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

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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 5$\log_{10}$ 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

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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/

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 ($^3PS^*$) 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 an UV-VIS spectrophotometer (Flame, Ocean Optics, USA). Light irradiance was adjusted to a standard irradiance of 100 mW/cm^2 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%.

Table 1
List of microbial strains used in this study.

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

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 1–2 × 10^6 CFU/mL of fungal cells and 1–2 × 10^8 CFU/mL of bacterial cells, respectively. Inocula were then diluted to a working concentration of 1–2 × 10^5 CFU/mL of fungi or 1–2 × 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/cm^2 steps (*A. baumannii, E. faecalis, E. faecium, E. coli* and *S. aureus*) or 5 J/cm^2 steps (*K. aerogenes, K. pneumoniae, P. aeruginosa, C. albicans* and *C. neoformans*). Radiant exposure was calculated as the product of irradiance (W/cm^2) 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 $\log_{10}$ 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 $\log_{10}$ 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]. LD_{90} 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.

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

$$LD_i = LD_{90}\left(-log_{10}\left(1 - \frac{i}{100}\right)\right)^{1/T} \quad \textbf{(2)}$$

where:

N_0 = initial microbial burden; N = final microbial burden; *Dose* = light exposure (*e.g.* J, J/cm^2, time units, *Absorbed Photons*/cm^3, *etc.*); LD_{90} = lethal dose for 90% of microbial burden (in light exposure units); T = tolerance factor; i = inactivation percentage (%).

Statistical differences of all LD_{90}, $LD_{99.9}$, $LD_{99.999}$ 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, ICBDVIM2). 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, LD_{90} 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 > 5$\log_{10}$ of burden reduction for radiant exposures greater than 40 J/cm^2 (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/cm^2 of irradiation at 100 mW/cm^2 (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 LD_{90}, $LD_{99.9}$ 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 LD_{90}. 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 (LD_{90}). 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 ~ 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: LD_{90} 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 LD_{90}; 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

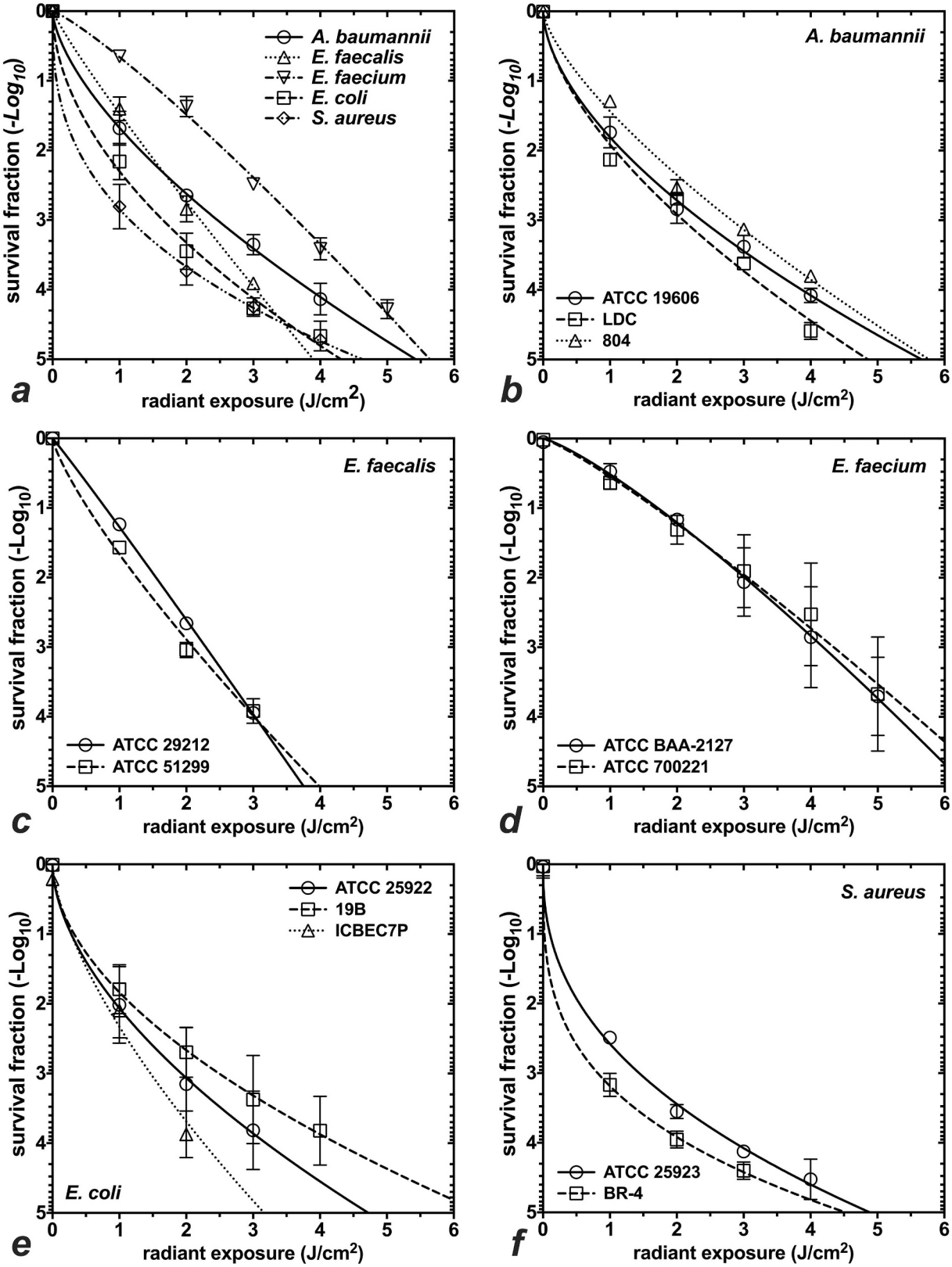

Fig. 1. Inactivation kinetics of bacterial species most sensitive to MB-aPDT (scale 1) in function of radiant exposure. Average inactivation kinetics data of all strains of each species in this board is presented in ***a***. Inactivation kinetics of each strain is also shown for *A. baumannii* (***b***), *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 μM costs fractions of US-

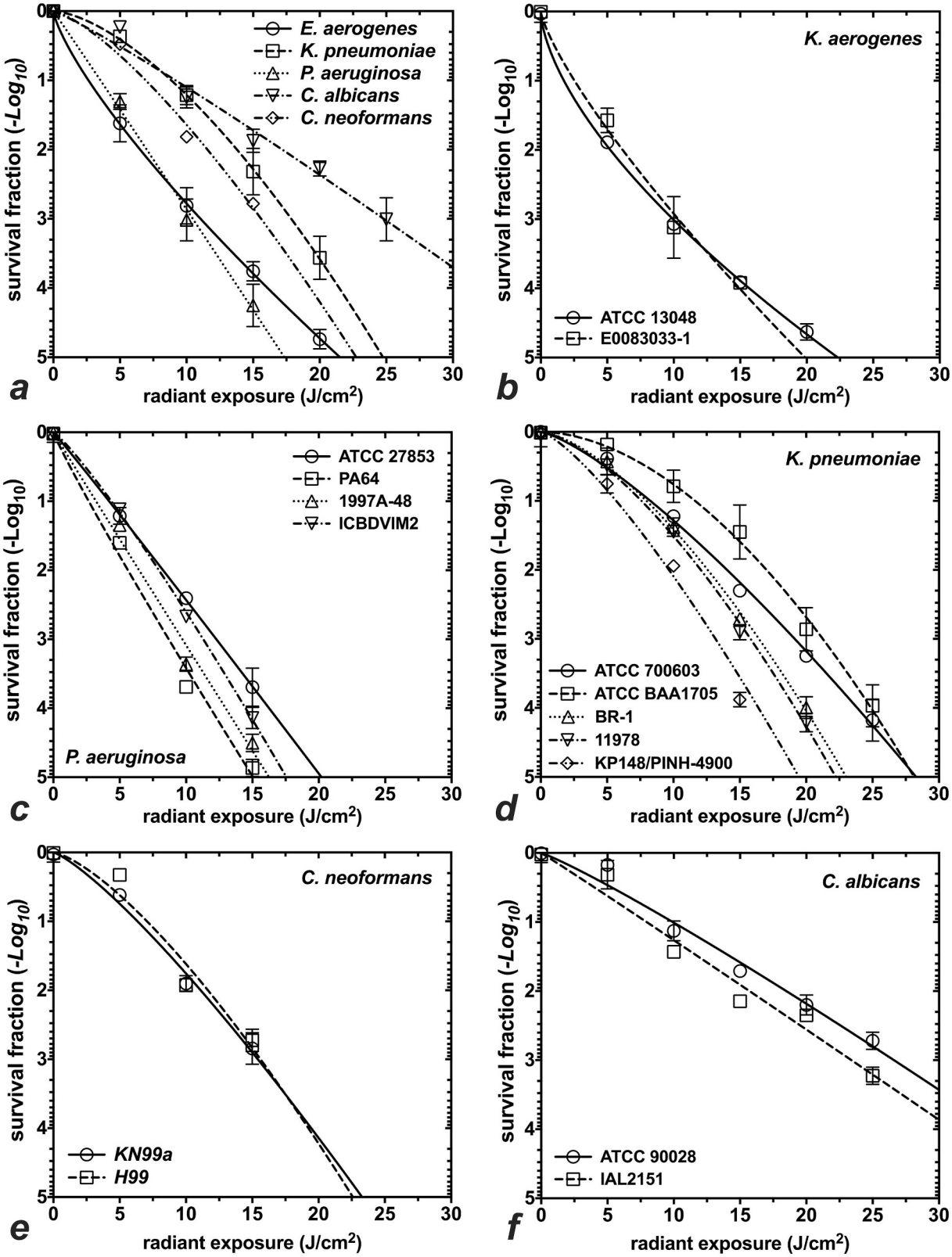

Fig. 2. Inactivation kinetics of bacterial and yeast species most tolerant to MB-aPDT (scale 5) in function of radiant exposure. Average inactivation kinetics data of all strains of each species in this board is presented in ***a***. Inactivation kinetics of each strain is also shown for *K. aerogenes* (***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

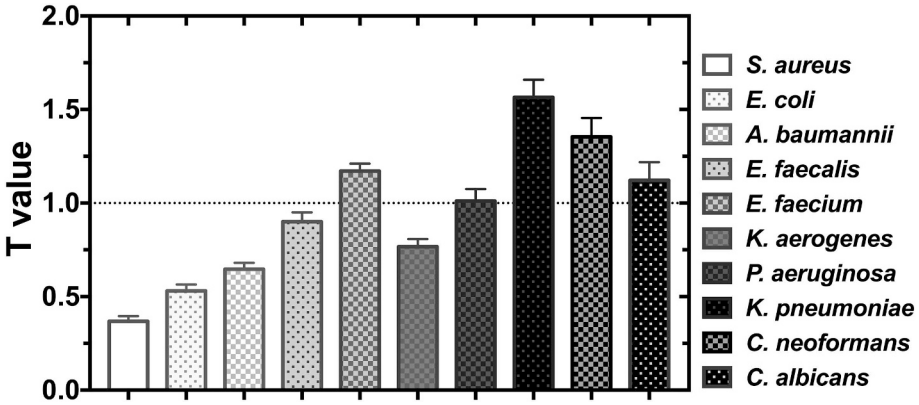

Fig. 3. Tolerance factor (T) of inactivation kinetics calculated for each tested species. If $T > 1$, microbial population is initially tolerant to aPDT but become increasingly sensitive. If $T < 1$, microbial population is initially sensitive, but some persistent cells remain more tolerant to inactivation 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

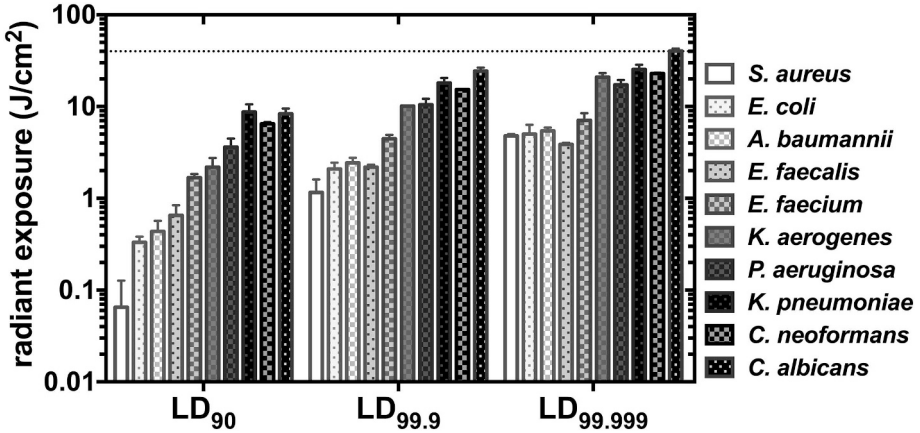

Fig. 4. Lethal dose values of aPDT calculated in function of radiant exposure. LD values correspond to percent of total microbial population reduction. Horizontal dotted line represents the maximum dose (40 J/cm^2) required for more than 5Log$_{10}$ of inactivation.

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 photoinactivation 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/cm^2, 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.

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Appendix A. Supplementary data

Supplementary material.

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