therapy (APDT) has been successfully used for over a century in modern Medicine to treat skin infections. During the 1900's, Dr. Niels Finsen performed pioneer experiments to investigate the interactions of monochromatic light with animal and microbial cells. The Finsen Method could successfully treat severe skin bacterial infections and soon became very popular in Northern Europe. In 1903 Finsen was awarded with the Nobel Prize *"in recognition of his contribution to the treatment of diseases, especially cutaneous tuberculosis, with concentrated light radiation, whereby he has opened a new avenue for medical science"* (Nobel Prize Website).

In fact, antimicrobial phototherapy, either with or without a PS, was flourishing as a prosperous method to combat skin infections until the 1930's, when Sir Alexander Fleming introduced the penicillin. Traditional antimicrobial chemotherapy was indeed more versatile to treat local and systemic infections, could be easily transported and used in distant locations (especially during war), was much more profitable for industries, and was yet effective against most pathogens.

The antibiotic era then overtook the scene and the focus on photodynamic therapy research and application shifted to the treatment of other conditions, such as cancer and age-related macular degeneration, with some appreciable success (29, 43). However, as a consequence of microbial drug-resistance emergence, APDT resurges nowadays as an interesting approach to overcome local infections and surface contamination (32, 39, 44-47). Examples of some potential APDT applications are illustrated in figure 4. Dental and oral soft tissues as well as dermatologic infections have been areas of special interest for antimicrobial APDT research due to easy access to light and high occurrence of biofilms and localized infections (32, 46-54). In fact, if light and photosensitizer are appropriately planned for a therapeutic protocol, the cytotoxic photodynamic dose required for microbial inactivation is, in general, lower than required to cause damage to host cells found in connective tissues (55, 56).

Figure 4. Potential clinical applications for antimicrobial photodynamic therapy. (Source: Caetano Padial Sabino)

APDT has an interesting feature that is not seen in other antimicrobial chemotherapies: the ability to inhibit virulence factors. Secreted virulence factors normally contain peptides and it is well known some amino acids (e.g. histidine, cysteine, tyrosine, tryptophan and methionine) are highly vulnerable to oxidation. This ability of APDT to destroy microbial toxins has been shown for lipopolysaccharides and proteases (57). It was proposed that this fact could explain the better wound healing observed when infected wounds were decontaminated by APDT compared to silver nitrate (58). In a similar view, we propose that APDT may inactivate resistance genes and their respective products, impairing microbial drug-resistance mechanisms.

2. Objectives

The main objective of this study was to assay the antimicrobial photodynamic efficacy and biochemical mechanisms of action against drug-resistant pathogens. For such task, we divided the main objective into four specific objectives, as follows:

1. A mathematical model to allow precise analysis and comparison of microbial photoinactivation kinetics;
2. An extensive analysis of APDT effectiveness against a broad-spectrum of global priority drug-resistant pathogens;
3. β -lactamase inhibition by APDT and antimicrobial blue light;
4. How the biological mechanisms of APDT avoid emergence of resistance.

The main results obtained through the investigation led by this thesis were divided into 4 scientific articles regarding each of the above-mentioned objectives. Each article is presented in the next section of this document.

3. Articles published or in process of publication

3.1. Main articles

1. Published. Title: **Inactivation kinetics and lethal dose analysis of antimicrobial blue light and photodynamic therapy**. Journal: *Photodiagnosis and Photodynamic Therapy*. Published in August 17th, 2019.
2. Published. Title: **Global priority multidrug-resistant pathogens do not resist photodynamic therapy**. Journal: *Journal of Photochemistry and Photobiology B: Biology*. Published in May 11th, 2020
3. Published. Title: **Antimicrobial blue light and photodynamic therapy inhibit clinically relevant β -lactamases with extended-spectrum (ESBL) and carbapenemase activity**. Journal: *Photodiagnosis and Photodynamic Therapy*. Published in November 4th, 2020
4. To be submitted. Expected title: **Deciphering the biochemical mechanisms of antimicrobial photodynamic therapy**. Journal: *Nature Microbiology*.

3.3. Article I

Photodiagnosis and Photodynamic Therapy 28 (2019) 186–191

Contents lists available at ScienceDirect

Photodiagnosis and Photodynamic Therapy

journal homepage: www.elsevier.com/locate/pdpdt

Inactivation kinetics and lethal dose analysis of antimicrobial blue light and photodynamic therapy

Caetano P. Sabino^{a,b,*}, Mark Wainwright^c, Carolina dos Anjos^d, Fábio P. Sellera^d,
Maurício S. Baptista^e, Nilton Lincopan^{b,f}, Martha S. Ribeiro^g

^a BioLambda, Scientific and Commercial LTD, São Paulo, SP, Brazil

^b Department of Clinical Analysis, School of Pharmaceutical Sciences, University of São Paulo, São Paulo, SP, Brazil

^c School of Pharmacy & Biomolecular Sciences, Liverpool John Moores University, Liverpool, UK

^d Department of Internal Medicine, School of Veterinary Medicine and Animal Science, University of São Paulo, São Paulo, SP, Brazil

^e Department of Biochemistry, Institute of Chemistry, University of São Paulo, São Paulo, SP, Brazil

^f Department of Microbiology, Institute for Biomedical Sciences, University of São Paulo, São Paulo, SP, Brazil

^g Center for Lasers and Applications, Nuclear and Energy Research Institute, National Commission for Nuclear Energy, São Paulo, SP, Brazil

ARTICLE INFO

Keywords:

Bacteria
Fungi
Microbial control
Photoantimicrobial
Photoinactivation
Photodynamic antimicrobial chemotherapy
Antimicrobial Photodynamic Therapy

ABSTRACT

Background: Antimicrobial Photodynamic therapy (A-PDT) has been used to treat infections. Currently, microbial inactivation data is reported presenting survival fraction averages and standard errors as discrete points instead of a continuous curve of inactivation kinetics. Standardization of this approach would allow clinical protocols to be introduced globally, instead of the piecemeal situation which currently applies.

Methods: To this end, we used a power-law function to fit inactivation kinetics and directly report values of lethal doses (LD) and a tolerance factor (T) that informs if inactivation rate varies along the irradiation procedure. A deduced formula was also tested to predict LD for any given survival fraction value. We analyzed the photoantimicrobial effect caused by red light activation of methylene blue (MB-APDT) and by blue light (BL) activation of endogenous microbial pigments against 5 clinically relevant pathogens.

Results: Following MB- APDT, *Escherichia coli* and *Staphylococcus aureus* cells become increasingly more tolerant to inactivation along the irradiation process ($T < 1$). *Klebsiella pneumoniae* presents opposite behavior, i.e., more inactivation is observed towards the end of the process ($T > 1$). *P. aeruginosa* and *Candida albicans* present constant inactivation rate ($T \approx 1$). In contrast, all bacterial species presented similar behavior during inactivation caused by BL, i.e., continuously becoming more sensitive to blue light exposure ($T > 1$).

Conclusion: The power-law function successfully fit all experimental data. Our proposed method precisely predicted LD and T values. We expect that these analytical models may contribute to more standardized methods for comparisons of photodynamic inactivation efficiencies.

1. Introduction

Photodynamic therapy (PDT) has been long studied and used to treat localized tumors and infections [1,2]. This light-based technology platform produces cytotoxic molecular species in a space-time controlled manner, i.e., in the absence of light, photosensitizer (PS) or oxygen, photodynamic reactions do not occur. The light-excited PS interacts with molecular oxygen, either by charge (type I reaction) or energy donation (type II reaction), forming a variety of reactive oxygen species (ROS) that can destroy bacteria, parasites, fungi, algae and viral particles [2–7].

The use of PSs thus offers an effective local – not just topical –

approach to infection control, often termed antimicrobial photodynamic therapy (APDT). Importantly, the agency of ROS here means that the conventional resistance status of the microbial target is unimportant. However, in order to provide photosensitization that is fit for purpose, the killing effects of PSs require proper quantification and benchmarking, e.g., the PS concentration and light dose required to destroy a given microbial burden at a certain rate. Standardization of this approach would allow clinical protocols to be introduced globally, instead of the piecemeal situation that currently applies.

According to the Second Law of Photochemistry, for each photon absorbed by a chemical system, only one molecule can be excited and subsequently undergo a photochemical reaction. Based on this

* Corresponding author at: BioLambda, Scientific and Commercial LTD, São Paulo, SP, Brazil.

E-mail address: caetanosabino@gmail.com (C.P. Sabino).

<https://doi.org/10.1016/j.pdpdt.2019.08.022>

Received 15 May 2019; Received in revised form 12 July 2019; Accepted 16 August 2019

Available online 17 August 2019

1572-1000/ © 2019 Elsevier B.V. All rights reserved.

principle, current literature supports photodynamic dosimetry in respect of the number of absorbed photons (*Absorbed Photons/cm³* instead of *J/cm²*) to provide a rather interpretable comparison of PS efficiency [8,9]. It has been proposed that using this method, problematic dosimetry due to variable PS concentration, optical path and excitation wavelength band can be minimized. However, some other problematic situations can be addressed by this method as well. If a filter effect is caused either by high cellular and/or PS concentrations, absorbed photon results may lead to divergent interpretations. Also, Prates et al. [10] have demonstrated that if the number of absorbed photons is kept constant but irradiance varies, the level of microbial inactivation also diverges [10]. These situations suggest the need for a more robust standard method, even though the number of absorbed photons per unit volume can be considered to represent an improvement on merely reporting inactivation as a function of radiant exposure.

Currently, the most accepted form of reporting microbial inactivation data in scientific articles is presenting survival fraction averages and standard errors as discrete points instead of a continuous curve of inactivation kinetics [9,10]. However, analysis of variance over individual points only allows the interpretation of whether those points present statistically significant differences among themselves. Therefore, if one intends to compare the potency of a set of variable antimicrobial photodynamic systems (i.e., different PSs, microbial species, light sources, etc.) this analysis may be misguided by local observation of a single point instead of the interpretation of a global kinetics rate. Therefore, this analytical method may lead to false-positive or -negative interpretations in respect to the overall phenomena of microbial inactivation kinetics.

To this end, we report a simple mathematical analysis of continuous bacterial inactivation kinetics curves. We analyzed the photodynamic killing effect caused by red light activation of methylene blue (MB) and by blue light activation of endogenous microbial photosensitive pigments. We expect that this method may assist in developing standardized and more insightful analysis of photoantimicrobial systems.

2. Material and methods

2.1. APDT experiments

In the present study we used the following strains from the American Type Culture Collection (ATCC): *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Klebsiella pneumoniae* (ATCC 700603), *Pseudomonas aeruginosa* (ATCC 27853) and *Candida albicans* (ATCC 90028).

Standard APDT susceptibility testing was carried out based on Prates et al. [10]. Inocula were prepared from log-phase overnight cultures. The turbidity of cell suspensions was measured in a spectrophotometer to obtain inocula at McFarland scale 0.5. The scale was calibrated to obtain an optical density of 0.09 at 540 nm and 625 nm resulting in $1-2 \times 10^6$ colony-forming units per milliliter (CFU/mL) of fungi cells, and $1-2 \times 10^8$ CFU/mL of bacterial cells, respectively. Inocula were diluted to a working concentration of $1-2 \times 10^5$ CFU/mL of fungi or $1-2 \times 10^7$ CFU/mL of bacteria.

MB hydrate (purity > 95%, Sigma-Aldrich, USA) was employed as the exogenous PS for this study. Before irradiation, cells were incubated with 100 μ M of MB in phosphate-buffered saline (PBS) for 10 min at room temperature and in the dark, to allow initial uptake. One-mL aliquots were individually placed in clean wells of a 12-well microplate. To avoid cross light exposure, each sample was kept in individual microtubes in the dark during pre-irradiation time and placed in the 12-wells plate only for irradiation.

A red LED probe (660 ± 10 nm, Prototype 1, BioLambda, Brazil) was positioned perpendicularly above each sample, keeping the beam diameter at the bottom of the well at 25 mm (which coincides to a single well diameter from the 12-wells plate). Red light irradiance was kept constant at 100 mW/cm² and radiant exposure levels varied

according to each microbial species sensitivity to MB-APDT as previously determined in pilot experiments.

A blue LED irradiator (415 ± 12 nm, LEDbox, BioLambda, Brazil) was placed below 12-well plates containing 1 mL of each microbial sample. In this case, no exogenous PS was added to the systems. Blue light irradiance was kept constant at 38.2 mW/cm². Radiant exposure levels varied according to each microbial species sensitivity to blue light inactivation as previously determined in pilot experiments.

Immediately after each irradiation process, bacterial suspensions were serially diluted in PBS to give dilutions from 10^{-1} to 10^{-6} times the original concentration. Ten- μ L aliquots of each dilution were streaked onto Mueller-Hinton agar plates, in triplicate, and incubated at 37 °C overnight. A similar procedure was performed for fungi. However, in this case dilutions were between 10^{-1} - 10^{-4} -fold and streaked onto Sabouraud dextrose agar. The colonies were counted and converted into CFU/mL for survival fraction analysis.

2.2. Data analysis

We adapted a power law function to fit inactivation kinetics data in respect to variable radiant exposure levels (equation (1)). Theoretical lethal dose (LD) for any given inactivation rate (i.e., % of bacterial or fungal survival fraction) was calculated according to equation (2). Fitting, residuals and derivatives were calculated using the Prism 7.0 (GraphPad, USA) interface.

$$\log_{10}\left(\frac{N_0}{N}\right) = \left(\frac{\text{Dose}}{\text{LD}_{90}}\right)^T \quad (1)$$

$$\text{LD}_i = \text{LD}_{90} \left(-\log_{10}\left(1 - \frac{i}{100}\right) \right)^{1/T} \quad (2)$$

where:

N_0 = initial microbial burden; N = final microbial burden; *Dose* = light exposure (e.g. J, J/cm², time units, *Absorbed Photons/cm³*, etc.); LD_{90} = lethal dose for 90% of microbial burden (in light exposure units); T = tolerance factor; i = inactivation percentage (%).

Since data analysis demands some strict procedures to be correctly performed, we organized it into 8 steps as presented in the spreadsheet available in the supplementary material.

Step 1. In Microsoft Excel spreadsheets, we organized the CFU/mL count averages for each experimental replicate according to the light dose delivered. Non-irradiated controls must be identified as 0 units of light dose. The other light doses used can be expressed in Joules, Joules/cm², number of absorbed photons or irradiation time (seconds, minutes or hours).

Step 2. We normalized all survival fraction data in relation to the average CFU/mL counts of non-irradiated controls (e.g., 0 Joules, Joules/cm², number of absorbed photons or irradiation time).

Step 3. Equation (1) only works for survival fraction data converted into Log₁₀ units. Therefore, all normalized survival fraction data were individually converted into Log₁₀ units before any fitting attempt.

Step 4. We calculated the survival fraction averages and standard deviations from the sets of experimental data of each group. These are the data that must be used in the data analysis software to fit the curve generated by equation (1).

Step 5. We created a new table and graph, organized as XY data configuration, using GraphPad Prism 7 software.

Step 6. After inserting all survival fraction data to Prism 7.0 XY-graph tables, we added equation (1) as an explicit equation for non-linear regressions. This step can be performed through Analyze > XY-analysis > Non-Linear-Regression-(curve fit). A new window will appear with a list of standard equations. Since Prism 7 does not have equation (1) as standard models for fitting data, we added it manually. For such, we clicked on the “+” button to “create a new equation”. On the “Equation” tab, equation type must be set as an explicit equation. In

Fig. 3. Non-linear regression parameters of inactivation kinetics obtained for each tested species. On the top, T values are presented for (a) MB-APDT and (b) blue light photoinactivation. Below are the LD₉₀ values calculated for (c) MB-APDT and (d) blue light photoinactivation. The presented values are means of constants and standard errors directly obtained by power law non-linear regressions.

Fig. 4. Lethal dose values calculated for 99.9% (3log₁₀) and 100% (7log₁₀ for bacteria and 5log₁₀ for yeast). On the left (a), calculated lethal doses are presented for MB-APDT groups and on the right (b) they are presented for blue light inactivation. The presented values are means and standard errors obtained from data of at least three independent experiments.

the same survival fraction level, this analysis can be used to establish the required light doses. Alternatively, this analysis can also calculate the dose required to achieve complete microbial inactivation (*i.e.*, LD₁₀₀), which is experimentally inviable to measure. In Fig. 4, we used data obtained from equation (1) (*e.g.*, LD₉₀ and T values) to calculate LD_{99.9} and LD₁₀₀ through equation (2). As expected, experimental groups with T < 1 presented much greater variations in between LD_{99.9} and LD₁₀₀ than groups with T > 1. The statistical results respective to data from Fig. 3 are presented in tables S5-6 from supplementary material.

For experimental verification of our proposed model, we compared photodynamic inactivation kinetics of MB-APDT and blue light using diverse species of clinically relevant pathogens. MB currently is the most broadly PS used in APDT studies while blue light inactivation is a promising antimicrobial platform using novel high-powered blue LEDs. These surrogates represent very different approaches to light-mediated microbial control and, yet, Eq. (1) successfully fit all tested data. We also showed that doses can be reported in time or energy units with no detriment of the analysis output. Thus, we expect that other PS classes should also be suitable for such analysis, and that this approach will allow the development of standardized protocols for photodynamic antimicrobial therapies. This way, future studies that choose to use this model could report quantitative data regarding LD₉₀ and T values in order to allow comparative analysis between different PS, light sources, irradiances, *etc.* We hope to motivate further studies to employ this method also for experiments regarding *in vitro* biofilms and *in vivo* infections.

4. Conclusion

We reported a mathematical model to fit and describe photoinactivation kinetics in interpretative and quantitative terms. A power-law function successfully fit all data from experiments performed with MB-APDT and blue light alone. A deduced formula could also be used to precisely predict lethal doses for any given survival fraction value. We truly expect that these analytical methods may contribute to a more standardized protocol for comparisons of photodynamic inactivation efficiency.

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.

Acknowledgments

We gratefully thank the Brazilian fostering agency FAPESP (grants 2013/07937-8, < GN1 > 2016/08593-9, < GN1 > 2016/25095-2, < GN1 > 2017/22406-0), CNPq (grants < GN2 > 465763/2014-6, < GN2 > 141901/2016-0, < GN2 > 312249/2017-9) < /GN2 > < /GN2 > < /GN2 > < /GN1 > < /GN1 > < /GN1 > and technical support offered by the start-up company BioLambda.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the

Fig. 1. Inactivation kinetics plots. On the top, survival fraction values are presented for (a) MB-APDT and (b) blue light photoinactivation. Below are the first derivatives (i.e., microbial inactivation rate) of each non-linear regression curves fitted for (c) MB-APDT and (d) blue light photoinactivation. The experimental data from a and b are the log reduction of normalized survival fraction and standard errors.

Fig. 2. Linearization of inactivation kinetics data by double-log transformations in a and b confirms the hypothesis of power law function fitting. Residuals of fitted data in c and d presented random distributions around the average, confirming data homogeneity and normality.

the “definition” field, we inserted the following formula: $Y = (x/LD_{90})^T$ (T). On the “Rules for Initial Values” tab, we set initial values of LD_{90} and T to be fit as 1. We clicked on “ok” and the new equation was created.

Step 7. If data fitting succeeded, calculated curves will be plotted over data-points in graphs. A table of results will be created on the left panel. This table will present the calculated averages and standard errors for LD_{90} and T.

Step 8. Back in the Microsoft Excel spreadsheet, $LD_{90,9}$ and LD_{100} values were calculated for each dataset using equation (2) in the following formula: $= (LD_{90}) * (-\log_{10}(1 - (i/100)))^{(1/T)}$ where i represents inactivation percentage (%). Due to the inocula used in this study, 100% of inactivation represents $7 \log_{10}$ (99.99999%) for bacteria and $5 \log_{10}$ (99.999%) for yeast.

All experiments were performed in triplicate at 3 different days ($n = 9$), except for MB-APDT against *K. pneumoniae*, which was performed in 4 different days ($n = 12$) due to a pilot study. Survival fraction data were analyzed by Shapiro-Wilk test to confirm normality. Fitted curves were also analyzed using F-test to check if any of the fitted curves are shared by different species. Lethal-dose and T value analysis were compared among species using one-way analysis of variance (ANOVA) with Bonferroni as post-hoc test for multiple comparisons. Results were considered significant if $p < 0.05$.

3. Results and discussion

The Weibull analysis is a well-known and accepted statistical method that uses a power-law function to describe breakdown kinetics of various materials. This analysis assumes that the survival curve is affected by cumulative distributions of damages that leads to lethal effects. Here we assumed that it properly describes effects such as the cumulative oxidative damage imposed by APDT over living cells [11].

Historically, this statistical model has been mostly employed in industries, such as aerospace and automotive, to estimate the reliability on lifespan of mechanical parts [12]. This mathematical function has been used to describe bacterial inactivation kinetics during thermal inactivation or gamma radiation, UV- and blue-light irradiation, free of exogenous PSs [13–16]. However, it has not so far been proposed as a method to standardize APDT sensitivity protocols.

Power-Law fit appears to represent a very good description for APDT inactivation kinetics of our data. Adjusted R^2 values always fluctuated above 0.95 (Table 1). These values represent very good results in relation to general non-linear curve fittings.

The F-test applied over non-linear regressions reported that each species dataset presents a unique inactivation kinetics curve (Fig. 1a–b). This means that even though some inactivation data points may not present statistical differences among species, the entire inactivation kinetics are not the same.

The first derivative of inactivation curves (Fig. 1c–d) further illustrates the variation in inactivation rates. This analysis shows how fast the inactivation occurs during the irradiation procedure. For MB-APDT, *E. coli* and *S. aureus* cells are inactivated rapidly in the beginning of the procedure but slower towards the end of the process. *K. pneumoniae* presents the exact opposite behavior. *P. aeruginosa* and *C. albicans*, however, present almost a constant inactivation rate. On the other hand, all bacterial species presented similar behavior during inactivation caused by blue light alone, i.e., slow initial inactivation but becoming continuously more sensitive to blue light exposure. Conversely,

C. albicans presented again almost a constant inactivation rate.

We also submitted inactivation data to double-log transformations in order to confirm data linearization. This is a standard empirical method used to confirm the feasibility of a power-law fit in experimental datasets. As a matter of fact, successful linearization (Fig. 2a–b) further proves the ability to describe photoinactivation kinetics assuming a Power-Law behavior, yet all residual dispersions presented random distributions (Fig. 2c–d).

Non-linear regression results are presented in Fig. 3 as values of the tolerance factor T and lethal doses for 90 percent ($1 \log_{10}$) of inactivation. The tolerance factor T informs the concavity of the inactivation curves; if $T > 1$, cells are initially tolerant to APDT but become increasingly sensitive; if $T < 1$, cells are initially very sensitive, but some persistent cells remain more tolerant to inactivation. Hence, the behavior observed at the inactivation rate curves (Fig. 1c–d) can be indicated by the T values (Fig. 3a–b).

For MB-APDT (Fig. 3a), *S. aureus* and *E. coli* $T < 1$ with no statistically significant difference among themselves; *P. aeruginosa* and *C. albicans* presented T values close to 1, with no statistical difference among themselves; *K. pneumoniae* presented a T value close to 1.5 and was statistically different from all other species treated by MB-APDT. For blue light inactivation, all species presented T values above 1, without any statistically significant differences in between them. These statistical analysis results are presented in tables S1–2 in supplementary material.

Lethal doses for 90% (i.e., $1 \log_{10}$) inactivation with MB-APDT (Fig. 3c) show that *E. coli* and *S. aureus* are the most sensitive and present statistically significant differences to all other species but not among themselves. *P. aeruginosa* presented an intermediate sensitivity to MB-APDT that was significantly different from all other species. *K. pneumoniae* and *C. albicans* are significantly more tolerant to MB-APDT than all other species but not amongst themselves. Even though no statistical differences were observed for T values of blue light inactivation (Fig. 3d), several particularities were reported for lethal dose values. *E. coli* and *S. aureus* are quite sensitive to blue light and present statistically similar behavior. However, *P. aeruginosa* seems to be the most sensitive species tested to blue light, although it did not show statistically significant differences relative to *S. aureus*. Such high sensitivity of *P. aeruginosa* to blue light may be linked to high yield production of pyoverdine, a naturally occurring fluorescent pigment that strongly absorbs 415 nm light and may undergo photodynamic reactions [17,18]. *K. pneumoniae* and *C. albicans* are significantly the most tolerant species to blue light and do not present statistical differences between themselves. These statistical results can be seen in tables S3–4 from supplementary material.

The concept of inactivation rate illustrated by the first derivative of inactivation curves can be specifically quantified by the tolerance factor, presented as T values (Fig. 3a). This is a dimensionless value that indicates the overall inactivation rate behavior. It describes whether cells are more tolerant to inactivation at the beginning of the irradiation process or at the end. Therefore, we can indicate the existence of microbial species with a constitutive tolerance ($T > 1$) that is soon depleted making cells become increasingly sensitive (e.g., MB-APDT for *E. coli* and *S. aureus*); or the presence of adapting or more persistent cells ($T < 1$) that remain harder to kill after a period of irradiation (e.g., blue light for bacteria). Microbial species with T values close to unity may represent an intermediate situation (e.g., *C. albicans* in both situations). The exact tolerance mechanisms responsible for these inactivation kinetics variations may have a multifactorial basis that leads to a constant inactivation rate.

A very useful aspect of using our proposed model is the ability to calculate lethal doses for any given level of survival fraction. Such information allows precise and direct comparisons in between experimental groups and also provides basis for future experimental planning. For example, if one is interested to analyze perspectives of microbial inactivation by APDT or blue light of different experimental groups at

Table 1
Adjusted R^2 value of each non-linear curve fit.

Species	<i>E. coli</i>	<i>S. aureus</i>	<i>P. aeruginosa</i>	<i>K. pneumoniae</i>	<i>C. albicans</i>
MB-APDT	0.9745	0.9955	0.9939	0.9834	0.9793
Blue Light	0.9691	0.9518	0.9805	0.9526	0.9756

online version, at doi:<https://doi.org/10.1016/j.pdpdt.2019.08.022>.

References

- [1] P. Agostinis, K. Berg, K.A. Cengel, T.H. Foster, A.W. Girotti, S.O. Gollnick, et al., Photodynamic therapy of cancer: an update, *CA Cancer J. Clin.* 61 (4) (2011) 250–281.
- [2] M. Wainwright, Photodynamic antimicrobial chemotherapy (PACT), *J. Antimicrob. Chemother.* 42 (1) (1998) 13–28.
- [3] T. Maisch, S. Hackbarth, J. Regensburger, A. Felgentrager, W. Baumler, M. Landthaler, et al., Photodynamic inactivation of multi-resistant bacteria (PIB) - a new approach to treat superficial infections in the 21st century, *J. Dermatol. Ges.* 9 (5) (2011) 360–366.
- [4] T. Dai, Y.Y. Huang, M.R. Hamblin, Photodynamic therapy for localized infections—state of the art, *Photodiagnosis Photodyn. Ther.* 6 (3–4) (2009) 170–188.
- [5] J.P. Lyon, L.M. Moreira, P.C. de Moraes, F.V. dos Santos, M.A. de Resende, Photodynamic therapy for pathogenic fungi, *Mycoses* 54 (5) (2011) e265–71.
- [6] R. Schmidt, Photosensitized generation of singlet oxygen, *Photochem. Photobiol.* 82 (5) (2007) 1161–1177.
- [7] F.P. Sellera, C.P. Sabino, M.S. Ribeiro, R.G. Gargano, N.R. Benites, P.A. Melville, et al., *In vitro* photoinactivation of bovine mastitis related pathogens, *Photodiagnosis Photodyn. Ther.* 13 (2016) 276–281.
- [8] B.C. Wilson, M.S. Patterson, L. Lilje, Implicit and explicit dosimetry in photodynamic therapy: a New paradigm, *Lasers Med. Sci.* 12 (3) (1997) 182–199.
- [9] F. Cieplik, A. Pummer, J. Regensburger, K.A. Hiller, A. Spath, L. Tabenski, et al., The impact of absorbed photons on antimicrobial photodynamic efficacy, *Front. Microbiol.* 6 (2015) 706.
- [10] R.A. Prates, E.G. Silva, A.M. Yamada, L.C. Suzuki, C.R. Paula, M.S. Ribeiro, Light parameters influence cell viability in antifungal photodynamic therapy in a fluence and rate fluence-dependent manner, *Laser Phys.* 19 (5) (2009) 1038–1044.
- [11] W. Weibull, A statistical distribution function of wide applicability, *J. Appl. Mech.* 18 (3) (1951) 293–297.
- [12] G.W. Weibull, Citation classic - a statistical distribution function of wide applicability, *Ce/Eng Tech. Appl. Sci.* (10) (1981) 18.
- [13] D.L. Holcomb, M.A. Smith, G.O. Ware, Y.C. Hung, R.E. Brackett, M.P. Doyle, Comparison of six dose-response models for use with food-borne pathogens, *Risk Anal.* 19 (6) (1999) 1091–1100.
- [14] X. Hu, P. Mallikarjunan, J. Koo, L.S. Andrews, M.L. Jahncke, Comparison of kinetic models to describe high pressure and gamma irradiation used to inactivate *Vibrio vulnificus* and *Vibrio parahaemolyticus* prepared in buffer solution and in whole oysters, *J. Food Prot.* 68 (2) (2005) 292–295.
- [15] K. McKenzie, M. Maclean, I.V. Timoshkin, E. Endarko, S.J. MacGregor, J.G. Anderson, Photoinactivation of bacteria attached to glass and acrylic surfaces by 405 nm light: potential application for biofilm decontamination, *Photochem. Photobiol.* 89 (4) (2013) 927–935.
- [16] H. Bozkurt, D.H. D'Souza, P.M. Davidson, Determination of thermal inactivation kinetics of hepatitis A virus in blue mussel (*Mytilus edulis*) homogenate, *Appl. Environ. Microbiol.* 80 (10) (2014) 3191–3197.
- [17] R.P. Elliott, Some properties of pyoverdine, the water-soluble fluorescent pigment of the pseudomonads, *Appl. Microbiol.* 6 (4) (1958) 241–246.
- [18] C. Propst, L. Lubin, Light-mediated changes in pigmentation of *Pseudomonas aeruginosa* cultures, *J. Gen. Microbiol.* 113 (2) (1979) 261–266.

3.4. Article II

Journal of Photochemistry & Photobiology, B: Biology 208 (2020) 111893

Contents lists available at ScienceDirect

Journal of Photochemistry & Photobiology, B: Biology

journal homepage: www.elsevier.com/locate/jphotobiol

Global priority multidrug-resistant pathogens do not resist photodynamic therapy

Caetano Padial Sabino^{a,b,*}, Mark Wainwright^c, Martha Simões Ribeiro^d, Fábio Parra Sellera^e, Carolina dos Anjos^e, Mauricio da Silva Baptista^f, Nilton Lincopan^{b,g}

^a BioLambda, Scientific and Commercial LTD, São Paulo, SP, Brazil.

^b Department of Clinical Analysis, Faculty of Pharmaceutical Sciences, University of São Paulo, São Paulo, Brazil.

^c School of Pharmacy & Biomolecular Sciences, Liverpool John Moores University, Liverpool, UK.

^d Center for Lasers and Applications, Nuclear, and Energy Research Institute, National Commission for Nuclear Energy, São Paulo, SP, Brazil.

^e Department of Internal Medicine, School of Veterinary Medicine and Animal Science, University of São Paulo, São Paulo, Brazil.

^f Department of Biochemistry, Institute of Chemistry, University of São Paulo, São Paulo, SP, Brazil.

^g Department of Microbiology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil.

ARTICLE INFO

Keywords:

Drug resistance
ESKAPE
Multidrug resistance
Photoinactivation

ABSTRACT

Microbial drug-resistance demands immediate implementation of novel therapeutic strategies. Antimicrobial photodynamic therapy (aPDT) combines the administration of a photosensitizer (PS) compound with low-irradiance light to induce photochemical reactions that yield reactive oxygen species (ROS). Since ROS react with nearly all biomolecules, aPDT offers a powerful multitarget method to avoid selection of drug-resistant strains. In this study, we assayed photodynamic inactivation under a standardized method, combining methylene blue (MB) as PS and red light, against global priority pathogens. The species tested include *Acinetobacter baumannii*, *Klebsiella aerogenes*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterococcus faecium*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Candida albicans* and *Cryptococcus neoformans*. Our strain collection presents resistance to all tested antimicrobials (> 50). All drug-resistant strains were compared to their drug-sensitive counterparts. Regardless of resistance phenotype, MB-aPDT presented species-specific dose-response kinetics. More than 5log₁₀ reduction was observed within less than 75 s of illumination for *A. baumannii*, *E. coli*, *E. faecium*, *E. faecalis* and *S. aureus* and within less than 7 min for *K. aerogenes*, *K. pneumoniae*, *P. aeruginosa*, *C. albicans* and *C. neoformans*. No signs of correlations in between drug-resistance profiles and aPDT sensitivity were observed. Therefore, MB-aPDT can provide effective therapeutic protocols for a very broad spectrum of pathogens. Hence, we believe that this study represents a very important step to bring aPDT closer to implementation into mainstream medical practices.

Original Article.

1. Introduction

The global crisis of antimicrobial resistance (AMR) is now accepted both by the research community and health authorities [1–4]. The lack of effective agents could mean the end of modern medicine worldwide, with simple infections again associated with high mortality rates and even routine surgical procedures becoming unsafe [4]. Access to effective conventional therapeutics is also becoming more difficult due to an insufficient pipeline of new drugs [5].

Among bacterial infections, resistance may – of course – be encountered anywhere, but the leading cause of nosocomial infections

globally is the ESKAPE group. The members of this group are commonly associated with multidrug-resistance and can thus circumvent the effects of a number of different classes of conventional antibacterial agents [6,7]. Consequently, some illnesses caused by these bacteria are very challenging to treat with the current armamentarium.

Such is the gravity of the situation that governments, healthcare providers and the pharmaceutical industry now recognize the requirement for non-conventional therapeutic approaches to combat AMR [4,8]. However, such alternatives must be robust, reliable and offer antimicrobial coverage exceeding that of conventional agents, particularly with respect to resistance. Biologicals such as vaccines and phage therapy are among alternatives proposed, but clearly both of these are highly specific in terms of the target rather than representing

* Corresponding author at: BioLambda, Scientific and Commercial LTD, São Paulo, SP, Brazil
E-mail address: caetanosabino@gmail.com (C.P. Sabino).

<https://doi.org/10.1016/j.jphotobiol.2020.111893>

Received 25 November 2019; Received in revised form 4 May 2020; Accepted 8 May 2020
Available online 11 May 2020

1011-1344/ © 2020 Elsevier B.V. All rights reserved.

generally useful antimicrobial approaches with non-conventional routes to target toxicity.

Antimicrobial photodynamic therapy (aPDT) is an interesting method to produce cytotoxic molecular species in a space-time-controlled manner. This light-based technology platform uses low-to-mid irradiance (*i.e.*, non-thermal) light in combination with a non-toxic compound, termed photosensitizer (PS), to treat localized infections [9,10]. Therefore, microbial inactivation mediated by photodynamic reactions may only occur where the photosensitizer is present and when it is being activated by light. The light-excited photosensitizer ($^3\text{PS}^*$) interacts with a biological substrate or molecular oxygen (O_2), either by charge or energy donation, producing a variety of reactive oxygen species (ROS) and radical reactions [11,12]. Products such as singlet oxygen or hydroxyl radicals are so reactive that in sufficient amounts, they can destroy any type of microbial cell regardless of taxonomy. Hence, this non-conventional mode of action provides effective kill regardless of conventional drug-resistance mechanisms [10,13–17].

In this study, we assayed the photodynamic antimicrobial efficiency of a broadly available photosensitizer drug (methylene blue, MB) and red light against bacterial and fungal species frequently associated with drug-resistance. We tested bacteria resistant to nearly all antibacterial drugs (> 50), including the entire *ESKAPE* group [1], WHO global priority pathogens [5,18] and azole-resistant fungi, to compare them to standard control strains. The study is therefore highly relevant in providing an examination of the efficacy of the photodynamic approach with a single agent against multiple, different microbial resistance mechanisms.

2. Material and Methods

2.1. Strains and Inocula Preparation

We used a collection of 23 strains from 8 bacterial species (*E. faecium*, *E. faecalis*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, *K. aerogenes* and *E. coli*), as well as 4 strains from 2 yeast species (*C. albicans* and *C. neoformans*). (See Table 1)

All bacterial strains were first subcultured from frozen vial stocks onto Muller-Hinton agar for 24 h at 37 °C. Yeast were subcultured onto Sabouraud dextrose agar under the same procedure. Individual colonies were then seeded into Muller-Hinton or Sabouraud dextrose broth and incubated overnight under shaking regimen (115 rpm) at 37 °C.

2.2. Qualitative Antimicrobial-Resistance Profile

Qualitative susceptibility assay was performed by Kirby-Bauer disk-diffusion method with interpretative criteria based on CLSI 2017 recommendations of inhibition diameter breakpoints [19]. We assayed a total of 43 representative antimicrobials against Gram-negative and 35 against Gram-positive bacteria. Minimum inhibitory concentration (MIC) assays were performed, as recommended by CLSI 2017, to determine the vancomycin-intermediate resistance of *S. aureus*, colistin resistance of *E. coli* and fluconazole resistance of fungi.

2.3. Photosensitizer and Light Source

We used the phenothiazine salt methylene blue hydrate (MB, purity $> 95\%$, Sigma-Aldrich) as a representative PS compound. Stock solutions of MB were prepared in type-1 Milli-Q water, at 10 mM. All MB stock solutions were filtered by 0.22 μm membrane for microbial decontamination, aliquoted into Eppendorf tubes and stored in the dark before use. MB working concentration was set at 100 μM in PBS. All experiments were performed with 1 mL of MB-inocula solution in wells of a 12-well cell culture plate (length of the optical path in solution was 2 mm).

We used a prototype LED system (660 ± 10 nm, LEDsaber Prototype 1, BioLambda, Brazil) that was previously characterized by

Table 1

List of microbial strains used in this study.

Bacterial Species	Strain	Capsule
Drug-sensitive control strains		
<i>Acinetobacter baumannii</i>	ATCC 19606	–
<i>Candida albicans</i>	ATCC 90028	–
<i>Cryptococcus neoformans</i>	KN99a	+
<i>Klebsiella aerogenes</i>	ATCC 13048	+
<i>Enterococcus faecalis</i>	ATCC 29212	–
<i>Enterococcus faecium</i>	ATCC BAA-2127	–
<i>Escherichia coli</i>	ATCC 25922	–
<i>Klebsiella pneumoniae</i> (ESBL, SHV-18)	ATCC 700603	+
<i>Pseudomonas aeruginosa</i>	ATCC 27853	+
<i>Staphylococcus aureus</i>	ATCC 25923	–
Drug-resistant strains (resistance phenotype)		
<i>Acinetobacter baumannii</i> (carbapenemase OXA-23)	LDC [65]	–
<i>Acinetobacter baumannii</i> (carbapenemase OXA-143)	804 [66]	–
<i>Candida albicans</i> (azole resistant)	IAL2151 [67]	–
<i>Cryptococcus neoformans</i> (azole resistant)	H99 [54]	+
<i>Klebsiella aerogenes</i> (carbapenemase NDM-1)	E0083033–1 [68]	+
<i>Enterococcus faecalis</i> (vancomycin-resistant VanB)	ATCC 51299	–
<i>Enterococcus faecium</i> (vancomycin-resistant VanA)	ATCC 700221	–
<i>Escherichia coli</i> (ESBL CTX-M-8)	19B [69]	–
<i>Escherichia coli</i> (ESBL CTX-M-1, colistin-resistant MCR-1)	ICBEC7P [70]	–
<i>Klebsiella pneumoniae</i> (metallo- β -lactamase IMP-1, ESBL CTX-M-2)	KP BR-1 [71]	+
<i>Klebsiella pneumoniae</i> (carbapenemase KPC-2)	ATCC BAA1705	+
<i>Klebsiella pneumoniae</i> (carbapenemase OXA-48)	11,978 [72]	+
<i>Klebsiella pneumoniae</i> (carbapenemase KPC-2, ESBL CTX-M-15)	KP148/PINH-4900 [73]	+
<i>Pseudomonas aeruginosa</i> (carbapenemase GES-5)	PA64 [74]	+
<i>Pseudomonas aeruginosa</i> (metallo- β -lactamase SPM-1)	1997A-48 [75]	+
<i>Pseudomonas aeruginosa</i> (metallo- β -lactamase VIM-2)	ICBDVIM2 [76]	+
<i>Staphylococcus aureus</i> (MRSA, VRSA)	VRSA BR-4 [77]	–

an UV-VIS spectrophotometer (Flame, Ocean Optics, USA). Light irradiance was adjusted to a standard irradiance of 100 mW/cm² measured at the sample bottom. Beam spot diameter was the same as a well of the 12-well plate (*i.e.*, 25 mm), with maximum irradiance variation between center and border below 10%.

2.4. aPDT Studies for Planktonic Suspensions

We performed standard aPDT susceptibility tests based on the study published by Sabino et al. [20]. Inocula were prepared from overnight broth cultures under shaking regimen. Inocula concentrations were adjusted to obtain OD of 0.09 at 540 nm and 625 nm resulting in $1\text{--}2 \times 10^6$ CFU/mL of fungal cells and $1\text{--}2 \times 10^8$ CFU/mL of bacterial cells, respectively. Inocula were then diluted to a working concentration of $1\text{--}2 \times 10^5$ CFU/mL of fungi or $1\text{--}2 \times 10^7$ CFU/mL of bacteria.

Before irradiation, cells were incubated with MB in phosphate-buffered saline (PBS) for 10 min at room temperature and in the dark, to allow initial cellular uptake. To avoid cross light exposure, 1 mL of each sample was placed in the 12-well plate only for irradiation procedure. Three types of controls were used for all 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).

Experimental procedure for irradiation was performed with variable radiant exposure. Radiant exposure levels were varied according to each microbial species sensitivity MB-aPDT as previously determined in pilot experiments. Basically, species were divided into 2 radiant

exposure ranges: 1 J/cm² steps (*A. baumannii*, *E. faecalis*, *E. faecium*, *E. coli* and *S. aureus*) or 5 J/cm² steps (*K. aerogenes*, *K. pneumoniae*, *P. aeruginosa*, *C. albicans* and *C. neoformans*). Radiant exposure was calculated as the product of irradiance (W/cm²) and the exposure time (seconds).

Following irradiation procedures, each bacterial suspension was serially diluted in PBS and 10 µL aliquots of each dilution were seeded onto Muller-Hinton (bacteria) or Sabouraud dextrose (yeast) agar plates in triplicate and incubated at 37 °C overnight. On the next day, colonies were counted and converted into normalized log₁₀ units of CFU/mL for survival fraction analysis.

2.5. Statistical Analysis

All data were obtained from at least three independent experiments, each performed in triplicate, resulting in a minimum of nine samples per group. Quantitative data are presented as log₁₀ of normalized means and standard deviation of means.

All inactivation kinetics data were fitted by power-law (eq. 1) for statistical comparison of each inactivation kinetics curve according to Sabino et al. [20]. LD₉₀ and T (tolerance factor) values were initially obtained for each strain by non-linear regression analysis. Then the same analysis was performed for the average inactivation kinetics of each species. Using the average inactivation kinetics of each species, we also calculated the LD_{99,9}, LD_{99,999} using eq. 2.

$$\log_{10} \left(\frac{N_0}{N} \right) = \left(\frac{\text{Dose}}{\text{LD}_{90}} \right)^T \quad (1)$$

$$\text{LD}_i = \text{LD}_{90} \left(-\log_{10} \left(1 - \frac{i}{100} \right) \right)^{1/T} \quad (2)$$

where:

N_0 = initial microbial burden; N = final microbial burden; Dose = light exposure (e.g. J, J/cm², time units, Absorbed Photons/cm², etc.); LD₉₀ = lethal dose for 90% of microbial burden (in light exposure units); T = tolerance factor; i = inactivation percentage (%).

Statistical differences of all LD₉₀, LD_{99,9}, LD_{99,999} and T values were then compared in between strains and species using one-way ANOVA with Tukey as post-test. Statistical results were considered significant if $p < .05$ and are presented in the supplementary information section.

3. Results

Antimicrobial susceptibility profiles of all strains are presented in **supplementary tables S1–3**. The inhibition diameters of unclassified antimicrobials are presented in numeric values (mm). For all species, we had one drug-sensitive strain and at least one multidrug-resistant (MDR) and/or extensively drug-resistant (XDR) representative strain [21]. Thus, we used ten drug-sensitive controls (*S. aureus* ATCC 29923; *E. faecium* ATCC BAA2127; *E. faecalis* ATCC 29212; *P. aeruginosa* ATCC 27853; *A. baumannii* ATCC 19606; *K. aerogenes* ATCC 13048; *E. coli* ATCC 25922; *K. pneumoniae* ATCC 700603; *C. albicans* ATCC 90028; *C. neoformans* KN99a), thirteen MDR (*S. aureus* VRSA-BR-4; *E. faecium* ATCC 700221; *E. faecalis* ATCC 51299; *K. pneumoniae* KP-BR-1, 11,978, ATCC BAA1705; *A. baumannii* 804, LDC; *K. aerogenes* E0083033–1; *E. coli* 19B, ICBEC7P; *C. albicans* IAL2151; *C. neoformans* H99) and four XDR strains (*K. pneumoniae* KP148/PINH-4900; *P. aeruginosa* 1997A-48, PA64, ICBVIM2). In a general perspective, resistance to all tested antibiotics except for linezolid was detected (tables S1–3). Fluconazole resistance was confirmed for *C. albicans* (IAL2151) and *C. neoformans* (H99).

A remarkable fact that can be extracted from our data (Fig. 1–2) is that microbial inactivation kinetics induced by MB-aPDT do not seem to depend on the conventional drug-resistance profile of different strains. At least for the representative MDR and XDR strains tested, current

drug-resistance does not impose any further challenges for MB-aPDT. For all tested strains, LD₉₀ and T values did not show any statistically significant differences within the same species. On the other hand, inactivation kinetics present great variations among different species of bacteria and yeast.

Regardless of taxonomy, all strains presented > 5log₁₀ of burden reduction for radiant exposures greater than 40 J/cm² (Fig. 1–2). However, *C. albicans*, *C. neoformans*, *K. pneumoniae*, *K. aerogenes* and *P. aeruginosa* presented a distinguishable tolerance to MB-aPDT. Light-doses required to inactivate 99.999% of these species were 2 to 10 times higher when compared to *A. baumannii*, *E. coli*, *E. faecalis*, *E. faecium* and *S. aureus*. However, it should be noted that this would not be problematic from a clinical viewpoint, since 40 J/cm² of irradiation at 100 mW/cm² (same irradiance as used in this study) would correspond to less than a 7-min procedure.

The microbial diversity assayed by this study in a standard manner allows us to observe some situations that diverge from some current thinking in the aPDT field. Based on our results, the classic generalization that aPDT sensitivity increases in the order fungi < Gram-negative < Gram-positive is no longer sustained [22]. The Gram-positives *E. faecalis* and *E. faecium*, for example, tend to be more tolerant to aPDT than Gram-negative species, such as *E. coli* and *A. baumannii*. Even though, *S. aureus*, *E. coli*, *A. baumannii*, *E. faecalis* and *E. faecium* do not present statistically significant differences for LD₉₀, LD_{99,9} and LD_{99,999} (supplementary table S4). In addition, the bacterium *K. pneumoniae* is slightly more tolerant than the *C. neoformans* yeast, but with no statistical significance. For LD_{99,999}, *C. neoformans* does not present statistically significant differences with the bacteria *K. aerogenes* and *P. aeruginosa*. The most tolerant species to MB-aPDT was *C. albicans*. It does not present statistically significant differences with *K. pneumoniae* at LD₉₀. However, it was significantly more tolerant than all other species at LD_{99,9} and LD_{99,999}.

Non-linear regression results for strain averages of each species are respectively presented in Figs. 3 and 4 as values of the tolerance factor (T), and lethal dose for 90% of inactivation (LD₉₀). The tolerance factor T (Fig. 3, statistical analysis in supplementary table S5) informs the concavity of the inactivation curves; if $T > 1$, the microbial population is initially tolerant to aPDT but becomes increasingly sensitive; if $T < 1$, the microbial population is initially sensitive, but some persistent cells remain more tolerant to inactivation as irradiation progresses. If $T = 1$, the microbial population presents a constant inactivation kinetics rate. These characteristics are clearly observable in Figs. 1 and 2, where *K. pneumoniae*, *P. aeruginosa*, *E. faecium*, *E. faecalis* and yeast presented T factors equal or greater than 1. Regarding *E. faecalis*, an interesting feature is highlighted by the use of non-linear regressions: by the beginning of irradiation process this species is more tolerant than *E. coli*, *S. aureus* and *A. baumannii*. However, due to the concavity of its inactivation kinetics curve (i.e., $T \sim 1$), at LD_{99,999} it becomes the most sensitive species to MB-aPDT (not statistically significant for *S. aureus*, *A. baumannii*, *E. coli* and *E. faecium*, supplementary table S4).

Regarding lethal doses, an interesting behavior occurs: LD₉₀ values present variations greater than 2 orders of magnitude among different species, such as the extreme case of *S. aureus* and *K. pneumoniae*, even though, due to variations in T values, all species seem to converge to closer LD_{99,9} and LD_{99,999} values (Fig. 4). Species with $T > 1$ tend to present higher LD₉₀; for those with $T < 1$ the opposite behavior is observed. Regarding the example of *S. aureus* and *K. pneumoniae* again, LD_{99,999} variation is reduced to less than one order of magnitude.

4. Discussion

Here we used drug-resistant phenotypes for all tested commercial antimicrobials (> 50), with a single exception for linezolid. If such drug-resistance profiles are detected in clinical cases, prognostics can hardly be optimistic. Consequently, cost and risk management of

Fig. 1. Inactivation kinetics of bacterial species most sensitive to MB-aPDT (scale 1) in function of radiant exposure. Average inactivation kinetics data of all strains of each species in this board is presented in **a**. Inactivation kinetics of each strain is also shown for *A. baumannii* (**b**), *E. faecalis* (**c**), *E. faecium* (**d**), *E. coli* (**e**) and *S. aureus* (**f**).

pathogens significantly increases.

Costs associated with drug-resistant infections could be significantly reduced if health insurance companies and public hospitals offered

aPDT as an option. Light sources can be reused countless times and PS doses of most commercially available dyes are relatively inexpensive. In our example, a one-mL dose of MB at 100 μ M costs fractions of US-

Fig. 2. Inactivation kinetics of bacterial and yeast species most tolerant to MB-aPDT (scale 5) in function of radiant exposure. Average inactivation kinetics data of all strains of each species in this board is presented in a. Inactivation kinetics of each strain is also shown for *K. aerogenes* (b), *K. pneumoniae* (c), *P. aeruginosa* (d), *C. neoformans* (e) and *C. albicans* (f).

dollar cents and can be purchased in most pharmacies as a standard drug to treat methemoglobinemia or carbon monoxide poisoning. MB is a particularly interesting photosensitizer since it is cost-effective, safe,

globally available and has been proved effective for several clinical applications of aPDT [10,13,23,24]. MB also presents intense light absorption properties (peak at 664 nm) in the optical window of light

Fig. 3. Tolerance factor (T) of inactivation kinetics calculated for each tested species. If $T > 1$, microbial population is initially tolerant to aPDT but become increasingly sensitive. If $T < 1$, microbial population is initially sensitive, but some persistent cells remain more tolerant to inactivation as irradiation progresses. If $T = 1$, microbial population presents a constant inactivation kinetics rate in a log-scale.

penetration into biological tissues (i.e., 600–1350 nm) [23,25]. Therefore, in our perspective, MB has great potential to be the first PS to be employed in mainstream medical procedures of aPDT applications.

Antimicrobial chemotherapeutic strategies generally target singular microbial molecules or metabolic pathway stages to achieve specific microbial inactivation. This approach facilitates the development or selection of resistant populations as they may be just a mutation away. Most antimicrobials are derivatives of natural fungal or bacterial metabolites that have been used by them to gain privileges over ecosystems. In this context, some microorganisms have naturally developed a resistance phenotype to also thrive in the environment.

Currently, hospital, farm and domestic effluents represent important environments that carry and gather resistance genes. Carbapenemases are being frequently detected in plasmids of Gram-negative clinical isolates of the *Enterobacteriaceae* family and non-fermenters such as *P. aeruginosa* and *A. baumannii* [26,27]. The genetic versatility of the *Enterobacteriaceae* is considered responsible for the global dissemination of KPC and has most likely enabled processes of adaptation and virulence expression in different ecosystems [28]. More worrisomely, the recent identification of the plasmid-mediated *mcr-1* gene, which confers resistance to polymyxins – a last-resort drug to treat carbapenem-resistant Gram-negative infections – has added another layer of complexity to therapeutic strategies for nosocomial infections.

Antimicrobial photodynamic therapy offers an effective strategy to challenge microbial resistance in local infections as it differs from traditional chemotherapy in one important point: it lacks molecular target specificity. Unlike traditional antimicrobial therapy, aPDT imposes its cytotoxic effects by high yields of ROS generation. ROS do not target a particular pathway but rather destroy proteins, nucleic acids and lipids indiscriminately. Due to their broad reactivity range, the biological

target of photodynamically-produced ROS will mostly depend on the cell/tissue compartment in which it was produced (i.e., where the PS accumulates) [29]. Given that in our system each MB molecule can theoretically produce more than 10^5 singlet oxygen molecules per second, microorganisms simply seem to not be equipped with enough antioxidant capacity to tolerate an attack of this magnitude [30]. Additionally, at low MB-aPDT doses (i.e. doses that are insufficient to present microbicidal effects), the exposed pathogens remain with transiently inhibited virulence factors, including increased antimicrobial sensitivity [31–34]. We previously reported that low-doses of systemic aPDT could temporarily inhibit the drug-resistance phenotype of vancomycin-resistant *E. faecium* and fluconazole-resistant *C. albicans* [31,32]. Therefore, the combination of aPDT with antimicrobial agents can potentially present synergistic activity or temporarily return drug-sensitivity. Furthermore, in combination with the innate immune system it may yet trigger responses that can lead to resolution of the infection [35].

In the related anticancer application, which uses different PS classes, mechanisms of resistance to PDT have already been reported [36]. A classic example is the constant failure to treat melanoma tumors [37]. Melanin is an antioxidant cellular defense and simultaneously blocks light propagation due to intense filter effect [38,39]. Drug sequestration in melanosomes has also been implicated with photosensitizers, as in cases of chemoresistance [40,41]. Similarly, melanization of *C. neoformans* yeast also increases its tolerance to photodynamic inactivation as seen in antifungal chemotherapy and upon gamma irradiation [34,42–44]. In a broader view, several microbial pigments can act as potent antioxidant defenses against environmental harms and as an immune system evasion strategy. Mycobacteria, staphylococci, chromobacteria and cryptococci can produce

Fig. 4. Lethal dose values of aPDT calculated in function of radiant exposure. LD values correspond to percent of total microbial population reduction. Horizontal dotted line represents the maximum dose (40 J/cm^2) required for more than 5Log_{10} of inactivation.

fair amounts of carotenoids and tryptophan-derived pigments to guarantee superior oxidative tolerance through ROS quenching [45]. However, it is well known by the aPDT community, and was further confirmed in our study, that the carotenoid-producing *S. aureus* is one of the most sensitive microorganisms to photo-oxidative inactivation with amphiphilic photosensitizers. Currently, it has not been established whether aPDT could lead to the selection for oxidative tolerant strains that overexpress pigment-producing enzymes and impair the technique effectiveness. Perhaps the most tolerant microbial species used in this study are protected by potent antioxidant defense systems.

Overexpression of ATP-binding cassette (ABC) transporters has been implicated as another resistance mechanism to aPDT [46]. Tumor and microbial cells can use this mechanism to tolerate chemotherapy and PDT [47–49]. ABC-transporters, as P-glycoprotein, are efficient efflux pumps that can inhibit the uptake of multiple drugs and PS that function inside the cell. To avoid or overcome this resistance mechanism, efflux pump inhibitors or PSs that function on the cell surface may be used [50–52]. Trindade et al. reported that MB was able to revert multidrug-resistance phenotypes of cancer cells via oxidation of efflux-pumps [53]. This suggests that synergistic activity between MB-aPDT and fluconazole, on azole-resistant *C. albicans*, may share the same mechanism [31,54].

Trindade et al. also reported that the MDR phenotype of cancer cells, mediated by efflux-pumps, does not impose any further challenge for MB-PDT inactivation [53]. As in our experiments, their results suggest that cellular sensitivity to MB-PDT are rather dependent on the species than the MDR phenotype. Here we used drug-resistant representatives for nearly all classes of antimicrobials. Resistance mechanisms to quinolones, phenicols, tetracyclines, aminoglycosides and fluconazole are highly associated with overexpression of drug-efflux pumps. Even though MB may also be a substrate of efflux proteins, no relevant differences between drug-sensitive and resistant strains could be observed.

In the microbiology community, a microbial strain is considered resistant to a certain antimicrobial if its MIC cannot be reached safely in patient's bloodstream via oral or intravenous administration. Therefore, a drug-resistant microorganism is untreatable by the use of certain systemically administered chemotherapeutic antimicrobials. Since aPDT is never systemically administered there is no influence on antimicrobial drug concentration in the bloodstream. Additionally, light dosimetry can always be increased to enhance aPDT microbicidal activity. On the other hand, tolerance is used as a relative term that describes a higher demand of light or photosensitizer dosimetry to reach a certain level of microbial inactivation. Hence, higher tolerance to aPDT does not mean that the effective dose cannot be reached. In regard to the tolerance factor (T) we use to describe inactivation kinetics, it only informs if some specific species is more tolerant to inactivation in the beginning or the end of a light-mediated microbicidal procedure.

Fungi and capsule-expressing Gram-negative bacteria, however, indeed present a higher tolerance to aPDT. Prates et al. demonstrated that capsule deletion in *C. neoformans* guarantees greater sensitivity to aPDT mediated by cationic PS, including MB [34]. Therefore, we hypothesized that the capsule barrier could protect microorganisms from aPDT, possibly reducing MB incorporation into the cytoplasm. A carbohydrate-rich capsule may act as a negatively charged electrostatic pool, diminishing intracellular incorporation of MB; or it may act by simply increasing cellular biomass, bringing further targets for non-specific photodynamic damage in non-vital structures. For fungi, however, a capsule does not seem to be as important as for bacteria. Even though it was demonstrated that capsule deletion in *C. neoformans* enhanced its sensitivity to aPDT, *C. albicans* does not produce a capsule and is more tolerant to aPDT than *C. neoformans*. We do not provide sufficient data to elucidate this finding, so we can only hypothesize that *C. albicans* may have cytosolic, mitochondrial and nuclear ROS defense that is less prevalent in *C. neoformans* [55,56].

Although our data seems to suggest that capsule expression in

bacterial results in higher aPDT tolerance, capsule presence in yeasts does not seem to increase aPDT tolerance. Capsule presence has been implicated as a relevant tolerance factor to aPDT, especially in the case of *C. neoformans* [34]. However, our data suggest that there must be other more relevant tolerance factors expressed by different species. Greater MB-aPDT tolerance may be rather related to cellular antioxidant systems, organelle compartmentalization and/or capacity to pump photosensitizers out of the cell through efflux systems [46].

Photosensitizers that preferentially undergo type I photodynamic reactions are more susceptible to microbial antioxidant defense since there are specific detoxifying enzymes for the photoproducts formed. Constitutive overexpression of superoxide dismutase, catalase, peroxiredoxin and glutathiones, or the accumulation of manganese ions can represent effective protection against oxidation by superoxide and hydroxyl radicals [57–59]. All of the mentioned features can be sufficient to impose challenges for PDT to treat tumors and microorganisms resistant to traditional chemotherapy and radiotherapy. However, since no enzymes capable of inactivating singlet oxygen efficiently have ever been reported, and since, in our hands, complete microbial inactivation required only around 7 min for the most tolerant species, it remains questionable whether any type of resistance to aPDT could ever be developed.

Here, we demonstrated that regardless of taxonomy or drug-resistance profile, all strains among each species are similarly sensitive to aPDT. Our results consequently support the proposition of aPDT as a consistent challenge against drug-resistance in local infections. MB-aPDT effects against these species have indeed been published elsewhere [10,16,24,60]. However, there was never a study performed with all of them being inactivated under standardized experimental parameters. It is widely known that the aPDT community lacks on standards for *in vitro* aPDT assays. Even though Cieplik et al. proposes a model of number of absorbed photons for more precise comparisons in between studies, changes in inocula concentration, photosensitizer concentration, solvent composition (e.g., water, PBS, saline solution, etc.), light irradiance, optical path, and other factors may yet lead to significantly diverging results [61–64]. Therefore, it is very unlikely that precise comparisons of inactivation kinetics data are achievable between two studies performed by different teams who varied any of the above-mentioned parameters and analyzed a reduced spectrum of species and light parameters. For example, such incapacity to compare results from different studies may have led to the persistently mistaken concept that Gram-positives are more sensitive than Gram-negatives that are more sensitive than fungi [22]. This mistaken information has persisted in our community for more than a decade whereas the current manuscript shows that Gram-positives can be more tolerant than Gram-negatives, which can also be more tolerant than fungi.

This information could be mistakenly interpreted again if we did not analyze inactivation kinetics as a curve fit instead of just reading individual data points. Hence, our standardized study of a broad spectrum of global priority pathogens does bring important insights about their sensitivity to MB-aPDT. Furthermore, we recently published a paper presenting a mathematical model of analysis for microbial photo-inactivation kinetics. So far, all studies used to compare inactivation kinetics are based on individual dose points, which is another issue that could lead to misleading statistical differences that do not represent the actual inactivation kinetics rate. Because of variable tolerance factors (T) the comparison of two different species could be misleading because some data points may not present significant differences, while others do (see the examples of *S. aureus* versus *E. faecalis* at 1 and 3 J/cm², Fig. 1). Because of this, several papers actually show significant differences between different strains of the same species. Even though many studies show that drug-resistant strains are also sensitive to aPDT, many of those do not compare with standard ATCC drug-sensitive controls. When they do, misleading statistics often show differences between strains because of the previously mentioned statistical issue of comparing a single dose point.

5. Conclusion

In summary, our study unequivocally demonstrates that antimicrobial photodynamic inactivation offers a powerful strategy to challenge microbial drug-resistance. For the first time, we compiled a large amount of data under a standardized method showing that MB-aPDT is effective against microorganisms that are resistant to more than 50 antimicrobial agents. Regardless of taxonomy or resistance phenotype, MB-aPDT presented consistent dose-response kinetics. Therefore, MB-aPDT can provide effective therapeutic protocols for a very broad spectrum of pathogens. This approach can be employed to significantly reduce the use of antimicrobial drugs and minimize the risk of us entering into a post-antimicrobial era. Hence, we believe that this study represents a very important step in bringing aPDT closer to implementation into mainstream medical practices.

Acknowledgements

We gratefully thank and technical support offered by the University of São Paulo, the Nuclear and Energy Research Institute and the start-up company BioLambda.

Funding

This work was supported by the São Paulo Research Foundation (FAPESP, grants 2016/25095–2, 2013/07937–8, 2017/22406–0 and 2016/08593–9) and by the National Council for Scientific and Technological Development (CNPq, grants 141901/2016–0 and 465763/2014–6, 312249/2017–9 and 433128/2018–6).

Transparency declarations.

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

Author statement.

Caetano Padiál Sabino: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Validation, Visualization, Roles/Writing – original draft, Writing – review & editing. **Mark Wainwright:** Formal analysis, Methodology, Visualization, Roles/Writing – original draft, Writing – review & editing. **Martha Simões Ribeiro:** Conceptualization, Formal analysis, Methodology, Supervision, Visualization, Roles/Writing – original draft, Writing – review & editing. **Fábio Parra Sellera:** Formal analysis, Methodology, Visualization, Roles/Writing – original draft, Writing – review & editing. **Carolina dos Anjos:** Investigation, Methodology, Visualization, Roles/Writing – original draft, Writing – review & editing. **Maurício da Silva Baptista:** Funding acquisition, Project administration, Resources, Supervision, Visualization, Roles/Writing – original draft, Writing – review & editing. **Nilton Lincopan:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Roles/Writing – original draft, Writing – review & editing.

Appendix A. Supplementary data

Supplementary material.

References

- [1] H.W. Boucher, G.H. Talbot, J.S. Bradley, J.E. Edwards, D. Gilbert, L.B. Rice, M. Scheld, B. Spellberg, J. Bartlett, Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America, *Clin. Infect. Dis.* 48 (2009) 1–12.
- [2] L.B. Rice, Unmet medical needs in antibacterial therapy, *Biochem. Pharmacol.* 71 (2006) 991–995.
- [3] L.B. Rice, Federal Funding for the Study of Antimicrobial Resistance in Nosocomial Pathogens: No ESKAPE, *J. Infect. Dis.* 197 (2008) 1079–1081, d.
- [4] World Health Organization, Antimicrobial resistance: global report on surveillance., 2014.
- [5] World Health Organization, Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics, 2017.
- [6] J.N. Pendleton, S.P. Gorman, B.F. Gilmore, Clinical relevance of the ESKAPE pathogens, *Expert Rev. Anti-Infect. Ther.* 11 (2013) 297–308.
- [7] L.B. Rice, Mechanisms of Resistance and Clinical Relevance of Resistance to β -Lactams, Glycopeptides, and Fluoroquinolones, in: Mayo Clin, Elsevier Inc., Proc., 2012, pp. 198–208.
- [8] World Health Organization, The evolving threat of antimicrobial resistance: Options for action, 2014.
- [9] F. Agostinis, K. Berg, K.A. Cengel, T.H. Foster, A.W. Girotti, S.O. Gollnick, S.M. Hahn, M.R. Hamblin, A. Juzeniene, D. Kessel, M. Korbelik, J. Moan, P. Mroz, D. Nowis, J. Piette, B.C. Wilson, J. Golab, Photodynamic therapy of cancer: an update, *CA Cancer J. Clin.* 61 (2011) 250–281.
- [10] M. Wainwright, Photodynamic antimicrobial chemotherapy (PACT), *J. Antimicrob. Chemother.* 42 (1998) 13–28.
- [11] C.S. Foote, Definition of type I and type II, *Photochem. Photobiol.* 54 (1991) 659.
- [12] M.S. Baptista, J. Cadet, P. Di Mascio, A.A. Ghogare, A. Greer, M.R. Hamblin, C. Lorente, S.C. Nunez, M.S. Ribeiro, A.H. Thomas, M. Vignoni, T.M. Yoshimura, Type I and II photosensitized oxidation reactions: guidelines and mechanistic pathways, *Photochem. Photobiol. Sci.* 93 (2017) 912–919.
- [13] T. Dai, Y.Y. Huang, M.R. Hamblin, Photodynamic therapy for localized infections-state of the art, *Photodiagn. Photodyn. Ther.* 6 (2009) 170–188.
- [14] X. Hu, Y.Y. Huang, Y. Wang, X. Wang, M.R. Hamblin, Antimicrobial photodynamic therapy to control clinically relevant biofilm infections, *Front. Microbiol.* 9 (2018) 1–24.
- [15] J.P. Lyon, L.M. Moreira, P.C.G. de Moraes, F.V. dos Santos, M.A. de Resende, Photodynamic therapy for pathogenic fungi, *Mycoses.* 54 (2011) 265–271.
- [16] T. Maisch, S. Hackbarth, J. Regensburger, A. Felgenträger, W. Bäuml, M. Landthaler, B. Röder, Photodynamic inactivation of multi-resistant bacteria (PIB) - a new approach to treat superficial infections in the 21st century, *J. Der Dtsch. Dermatologischen Gesellschaft.* 9 (2011) 360–366.
- [17] R. Schmidt, Photosensitized generation of singlet oxygen, *Photochem. Photobiol.* 82 (2007) 1161–1177.
- [18] E. Tacconelli, E. Carrara, A. Savoldi, S. Harbarth, M. Mendelson, D.L. Monnet, C. Pulcini, G. Kahlmeter, J. Kluytmans, Y. Carmeli, M. Ouellette, K. Outtersson, J. Patel, M. Cavalieri, E.M. Cox, C.R. Houchens, M.L. Grayson, P. Hansen, N. Singh, U. Theuretzbacher, N. Magrini, A.O. Aboderin, S.S. Al-Abri, N. Awang Jalil, N. Benzonana, S. Bhattacharya, A.J. Brink, F.R. Burkert, O. Cars, G. Cornaglia, O.J. Dyar, A.W. Friedrich, A.C. Gales, S. Gandra, C.G. Giske, D.A. Goff, H. Goossens, T. Gottlieb, M. Guzman Blanco, W. Hryniewicz, D. Kattula, T. Jinks, S.S. Kanj, L. Kerr, M.P. Kieny, Y.S. Kim, R.S. Kozlov, J. Labarca, R. Laxminarayan, K. Leder, L. Leibovici, G. Levy-Hara, J. Littman, S. Malhotra-Kumar, V. Manchanda, L. Moja, B. Ndoye, A. Pan, D.L. Paterson, M. Paul, H. Qiu, P. Ramon-Pardo, J. Rodriguez-Baño, M. Sanguinetti, S. Sengupta, M. Sharland, M. Si-Mehand, L.L. Silver, W. Song, M. Steinbakk, J. Thomsen, G.E. Thwaites, J.W. van der Meer, N. Van Kinh, S. Vega, M. V. Villegas, A. Wechsler-Fördös, H.F.L. Wertheim, E. Wesangula, N. Woodford, F.O. Yilmaz, A. Zorzet, Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis, *Lancet Infect. Dis.* 18 (2018) 318–327.
- [19] CLSI, Performance Standards for Antimicrobial Susceptibility Testing, 27th ed., Wayne, 2017.
- [20] C.P. Sabino, M. Wainwright, C. dos Anjos, F.P. Sellera, M.S. Baptista, N. Lincopan, M.S. Ribeiro, Inactivation kinetics and lethal dose analysis of antimicrobial blue light and photodynamic therapy, *Photodiagn. Photodyn. Ther.* 28 (2019) 186–191.
- [21] A.P. Magiorakos, A. Srinivasan, R.B. Carey, Y. Carmeli, M.E. Falagas, C.G. Giske, S. Harbarth, J.F. Hindler, G. Kahlmeter, B. Olsson-Liljequist, D.L. Paterson, L.B. Rice, J. Stelling, M.J. Struelens, A. Vatopoulos, J.T. Weber, D.L. Monnet, Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance, *Clin. Microbiol. Infect.* 18 (2011) 268–281.
- [22] M.R. Hamblin, T. Hasan, Photodynamic therapy: a new antimicrobial approach to infectious disease? *Photochem. Photobiol. Sci.* 3 (2004) 436.
- [23] J.P. Tardivo, A. Del Giglio, C.S. De Oliveira, D.S. Gabrielli, H.C. Junqueira, D.B. Tada, D. Severino, R. De Fátima Turchiello, M.S. Baptista, Methylene blue in photodynamic therapy: from basic mechanisms to clinical applications, *Photodiagn. Photodyn. Ther.* 2 (2005) 175–191.
- [24] M. Wainwright, T. Maisch, S. Nonell, K. Plaetzer, A. Almeida, G.P. Teges, M.R. Hamblin, Photoantimicrobials—are we afraid of the light? *Lancet Infect. Dis.* 17 (2017) e49–e55.
- [25] C.P. Sabino, A.M. Deana, T.M. Yoshimura, D.F.T. Da Silva, C.M. França, M.R. Hamblin, M.S. Ribeiro, The optical properties of mouse skin in the visible and near infrared spectral regions, *J. Photochem. Photobiol. B Biol.* 160 (2016) 72–78.
- [26] A. Potron, L. Poirel, P. Nordmann, Emerging broad-spectrum resistance in *Pseudomonas aeruginosa* and *Acinetobacter baumannii*: mechanisms and epidemiology, *Int. J. Antimicrob. Agents* 45 (2015) 568–585.
- [27] L.S. Tzouveleki, A. Markogiannakis, M. Psychogiou, P.T. Tassios, G.L. Daikos, Carbapenemases in *Klebsiella pneumoniae* and other Enterobacteriaceae: an evolving crisis of global dimensions, *Clin. Microbiol. Rev.* 25 (2012) 682–707.
- [28] L.N. Andrade, T. Curiao, J.C. Ferreira, J.M. Longo, E.C. Clímaco, R. Martinez, F. Bellissimo-Rodrigues, A. Basile-Filho, M.A. Evaristo, P.F. Del Peloso, V.B. Ribeiro, A.L. Barth, M.C. Paula, F. Baquero, R. Cantón, A.L. da C. Darini, T.M. Coque, Dissemination of bla_{NDP-2} by the Spread of *Klebsiella pneumoniae* Clonal Complex 258 Clones (ST258, ST11, ST437) and Plasmids (IncFII, IncN, IncL/M) among Enterobacteriaceae species in Brazil, *Antimicrob. Agents Chemother.* 55 (2011) 3579–3583.
- [29] B. Chen, B.W. Pogue, P.J. Hoopes, T. Hasan, Vascular and cellular targeting for photodynamic therapy, *Crit. Rev. Eukaryot. Gene Expr.* 16 (2006) 279–306.
- [30] I.E. Kochevar, R.W. Redmond, Photosensitized production of singlet oxygen, *Methods Enzymol.* 319 (2004) 20–28.
- [31] J. Chibebe Junior, C.P. Sabino, X. Tan, J.C. Junqueira, Y. Wang, B.B. Fuchs, A.O.

- Jorge, G. P., M.R. Hamblin, E. Mylonakis, Selective photoinactivation of *Candida albicans* in the non-vertebrate host infection model *Galleria mellonella*, *BMC Microbiol.* 13 (2013) 217.
- [32] J. Chibebe Junior, B.B. Fuchs, C.P. Sabino, J.C. Junqueira, A.O.C. Jorge, M.S. Ribeiro, M.S. Gilmore, L.B. Rice, E. Hamblin, Michael R Mylonakis, G.P. Tegos, Photodynamic and Antibiotic Therapy Impair the Pathogenesis of *Enterococcus faecium* in a Whole Animal Insect Model, *PLoS One*. 8 (2013) e55926.
- [33] I.T. Kato, Q. Naqvi, B.B. Fuchs, E. Mylonakis, G.P. Tegos, K. Mizuno, M.S. Ribeiro, M.R. Hamblin, R.A. Prates, Effect of virulence factors on the photodynamic inactivation of *Cryptococcus neoformans*, *PLoS One* 8 (2013) e54387.
- [34] R.A. Prates, B.B. Fuchs, K. Mizuno, Q. Naqvi, I.T. Kato, M.S. Ribeiro, E. Mylonakis, G.P. Tegos, M.R. Hamblin, Effect of virulence factors on the photodynamic inactivation of *Cryptococcus neoformans*, *PLoS One* 8 (2013) e54387.
- [35] M. Tanaka, P. Mroz, T. Dai, L. Huang, Y. Morimoto, M. Kinoshita, Y. Yoshihara, K. Nemoto, N. Shinomiya, S. Seki, M.R. Hamblin, Photodynamic therapy can induce a protective innate immune response against murine bacterial Arthritis via Neutrophil accumulation, *PLoS One*. 7 (2012).
- [36] A. Casas, G. Di Venosa, T. Hasan, A. Battle, Mechanisms of Resistance to Photodynamic Therapy, *Curr. Med. Chem.* 18 (2011).
- [37] L. Huang, Y. Xuan, Y. Koide, T. Zhiyentayev, M. Tanaka, M.R. Hamblin, Type I and type II mechanisms of antimicrobial photodynamic therapy: an in vitro study on gram-negative and gram-positive bacteria, *Lasers Surg. Med.* 44 (2012) 490–499.
- [38] N. Kollias, R.M. Sayre, L. Zeise, M.R. Chedekel, New trends in photobiology. Photoprotection by melanin, *J. Photochem. Photobiol. B Biol.* 9 (1991) 135–160.
- [39] Y. Wang, A. Casadevall, Susceptibility of melanized and nonmelanized *Cryptococcus neoformans* to nitrogen- and oxygen-derived oxidants, *Infect. Immun.* 62 (1994) 3004–3007.
- [40] R. Knörle, E. Schnitz, T.J. Feuerstein, Drug accumulation in melanin: an affinity chromatographic study, *J. Chromatogr. B Biomed. Appl.* 714 (1998) 171–179.
- [41] A. Radwa, T. Frąckowiak, H. Ibrahim, A. Aubry, R. Kaliszan, Chromatographic modelling of interactions between melanin and phenothiazine and dibenzazepine drugs, *Biomed. Chromatogr.* 9 (1995) 233–237.
- [42] D. Van Duijn, A. Casadevall, J.D. Nosanchuk, Reduces their susceptibilities to amphotericin B and Caspofungin, *Society*. 46 (2002) 3394–3400.
- [43] A. Khajro, R.A. Bryan, M. Friedman, R.M. Burger, Y. Levitsky, A. Casadevall, R.S. Magliozzo, E. Dadachova, Protection of Melanized *Cryptococcus neoformans* from lethal dose gamma irradiation involves changes in Melanin's chemical structure and Paramagnetism, *PLoS One* 6 (2011) e25092.
- [44] J.D. Nosanchuk, A. Casadevall, Impact of melanin on microbial virulence and clinical resistance to antimicrobial compounds, *Antimicrob. Agents Chemother.* 50 (2006) 3519–3528.
- [45] G.Y. Liu, V. Nizet, Color me bad : microbial pigments as virulence factors, *Trends Microbiol.* 17 (2009) 406–413.
- [46] R.A. Prates, I.T. Kato, M.S. Ribeiro, G.P. Tegos, M.R. Hamblin, Influence of multi-drug efflux systems on methylene blue-mediated photodynamic inactivation of *Candida albicans*, *J. Antimicrob. Chemother.* 66 (2011) 1525–1532.
- [47] P. Gros, Y. Ben Neriah, J.M. Croop, D.E. Housman, Isolation and expression of a complementary DNA that confers multidrug resistance, *Nature*. 323 (1986) 728–731.
- [48] D. Kessel, K. Woodburn, D. Skalkos, Impaired accumulation of a cationic photosensitizing agent by a cell line exhibiting multidrug resistance, *Photochem. Photobiol.* 60 (1994) 61–63.
- [49] R.A. Moorehead, S.G. Armstrong, B.C. Wilson, G. Singh, Cross-resistance to cisplatin in cells resistant to photofrin-mediated photodynamic therapy, *Cancer Res.* 54 (1994) 2556–2559.
- [50] J. Merlin, H. Gautier, M. Barberi-Heyob, M.-H. Teiten, F. Guillemin, The multidrug resistance modulator SDZ-PSC 833 potentiates the photodynamic activity of chlorin e6 independently of P-glycoprotein in multidrug resistant human breast adenocarcinoma cells, *Int. J. Oncol.* (2014) 733–739.
- [51] D. Preise, O. Mazor, N. Koudinova, M. Liscovitch, A. Scherz, Y. Salomon, Bypass of tumor drug resistance by Antivascular therapy I, *Neoplasia*. 5 (2003) 475–480.
- [52] G.P. Tegos, K. Masago, F. Aziz, A. Higginbotham, F.R. Stermitz, M.R. Hamblin, Inhibitors of bacterial multidrug efflux pumps potentiate antimicrobial Photoinactivation, *Antimicrob. Agents Chemother.* 52 (2008) 3202–3209.
- [53] G.S. Trindade, S.L.A. Farias, V.M. Rumjanek, M.A.M. Capella, Methylene blue reverts multidrug resistance: sensitivity of multidrug resistant cells to this dye and its photodynamic action, *Cancer Lett.* 151 (2000) 161–167.
- [54] I.T. Kato, R.A. Prates, C.P. Sabino, B.B. Fuchs, G.P. Tegos, E. Mylonakis, M.R. Hamblin, M.S. Ribeiro, Antimicrobial photodynamic inactivation inhibits *Candida albicans* virulence factors and reduces in vivo pathogenicity, *Antimicrob. Agents Chemother.* 57 (2013) 445–451.
- [55] E. Fréalle, C. Noël, E. Viscogliosi, D. Camus, E. Dei-Cas, L. Delhaes, Manganese superoxide dismutase in pathogenic fungi: an issue with pathophysiological and phylogenetic involvements, *FEMS Immunol. Med. Microbiol.* 45 (2005) 411–422.
- [56] P. González-Párraga, J.A. Hernández, J.C. Argüelles, Role of antioxidant enzymatic defences against oxidative stress (H2O2) and the acquisition of oxidative tolerance in *Candida albicans*, *Yeast*. 20 (2003) 1161–1169.
- [57] L.F. Agnez-Lima, J.T.A. Melo, A.E. Silva, A.H.S. Oliveira, A.R.S. Timoteo, K.M. Lima-Bessa, G.R. Martinez, M.H.G. Medeiros, P. Di Mascio, R.S. Galhardo, C.F.M. Menck, DNA damage by singlet oxygen and cellular protective mechanisms, *Mutat. Res.* 751 (2012) 15–28.
- [58] V.C. Culotta, M.J. Daly, Manganese complexes: diverse metabolic routes to oxidative stress resistance in prokaryotes and yeast, *Antioxid. Redox Signal.* 19 (2012) 933–944.
- [59] S.Y. Young, N.L. Young, Purification and some properties of superoxide dismutase from *Deinococcus radiophilus*, the UV-resistant bacterium, *Extremophiles*. 8 (2004) 237–242.
- [60] J. Nakonieczna, A. Wozniak, M. Pieranski, A. Rapacka-Zdonczyk, P. Ogonowska, M. Grinholc, Photoinactivation of ESKAPE pathogens: overview of novel therapeutic strategy, *Future Med. Chem.* 11 (2019) 443–461.
- [61] R.A. Prates, E.G. Silva, A.M. Yamada, L.C. Suzuki, C.R. Paula, M.S. Ribeiro, Light parameters influence cell viability in antifungal photodynamic therapy in a fluence and rate fluence-dependent manner, *Laser Phys.* 19 (2009) 1038–1044.
- [62] S.C. Nuñez, T.M. Yoshimura, M.S. Ribeiro, H.C. Junqueira, C. Maciel, M.D. Coutinho-Neto, M.S. Baptista, Urea enhances the photodynamic efficiency of methylene blue, *J. Photochem. Photobiol. B Biol.* 150 (2015) 31–37.
- [63] G.A. da Collina, F. Freire, V.S. Barbosa, C.B. Correa, H.R. Nascimento, A.C.R.T. Horliana, D. de F.T. Silva, R.A. Prates, C. Pavani, Photodynamic Antimicrobial Chemotherapy Action of Phenothiazinium D, *Photodiagnosis Photodyn. Ther.* 101612 (2019).
- [64] F. Cieplik, A. Pummer, J. Regensburger, K.A. Hiller, A. Späth, L. Tabenski, W. Buchalla, T. Maisch, The impact of absorbed photons on antimicrobial photodynamic efficacy, *Front. Microbiol.* 6 (2015).
- [65] L.M. Dalla-Costa, J.M. Coelho, H.A.P.H.M. Souza, M.E.S. Castro, C.J.N. Stier, K.L. Bragagnolo, A. Rea-Neto, S.R. Penteado-Filho, D.M. Livermore, N. Woodford, Outbreak of Carbapenem-Resistant *Acinetobacter baumannii* Producing the, *J. Clin. Microbiol.* 41 (2003) 3403–3406.
- [66] C.S. Antonio, P.R. Neves, M. Medeiros, E.M. Mamizuka, M.R.E. De Araújo, N. Lincopan, High prevalence of carbapenem-resistant *Acinetobacter baumannii* carrying the bla_{OXA-143} gene in Brazilian hospitals, *Antimicrob. Agents Chemother.* 55 (2011) 1322–1323.
- [67] N. Lincopan, A.M. Garmona-Ribeiro, E.M. Mamizuka, DOD/AMB: in vivo activity of a novel AMB formulation with synthetic cationic bilayer fragments, *Brazilian J. Microbiol.* 34 (2003) 131–134.
- [68] J.C. Campos, M.J.F. Da Silva, P.R.N. Dos Santos, E.M. Barros, M.D.O. Pereira, B.M.S. Seco, C.M. Magagnin, L.K. Leiroz, T.G.M. De Oliveira, C. De Faria-Júnior, L.T. Cerdeira, A.L. Barth, S.C.F. Sampaio, A.P. Zavascki, L. Poirel, J.L.M. Sampaio, Characterization of Tn3000, a transposon responsible for bla_{NDM-1} dissemination among Enterobacteriaceae in Brazil, Nepal, Morocco, and India, *Antimicrob. Agents Chemother.* 59 (2015) 7387–7395.
- [69] J. Aizawa, N. Neuwirt, L. Barbato, P.R. Neves, L. Leigue, J. Padilha, A.F.P. de Castro, L. Gregory, N. Lincopan, Identification of fluoroquinolone-resistant extended-spectrum β -lactamase (CTX-M-8)-producing *Escherichia coli* ST224, ST2179 and ST2308 in buffalo (*Bubalus bubalis*), *J. Antimicrob. Chemother.* 69 (2014) 2866–2869.
- [70] F.P. Sellera, M.R. Fernandes, L. Sartori, M.P.N. Carvalho, F. Esposito, C.L. Nascimento, G.H.P. Dutra, E.M. Mamizuka, P.J. Pérez-Chaparro, J.A. McCulloch, N. Lincopan, *Escherichia coli* carrying IncX4 plasmid-mediated mcr-1 and bla_{CTX-M-15} genes in infected migratory Magellanic penguins (*Spheniscus magellanicus*), *J. Antimicrob. Chemother.* 72 (2017) 1255–1256.
- [71] N. Lincopan, J.A. McCulloch, C. Reinert, A.C. Gales, E.M. Mamizuka, First isolation of Metallo- β -lactamase-producing multidrug-resistant *Klebsiella pneumoniae* from a patient in Brazil, *J. Clin. Microbiol.* 43 (2005) 516–519.
- [72] L. Poirel, C. Héritier, V. Torun, P. Nordmann, Emergence of Oxacillinase-mediated resistance to imipenem in *Klebsiella pneumoniae*, *Antimicrob. Agents Chemother.* 48 (2004) 15–22.
- [73] G.R. Francisco, M.F.C. Bueno, L. Cerdeira, N. Lincopan, S. Ienne, T.A. Souza, D. de Oliveira Garcia, Draft genome sequences of KPC-2- and CTX-M-15-producing *Klebsiella pneumoniae* ST437 isolated from a clinical sample and urban rivers in Sao Paulo, Brazil, *J. Glob. Antimicrob. Resist.* 16 (2019) 74–75.
- [74] M. Polotto, T. Casella, M.G. de Lucca Oliveira, F.G. Rúbio, M.L. Nogueira, M.T.G. de Almeida, M.C.L. Nogueira, Detection of *P. aeruginosa* harboring bla_{CTX-M-2}, bla_{GES-1} and bla_{GES-5}, bla_{AMP-1} and bla_{SPM-1} causing infections in Brazilian tertiary-care hospital, *BMC Infect. Dis.* 12 (2012).
- [75] M.A. Toleman, Molecular characterization of SPM-1, a novel metallo- β -lactamase isolated in Latin America: report from the SENTRY antimicrobial surveillance programme, *J. Antimicrob. Chemother.* 50 (2002) 673–679.
- [76] M.R. Fernandes, F.P. Sellera, Q. Moura, M.P.N. Carvalho, P.N. Rosato, L. Cerdeira, N. Lincopan, Zoonanthropotic transmission of drug-resistant *Pseudomonas aeruginosa*, Brazil, *Emerg. Infect. Dis.* 24 (2018) 1160–1162.
- [77] G.A. Oliveira, A.M. DellaAquila, R.L. Masiero, C.E. Levy, M.S. Gomes, L. Cui, H. Hiramatsu, E.M. Mamizuka, Isolation in Brazil of Nosocomial *Staphylococcus aureus* With Reduced Susceptibility to Vancomycin, in: *Infect. Control Hosp. Epidemiol.*, 2001: pp. 443–448.

Supplementary Material

Table S1. Antibigram profiles for strains of *S. aureus*, *E. faecium* and *E. faecalis*. Inhibition diameter of unclassified antimicrobials is presented in numeric values (mm).

	<i>S. aureus</i>		<i>E. faecium</i>		<i>E. faecalis</i>	
	ATCC 25923	VRSA BR-4	ATCC BAA2127	ATCC 700221	ATCC 29212	ATCC 51299
β-lactams and β-lactamase inhibitors						
amoxicillin	S	R	R	R	S	S
ampicillin	S	R	R	R	S	S
amp/sulbactam	S	R	R	R	S	S
oxacillin	S	R	R	R	R	R
penicillin	R	R	R	R	S	S
cephalothin	S	R	0	0	16	0
cefoxitin	S	R	0	0	0	0
ceftazidime	R	R	0	0	0	0
ceftiofur	S	R	0	0	20	0
imipenem	S	R	R	R	S	S
Glycopeptides						
teicoplanin	S	R	S	R	S	R
vancomycin	S	I	S	R	S	R
Aminoglycosides						
amikacin	S	R	R	R	R	R
kanamycin	S	R	R	R	R	R
streptomycin	21	0	R	R	R	R
gentamicin	S	R	R	R	R	R
tobramycin	S	R	R	R	R	R
Macrolides						
azithromycin	S	R	0	0	21	0
clarithromycin	S	R	0	0	29	0
erythromycin	S	R	R	R	S	R
Tetracyclines						
doxycycline	S	S	S	S	S	S
tetracycline	S	S	S	S	S	I
Quinolones and Fluoroquinolones						
ciprofloxacin	S	R	R	R	S	R
enrofloxacin	S	R	R	R	S	R
levofloxacin	S	R	R	R	S	R
moxifloxacin	S	R	R	R	S	R
norfloxacin	S	R	R	R	S	R
pefloxacin	29	0	R	R	S	R
<i>Misc.:</i> Phenicols, Nitrofurans, Lincosamides						
Folate Inhibitors, Streptogramins, Oxazolidinones						
chloramphenicol	S	I	S	S	S	R
florfenicol	31	40	35	35	31	37
linezolid	S	S	S	S	S	S
nitrofurantoin	S	S	S	R	S	S
Quinupristin/Dalfopristin	S	R	S	S	S	S
sulpha/trimethoprim	S	S	R	R	I	I
clindamycin	S	R	0	0	0	0

Table S2. Antibiogram profiles for strains of *K. pneumoniae* and *P. aeruginosa*. Inhibition diameter of unclassified antimicrobials is presented in numeric values (mm).

	<i>K. pneumoniae</i>					<i>P. aeruginosa</i>			
	ATCC 700603	KP148/ PINH-4900	KP BR-1	11978	ATCC BAA1705	ATCC 27853	1997A -48	PA64	ICBD VIM2
Penicillins and β-lactamase inhibitors									
amoxicillin	R	R	R	R	R	0	0	0	0
amoxicillin/clavulanic	S	R	R	R	R	0	0	0	0
ampicillin	R	R	R	R	R	0	0	0	0
amp/sulbactam	S	R	I	R	R	0	0	0	0
piper/tazobactam	S	R	R	R	R	S	S	R	R
ticarcillin/clavulanic	I	R	R	R	R	S	R	R	R
Cephems									
cefepime	S	R	R	R	R	S	R	R	R
cefotaxime	I	R	R	R	R	26	0	0	0
cefoxitin	I	R	R	R	R	0	0	0	0
ceftazidime	R	R	R	R	R	S	R	R	R
ceftiofur	S	R	R	R	R	24	0	0	0
ceftriaxone	I	R	R	R	R	26	0	0	0
cephalothin	S	R	R	R	R	0	0	0	0
Monobactam									
aztreonam	R	R	R	R	R	I	I	R	R
Carbapenems									
ertapenem	S	R	R	R	R	21	0	0	0
imipenem	S	R	R	R	R	S	R	R	R
meropenem	S	R	R	R	I	S	R	R	R
Aminoglycosides									
amikacin	S	S	S	S	R	S	S	R	S
streptomycin	S	S	S	S	S	24	0	14	16
gentamicin	I	S	S	R	S	S	R	R	S
kanamycin	I	R	I	R	R	0	10	0	27
neomycin	22	11	20	23	15	13	15	16	30
netilmicin	S	R	S	R	R	S	S	R	R
tobramycin	S	I	S	R	R	S	S	R	S
Tetracyclines									
doxycycline	S	R	S	S	S	13	0	0	0
minocycline	R	R	S	I	S	13	10	0	0
tetracycline	S	R	S	S	S	17	15	15	13
Quinolones and Fluoroquinolones									
nalidixic acid	I	R	R	R	R	0	0	0	0
ciprofloxacin	S	R	R	S	R	S	R	R	R
enrofloxacin	S	R	R	S	R	13	0	0	0
levofloxacin	S	R	R	R	R	S	R	R	R
norfloxacin	S	R	R	S	R	S	R	R	R
ofloxacin	R	R	R	S	R	S	R	R	R
pefloxacin	R	R	R	R	R	19	0	0	0
Misc.: Lipopeptides, Phenolics, Nitrofurans, Folate Inhibitors, Fosfomycin, Glycylcycline									
colistin	12	0	14	13	13	S	S	S	S
chloramphenicol	S	R	S	R	R	0	0	0	0
florfenicol	20	27	30	27	22	14	0	0	0
fosfomycin	I	R	S	S	S	37	24	28	16
nitrofurantoin	I	R	R	S	R	0	0	0	0
polymyxin B	17	14	17	15	17	S	S	S	S
rifampicin	18	20	18	17	17	18	19	11	25
sulpha/trimethoprim	S	R	S	S	R	R	R	R	R
tigecycline	S	S	S	S	S	15	15	0	16

Table S3. Antibigram profiles for strains of *A. baumannii*, *K. aerogenes* and *E. coli*. Inhibition diameter of unclassified antimicrobials is presented in numeric values (mm).

	<i>A. baumannii</i>			<i>K. aerogenes</i>		<i>E. coli</i>		
	ATCC 19606	804	LDC	ATCC 13048	E00830 33-1	ATCC 25922	19B	ICBE C7P
Penicillins and β-lactamase inhibitors								
amoxicillin	11	0	0	R	R	S	R	R
amoxicillin/clavulanic	17	0	0	R	R	S	S	R
ampicillin	0	0	0	R	R	S	R	R
amp/sulbactam	S	S	S	S	R	S	R	R
piper/tazobactam	S	R	R	S	R	S	S	R
ticarcillin/clavulanic	S	R	R	S	R	S	I	R
Cephems								
cefepime	S	R	S	S	R	S	R	R
cefotaxime	S	R	R	S	R	S	R	R
cefoxitin	12	0	0	R	R	S	S	S
ceftazidime	S	R	R	S	R	S	S	R
ceftiofur	24	0	0	S	R	S	I	R
ceftriaxone	I	R	R	S	R	S	R	R
cephalothin	0	0	0	R	R	S	R	R
Monobactam								
aztreonam	28	12	19	S	R	S	S	R
Carbapenems								
ertapenem	26	0	0	S	R	S	S	S
imipenem	S	R	R	S	R	S	S	S
meropenem	S	R	R	S	R	S	S	S
Aminoglycosides								
amikacin	S	S	R	S	S	S	S	R
streptomycin	11	17	21	S	S	S	R	S
gentamicin	S	S	R	S	R	S	R	R
kanamycin	28	25	0	S	R	S	I	S
neomycin	21	28	14	22	23	28	21	22
netilmicin	S	S	S	S	S	S	S	S
tobramycin	S	S	I	S	S	S	I	S
Tetracyclines								
doxycycline	S	S	S	S	R	S	S	R
minocycline	S	S	S	S	R	S	S	R
tetracycline	S	S	S	S	R	S	S	R
Quinolones and Fluoroquinolones								
nalidixic acid	29	0	0	S	R	S	R	R
ciprofloxacin	S	R	R	S	R	S	R	R
enrofloxacin	40	0	19	S	R	S	R	R
levofloxacin	S	S	S	S	R	S	R	R
norfloxacin	25	0	0	S	R	S	R	R
ofloxacin	34	0	10	S	R	S	R	R
pefloxacin	31	0	0	28	0	0	0	0
Misc.: Lipopeptides, Phenolics, Nitrofurans, Folate Inhibitors, Fosfomycin, Glycylcycline								
colistin	13	13	14	13	13	S	S	R
chloramphenicol	18	0	0	S	R	S	S	S
florfenicol	22	0	0	27	28	38	35	36
fosfomycin	18	21	20	S	S	S	S	S
nitrofurantoin	24	0	0	S	R	S	S	S
polymyxin B	17	18	17	18	15	S	S	R
rifampicin	27	19	22	20	0	18	22	20
sulpha/trimethoprim	S	S	R	S	R	S	S	R
tigecycline	22	28	26	S	S	S	S	S

Table S4. One-way ANOVA results for comparisons of LD₉₀, LD_{99.9} and LD_{99.999} values obtained from average inactivation kinetics of each species. Non-significant differences are indicated as NS.

LD₉₀	<i>E. coli</i>	<i>A. baumannii</i>	<i>E. faecalis</i>	<i>E. faecium</i>	<i>K. aerogenes</i>	<i>P. aeruginosa</i>	<i>K. pneumoniae</i>	<i>C. neoformans</i>	<i>C. albicans</i>
<i>S. aureus</i>	NS	NS	NS	NS	NS	<0.05	<0.0001	<0.0001	<0.0001
<i>E. coli</i>	–	NS	NS	NS	NS	<0.01	<0.0001	<0.0001	<0.0001
<i>A. baumannii</i>	–	–	NS	NS	NS	<0.05	<0.0001	<0.0001	<0.0001
<i>E. faecalis</i>	–	–	–	NS	NS	<0.05	<0.0001	<0.001	<0.0001
<i>E. faecium</i>	–	–	–	–	NS	NS	<0.0001	<0.01	<0.0001
<i>K. aerogenes</i>	–	–	–	–	–	NS	<0.0001	<0.01	<0.0001
<i>P. aeruginosa</i>	–	–	–	–	–	–	<0.0001	<0.05	<0.001
<i>K. pneumoniae</i>	–	–	–	–	–	–	–	NS	NS
<i>C. neoformans</i>	–	–	–	–	–	–	–	–	NS
LD_{99.9}	<i>E. coli</i>	<i>A. baumannii</i>	<i>E. faecalis</i>	<i>E. faecium</i>	<i>K. aerogenes</i>	<i>P. aeruginosa</i>	<i>K. pneumoniae</i>	<i>C. neoformans</i>	<i>C. albicans</i>
<i>S. aureus</i>	NS	NS	NS	NS	<0.001	<0.0001	<0.0001	<0.0001	<0.0001
<i>E. coli</i>	–	NS	NS	NS	<0.001	<0.0001	<0.0001	<0.0001	<0.0001
<i>A. baumannii</i>	–	–	NS	NS	<0.001	<0.0001	<0.0001	<0.0001	<0.0001
<i>E. faecalis</i>	–	–	–	NS	<0.01	<0.001	<0.0001	<0.0001	<0.0001
<i>E. faecium</i>	–	–	–	–	<0.05	<0.01	<0.0001	<0.0001	<0.0001
<i>K. aerogenes</i>	–	–	–	–	–	NS	<0.001	<0.05	<0.0001
<i>P. aeruginosa</i>	–	–	–	–	–	–	<0.0001	<0.05	<0.0001
<i>K. pneumoniae</i>	–	–	–	–	–	–	–	NS	<0.01
<i>C. neoformans</i>	–	–	–	–	–	–	–	–	<0.001
LD_{99.999}	<i>E. coli</i>	<i>A. baumannii</i>	<i>E. faecalis</i>	<i>E. faecium</i>	<i>K. aerogenes</i>	<i>P. aeruginosa</i>	<i>K. pneumoniae</i>	<i>C. neoformans</i>	<i>C. albicans</i>
<i>S. aureus</i>	NS	NS	NS	NS	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
<i>E. coli</i>	–	NS	NS	NS	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
<i>A. baumannii</i>	–	–	NS	NS	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
<i>E. faecalis</i>	–	–	–	NS	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
<i>E. faecium</i>	–	–	–	–	<0.001	<0.001	<0.0001	<0.0001	<0.0001
<i>K. aerogenes</i>	–	–	–	–	–	NS	NS	NS	<0.0001
<i>P. aeruginosa</i>	–	–	–	–	–	–	<0.001	NS	<0.0001
<i>K. pneumoniae</i>	–	–	–	–	–	–	–	NS	<0.0001
<i>C. neoformans</i>	–	–	–	–	–	–	–	–	<0.0001

Table S5. One-way ANOVA results for comparisons of tolerance factor (T) values obtained from average inactivation kinetics of each species. Non-significant differences are indicated as NS.

T	<i>E. coli</i>	<i>A. baumannii</i>	<i>E. faecalis</i>	<i>E. faecium</i>	<i>K. aerogenes</i>	<i>P. aeruginosa</i>	<i>K. pneumoniae</i>	<i>C. neoformans</i>	<i>C. albicans</i>
<i>S. aureus</i>	NS	NS	<0.01	<0.001	NS	<0.001	<0.0001	<0.0001	<0.001
<i>E. coli</i>	–	NS	NS	<0.01	NS	<0.01	<0.0001	<0.001	<0.05
<i>A. baumannii</i>	–	–	NS	<0.01	NS	<0.05	<0.0001	<0.001	<0.05
<i>E. faecalis</i>	–	–	–	NS	NS	NS	<0.001	NS	NS
<i>E. faecium</i>	–	–	–	–	<0.05	NS	NS	NS	NS
<i>K. aerogenes</i>	–	–	–	–	–	NS	<0.0001	<0.01	NS
<i>P. aeruginosa</i>	–	–	–	–	–	–	<0.001	NS	NS
<i>K. pneumoniae</i>	–	–	–	–	–	–	–	NS	<0.05
<i>C. neoformans</i>	–	–	–	–	–	–	–	–	NS

3.5. Article III

Photodiagnosis and Photodynamic Therapy 32 (2020) 102086

Contents lists available at ScienceDirect

Photodiagnosis and Photodynamic Therapy

journal homepage: www.elsevier.com/locate/pdpdt

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

Carolina dos Anjos^a, Fábio P. Sellera^a, Martha S. Ribeiro^b, Maurício S. Baptista^c,
Fabio C. Pogliani^a, Nilton Lincopan^{d,e}, Caetano P. Sabino^{e,f,*}

^a Department of Internal Medicine, School of Veterinary Medicine and Animal Science, University of São Paulo, São Paulo, Brazil

^b Center for Lasers and Applications, Nuclear, and Energy Research Institute, National Commission for Nuclear Energy, São Paulo, SP, Brazil

^c Department of Biochemistry, Institute of Chemistry, University of São Paulo, São Paulo, SP, Brazil

^d Department of Microbiology, Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, Brazil

^e Department of Clinical Analysis, Faculty of Pharmaceutical Sciences, University of São Paulo, São Paulo, Brazil

^f BioLambda, Scientific and Commercial LTD, São Paulo, SP, Brazil

ARTICLE INFO

Keywords:

Antibiotic resistance
 β -lactams
Enzymatic inactivation
Photoantimicrobial
Photoinactivation
Visible light
Antimicrobial Photodynamic Therapy

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 ($\lambda = 410 \pm 10$ nm) alone or red LED light ($\lambda = 660 \pm 10$ 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 carbapenemase- 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

* Corresponding author.

E-mail address: caetanosabino@gmail.com (C.P. Sabino).

<https://doi.org/10.1016/j.pdpdt.2020.102086>

Received 17 August 2020; Received in revised form 28 September 2020; Accepted 26 October 2020

Available online 4 November 2020

1572-1000/© 2020 Elsevier B.V. All rights reserved.

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_PDMV000000000.1) 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 10,000g 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 ($\lambda = 625$ 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 $100 \mu\text{M}$ 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 $3\log_{10}$ ($\text{LD}_{99.9}$) 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 $\text{LD}_{99.9}$ 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 (16,000g, 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 $\pm$ standard deviation.

2.4. Statistical analyses

Statistical analysis of $\text{LD}_{99.9}$ 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 ICBKPS3.2 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/cm²). 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^a.

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

^a The values are showed in radiant exposure (J/cm²) 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 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.

Funding

This work was supported by the São Paulo Research Foundation (FAPESP, grants 2016/25095-2, 2017/22406-0 and 2019/10851-4), by the Brazilian National Council for Scientific and Technological Development (CNPq, grant 141901/2016-0), and Photonics Institute (INFO, grant 465763/2014-6). We also recognized the technical support offered by the start-up company BioLambda. N.L. is a research fellow of CNPq (grant 312249/2017-9).

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.

Acknowledgements

We thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for research grants; BioLambda start-up company by technical support and light sources providing.

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>.

References

- [1] K. Bush, P.A. Bradford, Epidemiology of β -lactamase-producing pathogens, Clin. Microbiol. Rev. 33 (2020) 1–37, <https://doi.org/10.1128/CMR.00047-19>.
- [2] E. Tacconelli, E. Carrara, A. Savoldi, S. Harbarth, M. Mendelson, D.L. Monnet, et al., Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis, Lancet Infect. Dis. 18 (2018) 318–327, [https://doi.org/10.1016/S1473-3099\(17\)30753-3](https://doi.org/10.1016/S1473-3099(17)30753-3).
- [3] K. Bush, Past and present perspectives on β -lactamases, Antimicrob. Agents Chemother. 62 (2018) 1–20, <https://doi.org/10.1128/AAC.01076-18>.
- [4] H. Wilson, M.E. Török, Extended-spectrum β -lactamase-producing and carbapenemase-producing Enterobacteriaceae, Microb Genomics (2018) 4, <https://doi.org/10.1099/mgen.0.000197>.
- [5] I.G.H. Rood, Q. Li, Review: Molecular detection of extended spectrum- β -lactamase- and carbapenemase-producing Enterobacteriaceae in a clinical setting, Diagn. Microbiol. Infect. Dis. 89 (2017) 245–250, <https://doi.org/10.1016/j.diagmicrobio.2017.07.013>.
- [6] R. Onori, S. Gaiarsa, F. Comandatore, S. Pongolini, S. Brisse, A. Colombo, et al., Tracking nosocomial *Klebsiella pneumoniae* infections and outbreaks by whole-genome analysis: small-scale Italian scenario within a single hospital, J. Clin. Microbiol. 53 (2015) 2861–2868, <https://doi.org/10.1128/JCM.00545-15>.
- [7] J.A. Ramos-Castaneda, A. Ruano-Ravina, R. Barbosa-Lorenzo, J.E. Paillier-Gonzalez, J.C. Saldana-Campos, D.F. Salinas, et al., Mortality due to KPC carbapenemase-producing *Klebsiella pneumoniae* infections: systematic review and meta-analysis: mortality due to KPC *Klebsiella pneumoniae* infections, J. Infect. 76 (2018) 438–448, <https://doi.org/10.1016/j.jinf.2018.02.007>.
- [8] A.J. Mathers, G. Peirano, J.D.D. Pitout, The role of epidemic resistance plasmids and international high- risk clones in the spread of multidrug-resistant Enterobacteriaceae, Clin. Microbiol. Rev. 28 (2015) 565–591, <https://doi.org/10.1128/CMR.00116-14>.
- [9] C. dos Anjos, C.P. Sabino, V. Bueris, M.R. Fernandes, F.C. Pogliani, N. Lincopan, et al., Antimicrobial blue light inactivation of international clones of multidrug-resistant *Escherichia coli* ST10, ST131 and ST648, Photodiagnosis Photodyn. Ther. 27 (2019) 51–53, <https://doi.org/10.1016/j.pdpdt.2019.05.014>.
- [10] J. Nakonieczna, A. Wozniak, M. Pieranski, A. Rapacka-Zdonczyk, P. Ogonowska, M. Grinholc, Photoinactivation of ESKAPE pathogens: overview of novel therapeutic strategy, Future Med. Chem. 11 (2019) 443–461, <https://doi.org/10.4155/fmc-2018-0329>.
- [11] Y. Feng, A. Palanisami, S. Ashraf, B. Bhayana, T. Hasan, Photodynamic inactivation of bacterial carbapenemases restores bacterial carbapenem susceptibility and enhances carbapenem antibiotic effectiveness, Photodiagnosis Photodyn. Ther. (2020), 101693, <https://doi.org/10.1016/j.pdpdt.2020.101693>.
- [12] R.P. Ambler, The structure of β -lactamases, Philos. Trans. R. Soc. London B, Biol. Sci. 289 (1980) 321–331, <https://doi.org/10.1098/rstb.1980.0049>.
- [13] B.D. Jett, K.L. Hatter, M.M. Huycke, M.S. Gilmore, Simplified agar plate method for quantifying viable bacteria, Biotechniques 23 (1997) 648–650, <https://doi.org/10.2144/97234bm22>.
- [14] C.P. Sabino, M. Wainwright, C. dos Anjos, F.P. Sella, M.S. Baptista, N. Lincopan, et al., Inactivation kinetics and lethal dose analysis of antimicrobial blue light and photodynamic therapy, Photodiagnosis Photodyn. Ther. 28 (2019) 186–191, <https://doi.org/10.1016/j.pdpdt.2019.08.022>.
- [15] C.H. O'Callaghan, A. Morris, S.M. Kirby, A.H. Shingler, Novel method for detection of β -lactamases by using a chromogenic cephalosporin substrate, Antimicrob. Agents Chemother. 1 (1972) 283–288, <https://doi.org/10.1128/AAC.1.4.283>.
- [16] C. dos Anjos, C.P. Sabino, F.P. Sella, F. Esposito, F.C. Pogliani, N. Lincopan, Hypervirulent and hypermucoviscous strains of *Klebsiella pneumoniae* challenged by antimicrobial strategies using visible light, Int. J. Antimicrob. Agents (2020), 106025, <https://doi.org/10.1016/j.ijantimicag.2020.106025>.
- [17] K.M. Papp-Wallace, A. Endimiani, M.A. Taracila, R.A. Bonomo, Carbapenems: past, present, and future, Antimicrob. Agents Chemother. 55 (2011) 4943–4960, <https://doi.org/10.1128/AAC.00296-11>.
- [18] J.M. Munita, C.A. Arias, Mechanisms of antibiotic resistance, Microbiol. Spectr. 4 (2016) 1–24, <https://doi.org/10.1128/microbiolspec.VMBF-0016-2015>.
- [19] D.L. Paterson, R.A. Bonomo, Extended-spectrum β -lactamases: a clinical update, Clin. Microbiol. Rev. 18 (2005) 657–686, <https://doi.org/10.1128/CMR.18.4.657-686.2005>.
- [20] W.H. Sheng, R.E. Badal, P.R. Hsueh, Distribution of extended-spectrum β -lactamases, AmpC β -lactamases, and carbapenemases among Enterobacteriaceae isolates causing intra-abdominal infections in the Asia-Pacific region: results of the study for monitoring antimicrobial resistance trends (SMART), Antimicrob. Agents Chemother. 57 (2013) 2981–2988, <https://doi.org/10.1128/AAC.00971-12>.
- [21] C.P. Sabino, A.R. Ball, M.S. Baptista, T. Dai, M.R. Hamblin, M.S. Ribeiro, et al., Light-based technologies for management of COVID-19 pandemic crisis, J. Photochem. Photobiol. B (2020), <https://doi.org/10.1016/j.jphotobiol.2020.111999>, In press.

3.6. Article IV

Expected title: **Protein oxidation is the primary cause of bacterial inactivation induced by antimicrobial photodynamic therapy**. Journal: To be submitted to Nature Microbiology.

Protein oxidation is the primary cause of bacterial inactivation induced by antimicrobial photodynamic therapy

Sabino, C.P.^{1,2}; Ribeiro, M.S.³; Wainwright, M.⁴; dos Anjos, C.^{5,6,7}; Sellera, F.P.^{5,8}; Dropa, M.⁹; Nunes, N.B.¹⁰; Brancini, G.T.P.¹⁰; Braga, G.U.L.¹⁰; Arana-Chavez, V.E.¹¹; Freitas, R.O.¹²; Lincopan, N.^{2,13}; Baptista, M.S.¹⁴

¹BioLambda, Scientific and Commercial Ltd., São Paulo, SP, Brazil, 05595-000

²Department of Clinical and Toxicological Analysis, School of Pharmaceutical Sciences, University of São Paulo, São Paulo, SP, Brazil, 05508-000

³Center for Lasers and Applications, Energy and Nuclear Research Institute, São Paulo, SP, Brazil, 05508-000

⁴School of Pharmacy & Biomolecular Sciences, Liverpool John Moores University, Liverpool, UK

⁵Department of Internal Medicine, School of Veterinary Medicine and Animal Science, University of São Paulo, São Paulo, SP, Brazil, 05508-270

⁶Wellman Center for Photomedicine, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts, USA

⁷Vaccine and Immunotherapy Center, Massachusetts General Hospital, Harvard Medical School, Boston, USA.

⁸School of Veterinary Medicine, Metropolitan University of Santos, Santos, SP, Brazil, 11080-300.

⁹Public Health Laboratory, School of Public Health, Universidade de São Paulo, São Paulo, Brazil

¹⁰Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, 14040-903, Brazil

¹¹Department of Biomaterials and Oral Biology, University of São Paulo, São Paulo, São Paulo, Brazil

¹²Brazilian Synchrotron Light Laboratory, Brazilian Center for Research in Energy and Materials, 13083-970, Campinas, SP, Brazil, 13083-970

¹³Department of Microbiology, Institute for Biomedical Sciences, University of São Paulo, São Paulo, SP, Brazil, 05508-000

¹⁴Department of Biochemistry, Institute of Chemistry, University of São Paulo, São Paulo, SP, Brazil, 05513-970

Abstract

The unbridled dissemination of multidrug-resistant pathogens is a major threat to global health and urgently demands novel therapeutic alternatives. Following the current trend, the WHO expects that therapeutic options will run out by 2050. Antimicrobial photodynamic therapy (aPDT) has been developed as a promising approach to treat localized infections regardless of drug resistance profile or taxonomy. Even though this technique has been known for more than a century, discussions and speculations regarding the biochemical mechanisms of microbial inactivation have never reached a consensus on what is the primary cause of cell death. Since photochemically generated oxidants promote ubiquitous reactions towards several biomolecules, researchers simply assumed that all cellular structures are equally damaged. In this study, biochemical, molecular biology, and advanced microscopy techniques were employed to investigate if protein, membrane, or DNA damage correlates better with dose-dependent microbial inactivation kinetics. Results showed that although mild membrane permeabilization and late DNA damage occurs, no correlation with inactivation kinetics was found. On the other hand, protein degradation was analyzed by 3 different methods and showed a dose-dependent trend that matches microbial inactivation kinetics. Through this report, we expect to provide a better mechanistic understanding of aPDT for the development of optimized photosensitizing drugs and also rationally propose synergistic combinations with antimicrobial chemotherapy.

Introduction

The catastrophic emergence of microbial drug resistance (1-3) is a global health priority (4-7). This scenario is likely to worsen following the COVID-19 pandemic crisis since antibiotics have been widely used to treat bacterial co-infections and/or secondary infections. The major concern on drug resistance dissemination relies on the rapid bacterial ability to acquire resistance genes from prior generations as well as from other strains and species (6, 8-12). A post-antibiotic era could mean the end of current ideas of modern medicine due to the consequent inability to treat common minor infections or to support other areas such as transplants and oncology (13).

Antimicrobial photodynamic therapy (aPDT) has been successfully used for over a century in modern medicine to treat superficial infections (3, 13, 14). It is an elegant method to produce many strong oxidant substances (mainly excited-state species and free-radicals) in a space-time-controlled manner that is triggered by light. Such a mechanism of action can simultaneously damage several vital structures (*e.g.*, proteins, nucleic acids, and membranes) leaving a questionable hypothesis on whether microbes could ever develop a resistance mechanism. On the other hand, aPDT has the broadest action-spectrum possible, as it can neutralize any type of pathogen, such as bacteria, fungi, algae, protozoa, viral particles, and prions (1, 14-19). Currently, the convergence of two independent phenomena has provided an exponential interest in aPDT: the outbreak of drug-resistant microbes demanding alternative therapeutic strategies and the photonic revolution that allow the development of new cost-effective antimicrobial technologies that are not sensitive to the selection of resistant strains.

Despite the great potential of aPDT, the photoactivated drugs, generally referred to as photosensitizers (PS), that are being used today are the same as those developed decades or even more than a century ago. Yet, the mechanistic understanding of cell killing remains unsophisticated. Current hypotheses are generic, regarding photosensitizer action as electron-transfer (type I) and energy transfer (type II) - promoted mechanisms of oxidation (20) and that positively-charged (cationic) PS benefit from electrostatic interactions with the negatively-charged (anionic) surface moieties of Gram-negative, Gram-positive bacteria and fungi, whereas repulsion may occur with anionic PS. More detailed mechanistic information is necessary to rationally develop optimized PS for antimicrobial applications so that synergistic applications may be developed with classic antimicrobial chemotherapeutics, *i.e.*, if a

PS is optimized for protein damage, this could be combined with a cell wall-targeting chemotherapeutic antimicrobial.

Although the strong oxidants generated during aPDT can damage various vital cellular structures, such as membranes, proteins, and DNA, the sites of critical damage, leading to microbial inactivation have not been established. Thus, major damage at membranes (21), proteins (22, 23), or nucleic acids (24) has never been specifically linked to cell death.

The present work reports an experimental protocol that allows deeper insights into the biochemical mechanisms of antimicrobial photodynamic inactivation, demonstrating whether microbial inactivation is primarily caused by the degradation of the cell membrane, proteins, or nucleic acids. The protocol may allow future studies to explore the development of optimized PS, rational combination with other antimicrobials, and pave the way to improved therapeutic outcomes.

Results & Discussion

Methylene blue (MB) was employed as a model photosensitizing drug because it is the most popular and broadly available photosensitizer used for aPDT. To match the maximum absorption peak of MB monomers at 664 nm a customized LED system with very close peak emission at 662(21) nm was used. The spectral characteristics of LED and MB are plotted in **Supplementary Figure 1a**. Using equation 2, the number of absorbed photons were calculated as a function of wavelength (**Supplementary Figure 1b**). Results indicate that 64.6 % of incident light was absorbed, resulting in the absorption of 2.62×10^{22} photons per second. According to the molarity of the experimental solution, it is estimated that, on average, each MB molecule is being excited 4.3×10^5 times per second. Since the singlet oxygen yield of MB has an average efficiency of 50 %, each molecule would be able to produce up to 2.1×10^5 singlet oxygen molecules per second. No microbial cell is expected to tolerate such an oxidative insult for long nor to be able to develop an effective resistance mechanism to such an approach.

Inactivation kinetics curves of all three *Klebsiella pneumoniae* strains tested in this study are presented in **figure 1**. Lethal doses for 90% of inactivation were 4.3 ± 0.3 J/cm² for ATCC 700603, 4.7 ± 0.4 J/cm² for ATCC BAA1705 and 6.4 ± 0.6 J/cm²

for BR-1 with statistically significant differences between BR-1 and ATCC 700603 or ATCC BAA1705. The tolerance factor (T) was 0.81 ± 0.04 for ATCC 700603, 0.93 ± 0.07 for ATCC BAA1705, and 1.1 ± 0.1 for BR-1 only with statistically significant differences between BR-1 and ATCC 700603. Even though some statistically significant differences were found, it is noticeable that inactivation kinetics rates were highly similar amongst all strains. This data is in agreement with a recently published study that compared the inactivation kinetics of multidrug-resistant (MDR) bacteria and yeast concerning their drug-sensitive counterparts (16). Here it was shown that there is no correlation between photosensitivity and drug resistance profiles, whereas aPDT inactivation kinetics is strongly correlated with microbial species but not strain.

Figure 1. Inactivation kinetics of aPDT against *K. pneumoniae* strains. Observe that all strains had very similar inactivation kinetics curves and the lethal dose for 90% of inactivation fluctuated around 5 J/cm².

Analysis via fluorescence-lifetime imaging microscopy (FLIM) indicates that the PS effectively permeates the microbial cells and interacts with DNA and intracellular proteins. Fluorescence lifetime for PS molecules embedded in the cytoplasm is 0.6 ns and for molecules interacting with DNA, it becomes 1.8 ns (**Fig. 2**). Compartmentalized *Candida albicans* cells demonstrate longer PS fluorescence lifetimes in DNA-rich compartments, such as nuclei and mitochondria (**Fig. 2b**). This is also seen inside *K. pneumoniae* cytosol (**Fig. 2e**). It is well-known that MB intercalates with DNA double-

strands allowing more stable excited states by shielding vibronic interactions with water (25). MB intercalation in GC pairs of the DNA sequence strongly affects its fluorescence lifetime due to Coulomb and van der Waals interactions (26). Intercalation in AT pairs also occurs, but hydrogen bonding is not possible because of the absence of donors inside the minor groove (27, 28). Thus, the MB fluorescence lifetime is longer when intercalated in DNA than when solvated by water or interacting with other biomolecules. This is the first time that MB distribution and interaction inside yeast and bacterial cells has been reported in FLIM photomicrographs.

Figure 2. Fluorescence-lifetime imaging microscopy (FLIM) images of MB interacting with *C. albicans* (a, b, c) and *K. pneumoniae* (d, e, f) cells. Images on a and d show the fluorescence signal of MB interacting with proteins in cytosol presented an average lifetime of 0.6 ns. On c and e, stabilized MB interacting with DNA presented a three-times higher fluorescence lifetime with an average of 1.8 ns. Images c and f show the merged fluorescence signal in each cell type, whereas *C. albicans* organelles facilitate the specificity visualization of this technique. Scale bars represent 2 μ m.

The characteristic singlet oxygen phosphorescence peak was observed around 1277 nm and its normality was confirmed by Gaussian fit (**Supplementary Fig. 2.a**). According to exponential fit parameters, phosphorescence lifetime fluctuated around 45 μ s and the addition of 10^8 CFU/mL was not enough to quench the phosphorescence in observable levels when measurements were performed in solution (**Supplementary Fig. 2b**). The fact that singlet oxygen can be generated

within bacterial cells indicates not only that it may be involved itself in the cell-killing but, perhaps more importantly, that triplets are generated in abundant levels close to the molecular targets, being therefore prone to initiate contact-dependent reactions with relevant biomolecules, such as proteins, nucleic acids, carbohydrates and membranes constituents (45).

Nanoscopy analysis performed by TEM and AFM-IR comparing non-treated cells to those exposed to 100 J/cm² of aPDT are presented in **figure 3**. According to **equation 4**, the dose required to inactivate 100% (*i.e.*, 9log₁₀) of cells is 64 J/cm² for ATCC 700603, 50 J/cm² for ATCC BAA1705 and 45 J/cm² for BR-1 strains, respectively. Even though cells were treated with a light dose much higher than necessary to inactivate 100% of cells, TEM and AFM-IR images revealed only moderate differences concerning non-treated cells. Cell membranes did not rupture, releasing intracellular content and cellular morphology was well preserved. However, aPDT-treated cells presented a tendency to aggregate protein content around the intracellular periphery leaving DNA content accumulated in the central region of the cell (indicated by arrows). Since the AFM-IR laser was tuned to be centered at the amide 1 absorption band (1640 cm⁻¹), it is also possible to confirm that such DNA rich areas contain very small quantities of proteins. TEM images also allow the visualization of DNA strands in the same region.

Figure 3. Nanoscopy images of (left) control and (right) aPDT-treated cells of *K. pneumoniae* at 100J/cm². Transmission electron microscopy (TEM) images (**a** and **b**) were acquired at 80,000x original magnification. Atomic force microscopy coupled to infrared-spectroscopy (AFM-IR) images (**c** and **d**) were acquired in contact mode. Observe that aPDT-treated cells tend to aggregate protein content around the intracellular periphery leaving DNA content accumulated in the central region of the cell (indicated by arrows).

Since PS molecules are accumulated on DNA (**Fig. 2**), and it was calculated that in this system each PS molecule might be excited around 4.3×10^5 times per second, it is reasonable to suppose that DNA damage can be expressive. Purine bases are common targets for singlet oxygen and hydroxyl radicals while the ribose-phosphate backbone structure is mostly sensitive to hydroxyl-radicals (29). To elucidate these valuable findings, the DNA damage of chromosomes and plasmids were investigated by pulsed-field gel electrophoresis (PFGE) and also important drug resistance genes (*e.g.*, *bla*_{IMP-1} and *bla*_{KPC-2}) by PCR (**Fig. 4**). Considering the singlet oxygen formation rate above-mentioned, it is unlikely that microbial cells can repair the DNA damage at a sufficient rate to resist inactivation.

PFGE analysis of the degradation of chromosomal DNA (**Fig. 4a**) presented a loss of band pattern, forming a smear aspect at 10 J/cm². At 50 and 100 J/cm², DNA is fragmented in such small sections that they were nearly not retained in the PFGE matrix. Assuming that single event DNA damage can impair DNA-polymerase or ribosomal activity, the amplification of DNA in genes related to carbapenemase resistance following crescent doses of aPDT was attempted. Dose-dependent inhibition of DNA amplification is observable (**Fig. 4b-c**). The inhibition level was also dependent on the amplicon sequence size. The more sensitive *bla*_{KPC-2} amplicon is 799bp long and began presenting amplification inhibition at 20 J/cm² while *bla*_{IMP-1} is half as long (399bp) and presented the same level of amplification inhibition at 40 J/cm². It indicates that damage probability must be directly proportional to the DNA sequence size or – in other words – simply the number of targets. Consequently, hit points for oxidative damage during aPDT must have a greater impact on a target the size of chromosomal DNA. However, comparing these dose-dependent DNA degradation results to the cellular inactivation kinetics (**Fig. 1**) it remains questionable whether DNA damage contributes directly to microbial inactivation since the LD₉₀ is reached at around only 5 J/cm². This suggests that intense DNA damage only occurs after bacterial cells are already dead.

Figure 4. Analysis of DNA damage by electrophoresis. In **a** it is presented the PFGE result of the total *K. pneumoniae* DNA. C– refers to negative (sham) controls, CL are controls only treated with 100 J/cm² of light, 0 are controls only treated with PS for 1 h and L are molecular weight reference ladders of 5Kb. Double-strand DNA breaks induced by aPDT caused the formation of smaller fragments of random sizes giving the smear impression seen in group 10 J/cm². In higher dose groups (e.g., 50 and 100 J/cm²), such small fragments were formed that no retention is visible in the electrophoresis gel. In **b** and **c**, PCR amplification products of *K. pneumoniae* carbapenemase-encoding genes before and after aPDT. Greyscale fluorescence images allow clear visualization of amplicon size and dose-dependent fading of PCR-amplification bands induced by aPDT.

Very interesting results were obtained on protein degradation analysis (**Fig. 5a-c**). Significant loss of protein content (**Fig. 5a**) and increase of protein carbonylation (**Fig. 5b**) were observed, which continuously progressed in a dose-dependent manner. The results show a tendency towards significantly higher protein carbonylation already at 2.5 J/cm² and continuous increases with no saturation point over the analyzed range. Longer exposures to light (e.g., 7.5 and 10 J/cm²) lead to such severe protein loss that the precision of carbonyl content quantification was reduced, although protein loss is itself an indicator of extensive protein oxidation (30).

The formation of carbonyl groups resulting from the photo-oxidation of proteins has been extensively reported and is thought to be a result of side-chain oxidation of susceptible amino acids by reactive oxygen species (22, 23, 30). Therefore, the extent to which photodynamic treatment affected protein carbonyl content was evaluated using a dose-dependent analysis (**Fig. 5b**). Protein loss is another known consequence of photodynamic treatment as oxidative damage to proteins can lead to

both aggregation and fragmentation (22, 23). Protein aggregation and cross-linking normally occur after the formation of reactive carbonyl functions on amino acid residues followed by a nucleophilic attack by the sidechains of His, Lys, Arg, and Cys (22, 31, 32). Fragmentation is thought to be a consequence of protein peroxides generated after singlet oxygen reaction with Tyr, His, and Trp residues. The catalyzed decomposition of such peroxides by metals can yield radicals that may propagate the oxidations through chain reactions (33). These can generate α -carbon radicals via hydrogen abstraction which, in turn, results in the formation of Schiff bases involving the amide nitrogen on peptide bonds, ultimately leading to backbone cleavage (34).

Figure 5. Degradation kinetics of proteins presented as (a) loss of protein content, (b) carbonylation of proteins, and (c) inhibition β -lactamase enzymatic activity (SHV-18, produced by *K. pneumoniae* ATCC 700603). Membrane permeability assay (d) was measured by *N*-phenyl-1-naphthylamine (NPN) uptake. C- are negative (sham) controls, C+ are positive controls for membrane damage treated with propyl alcohol at 70% for 15 min, CL are controls only treated with 20 J/cm² of light, 0 are controls only treated with 100 μ M of MB for 1 h. Figures demonstrate that protein degradation continuously increases in a dose-dependent manner while cell permeability is significantly increased at the beginning of the irradiation process (2.5 J/cm²) but remains unchanged until 20 J/cm². Single asterisks (*) represent $p < 0.05$, double asterisks (**) represent $p < 0.01$, triple asterisks (***) represent $p < 0.001$, and quadruple asterisks (****) represent $p < 0.0001$ differences compared to the negative control group (C-).

Regarding the enzymatic activity of β -lactamase (**Fig. 5c**), a continuous dose-response trend of enzymatic inhibition is presented in a similar pattern as loss of protein content. Measurements of enzymatic activity precisely confirm the effects of protein loss and carbonylation over protein function. Yet, loss of β -lactamase activity may be a very important factor allowing increased sensitivity to antibiotic therapy of drug-resistant strains that were exposed to sublethal doses of aPDT. In this regard, *K. pneumoniae* strains carrying carbapenemase genes have been under the spotlight of scientific awareness, being globally classified by the WHO as critical-priority pathogens (10, 12). The production of ESBLs and carbapenemases - antimicrobial-cleaving enzymes without ATPase activity - are important examples of what leads to such relevance towards MDR and extensively-drug resistant (XDR) phenotypic profiles. In practical terms, the existence of such phenotypes restrains the use of most commercially-available antibiotic options (35). Therefore, the necessity of novel antimicrobial strategies that are not susceptible to microbial resistance, such as aPDT, is an urgent demand.

Membrane permeability, on the other hand, rapidly increases and saturates in intermediary levels leaving no dose-dependent pattern (**Fig. 5d**). All aPDT experimental data presented statistically significant differences compared to both, C- and C+, indicating that mild membrane damage occurs but is not as intense as that caused by exposure to 70% propyl alcohol. However, TEM and AFM-IR images (**Fig. 3**) confirmed that membrane rupture does not occur even in much higher aPDT doses (100 J/cm^2). Therefore, membrane and even DNA damage may represent contributing factors to bacterial inactivation by aPDT, although protein damage appears to be the principal cause of cell death.

Membrane damage levels imposed by photodynamic reactions of phenothiazinium compounds have previously been related to the amphiphilicity of the PS molecule. More lipophilic molecules, such as dimethyl methylene blue (DMMB) and DO15, can efficiently induce membrane rupture while the more hydrophilic MB and toluidine blue are not nearly as potent in achieving this effect (21). However, although moderate levels of membrane permeabilization (**Fig. 5d**) were observed, there were no signs of membrane rupture, even after exposure to aPDT doses much higher than those necessary to induce complete microbial inactivation (see **Fig. 3b and d**).

Antimicrobial chemotherapeutic strategies generally target singular microbial biomolecules or metabolic pathways to achieve specific microbial inactivation. This approach facilitates the development or selection of resistant populations as these may be a few mutations away. Most antimicrobials are derivatives of natural fungal or bacterial metabolites that have been used by these microbes to survive in their ecosystems. In this context, some microorganisms have naturally developed intrinsic resistance phenotypes also to thrive in the environment. Hence, genes of naturally occurring drug resistance can already be available via vertical and horizontal transmission, based on the microbial ability to acquire function through the uptake of mobile genetic elements (e.g., transformation, transduction, or conjugation). Membrane rupture may facilitate the horizontal transmission of resistance genes by environmental dissemination of genetic material. Thus, from the current perspective, aPDT works in an ideal situation where inactivated cells keep their damaged DNA and protein content sealed inside the cell.

In summary, even though the cytotoxic effects of aPDT towards bacteria have been known for over a century, no one has ever stated the primary *causa mortis* of bacterial cells. Here, it was observed that the PS effectively permeates microbial cell membranes and even binds with DNA molecules and leads to significant damages to both structures. However, by comparing the dose-dependent degradation of DNA, membrane, and protein structures, it is clear that protein damage gives the best correlation with cell killing.

A remarkable interpretation of this mechanism of action is that microbial inactivation can be achieved while damaged and non-functional drug resistance enzymes and genes remain sealed inside the plasma membrane. This fact suggests that aPDT can effectively inactivate drug-resistant microorganisms and simultaneously block drug resistance transmission and dissemination. Consequently, aPDT should become an even more interesting approach shortly as antimicrobial resistance is only expected to increase. Finally, it is suggested that the results presented can provide a better mechanistic understanding of aPDT for the development of optimized photosensitizing drugs and also rationally support synergistic combinations with conventional antimicrobial chemotherapeutics.

Acknowledgements

We gratefully thank and technical support offered by the University of São Paulo, the Nuclear and Energy Research Institute, Brazilian Center for Research in Energy and Materials, and the start-up company BioLambda.

Funding

This work was supported by the São Paulo Research Foundation (FAPESP, grants 2019/10851-4, 2016/25095-4, 2016/25095-2, 2013/07937-8, 2017/22406-0 and 2016/08593-9) and by the National Council for Scientific and Technological Development (CNPq, grants 141901/2016-0, 465763/2014-6, 312249/2017-9, 315738/2018-9 and 433128/2018-6).

Materials & Methods

Photosensitizer and light source

Methylene blue (MB, Sigma-Aldrich, USA) as a standard PS, whereby microbial inactivation was calculated based on light radiant exposure (equation 1, J/cm^2) and the number of photons absorbed per volume (equation 2, $\text{AbsorbedPhotons}/\text{cm}^3$). Stock solutions of MB were prepared in type-1 Milli-Q water, at 10 mM. The MB stock solutions were filtered by 0.22 μm membrane for decontamination and stored at 4° C in the dark before use.

The LED system (LEDsaber Prototype 1, BioLambda, Brazil) to sample distance was adjusted to achieve a beam-spot diameter of 25 mm, which covered the entire bottom of a well in a 12-well plate. Light irradiance was adjusted to a standard radiant exposure rate (dR/dt) of 100 mW/cm^2 (measured at the sample bottom) and beam divergence of 45°. Beam spot on samples was highly homogeneous, with irradiance divergence between center and border below 10%. The LED spectral emission was measured by a UV-VIS spectrophotometer (Flame, Ocean Optics, USA).

Radiant exposure [J/cm^2] is calculated as the product of mean optical power ($\bar{P}$) [mW] and exposure time [s], divided by the beam area [cm^2]. To estimate the number of absorbed photons per volume of solution [$\text{V} - \text{cm}^3$], the PS absorption spectrum $A_{(\lambda)}$ and the spectral radiant power emitted by the LED system $P_{(\lambda)}$ were

measured. The absorption spectrum of MB was measured in a plate-reader spectrophotometer (SpectraMax M4, Molecular Devices, USA). MB concentration in phosphate-buffered saline (PBS) was 100 µM and the optical path was 2 mm (corresponding to the height of 1 mL solution in a 25 mm cylindric well). Moreover, the universal constant of the velocity of light in vacuum ($c_0 = 299,792,458$ m/s) and the Planck constant ($h = 6.62606957 \times 10^{-34}$ J.s) were used.

$$Radiant\ Exposure = \frac{\bar{P} \cdot t}{Area} \quad \text{equation (1)}$$

$$\frac{AbsorbedPhotons}{s \cdot cm^3} = \int_{\lambda_0}^{\lambda} \frac{P_{(\lambda)} \cdot 10^{A(\lambda)}}{V \cdot c_0 \cdot h} d\lambda \quad \text{equation (2)}$$

The inactivation kinetics analysis method described by Sabino et al. (36) was used as follows in equation 3. Curve fitting and numeric operations were calculated in Prism 7.0 (GraphPad, USA) interface.

$$\log_{10} \left(\frac{N_0}{N} \right) = \left(\frac{Dose}{LD_{90}} \right)^T \quad \text{equation (3)}$$

$$LD_i = LD_{90} \left(-\log_{10} \left(1 - \frac{i}{100} \right) \right)^{1/T} \quad \text{equation (4)}$$

where:

N_0 = initial microbial burden; N = final microbial burden; $Dose$ = radiant exposure (J/cm^2); LD_{90} = lethal dose for 90% of microbial burden (J/cm^2); T = tolerance factor; i = inactivation percentage (%).

aPDT – Sample irradiation studies

K. pneumoniae was used as the bacterial model due to its profound association with extensive drug-resistance. The ATCC 700603 strain *K. pneumoniae* expressing SHV-18 extended-spectrum β-lactamase (ESBL) was used in all experiments except for polymerase chain reaction (PCR) analysis where KPBr-1 (GenBank accession number: NZ_PDMV00000000.1) and ATCC BAA1705 strains expressing IMP-1 and KPC-2 carbapenemases, respectively, were employed.

Standard aPDT susceptibility test was performed based on Sabino *et al.* (36). Inocula were prepared from log-phase overnight bacterial cultures. Cells were then washed twice in PBS (dibasic phosphate = 2.65 g/L, monobasic phosphate = 0.358 g/L, NaCl = 8.183 g/L and milli-Q water). The turbidity of cell suspensions in PBS was measured in a spectrophotometer to obtain inocula at McFarland scale 4. The scale was calibrated to obtain OD of 0.67 at 625 nm resulting in $1-2 \times 10^9$ CFU/mL of working concentration of planktonic bacteria.

For bacterial inactivation kinetics assays, aPDT groups were subdivided into 5 groups of variable radiant exposures in steps of 5 J/cm². For membrane, protein, and DNA degradation kinetics assays, radiant exposure steps were set according to the detection sensitivity of each method that was predetermined in pilot studies. To avoid cross light exposure, each sample was kept in individual microtubes in the dark during the pre-irradiation time and placed in the 12-wells plate only for irradiation.

For bacterial survival analysis, the simplified method of colony counting established by Jett *et al.* (37) was used. Immediately after irradiation, bacterial suspensions were serially diluted in PBS to give dilutions from 10^{-1} to 10^{-6} times the original concentration. Ten- μ L aliquots of each dilution were streaked onto Muller-Hinton agar plates, in triplicates, and incubated at 37° C overnight. The colonies were counted and converted into normalized log₁₀ units of CFU/mL (colony-forming units per milliliter).

Singlet oxygen detection in solution

Phosphorescence intensity and the lifetime of singlet oxygen (¹O₂) produced were measured using a near-infrared (NIR) spectrophotometer. In this assay, the MB solution was prepared in deuterium oxide (D₂O, 99% pure, Sigma-Aldrich), to optimize ¹O₂ phosphorescence signal (peak emission $\cong$ 1270 nm). MB solutions [100 μ M] were prepared either alone or in the presence of 10^8 CFU/mL of *K. pneumoniae*.

MB excitation was pumped by a Nd:YAG tunable laser (λ = 664 nm, Surrelite III, Continuum). Radiation emitted from samples was first filtered through a silicon cutoff filter and subsequently wavelength-resolved with the use of a monochromator (Model M300, Edinburgh Instruments). The detection of the emitted NIR light was performed with the use of a liquid nitrogen-refrigerated NIR-sensitive photomultiplier (Model R5509, Hamamatsu Photonics) with low dark noise (<500 cps), connected to

a personal computer via a 300-MHz acquisition MSA board (Becker & Hickl GmbH) (38).

Fluorescence-lifetime imaging confocal microscopy

Photosensitizer accumulation, localization, and interaction with biomolecules were observed through fluorescence-lifetime imaging confocal microscopy (FLIM). Overnight log-phase cultures of *C. albicans* (ATCC 90028) and *K. pneumoniae* (ATCC 700603) were employed. *C. albicans* was diluted in PBS to reach a working concentration at $1-2 \times 10^7$ CFU/mL, and *K. pneumoniae* was used at $1-2 \times 10^9$ CFU/mL. MB was used in a working concentration of 100 μ M in PBS. Cells were not washed to remove MB from supernatant for imaging because fluorescence background (*i.e.*, outside cells) was used as intensity and lifetime controls.

Time-resolved fluorescence detection was carried out by a confocal laser scanning microscope (MicroTime 200, PicoQuant, Germany). The fluorescence system was set with 700(20) nm band-pass filters, short pulse (<1 ns) 638 nm excitation lasers, and 60x objective lens to analyze samples. Obtained images and data were analyzed by proper software (SymPhoTime 64, PicoQuant, Germany) to extract information regarding MB fluorescence lifetime.

Nanoscopy techniques - Transmission electron microscopy and atomic force microscopy coupled with infrared spectroscopy

Nanoscopy techniques were employed to visualize ultrastructural damages of treated cells. Negative controls (C-) of viable *K. pneumoniae* (ATCC 700603) cells suspended in PBS were compared to samples treated with 100 J/cm² of aPDT. Such a high dose was used to search for the most pronounced ultrastructural damages. Samples were fixed in 2 % glutaraldehyde kept overnight at 4° C. Then, samples were centrifuged at 10,000 g for 10 min and washed in 0.1 M sodium cacodylate buffer (pH 7.2) twice. Pellets were post-fixed in 2 % osmium tetroxide in sodium cacodylate buffer for 8 hours. Samples were then serially dehydrated in ethanol, and embedded in Spurr resin (Tousimis, USA). Ultrathin slices (<100 nm) were cut by diamond blades of an ultramicrotome (Leica, Germany).

Transmission electron microscopy (TEM) samples were placed on 200-mesh copper grids and post-stained with uranyl acetate and lead citrate solution. Sample visualization was performed on a transmission electron microscope (Model 1010,

JEOL, Japan). Atomic force microscopy coupled to infrared spectroscopy (AFM-IR) samples were placed over a flat silicon crystal previously coated with 100 nm of gold by sputtering. AFM-IR sample visualization was performed by an atomic force microscope coupled with an infrared tunable laser (NanoIR2-s, Anasys, USA) set at the center of the amide I absorption band (1640 cm^{-1}). False-color scale images for AFM-IR were processed by ImageJ software (NIH, USA).

Pulsed-field gel electrophoresis - Damage of chromosomal DNA

Pulsed-field gel electrophoresis (PFGE) analysis was used to assay double-strand breaks of *K. pneumoniae* DNA. 3 control groups were employed: a negative control (C-) of viable cells suspended in PBS; a light control (CL) of cells only exposed to 100 J/cm^2 ; and a non-irradiated control (0) exposed only to MB at $100\text{ }\mu\text{M}$ for 60 min. aPDT experimental groups were prepared as described for inactivation studies. However, in this assay, samples were exposed to 2.5, 5, 10, 50 or 100 J/cm^2 of radiant exposure.

Following irradiation, samples were immediately transferred to Eppendorf tubes that were kept in the dark at $0-4^{\circ}\text{C}$. Then, samples were washed twice with cold PBS. After the third centrifugation at $6,000\text{ g}$, cells were resuspended in $200\text{ }\mu\text{L}$ of TE I buffer (Tris-HCl 10 mM , EDTA 1 mM , pH 7.5). Eighty- μL of samples were transferred into $320\text{ }\mu\text{L}$ of agarose ($1\text{ }\%$ m/v, $50-60^{\circ}\text{C}$) and casted into proper plug molds. Plugs were then immersed into a lysis buffer (EDTA 50 mM , Tris-HCl 50 mM , SDS $20\text{ }\%$, sarkosyl 1% , proteinase K 20 mg/mL , pH 7.5) for 2 h at 55°C . After lysis, plugs were washed twice in type I Milli-Q water and four times in TE I buffer at 55°C with intervals of 15 min.

For DNA cleavage, plugs were transferred into microtubes containing 10 U of XbaI restriction enzyme in TE I buffer and were incubated at 37°C for 18 h. After incubation, plugs and molecular weight standards (multimeric phase lambda DNA ladder 48, 5Kb, Bio-Rad, USA) were inserted into 1% agarose gel and immersed in TBE buffer (Tris-borate 45 mM , EDTA 1 mM , pH 8.0). Electrophoresis was performed by proper equipment (Chef Mapper, Bio-Rad, USA) configured as 6 V/cm at 14°C for 22 h, with pulse intervals between 3.51 and 30.82 s. DNA was stained with ethidium bromide ($1\text{ }\mu\text{g/mL}$, Sigma-Aldrich, USA) and fluorescence images were acquired (Epi Chemi II Darkroom, UVP Bioimaging Systems, USA).

Polymerase chain reaction (PCR) assay – damage of specific genes

DNA double-strand breaks impair DNA/RNA-polymerase activity, thereby blocking gene expression and replication or amplification (39). Hence, double-strand breaks can be monitored via reduction of amplification yields of PCR reactions. Based on this principle, semi-quantitative PCR-based analysis was performed to evaluate the viability of resistance genes.

The final cell suspension was treated with aPDT under five different radiant exposures (10, 20, 30, 40 or 50 J/cm²) or incubated with PS in the dark or treated only with light. Cells treated only with MB (0 J/cm²) or PBS alone (C–) were used as control genes to validate the findings by comparison.

After the irradiation procedure, suspensions were washed in TBE and total nucleic acid content was extracted by boiling assay at 100° C for 15 min, in a dry block. Samples were then centrifuged at 4° C for 15 min, at 10,000 g. Total DNA was extracted from KPC-2- and IMP-1-producing *K. pneumoniae* strains (40). The amplification of the β -lactamase encoding genes was performed according to previously established primers and conditions (41-43). PCR products were stained with GelRed (Uniscience, USA) and separated by electrophoresis in agarose gel at 1%, together with a molecular weight marker of 100 bp (New England Biolabs, USA).

Total protein and carbonyl quantification assays

Growth was performed in Mueller-Hinton broth for 20 h at 37 °C and 200 rpm. Cells were pelleted by centrifugation (6,000 g, 10 min) and washed twice with PBS. Cell density was adjusted to OD_{625nm} = 0.7 in PBS with the help of a spectrophotometer to yield 1-2 x 10⁹ CFU/mL. A total of 10.8 mL of cell suspension was used for each treatment and control group.

aPDT samples were exposed to 2.5, 5, 7.5 or 10 J/cm² of aPDT. We also had control groups prepared without MB that were not exposed to light (C–) and that were exposed to 10 J/cm² (CL). After irradiation, sample groups were washed twice with PBS as described previously. Each resulting cell pellet was suspended in 60 μ L of sample diluent from the OxiSelect™ Protein Carbonylation Fluorimetric Assay (STA-307, Cell Biolabs Inc., USA). Cell disruption was achieved with an ultrasound cell homogenizer by pulsing cells for 5 s at 90 W of power (40 kHz, Sonifier SLPe, Soni-Tech, Brazil), followed by 5 min incubation on ice. Five cycles of pulsing-cooling were performed. Cell debris were removed by centrifugation (10,000 g, 4 °C, 5 min). A total

of 50 μL were used as input and protein carbonylation analysis was performed according to the manufacturer's instructions. The assay requires protein quantification to be performed at the end of the procedure to normalize carbonyl content (nmol/mL) to protein amount (mg/mL). This quantification was carried out with Bradford Assay (Bio-Rad, USA).

β -lactamase inhibition assay

3 control groups were used: a negative control (C $^-$) of viable cells suspended in PBS; a light control (CL) of cells only exposed to 10 J/cm 2 without MB; and a non-irradiated control (0) exposed only to methylene blue for 60 min. aPDT experimental groups were prepared as described in the "aPDT studies for planktonic suspensions" section. For this assay, samples were exposed to 2.5, 5, 7.5 or 10 J/cm 2 of aPDT. Following irradiation, each sample was immediately transferred to an Eppendorf tube that was kept in the dark at 0-4 $^\circ$ C. Then, the samples were washed twice with fresh PBS.

β -lactamase activity assay (ab197008, ABCAM, UK) was performed as instructed by the manual. Briefly, pellets were weighed and suspended in the kit's assay buffer with 5 μL per milligram of the pellet. Samples were sonicated for 5 min and centrifuged at 16,000 g at 4 $^\circ$ C for 20 min. Two- μL of supernatant were transferred into 96-well plates containing 96 μL of assay buffer and 2 μL of nitrocefin. Plates were immediately placed in a proper spectrophotometer (SpectraMax M4, Molecular Devices, USA) and absorbance at 490 nm was measured every minute for 60 min. Measurement time-points were determined by signal saturation of C $^-$. Absorbance data were normalized to C $^-$.

Membrane damage assays

To verify the membrane integrity after aPDT, quantitative methods using *N*-phenyl-1-naphthylamine (NPN, Sigma-Aldrich, USA) were employed. NPN becomes fluorescent when inserted into the internal face of cell membranes and, therefore, it signalizes variations in permeability of such structures. NPN is an especially interesting probe for this study since it does not present any fluorescence excitation and emission overlaps with MB.

For these experiments, there were 4 control groups: a negative control (C $^-$) of viable cells suspended in PBS; a positive membrane damage control (C $^+$) of cells

inactivated in 70% (v/v) propyl alcohol for 15 min; a light control (CL) of cells only exposed to 20 J/cm² without MB; and a non-irradiated control (0) exposed only to MB for 60 min. The aPDT experimental groups were prepared as described previously. For these membrane damage assays, samples were exposed to 2.5, 5, 10 or 20 J/cm². Following irradiation, samples were immediately transferred to Eppendorf tubes that were kept in the dark at 0-4° C. Then, samples were washed twice with fresh PBS before they were probed with NPN.

Sample sets were distributed in 96-well plates and NPN was added to reach a final concentration of 15 µM. Plates were then kept in the dark at room temperature for 15 min. Arbitrary fluorescence intensity was measured in a proper plate reader (SpectraMax M4, Molecular Devices, USA). NPN fluorescence was excited at 350 nm and measured at 415 nm.

Statistical analysis

Experiments were performed in technical and biological triplicates for all quantitative assays. Quantitative data are presented as means and standard deviation of means. Statistical analysis for quantifications of protein content, protein carbonylation, β-lactamase inhibition, and membrane permeability was evaluated by multiple unpaired T-tests with comparisons to C-. Statistical differences were considered significant if $p < 0.05$. Prism 6.0 (GraphPad, USA) was used for data analysis.

References

1. Dai T, Huang YY, Hamblin MR. Photodynamic therapy for localized infections--state of the art. Photodiagnosis and photodynamic therapy. 2009;6(3-4):170-88.
2. Kharkwal GB, Sharma SK, Huang YY, Dai T, Hamblin MR. Photodynamic therapy for infections: clinical applications. Lasers in surgery and medicine. 2011;43(7):755-67.
3. St Denis TG, Dai T, Izikson L, Astrakas C, Anderson RR, Hamblin MR, et al. All you need is light: antimicrobial photoinactivation as an evolving and emerging discovery strategy against infectious disease. Virulence. 2011;2(6):509-20.

4. Boucher HW, Talbot GH, Bradley JS, Edwards JE, Gilbert D, Rice LB, et al. Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*. 2009;48(1):1-12.
5. Maisch T, Hackbarth S, Regensburger J, Felgentrager A, Baumler W, Landthaler M, et al. Photodynamic inactivation of multi-resistant bacteria (PIB) - a new approach to treat superficial infections in the 21st century. *Journal der Deutschen Dermatologischen Gesellschaft = Journal of the German Society of Dermatology : JDDG*. 2011;9(5):360-6.
6. Organization WH. The evolving threat of antimicrobial resistance: Options for action [website]. WHO Publ.; 2014 [Available from: <http://www.ijmr.org.in/article.asp?issn=0971-5916;year=2014;volume=139;issue=1;spage=182;epage=183;aulast=Kapi>.
7. Laxminarayan R, Duse A, Wattal C, Zaidi AK, Wertheim HF, Sumpradit N, et al. Antibiotic resistance-the need for global solutions. *Lancet Infect Dis*. 2013;13(12):1057-98.
8. Willems RJ, Hanage WP, Bessen DE, Feil EJ. Population biology of Gram-positive pathogens: high-risk clones for dissemination of antibiotic resistance. *FEMS Microbiol Rev*. 2011;35(5):872-900.
9. Rice LB. Mechanisms of resistance and clinical relevance of resistance to beta-lactams, glycopeptides, and fluoroquinolones. *Mayo Clin Proc*. 2012;87(2):198-208.
10. Tzouveleakis LS, Markogiannakis A, Psychogiou M, Tassios PT, Daikos GL. Carbapenemases in *Klebsiella pneumoniae* and other Enterobacteriaceae: an evolving crisis of global dimensions. *Clin Microbiol Rev*. 2012;25(4):682-707.
11. Carattoli A. Plasmids and the spread of resistance. *Int J Med Microbiol*. 2013;303(6-7):298-304.
12. Potron A, Poirel L, Nordmann P. Emerging broad-spectrum resistance in *Pseudomonas aeruginosa* and *Acinetobacter baumannii*: Mechanisms and epidemiology. *Int J Antimicrob Agents*. 2015;45(6):568-85.
13. Wainwright M, Maisch T, Nonell S, Plaetzer K, Almeida A, Teges GP, et al. Photoantimicrobials-are we afraid of the light? *Lancet Infect Dis*. 2017;17(2):e49-e55.

14. Hamblin MR, Hasan T. Photodynamic therapy: a new antimicrobial approach to infectious disease? *Photochem Photobiol Sci.* 2004;3(5):436-50.
15. Wainwright M. Photodynamic antimicrobial chemotherapy (PACT). *The Journal of antimicrobial chemotherapy.* 1998;42(1):13-28.
16. Sabino CP, Wainwright M, Ribeiro MS, Sellera FP, Dos Anjos C, Baptista MDS, et al. Global priority multidrug-resistant pathogens do not resist photodynamic therapy. *J Photochem Photobiol B.* 2020;208:111893.
17. Sabino CP, Sellera FP, Sales-Medina DF, Machado RRG, Durigon EL, Freitas-Junior LH, et al. UV-C (254 nm) lethal doses for SARS-CoV-2. *Photodiagnosis Photodyn Ther.* 2020;32:101995.
18. Dos Anjos C, Sellera FP, Gargano RG, Lincopan N, Pogliani FC, Ribeiro MG, et al. Algicidal effect of blue light on pathogenic *Prototheca* species. *Photodiagnosis Photodyn Ther.* 2019;26:210-3.
19. Cabral FV, Sabino CP, Dimmer JA, Sauter IP, Cortez MJ, Ribeiro MS. Preclinical Investigation of Methylene Blue-mediated Antimicrobial Photodynamic Therapy on Leishmania Parasites Using Real-Time Bioluminescence. *Photochem Photobiol.* 2020;96(3):604-10.
20. Baptista MS, Cadet J, Di Mascio P, Ghogare AA, Greer A, Hamblin MR, et al. Type I and Type II Photosensitized Oxidation Reactions: Guidelines and Mechanistic Pathways. *Photochem Photobiol.* 2017;93(4):912-9.
21. Bacellar IO, Pavani C, Sales EM, Itri R, Wainwright M, Baptista MS. Membrane damage efficiency of phenothiazinium photosensitizers. *Photochemistry and photobiology.* 2014;90(4):801-13.
22. Pattison DI, Rahmanto AS, Davies MJ. Photo-oxidation of proteins. *Photochem Photobiol Sci.* 2012;11(1):38-53.
23. Davies MJ. Singlet oxygen-mediated damage to proteins and its consequences. *Biochem Biophys Res Commun.* 2003;305(3):761-70.
24. Wang J, Wang S, Zhong C, Tian T, Zhou X. Novel insights into a major DNA oxidative lesion: its effects on Z-DNA stabilization. *Org Biomol Chem.* 2015;13(34):8996-9.
25. Nogueira JJ, Oppel M, Gonzalez L. Enhancing intersystem crossing in phenothiazinium dyes by intercalation into DNA. *Angew Chem Int Ed Engl.* 2015;54(14):4375-8.

26. Rohs R, Sklenar H. Methylene blue binding to DNA with alternating GC base sequence: continuum treatment of salt effects. *Indian J Biochem Biophys.* 2001;38(1-2):1-6.
27. Rohs R, Sklenar H. Methylene blue binding to DNA with alternating AT base sequence: minor groove binding is favored over intercalation. *J Biomol Struct Dyn.* 2004;21(5):699-711.
28. Nogueira JJ, Gonzalez L. Molecular dynamics simulations of binding modes between methylene blue and DNA with alternating GC and AT sequences. *Biochemistry.* 2014;53(14):2391-412.
29. Agnez-Lima LF, Melo JT, Silva AE, Oliveira AH, Timoteo AR, Lima-Bessa KM, et al. DNA damage by singlet oxygen and cellular protective mechanisms. *Mutat Res.* 2012.
30. Davies MJ. Protein oxidation and peroxidation. *Biochem J.* 2016;473(7):805-25.
31. Liu F, Lu W, Fang Y, Liu J. Evolution of oxidation dynamics of histidine: non-reactivity in the gas phase, peroxides in hydrated clusters, and pH dependence in solution. *Phys Chem Chem Phys.* 2014;16(40):22179-91.
32. Shen HR, Spikes JD, Kopecková P, Kopecek J. Photodynamic crosslinking of proteins. II. Photocrosslinking of a model protein-ribonuclease A. *J Photochem Photobiol B.* 1996;35(3):213-9.
33. Wright A, Hawkins CL, Davies MJ. Photo-oxidation of cells generates long-lived intracellular protein peroxides. *Free Radic Biol Med.* 2003;34(6):637-47.
34. Gomyo T, Fujimaki M. Studies on Changes of Protein by Dye Sensitized Photooxidation. *Agricultural and Biological Chemistry.* 1970;34(2):7.
35. WHO. Antimicrobial resistance: global report on surveillance [Global Report on Surveillance]. Geneva: World Health Organization; 2014.
36. Sabino CP, Wainwright M, Dos Anjos C, Sellera FP, Baptista MS, Lincopan N, et al. Inactivation Kinetics and Lethal Dose Analysis of Antimicrobial Blue Light and Photodynamic Therapy. *Photodiagnosis Photodyn Ther.* 2019.
37. Jett BD, Hatter KL, Huycke MM, Gilmore MS. Simplified agar plate method for quantifying viable bacteria. *Biotechniques.* 1997;23(4):648-50.
38. Oliveira CS, Turchiello R, Kowaltowski AJ, Indig GL, Baptista MS. Major determinants of photoinduced cell death: Subcellular localization versus photosensitization efficiency. *Free radical biology & medicine.* 2011;51(4):824-33.

39. Laval J. Role of DNA repair enzymes in the cellular resistance to oxidative stress. *Pathol Biol (Paris)*. 1996;44(1):14-24.
40. Chapman PA, Ellin M, Ashton R, Shafique W. Comparison of culture, PCR and immunoassays for detecting *Escherichia coli* O157 following enrichment culture and immunomagnetic separation performed on naturally contaminated raw meat products. *Int J Food Microbiol*. 2001;68(1-2):11-20.
41. Woodford N. Rapid characterization of beta-lactamases by multiplex PCR. *Methods Mol Biol*. 2010;642:181-92.
42. Lincopan N, McCulloch JA, Reinert C, Cassettari VC, Gales AC, Mamizuka EM. First isolation of metallo-beta-lactamase-producing multiresistant *Klebsiella pneumoniae* from a patient in Brazil. *J Clin Microbiol*. 2005;43(1):516-9.
43. Oliveira S, Moura RA, Silva KC, Pavez M, McCulloch JA, Dropa M, et al. Isolation of KPC-2-producing *Klebsiella pneumoniae* strains belonging to the high-risk multiresistant clonal complex 11 (ST437 and ST340) in urban rivers. *J Antimicrob Chemother*. 2014;69(3):849-52.

Supplementary Material

Supplementary Figure 1. Spectral characteristics of the LED system and methylene blue. (a) Spectral emission of LED system and absorption of MB. (b) Spectrum resolved light absorption of our irradiation system.

Supplementary Figure 2. Singlet oxygen direct detection by phosphorescence emission. (a) Phosphorescence spectrum of singlet oxygen in PBS solution prepared with D₂O with or without bacterial inoculum presented peak emission at 1277 nm and (b) lifetime of $45.2 \pm 0.5 \mu$ s.

3.2. Scientific awards, book chapters, patents and secondary articles

Besides the main publications mentioned in the previous section, 17 other research articles, 10 book chapters, 4 patents and 4 scientific awards were obtained during the period of this study (2016–2021).

3.2.1. List of Secondary Articles

1. **Sabino, Caetano P.**; Deana, Alessandro M. ; Yoshimura, Tania M. ; Da Silva, Daniela F.T. ; França, Cristiane M. ; Hamblin, Michael R. ; Ribeiro, Martha S. . **The optical properties of mouse skin in the visible and near infrared spectral regions.** *Journal of Photochemistry and Photobiology B-Biology*, v. 160, p. 72-78, 2016.
2. Yoshimura, Tania M.; **Sabino, Caetano P.**; Ribeiro, Martha S. . **Photobiomodulation reduces abdominal adipose tissue inflammatory infiltrate of diet-induced obese and hyperglycemic mice.** *Journal of Biophotonics*, v. 1, p. 1-1, 2016.
3. Baptista, Alessandra; **Sabino, Caetano P.**; Núñez, Silvia C.; Miyakawa, Walter; Martin, Airton A.; Ribeiro, Martha S. . **Photodynamic damage predominates on different targets depending on cell growth phase of *Candida albicans*.** *Journal of Photochemistry and Photobiology B-Biology*, v. 1, p. 01-10, 2017.
4. Esposito, Fernanda ; Fernandes, Miriam R. ; Lopes, Ralf ; Muñoz, Maria ; **Sabino, Caetano P.** ; Cunha, Marcos P. ; Silva, Ketrin C. ; Cayô, Rodrigo ; Martins, Willames M. ; Moreno, Andrea M. ; Knöbl, Terezinha ; Gales, Ana C. ; Lincopan, Nilton . **Detection of Colistin-Resistant MCR-1-Positive *Escherichia coli* Using Inhibition by EDTA and Zeta Potential Assays.** *Journal of Clinical Microbiology*, v. 1, p. JCM.00835-17-385, 2017.
5. Suzuki, Luis C.; Kato, Ilka T.; Prates, Renato A.; **Sabino, Caetano P.**; Yoshimura, Tania M.; Silva, Tamires O.; Ribeiro, Martha S. . **Glucose modulates antimicrobial photodynamic inactivation of *Candida albicans* in biofilms.** *Photodiagnosis and Photodynamic Therapy*, v. 17, p. 173-179, 2017.
6. Fernandes, Miriam R. ; Sellera, Fábio P. ; Esposito, Fernanda ; **Sabino, Caetano P.** ; Cerdeira, Louise ; Lincopan, Nilton . **Colistin-Resistant mcr-1 -**

- Positive *Escherichia coli* in Public Beaches, an Infectious Threat Emerging in Recreational Waters.** *Antimicrobial Agents and Chemotherapy*, v. 1, p. AAC.00234-17-2, 2017.
7. Ribeiro, M. S.; Gargano, Ronaldo G.; Dos Anjos, Carolina; Pogliani, Fabio C.; Sellera, Fábio P.; **Sabino, Caetano P.. Clinical challenges of antimicrobial photodynamic therapy for bovine mastitis.** *Photodiagnosis and Photodynamic Therapy*, v. 21, p. 327-327, 2018.
 8. Dos Anjos, Carolina ; **Sabino, Caetano P.** ; Bueris, Vanessa ; Fernandes, Miriam R. ; Pogliani, Fabio C. ; Lincopan, Nilton ; Sellera, Fábio P. . **Antimicrobial blue light inactivation of international clones of multidrug-resistant *Escherichia coli* ST10, ST131 and ST648.** *Photodiagnosis and Photodynamic Therapy*, v. 1, p. 1, 2019.
 9. Dos Anjos, Carolina ; Sellera, Fábio P.; Gargano, Ronaldo G.; Lincopan, Nilton ; Pogliani, Fabio C.; Ribeiro, Marcio G.; Jagielski, Tomasz ; **Sabino, Caetano P.. Algicidal effect of blue light on pathogenic *Prototheca* species.** *Photodiagnosis and Photodynamic Therapy*, v. 26, p. 210-213, 2019.
 10. Dimmer, Jesica ; Cabral, Fernanda V. ; **Sabino, Caetano P.**; Silva, Camila Ramos ; Núñez-Montoya, Susana C. ; Cabrera, José Luis ; Ribeiro, Martha S. . **Natural anthraquinones as novel photosensitizers for antiparasitic photodynamic inactivation.** *Phytomedicine*, v. 1, p. 152894, 2019.
 11. Sellera, Fábio P. ; Fernandes, Miriam R. ; **Sabino, Caetano P.** ; De Freitas, Laura M. ; Da Silva, Luciano C.B.A. ; Pogliani, Fabio C. ; Ribeiro, Martha S. ; Hamblin, Michael R. ; Lincopan, Nilton . **Effective treatment and decolonization of a dog infected with carbapenemase (VIM-2)-producing *Pseudomonas aeruginosa* using probiotic and photodynamic therapies.** *Veterinary Dermatology*, v. 1, p. 1-5, 2019.
 12. Dos Anjos, C. ; Sellera, F.P. ; De Freitas, L.M. ; Gargano, R.G. ; Telles, E.O. ; Freitas, R.O. ; Baptista, M.S. ; Ribeiro, M.S. ; Lincopan, N. ; Pogliani, F.C. ; **Sabino, C.P. . Inactivation of milk-borne pathogens by blue light exposure.** *Journal of Dairy Science*, v. 1, p. 1-8, 2019.
 13. Fernandes, Miriam R. ; Sellera, Fábio P. ; Moura, Quêzia ; Esposito, Fernanda ; **Sabino, Caetano P.** ; Lincopan, Nilton . **Identification and genomic features of halotolerant extended-spectrum- β -lactamase (CTX-M)-producing**

- Escherichia coli in urban-impacted coastal waters, Southeast Brazil.** *Marine Pollution Bulletin*, v. 150, p. 110689-110693, 2019.
14. **Sabino, Caetano P.**; Ball, Anthony R. ; Baptista, Mauricio S. ; Dai, Tianhong ; Hamblin, Michael R. ; Ribeiro, Martha S. ; Santos, Ana L. ; Sellera, Fábio P. ; Tegos, George P. ; Wainwright, Mark . **Light-based technologies for management of COVID-19 pandemic crisis.** *Journal of Photochemistry And Photobiology B-Biology*, v. 1, p. 111999, 2020.
 15. **Sabino, Caetano P.**; Sellera, Fábio P. ; Sales-Medina, Douglas F. ; Machado, Rafael Rahal Guaragna ; Durigon, Edison Luiz ; Freitas-Junior, Lucio H. ; Ribeiro, Martha S. . **UV-C (254-nm) lethal doses for SARS-CoV-2.** *Photodiagnosis and Photodynamic Therapy*, v. 32, p. 101995, 2020.
 16. Cabral, Fernanda V. ; **Sabino, Caetano P.** ; Dimmer, Jesica A. ; Sauter, Ismael P. ; Cortez, Mauro J. ; Ribeiro, Martha S. . **Preclinical Investigation of Methylene Blue-mediated Antimicrobial Photodynamic Therapy on Leishmania Parasites Using Real-Time Bioluminescence.** *Photochemistry and Photobiology*, v. 96, p. 604-610, 2020.
 17. Dos Anjos, Carolina ; **Sabino, Caetano P.** ; Sellera, Fábio P. ; Esposito, Fernanda ; Pogliani, Fabio C. ; Lincopan, Nilton . **Hypervirulent and hypermucoviscous strains of Klebsiella pneumoniae challenged by antimicrobial strategies using visible light.** *International Journal of Antimicrobial Agents*, v. 1, p. 106025, 2020.

3.2.2. List of Patents

1. **Sabino, C. P.**; Nogueira, A. P. ; Vidal, J. T. **Método E Aparelho Para Desinfecção De Máscaras De Proteção Respiratória.** 2020, Brasil. Patente: Privilégio de Inovação. Número do registro: BR10202000955, Instituição de registro: INPI - Instituto Nacional da Propriedade Industrial. Depósito: 13/05/2020
2. **Sabino, C.P.**; Mosca, R. C. ; Nogueira, A. P. ; Vidal, J. T. ; Tardivo, J. P. . **Dispositivo Emissor se Luz para Visualização de Substâncias Naturalmente Fluorescentes Presentes na Pele e Seus Anexos.** 2019, Brasil. Patente: Privilégio de Inovação. Número do registro:

BR1020190212624, Instituição de registro: *INPI - Instituto Nacional da Propriedade Industrial*. Depósito: 22/07/2019

3. **Sabino, C. P.**; Sellera, F. ; Anjos, C. ; Nogueira, A. P. ; Vidal, J. T. ; Freitas, R. O. . **Método e Sistemas para Inativação de Microrganismos em Fluidos Usando Radiação Visível**. 2019, Brasil. Patente: Privilégio de Inovação. Número do registro: BR10201901740, Instituição de registro: *INPI - Instituto Nacional da Propriedade Industrial*. Depósito: 21/08/2019
4. Mosca, R. C. ; **Sabino, Caetano P** ; Nogueira, A. P. ; Vidal, J. T. **Dispositivo Emissor De Luz Intraoral Conformável À Cavidade Oral**. 2019, Brasil. Patente: Privilégio de Inovação. Número do registro: BR10201901611, Instituição de registro: *INPI - Instituto Nacional da Propriedade Industrial*. Depósito: 02/08/2019

3.2.3. List of Book chapters

1. Ribeiro, Martha S. ; **Sabino, Caetano P**. **Dosimetria na Terapia Fotodinâmica Antimicrobiana**. In: Núñez SC, Ribeiro MS, Garcez AS. (Org.). *Terapia fotodinâmica antimicrobiana na Odontologia*. 2ed. Rio de Janeiro: Elsevier, 2019, p. 23-32.
2. **Sabino, Caetano P.**; Hamblin, Michael R . **Photophysical and Photochemical Mechanisms**. In: Fabio P. Sellera; Cristiane L. Nascimento; Martha S. Ribeiro. (Org.). *Photodynamic Therapy in Veterinary Medicine: From Basics to Clinical Practice*. 1ed.Cham: Springer, 2016, v. 1, p. 11-24.
3. **Sabino, Caetano P.**; Hamblin, Michael R . **Molecular Damage**. In: Fabio P. Sellera; Cristiane L. Nascimento; Martha S. Ribeiro. (Org.). *Photodynamic Therapy in Veterinary Medicine: From Basics to Clinical Practice*. 1ed.Cham: Springer, 2016, v. 1, p. 46-56.
4. **Sabino, Caetano P.**; Hamblin, Michael R . **Cellular Damage**. In: Fabio P. Sellera; Cristiane L. Nascimento; Martha S. Ribeiro. (Org.). *Photodynamic Therapy in Veterinary Medicine: From Basics to Clinical Practice*. 1ed.Cham: Springer, 2016, v. 1, p. 57-72.
5. Sellera, F. ; **Sabino, Caetano P.**; Hamblin, M. R. . **History of Photodynamic Therapy**. In: Fabio P. Sellera; Cristiane L. Nascimento; Martha S. Ribeiro.

- (Org.). *Photodynamic Therapy in Veterinary Medicine: From Basics to Clinical Practice*. 1ed.Cham: Springer, 2016, v. 1, p. 1-10.
6. Hamblin, Michael R ; **Sabino, Caetano P. . Photosensitizers**. In: Fabio P. Sellera; Cristiane L. Nascimento; Martha S. Ribeiro. (Org.). *Photodynamic Therapy in Veterinary Medicine: From Basics to Clinical Practice*. 1ed.Cham: Springer, 2016, v. 1, p. 25-44.
 7. Ribeiro, Martha S. ; **Sabino, Caetano P. . Multimodality Dosimetry**. In: Fabio P. Sellera; Cristiane L. Nascimento; Martha S. Ribeiro. (Org.). *Photodynamic Therapy in Veterinary Medicine: From Basics to Clinical Practice*. 1ed.Cham: Springer, 2016, v. 1, p. 93-110.
 8. Sellera, Fábio P. ; Pogliani, Fábio C. ; **Sabino, Caetano P. . Other Practices in PDT**. In: Fabio P. Sellera; Cristiane L. Nascimento; Martha S. Ribeiro. (Org.). *Photodynamic Therapy in Veterinary Medicine: From Basics to Clinical Practice*. 1ed.Cham: Springer, 2016, v. 1, p. 197-208.
 9. Sellera, Fábio P.; Nascimento, Cristiane L.; Pogliani, F. ; **Sabino, Caetano P. ; Ribeiro, Martha S. . Future Perspectives**. In: Fabio P. Sellera; Cristiane L. Nascimento; Martha S. Ribeiro. (Org.). *Photodynamic Therapy in Veterinary Medicine: From Basics to Clinical Practice*. 1ed.Cham: Springer, 2016, v. 1, p. 209-222.
 10. Hamblin, Michael R ; **Sabino, Caetano P. . Systemic Effects**. In: Fabio P. Sellera; Cristiane L. Nascimento; Martha S. Ribeiro. (Org.). *Photodynamic Therapy in Veterinary Medicine: From Basics to Clinical Practice*. 1ed.Cham: Springer, 2016, v. 1, p. 73-92.

3.2.4. List of scientific awards

1. **IPA Student and Post-Doctoral Award**, *International Photodynamic Association (IPA)*, USA, 2019.
2. **Leaders in Innovation Fellowships**, *Newton Fund, Royal Academy of Engineering*, UK, 2019.
3. **Gold Medal - University of São Paulo Team**, *International Genetically Engineered Machine Competition (iGEM)*, USA, 2019.

4. **ASM Student and Post-Doctoral Award**, *American Society for Microbiology (ASM)*, USA, 2017.

4. General conclusions

Antimicrobial photodynamic inactivation offers a powerful strategy to challenge microbial resistance of local infections. We first proved that a simple mathematical model can provide accurate information regarding photoinactivation kinetics profiles (Article 1). Then, the mathematical method was used to compare the inactivation kinetics of drug-resistant bacteria and yeast in relation to their drug-sensitive counterparts. This later paper proved that there is no correlation in between photosensitivity and drug resistance profiles, whereas APDT inactivation kinetics is strongly correlated with microbial species but not strain. Afterwards we showed that APDT inhibits β -lactamase activity, including ESBL and carbapenemases, giving the possibility of drug-resistance inhibition and suggesting synergistic combinations of MB-APDT and β -lactam antibiotic therapy. Finally, we demonstrated the biochemical mechanisms of MB-APDT that have remained as mystery for nearly one century. We proved that the major cause of bacterial inactivation by MB-APDT is primarily associated with protein damage while membrane and DNA damages are secondary effects. All together, these facts suggest that MB-APDT can effectively inactivate drug-resistant microorganisms and simultaneously block drug-resistance expression, transmission and dissemination.

5. Future perspectives

In 2017, I founded a startup company named BioLambda to develop specialized light sources for scientific, clinical and industrial applications. So far, we have developed more than 5 scientific equipment models that are already performing experiments for researchers based in Brazil, USA, Portugal, France, South Africa, Japan and Australia.

We have also developed a clinical equipment, with a stage 1 PIPE-FAPESP grant, to treat localized infections using APDT. It has unique capacities to perform APDT and photobiomodulation on body surfaces that are up to 1,000 cm² at once, with automatic dosimetry calibration based on distance sensors. This equipment will be

used in a clinical trial – organized by Dr. João Paulo Tardivo – aiming to prevent amputations of more than 300 patients suffering from diabetic foot disease.

In the beginning of COVID-19 pandemics, we also developed 3 series of biosafety equipments that use ultraviolet C light to disinfect 1) surfaces, 2) air and 3) N95 masks. We even won a challenge promoted by Vale do Rio Doce and could donate 17 equipments to public hospitals. Such equipments and other customized industrial systems have also been commercialized to large companies such as, Colgate-Palmolive, Embraer, Hospital Albert Einstein, O Boticário, JBS, BRF and others.

On the academic side, I intend to keep interactions with students and other researchers as a regular practice to perhaps help others to pursue a scientific entrepreneur career. I also intend to keep publishing original research, patents and books of discoveries that come from interactions in between universities, research institutes and companies.

Over the next years I'll focus my energy into bringing Biophotonics to our society, either as a teacher, advisor or inventor. My ultimate goal will always be to bring our science and technology to the people who need it.

References

1. Boucher HW, Talbot GH, Bradley JS, Edwards JE, Gilbert D, Rice LB, et al. Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*. 2009;48(1):1-12.
2. Maisch T, Hackbarth S, Regensburger J, Felgentrager A, Baumler W, Landthaler M, et al. Photodynamic inactivation of multi-resistant bacteria (PIB) - a new approach to treat superficial infections in the 21st century. *Journal der Deutschen Dermatologischen Gesellschaft = Journal of the German Society of Dermatology : JDDG*. 2011;9(5):360-6.
3. Organization WH. The evolving threat of antimicrobial resistance: Options for action [website]. WHO Publ.; 2014 [Available from: <http://www.ijmr.org.in/article.asp?issn=0971-5916;year=2014;volume=139;issue=1;spage=182;epage=183;aulast=Kapi>].

4. Laxminarayan R, Duse A, Wattal C, Zaidi AK, Wertheim HF, Sumpradit N, et al. Antibiotic resistance-the need for global solutions. *Lancet Infect Dis*. 2013;13(12):1057-98.
5. Woolhouse M, Farrar J. Policy: An intergovernmental panel on antimicrobial resistance. *Nature*. 2014;509(7502):555-7.
6. Verhoef TJ, Morris S. Cost-effectiveness and pricing of antibacterial drugs. *Chem Biol Drug Des*. 2015;85(1):4-13.
7. Ciorba V, Odone A, Veronesi L, Pasquarella C, Signorelli C. Antibiotic resistance as a major public health concern: epidemiology and economic impact. *Ann Ig*. 2015;27(3):562-79.
8. Fair RJ, Tor Y. Antibiotics and bacterial resistance in the 21st century. *Perspect Medicin Chem*. 2014;6:25-64.
9. Pendleton JN, Gorman SP, Gilmore BF. Clinical relevance of the ESKAPE pathogens. *Expert Rev Anti Infect Ther*. 2013;11(3):297-308.
10. Rice LB. Federal funding for the study of antimicrobial resistance in nosocomial pathogens: no ESKAPE. *J Infect Dis*. 2008;197(8):1079-81.
11. Rice LB. Progress and challenges in implementing the research on ESKAPE pathogens. *Infect Control Hosp Epidemiol*. 2010;31 Suppl 1:S7-10.
12. Rice LB. Mechanisms of resistance and clinical relevance of resistance to beta-lactams, glycopeptides, and fluoroquinolones. *Mayo Clin Proc*. 2012;87(2):198-208.
13. Rossi F. The challenges of antimicrobial resistance in Brazil. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*. 2011;52(9):1138-43.
14. Nawar T, Kanafani ZA. Telavancin (VIBATIV) for the treatment of complicated skin and skin structure infections. *Expert Rev Anti Infect Ther*. 2015;13(7):825-33.
15. Willems RJ, Hanage WP, Bessen DE, Feil EJ. Population biology of Gram-positive pathogens: high-risk clones for dissemination of antibiotic resistance. *FEMS Microbiol Rev*. 2011;35(5):872-900.
16. Lindenstrauss AG, Pavlovic M, Bringmann A, Behr J, Ehrmann MA, Vogel RF. Comparison of genotypic and phenotypic cluster analyses of virulence determinants and possible role of CRISPR elements towards their incidence in *Enterococcus faecalis* and *Enterococcus faecium*. *Syst Appl Microbiol*. 2011;34(8):553-60.

17. Keen EF, 3rd, Robinson BJ, Hospenthal DR, Aldous WK, Wolf SE, Chung KK, et al. Prevalence of multidrug-resistant organisms recovered at a military burn center. *Burns*. 2010;36(6):819-25.
18. Rice LB. Unmet medical needs in antibacterial therapy. *Biochemical pharmacology*. 2006;71(7):991-5.
19. Tzouvelekis LS, Markogiannakis A, Psychogiou M, Tassios PT, Daikos GL. Carbapenemases in *Klebsiella pneumoniae* and other *Enterobacteriaceae*: an evolving crisis of global dimensions. *Clin Microbiol Rev*. 2012;25(4):682-707.
20. Carattoli A. Plasmids and the spread of resistance. *Int J Med Microbiol*. 2013;303(6-7):298-304.
21. Potron A, Poirel L, Nordmann P. Emerging broad-spectrum resistance in *Pseudomonas aeruginosa* and *Acinetobacter baumannii*: Mechanisms and epidemiology. *Int J Antimicrob Agents*. 2015;45(6):568-85.
22. Chagas TP, Seki LM, da Silva DM, Asensi MD. Occurrence of KPC-2-producing *Klebsiella pneumoniae* strains in hospital wastewater. *J Hosp Infect*. 2011;77(3):281.
23. Montezzi LF, Campana EH, Correa LL, Justo LH, Paschoal RP, da Silva IL, et al. Occurrence of carbapenemase-producing bacteria in coastal recreational waters. *Int J Antimicrob Agents*. 2015;45(2):174-7.
24. Picao RC, Cardoso JP, Campana EH, Nicoletti AG, Petrolini FV, Assis DM, et al. The route of antimicrobial resistance from the hospital effluent to the environment: focus on the occurrence of KPC-producing *Aeromonas* spp. and *Enterobacteriaceae* in sewage. *Diagn Microbiol Infect Dis*. 2013;76(1):80-5.
25. Oliveira S, Moura RA, Silva KC, Pavez M, McCulloch JA, Dropa M, et al. Isolation of KPC-2-producing *Klebsiella pneumoniae* strains belonging to the high-risk multiresistant clonal complex 11 (ST437 and ST340) in urban rivers. *The Journal of antimicrobial chemotherapy*. 2014;69(3):849-52.
26. Martins N, Ferreira IC, Barros L, Silva S, Henriques M. Candidiasis: predisposing factors, prevention, diagnosis and alternative treatment. *Mycopathologia*. 2014;177(5-6):223-40.
27. Gullo A. Invasive fungal infections: the challenge continues. *Drugs*. 2009;69 Suppl 1:65-73.
28. Xie JL, Polvi EJ, Shekhar-Guturja T, Cowen LE. Elucidating drug resistance in human fungal pathogens. *Future Microbiol*. 2014;9(4):523-42.

29. Agostinis P, Berg K, Cengel KA, Foster TH, Girotti AW, Gollnick SO, et al. Photodynamic therapy of cancer: an update. *CA Cancer J Clin*. 2011;61(4):250-81.
30. Wainwright M. Photodynamic antimicrobial chemotherapy (PACT). *The Journal of antimicrobial chemotherapy*. 1998;42(1):13-28.
31. Wainwright M, Maisch T, Nonell S, Plaetzer K, Almeida A, Tegos GP, et al. Photoantimicrobials-are we afraid of the light? *Lancet Infect Dis*. 2017;17(2):e49-e55.
32. Dai T, Huang YY, Hamblin MR. Photodynamic therapy for localized infections--state of the art. *Photodiagnosis and photodynamic therapy*. 2009;6(3-4):170-88.
33. Lyon JP, Moreira LM, de Moraes PC, dos Santos FV, de Resende MA. Photodynamic therapy for pathogenic fungi. *Mycoses*. 2011;54(5):e265-71.
34. Schmidt R. Photosensitized Generation of Singlet Oxygen. *Photochemistry and photobiology*. 2007;82(5):1161-77.
35. Sabino CP, Wainwright M, Ribeiro MS, Sellera FP, Dos Anjos C, Baptista MDS, et al. Global priority multidrug-resistant pathogens do not resist photodynamic therapy. *J Photochem Photobiol B*. 2020;208:111893.
36. Cabral FV, Sabino CP, Dimmer JA, Sauter IP, Cortez MJ, Ribeiro MS. Preclinical Investigation of Methylene Blue-mediated Antimicrobial Photodynamic Therapy on Leishmania Parasites Using Real-Time Bioluminescence. *Photochem Photobiol*. 2020;96(3):604-10.
37. Hamblin MR, Hasan T. Photodynamic therapy: a new antimicrobial approach to infectious disease? *Photochemical & photobiological sciences : Official journal of the European Photochemistry Association and the European Society for Photobiology*. 2004;3(5):436-50.
38. Lei W, Zhou Q, Jiang G, Zhang B, Wang X. Photodynamic inactivation of *Escherichia coli* by Ru(II) complexes. *Photochemical & photobiological sciences : Official journal of the European Photochemistry Association and the European Society for Photobiology*. 2011;10(6):887-90.
39. Sabino CP, Ball AR, Baptista MS, Dai T, Hamblin MR, Ribeiro MS, et al. Light-based technologies for management of COVID-19 pandemic crisis. *J Photochem Photobiol B*. 2020;212:111999.
40. Sellera FbP, Nascimento CLI, Ribeiro MSe, Sabino CP, Hamblin MR, Buchholz J, et al. *Photodynamic Therapy in Veterinary Medicine: From Basics to Clinical Practice*. 1st ed. Gewerbestrasse 11, 6330 Cham, Switzerland: Springer Nature; 2016. 228 p.

41. Sayed Z, Harris F, Phoenix DA. A study on the bacterial photo-toxicity of phenothiazinium based photosensitisers. *FEMS immunology and medical microbiology*. 2005;43(3):367-72.
42. Ziegelhoffer EC, Donohue TJ. Bacterial responses to photo-oxidative stress. *Nature reviews Microbiology*. 2009;7(12):856-63.
43. Prasad PS, Schwartz SD, Hubschman JP. Age-related macular degeneration: current and novel therapies. *Maturitas*. 2010;66(1):46-50.
44. Kharkwal GB, Sharma SK, Huang YY, Dai T, Hamblin MR. Photodynamic therapy for infections: clinical applications. *Lasers in surgery and medicine*. 2011;43(7):755-67.
45. St Denis TG, Dai T, Izikson L, Astrakas C, Anderson RR, Hamblin MR, et al. All you need is light: antimicrobial photoinactivation as an evolving and emerging discovery strategy against infectious disease. *Virulence*. 2011;2(6):509-20.
46. Tardivo JP, Wainwright M, Baptista MS. Local clinical phototreatment of herpes infection in Sao Paulo. *Photodiagnosis Photodyn Ther*. 2012;9(2):118-21.
47. Tardivo JP, Wainwright M, Baptista M. Small scale trial of photodynamic treatment of onychomycosis in Sao Paulo. *J Photochem Photobiol B*. 2015;150:66-8.
48. Fimple JL, Fontana CR, Foschi F, Ruggiero K, Song X, Pagonis TC, et al. Photodynamic treatment of endodontic polymicrobial infection in vitro. *Journal of endodontics*. 2008;34(6):728-34.
49. Garcez AS, Ribeiro MS, Tegos GP, Nunez SC, Jorge AO, Hamblin MR. Antimicrobial photodynamic therapy combined with conventional endodontic treatment to eliminate root canal biofilm infection. *Lasers in surgery and medicine*. 2007;39(1):59-66.
50. Sabino CP, Garcez AS, Nunez SC, Ribeiro MS, Hamblin MR. Real-time evaluation of two light delivery systems for photodynamic disinfection of *Candida albicans* biofilm in curved root canals. *Lasers Med Sci*. 2014.
51. Baptista A, Kato IT, Prates RA, Suzuki LC, Raele MP, Freitas AZ, et al. Antimicrobial photodynamic therapy as a strategy to arrest enamel demineralization: a short-term study on incipient caries in a rat model. *Photochemistry and photobiology*. 2012;88(3):584-9.
52. Fontana CR, Abernethy AD, Som S, Ruggiero K, Doucette S, Marcantonio RC, et al. The antibacterial effect of photodynamic therapy in dental plaque-derived biofilms. *Journal of periodontal research*. 2009;44(6):751-9.

53. Tardivo JP, Del Giglio A, de Oliveira CS, Gabrielli DS, Junqueira HC, Tada DB, et al. Methylene blue in photodynamic therapy: From basic mechanisms to clinical applications. *Photodiagnosis and Photodynamic Therapy*. 2005;2(3):175-91.
54. Tardivo JP, Baptista MS. Treatment of osteomyelitis in the feet of diabetic patients by photodynamic antimicrobial chemotherapy. *Photomed Laser Surg*. 2009;27(1):145-50.
55. Arenas Y, Monro S, Shi G, Mandel A, McFarland S, Lilge L. Photodynamic inactivation of *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* with Ru(II)-based type I/type II photosensitizers. *Photodiagnosis and photodynamic therapy*. 2013;10(4):615-25.
56. Rodrigues GB, Dias-Baruffi M, Holman N, Wainwright M, Braga GU. In vitro photodynamic inactivation of *Candida* species and mouse fibroblasts with phenothiazinium photosensitisers and red light. *Photodiagnosis and photodynamic therapy*. 2013;10(2):141-9.
57. Komerik N, Wilson M, Poole S. The effect of photodynamic action on two virulence factors of gram-negative bacteria. *Photochemistry and photobiology*. 2000;72(5):676-80.
58. Hamblin MR, Zahra T, Contag CH, McManus AT, Hasan T. Optical monitoring and treatment of potentially lethal wound infections in vivo. *J Infect Dis*. 2003;187(11):1717-25.