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Fotoinaktywacja światłem widzialnym jako narzędzie uwrażliwienia wielolekoopornych patogenów na działanie antybiotyków

Photodynamic inactivation as a tool for sensitization of multidrug resistant pathogens to antimicrobials

Praca przedstawiona
Radzie Dyscypliny Nauki Biologiczne Uniwersytetu Gdańskiego
celem uzyskania stopnia doktora
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Z całego serca pragnę podziękować…

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STRESZCZENIE PRACY

Tematyka niniejszej rozprawy doktorskiej skupiona jest na wielolekoopornych drobnoustrojach, które należą do tak zwanej grupy ESKAPE. Nazwa ta stanowi akronim mikroorganizmów z podwyższonej grupy ryzyka, które są w stanie dzięki nabytym mechanizmom z łatwością uciec od biobójczej aktywności antybiotyków: ***E****nterococcus* spp.*, **S**taphylococcus aureus, **K**lebsiella pneumoniae, **A**cinetobacter baumannii, **P**seudomas aeruginosa i **E**nterobacter cloacae.* Według raportu O' Neil z 2016 roku *The Review on Antimicrobial Resistance (AMR)* liczba śmiertelnych przypadków spowodowanych lekopornością drobnoustrojów może znacznie przekraczać liczbę zgonów spowodowanych chorobami nowotworowymi do 2050 roku. Ponadto, liczba przypadków śmiertelnych na skutek AMR, również w roku 2050 może wynosić aż 10 milionów rocznie, jeżeli żadne działania nie zostaną podjęte w celu zatrzymania postępującego problemu antybiotykooporności. W obliczu postępującego kryzysu, wszelkie działania, które prowadzą do zminimalizowania stosowania antybiotyków, bądź prowadzą do eliminacji drobnoustrojów na innej drodze działania stają się atrakcyjnymi narzędziami do walki z AMR.

Niniejsza praca skupiona jest na wykorzystaniu inaktywacji fotodynamicznej celem uwrażliwienia izolatów klinicznych z grupy ESKAPE na działanie antybiotyków. Inaktywacja fotodynamiczna (ang. *Antimicrobial Photodynamic Inactivation,* aPDI*)* opiera się na wykorzystaniu światła widzialnego z zakresu 380 nm - 740 nm, tlenu oraz fotouczulaczy, zarówno egzogennych jak i endogennych w przypadku inaktywacji światłem niebieskim (ang. *Antimicrobial Blue Light Inactivation,* aBL*).* Na skutek fotowzbudzenia tych związków powstają Reaktywne Formy Tlenu (RFT), które to z kolei prowadzą do uszkodzenia różnych struktur w komórkach bakteryjnych i docelowo również do jej śmierci. Liczne prace naukowe wskazują na skuteczność zastosowania fotoinaktywacji aPDI/aBL oraz antybiotyków w celu eradykacji lekoopornych drobnoustrojów. Jednakże, wiele z tych prac w niewłaściwy sposób, tj. z użyciem niewłaściwych metod testowania synergii dowiodło o skuteczności łączenia ze sobą tych dwóch monoterapii.

Przeprowadzone w niniejszej pracy doktorskiej badania, zostały przeprowadzone w oparciu o stworzony przeze mnie protokół testowania interakcji pomiędzy fotoinaktywacją a antybiotykami, opierający się na wykorzystaniu rekomendowanych metod oraz antybiotyków z różnych klas i kategorii oraz o różnych mechanizmach działania. Właściwie wdrożony protokół testowania synergii daje pełny obraz możliwości jakie niesie ze sobą fotoinaktywacja aPDI/aBL jako narzędzie uwrażliwiania drobnoustrojów z grupy ESKAPE o zróżnicowanych profilach oporności. Ponadto, dodatkowymi celami niniejszej pracy

była próba wyjaśnienia mechanizmów stojących za efektem synergistycznym pomiędzy aBL/aPDI, a antybiotykami, określenie wymiernego wpływu działania fotoinaktywacji na testowane drobnoustroje oraz ocena foto- i cyto-toksyczności jaką niesie ze sobą stosowanie światła widzialnego (niebieskiego). Ostatnim elementem badań była weryfikacja potencjału wykorzystania łączonej terapii (fotoinaktywacji i antybiotyku) z wykorzystaniem mysiego modelu *in vivo* rany zakażonej *Staphylococcus aureus* oraz *Pseudomonas aeruginosa.*

Otrzymane przeze mnie wyniki badań naukowych prezentowane w niniejszej rozprawie doktorskiej zostały opublikowane w 5 załączonych publikacjach naukowych. Całość stanowi zbiór rezultatów spójnych tematycznie.

ABSTRACT

This doctoral dissertation is focused on multidrug resistant microorganisms that belong to the ESKAPE group. ESKAPE is an acronym for a group of microorganisms (***E****nterococcus* spp.*,* ***S****taphylococcus aureus,* ***K****lebsiella pneumoniae,* ***A****cinetobacter baumannii,* ***P****seudomonas aeruginosa and* ***E****nterobacter cloacae*) that have an increased risk due to acquired mechanisms and can easily escape from the biocidal activity of antibiotics. According to the data presented in the Antimicrobial Resistance (AMR) report, the number of deaths from drug-resistant microorganisms could exceed the number of deaths from cancer by 2050. In addition, the number of deaths from AMR in 2050 could also be as high as 10 million per year if no action is taken to stop the progressing problem of antibiotic resistance. In face of the progressing crisis, all activities that help minimize the use of antibiotics or eliminate microorganisms with a different mode of action have become attractive tools to fight AMR.

This work focuses on the use of photodynamic inactivation to sensitize clinical isolates from the ESKAPE group to the action of antibiotics. Photodynamic inactivation (aPDI) is based on the use of visible light in the range of 380 nm - 740 nm, oxygen and photosensitizing compounds (both exogenous or endogenous) in the case of antimicrobial blue light inactivation (aBL). As a result of the excitation of these compounds, reactive oxygen species (ROS) are created, which in turn cause various structures in bacterial cells to become damaged and ultimately perish. Numerous scientific studies have demonstrated the effectiveness of utilizing aPDI/aBL photoinactivation and antibiotics to eradicate multidrug-resistant microorganisms. However, many of these experiments were performed in the wrong way, i.e., using inappropriate synergy testing methods, which has evidenced that combining these two monotherapies is efficient.

The research presented in this doctoral dissertation was carried out based on my own protocol for testing the interaction between photoinactivation and antibiotics, the use of recommended methods and implementation of antibiotics, which originated from various classes and categories and exhibited various mechanisms of activity. A properly implemented synergy testing protocol provides a full picture of the possibilities of aPDI/aBL photoinactivation as a tool to sensitize ESKAPE microorganisms with different resistance profiles. Moreover, another aim of this study was to explain the mechanisms behind the synergistic effect between aBL/aPDI and antibiotics and to determine the measurable impact of photoinactivation on the tested microorganisms. An additional objective of this dissertation was to verify the photo- and cytotoxicity of the potential use of visible light (blue) against eukaryotic and prokaryotic cells. The last element of the research was verifying the potential of combined therapy (photoinactivation and

antibiotics) using an *in vivo* mouse model infected with *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

The results of my research that are presented in this doctoral dissertation have been published in 5 scientific publications, which are attached. The whole work is a set of thematically coherent results.

Chapter I

INTRODUCTION

During his Nobel Prize lecture in 1945, Alexander Fleming prophetically warned about overusing Penicillium, which he had discovered. "*It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them (...) Then, there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to nonlethal quantities of the drug make them resistant"*. Many years after this quote was said, many microorganisms transitioned from being primary nonsusceptible to an antimicrobial agent to being susceptible – this is called **antimicrobial resistance (AMR)**. Resistance to antibiotics is a natural evolutionary process that was dangerously accelerated by the inappropriate prescription of antibiotics or overusing antibiotics in animals (e.g., to promote animal growth) and humans (sharing or using leftovers of antibiotics) in different sectors[1]. According to the World Health Organization, antibiotic resistance influences the increased medical costs, prolonged hospitalization of patients and increased mortality[2]. The prognosis related to AMR indicates that if no action is taken, the number of deaths as a result of AMR can reach 10 million by 2050[3].

Due to their multiple mechanisms of resistance, multidrug-resistant (MDR) microorganisms are currently nonsusceptible to multiple antimicrobial agents; therefore, international standards that more accurately and precisely define the categories of resistance have been created. According to the data published in 2016 by Magiorakos et al., there are 3 main categories of resistance in the acquired resistance profiles for microorganisms that are involved in health-care infections and are therefore prone to multidrug resistance (*Staphylococcus aureus*, *Pseudomonas aeruginosa, Enterobacter* spp., *Acinetobacter* spp. and *Enterobacteriaceae*)[4]. According to this classification, MDR (multidrug-resistant) isolates are nonsusceptible to at least one agent in three or more antimicrobial categories; extensively drug-resistant (XDR) isolates are nonsusceptible to at least one agent in all but two or fewer antimicrobial categories (isolates remain susceptible to only one or two antimicrobial categories)[4,5]. The third category describes microorganisms as nonsusceptible to all available antimicrobial agents; therefore, this group is called the pandrug-resistant (PDR) group[4]. The complexity and relationship of these categories are presented in **Błąd! Nie można odnaleźć źródła odwołania.**. In the environment, there are also microorganisms that are not classified as MDR pathogens. These microorganisms are susceptible to available antibiotics; therefore, they are not in the group of increased risk. MDR

microorganisms are sensitive to broad antimicrobial categories, and each of the following groups lack a number of available treatment possibilities; thus, at the "end" of this classification, we can distinguish strains that are resistant to all available antibiotics (PDRs).

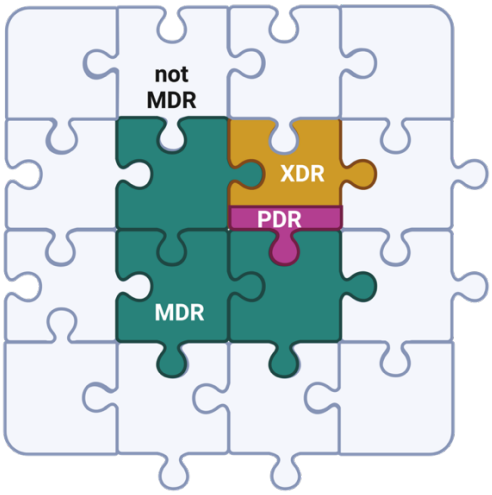

Figure 1. Schematic relationship of the categories of resistance according to Magiorakos et al. 2016

Along with antibiotic resistance, special attention is focused on the group of ESKAPE alarm pathogens that are responsible for hospital- and community-acquired infections. On the other hand, this group of emerging pathogens has the ability to "escape" the biocidal action of antibiotics, as they possess multiple virulence factors that participate in transition and pathogenesis[6]. The acronym ESKAPE represents the following MDR alarming pathogens: ***E****nterococcus faecium*, ***S****taphylococcus aureus*, ***K****lebsiella pneumoniae*, ***A****cinetobacter baumannii*, ***P****seudomonas aeruginosa*, and ***E****nterobacter* species[7].

Enterococcus faecium is a Gram-positive opportunistic microorganism that is involved in hospital-acquired infections, especially in immunocompromised patients[6]. This microorganism is also responsible for urinary tract infections, endocarditis, meningitis and septicaemia[8,9]. Vancomycin-resistant (VRE) *E. faecium* is responsible for one-third of nosocomial bloodstream infections in the United States[7]. Among enterococcal species, the second species (*E. faecalis*) is also relevant regarding AMR due to endodontic infection and the periarticular inflammatory response; nevertheless, this pathogen is less frequently associated with VRE infections[10]. Moreover, *E. faecalis* is responsible for 80-95% of bacterial infections in comparison to *E. faecium* (5-10%)[11]. However, the level of resistance differs between these two species. It is also worth mentioning that *Enterococcus* spp. are naturally resistant to cephalosporins, and their overuse has contributed to the development of VRE isolates[12]. *E. faecium* is intrinsically resistant to penicillins, aminoglycosides (tobramycin, kanamycin) and imipenem; for example, *E. faecalis* is resistant to lincosamides (clindamycin, streptogramin A and B)[13,14].

One of the most important Gram-positive representatives in the ESKAPE group is *Staphylococcus aureus*, which colonizes 25-30% of healthy individuals (in their skin, gastrointestinal tract or oropharynx)[8,9]. The most alarming *S. aureus* isolates are methicillin-resistant *Staphylococcus aureus* (MRSA) strains that are resistant to various ß-lactam antibiotics, such as methicillin, oxacillin, cloxacillin or cephalosporins[9]. Methicillin-susceptible *S. aureus* (MSSA) and MRSA isolates are responsible for pneumonia, osteoarticular infections, osteomyelitis and endocarditis[8,15]. In hospital settings, other drug-resistant *staphylococcal* isolates responsible for infections were detected, i.e., vancomycin-intermediate *S. aureus* (VISA) and vancomycin-resistant *S. aureus* (VRSA). Apart from the resistance of *S. aureus* to multiple antibiotics, the variety of diseases caused by this pathogen is related to the wide range of its virulence factors[16]. For example, golden pigment-staphyloxanthin, exotoxins (for example α, ß, γ and Panton-Valentine leucocidin), enterotoxins (e.g., SEA, SEB), protein A, or fibronectin binding[16,17]. Almost 20,000 people died in 2017 due to MRSA strains in the USA, and the mortality rate in patients infected with MRSA was 2-3 times higher than that in those infected with MSSA[18,19].

Klebsiella pneumoniae is a Gram-negative microorganism that is a part of the human and animal intestine microbiomes; on the other hand, it is responsible for respiratory tract infections, pneumonia, bacteremia, catheter-associated urinary tract infections and meningitis[9,20,21]. In health care settings, this microorganism is spreading via person-to-person contact, by ventilator contaminations, intravenous catheters or infections via injury or surgery wounds. Moreover, *K. pneumoniae* is naturally resistant to amoxicillin and carbenicillin and is capable of producing multiple virulence factors, e.g., capsules, endotoxins, adhesins or siderophores (aerobactin and salmochelin)[22,23]. The most important virulence factor, capsule, enables *K. pneumoniae* to inhibit the response of the host immunological system and indicates that this microorganism is less susceptible to phagocytosis[20,22]. According to the data published by the Centers for Disease Control and Prevention, infections caused by hospital-acquired carbapenem-resistant *K. pneumoniae* in the USA in 2013 were estimated to be more than approximately 7,000 cases[24].

Acinetobacter baumannii is a Gram-negative nonfermentive opportunistic coccobacillus that for a long time was considered a low-grade pathogen; however, due to the rapid development of resistance among this strain, it has become a serious threat to human life[25]. This microorganism is associated with pneumonia and urinary and bloodstream infections (in 10%–15% of cases, it is due to invasive procedures, intravascular/respiratory catheters, or cannulas)[26]. Carbapenem-resistant *A. baumannii* isolates were responsible for more than 8,000 infections in

hospitalized patients and 700 deaths in 2017 according to the data published by the Centers for Disease Control and Prevention[27]. This microorganism also has the ability to survive in the inanimate environment up to 5 months; therefore, *A. baumannii* is the main source of infections among injured soldiers and is referred to as "Iraqibacter" [6,28].

Another member of the ESKAPE group is Gram-negative *Pseudomonas aeruginosa*, which is an opportunistic pathogen that is responsible for hospital-acquired infections, e.g., urinary tract, bloodstream and soft tissue infections, and more importantly, it is involved in chronic lung infections, such as cystic fibrosis (CF)[29,30]. The ability to produce various virulence factors, e.g., flagella, type IV pili, pigments (pyocyanin, pyoverdine), proteases and phospholipases, enables this microorganism to colonize and cause successful infections in various environments[31]. *P. aeruginosa* also characterizes instinct resistance to the first- and second-generation cephalosporins, tetracyclines and sulfonamides[32]. Another significant aspect of this microorganism is the production of biofilms, which play a crucial role in pathogenesis, enabling the microorganisms to colonize urinary catheters and contact lenses and providing protection from unfavorable conditions[33].

The last microorganism in the ESKAPE group is *Enterobacter* spp. which, together with *K. pneumoniae,* is a part of the *Enterobacteriaceae* family. The *Enterobacter cloacae* complex comprises the following Gram-negative bacilli: *E. cloacae*, *E. nimipressuralis*, *E. asburiae*, *E. hormaechei*, *E. kobei* and *E. ludwigii*[34]. The most clinically important microorganism among this complex is *E. cloacae*; this strain is the 3rd most frequent cause of bloodstream infections among *Enterobacterales*[15]. Moreover, it is well known that *E. cloacae* consists of human microflora in 40-80% of individuals and colonizes the gastrointestinal tract; however, there is a lack of data concerning virulence factors of this pathogen[35]. The intrinsic resistance to amoxicillin, first-generation cephalosporins, cefoxitin and ampicillin is another feature of *E. cloacae* isolates[35,36].

The spreading of AMR is caused by horizontal gene transfer (acquisition of foreign DNA with the material), which occurs via transduction, transformation or conjugation[37]. The resistance mechanisms resulting from the ingestion of mutant genetic material can be divided into the following main categories: i) modification of drug target site; ii) reduction in antibiotic penetration and accumulation; iii) inactivation/alteration of antibiotics; and iv) biofilm formation. One of the best known mechanisms of resistance is related with the removal of the antibiotic from the cells (efflux pump), the presence of enzymes that cause drug degradation, reduction of the influx of the antibiotic or antibiotic modifying enzymes (Figure 2).

Alternative approaches to combat AMR and "superbugs" can be based on i) modifying the cell phenotype to increase the sensitivity to antibiotics or to the immune system of a host; ii) photosensitizing resistant phenotypes; and iii) using bacteriophages as a conventional treatment[38]. Other possibilities involve antimicrobial peptides (AMPs) (which are isolated from bacteria, fungi or plants) or their synthetic mimics, innate defense regulatory peptides (IDRP), silver nanoparticles (AgNPs), natural products (e.g., resveratrol, quercetin), bacteriocins or even probiotics[39,40].

Antimicrobial photodynamic inactivation (aPDI) and antimicrobial blue light inactivation (aBL) are light-based technologies that involve the following simple components: visible light (ranging from 380 nm to 740 nm), oxygen and exogenous photosensitizing agent (PS). Additionally, the common hypothesis regarding aBL is based on the presence of endogenous chromophores in bacterial cells that act like PS particles [41,42]. Absorption of photons by exo- and endogenous PS causes the photosensitizer to excite to the singlet state ($^1PS^*$), which then becomes the excited triplet state ($^3PS^*$). Then, two photochemical reactions can occur in the environment; thus, in the type I reaction, the electrons from the excited triplet state of PS are transferred to the substrate, leading to the production of oxygen radicals, such as superoxide ($^\bullet O_2^-$), hydrogen peroxide (H_2O_2) or hydroxyl radicals ($^\bullet OH$)[43]. A type II reaction results in the production of singlet oxygen ($^1O_2^-$) via energy transfer from the triplet state of PS to the oxygen molecule[44]. Interestingly, there is also evidence of another type of reaction (type III), which is an oxygen-independent process[45]. Reactive oxygen species (ROS) that are produced in both photochemical reactions lead to various changes in bacterial cells. In Gram-positive cells, phospholipids and proteins may be primarily affected by photoinactivation due to the interaction between PS and cell structures. Furthermore, similar to Gram-negative species, LPS (lipopolysaccharide) and proteins can be major cellular targets of photoinactivation, which are presented in Figure 2[46]. ROS action can lead to lipid peroxidation, protein oxidation (the generation of protein carbonyls) and DNA damage[43]. Overall, the destruction of cellular components via oxidative stress may result in cell death. Photoinactivation is considered a multitargeted procedure; however, there are possible cellular targets of ROS action. Figure 2 presents the potential targets of photoinactivation.

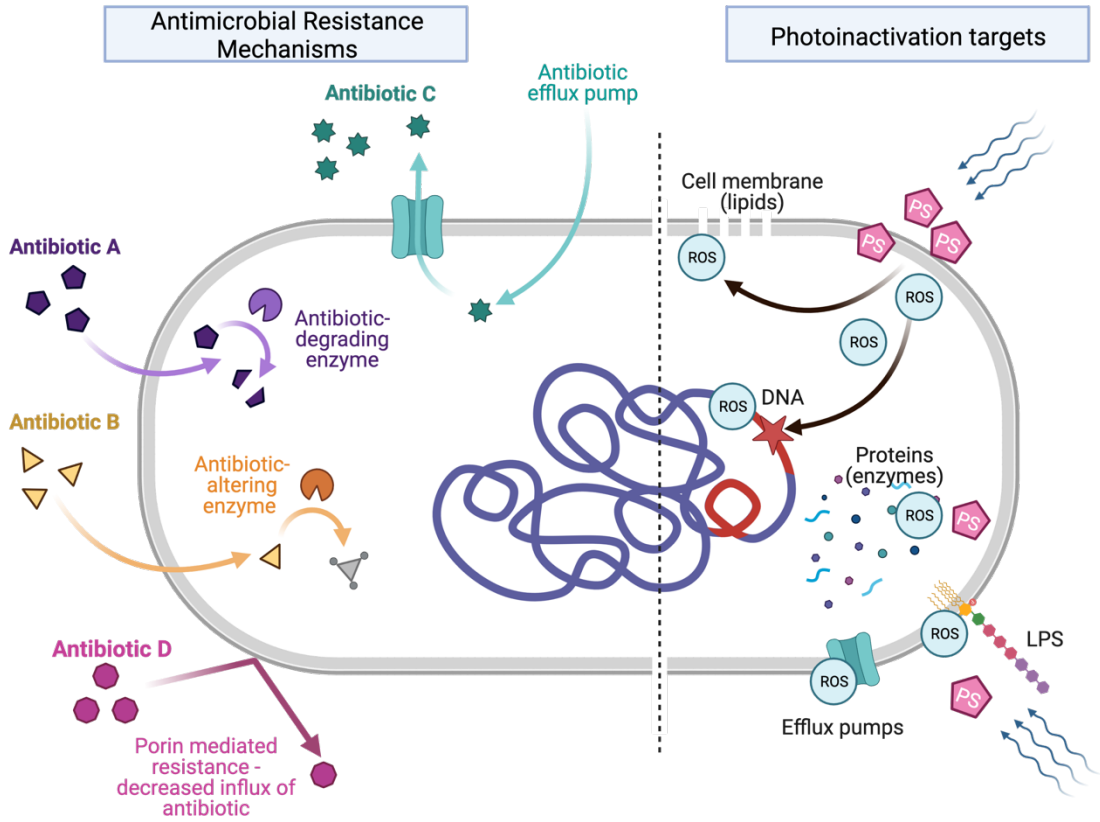

Figure 2 Antimicrobial resistance mechanism and photoinactivation targets

There are multiple classes of exogenous photosensitizing agents that are widely used in aPDI. The agents differ not only in structure but also in light and dark toxicity, molar absorption coefficient at a particular wavelength, penetration properties and quantum yield, and these agents produce ROS upon irradiation[47,48]. Xahnthenes (Rose Bengal), derivatives of phenothiazines (Toluidine blue O), chlorins (chlorin-e6), porphyrins (hematoporphyrin), porphyrin derivatives (Tetra-Py+-Me), fullerene derivatives (N-methylpyrrolidinium fullerene) or boron dipyrromethene derivatives (BODIPY) were present multiple times as efficient photosensitizers in aPDI[49–53].

On the other hand, endogenous chromophores present in bacterial cells and implemented in aBL photoinactivation have been identified in multiple studies. To date, the mechanism of this process is not fully understood; however, various studies have indicated that the presence of porphyrins and/or flavins in *P. aeruginosa*, *A. baumannii, Candida albicans*, *A. actinomycetemcomitans* and coproporphyrin (I and III) in *Helicobacter pylori* is crucial in the photoinactivation process[54,55].

In comparison to routinely used antibiotic treatment, photodynamic inactivation, which is a tool for bacterial eradication, possesses many positive features worth emphasizing. First, phototreatment does not have one specific

target of action in contrast to antibiotics, which are targeted to a specific cellular component in microorganisms[56]. Therefore, developing or inducing resistance to photoinactivation by exposing microorganisms to light conditions multiple times is unlikely to occur[57]. Experiments performed by our team clearly indicate that the only risk of exposing Gram-positive (*S. aureus*, *E. faecium*) and Gram-negative (*P. aeruginosa, E. coli*, *K. pneumoniae*) species to multiple exposures of photoinactivation is the development of tolerance and no resistance to this light-based approach[58,59]. However, this problem can be overcome by applying an increased dose of phototreatment[58,59]. Furthermore, photoinactivation is applicable to inactivate multidrug resistant microorganisms; thus, the resistance profile is not a limitation for this method[60]. Moreover, the mode of action due to the uptake of photosensitizers by microorganisms and the production of ROS is relatively faster than the duration of action of the antibiotic[61]. The low cost of photoinactivation, slight side effects and safety to human tissues are other advantages that clearly show the possibilities and safety of using inactivation in clinical practice[62].

Despite the many advantages of photoinactivation, as with any treatment strategy (e.g., antibiotics), photoinactivation also involves drawbacks. First, ROS are not produced when the light source is off; thus, the activity of this method is completed with the exclusion of the radiation source (e.g., LED lamp)[62]. Second, the penetration of light into the site of treatment is low and the photosensitizers among the Gram-negative and Gram-positive microorganisms treated exhibit divergent specificity upon photoinactivation, which are other barriers that hinder the effectiveness of this method[63]. The last disadvantage that is worth emphasizing is that photoinactivation works in the place at which the photosensitizer and light are applied; therefore, it is useful to treat localized and not systemic infections[38]. To date, there have been many clinical trials involving photoinactivation in the treatment of acne vulgaris, periodontal diseases, halitosis, ulcers on the skin or peri-implantitis[64–67]. As a monotherapy, photoinactivation is implemented on the commercial market, and at the moment, there are many solutions aimed at treating skin problems in humans and animals[68–70]. It is worth emphasizing that many of the available treatments involve the UV spectrum (e.g., psoralen with UV-a; PUVA); however, these wavelengths are much more harmful to skin than the visible light used for photoinactivation[45].

Due to limitations of photoinactivation as a single monotherapy and the risk of incomplete eradication in the site, the combination of a light-based treatment and antibiotics is a promising approach. Confirmation of this statement can be easily found in the literature data, and there are many works that address this issue and present results of *in vitro* and *in vivo* tests as well as

the usefulness of these treatments in clinical applications[71–73]. However, multiple studies have been performed with inappropriate methodology and a limited number of antibiotics when establishing the effectiveness of combined treatment. **Considering the current literature reports and deficiencies in research on combined therapy, which resulted from a lack of accuracy and completeness, this doctoral dissertation thoroughly verified and examined the effectiveness of photoinactivation in sensitizing pathogens from the ESKAPE group, so that both of these monotherapies can be used in combination to fight with multidrug-resistant microorganisms.**

HYPOTHESIS AND AIMS OF THE WORK

The presented doctoral thesis focuses on verifying whether antimicrobial photodynamic inactivation (aPDI) and antimicrobial blue light (aBL) can be effective methods for sensitizing the clinical isolates in the ESKAPE group to antibiotics. The second part of the thesis is focused on searching for the mechanism of aBL/aPDI, which could be responsible for the synergies between light and antibiotics. The third part of the doctoral thesis examines the influence of photoinactivation conditions on prokaryotic and eukaryotic cells in regard to photo- and cytotoxicity.

In connection with the research hypothesis, the following goals were formulated in this work:

1. Develop an appropriate protocol for determining the synergy between photoinactivation (aBL/aPDI) and antibiotics.

2. Determine the biocidal activity of aPDI and aBL against 12 ESKAPE clinical isolates with a multidrug-resistance profile.

3. Examine the effect of photoinactivation on the antibiotic resistance of microorganisms belonging to the ESKAPE group, i.e., whether there is a synergistic effect between photoinactivation and antibiotics.

4. Determine the effect of inactivation (aBL) on prokaryotic and eukaryotic cells to determine the cyto- and phototoxic effects.

5. Determine the influence of photoinactivation (aBL/aPDI) on cell membrane integrity and the production of reactive oxygen species.

6. Determine the impact of endogenous porphyrin synthesis in *Staphylococcus aureus* cells on the sensitization process upon aBL exposure.

7. Verify the potency of combined treatment (aBL and antibiotic) *in vivo* with a mouse model of wounds infected with *Staphylococcus aureus* and *Pseudomonas aeruginosa.*

The results of my research that are presented in this doctoral dissertation have been published in 5 scientific publications, which are attached. The whole work is a set of thematically coherent results.

Chapter II

Combined Antimicrobial Activity of Photodynamic Inactivation and Antimicrobials– *State of the Art*

1. Summary of the publication

The successful treatment of many infectious diseases is based on applying two antimicrobial agents or factors that, compared to monotherapies, increase the bactericidal effectiveness when used in combination. For example, gram-negative sepsis or enterococcal endocarditis treatment would not be as effective without the implementation of two antibiotics[74]. The efficacy of two combined antibiotics or other agents is called the synergistic effect, whereas the opposite result is called the antagonistic effect. The primary methods of synergy testing in *in vitro* conditions are as follows: time-kill curve assays, checkerboard method, E-test and multiple-combination of bactericidal antimicrobial testing (MCBT)[75]. Various studies have demonstrated the synergistic effect between antimicrobial photoinactivation and antibiotics as a strategy to eradicate reference and clinical isolates of Gram-positive and Gram-negative species[71]. Reliable conclusions based on the literature analysis and comparisons of the experimental outcomes indicate that the synergy between photoinactivation and antibiotics involves risks due to the lack of an appropriate methodology for synergy testing and the differences in experimental protocols presented in various publications.

To compare the obtained experimental data and assess the reliable differences during the synergy study, I created and published recommendations that allowed me to analyze and investigate the interactions between light and antimicrobial agents. **Publication no. 1,** which is entitled *Combined Antimicrobial Activity of Photodynamic Inactivation and Antimicrobials–State of the Art,* is focused on the analysis of literature data in terms of the methodology of synergy testing between antimicrobial photodynamic inactivation and antibiotics in *in vitro* conditions (biofilm, planktonic cultures) and *in vivo* conditions. Reliable conclusions should be drawn only when the synergy analysis investigates the appropriate methods. Most of the analyzed studies (18 out of 27) confirmed that photoinactivation and antibiotics offer an effective and successful method to eradicate microorganisms; however, in many of the works, an appropriate methodology was not used to demonstrate the synergistic interaction. It is worth also mentioning that this state-of-the-art review also focuses on the mechanisms underlying the effectiveness of photoinactivation and antibiotics.

2. Publication

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Combined Antimicrobial Activity of Photodynamic Inactivation and Antimicrobials–State of the Art

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Antimicrobial photodynamic inactivation (aPDI) is a promising tool for the eradication of life-threatening pathogens with different profiles of resistance. This study presents the state-of-the-art published studies that have been dedicated to analyzing the bactericidal effects of combining aPDI and routinely applied antibiotics in *in vitro* (using biofilm and planktonic cultures) and *in vivo* experiments. Furthermore, the current paper reviews the methodology used to obtain the published data that describes the synergy between these antimicrobial approaches. The authors are convinced that even though the combined efficacy of aPDI and antimicrobials could be investigated with the wide range of methods, the use of a unified experimental methodology that is in agreement with antimicrobial susceptibility testing (AST) is required to investigate possible synergistic cooperation between aPDI and antimicrobials. Conclusions concerning the possible synergistic activity between the two treatments can be drawn only when appropriate assays are employed. It must be noticed that some of the described papers were just aimed at determination if combined treatments exert enhanced antibacterial outcome, without following the standard methodology to evaluate the synergistic effect, but in most of them (18 out of 27) authors indicated the existence of synergy between described antibacterial approaches. In general, the increase in bacterial inactivation was observed when both therapies were used in combination.

Keywords: antimicrobials, antimicrobial photodynamic inactivation, photoinactivation, photosensitizers, synergy

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INTRODUCTION

Almost 89 years have passed since Alexander Fleming discovered penicillin–the antibiotic that revolutionized medicine–and contributed to research associated with the golden age of antibiotics (Davies, 2006; Tan and Tatsumura, 2015). Microbiologists and clinicians are currently struggling with the increasing frequency of drug resistance among pathogenic bacteria (Nakonechny and Nisnevitch, 2011). According to the antimicrobial resistance report published in 2016, the number of deaths caused each year by pathogenic bacteria will increase to 10 million by 2050 if no actions are taken (O'Neill, 2016); scientists are thus now focused on finding new biocidal substances or methods to effectively cope with emerging drug resistance. A few of the most recent examples include (i) the discovery of a new antibiotic by researchers at Rutgers University–pseudouridimycin, produced by microorganisms isolated from soil (Maffioli et al., 2017), (ii) the acquisition of a new class of antibiotics

(Teixobactin) from the soil bacterium *Eleftheria terrae* (Fiers et al., 2017), and (iii) the discovery of the cathelicidins by researchers at Sydney University-these antimicrobial peptides are acquired from Tasmanian devil and active against gram-positive and gram-negative bacteria (Peel et al., 2016). These new compounds effectively fight against drug-resistant bacteria. However, the problem of rapidly growing resistance is still present and unsolved. Scientists engaging this problem should focus on alternative approaches to eradicating pathogenic bacteria (Wainwright et al., 2016). Antimicrobial photodynamic inactivation (aPDI), also known as a photodynamic antimicrobial therapy (PACT) and photodynamic inactivation (PDI), is an alternative method to fight resistant microorganisms, including bacteria, fungi, parasites and viruses (Awad et al., 2016; Hamblin, 2016). The aPDI method requires the presence of oxygen, a non-toxic photosensitizer (PS) and light. The PS is activated by the absorption of a photon with a specific wavelength, and this absorption leads to the formation of short-lived excited states of the PS. These states are then transformed to a triplet excited state, which further progresses along two separate photochemical pathways. In a type 1 mechanism, an electron is transferred from the triplet state of the PS and promotes the creation of reactive oxygen species (ROS), e.g., hydroxyl radicals ($HO^{\cdot}$). In a type 2 mechanism, the energy from the triplet state of the PS is transferred to produce singlet oxygen radicals ($^1O_2$). These compounds promote oxidative stress, which results in DNA damage and the destruction of cell envelopes, lipids and other components whose dysfunction finally leads to cell death. Moreover, aPDI confers numerous positive effects. The main advantage of aPDI is that bacterial resistance does not develop as a result of the treatment, which is due to the production of widely acting and indirectly targeted ROS during aPDI (Denis and Hamblin, 2011). Next, aPDI could affect the activity and/or production of numerous virulence factors, leading to decreased bacterial pathogenicity unlike antibiotic therapy, which can promote the production of virulence factors and lead to an increase in their release upon antibiotic treatment (Kharkwal et al., 2012; Fila et al., 2016; Dai, 2017; Wang et al., 2017). Furthermore, the aPDI is not cyto- and phototoxic toward eukaryotic cells in a wide therapeutic window and does not promote mutagenic effects in treated eukaryotic and prokaryotic cells (Grinholc et al., 2015).

The aPDI method has been repeatedly demonstrated in the literature to have many more advantages than individual routine antibiotic therapies. First, this method functions in a short time and limited space, potentially inactivating only the microorganisms that are present in the infection site without negatively influencing physiological flora (Ryskova et al., 2010). Second, the literature does not show that aPDI leads to the development of resistance against aPDI. Numerous studies have shown that habituation or incubation of bacterial cells with sublethal aPDI doses does not result in the development of resistance against phototreatments (Cassidy et al., 2010; Tavares et al., 2010). The main targets for aPDI are various structures and components of bacterial cells instead of one major target (as in the case of antibiotics), which reduces the possibility of developing resistance against such approaches (Maisch, 2015). Finally, the biocidal factors in aPDI are ROS and singlet oxygen; mechanisms of resistance against these species have not been discovered. Another unquestionable advantage of aPDI is its effectiveness in the inactivation of numerous virulence factors (Fila et al., 2016). The aPDI method may decrease the activity of proteases, lipases, secreted toxins, etc., (Fila et al., 2016).

In most research in this area, aPDI has been proposed as an alternative treatment option that acts independently and in isolation from complementary antimicrobial approaches, e.g., antibiotic therapy. Use of aPDI alone should lead to the successful eradication of pathogenic microorganisms from the site of infection. However, the achievement of satisfactory clinical effects with photodynamic inactivation, understood as the total eradication of microorganisms at the site of infection, is extremely difficult and rarely described despite numerous works carried out worldwide. Two important limitations of the aPDI method are its lower bactericidal efficacy against microorganisms growing in biofilms and the fact that the efficiency it exhibits in *in vitro* studies rarely translates into animal models. Even after the effective elimination of microorganisms from the site of infection, the regrowth of microorganisms and recurring development of the infection are observed 24 h post-treatment. Nevertheless, we are deeply convinced that photoinactivation has many advantages that make it an attractive tool for a comprehensive fight against multiresistant human pathogens. We therefore suggest the use of the unquestionable advantages of photodynamic inactivation to sensitize multidrug-resistant microorganisms to chemotherapeutic agents by pairing it with routinely used antibiotics. This approach allows the use of antimicrobial agents to which bacteria express high resistance and leads to significant decreases in the MIC, enabling the eradication of microorganisms and inhibiting the regrowth of microbes at the infection site.

The most recent discovery concerning combined aPDI and antibiotics indicate that photoinactivation renders microbes susceptible for routinely used antimicrobials (Fila et al., 2016). If this phenomenon is confirmed using appropriate methodology and translated into *in vivo* and clinic applications, this approach could significantly reduce the rate of emerging drug resistance among pathogens due to the reduced use of antimicrobials employed in the treatment. Reviewing existing publications and searching for evidence-based proof of synergism between aPDI and antimicrobial activities are thus important, as is using appropriate experimental approaches for studying the synergy between these two treatments.

APPROVED METHODOLOGY FOR SYNERGY TESTING

According to the *American Society for Microbiology*, only a few experimental procedures are adequate for determining synergistic effects between various antibacterial approaches (http://www.aac.asm.org). These methods include using (i) disk-diffusion assays, (ii) *E*-tests for antibiotic susceptibility testing, (iii) checkerboard assays, (iv) post-antibiotic effects (PAEs), and

(v) the Bliss model for synergy testing in biofilm cultures (Habash et al., 2014).

Disk-Diffusion Assay

This technique is a simple approach to test antimicrobial susceptibility in routine clinical microbiology laboratories (Matuschek et al., 2014). This assay operates by the diffusion of an antimicrobial agent from a disk to solid medium (typically M-H medium), which leads to the formation of circular zones of growth inhibition (Kuper et al., 2012). According to the EUCAST (European Committee On Antimicrobial Susceptibility Testing) guidelines, the disk-diffusion methodology includes the use of overnight bacterial inocula or colonies that are suspended in saline to obtain bacterial suspensions with an optical density of 0.5 McFarland (Matuschek et al., 2014). Bacterial suspensions are placed on M-H plates at some point from 15 to 60 min after preparation. The disks are then placed on M-H medium 15 min after incubation, and the incubation of antibiograms is performed at $35 \pm 1^{\circ}C$ for 16-20 h. The measurements of growth inhibition zones and interpretation of results are based on the EUCAST breakpoint tables and additional instructions (http://www.eucast.org). AST guidelines provide no indication about the change in inhibition zone value that justifies considering synergy, thus, any statistically significant change in growth inhibition zone could potentially indicate synergy.

E-test

The *E*-test, known also as the epsilometer test, is also based on the diffusion of an antimicrobial agent in culture medium, but in contrast to disk-diffusion assays, this quantitative technique can be used to estimate MIC values. As in disk-diffusion assays, this method typically uses M-H medium and appropriate incubation conditions (Kuper et al., 2012). MIC values are validated by identifying the intersecting areas of growth inhibition on *E*-test strip scales (Kuper et al., 2012). This method defines synergy as a ≥ 3 dilutions in MIC, additivity as a decrease of ≥ 2 but < 3 dilutions and indifference as a decrease of < 2 dilutions in the MIC. Antagonism is defined as an increase of ≥ 3 dilutions of the MIC.

Checkerboard Assay

This method is often used to determine the interaction between and potency of two or even three factors. Serial 2-fold dilutions of tested compounds are prepared in 2-dimensional fashion in one microtiter plate(Jenkins and Schuetz, 2012). The type of interaction is determined based on the assessment of the fractional inhibitory concentration index (FICI) for each tested antimicrobial agent (FIC_A, FIC_B) (Rybak et al., 2014). However, these values are appointed for those concentrations of compounds which administered together lead to the inhibition of the bacterial growth; next, these values are compared with the MIC values for each agent tested separately (Doern, 2014). Thus, the determination of interaction is based on FICI value which is calculated as follow: $\sum \text{FICI} = FIC_A + FIC_B$, where FIC_A equals the MIC of drug A in combination divided by the MIC of drug A alone and FIC_B equals the MIC of drug B in combination divided by the MIC of drug B alone (Jenkins and Schuetz, 2012). The most recent guidelines given by the British Society for Antimicrobial Chemotherapy concerning checkerboard assays stated that based on this assay, one could determine the two following interactions: (i) synergy (when FICI is ≤ 0.5) and (ii) antagonism (when FICI is > 4.0). No other interactions, such as indifference, are defined by this method (Odds, 2003).

Time-Kill Assay (TKA)

TKAs are performed in large volumes (>10 ml) in glass beakers where the bacterial inoculum is placed into broth that contains the desired concentration of antimicrobials. The inoculum is then incubated for 48 h, and 0.5 ml aliquots are periodically collected and plated for colony count determinations. These samplings generally occur at 4, 8, 10, 12, and 24 h. The time-kill colony counts are then graphically represented as a function of time. Synergy occurs in time-kill assays when the results of the antimicrobial combination are $> 2 \log_{10}$ greater than the results of the combination's most active constituent (Boluki et al., 2017).

Post-antibiotic Effect (PAE)

PAEs are defined as delays in bacterial regrowth after a brief exposure to an antibiotic at a specific concentration (MIC). A bacterial inoculum is exposed to multiple MIC dilutions of antibiotics that are later removed or inactivated. Next, the regrowth of bacterial cells resuspended in antibiotic-free medium is monitored every 0.5–2 h. The post-antibiotic effect is defined based on the following formula: $PAE = T - C$, where T is the estimated time for a bacterial culture population to increase by $1 \log_{10}$ of viable cells from the number of bacterial cells that were present after the chemotherapeutic agent had been removed and C is the time of growth of untreated control cells. The difference in the time that a microorganism requires after contact with an antibiotic to increase its number of viable cells 10-fold compared to the time that untreated bacteria require is described as the PAE. This effect can depend on several factors: the bacterial species, antibiotic concentrations and the time of exposure of bacterial cells to chemotherapeutical agents (Odenholt, 2001).

Bliss Model

The following formula is used for the Bliss model: $S = (f_{X0}/f_{00})(f_{0Y}/f_{00})\text{-}(f_{XY}/f_{00})$, where f_{XY} refers to the biofilm biomass in the presence of the combined treatment at concentration X for chemotherapeutic A and concentration Y for chemotherapeutic B, f_{X0} and f_{0Y} refer to the biofilm biomass in the presence of the individual treatments at concentrations of X and Y, respectively, f_{00} refers to the biofilm biomass in the absence of treatments, and S corresponds to the degree of synergy. Positive values of S reflect synergy, while a negative value of S reflects an antagonistic interaction. This methodology was used successfully for investigating the influence of combined factors on *Pseudomonas aeruginosa* biofilms with the application of aPDI (Habash et al., 2014).

Only 5 of 27 studies reviewed within the current paper and concerning combined aPDI and antimicrobial treatments were performed in accordance with the approved methodology. Moreover, the most recent guidelines indicate that the synergy can be concluded when it is defined by use of two or more of

the abovementioned methods. None of the available published studies meet these requirements.

OTHER METHODOLOGY USED FOR SYNERGY AND/OR COMBINED EFFECT TESTING

Unfortunately, most of the studies describing the use of combined aPDI/antimicrobial treatments were not designed in accordance with approved standards. The potential synergistic interactions between aPDI and antimicrobials were reported after the use of custom-made methodology.

The most often used method for defining synergy and/or combined effect is a serial dilution of bacterial suspensions irradiated in the presence of antibiotics at different concentrations and the subsequent calculation of the number of CFU/ml. A reduction in the viable counts of bacterial cells of 6 or more $\log_{10}$ defines a synergistic interaction (Pérez-Laguna et al., 2017). The same method was used by the Cassidy group, who described a synergistic effect as a reduction in viable counts by $\geq$ 2 $\log_{10}$ more than the reduction in counts by the most active single agent (Cassidy et al., 2012). The same methodology was employed by Ronqui et al. (2016), who stated that a synergistic effect was present in a combined aPDI and antibiotic treatment against biofilm cultures based on comparing the level of reduction of bacterial cells receiving monotherapy (aPDI) to the reduction level obtained using the combined treatment. A difference in reduction of 0.6 $\log_{10}$ between these two groups was defined to represent a synergistic effect.

It must be stated that all the studies testing the efficiency of the combined therapy to inactivate bacteria and/or detecting the synergic effect of both therapies are highly appreciated. It must be noted that some of described papers were just aimed at determination whether the combined treatments exert enhanced antibacterial outcome with no interest in synergy testing, but most of them indicated the existence of synergy between described antibacterial approaches. However, omitting what was the purpose of performed researches, it is worth to underline that if aPDI is expected to gain the attention of international microbiologists and clinicians communities, it must be tested with the standard and approved methodology. One should be aware that even if the antimicrobials and aPDI reveal enhanced bacterial killing when acting together, the results will only be reliable and convincing if they are confirmed with the employment of approved standards.

ANTIMICROBIAL PHOTODYNAMIC INACTIVATION COMBINED WITH ANTIMICROBIALS

In Vitro Studies: Planktonic Cultures

Endogenously Produced Porphyrins

Microorganisms, due to the presence of haem synthesis pathway, are able to produce and accumulate endogenous porphyrins. Their production could be increased with the administration of the appropriate precursor, i.e., delta-aminolevulinic acid (ALA). These endogenously produced porphyrins could serve as a photosensitizer and were used repeatedly in eradication of numerous bacterial pathogens by inducing photochemical damages (Hamblin and Hasan, 2004; Grinholc et al., 2008). Reznick et al. (2013) published data in 2013 indicating that a combined treatment of visible light irradiation and gentamycin results in increased antibacterial effects against *Pseudomonas aeruginosa*. During their experiments, bacteria were irradiated with continuous or pulsed-switched light in the presence or absence of gentamicin for 24 h. Treating bacteria separately with gentamycin and green light (λ = 532 nm) in two exposure modes of irradiation did not reduce the number of viable counts (Reznick et al., 2013). The application of continuous or pulsed-switched light in combination with gentamycin for 24 h gave an 8 $\log_{10}$ greater reduction in viable counts than individual treatments (Reznick et al., 2013). Endogenous porphyrins were also used in eradication of *Clostridium difficile* which is an etiological agent of pseudomembranous colitis and is responsible for opportunistic infections in intensive care units, which are mainly caused by the eradication of natural flora as a result of antibiotic administration (Musher et al., 2013; De Sordi et al., 2015). Choi et al. (2015) proved that application of aPDI in combination with tetracycline (0.5 mg/ml) gave a 2 $\log_{10}$ increased reduction in viable count after 5 min of irradiation and 3 $\log_{10}$ after 10 min of light exposure. In addition, this effect could be further enhanced (4 $\log_{10}$ greater than the count reduction in the control group) when chitosan was applied (Choi et al., 2015). The application of aPDI in combination with tetracycline (1.0 mg/ml) or chloramphenicol reduced the number of viable counts for *C. difficile* by 7 $\log_{10}$ more than UVA monotherapy. Interestingly, Fila et al. (2016) proved in 2016 that the application of blue light (λ = 410 nm) with the presence of intracellular photosensitizing compound eradicates planktonic cultures of *P. aeruginosa* strains that presented multidrug resistance (MDR) and extensive drug resistance (XDR) profiles. Moreover, a combined application of sublethal dose of blue light (10 J/cm^2) and tested antibiotics (gentamicin, meropenem, or ceftazidime) reduced the minimal inhibitory concentration (MIC) by 2- to 64-fold more than individual treatments. The synergistic effect of light and antimicrobial applications was estimated using a checkerboard assay, which is a reliable technique for testing synergetic or antagonistic interactions. This evidence was the first to indicate the synergistic effect of combining blue light and antibiotic treatments for *P. aeruginosa* (Fila et al., 2016). Another study of combining aPDI with antibiotics was presented by Pereira et al. (2017a) in 2017; this study proved that irradiation of *Escherichia coli* and *Staphylococcus aureus* with blue (470 nm) or red light (625 nm) for 10 min in the presence of ciprofloxacin (5 mg) is more effective than antibiotic monotherapy. Moreover, the presence of norfloxacin (10 mg) with blue or red irradiation also exerted the positive effect of combined treatment on *S. aureus*, displayed as an increase in the sizes of inhibition zones on antibiogram plates (Pereira et al., 2017a). An interesting case of combined aPDI/antimicrobial therapy was reported by Jeong et al. (2017), whose group used *Propionibacterium acnes* and erythromycin-loaded liposomes (OELL) in their experiments. Irradiation of bacterial cells with light (200 s) in the presence of

liposomes containing erythromycin at a concentration of 1 μg/ml reduced the viable cell count by 1.99 $\log_{10}$ more than laser monotherapy (Jeong et al., 2017).

Exogenously Administered Porphyrin-Based PS

An excellent example of the synergistic effect of combining light, exogenous photosensitizer and antimicrobial therapy on biofilms and planktonic cultures was presented by Iluz et al. (2018) in 2018. When the planktonic cultures of *S. aureus* were treated with deuteroporphyrin IX (DP) and oxacillin and irradiated with a light dose of 46 J/cm^2, a synergistic interaction was observed for DP (2-9 μM) and oxacillin (MIC 250 μM) based on checkerboard assays. The synergistic effect with oxacillin (1 μg/ml) was also represented by changes in the survival rate of bacterial cells. Irradiation with a light dose of 46 J/cm^2 with DP (17 μM) completely eradicated bacterial cells, and the synergistic effect was still present at lower concentrations of DP (4 μM), but the number by which the viable bacterial cell count was reduced was lower (∼6 $\log_{10}$) than that observed with the higher dose of DP (Iluz et al., 2018). Interestingly, Iluz et al. (2018) verified how long the synergistic interaction for DP-aPDI treatments remains after their application and proved that the absence of light treatment and exposure to oxacillin leads to a smaller reduction in the number of bacterial cells. The application of aPDI in combination with oxacillin (4 μM) increased the reduction in the viable counts of planktonic cultures by 6 $\log_{10}$ over the reduction achieved by independent treatments. In 2013, Sana S. Dastgheyb presented results for this combined treatment against *E. coli*, *Staphylococcus epidermidis*, and a methicillin-resistant *S. aureus* (MRSA) clinical isolate. Compared to the treatment of bacteria with only antibiotics (vancomycin and ceftriaxone), which did not affect cell viability, the exposure of *S. aureus* to 5 h of irradiation in the presence of porphyrin *meso*-tetrakis(4-aminophenyl) porphyrin (TAPP) gave a 1-1.5 $\log_{10}$ reduction in viable counts. Combining aPDI treatment with ceftriaxone and vancomycin reduced the cell viability by a further 1-2 $\log_{10}$ from its value in control groups. Higher bactericidal effectiveness was obtained for tobramycin and chloramphenicol (2.5 and 1 $\log_{10}$ reductions in viable counts, respectively). The application of light and PSs to antibiotic treatments increased antibacterial efficacy by a further 2.5 $\log_{10}$ for tobramycin and 3 $\log_{10}$ for chloramphenicol over the efficacy in probes where only antibiotics were used. Furthermore, to investigate the type of interactions (synergism and antagonism) for tobramycin, chloramphenicol, PSs and light, a checkerboard assay was prepared for all tested strains. An *S. aureus* strain was used as a reference: when both antibiotics were used as a treatment, an additive effect was observed. The combination of light and both chemotherapeutics had also an additive effect for an *E. coli* reference strain. The synergistic effect of combined therapy was proven for *S. epidermidis* and the MRSA clinical isolate. The types of interactions were defined using the checkerboard assay and established by measuring FICI range (Dastgheyb et al., 2013). The application of aPDI in combination with different antibiotics (tobramycin, ceftriaxone, vancomycin, or chloramphenicol) increased the reduction in viable counts from 0.5 to 3 $\log_{10}$ over the reduction achieved with individual treatments. The effects of the synergistic interaction between treatment with light and antibiotics on multidrug resistant bacterial strains isolated from hospital wastewater and patients have been reported (Almeida et al., 2014). For all tested strains isolated from patients, irradiation with white light and *meso*-tetrakis(1-methylpyridinium)porphyrin (Tetra-Py$^+$-Me) (5 μM) reduced the number of viable cells by 6-8 $\log_{10}$ after 270 min of exposure to light, while a significant reduction (by 4-5 $\log_{10}$) had already occurred after 90-180 min. In the case of strains isolated from hospital water probes containing the same species of microbial pathogens, the bactericidal effect (4 $\log_{10}$ reduction in viable counts) was observed after just 30 min of irradiation with Tetra-Py$^+$-Me (5 μM). Moreover, adding ampicillin (32 μg/ml) to an *E. coli* suspension reduced the number of viable cells by a further 1 $\log_{10}$ after 180 min and 2 $\log_{10}$ after 270 min of irradiation from the number of viable cells irradiated without the presence of antibiotic. Adding chloramphenicol (32 μg/ml) and exposing the bacterial suspension to light and PS for 270 min reduced the number of viable cells by 2 $\log_{10}$ more than treatment with only light and Tetra-Py$^+$-Me (Almeida et al., 2014). The positive effects of combining aPDI and antibiotic therapy *in vitro* and *ex vivo* were also reported by Branco et al. (2018) in 2018. In *in vitro* experiments, a reference *S. aureus* strain was irradiated with white light and Tetra-Py$^+$-Me (5 μM) for 180 min with a variety of antibiotics: chloramphenicol (0.25 μM), kanamycin (2 μg/ml), penicillin G (0.125 μg/ml) and ampicillin (0.25, 0.5 and 1 μg/ml). After 180 min of irradiation, the number of viable cells was reduced by 8 $\log_{10}$ for all antibiotics (Branco et al., 2018). Compared with monotherapy with DP, the application of aPDI in combination with ampicillin at its highest concentration (1.0 μg/ml) improved the reduction in viable counts for planktonic cultures by 4 $\log_{10}$ (Branco et al., 2018).

Phenothiazines

The enhanced effectiveness of aPDI with phenothiazinium PS and antibiotics was also demonstrated by M.H. Shih and F.C. Huang in 2010 using *Mycobacterium fortuitum* in *in vitro* and *in vivo* experiments. Monotherapy (100 J/cm^2, 50 μg/ml methylene blue, MB) resulted in reducing the number of colony forming units (CFUs) by 2-3 $\log_{10}$ from their number in untreated cells. A synergistic effect was obtained in *in vitro* studies when a light dose of 100 J/cm^2 was applied with MB (50 μg/ml) after bacteria were incubated for 72 h with antimicrobial agents. The presence of antibiotics (amikacin, ciprofloxacin, or moxifloxacin) in respective concentrations of 0.5, 0.06 and 0.06 μg/ml reduced the mycobacterial cell viability by 2 $\log_{10}$ from its value in untreated cultures (Shih and Huang, 2011). The application of aPDI in combination with antibiotics in *in vitro* experiments improved the cell count reduction by a further 2 $\log_{10}$ over aPDI or antibiotic monotherapy (moxifloxacine). Another example of synergistic interactions between aPDI and antibiotic was presented by Ronqui et al. (2016) in 2016. The main subjects of their research were *S. aureus* and *E. coli.* Additionally, the appropriate treatment order for ciprofloxacin and red light with MB was verified in experiments. For *S. aureus*, when the application of ciprofloxacin (0.6 μg/ml)

preceded irradiation with MB (50 μg/ml), the viable cell count reduction was ∼5 $\log_{10}$. The same $\log_{10}$ reduction was reached for *E. coli* with an antibiotic concentration of 0.004 μg/ml and the same light dose (2.8 J/cm^2) in comparison to *E. coli* receiving monotherapy with MB. However, when ciprofloxacin (0.004 μg/ml) was administered after light irradiation in the presence of MB (50 μg/ml), the viability of *S. aureus* cells was reduced by ∼6 $\log_{10}$. No change in viable cell reduction was reported for *E. coli* upon different drug administration. The application of aPDI in combination with ciprofloxacin thus improved the reduction in viable counts for *E. coli* and *S. aureus* by 5-6 $\log_{10}$ over the reduction from light monotherapy. Another interesting application of combining aPDI with MB and antibiotic treatment was described by Oppezzo and Forte Giacobone (2017) in 2017. They used aPDI with antibiotic against persistent bacteria. Persistent microorganisms can survive the lethal effects of antibiotic treatments as a result of reversible and temporary phenotypic alteration (Oppezzo and Forte Giacobone, 2017). A *P. aeruginosa* strain was treated for as long as 180 min with visible light in the presence of a PS (MB, 15 μM), and ofloxacin was added immediately thereafter to the inoculum. The same experiment was conducted against persistent cells that tolerate ofloxacin. The antibiotic was first added to the same final concentration, and after 50 min of incubation, MB was administered. Exposure to light was initiated at minute 60 of the experiment and lasted up to 240 min. The survival fraction when ofloxacin was added at the beginning of the experiment was significantly lower at 240 min than tests when the antibiotic was added at 90 min, clearly indicating that the chemotherapeutic agent exerted a greater effect when combined with aPDI, even when treated cells were tolerant to the agent, i.e., in the case of persistent cells of *P. aeruginosa* (Oppezzo and Forte Giacobone, 2017). The application of aPDI in combination with antibiotic on persistent bacteria that tolerate ofloxacin reduced the viable counts by 6 $\log_{10}$ more than monotherapy (antibiotic treatment). The first literature evidence of a combined aPDI/antibiotics treatment against pandrug-resistant *Acinetobacter baumannii* was presented by Boluki et al. (2017) in 2017. The presented research also aimed at studying whether aPDI affects the level of expression of *pmrA* and *pmrB* genes, which are responsible for *Acinetobacter* resistance to colistin. The author stated that the exposure of *A. baumannii* to Toluidine Blue O (TBO) (50 mg/l) and light-emitting diode (LED) light for 60 and 90 s resulted in increased bacterial drug susceptibility, which was evidenced by disk-diffusion antibiograms using colistin, ceftazidime, piperacillin and doripenem. An aPDI treatment was also successful with regard to the expression of two genes responsible for colistin resistance. The expression of *pmrA* and *pmrB* was 6.1- and 4.9-fold lower, respectively, in cells treated with aPDI with TBO (0.37 mg/ml) and light (180 J/cm^2) than in untreated cells, indicating that aPDI influenced the expression of genes responsible for the production of lipid A (a constituent of lipopolysaccharide, LPS) which is strictly linked with resistance to colistin (Boluki et al., 2017). These results may suggest a mechanism underlying the synergy between antimicrobials and light therapy. Most experiments in the literature have been performed with *S. aureus,* which is the main etiological agent responsible for nosocomial, mucosal and cutaneous infections (Navidinia, 2016; Pérez-Laguna et al., 2017). Multidrug-resistant strains, i.e., methicillin-resistant *S. aureus* (MRSA), play a major role in life-threatening infections (Orrett and Land, 2006).

Rose Bengal (RB)

In 2017, Pérez-Laguna et al. (2017) published data presenting the bactericidal effectiveness of aPDI used in conjunction with mupirocin and linezolid on a reference *S. aureus* strain. Irradiation of planktonic cultures was performed using two light sources (LEDs and white metal halide (WMH) lamps) with rose bengal (RB) or methylene blue as PSs. Irradiation with the LED light (18 and 37 J/cm^2) and the WMH lamp (37 J/cm^2) was performed with tested antibiotics at two concentrations (1 and 10 μg/ml). Complete eradication was observed in all experiments but for different concentrations of PSs in combination with mupirocin and linezolid. The most pronounced results were obtained when the concentration of antibiotics was 10 μg/ml (Pérez-Laguna et al., 2017). The application of aPDI in combination with mupirocin or linezolid improved cell viability reduction by a further 2-6. $\log_{10}$ over the reduction by aPDI and antibiotic monotherapy. The amount of increased reduction in the combined treatment depended on the fluence and light source (Pérez-Laguna et al., 2017) The most current report published in 2018 indicated that combined treatment of aPDI and gentamycin was effective against *S. aureus* biofilm and planktonic cultures (Pérez-Laguna et al., 2018). Light irradiation (18 J/cm^2) of planktonic cultures administered with rose bengal (0.03 μg/ml) resulted in ∼2 $\log_{10}$ reduction in viable cells whereas the combined treatment with the presence of antibiotic in two concentrations (1 and 10 μg/ml) reached the viability reduction by ∼4 and 5 $\log_{10}$ units (Pérez-Laguna et al., 2018). Experiments conducted by Pérez-Laguna et al. (2018) proved that combined treatment was more effective in comparison to aPDI monotherapy. The first *in vitro* study that presented the influence of combining aPDI and antibiotics in planktonic cultures was described by Cahan et al. (2010) in 2010. The effectiveness of aPDI increased when conjugates of PS and antimicrobials were used, i.e., kanamycin and RB (RBLKAN) or 6-penicillic acid and RB (RBLPA) (Fiebelkorn et al., 2003; Cahan et al., 2010). Irradiation of *S. aureus* with red light (2 J/cm^2) and treatment with RB gave only 1 $\log_{10}$ reduction in viable count, whereas the presence of the conjugates RBLKAN and RBLPA (0.078 μM) decreased the viable count by 7 and 5 $\log_{10}$, respectively from its value in cultures treated only with light and a PS (Cahan et al., 2010). When *E. coli* was treated with aPDI (16 J/cm^2) and RB, the number of viable cells decreased by 3 $\log_{10}$; treatment with the RBLKAN conjugate (20 μM) decreased the viable cell count by 5 $\log_{10}$ further than monotherapy (aPDI) (Cahan et al., 2010). A combination of phototherapy with routinely applied antibiotics is a method leading to the complete eradication of this widespread pathogen. Cahan et al. (2010) proved the effectiveness of combining aPDI with antibiotics (which were administered as conjugates), improving the viable cell reduction by 5-8 $\log_{10}$ from reduction achieved with monotherapy with light and RB.

Most of the studies mentioned above reported enhanced bactericidal outcomes if combined aPDI/antimicrobial

treatments were employed. Contradictory results have been reported only by Ramìrez et al. (2015), who demonstrated antagonistic interactions when using this combined treatment against *A. baumannii*. The application of blue and white light resulted in growth inhibition zones on petri dishes with LB medium smaller than those of bacteria untreated with light. Similar effects were reported in the case of green light irradiation. This phenomenon was especially observed in the cases of two antibiotics: minocycline and tigecycline. Interestingly, inhibition zones did not change when red light was used. When another medium was used, e.g., Mueller-Hinton (M-H) or blood agar, inhibition zones for the tested antimicrobials did not differ between control and irradiated samples. The same conclusions concerning increased resistance to both antibiotics after irradiation with blue light were drawn when liquid LB medium was used. For example, MIC values changed from 0.125 to 128 μg/ml for *A. baumannii* A42 after treatment with minocycline and blue light. The mentioned investigation was performed using a few clinically important *Acinetobacter* species such as *A. radioresistens* (Ar181L), *A. nosocomialis*, *A. calcoaceticus,* and *A. soli* and *E. coli, Klebsiella pneumoniae* and *Enterobacter cloacae* and blue light irradiation affected antimicrobial susceptibility to minocycline and tigecycline (Ramìrez et al., 2015). The application of aPDI in combination with minocycline increased the MIC for *A. baumannii* strains between 16 and 128-fold over its value resulting from individual treatments.

Table 1 summarizes the published results concerning the efficacy of treating planktonic cultures *in vitro* with a combination of aPDI and antimicrobials.

In Vitro Studies: Biofilm Cultures

Most of the microorganisms grow as biofilms in their natural habitats. These biological conglomerates consist of bacterial communities existing in a matrix composed of polysaccharides, lipids, proteins and extracellular DNA (Santajit and Indrawattana, 2016). This microenvironment constitutes a mechanical and biochemical protection from PSs or antibiotics at concentrations as high as 1,000 times those that affect planktonic cultures, challenging the treatment of infections (Fu et al., 2013; Abouelfetouh et al., 2016).

Endogenously Produced Porphyrins

The first published experimental data describing the inactivation of *S. aureus* biofilm cultures with two sources of light were reported by Krespi et al. (2011); they reported the application of two different lasers and ciprofloxacin accompanied by the presence of endogenous porphyrins. A shockwave (SW) laser was used for biofilm disruption, and a near-infrared (NIR) laser was used for the eradication of bacterial cells that persist in planktonic cultures. Irradiation of *S. aureus* biofilms with both lasers and treatment with ciprofloxacin (0.3 mg/L) after the planktonic bacteria had been rinsed reduced the viable cell count by 85%, while irradiation of the bacterial cultures before rinsing gave only 66% reduction under the same experimental conditions. Furthermore, when the SW laser was used with the addition of ciprofloxacin (0.3 mg/L), biofilm and planktonic cultures were reduced by 64%, whereas biofilm cultures (after rinsing the planktonic bacteria) were reduced by 81% from the control group value (Krespi et al., 2011). The combined treatment with ciprofloxacin and both lasers was effective against *S. aureus* biofilms, reducing the biofilm cell counts by 81% from its value for untreated cells and by 44% from its value for cells receiving monotherapy with ciprofloxacin (Krespi et al., 2011). In other studies, Barra et al. (2015) and Zhang et al. (2017) presented results of experiments which were conducted with the presence of delta- aminolevulinic acid and antibiotics against *S. aureus*. Barra et al. (2015) reported a synergistic effect from the use of a combined aPDI and gentamycin treatment against three representatives belonging to *Staphylococcus*. In this study, 5-aminolevulinic acid (5-ALA) was used as a precursor for endogenous porphyrin production. A quantitative analysis of biofilms demonstrated that exposure to 2 μg/ml gentamycin followed by light irradiation (500 J/cm^2) resulted in total eradication of *Staphylococcus haemolyticus*; however, the same level of reduction was obtained after monotherapy with light (500 J/cm^2). When the *S. aureus* and *S. epidermidis* biofilms were exposed solely to light treatment (250 J/cm^2), cell survival was reduced by 40 and 60%, respectively. However, the addition of gentamycin reduced biofilm culture counts by a further 20 and 15%, respectively, from their values in biofilms receiving aPDI alone (Barra et al., 2015). The influence of aPDI and an antibiotic on MRSA and methicillin-sensitive *S. aureus* (MSSA) biofilm cultures was described by Zhang et al. (2017), who used 5-ALA during photochemical reactions. Irradiation with a light dose of 360 J/cm^2 and a subsequent 2 h incubation with 5-ALA (10 mM) gave an ∼2 $\log_{10}$ reduction in the viable count of biofilm cells (Zhang et al., 2017). However, the effectiveness of this combined treatment was strain dependent. When antibiotics were present at very high concentrations (10x MIC), less biofilm was observed when aPDI was used with netilmicin, vancomycin and cefaclor for 7, 8 and 5 of 15 biofilm cultures, respectively (Zhang et al., 2017). The highest reduction in viable cells as a result of combined treatment, in comparison to monotherapy with light, was an ∼2 $\log_{10}$ reduction in viable counts for biofilms (Zhang et al., 2017).

Exogenously Administered Porphyrin-Based PS

The results presented in section *In vitro* studies: planktonic cultures: Exogenously administered porphyrin-based PS demonstrated a synergistic interaction between DP-aPDI and oxacillin for planktonic *S. aureus* cultures, which was also shown for biofilms (Iluz et al., 2018). Experiments conducted under shear flow conditions demonstrated that irradiation with a light dose of 15 J/cm^2 and treatment with 17 μM deuteroporphyrin in the presence of oxacillin (1 μM) reduced the biofilm mass significantly more than treatment of biofilms with only oxacillin or DP-aPDI (Iluz et al., 2018). The most efficient reduction in viability came from a combination of aPDI and antibiotic applied to a MRSA biofilm (4 $\log_{10}$ greater than biofilms treated solely with light and DP). The reduction in viable cells in biofilms of MSSA or the heterogeneous vancomycin-intermediate *S. aureus* (h-VISA) receiving this treatment was ∼2 $\log_{10}$ greater than in biofilms treated with only aPDI (Iluz et al., 2018).

TABLE 1 | Summary of combined aPDI/antimicrobial treatments against planktonic cultures - *in vitro* experiments.

References	Species	Photosensitizer/ compound	Source of light	Wavelength [nm]	Intensity [mW/cm^2]	Antibiotics	Max. viability reduction in comparison to monotherapy (light)	Max. viability reduction in comparison to monotherapy (antibiotic)	Applied methodology for determination of combined effect
Reznick et al., 2013	*P. aeruginosa*	–	Nd:YAG laser-continuous	532	100	Gentamycin	8 $\log_{10}$	7.5 log	Bacterial viability (CFU/ml)
			Nd: YAG laser-Pulsed-Q-switched	532	106		8 $\log_{10}$	7.5 log	
Choi et al., 2015	*C. difficile*		UVA lamp UV801KL	315-400	No data	Tetracycline	7 $\log_{10}$	8 $\log_{10}$	Bacterial viability (CFU/ml)
Ramìrez et al., 2015	*A. baumannii*		9 LED emitting diodes (three-LED module strips emitting blue, green or red light)	No data	No data	Tigecycline minocycline	No reduction	No reduction	MIC determination
Fila et al., 2016	*P. aeruginosa* (XDR, MDR)		Single-emitter diode lamp	405	15.7	Gentamicin	No data	128 x MBC[a] reduction	Checkerboard assay
						Meropenem	No data	4 x MBC reduction	
						Ceftazidime	No data	32 x MBC reduction	
Pereira et al., 2017a,b	*E. coli S. aureus*		LED	470 625	no data	Ciprofloxacin	No data	Inhibition zone increased (≥3 mm)	Disk diffusion assay
						Norfloxacin	No data	Inhibition zone increased (3 mm)	
Jeong et al., 2017	*P. acnes*		Fiber-Coupled Laser System	671	20	Erythromycin (loaded in liposomes)	1.99 $\log_{10}$	No data	Bacterial viability (CFU/ml)
Iluz et al., 2018	*S. aureus* (MRSA)	DP	Blue light tube TL 20 W/03 ES	360-460	No data	Oxacillin	~6 $\log_{10}$	~10 $\log_{10}$	Checkerboard assay
Shih and Huang, 2011	*M. fortuitum*	MB	Metal halogen lamp	560-780	100	Ciprofloxacin	~2 $\log_{10}$	~0.5 $\log_{10}$	Bacterial viability (CFU/ml)
						Moxifloxacin	~1.5 $\log_{10}$	~2 $\log_{10}$	
						Amikacin	~1 $\log_{10}$	~1 $\log_{10}$	
Ronqui et al., 2016	*S. aureus E. coli*		Red LED	660	No data	Ciprofloxacin	~5 $\log_{10}$	No data	Bacterial viability (CFU/ml)
							~6 $\log_{10}$	No data	
Oppezzo and Forte Giacobone, 2017	*P. aeruginosa*		LED	637	44	Ofloxacin	~3 $\log_{10}$	~6 $\log_{10}$	Bacterial viability (log N/N_0)

(Continued)

TABLE 1 | Continued

References	Species	Photosensitizer/ compound	Source of light	Wavelength [nm]	Intensity [mW/cm^2]	Antibiotics	Max. viability reduction in comparison to monotherapy (light)	Max. viability reduction in comparison to monotherapy (antibiotic)	Applied methodology for determination of combined effect
Pérez-Laguna et al., 2017	*S. aureus*		LED	625 515 ± 10	7.0 5.8	Mupirocin linezolid	~3 $\log_{10}$ ~2 $\log_{10}$	~6 $\log_{10}$ ~6 $\log_{10}$	Bacterial viability (CFU/ml)
			White metal halide (WMH) lamp	420-700	90	Mupirocin linezolid	~5 $\log_{10}$ ~4 $\log_{10}$	~6 $\log_{10}$ ~6 $\log_{10}$	
	S. aureus	RB	LED	625 515 ± 10	7.0 5.8	Mupirocin linezolid	4.5 $\log_{10}$ 3 $\log_{10}$	~7 $\log_{10}$ ~7 $\log_{10}$	Bacterial viability (CFU/ml)
			White metal halide (WMH) lamp	420-700	90	Mupirocin linezolid	5.5 $\log_{10}$ 5.5 $\log_{10}$	~6.5 $\log_{10}$ ~7 $\log_{10}$	
Pérez-Laguna et al., 2018	*S. aureus*		Green LED-lamp	515 ± 10	5.8	Gentamycin	6 $\log_{10}$	~5.5 $\log_{10}$	Bacterial viability (CFU/ml)
			White metal halide (WMH) lamp	420-700	90	Gentamycin	6 $\log_{10}$	~5.5 $\log_{10}$	
Cahan et al., 2010	*S. aureus E. coli*	RB conjugate with kanamycin and 6-aminopenicillinic acid	3 x White luminescent lamps (18 W)	400-700	1.4-1.7	Kanamycin, aminopenicillanic acid	~7 $\log^b_{10}$ ~5 $\log^c_{10}$ ~5 $\log^b_{10}$	~8 $\log^b_{10}$ ~6 $\log^c_{10}$ ~6 $\log^b_{10}$	Bacterial viability (CFU/ml)
Dastgheyb et al., 2013	*S. aureus* (MRSA)	TAPP	Sylvania white light (100 W, 120 V)	No data	No data	Tobramycin	~3 $\log_{10}$	~2.5 $\log_{10}$	Checkerboard assay
Boluki et al., 2017	*A. baumannii*	TBO	LED FotoSan 630 nm LAD	630	2.000-4.000	Chloramphenicol Piperacillin ceftazidime doripenem colistin	~2 $\log_{10}$ No data	~3 $\log_{10}$ No data	Disk diffusion assay
Almeida et al., 2014	*E. coli*	TMPyP	White light lamp (OSRAM 21)	380-770	4	Ampicillin Chloramphenicol	2 $\log_{10}$ 2 $\log_{10}$	~8 $\log_{10}$ ~8 $\log_{10}$	Bacterial viability (CFU/ml)
Branco et al., 2018	*S. aureus*		OSRAM 21	380-700 400-800	4	Ampicillin Chloramphenicol Tetracycline Penicillin G Kanamycin	~8 $\log_{10}$ ~8 $\log_{10}$ ~8 $\log_{10}$ ~8 $\log_{10}$ ~8 $\log_{10}$	~7 $\log_{10}$ ~8 $\log_{10}$ ~7.3 $\log_{10}$ ~8 $\log_{10}$ ~7.4 $\log_{10}$	Bacterial viability (CFU/ml)

[a] *Minimal Bactericidal Concentration.*
[b] *Kanamycin conjugate.*
[c] *Penicillinic acid conjugate.*

In 2009, Di Poto et al. (2009) as a first reported that aPDI combined with TMP and antibiotics exhibited increased effects against biofilms in *S. aureus* cultures. Combining the irradiation of biofilms with light doses ranging from 150 to 200 J/cm^2 and the administration of 10 μM *meso*-tetrakis(n-methyl-4-pyridyl)porphine tetra tosylate (TMP), a PS, resulted in survival rates that were 30-70-fold lower than in untreated cultures. Moreover, when vancomycin was added after irradiation, the number of viable cells was reduced a further 5-fold from its value in samples treated only with aPDI. The vancomycin MIC value for biofilm cells not treated with aPDI was 10^3-10^4 higher than the MIC value after light treatment. The application of aPDI in combination with vancomycin gave a 5 $\log_{10}$ increased reduction in survival fraction over the reduction from independent treatments, which indicates the success of combining aPDI and antibiotic therapy (Di Poto et al., 2009).

Phenothiazines

A synergistic effect between aPDI and antibiotic therapy was also observed by Ronqui et al. (2016) when they used *E. coli* and *S. aureus* in both planktonic and biofilm cultures. The synergistic effect against planktonic cultures was determined for two different ciprofloxacin applications, one before and one after irradiation. In the case of biofilm cultures, the antibiotic was applied after aPDI treatment. The mode of aPDI and ciprofloxacin administration did not significantly affect the results in planktonic cultures. Irradiating *S. aureus* with a light dose of 2.8 J/cm^2 in the presence of MB (50 μg/ml) and ciprofloxacin (0.5 μg/ml) resulted in a 5 $\log_{10}$ reduction in the viable cell count. In the case of *E. coli*, only a 1 $\log_{10}$ reduction was observed when the highest concentration of antibiotic was applied (50 μg/ml) after irradiation. When ciprofloxacin was administered before the aPDI treatment (2.8 J/cm^2), the number of viable cells was reduced by 6 and 4 $\log_{10}$ for *S. aureus* and *E. coli*, respectively, but only when the highest concentration of PS was used (200 μg/ml). The order of application of antibiotic and aPDI significantly influenced the results only in case of *S. aureus*. In the case of biofilm cultures, a combined ciprofloxacin and aPDI treatment reduced the viable counts of *S. aureus* by 1 $\log_{10}$ more than treatment with only aPDI. For *E. coli* biofilms, the reduction of cell viability was 2.4 $\log_{10}$ greater than that in samples treated with an aPDI monotreatment. These results indicate the synergistic effect of the aPDI/ciprofloxacin combination against gram-positive and gram-negative microorganisms (Ronqui et al., 2016). The most effective biofilm inactivation resulted from a combination of aPDI and ciprofloxacin, which reduced viable cell counts for S. *aureus* by 1 $\log_{10}$ and for *E. coli* by 2.4 $\log_{10}$ more than monotherapy. The first published evidence stating the existence of a synergistic interaction between antimicrobials and aPDI was reported by Cassidy et al. (2012), who were focused on the *Burkholderia cepacia* complex, which is responsible for chronic cystic fibrosis pulmonary infections. For biofilm cultures, the assignment of synergy, antagonism and indifference to combined treatments was performed based on changes in a total viable count (synergy defined as a $\geq$2 $\log_{10}$ decrease in viable count; indifference $<$1 $\log_{10}$ change in viable count; antagonism defined as a $\geq$2 $\log_{10}$ increase in viable count). Planktonic cultures were also treated with light and PSs (TMP or MB). The aPDI monotherapy with MB resulted in a more than 3 $\log_{10}$ reduction for 4 of 6 tested *Burkholderia* strains (Cassidy et al., 2012). For biofilm cultures of *B. cenocepacia* (LMG 16659) and *B. multivorans* (LMG 18822), the highest reduction in viable counts was obtained when MB was used as a PS (5.09 and 4.53 $\log_{10}$, respectively). When only antibiotic was applied to biofilms, the bactericidal effect (a reduction by 3 $\log_{10}$) was determined for tobramycin in 5 of 6 tested strains and for meropenem, ciprofloxacin and piperacillin-tazobactam in 3 of 6 tested strains. For all strains and antibiotics (ceftazidime, chloramphenicol and ciprofloxacin), the combination of MB-aPDI and antibiotic reduced the viable counts more than antibiotic alone. Nevertheless, the synergistic effect was only observed for 3 isolates when a combined aPDI and chloramphenicol treatment was applied. The indifferent effect was dominant for treatments with a combination of aPDI and chemotherapeutical agents eradicating *B. cepacia* genomovars (Cassidy et al., 2012). The application of aPDI in combination with tobramycin or chloramphenicol increased the reduction in viable counts by ∼4.5 $\log_{10}$ and 4 $\log_{10}$, respectively, over the reduction achieved by independent treatments. In 2017, Kashef et al. (2017) described the application of aPDI and linezolid to *S. aureus* biofilm cultures. For this purpose, TBO and MB were used as PS. Treatment of biofilms with only aPDI reduced the bacterial burden by no more than 0.6 $\log_{10}$ for MB and 0.7 $\log_{10}$ for TBO (Kashef et al., 2017). Similar effects were observed during exposure of *S. aureus* biofilms to only linezolid (0.7 $\log_{10}$ reduction). However, a combination of antibiotic and aPDI treatment increased the reduction in viable cell counts. When *S. aureus* strains were irradiated in the presence of TBO, the biofilm cell counts were reduced by 2.1–2.6 $\log_{10}$ by a preincubation with linezolid at a concentration of 1.6 mg/ml. A treatment combining irradiation of *S. aureus* biofilms with a light dose of 54.6 J/cm^2, administration of MB and pretreatment with the same concentration of antibiotic reduced cell survival by 1.2 $\log_{10}$ (Kashef et al., 2017). A combination of aPDI with linezolid and MB increased a reduction in survival fraction by 2 $\log_{10}$ over the reduction caused by monotherapy but only against biofilms of one *S. aureus* strain (UTMC 1440).

Rose Bengal

Results by Perez-Laguna group presented in section Rose Bengal (RB) concerning the aPDI/gentamycin combined inactivation of *S. aureus* planktonic cultures were also confirmed for biofilms (Pérez-Laguna et al., 2018). The bactericidal effectiveness of light irradiation (18 J/cm^2) and rose bengal (64 μg/ml) was lower than in case of planktonic cultures and reached 3.0 $\log_{10}$ reduction in viable counts. Nevertheless, the additional administration of gentamycin to aPDI treatment resulted in enhanced bactericidal effect finally leading to 6 $\log_{10}$ reduction in survival fraction. Combined aPDI and gentamycin treatment against *S. aureus* biofilm cultures was 2-fold more effective than aPDI monotherapy (Pérez-Laguna et al., 2018).

Table 2 summarizes the published results concerning the efficacy of combined aPDI/antimicrobial treatment *in vitro* for biofilm cultures.

TABLE 2 | Summary of combined aPDI/antimicrobial treatments against biofilm cultures—*in vitro* experiments.

References	Species	Photosensitizer/ compound	Source of light	Wavelength [nm]	Intensity [mW/cm^2]	Antibiotics	Max. viability reduction in comparison to monotherapy (light)	Max. viability reduction in comparison to monotherapy (antibiotic)	Applied methodology for determination of combined effect
Krespi et al., 2011	*S. aureus* (MRSA)	–	Q-switched Nd-YAG SW laser, NIR diode laser	940	7894.7 (calculated)	Ciprofloxacin	47% (SW + NIR laser)	37%	Optical density measurement
Barra et al., 2015	*S. aureus* *S. epidermidis* *S. haemolyticus*	5-ALA	LED	630	No data	Gentamycin	20% 15% 25%	No data No data No data	Residual cell survival (CFU/ml)
Zhang et al., 2017	*S. aureus* (MRSA, MSSA)		LED	633 ± 10	No data	Netilmicin	~2 $\log_{10}$	No data	Bacterial viability (CFU/ml)
						Vancomycin	~2 $\log_{10}$	No data	
						Cefaclor	~1.5 $\log_{10}$	No data	
Iluz et al., 2018	*S. aureus* (MRSA, VISA, h-VISA)	DP	Blue light tube TL 20 W/03 ES	360-460	No data	Oxacillin	4 $\log_{10}$ 1.5 $\log_{10}$ 2 $\log_{10}$	– 5.5 $\log_{10}$ –	Bacterial viability (CFU/ml)
Ronqui et al., 2016	*S. aureus E. coli*	MB	Red LED	660	No data	Ciprofloxacin	1 $\log_{10}$ 2.4 $\log_{10}$	No data No data	Bacterial viability (CFU/ml)
Cassidy et al., 2012	*B. cepacia* *B. multivorans* *B. cenocepacia*		Paterson Lamp	635	200	Piperacillin-tazobactam	~2 $\log_{10}$ ~2.6 $\log_{10}$ –	~1 $\log_{10}$ ~2 $\log_{10}$ ~1.5 $\log_{10}$	Bacterial viability (CFU/ml)
						Meropenem	~2 $\log_{10}$ ~3 $\log_{10}$ ~0.9$\log_{10}$	1 $\log_{10}$ ~2 $\log_{10}$ ~3.1 $\log_{10}$	
						Ceftazidime	~1 $\log_{10}$ ~1.2 $\log_{10}$ ~0.4 $\log_{10}$	~2 $\log_{10}$ ~1.8 $\log_{10}$ ~2.3 $\log_{10}$	
						Tobramycin	3.5 $\log_{10}$ 4.5 $\log_{10}$ ~2.3 $\log_{10}$	~2 $\log_{10}$ 1.6 $\log_{10}$ ~0.9 $\log_{10}$	
						Chloramphenicol	~4 $\log_{10}$ ~3.1 $\log_{10}$ ~1.6 $\log_{10}$	~5 $\log_{10}$ ~3.8 $\log_{10}$ ~3.6 $\log_{10}$	
						Ciprofloxacin	~2 $\log_{10}$ ~2.8 $\log_{10}$ ~1.2 $\log_{10}$	~1.5 $\log_{10}$ ~3.3 $\log_{10}$ ~2.6 $\log_{10}$	

(Continued)

TABLE 2 | Continued

References	Species	Photosensitizer/ compound	Source of light	Wavelength [nm]	Intensity [mW/cm^2]	Antibiotics	Max. viability reduction in comparison to monotherapy (light)	Max. viability reduction in comparison to monotherapy (antibiotic)	Applied methodology for determination of combined effect
Kashef et al., 2017	*S. aureus*		Diode laser	660 630	91 26	Linezolid	~0.6 $\log_{10}$	~0.5 $\log_{10}$	Bacterial viability (CFU/ml)
Pérez-Laguna et al., 2018	*S. aureus*	RB	Green LED-lamp	515 ± 10	5.8	Gentamycin	~3.5 $\log_{10}$	No data	Bacterial viability (CFU/cm^2)
Kashef et al., 2017	*S. aureus*	TBO	Diode laser	660 630	91 26	Linezolid	~2 $\log_{10}$	~2 $\log_{10}$	Bacterial viability (CFU/ml)
Di Poto et al., 2009	*S. aureus*	TMP	Tungsten lamp	400-800	166	Vancomycin	5 $\log_{10}$	No data	Reduction of surviving fraction

In Vivo Studies

Fullerene Derivatives

Only a few published reports concern the combined use of aPDI and antimicrobials in *in vivo* experiments. On the other hand, many studies describe the bactericidal efficacy of light therapies, employing various biomaterials, *ex vivo* tissues and animal models (Dai et al., 2009). In 2010, Lu et al. (2010) described the use of a mouse model to evidence the synergistic effect between aPDI and antibiotic treatment. *In vitro* analysis confirmed the high bactericidal effectiveness of aPDI against the tested strains, *P. aeruginosa* and *Proteus mirabilis* (30 μM sensitizer, fullerene derivative BF6 and irradiation with a light dose of 10 J/cm^2). This approach reduced bacterial viability by >6 $\log_{10}$ in *P. aeruginosa* and totally eradicated *Proteus mirabilis* when the concentration of PS was 100 μM. *In vivo* experiments with fullerene derivative (180 J/cm^2) were performed using the *Proteus mirabilis* wound infection model. Application of aPDI increased animal survival by 82% from its value in untreated animals. In the *P. aeruginosa* wound infection model, the bacterial burden was reduced 95% as a result of using aPDI with BF6 and an irradiance dose of 180 J/cm^2. Despite an effective reduction of the bacterial load, *P. aeruginosa* survived the treatment, and after 3 days, all mice died from sepsis. An antibiotic treatment was used to increase the bactericidal efficacy of aPDI. A combined treatment using tobramycin (6 mg/kg each day) with light irradiation resulted in the survival of 60% of the infected animals; in contrast, 8% of mice treated with only tobramycin survived. Moreover, the infected wound was clear after 10 days, and no bacterial load was detected, indicating total eradication (Lu et al., 2010). These results are excellent evidence that indicate that combining light and antimicrobials can augment efficacy in both *in vitro* and *in vivo* studies.

Phenothiazines

Another combined treatment used in *in vivo* experiments refers to a method presented in the section *In vitro* studies: planktonic cultures: Phenothiazines *in vitro* results for the eradication of *Mycobacterium fortuitum* (Shih and Huang, 2011). White rabbits were used as a model of mycobacterial keratitis, and contact lenses infected with *M. fortuitum* cells were applied to their eyes. Treatment with only amikacin (20 mg/ml amikacin; 4 doses a day/7 days) gave an ∼1 $\log_{10}$ reduction in viable bacterial cells in corneas. However, 7 days of a combined treatment (light dose of 97.5 J/cm^2, MB 0.5% and amikacin 20 mg/ml; 4 doses a day/7 days) increased the reduction in the number of *M. fortuitum* cells in corneas by a further 0.91 $\log_{10}$ over the reduction in a group treated with only monotherapy (Shih and Huang, 2011). The use of a non-mammalian *in vivo* model was described by Chibebe Junior et al. (2013), who employed larvae of the greater wax moth, *Galleria mellonella,* in their *in vivo* experiments. A bacterial inoculum containing *Enterococcus faecium* or *E. faecalis* was injected into larvae hemocoel, and the antibacterial agents were administered within 2 h post-inoculation. PS (MB) was applied 90 min after bacterial cell injection, and irradiation with non-coherent red light of different fluences (0.9-18 J/cm^2) was performed 30 min after infected *G. mellonella* had been administered with PS. All infected larvae except those infected

with vancomycin-resistant *E. faecium* survived as a result of aPDI application. This species was next used to evaluate the bactericidal efficacy of the sequential application of aPDI and antimicrobials. Applying aPDI in combination with antibiotic made the survival rate of *G. mellonella* larger than that of organisms receiving only aPDI or vancomycin treatment (Chibebe Junior et al., 2013).

On other hand, Tanaka et al. (2013) reported in 2012 that the combined use of aPDI and antibiotics had the opposite effect. A MRSA mouse arthritis model was used in these studies. Based on previous experiments conducted by co-authors, a group of infected mice treated simultaneously with aPDI (with MB) and antibiotics were expected to yield the best results. However, therapeutic efficacy was not enhanced when linezolid was used. Nevertheless, when vancomycin was administered, the infection was reduced in intensity after 5 and 7 days. Irradiation with a light dose of 50 J/cm^2 in the presence of MB (100 μM) without the administration of antibiotic totally eradicated pathogens, and no regrowth occurred in the first day after the treatment. A combined treatment did not result in such a positive effect. Even at day 7 of the experiment, the infection and bacterial load were still observed at the infection site. The authors concluded that the failure of combined treatment could result from an inhibition of neutrophil infiltration that was driven by light and antibiotic exposure. The reduced level of inflammatory cytokines caused by antibiotic administration contributed to the inhibition of cytokines, which are present as a result of aPDI (Tanaka et al., 2013).

Table 3 summarizes the published results concerning the efficacy of combined aPDI/antimicrobial treatments for *in vivo* models.

TABLE 3 | Summary of combined aPDI/antimicrobial treatment—*in vivo* experiments.

References	Species	Model organism	Photosensitizer/ compound	Source of light	Wavelength [nm]	Intensity [mW/cm^2]	Antibiotics	Combined treatment outcome	Max. viability reduction in comparison to monotherapy (light)	Max. viability reduction in comparison to monotherapy (antibiotic)	Applied methodology for determination of combined effect
Lu et al., 2010	*P. aeruginosa* *Proteus mirabilis*	Mouse *Mus musculus*	BF6	Non-coherent Lamp, white light bandpass filter	400-700	200	Tobramycin	60% survival of mice after combined treatment	60% higher survival of mice	40% higher survival of mice	Bioluminescence imaging
Shin and Huang, 2011	*M. fortuitum*	Rabbit *Oryctolagus cuniculus*	MB	AlGaInP visible laser	650	100	Amikacin	2.1 $\log_{10}$ reduction of bacterial cells after combined treatment	No data	~1 $\log_{10}$	Quantitative analysis of viable colonies -bacterial viability (CFU)
Chibebe Junior et al., 2013	*E. faecium*	*Galleria mellonella*		Non-coherent	660 ± 15	No data	Vancomycin	6-fold increase in the time of survival of the larvae	~2.5-fold increase in the time of survival of the larvae	~1.7-fold increase in the time of survival of the larvae	Larvae viability (% survival)
Tanaka et al., 2013	*S. aureus* (MRSA)	Mouse *Mus musculus*		Xenon light source	660 ± 15	100	Vancomycin linezolid	Approx. 45-fold and 25-fold greater bioluminescence intensity (RLU)	25-fold greater RLU signal	2-fold greater RLU signal	Bioluminescence imaging
									20- fold greater RLU signal	~1-fold greater RLU signal	

Clinical Application

Endogenously Produced Porphyrins

In the case of clinical applications, aPDI, especially with the administration of 5-ALA, has been widely described in the treatment of skin infections such as acne vulgaris or psoriasis (Maisch et al., 2004). Nevertheless, the clinical studies that refer to the addition of chemotherapeutic agents during light therapy have also been reported. In 2017, four cases of patients with different skin disorders caused by *Mycobacterium* species (*M. chelonae*, *M. gordonae*, *M. gilvum,* and *M. fortuitum*) were treated with 5-ALA aPDI and antibiotic. Skin lesions of these patients were impregnated with 20% 5-ALA and irradiated with one dose of red light (100 J/cm^2). This procedure was repeated every 10 days for 3-5 sessions with a combination of antibiotics (e.g., clarithromycin, moxifloxacin hydrochloride, or amikacin). All of the patients did not present any signs of recurrence 3 months after with combined treatment (Sun et al., 2017). More evidence of the enhanced bactericidal efficacy of an aPDI and antibiotics combination was presented by one patient with multiple skin abscesses caused by *M. fortuitum*. The same light source mentioned above and 20% 5-ALA were applied to their left hand every 10 days for 4 sessions with antibiotic therapy (clarithromycin, rifampin, levofloxacin, and ethambutol hydrochloride), while the right hand received the combined treatment in only two sessions and only after treatment with

TABLE 4 | Summary of combined aPDI/antimicrobial treatments—clinical applications.

References	Case no.	Species	Photosensitizer/ compound	Source of light	Wavelength [nm]	Intensity [mW/cm^2]	Dose of light-(number of sessions)	Antibiotics	Result of combined treatment
Gong et al., 2016	1.	*M. fortuitum*	5-ALA	Laser optical fiber, Red LED-IB	635,633	– 84	– 100 J/cm^2 - (2)	Clarithromycin, rifampin, levofloxacin, ethambutol hydrochloride	Cure
Sun et al., 2017	2.	*M. chelonae*		Red LED-IB	633	84	100 J/cm^2 - (5)	Clarithromycin, moxifloxacin, amikacin, imipenem cilastatin sodium	Cure
	3.	*M. gordonae*					100 J/cm^2 - (3)	Clarithromycin, moxifloxacin, amikacin, sulfamethoxazole	Cure
	4.	*M. gilvum*					100 J/cm^2 - (3)	Moxifloxacin, clarithromycin	Cure
	5.	*M. fortuitum*					100 J/cm^2 - (4)	Amikacin, moxifloxacin, clarithromycin, rifampicin, ethambutol hydrochloride, levofloxacin	Cure
Xu et al., 2017	6.	*P. acnes*		Red Laser emitted diode LED	633	20-100	120 J/cm^2 - (4)	Minocycline	–

antibiotics. After each session of treatment, the area of lesion had significantly decreased in the left hand, while the significant effectiveness of aPDI was observed for the right hand after the first application of aPDI. Skin abscesses caused by *M. fortuitum* were effectively healed during the combined treatment, and no adverse reaction was observed after 3 months (Gong et al., 2016). Next, the bactericidal effectiveness of combined therapy using 5-ALA and antibiotics (minocycline) in acne vulgaris treatment was presented by Xu et al. (2017) in 2017. Forty eight patients were treated with minocycline (100 mg/day for 4 weeks) and once a week lesions were irradiated with light dose of 120 J/cm^2 after skin incubation with 5% 5-ALA. Second group of patients was administered only with minocycline 100 mg/day for 4 weeks. Eight weeks after the treatment the effectiveness of combined therapy was higher than in case of minocycline monotherapy reaching the 80% reduction of inflammatory lesions (the reduction of lesions in minocycline monotherapy reached 50%) (Xu et al., 2017).

Table 4 summarizes the published results concerning the efficacy of combined aPDI/antimicrobial treatments in clinical studies.

MECHANISMS UNDERLYING COMBINED APDI/ANTIMICROBIALS TREATMENT

The synergistic effects are often spectacular and indicate a high reduction in the MIC for microorganisms that had earlier manifested a significant level of resistance to an antibiotic (Fila et al., 2016). This phenomenon might result from the increased permeability of the cell envelope as a result of photoinactivation inducing its damage, which leads to greater antibiotic penetration into bacterial cells (Dai, 2017). Moreover, the enhanced bactericidal effect of antimicrobials in response to aPDI treatment might have been explained in the case of biofilm cultures by their disruption by different sources of light (e.g., shockwave laser), which could potentiate the action of antimicrobial agents (Krespi et al., 2011; Dai, 2017). Another possible mechanism underlying the synergistic effect of aPDI/antibiotic combinations is the oxidative stress that results from photochemical reactions inhibiting the expression of genes that are responsible for the antibiotic resistance; this mechanism was presented in research into a colistin-resistant *A. baumannii* strain (Boluki et al., 2017). The presence of these genes in other microorganisms (e.g., *K. pneumoniae*, *E. coli,* and *P. aeruginosa*) was also reported; this presence can explain the synergistic cooperation described for other bacterial species. The *mcr-1* gene is responsible for the modification of lipid A (phosphoethanoloamine), which leads to increased resistance to colistin, but this reaction can be reversed when the expression of this gene is inhibited (Boluki et al., 2017; Liu et al., 2017). The aPDI method probably leads to downregulated expression of these genes and the consequent reduced colistin resistance (Boluki et al., 2017). Possible explanations for aPDI/antimicrobial synergy include the ability of singlet oxygen and hydroxyl radicals to influence cellular homeostasis, the synthesis of nucleic acids (DNA and RNA),

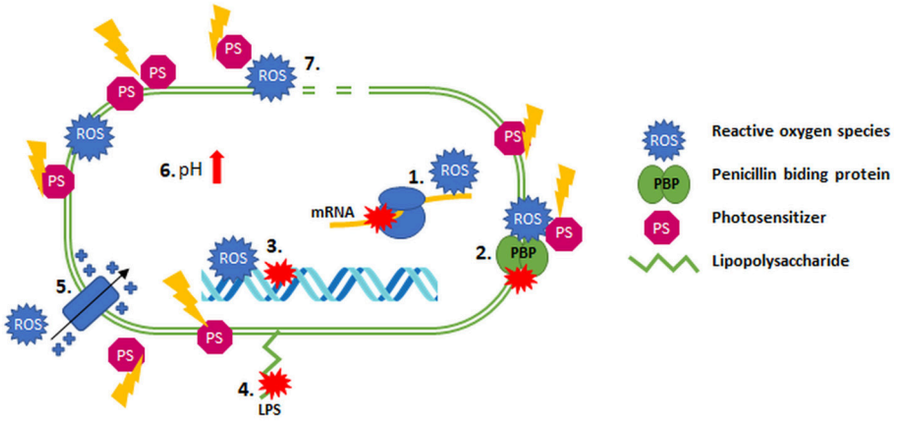

FIGURE 1 | Possible mechanisms of synergy between aPDI and antibiotics. (1) mRNA inhibition; (2) dysfunction PBP; (3) DNA damage/inhibition of synthesis; (4) modification of LPS; (5) depolarization of the membrane; (6) alkalization of the cytoplasm; (7) permeabilization of the membrane.

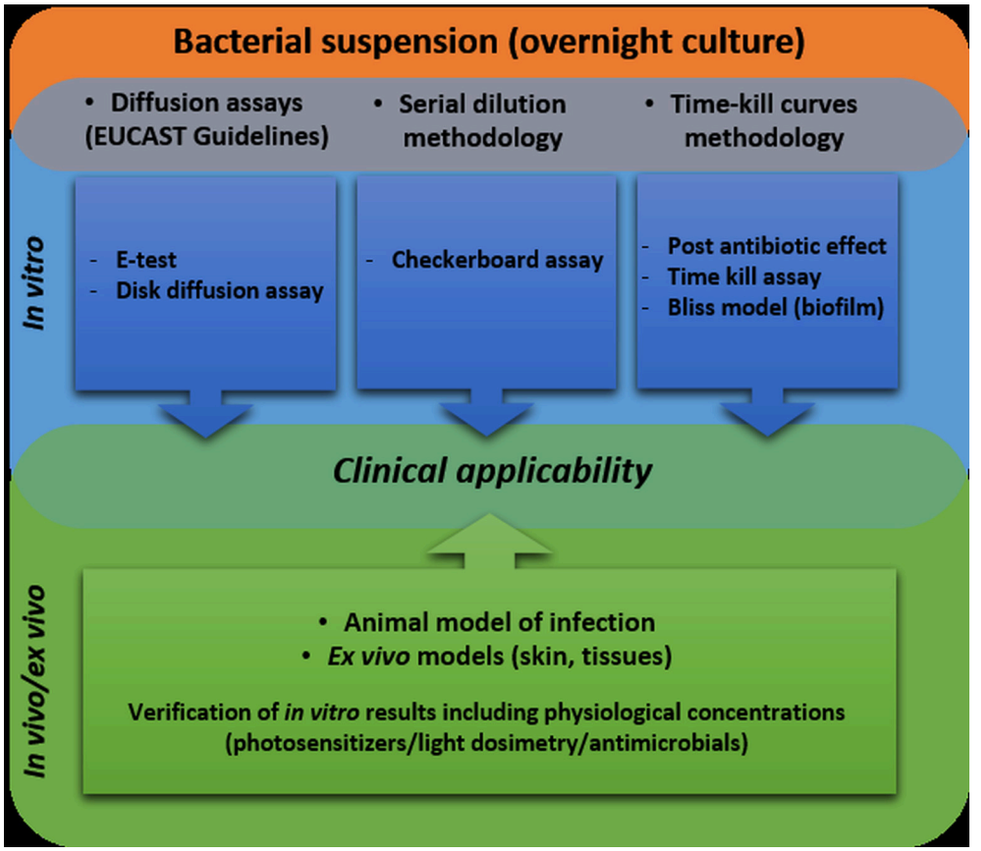

FIGURE 2 | Workflow including recommended methodologies for testing the synergy between antimicrobial photodynamic inactivation and antibiotics.

the alkalization of the cytoplasm and even the depolarization of the membrane (Pereira et al., 2017b). ROS can potentiate killing when antimicrobial agents such as ciprofloxacin, gentamycin, and fluoroquinolones are used (reported by the Brynildsen group in 2013; Brynildsen et al., 2013). In addition, the lower pH level in *Mycobacterium smegmatis* cells contributed to the increased sensitivity of bacterial cells to antibiotic treatment (Bartek et al., 2016). However, the possible connection between the production of ROS and increased pH levels is unexplained. The higher efficiencies resulting from combined treatments can be further

explained by the hypothesis that PSs (e.g., MB) at very high concentrations can be substrates for efflux pumps, which might result in a competition between PSs and antimicrobial agents that increases the uptake of antibiotic by bacterial cells after the permeabilization of their membrane (Shih and Huang, 2011). Another possible explanation for the synergy of the combined treatment originates from the ROS production that occurs as a result of both aPDI and antibiotic treatments. ROS are involved in an alternative mechanism of action of numerous antimicrobials (Van Acker and Coenye, 2017). The aPDI method could thus simply potentiate the oxidative stress induced by antibiotic administration, leading to enhanced bactericidal effects and synergy. However, the mediation of the production and importance of the production of ROS by antibiotic action has been the subject of many disputes in the literature. Many studies report the production of ROS as a mechanism employed by antibiotics (Van Acker and Coenye, 2017), but contradictory data supports the lack of ROS-related mechanisms of antibiotic action in these cases (Liu and Imlay, 2013). Another possible mechanism involves the bactericidal effectiveness of aPDI toward persistent cells. Persistent microorganisms survive lethal effects of antibiotic treatments as a result of reversible and temporary phenotypic alterations (Cohen et al., 2013; Oppezzo and Forte Giacobone, 2017). This fact should be especially considered in *in vivo* studies because the presence of persistent cells can decrease the ratio of aPDI treatment effectiveness (recurrence of infection and bacterial growth). The fact that aPDI decreased the level of persistent cells could explain the higher efficiency of antimicrobial action. Exposure of bacterial cells to white, blue or red light clearly may significantly influence their susceptibility to antibiotics. This idea may be further supported by the presence of growth factors during pathogen incubation. For example, the concentration of iron in a culture medium or the temperature of incubation can significantly influence results. This influence was demonstrated by experiments performed by Ramìrez et al. (2015) in 2015 and should be considered significant during synergy testing. The possible mechanisms underlying the synergistic effects of aPDI and antimicrobial agents are visualized in **Figure 1**.

The different aspects and factors described above are probable explanations of why antimicrobial agents work more efficiently when combined with aPDI, which was evidenced many times in the literature and discussed in this paper. The development of the alternative approach of combining aPDI and antibiotics therefore seems to be justified and desired. The combined treatment leads to not only the increased effectiveness of aPDI and antibiotics but also the decreased dosage of these chemotherapeutical agents, which may greatly slow the increasing rate of drug resistance (Dai, 2017).

CONCLUSIONS

It is worth to underline that some of the papers described within the current review were just aimed at determination if combined treatments exert enhanced antibacterial outcome, without following the standard methodology to evaluate the synergistic effect, but in most of them (18 out of 27) authors of the cited papers indicated the existence of synergy between described antibacterial approaches. Most of the reported studies describing the combined aPDI/antibiotic treatment did not comply with the imposed standards for scientific literature that aim at analyzing the synergistic interactions between different biocidal approaches. The determination of synergistic interactions, which is especially desirable in the case of antibiotics and aPDI, will be possible only when the research is consistent with the existing guidelines. Following these guidelines may also be very helpful when comparing results obtained by different scientific groups and useful in defining reliable conclusions. We also emphasize here that a gold standard for the study of procedures involving light therapy and antibiotic interactions is lacking, thus comparing results obtained during aPDI by different scientific groups is very difficult. To facilitate adequate comparisons of results, we thus believe that antimicrobial susceptibility testing (AST) (even when combined with aPDI) should be performed in accordance with EUCAST or Clinical and Laboratory Standards Institute protocols. The employment of various antimicrobials exhibiting different mechanisms of action and aiming at various cellular targets is significant for synergy testing.

In general, the increase in bacterial inactivation was observed when both therapies were used in combination. Moreover, it is significant to indicate that beside the increase in bacterial inactivation with the combined therapy, the potential reduction in treatment time or/and in reduction in bacterial resistance development to antibiotics can be also expected when the combined therapy is used due to the reduced use of antimicrobials employed in the treatment.

One could expect that taking into consideration the described within this review paper published works it should be possible to draw constructive conclusions. Unfortunately, the lack of unified research methodology conducted in accordance with the available standards makes it impossible to reliably compare the results of the work obtained by various research groups. Looking for a combined/synergistic effect of various antibiotics (in accordance with applicable standards), the majority of experimental conditions are clearly defined, e.g., what species of bacteria is to be used, which strain that is characterized with the appropriate drug resistance profile should be employed, what antibiotic concentrations justify the inference about the increased bacterial effect of combined therapy etc. In the case of research on aPDI/antimicrobials combined treatment, the above mentioned parameters are set based on researchers' assumptions and experience. One can freely choose (i) a set of species and strains of microorganisms, regardless of the profile of their drug resistance; (ii) antibiotics and their concentrations; (iii) culture conditions, i.e., media, time and temperature of incubation; (iv) bacterial inoculum etc., which makes it difficult to draw constructive conclusions. In general, it is obvious that the degree of microbial inactivation in combined aPDI/antimicrobials treatment is significantly improved in accordance to monotherapies. Nevertheless, it is worth noting that in the case of some studies, such an enhanced effect was noted for concentrations of antibiotics equal to 10xMIC (Zhang et al., 2017) or 100xMIC (Di Poto et al., 2009), and in other works the same effect was obtained for sub-MIC concentrations

equal to ½ or ¼ × MIC (Ronqui et al., 2016). It makes significant difference. Some studies present the effect of increased inactivation using wild-type (Fila et al., 2016; Kashef et al., 2017), antibiotic susceptible microbial strains (Branco et al., 2018), in other works this applies to multi-drug resistant isolates (Fila et al., 2016; Boluki et al., 2017; Iluz et al., 2018). The same problems can be identified when determining the conditions of aPDI. They largely stem from the experience and assumptions of the researchers. We hope that the indication of the above problems will convince research groups involved in a combined aPDI/antimicrobials treatment with the necessity to apply a unified research methodology based on available AST standards.

Being aware of the existing issues, we created a workflow that shows the appropriate methodologies for synergy testing (**Figure 2**). An ideal approach would be an attempt to use as many as possible *in vitro* as well as *in vivo/ex vivo* tests to assess the synergistic interaction between tested antimicrobial approaches. We are convinced that only synergistic interactions that are confirmed in the maximum number of tests have a chance to be confirmed in clinical applications. In our studies, we repeatedly faced the problem that both various antibiotics and photosensitizers could reveal synergistic interactions when studied with some tests and simultaneously, other assays indicated the lack of such interaction. Therefore, our proposal is to use the largest possible number of *in vitro* tests before going into trials in *in vivo* and clinical applications. More importantly, we strongly believe that having a system of proposed methods will improve the research linking the problem of MDR and the clinical applicability of photodynamic inactivation.

In the current paper, we attempted to raise awareness of a problem and note the possible experimental approaches that will bring us closer to a final verification of which antimicrobials interact synergistically with aPDI and finally lead to enhanced bactericidal effectiveness.

AUTHOR CONTRIBUTIONS

AW wrote the draft of the manuscript. MG has been involved in the coordination, conception, and design of the study and helped drafting manuscript. All of the authors have read and approved the final manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Chapter III

Antimicrobials Are a Photodynamic Inactivation Adjuvant for the Eradication of Extensively Drug-Resistant Acinetobacter baumannii

1. Summary of the publication

Acinetobacter baumannii is a Gram-negative coccobacillus that is responsible for hospital-acquired infections, especially in intensive care units, due to its ability to survive on various hospital surfaces and inanimate objects[2,3]. More than 45% of *A. baumannii* isolates are resistant to carbapenems, and according to the World Health Organization, *A. baumannii* is listed as a critical multidrug-resistant pathogen[76].

To conduct investigations for my doctoral thesis hypothesis, I chose the Gram-negative bacterium *A. baumannii*, as it is the first microorganism in the ESKAPE group. The results obtained from this study are presented in **publication no. 2.** The microorganisms used in this manuscript had an extensively drug-resistant (XDR) profile and were isolated from tracheal secretions and wounds from Intensive Care Unit (ICU) patients.

The first step of my research was to examine the effectiveness of aBL and aPDI with endogenous and exogenous photosensitizers (Rose Bengal) on the survival rate of two *A. baumannii* isolates (nos. 127 and 128). The effectiveness of photoinactivation was measured by calculating the changes in the survival rate of microorganisms (CFU/ml). The experiments performed indicated that XDR clinical isolates *A. baumannii* no. 127 and no. 128 are susceptible to phototreatment with aBL and aPDI with Rose Bengal; thus, the changes in the resistance profile of those pathogens could be investigated.

Applying sublethal doses of aBL caused the resistance profiles to change in both XDR isolates, which was confirmed with 4 recommended methods of synergy testing. The first two diffusion assays, E-TEST and SensiDiscs, indicated an increased susceptibility to DOX, IPM, CST, GEN, and SAM; however, interestingly, sensitization did not occur in all of the tested methods, and sensitization was different among isolates. Similar conclusions were made during the checkerboard assay, which was the third method of synergy testing. Synergies between aBL and antibiotics were observed for DOX and when aPDI was implemented as phototreatment only for CST in the case of two isolates. The last method of verification of synergy was the time-kill assay, which examined the postantibiotic effect. This method, which is based on measuring the bacterial

growth curves, revealed that most of the implemented antibiotics with aBL/aPDI influence bacterial growth, leading to delay, and this suggested that synergy and the process of sensitization occurred after aBL/aPDI was applied.

Searching for the mechanism of the identified synergies for light (aBL/aPDI) and antibiotics (CST, DOX) for *A. baumannii* isolates became an additional goal of my work in **publication no. 2**. During the photoinactivation process, ROS, such as singlet oxygen ($^1O_2$), hydroxyl radicals ($OH^{\cdot}$), superoxide anion ($O_2^{-\cdot}$) or hydrogen peroxide (H_2O_2), are produced. Moreover, it was stated multiple times in the literature that antibiotics, such as tetracyclines, can absorb photons and undergo excitation to produce ROS, indicating that synergies could be explained by this hypothesis. To verify this idea, I implemented the fluorescent probe APF 3-(p-aminophenyl) fluorescein in my research; thus, I was able to identify the hydroxyl radicals with this compound. **The obtained results indicated that upon aBL/aPDI exposure, compared to conditions without antibiotics in the cell environment, ROS are produced when antibiotics are present with exo- and endogenous photosensitizers and light.**

2. Publication

frontiers in Microbiology

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Antimicrobials Are a Photodynamic Inactivation Adjuvant for the Eradication of Extensively Drug-Resistant *Acinetobacter baumannii*

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The worldwide emergence of extensively drug resistant (XDR) *Acinetobacter baumannii* has reduced the number of antimicrobials that exert high bactericidal activity against this pathogen. This is the reason why many scientists are focusing on investigations concerning novel non-antibiotic strategies such as antimicrobial photodynamic inactivation (aPDI) or the use of antimicrobial blue light (aBL). Therefore, the aim of the current study was to screen for antimicrobial synergies of routinely used antibiotics and phototherapies, including both aPDI involving exogenously administered photosensitizing molecules, namely, rose bengal, and aBL, involving excitation of endogenously produced photoactive compounds. The synergy testing was performed in accordance with antimicrobial susceptibility testing (AST) standards, including various methodological approaches, i.e., antibiotic diffusion tests, checkerboard assays, CFU counting and the evaluation of postantibiotic effects (PAEs). We report that combining antimicrobials and aPDI/aBL treatment led to a new strategy that overcomes drug resistance in XDR *A. baumannii*, rendering this pathogen susceptible to various categories of antibiotics. Sublethal aPDI/aBL treatment in the presence of sub-MIC levels of antimicrobials effectively killed *A. baumannii* expressing drug resistance to studied antibiotics when treated with only antibiotic therapy. The susceptibility of XDR *A. baumannii* to a range of antibiotics was enhanced following sublethal aPDI/aBL. Furthermore, 3′-(*p*-aminophenyl) fluorescein (APF) testing indicated that significantly increased reactive oxygen species production upon combined treatment could explain the observed synergistic activity. This result represents a conclusive example of the synergistic activity between photodynamic inactivation and clinically used antimicrobials leading to effective eradication of XDR *A. baumannii* isolates and indicates a potent novel therapeutic approach.

Keywords: *Acinetobacter baumannii*, antimicrobials, antimicrobial blue light, photodynamic inactivation, rose bengal, synergy

INTRODUCTION

Acinetobacter baumannii is a threatening human pathogen. A key component of its pathogenicity is its outstanding capability to acquire resistance (Spellberg and Bonomo, 2014). Pan-drug resistant (PDR) strains that express resistance to all clinically available antibiotics are of particular concern (Valencia et al., 2009). A lack of effective antimicrobials has forced the need for the development of novel strategies to control *A. baumannii* infections. One of these approaches is antimicrobial photodynamic inactivation (aPDI) or antimicrobial blue light (aBL) (Nitzan et al., 1998; Dai et al., 2009; Cai et al., 2012; Huang et al., 2014; Yuan et al., 2017; Yang et al., 2018). These strategies exert high bactericidal efficacy toward various microbes regardless of antibiotic resistance. Moreover, the acquisition of resistance to such a method is unlikely due to the nature of the multi-targeted process (Maisch, 2015). Briefly, the mechanism of aPDI involves a combination of non-toxic photosensitizers (PSs) and visible light (Wainwright, 1998). In the presence of oxygen, light induces the formation of reactive oxygen species (ROS) by energy or electron transfer from the PS excited state; these ROS can oxidize numerous cell biomolecules, leading to bacterial killing (Grinholc et al., 2015).

The most recent discoveries concerning aPDI or aBL indicates that photoinactivation renders microbes susceptible to clinically used antimicrobial agents (Wozniak and Grinholc, 2018). Nevertheless, only limited studies aimed at analyzing the synergistic interactions between bactericidal approaches have complied with the standards imposed for scientific literature. Thus, it was barely possible to draw reliable conclusions indicating possible synergies between photoinactivation and antimicrobials. Photoinactivation of microorganisms can damage the cell envelope, genetic material or both simultaneously (Grinholc et al., 2015); thus, in the present study, we focused on analyzing whether the synergistic effect between aPDI/aBL and antimicrobials occurs and whether it is influenced by the administration of an exogenous PS such as rose bengal (RB) or thus of endogenously produced PSs such as porphyrins, which we excited with very intense blue light (aBL). Next, to provide accurate and reliable evidence that photoinactivation indeed renders microbes susceptible to antimicrobials and acts synergistically with antibiotics, in the current work, two XDR *A. baumannii* isolates together with numerous synergy testing assays guidelines from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical Laboratory and Standards Institute (CLSI) were employed. In addition, within the current study, the interaction of aPDI/aBL with chemotherapeutic agents (from all antibiotic classes and covering all mechanisms of action) listed by the National and European Centers for Antimicrobial Susceptibility Testing (AST) was investigated.

MATERIALS AND METHODS

Strains and Culture Conditions

Acinetobacter baumannii strains (no. 127, 128) were isolated from tracheal secretions and wounds from ICU patients at University Medical Center Freiburg. The profiles of resistance showed that both strains have XDR profiles (Magiorakos et al., 2012). *A. baumannii* strains were cultivated at 37°C in tryptic soy broth (TSB, bioMérieux, France) for 16 – 20 h under aerobic conditions in an orbital incubator (Innova 40, Brunswick, Germany) at 150 rpm. Moreover, two ATCC reference strains were used as a quality control for AST, i.e., *P. aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922.

Antimicrobial Susceptibility Testing (AST)

AST protocols followed EUCAST guidelines. The antimicrobial agents listed in **Table 1** were used (Sigma-Aldrich, Germany). For AST, ETEST^{TM} (bioMérieux, France) and Sensi-Disc^{TM} (Becton Dickinson, United States) were used. Each experiment was performed in three repetitions at different time. Interpretation of the results was performed using EUCAST breakpoint tables (Version 8.1).

Minimal Inhibitory Concentration of aBL/aPDI

The minimal inhibitory dose of aBL and aPDI was defined as the amount of light and/or PS that inhibits the growth of bacteria under experimental conditions complementary to the AST. For aBL, light with a wavelength of 411 nm was used; for aPDI treatment, the light at 515 nm and RB (5 μM) were used. Overnight bacterial cultures were adjusted in fresh MHB medium to 0.5 McFarland, 10-fold diluted and finally transferred with or without PS to a 96-well plate. For MIC estimation of aBL, light (18.2, 36.4, 54.5, 72.7, 90.9 J/cm^2) was delivered for three independent biological samples. In the case of the MIC of aPDI, light doses of 20, 40, 60, 70, 80, 90, and 100 J/cm^2 (70 mW/cm^2) were delivered to three independent biological samples. After phototreatment, plates were kept in dark at 37°C for 16 – 20 h (Termaks, Norway), followed by aBL/aPDI MIC determination via measuring the medium turbidity.

Light Sources

Illumination was performed with two light-emitting diode (LED) light sources, emitting blue (λ_{max} 411 nm, irradiance 130 mW/cm^2, full width at half maximum (FWHM) 17 nm) and green light (λ_{max} 515 nm, irradiance 70 mW/cm^2, FWHM 33 nm) (SecureMedia, Poland). The full characteristics of the light sources were recently published by Ogonowska et al. (2018).

Photosensitizer

RB [4,5,6,7-tetrachloro-2′,4′,5′,7′-tetraiodofluorescein disodium salt (Sigma-Aldrich, Germany)] was dissolved in sterile water at a 1 mM concentration and kept in the dark at −20°C. For photodynamic inactivation, RB was used in two final concentrations, 5 and 10 μM.

aBL/aPDI Treatment

Overnight bacterial cultures adjusted to 5 × 10^7 CFU/ml were transferred to a 96-well plate alone or in combination with PS. The aPDI samples treated with RB were incubated at room temperature in the dark (15 min) and then irradiated with

TABLE 1 | Minimal inhibitory concentrations for antimicrobials and light conditions.

Antibiotic target	Antimicrobial category	Antibiotic	*A. baumannii* 127	*A. baumannii* 128
			MIC [μg/ml][a]	MIC [μg/ml]
Protein synthesis (50S)	Lincosamides	Clindamycin	NR	
	Macrolides	Erythromycin	NR	
	Phenicols	Chloramphenicol	NR	
	Streptogamins	Quinupristin-dalfopristin	NR	
Protein synthesis (30S)	Aminoglycosides	Gentamycin	1024 (R)	1024 (R)
	Tetracyclines	Doxycycline (NR)	32	64
	Aminoglycosides	Tobramycin	≥16 (R)	≥16 (R)
	Glycylcyclines	Tigecycline	NR	
70S initiation complex	Oxazolidinones	Linezolid	NR	
Folic acid metabolism	Folate pathway inhibitors	Trimethoprim-sulfamethoxazole	512 (R)	1024 (R)
DNA-directed RNA polymerase	Ansamycins	Rifampicin	NR	
DNA gyrase	Fluoroquinolones	Ciprofloxacin	32 (R)	128 (R)
Cell-wall synthesis	Carbapenems	Imipenem	32 (R)	32 (R)
	Antipseudomonal penicillins + β-lactamase inhibitor	Piperacillin-tazobactam	512	256
	Extended-spectrum cephalosporins	Ceftazidime (NR)	512	256
	Penicillins + β-lactamase inhibitor	Ampicillin-sulbactam	128	64
	Extended-spectrum cephalosporins	Cefotaxime	NR	
	Carbapenems	Meropenem	≥16 (R)	≥ 16 (R)
	Phosphonic acid	Fosfomycin	NR	
	Monobactam	Aztreonam	NR	
Cell membrane	Polymyxins	Colistin	2 (S)	2 (S)
			Light dose [J/cm^2]	
Phototherapy	aBL	Blue light (411 nm)	72.7	72.7
	aPDI	Green light (515 nm) + rose bengal (5 μM)	80	90

NR, not recommended; S, susceptible; R, resistant. [a]MIC values were determined within the current study.

different light doses up to 300 J/cm^2. The aBL samples without RB were illuminated with different light doses, with the highest value being 109.1 J/cm^2. After illumination, a 10-μl aliquot was transferred to PBS, serially diluted and streaked horizontally on TSA plates. The control consisted of untreated bacteria. TSA plates were incubated at 37°C for 16 – 20 h, and then CFU were counted. Each experiment was performed in three independent replicates.

Determination of the Sublethal and Lethal Doses of aBL/aPDI

Estimation of the sublethal (reduction of 0.5 – 2 $\log_{10}$ in CFU/ml) and lethal (reduction $\geq$ 3 $\log_{10}$ in CFU/ml) photodynamic (aBL/aPDI) treatments were assessed as the changes in survival rate of treated bacteria vs. untreated bacteria (Barry and Lasner, 1979; Dodd et al., 1997; Kohanski et al., 2010; Latimer et al., 2012; Andersson and Hughes, 2014; Amin et al., 2016; Fila et al., 2018).

Synergy Testing

There are only a few approved methods for synergy testing that give reliable results, according to the *American Society for Microbiology*[1]: (i) disk diffusion assay; (ii) ETEST; (iii) time-kill assay [e.g., PAE (postantibiotic effect)]; and (iv) checkerboard assay (Doern, 2014). For experiments involving aPDI/aBL and antimicrobials, all of the recommended methods were used, and the survival rate of bacterial cells (CFU/ml) and the optical density (OD_{580}) were determined.

Diffusion Assays

Bacterial cultures (5 × 10^6 CFU/ml) were ready to use within 15 min of preparation. For experiments concerning the combined aPDI treatment, the bacterial cultures were transferred to 24-well plates with RB (1 ml per well) to receive a final PS concentration of 5 or 10 μM and then incubated for 15 min. Next, samples were treated with 515 nm light. In the case of aBL, the bacterial cultures were irradiated with 411 nm light. After phototreatment, samples were streaked on Mueller-Hinton agar plates (MHE, bioMerieux, France). Then, Sensi-Discs™ and ETESTs were placed on MHE agar plates and incubated for the next 15 min at room temperature. Next, plates were incubated at 37°C for 16 h. The control consisted of bacteria not treated with aPDI/aBL. For

[1]https://aac.asm.org/

the disk diffusion method, the synergistic effect was considered positive only when the differences in inhibition zones between the control and aBL/aPDI treatments were ≥ 2 mm. In the case of the ETEST, the synergy was defined as positive only when the MIC was 2-fold lower than the MIC for the control (untreated cells).

Checkerboard Assay

Antimicrobial blue light

The bacterial suspension (5×10^6 CFU/ml) was transferred with antibiotics to a 96-well plate to achieve the following concentrations in each row: 2 × MIC, MIC, ½ × MIC, ¼ × MIC, and 0 × MIC, indicating the control. Next, plates were incubated in the dark for 30 min, followed by separate irradiation of each column of a 96-well plate with the following doses of aBL: 0 × MIC, ⅛ × MIC, ¼ × MIC, ½ × MIC, MIC, and 2 × MIC. After exposure to aBL, plates were incubated for 16 h at 37°C. Next, the optical density was measured at 580 nm with a plate reader (Victor 1420 multilabel counter, Perkin Elmer, United States). The control group consisted of bacterial cells not treated with aBL. Each experiment was performed in three independent replicates.

Antimicrobial photodynamic inactivation

When aPDI was combined with antimicrobials, the rows of 96-well plates were filled with bacterial suspension combined with antibiotics in various concentrations (2 × MIC, MIC, ½ × MIC, ¼ × MIC, and 0 × MIC). Additionally, the wells in columns were 2-fold diluted with RB to obtain final PS concentrations of 2 × MIC, MIC, ½ × MIC, ¼ × MIC, ⅛ × MIC, and 0 × MIC. The prepared plate was incubated for 30 min in the dark, and the samples were then irradiated with 515 nm light. The plates were then incubated at 37°C for 16 h, and the optical density was measured at 580 nm. The control group consisted of a bacterial suspension administered with RB but not treated with light.

The interaction of two tested compounds was defined based on the fractional inhibitory concentration index (FIC_I), which was also defined for each tested agent separately (FIC_A, FIC_B). $FIC_{A/B}$ is equal to the MIC value of drug A/B when used in combination divided by the MIC of drug A/B alone. The FIC_I was calculated as follows: $\Sigma FIC_I = FIC_A + FIC_B$. Regarding the guidelines, the interaction between two tested factors can be defined as synergy when FIC_I is ≤ 0.5 or as antagonism when $FIC_I > 4.0$ (Odds, 2003).

Estimation of Posttreatment Survival Rate (CFU/ml)

To estimate the changes in the survival rate of tested *A. baumannii* isolates during the checkerboard assay procedure, 10 µL of each sample was transferred to PBS 30 min after light treatment, serially diluted, streaked on TSA plates and then incubated at 37°C for 16 h. Next, the colonies were counted (CFU/ml). A synergy was confirmed when the survival rates for the combination of aBL/aPDI and antibiotic were decreased in reference to the control curve, indicating the effect of light monotherapy (aPDI/aBL).

Postantibiotic Effect

Overnight culture was diluted 1:20 (v/v) in TSB medium and then pretreated for 2 h with antibiotic/photosensitizer combinations as follows: (a) aPDI/aBL (MIC), (b) antibiotic (½ MIC), and (c) aPDI/aBL (MIC) + antibiotic (½ MIC). Next, PS/antibiotics were removed by two PBS washing steps. Samples a and c were transferred to a 24-well plate and singly irradiated with the MIC dose of aPDI/aBL. The control group of bacterial cells was not-treated with light or antimicrobial agents. Subsequently, 10-µl aliquot was serially diluted, streaked on TSA plates and incubated for 16 h at 37°C. Samples loaded into 24-well plates were placed in an EnVision Multilabel Plate Reader (PerkinElmer, United States), and the optical density (λ 600 nm) was measured every 40 min for 20 h (30 repetitions).

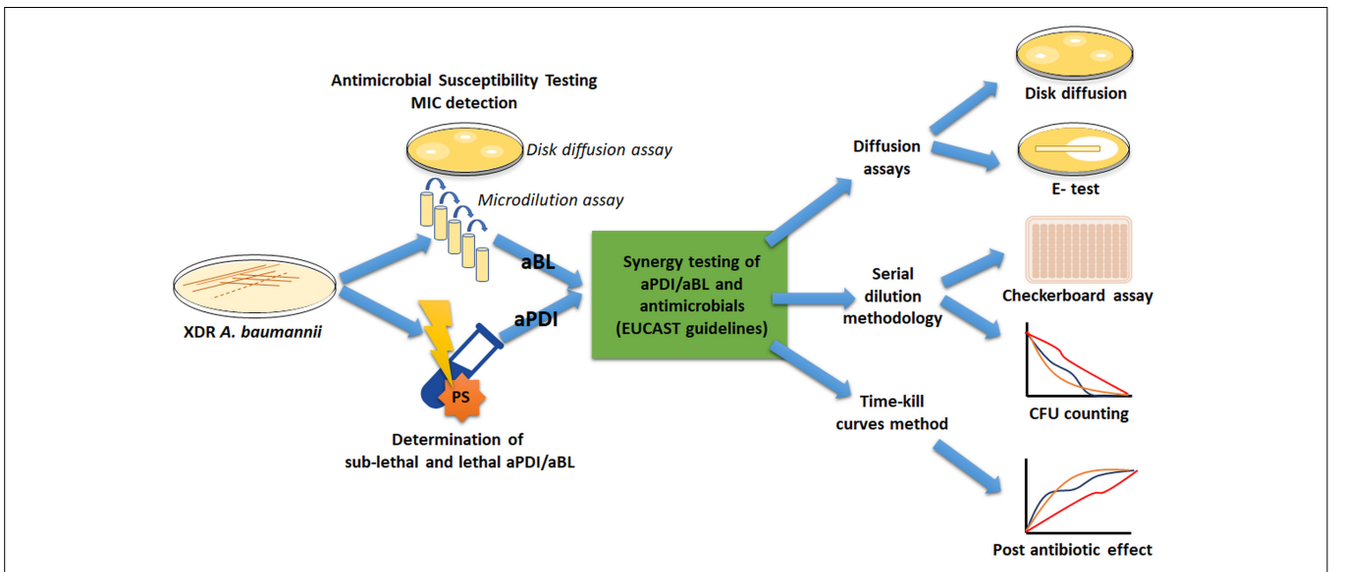

FIGURE 1 | The experimental workflow.

Next, growth curves of bacterial cells exposed to combined treatments were compared to those of the control as well as to curves representing monotreatments (MIC aPDI/aBL, ½ MIC antibiotic). The presence of PAE (time of delayed bacterial recovery during the growth vs. time curves) indicated a possible synergistic effect. The PAE can be calculated with the following formula: (Δt) $PAE = T - C$, where T is the time the bacterial population requires to reach half the maximum optical density after the tested compound (e.g., antibiotic) is removed and C is the time required for untreated cells to reach half of the maximum absorbance (Odenholt, 2001). A synergistic effect was considered significant when the PAE parameter $\Delta t \geq 3$ h and partial when 1.5 h $\leq \Delta t >$ 2.9 h. Colony counting was necessary to establish the viable cell number in tested samples and controls.

ROS Detection

Reactive oxygen species detection was performed using 3′-(p-aminophenyl) fluorescein (APF, Thermo Fisher Scientific, United States), which is a fluorescent indicator of hydroxyl radicals (•OH). In addition, APF may also be used to detect exclusively singlet oxygen when administered with DMSO (0.1%). The protocol described by Price et al. allows the quenching of the fluorescence linked to the hydroxyl radicals (Price et al., 2009). Thus, the detection of ROS was carried out both in the absence and presence of 0.1% DMSO for combined aBL/aPDI treatment, monotreatments (aPDI/aBL) and untreated, control samples. The concentrations of APF, CST, and DOX were 10 μg/ml, 2 μg/ml, and 32 μg/ml, respectively. RB was used at a concentration of 5 μM. Combined samples were prepared in PBS in black 96-well plates and then incubated for 15 min in the dark at room temperature. Next, a 515 nm light dose of 90 J/cm^2 was delivered. In the case of aBL, samples were exposed to a 411 nm light dose of 90.1 J/cm^2. Fluorescence measurements were performed immediately after aPDI/aBL irradiation with an EnVision Multilabel Plate Reader (PerkinElmer, United States) at emission/excitation wavelengths of 490/515 nm.

RESULTS

Experimental Workflow

To meet the international standards for synergy testing, numerous official AST procedures were employed to ensure that reliable conclusions were drawn; thus, we introduce a general workflow diagram to facilitate following the obtained results (**Figure 1**).

Two XDR *A. baumannii* isolates (nos. 127 and 128) were employed. The first stage was to characterize the drug resistance profile of *A. baumannii* isolates, followed by antimicrobial MIC evaluation. Next, overnight bacterial cultures were treated with different light doses and/or PS (i.e., RB) concentrations to determine both lethal and sublethal photo treatment conditions. The identification of sublethal doses was required because adequate synergy testing needs to be performed with living cells. Afterward, combined sublethal aPDI/aBL and sub-MIC doses of antimicrobials were investigated to find possible synergies. For proper implementation of synergy testing, various standard methodologies were used.

Identification of Lethal and Sublethal Treatments

Adequate synergy testing required the preliminary characteristics of the studied *A. baumannii* isolates regarding their drug resistance profiles as well as their response to aPDI and aBL treatments. Detailed characteristics are presented in **Table 1**.

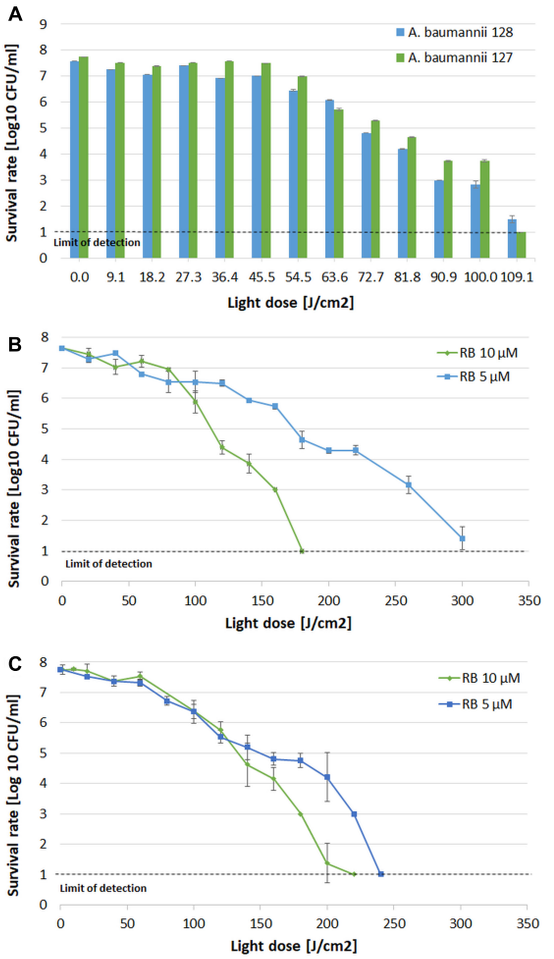

FIGURE 2 | Influence of aBL and aPDI on *A. baumannii* strains. **(A)** aBL treatment of *A. baumannii* isolates. Light doses ranging from 9.1 to 109.1 J/cm^2 (irradiance 130 mW/cm^2, irradiation time from 79 to 952 s, λ 411 nm) were applied to two XDR strains (blue bars – strain no. 128; green bars – strain no. 127. **(B)** aPDI treatment of *A. baumannii* strain no. 128. Light doses ranging from 20 to 300 J/cm^2 (irradiance 70 mW/cm^2, irradiation time from 303 to 4545 s, λ 515 nm) and two rose bengal concentrations were tested (green diamonds – 10 μM; blue squares – 5 μM). **(C)** aPDI treatment of *A. baumannii* strain no. 127. Light doses ranging from 20 to 240 J/cm^2 (irradiance 70 mW/cm^2, irradiation time from 303 to 3636 s; λ 515 nm) and two rose bengal concentrations were tested (green diamonds – 10 μM; blue squares – 5 μM). The detection limit was 10 CFU/ml. The values are the means of three separate experiments. Values were combined by a line for better visualization of the data.

TABLE 2 | Sublethal aPDI impacts on *A. baumannii* drug susceptibility.

		aPDI (λ 515 nm)											
	Antibiotic	**Control**		**Light (5 J/cm^2) + RB (10 μM)**		**Light (10 J/cm^2) + RB (10 μM)**		**Light (11.25 J/cm^2) + RB (5 μM)**		**Light (18.0 J/cm^2) + RB (5 μM)**		**Light (22.5 J/cm^2) + RB (5 μM)**	
		Disk diffusion [mm]	***E*-test [μg/ml]**	**Disk diffusion [mm]**	***E*-test [μg/ml]**	**Disk diffusion [mm]**	***E*-test [μg/ml]**	**Disk diffusion [mm]**	***E*-test [μg/ml]**	**Disk diffusion [mm]**	***E*-test [μg/ml]**	**Disk diffusion [mm]**	***E*-test [μg/ml]**
A. baumannii 127	GEN	6 (R)	≥256 (R)	6 (R)	≥256 (R)	6 (R)	≥256 (R)	6 (R)	≥256 (R)	6 (R)	≥256 (R)	6 (R)	24 (R)
	DOX (NR)	6	128	6	128	7.4	128	7.3	32	9.1	32	10.4	6
	SXT	6 (R)	≥32 (R)	6 (R)	≥32 (R)	6 (R)	≥32 (R)	6 (R)	≥32 (R)	6 (R)	≥32 (R)	6 (R)	≥32 (R)
	CIP	6 (R)	≥32 (R)	6 (R)	≥32 (R)	6 (R)	≥32 (R)	6 (R)	≥32 (R)	6 (R)	≥32 (R)	6 (R)	≥32 (R)
	IPM	6.8 (R)	≥32 (R)	7.9 (R)	24 (R)	10 (R)	≥32 (R)	10.2 (R)	≥32 (R)	11.6 (R)	12 (R)	12.2 (R)	8 (R)
	TZP	6	≥256	6	≥256	6	≥256	6.9	≥256	8.4	≥256	6.7	≥256
	CAZ (NR)	6	≥256	6	≥256	6	≥256	6	≥256	6	≥256	6	≥256
	SAM	6.5	48	7.1	24	7.7	32	9.7	32	10.0	12	11.2	12
	CST	14.0	0.094 (S)	14.6	0.125 (S)	14.3	0.125 (S)	13.9	0.125 (S)	16.7	0.125 (S)	18.3	0.094 (S)
	Antibiotic	**Control**		**Light (5 J/cm^2) + RB (10 μM)**		**Light (10 J/cm^2) + RB (10 μM)**		**Light (12.5 J/cm^2) + RB (5 μM)**		**Light (20.0 J/cm^2) + RB (5 μM)**		**Light (22.5 J/cm^2) + RB (5 μM)**	
A. baumannii 128	GEN	6 (R)	≥256 (R)	6 (R)	≥256 (R)	6 (R)	≥256	6 (R)	≥256 (R)	6 (R)	128 (R)	6 (R)	64 (R)
	DOX (NR)	6.6	48	6.2	24	7.1	12	7.8	24	9.1	8	11.6	6
	SXT	6 (R)	≥32 (R)	6 (R)	≥32 (R)	6 (R)	≥32 (R)	6 (R)	≥32 (R)	6 (R)	≥32 (R)	6 (R)	≥32 (R)
	CIP	6 (R)	≥32 (R)	6 (R)	≥32 (R)	6 (R)	≥32	6 (R)	≥32 (R)	6.1 (R)	12 (R)	6 (R)	32 (R)
	IPM	7.3 (R)	≥32 (R)	8.3 (R)	≥32 (R)	8.2 (R)	12	8.9 (R)	≥32 (R)	9.8 (R)	8 (S/R)	14 (R)	2 (S)
	TZP	6	≥256	6	≥256	6	≥256	6	≥256	6.1	≥256	10.0	≥256
	CAZ (NR)	6	≥256	6	≥256	6	≥256	6	≥ 256	6	≥256	6	64
	SAM	8.0	48	9.2	44	7.4	16	8.8	32	9.1	24	14.8	12
	CST	13.7	0.094 (S)	15	0.19 (S)	13.6	0.094 (S)	14.1	0.094 (S)	15.9	0.094 (S)	16.9	0.094 (S)

NR, not recommended; R, resistant; S, susceptible; CAZ, ceftazidime; CIP, ciprofloxacin; CST, colistin; DOX, doxycycline; GEN, gentamycin; IPM, imipenem; SAM, ampicillin-sulbactam; SXT, trimethoprim-sulfamethoxazole; TZP, piperacillin-tazobactam.

The results indicated that both endogenously- (aBL) and exogenously administered PS (aPDI)-based phototreatments could reach high bactericidal efficacy, leading to a reduction in cell viability by ≥6 $\log_{10}$ units (**Figure 2**).

In the case of aBL (**Figure 2A**), the sublethal light dose was defined as 63.6 J/cm^2, which reduced bacterial viability by 1.5 and 2 $\log_{10}$ (in the cases of *A. baumannii* no. 128 and 127, respectively). When considering aPDI treatment with two studied RB concentrations vs. light doses, different combinations could define sublethal conditions (**Figures 2B,C**). In the case of 5 μM RB, the sublethal aPDI could be defined as 160 and 100 J/cm^2, resulting in cell viability reduction by 1.9 and 1.4 $\log_{10}$ units (in the cases of *A. baumannii* no. 128 and no. 127, respectively). When 10 μM RB was used, the sublethal light dose was defined as 100 and 120 J/cm^2, which led to viable cell reduction by 1.7 and 2 $\log_{10}$ units (for *A. baumannii* no. 128 and 127, respectively).

Diffusion Based Assays for Synergy Testing

First-line screening for potent synergies of antimicrobials was performed by employing diffusion-based techniques. The results indicated that in the case of both photobased treatments, the employment of sublethal aBL/aPDI conditions influenced *A. baumannii* susceptibility to numerous routinely used antimicrobials, resulting in larger growth inhibition zones (in the case of the disk-diffusion assay) and decreased MICs (for the ETEST) (**Tables 2**, **3**). Though the impact of aPDI on *A. baumannii* drug susceptibility was observed in the cases of numerous antimicrobial agents (i.e., gentamycin, doxycycline, imipenem, ampicillin/sulbactam, and colistin), the most pronounced effect was reported for gentamycin; in this case, sublethal aPDI treatment reduced the MIC values 42-fold for *A. baumannii* no. 127 isolate from 1024 μg/ml (as stated in **Table 1**) to 24 μg/ml. Similar results were reported for aBL treatment (**Table 3**). Sublethal aBL levels resulted in larger growth inhibition zones for the *A. baumannii* no. 128 isolate (i.e., from 7.3 to 8.1 mm and from 13.7 to 14.2 mm, in the cases of imipenem and colistin, respectively) and in case of *A. baumannii* no. 127 decreased MICs (i.e., reduction in MIC from 128 to 64 μg/ml and from 48 to 32 μg/ml in the cases of doxycycline and ampicillin/sulbactam, respectively) (**Table 3**).

Serial Dilution Methodology for Synergy Testing

To further confirm and/or detect other synergies, the serial dilution methodology was employed. **Figure 3** exemplifies the checkerboard assay. The obtained results indicate that with the employment of phototreatment/antimicrobial combinations,

TABLE 3 | Sublethal and lethal aBL impact on *A. baumannii* drug susceptibility.

		aBL (λ 411 nm)								
	Antibiotic	**Control**		**36.4 J/cm^2**	**54.5 J/cm^2**	**72.7 J/cm^2**	**90.9 J/cm^2**		**109.1 J/cm^2**	
		Disk diffusion [mm]	***E*-test [μg/ml]**	**Disk diffusion [mm]**	**Disk diffusion [mm]**	**Disk diffusion [mm]**	**Disk diffusion [mm]**	***E*-test [μg/ml]**	**Disk diffusion [mm]**	***E*-test [μg/ml]**
A. baumannii 127	GEN	6 (R)	≥256 (R)	6 (R)	6 (R)	6 (R)	6 (R)	≥256 (R)	6 (R)	≥256 (R)
	DOX (NR)	6	128	6.5	6	6	6	64	6	48
	SXT	6 (R)	≥32 (R)	6 (R)	6 (R)	6 (R)	6 (R)	≥32 (R)	6 (R)	≥32 (R)
	CIP	6 (R)	≥32 (R)	6 (R)	6 (R)	6 (R)	6 (R)	≥32 (R)	6 (R)	≥32 (R)
	IPM	6.8 (R)	≥32 (R)	8.2	8.0	7.6	7.7	≥32	8.6 (R)	≥32 (R)
	TZP	6	≥256	6	6	6	6	≥256	6	≥256
	CAZ (NR)	6	≥256	6	6	6	6	≥256	6	≥256
	SAM	6.5	48	7.8	7.3	7.5	8.1	32	6	24
	CST	14.0	0.094 (S)	14.0	14.3	14.1	15.4	0.125 (S)	16	0.125 (S)
A. baumannii 128	GEN	6 (R)		≥256 (R)	6 (R)	6 (R)	6 (R)		6 (R)	
	DOX (NR)	6.6	48	6.1	7.8	7.8	7.1	256 (R)	6 (R)	256 (R)
	SXT	6 (R)	≥32 (R)	6 (R)	6 (R)	6 (R)	6 (R)	≥32	7.2	16
	CIP	6 (R)	≥32 (R)	6 (R)	6 (R)	6	6	≥32 (R)	6 (R)	≥32 (R)
	IPM	7.3 (R)	≥32 (R)	7.8 (R)	8.1 (R)	8.2 (R)	8.8 (R)	≥32	6 (R)	≥32 (R)
	TZP	6	≥256	6	6	6	6	≥32 (R)	9.1 (R)	≥32 (R)
	CAZ (NR)	6	≥256	6	6	6	6	≥256	6	≥256
	SAM	8.0	48	7.6	7.2	7.5	7.2	≥256	6	≥256
	CST	13.7	0.094 (S)	13.8	14.2	14.7	13.9	48	8.9	32

NR, not recommended; R, resistant; S, susceptible; CAZ, ceftazidime; CIP, ciprofloxacin; CST, colistin; DOX, doxycycline; GEN, gentamycin; IPM, imipenem; SAM, ampicillin-sulbactam; SXT, trimethoprim-sulfamethoxazole; TZP, piperacillin-tazobactam.

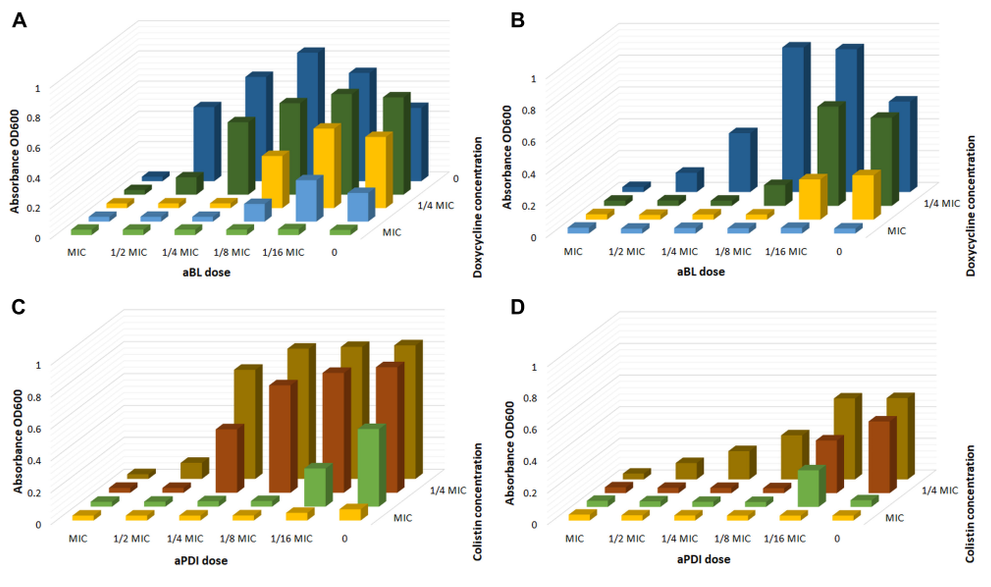

FIGURE 3 | Checkerboard analysis. **(A)** Checkerboard analysis of aBL/doxycycline combined treatment for *A. baumannii* no. 128. **(B)** Checkerboard analysis of aBL/doxycycline treatment for *A. baumannii* no. 127. **(C)** Checkerboard analysis of aPDI/colistin combined treatment for *A. baumannii* no. 128. **(D)** Checkerboard analysis of aPDI/colistin treatment for *A. baumannii* no. 127.

successful *A. baumannii* eradication was achieved with the use of as little as 1/4 of the MIC of doxycycline and colistin together with 1/4 of the MIC of aBL and 1/8 of the MIC of aPDI (**Figure 3**).

Checkerboard FIC calculations confirmed the existence of synergistic interactions when phototreatment was used in combination with doxycycline, imipenem or colistin;

TABLE 4 | Checkerboard FIC calculation.

Antibiotic	*A. baumannii* 127		*A. baumannii* 128	
	aBL	aPDI	aBL	aPDI
GEN	>0.5[a]	>0.5	>0.5	>0.5
DOX	**0.375**	>0.5	**0.5**	**0.375**
SXT	**0.5**	>0.5	>0.5	>0.5
CIP	>0.5	>0.5	>0.5	>0.5
IPM	>0.5	>0.5	>0.5	**0.375**
TZP	>0.5	>0.5	>0.5	>0.5
CAZ	>0.5	>0.5	>0.5	>0.5
SAM	>0.5	>0.5	>0.5	>0.5
CST	>0.5	**0.375**	>0.5	**0.375**

Bold indicates possible synergistic interactions. [a]FICI index; FICI = $FIC_a + FIC_b$; CAZ, ceftazidime; CIP, ciprofloxacin; CST, colistin; DOX, doxycycline; GEN, gentamycin; IPM, imipenem; SAM, ampicillin-sulbactam; SXT, trimethoprim-sulfamethoxazole; TZP, piperacillin-tazobactam.

furthermore, the results indicated a synergy between aBL and trimethoprim/sulfamethoxazole treatments (**Table 4**).

Along with checkerboard analysis, direct post-treatment probing and CFU counting were performed. The obtained results are exemplified by aBL/doxycycline and aPDI/colistin combinations (**Figure 4**). The obtained results clearly indicate that combined treatment led to more effective bacterial killing of both *A. baumannii* isolates with the employment of decreased antibiotic concentrations as well as lower aBL/aPDI doses (**Figure 4**). For all other combinations, the obtained results are summarized in **Table 6**, where all data from synergy testing via all included assays are shown. If characteristic "shifting" of bacterial survival rate curves was reported, the combination was marked with a "+" to indicate possible synergistic interaction.

Time-Kill Curves for Synergy Testing

Finally, possible synergies were confirmed and newly detected with PAE testing. The characteristic "shifting" of growth curves of *A. baumannii* pre-exposed with a combined treatment indicates that this approach delayed bacterial recovery (**Figure 5**). **Figure 5** only show the results for aBL/doxycycline and aPDI/colistin combinations. In the case of *A. baumannii* no. 127, it is clear that only combined aBL/DOX and aPDI/CST treatment leads to delayed bacterial recovery resulting in PAEs of approximately 330 and 210 min, respectively (**Figures 5B,D**). In the case of *A. baumannii* no. 128, a clear indication of synergy was observed only for the aBL/DOX combination (**Figure 5A**); however, only a limited PAE was found for the aPDI and CST combined treatment (**Figure 5C**). This case exemplifies ambiguous results, which were marked "+/−" in the summary tables (**Tables 5**, **6**). For all other combinations, the results are summarized in **Tables 5**, **6**. If characteristic "shifting" of bacterial growth curves was reported, the combination was marked with "+" to indicate a possible synergistic interaction.

The most pronounced indication concerned a aBL/aPDI and DOX combined treatment (**Table 5**). Clear "shifting" and a significant Δt (PAE) were observed for exposure to the combination of DOX and either aBL or aPDI. The same result was reported in the case of aBL/SAM and aBL/IPM combinations. Other inconclusive results were found for aBL treatment combined with GEN, SXT or CIP. In these cases, the

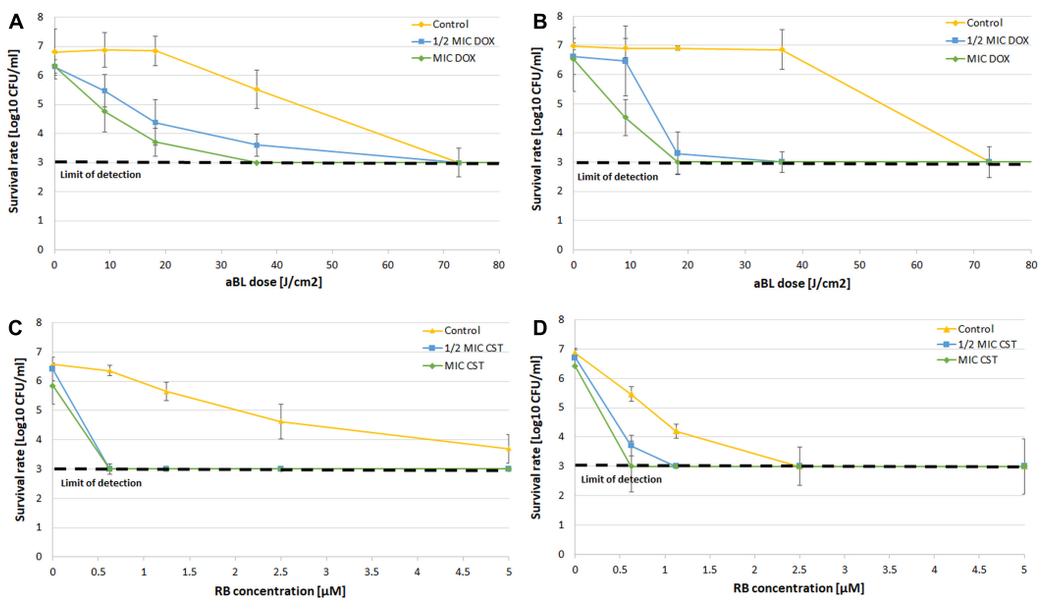

FIGURE 4 | CFU counting after combined treatment. **(A)** Survival rate analysis of aBL/doxycycline combined treatment for *A. baumannii* no. 128. **(B)** Survival rate analysis of aBL/doxycycline treatment for *A. baumannii* no. 127. **(C)** Survival rate analysis of aPDI/colistin combined treatment for *A. baumannii* no. 128. **(D)** Survival rate analysis of aPDI/colistin treatment for *A. baumannii* no. 127. The detection limit was 1000 CFU/ml. The values are the means of three separate experiments. Control constituted of aBL or aPDI monotherapy, with no antibiotic administration. Values were combined by a line for better visualization of the data.

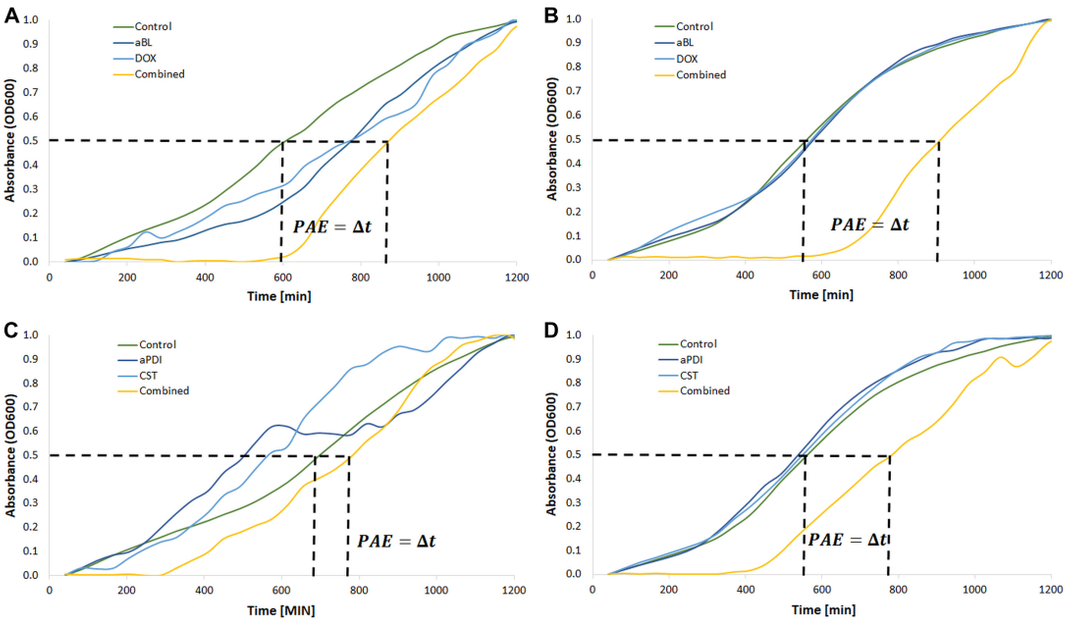

FIGURE 5 | Postantibiotic effect testing. **(A)** Growth curve analysis of aBL/doxycycline combined treatment for *A. baumannii* no. 128. **(B)** Growth curve analysis of aBL/doxycycline treatment for *A. baumannii* no. 127. **(C)** Growth curve analysis of aPDI/colistin combined treatment for *A. baumannii* no. 128. **(D)** Growth curve analysis of aPDI/colistin treatment for *A. baumannii* no. 127. Phototreatments (aBL and aPDI) were employed with MIC doses. Antibiotics (doxycycline, DOX; colistin, CST) were administered at ½ MIC. Only one representative curve was presented.

indisputable results were observed for only one of two studied *A. baumannii* isolates. The same was observed for the aPDI and SXT combination. Significant strain dependence was reported for aBL/CST, aPDI/GEN and aPDI/CST combined treatments.

Possible aBL/aPDI and Antimicrobials Synergies

All data collected within the current study are summarized in **Table 6** to provide better insight into the possible synergies (**Table 6**). The most potent strain-independent synergies are marked with gray.

Some antimicrobials (i.e., DOX) interact synergistically with both aBL and aPDI treatments, and other interactions were observed when endogenously produced or exogenously administered PSs were involved in photodynamic inactivation (i.e., IPM and SAM with aBL treatment and CST with aPDI) (**Table 6**). Interestingly, some antimicrobials had synergies with phototreatments, but they were strain-dependent suggesting that no general conclusion concerning the possible synergy could be drawn (i.e., SXT and CST when combined with aBL for *A. baumannii* nos. 127 and 128, respectively, or IPM and SAM interacting synergistically with aPDI in case of *A. baumannii* no. 128) (**Table 6**). As expected, various synergy testing methods detected different synergistic interactions, indicating that the employment of various techniques is mandatory.

TABLE 5 | Postantibiotic effect on *A. baumannii* clinical isolates.

Antibiotic	*A. baumannii* 127		*A. baumannii* 128	
	aBL	aPDI	aBL	aPDI
GEN	+	+	+/–	–
DOX	+	+	+	+
SXT	+	+	+/–	+/–
CIP	+	–	+/–	+/–
IPM	+	–	+	+/–
TZP	–	+/–	–	–
CAZ	–	+/–	–	–
SAM	+	+/–	+	–
CST	–	+	+	–

(+) synergistic effect; (+/–) partial synergy; (–) no synergistic effect. CAZ, ceftazidime; CIP, ciprofloxacin; CST, colistin; DOX, doxycycline; GEN, gentamycin; IPM, imipenem; SAM, ampicillin-sulbactam; SXT, trimethoprim-sulfamethoxazole; TZP, piperacillin-tazobactam.

Increased ROS Generation Could Explain the Mechanism Underlying the Observed Synergies

To investigate whether increased ROS production is responsible for the synergies between aBL/aPDI and antimicrobials, the level of ROS generated upon combined treatment was examined for four combined treatments (**Figure 6**).

As expected, effective ROS generation was observed upon only photodynamic treatment. Interestingly, the level of generated ROS was significantly increased when a combined treatment was employed (**Figure 6**). The most pronounced increase in ROS generation was reported for the aBL and DOX combination

TABLE 6 | Summarized results of synergy testing.

Antibiotic	*A. baumannii* 127									
	aBL					aPDI				
	E – test	Disk diffusion	Checkerboard assay	Survival rate	Post antibiotic effect	*E*- test	Disk diffusion	Checkerboard assay	Survival rate	Post antibiotic effect
GEN	+	−	−	+/−	+	−	−	−	−	+
DOX	+	+	+	+	+	+	+	−	+	+
SXT	−	−	+	+	+	−	−	−	−	+
CIP	−	−	−	+	+	−	−	−	−	−
IPM	+	+	−	+/−	+	+	−	−	−	−
TZP	−	−	−	+	+	−	−	−	−	+/−
CAZ	−	−	−	+/−	−	−	−	−	−	+/−
SAM	+	+	−	−	+	−	+	−	−	+/−
CST	−	−	−	+	−	−	+	+	+	+
	A. baumannii 128									
GEN	−	+	−	−	+/−	+	−	−	−	−
DOX	+	−	+	+	+	+	+	+	+	+
SXT	−	−	−	−	+/−	−	−	−	−	+/−
CIP	−	−	−	−	+/−	−	−	−	−	+/−
IPM	−	+	−	+	+	+	+	+	−	+/−
TZP	−	−	−	−	−	−	+	−	−	−
CAZ	−	−	−	−	−	+	−	−	−	−
SAM	+	−	−	+/−	+	+	+	−	+/−	−
CST	−	+	−	+	+	−	+	+	+	+/−

CAZ, ceftazidime; CIP, ciprofloxacin; CST, colistin; DOX, doxycycline; GEN, gentamycin; IPM, imipenem; SAM, ampicillin-sulbactam; SXT, trimethoprim-sulfamethoxazole; TZP, piperacillin-tazobactam.

(**Figure 6A**), but a similar effect was observed for all studied combined approaches. This discovery supported one of several possible mechanisms underlying the observed synergies.

DISCUSSION

The most recent discoveries concerning combinations of aBL/aPDI and antibiotics indicate that photoinactivation sensitizes microorganisms to routinely used antimicrobials [most recently reviewed by Wozniak and Grinholc (2018)]. If it is confirmed with the employment of approved methodology and translated into *in vivo* and clinical applications, this approach could improve the clinical outcome of i.e., wound infections caused by MDR pathogens and might reduce usage of antibiotics in the long term. Only a limited number of methodologies are adequate for investigation of synergistic interactions between various antibacterial approaches. This recommendation explains the employment of all the indicated methods within the current study. Moreover, the current study is the first to describe aBL/aPDI interactions with antimicrobials covering all antibiotic categories as well as all antimicrobial mechanisms of action.

The significant bactericidal efficacy of both aBL and aPDI against *A. baumannii* was repeatedly reported in numerous published *in vitro* and *in vivo* studies (Dai et al., 2009; Huang et al., 2014; Maisch et al., 2014; Zhang et al., 2014; Yuan et al., 2017; Yang et al., 2018). However, the first published evidence of a combined aPDI/antibiotic approach being used against pandrug-resistant *A. baumannii* was presented by Boluki et al. (2017). Their research evidenced that aPDI affects the expression level of genes responsible for *Acinetobacter* resistance to colistin, i.e., *pmrA* and *pmrB*. In the case of other microbial species, the enhanced bacterial killing of the combined approach was frequently reported using *in vitro* planktonic (Almeida et al., 2014; Fila et al., 2017; Branco et al., 2018) biofilm (Perez-Laguna et al., 2017; Zhang et al., 2017) and *in vivo* models (Lu et al., 2010; Chibebe et al., 2013). The mentioned studies indicate that employing various culture media and experimental conditions, one could report different results; thus, it is mandatory to utilize standardized and approved methodology for synergy testing, which was the issue of prime importance within the current study.

The mechanisms underlying aBL/aPDI and antimicrobial interactions have never been identified, although some hypotheses have already been drawn (Wozniak and Grinholc, 2018). First, the synergistic effects may result from the increased permeability of the cell envelope resulting from aBL/aPDI-induced damage of this structure, which leads to increased antibiotic uptake into bacterial cells (Hewelt-Belka et al., 2016; Kossakowska-Zwierucho et al., 2016; Dai, 2017). Another possible mechanism could be the oxidative stress resulting from photochemical reactions and inhibiting the expression

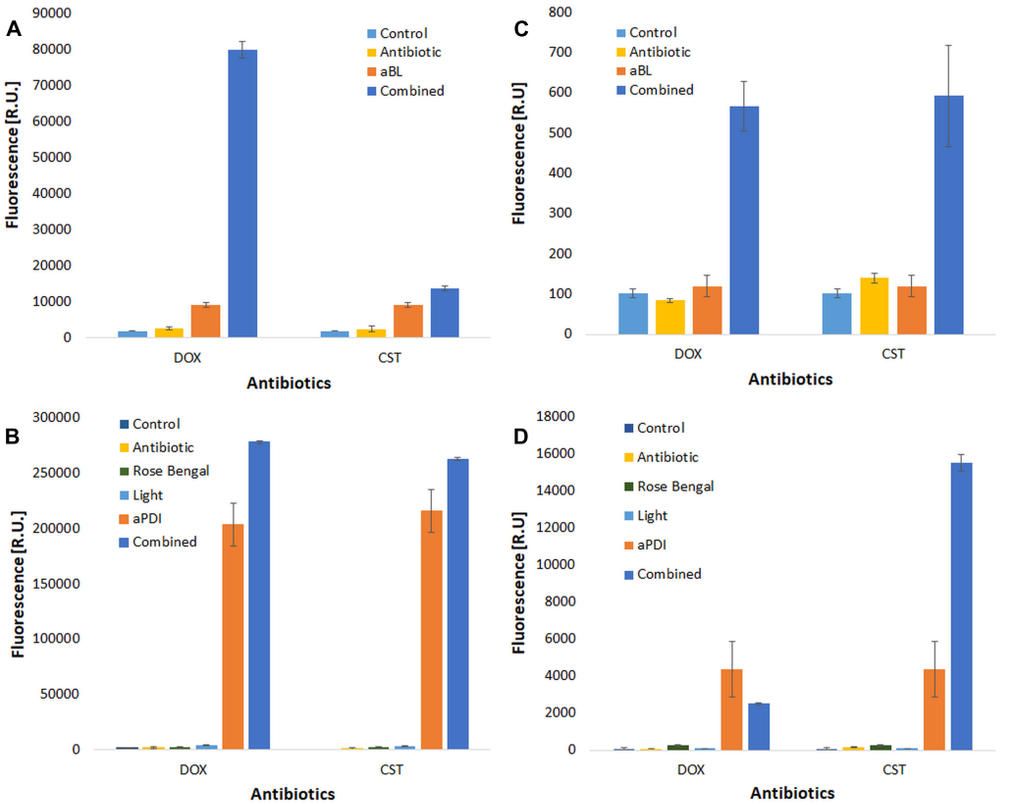

FIGURE 6 | ROS detection. ROS detection was performed for two antimicrobials (doxycycline, DOX and colistin, CST). Cell-free suspensions of antimicrobials and/or rose bengal were incubated with ROS-detecting fluorescent probes to detect hydroxyl radicals (•OH) [panels **(A)** aBL treatment, and **(B)** aPDI treatment] and singlet oxygen [**(C)** aBL treatment, and **(D)** aPDI treatment] upon irradiation. The values are the means of three separate experiments.

of genes determining microbial drug resistance (Boluki et al., 2017). Furthermore, the high bactericidal efficacy of combined approach can be explained by the fact that PSs can be substrates for efflux pumps. This competition between PSs and antimicrobials leads to increased uptake of antibiotics after the permeabilization of bacterial cell envelopes (Shih and Huang, 2011). In addition and most recently, He et al. (2018) published results confirming that some antimicrobials may express dual activity (He et al., 2018). They reported that tetracyclines may function as dual-action light-activated antibiotics expressing photosensitizing activity; this phenomenon may thus explain the synergy between aBL/aPDI and DOX within the current study. Another possible explanation for the combined treatment synergy could be concluded from the fact that both aBL/aPDI and antibiotic treatments lead to increased ROS production (Van Acker and Coenye, 2017); thus, phototherapy may lead to increased bactericidal efficacy and synergy via potentiation of the oxidative stress induced by antibiotic administration. It is only a hypothesis, as the mediation of ROS by antibiotic action has been an issue of concern in numerous literature studies. Within the current study, an effort was made to determine the role of ROS in the enhanced bacterial killing by combined treatments. The obtained data confirmed that increased ROS generation occur upon combined aBL/aPDI and antimicrobial treatment, indicating a possible explanation for the mechanism underlying this interaction.

CONCLUSION

The described above issues indicate possible explanations for increased bactericidal efficacy of aBL/aPDI and antimicrobials when administered in combination; thus, the development of an alternative combined aBL/aPDI and antibiotics treatment seems to be justified and desired. The combined approach results not only in increased antimicrobial efficacy of but also decreased concentrations of antimicrobials, which may greatly slow the increasing rate of drug resistance (Dai, 2017).

ETHICS STATEMENT

All isolated strains were collected during routine sampling. The current study only describes a collection of bacteria that comprised strains obtained from patients. Data collected from patients were anonymized and restricted to the information of the type of specimen and infection the strains were isolated from. Ethical approval was therefore not required. Moreover, the manuscript contains no data concerning animal studies,

studies involving human subjects or inclusion of identifiable human data or clinical trials; thus, no ethical approval was required.

AUTHOR CONTRIBUTIONS

AW did the experimental work and participated in conception of the study. AR-Z performed studies concerning rose Bengal checkerboard analysis. NM participated in the data interpretation and critical manuscript review. MG has been involved in the coordination, conception, and design of the study and wrote the manuscript. All of the authors have read and approved the final manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Chapter IV

Antimicrobial Photodynamic Inactivation Affects the Antibiotic Susceptibility of *Enterococcus* spp. Clinical Isolates in Biofilm and Planktonic Cultures

1. Summary of the publication

The objective of the following publication involved two nosocomial microorganisms, *Enterococcus faecium* and *Enterococcus faecalis,* which are capable of acquiring rapid mechanisms of antibiotic resistance[77]. The prevalence of infections caused by vancomycin-resistant enterococci (VRE) forces the use of alternative therapeutic options to avoid the use of antibiotics or change the bacterial phenotype to decrease the antibiotic concentration in the treatment. The photoinactivation of *E. faecium* and *E. faecalis* clinical isolates and reference strains has been confirmed in many photochemical studies. For example, the bacterial viability of *E. faecalis* (ATCC 29212) was reduced by 9.98 $\log_{10}$ CFU/ml in a study that involved methylene blue as a PS[78], and in another study, the clinical isolate *E. faecium* (EFM_513) was less sensitive to photoinactivation with aBL; thus, the viability was reduced by 1.9 $\log_{10}$ CFU/ml[79].

Within **publication no. 3,** the effect of antimicrobial photoinactivation (aPDI) with Rose Bengal (RB) and second photosensitizer (FL) fullerene as PS in the presence of green was investigated with clinical isolates of *E. faecium* and *E. faecalis* in planktonic and biofilm cultures. It is worth emphasizing that aBL photoinactivation was not implemented in the following research due to inefficient responses of those microorganisms to blue light conditions. **Photoinactivation with RB and FL led to a decrease in the survival rate of planktonic cultures** in both the tested isolates from 2.5–6 $\log_{10}$ CFU/ml and a maximum of 5 $\log_{10}$ CFU/ml, respectively, therefore confirming the efficacy of photoinactivation with these two photosensitizers against clinical isolates *Enterococcus* spp. **Changes in the resistance profile that resulted from the application of sublethal doses of aPDI(RB) and aPDI(FL) were confirmed** with the recommended methods of synergy testing that were highlighted in **publication no. 1**. The prevalence of synergies was observed for *E. faecalis* when diffusion methods (disks and E-TEST) were performed. In the present work, *E. faecalis* better responds to photoinactivation treatments, which results in

increased synergies among the combined treatment in the checkerboard assay and postantibiotic effect.

Compared to planktonic cultures, biofilm cultures are more resistant to photoinactivation and antibiotic treatments[80]. Within **publication no. 3,** I attempted to investigate the effect of combined treatment aPDI (RB) and two antibiotics, ciprofloxacin (CIP) and streptomycin (STR), on mature biofilm culture of *E. faecalis*. Even though this isolate exhibited a high level of resistance to STR, the synergy in the combined treatment with the implementation of aPDI(RB) was confirmed in planktonic and biofilm cultures. Biofilm cultures were grown on polycarbonate coupons and were exposed to aPDI(RB) and/or antibiotic conditions.

In contrast, the changes in the survival rate of cells after exposure to mature biofilm were measured as a $\log_{10}$ CFU/ml/cm^2. Differences **in the survival rate of biofilm cultures grown on coupons were the most prevalent for the combined treatment (aPDI (RB) and streptomycin) compared to treatment with antibiotics only as monotherapy or control**. **This is the first doctoral thesis evidence that the synergy between photoinactivation and antibiotics can occur in mature biofilm cultures**.

The mechanism of the synergy between light and antimicrobials is not fully understood, which is similarly described in **publication no 2.** Whether this phenomenon can accompany increased ROS production in the presence of antibiotics, such as gentamycin (GEN), tigecycline (TGC) or ciprofloxacin (CIP), was verified. ROS production was detected with aminophenyl-fluoresceine (APF) and the following fluorescent probes: Singlet Oxygen Sensor Green (SOSG) and dichlorofluorescein (DCF). Increased production of ROS occurred for the DCF probe when CIP was present in a cell environment with aPDI(RB); however, this level was not significantly higher than the ROS amount produced in aPDI alone. Fluorescent probes aimed at detecting singlet oxygen (SOSG) indicated that the fluorescence signal was increased when aPDI(RB) was combined with CIP compared to the fluorescence signal obtained with aPDI alone. This observation confirms that the increased production of singlet oxygen via light-assisted antibiotic (CIP) can be the mechanism of synergy with light for this antimicrobial agent. The last conclusion drawn from **publication no. 3** resulted from the use of fluorescent probes to measure cell damage after photoinactivation. The fluorescence signal from the SYTOX green probe revealed increased cell membrane permeabilization; thus, DNA release from the cells occurred upon aPDI treatment; however, the antibiotics TGC, CIP, and GEN did not enhance this process.

2. Publication

biomolecules

MDPI

Article

Antimicrobial Photodynamic Inactivation Affects the Antibiotic Susceptibility of *Enterococcus* spp. Clinical Isolates in Biofilm and Planktonic Cultures

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Abstract: *Enterococcus faecium* and *Enterococcus faecalis* are opportunistic pathogens that can cause a vast variety of nosocomial infections. Moreover, *E. faecium* belongs to the group of ESKAPE microbes, which are the main cause of hospital-acquired infections and are especially difficult to treat because of their resistance to many antibiotics. Antimicrobial photodynamic inactivation (aPDI) represents an alternative to overcome multidrug resistance problems. This process requires the simultaneous presence of oxygen, visible light, and photosensitizing compounds. In this work, aPDI was used to resensitize *Enterococcus* spp. isolates to antibiotics. Antibiotic susceptibility testing according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations was combined with synergy testing methods recommended by the American Society for Microbiology. Two clinical isolates, *E. faecalis* and *E. faecium*, were treated with a combination of aPDI utilizing rose bengal (RB) or fullerene (FL) derivative as photosensitizers, antimicrobial blue light (aBL), and 10 recommended antibiotics. aPDI appeared to significantly impact the survival rate of both isolates, while aBL had no significant effect. The synergy testing results differed between strains and utilized methods. Synergy was observed for RB aPDI in combination with gentamycin, ciprofloxacin and daptomycin against *E. faecalis*. For *E. faecium*, synergy was observed between RB aPDI and gentamycin or ciprofloxacin, while for RB aPDI with vancomycin or daptomycin, antagonism was observed. A combination of FL aPDI gives a synergistic effect against *E. faecalis* only with imipenem. Postantibiotic effect tests for *E. faecium* demonstrated that this isolate exposed to aPDI in combination with gentamycin, streptomycin, tigecycline, doxycycline, or daptomycin exhibits delayed growth in comparison to untreated bacteria. The results of synergy testing confirmed the effectiveness of aPDI in resensitization of the bacteria to antibiotics, which presents great potential in the treatment of infections caused by multidrug-resistant strains.

Keywords: antimicrobials; biofilm flow system; CDC bioreactor; *Enterococcus faecium*; *Enterococcus faecalis*; fullerene; photodynamic inactivation; rose bengal; synergy

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1. Introduction

Most Enterococci cause a vast variety of nosocomial infections of soft tissues, abscesses, urinary tract infections or even endocarditis, which overall are caused by *E. faecalis* and *E. faecium* [1]. *E. faecalis* is known as an etiological agent of opportunistic infections including bacteremia, endocarditis, meningitis, and urinary tract and bloodstream infections [2]. *Enterococcus* species, especially *E. faecalis*, are also associated with persistent endodontic infections. The most important antibiotics against which these microorganisms express resistance are β-lactams (penicillin), cephalosporines, lincosamides, streptogramins, and aminoglycosides, whereas they can also acquire resistance to glycopeptides (e.g., vancomycin, VAN) or macrolides. The first occurrence of resistance to VAN was observed in

1980, and to date, this resistance has spread massively among *E. faecium* isolates. This urgent problem of resistance is associated with an increasing number of nosocomial infections linked with VAN-resistant *E. faecium*. These reasons explain why this organism belongs to the group of ESKAPE microbes, which are the main cause of hospital-acquired infections and are especially difficult to treat because of their resistance to many antibiotics [3]. It is worth mentioning that planktonic cultures possess a drug resistance 100 to 1000 times lower than that of biofilms, and still increasing the antimicrobial resistance crisis is an additional force to find new alternatives to currently used bactericidal methods [4–6]. Moreover, increasing tolerance of hospital-acquired *E. faecium* strains to handwash alcohols is another problem that requires additional procedures to prevent transmission of this pathogen in the hospital setting [7]. Antimicrobial photodynamic inactivation (aPDI) seems to match perfectly as a potential candidate method for bactericidal action against planktonic and biofilm cultures. The method requires visible light, oxygen, and a photosensitizer (PS) [8]. Absorption of photons by photosensitizing agents leads to the formation of excited states of such compounds, which through further photochemical reactions lead to the production of highly toxic reactive oxygen species (ROS) or singlet oxygen [9,10]. Such products of photooxygenation can interact with DNA, lipids and proteins, leading to cell death. The appropriate degree of photoinactivation can act as a 'tool' for sensitization of microorganisms to antimicrobials, which was demonstrated in a previous paper published by our team for *Acinetobacter baumannii* [11]. In the current research, exogenous PSs (rose bengal (RB) and fullerene (FL) derivative) with visible green light were used as tools for the 'sensitization' of *Enterococcus* clinical isolates to routinely used antibiotics.

2. Results

2.1. aPDI Significantly Influences the Survival Rate of Planktonic Cultures of Enterococcus Species

The application of green light with RB revealed that the PS even at very low concentrations (0.1 μM) with a dose of green light irradiation (6.4 J/cm^2) was able to reduce *E. faecium* viability by approx. 2.5 $\log_{10}$ CFU/mL (Figure 1A). The second PS, fullerene (FL), was administered at different concentrations (ranging from 0.15 μM to 0.5 μM) and to obtain a 5 $\log_{10}$ reduction with the same light dose (6.4 J/cm^2) it required 0.5 μM concentration. Similar results were obtained for the second isolate (*E. faecalis*) when RB was present at a concentration of 0.1 μM, and the highest reduction (approx. 6 $\log_{10}$ CFU/mL) was detected after the application of 6.4 J/cm^2 of green light (Figure 2A). For FL, the highest reduction was obtained when the PS was applied at concentrations of 0.5 μM. After administration of a 6.4 J/cm^2 light dose, the reduction was estimated to be approx. 5 $\log_{10}$ CFU/mL (Figures 1B and 2B).

The results presented above clearly indicate that both RB and FL based aPDI may lead to effective inactivation of two tested *Enterococcus* species. For further experiments sublethal treatments marked with bold frames were used.

2.2. Identification of MIC of Treatments

Adequate synergy testing required the preliminary characteristics of the studied *E. faecium* and *E. faecalis* regarding their antibiotic resistance profiles and their response to aPDI treatment. Applied techniques have indicated that both clinical isolates are multidrug-resistant pathogens; therefore, they are resistant to multiple antimicrobial agents (e.g., STR, AMP, DAP) [12], covering all possible drug categories and all mechanisms of action. Detailed characteristics are presented in Table 1.

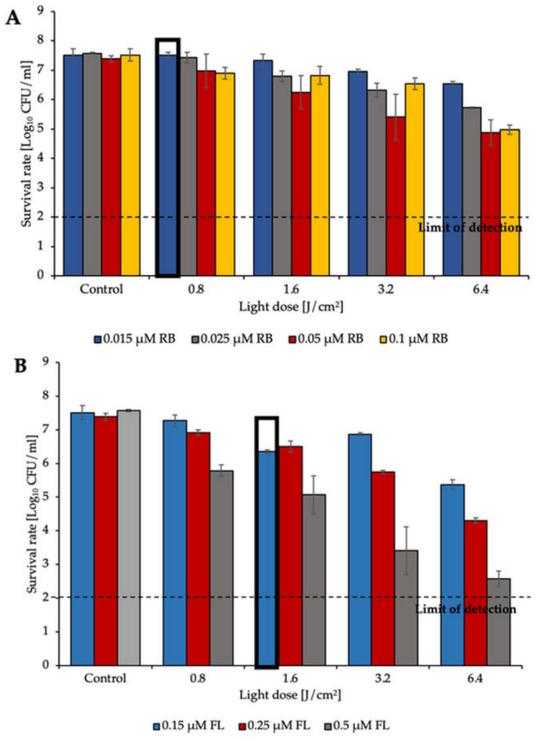

Figure 1. aPDI inactivation of *E. faecium* with various doses of green light and (**A**) RB concentrations (0.015, 0.025, 0.05, and 0.1 µM) or (**B**) FL concentrations (0.15, 0.25, and 0.5 µM). The experiment was performed in three biological replicates. The detection limit was 100 CFU/mL. Bold frames indicate sublethal treatment.

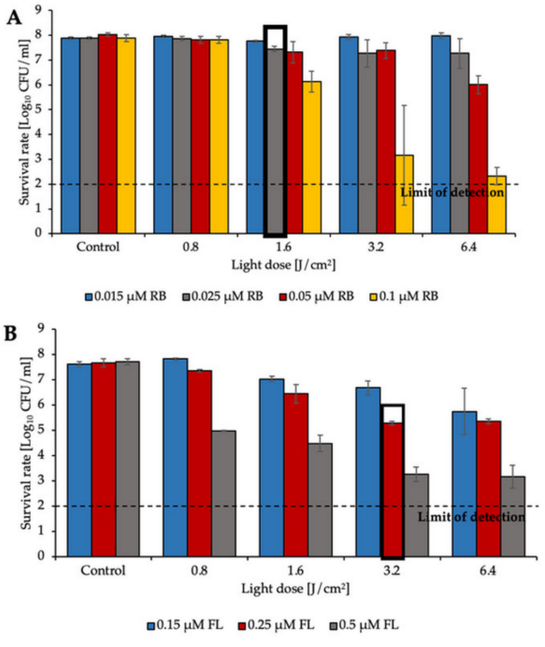

Figure 2. aPDI inactivation of *E. faecalis* with various doses of green light and (**A**) RB concentrations (0.015, 0.025, 0.05, and 0.1 µM) or (**B**) FL concentrations (0.15, 0.25, and 0.5 µM). The experiment was performed in three biological replicates. The detection limit was 100 CFU/mL. Bold frames indicate sublethal treatment.

Table 1. MIC for antimicrobials and light.

Antibiotic Target	Antimicrobial Category	Antibiotic	*E. faecalis*	*E. faecium*
			MIC (μg/mL)	MIC (μg/mL)
Protein synthesis (30S)	Aminoglycosides	Gentamycin	64	32
	Tetracyclines	Doxycycline	16/8	32
	Streptomycin	Streptomycin	256 (R)	1024 (R)
	Glycylcyclines	Tigecycline	1 (R)	8/4 (R)
70S initiation complex	Oxazolidinones	Linezolid	2	1
DNA gyrase	Fluoroquinolones	Ciprofloxacin	2	2
Cell-wall synthesis	Carbapenems	Imipenem	1/0.5	16/8 (R)
	Glycopeptides	Vancomycin	1	2
	Penicillins	Ampicillin	>64 (R)	>1024 (R)
Cell membrane	Lipopeptides	Daptomycin	128 (R)	64 (R)
Phototherapy	aPDI (FL)	Green light + FL	28.6 J/cm^2 + 10 μM FL *(3.6 J/cm^2 + 0.625 μM FL)* [1]	28.6 J/cm^2 + 10 μM FL *(3.6 J/cm^2 + 0.312 μM FL)*
	aPDI (RB)	Green light + RB	15.9 J/cm^2 + 1 μM RB *(7.95 J/cm^2 + 0.5 μM RB)*	15.9 J/cm^2 + 1 μM RB *(15.9 J/cm^2 + 0.5 μM RB)*

[1] Italic font indicates the sublethal conditions used for post antibiotic effect (PAE) testing.

For all of the antibiotics as well as for the photoinactivation conditions, the MIC values for both tested clinical isolates were determined. In the next set of experiments, the MIC values were used to evaluate the synergy between tested monotreatments, which was performed with the recommended methods for synergy testing (e.g., antimicrobial susceptibility testing, checkerboard assay, time-kill assay).

2.3. Diffusion-Based Assays Confirm aPDI/Antimicrobial Synergy

The results indicated that in the case of both phototreatments, the employment of sublethal aPDI conditions influenced the susceptibility to numerous routinely used antimicrobials, resulting in larger growth inhibition zones (in the case of the disk diffusion assay) and decreased MICs (for the E-test). The results regarding synergy testing with diffusion methods are presented in Table 2. The disk diffusion assay revealed that after treatment with aPDI, *E. faecalis* became more sensitive to STR and TGC; thus, the zones of inhibition increased by greater than or equal to 2 mm, whereas the MIC values from the E-test decreased by a minimum of 2-fold in comparison to the control, confirming the synergistic effect between aPDI and antibiotics. aPDI treatment also influenced changes in susceptibility to DOX (e.g., the inhibition zone increased from 9.4 mm to 11.3 mm) and to IPM and AMP (an increase in the inhibition zone was detected, whereas the MIC values from the E-test remained unchanged). In contrast, *E. faecium* did not respond in a similar manner to aPDI treatment. Synergy was observed for aPDI (RB) treatment with GEN (the MIC value decreased from 6 to 3 μg/mL) and TGC (the inhibition zone increased from 28.5 mm to 32.6 mm). For aPDI (FL) treatment, synergy was indicated only for DOX based on a reduction in the MIC value for the E-test from 32 to 16 μg/mL. Differences resulting from the obtained results indicate the necessity of applying multiple approaches for synergy testing; thus, one method is not sufficient to confirm the research assumptions. In addition, as light alone treatment (with no PS administration) as well as PS alone (with no light excitation) exerted no change in microbial antibiotic susceptibility, these control conditions were not included within the Table 2.

Table 2. Antimicrobials MIC change upon sublethal aPDI treatments.

Antibiotic	*E. faecalis*						*E. faecium*					
	Control		aPDI (RB)		aPDI (FL)		Control		aPDI (RB)		aPDI (FL)	
	DF [1]	E-Test	DF	E-Test	DF	E-Test	DF	E-test	DF	E-Test	DF	E-Test
GEN	10 [2]	12 [3]	9.8	8	10.8	8	16.5	6	17.8	3	15.8	4
STR	11	256	**13.9**	**128** [4]	**15**	**128**	10	1024	10.9	≥1024	8.6	≥1024
TGC	22.4	19	**24.7**	**0.64**	**25.5**	≥256	28.5	0.064	32.6	0.047	26	0.64
DOX	9.4	32	**11.3**	**16**	10.9	**16**	13	32	10.9	32	11.6	**16**
LZD	24.7	2	24	1.5	25.9	2	29.6	1	31	0.75	28.7	1
CIP	20.5	0.75	21.8	0.5	**22.7**	0.5	22.1	0.5	22.7	0.5	21.4	0.5
IMP	29.3	0.75	**31.2**	0.75	29.5	0.75	10.5	32	6	32	8.9	≥32
VAN	13.4	2	14	2	13.8	2	21	0.5	21.1	0.38	18	0.38
AMP	8.4	0.5	**12.2**	0.5	**11**	0.75	6	2	6	3	6	-
DAP	-	1	-	1	-	1	-	2	-	1.5	-	1.5
Q-D	11.5	-	12.1	-	12.2	-	17.7	-	16.1	-	17.7	-

[1] Disk diffusion; [2] Expressed in mm; [3] Expressed in μg/mL; [4] Bold font indicates significant change in MIC upon sublethal aPDI treatments; Abbreviations: GEN, gentamycin; STR, streptomycin; TGC, tigecycline; DOX, doxycycline; LZD, linezolid; CIP, ciprofloxacin; IMP, imipenem; VAN, vancomycin; AMP, ampicillin; DAP, daptomycin; Q-D, quinupristin-dalfopristin (Synercid); FL, fullerene; RB, rose bengal.

2.4. Serial Dilution Methods Demonstrate aPDI/Antimicrobial Synergy

The checkerboard assay method indicated that aPDI (RB) has a synergistic effect with GEN, CIP, and DAP. This conclusion was based on the FICI, the value of which was estimated as 0.38, 0.38, and 0.16 for GEN, CIP, and DAP, respectively, for *E. faecalis*. aPDI (FL) indicated synergy only with IMP (FICI = 0.25). For *E. faecium*, it was observed that aPDI (RB) has an antagonistic effect when combined with VAN/DAP. The FICI value was 8.5 and 5.25 for VAN and DAP, respectively, whereas for combined treatment with CIP and GEN, it was estimated to be 0.5, indicating synergy with aPDI (RB). A similar conclusion for *E. faecium* could also be drawn for aPDI (FL) combined with LZD. All results from the checkerboard assay are presented in Table 3. In addition, as light alone treatment (with no PS administration) as well as PS alone (with no light excitation) exerted no change in microbial antibiotic susceptibility, these control conditions were not included within the Table 3.

Table 3. Checkerboard FICI calculation.

Antibotic	*E. faecalis*		*E. faecium*	
	aPDI (RB)	aPDI (FL)	aPDI (RB)	aPDI (FL)
GEN	**0.38** [1]	>0.5	**0.5**	>0.5
STR	>0.5	>0.5	>0.5	>0.5
TGC	>0.5	>0.5	>0.5	>0.5
DOX	>0.5	>0.5	>0.5	>0.5
LZD	>0.5	>0.5	>0.5	**0.5**
CIP	**0.38**	>0.5	**0.5**	>0.5
IMP	>0.5	**0.25**	>0.5	>0.5
VAN	>0.5	>0.5	**8.5**	>0.5
AMP	>0.5	>0.5	>0.5	>0.5
DAP	**0.16**	>0.5	**5.25**	>0.5
Q-D	-	-	-	-

[1] Bold indicates possible synergistic interactions; GEN, gentamycin; STR, streptomycin; TGC, tigecycline; DOX, doxycycline; LZD, linezolid; CIP, ciprofloxacin; IMP, imipenem; VAN, vancomycin; AMP, ampicillin; DAP, daptomycin; Q-D, quinupristin-dalfopristin (Synercid); FL, fullerene; RB, rose bengal.

2.5. Time–Kill Curve Assay Confirms aPDI/Antimicrobial Synergy

The time–kill assay, i.e., post antibiotic effect (PAE), represented another method to investigate the synergy or other interactions between aPDI (RB)/aPDI (FL) and antibiotics. For both *E. faecium* and *E. faecalis*, a synergistic effect was observed for all of the tested antibiotics (with the exception of AMP and Q-D) when combined with aPDI (FL). A characteristic "shift" of the growth curve was detected both for FL and RB aPDI and most

of antibiotics; however, only four representatives were used for visualization, i.e., aPDI (FL)/GEN (Figure 3A), aPDI (FL)/LZD (Figure 3B), aPDI (RB)/DOX (Figure 3C) and aPDI (RB)/DAP (Figure 3D). In addition, as light alone treatment (with no PS administration) as well as PS alone (with no light excitation) exerted no change in microbial antibiotic susceptibility, these control conditions were not included within the Figure 3.

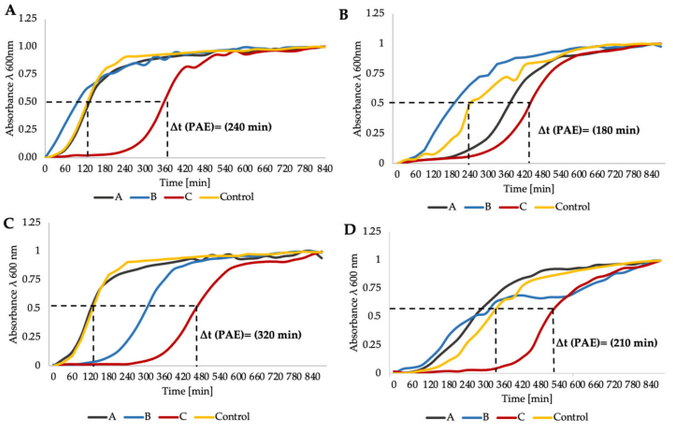

Figure 3. Postantibiotic effect testing. (**A**) Growth curve analysis of aPDI (FL)/GEN combined treatment for *E. faecalis*; (**B**) Growth curve analysis of aPDI (FL)/LZD treatment for *E. faecium*; (**C**) Growth curve analysis of aPDI (RB)/DOX treatment for *E. faecalis*; (**D**) Growth curve analysis of aPDI (RB)/DAP treatment for *E. faecium*. Phototreatments (aPDI (RB)/(FL)) were employed with 1/2 MIC doses and are presented on graphs with symbols (A). Antibiotics (LZD, DAP, GEN, and DOX) were administered at the MIC and are represented in the figure by symbol B. The combination of light and antibiotics is presented as symbol C (1/2MIC aPDI + MIC antibiotic). Only one representative curve is presented.

2.6. aPDI/Antimicrobials Exerts Numerous Synergies

All of the tests regarding synergy testing between antibiotics and aPDI revealed that for *E. faecium*, the prevalence of synergy was indicated for GEN with aPDI (RB) and for DOX combined with aPDI (FL) (Table 4). *E. faecalis* responded better to photoinactivation, which was reflected in the increased number of observed synergies between aPDI and antimicrobials. For example, after application of aPDI, increased susceptibility was indicated for two antibiotics, namely, STR and TGC, which was confirmed with multiple methods (Table 5).

Table 4. Summarized results of synergy testing for *E. faecium*.

Antibiotic	aPDI (RB)				aPDI (FL)			
	DF [1]	E-Test	Checkerboard Assay	PAE	DF	E-Test	Checkerboard Assay	PAE
GEN	-	+	+	+	-	-	-	+
STR	-	-	-	+	-	-	-	+/-
TGC	+	-	-	+	–	–	-	+
DOX	-	-	-	+/-	-	+	-	+
LZD	-	-	-	-	-	-	+	+
CIP	-	-	+	-	-	-	-	+
IMP	–	-	-	-	-	-	-	+
VAN	-	-	–	-	–	-	-	+
AMP	-	-	-	-	-	-	-	+/-
DAP		-	–	+/-		-	-	+/-
Q-D	-		ND	ND	-		ND	ND

[1] Disk diffusion; GEN, gentamycin; STR, streptomycin; TGC, tigecycline; DOX, doxycycline; LZD, linezolid; CIP, ciprofloxacin; IMP, imipenem; VAN, vancomycin; AMP, ampicillin; DAP, daptomycin; Q-D, quinupristin-dalfopristin (Synercid); PAE, post antibiotic effect; FL, fullerene; RB, rose bengal; ND, not defined. (+), synergy; (+/-), partial synergy; (-) no synergistic effect; (–) antagonism.

Table 5. Summarized results of synergy testing for *E. faecalis*.

Antibiotic	aPDI (RB)				aPDI (FL)			
	DF [1]	E-Test	Checkerboard Assay	PAE	DF	E-Test	Checkerboard Assay	PAE
GEN	-	-	+	-	-	-	-	+
STR	+	+	-	+	+	+	-	+
TGC	+	+	-	+	+	–	-	+
DOX	+/-	+	-	+	-	+	-	+
LZD	-	-	-	-	-	-	-	+
CIP	-	-	+	-	+	-	-	+
IMP	+/-	-	-	-	-	-	+	+
VAN	-	-	-	-	-	–	+	+
AMP	+	-	-	+	+	-	-	-
DAP		-	+	-			-	+
Q-D	-		ND	ND	-	-	ND	ND

[1] Disk diffusion; GEN, gentamycin; STR, streptomycin; TGC, tigecycline; DOX, doxycycline; LZD, linezolid; CIP, ciprofloxacin; IMP, imipenem; VAN, vancomycin; AMP, ampicillin; DAP, daptomycin; Q-D, quinupristin-dalfopristin (Synercid); PAE, post antibiotic effect; FL, fullerene; RB, rose Bengal; ND, not defined; (+), synergy; (+/-), partial synergy; (-) no synergistic effect; (–) antagonism.

2.7. aPDI/Antimicrobial Synergy Can Be Reached in the Mature Biofilm Model

For the experimental procedures, RB was applied at a 10-fold higher concentration (5 μM) than that in the planktonic culture, whereas STR and CIP were applied at concentrations of 3× MIC and 5× MIC, respectively. Coupons with biofilms were irradiated twice from each side with a dose of green light of 7.95 J/cm^2. Increased concentrations of all compounds are associated with a higher resistance of biofilm cultures to the treatment conditions. aPDI of *E. faecalis* biofilm culture with RB reduced the bacterial viability by 3.1 $\log_{10}$ CFU/cm^2, and when combined with 3× MIC of STR, the reduction increased to 4.4 $\log_{10}$ CFU/cm^2. The addition of 5× MIC of CIP with a PS reduced the viable cell count by 2.9 $\log_{10}$ CFU/cm^2 (Figure 4). The results estimated by CFU/cm^2 counting were confirmed by confocal laser scanning microscopy (CLSM) images of stained biofilm cells before and after mono- and combined aPDI therapy. The images of coupons (Figure 5A–D) with biofilms revealed that the combination of aPDI (RB) with CIP (Figure 5B) or STR (Figure 5C) led to an increased presence of red fluorescent cells, which indicated biofilm damage upon treatment.

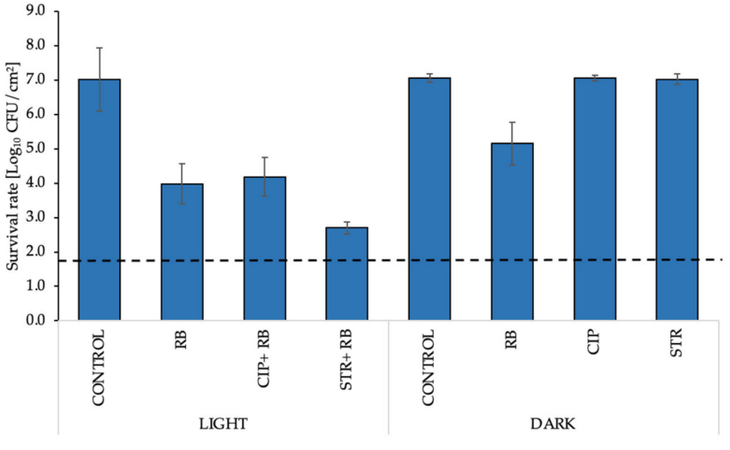

Figure 4. Assessment of the survival rate of *E. faecalis* biofilms grown on coupons and treated under various conditions: control (nontreated); RB (5 μM); CIP (5× MIC); STR (3× MIC) in dark or treated with green light (7.95 J/cm^2). For each condition, three coupons were analyzed. The detection limit was 39.5 CFU/cm^2.

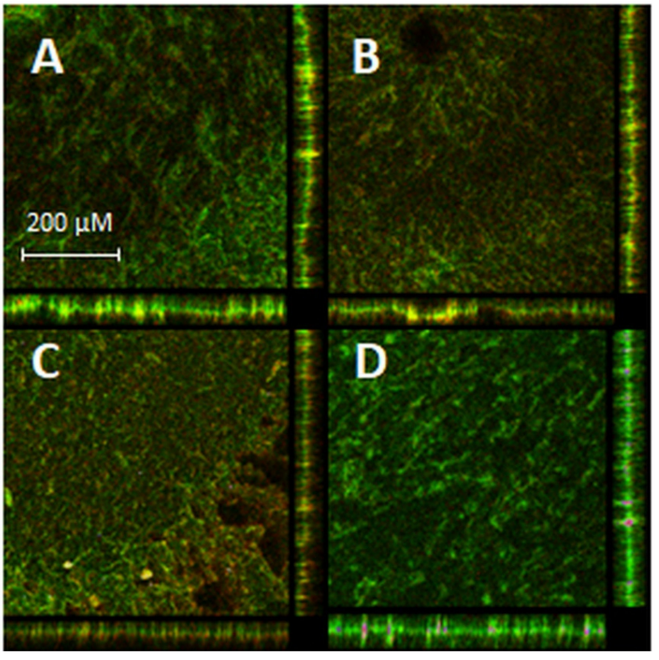

Figure 5. CLSM assessment of *E. faecalis*-treated biofilms. Biofilms were grown for 24 h on coupons, exposed to RB (5 µM) for 15 min and irradiated twice with a dose of green light at 7.95 J/cm^2. Biofilms were stained with the BacLight Live/Dead kit. Panel (**A**): biofilm exposed to aPDI (RB); panel (**B**): aPDI (RB)-treated biofilm exposed to 5× MIC of CIP; panel (**C**): aPDI (RB)-treated biofilm exposed to 3× MIC of STR; panel (**D**): control (nontreated biofilm).

This is the first report of a resensitization of cells growing as a mature biofilm to antibiotic treatment upon photoinactivation. These important results were confirmed by CFU/cm^2 determination and confocal microscopy analysis. We were able to observe the bactericidal effect (approx. 4 $\log_{10}$ CFU/cm^2 viability reduction) of the aPDI (RB) and STR combination on biofilm cells.

2.8. Increased ROS Generation Can Explain the Mechanism Underlying the Observed Synergies

To investigate whether combinations of antibiotics and photoinactivation can lead to increased production of ROS as well as singlet oxygen, various fluorescent probes were used. Application of various fluorescent probes, i.e., dichlorofluorescein (DCF) and 3′-(*p*-Aminophenyl) fluorescein (APF), is associated with different fluorescence responses of these compounds to ROS. Specific ROS (e.g., hydroxyl radicals) lead to different levels of fluorescence for each probe. From the literature data, it is well known that many antibiotics can exert their bactericidal activity due to stimulation of ROS formation [13]. To investigate whether this phenomenon could also be observed for combined aPDI/antimicrobial treatment, combinations of antibiotics—such as TGC, GEN, and CIP (at MIC concentrations)—with aPDI at dose of MIC were tested. After exposure of bacterial cells to the tested antibiotics and MIC dose of aPDI (RB), increased production of ROS was detected only for GEN. For CIP and TGC, exposure to the combined treatment did not reveal the additional production of ROS (Figure 6A). The observed effect could explain the synergy between GEN and aPDI (RB) in an in vitro model of *E. faecalis* eradication. Another fluorescent probe that was used in the experiment is also strictly associated with the production of various ROS. APF was tested with the same antimicrobials as described above under the same experimental conditions. The results of this experiment did not confirm any increased ROS production upon treatment with aPDI (RB) and GEN, CIP, or TGC (data not shown). For the detection of singlet oxygen, a SOSG probe was used to test the synergy between TGC or CIP and aPDI (Figure 6B). This experiment revealed increased singlet oxygen production

(represented by the highest fluorescence level) after exposure to the combination of aPDI (RB) and CIP. The signal was higher than that with both aPDI (RB) monotherapy and the combination of aPDI (RB) and TGC. For the three different probes, increased ROS and singlet oxygen production was confirmed for the two different antimicrobials, indicating that increased levels of ROS and/or singlet oxygen may be responsible for the synergistic effect of the combined treatment.

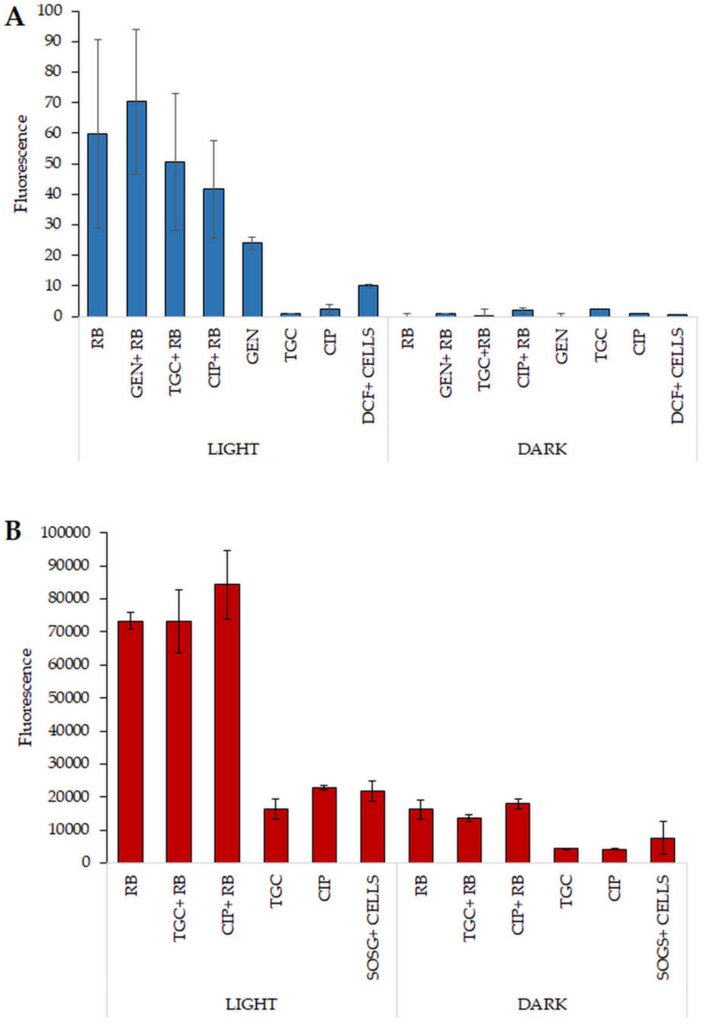

Figure 6. Reactive oxygen species and singlet oxygen identification. (**A**) Exposure of *E. faecalis* to various oxidative stress conditions and antibiotic monotherapies at MIC concentrations. For experimental purposes, dark controls of the tested combinations were also analyzed. The fluorescence of DCF was observed using wavelengths of 521 nm (emission) and 488 nm (excitation) with an EnVision multilabel plate reader (PerkinElmer, Waltham, MA, USA). The values are the mean of three independent experiments. (**B**) Cell suspensions of *E. faecalis* were exposed to mono- and combination therapies to detect singlet oxygen production. Fluorescence was measured at excitation/emission wavelengths of 505/523 nm with an EnVision multilabel plate reader (PerkinElmer).

2.9. Increased Permeabilization Could Explain the Mechanism of the Observed Synergies

To investigate whether aPDI (RB) can lead to permeabilization of the bacterial membrane, SYTOX Green was used. Increased permeabilization could result in more efficient antibiotic penetration into bacterial cells, leading to increased damage and cell death, thus explaining the phenomenon of synergy. For this purpose, SYTOX Green was used as a high-affinity nucleic acid compound that can interact with intracellular DNA [14]. The leak-

age of DNA is a result of the cell permeabilization process upon photoinactivation. When aPDI (RB) was applied, the most severe damage to the cell membrane was observed under this condition (Figure 7); thus, we are convinced that the increased permeabilization may be the most important reason for the observed synergistic effect between antimicrobials and aPDI as it may result in increased antibiotic uptake.

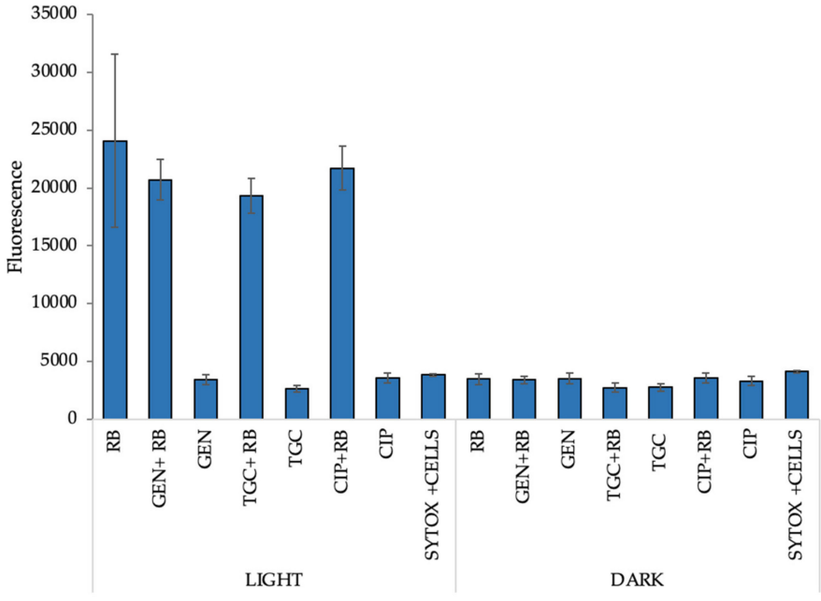

Figure 7. *E. faecalis* cell membrane integrity. Samples were treated with aPDI and aPDI combined with antibiotics at MIC concentrations and exposed to the SYTOX Green label. Additionally, the control for cells and labels was prepared (SYTOX + cells). The absorbance was measured with an EnVision multiplate reader (PerkinElmer) with 504/523 nm excitation/emission filters. The experiment was performed in three independent biological replicates.

3. Discussion

Disturbance of oral human microflora can rapidly influence the growth and spread of nosocomial pathogens—e.g., *E. faecium*, *Streptococcus mutans*, *Porphyromonas ginvigalis*, and *Lactobacillus gasserii*—leading to the development of intraoral diseases. It is worth mentioning here that *E. faecalis* is commonly detected in persistent infections after failed endodontic treatments, and *E. faecium* is mainly associated with infections caused by the use of indwelling medical devices, e.g., central venous and urinary catheters [15,16]. Before the era of widespread application of antibiotics, most bacterial infections were fatal for patients. The discovery of the bactericidal or bacteriostatic activity of some compounds was shown to be a very effective therapeutic solution. Since then, antibiotics have been used to treat infections caused by many types of bacteria. However, there has now been an increase in the incidence of diseases caused by microbes resistant to many types of therapeutics and a decline in the number of new antibiotics introduced. Hence, this kind of therapy will be ineffective in the future. Antimicrobial photoinactivation of bacteria (aPDI) is a promising approach, but it also has a few limitations (e.g., depth of penetration of light); regardless, many positive applications and evidence of success have been observed. Photoinactivation is often presented as a method in the treatment of peri-implantitis, tooth canal infections, and other oral infections [17,18].

The first case of the significant potential of aPDI in sensitizing *Enterococcus* spp. strains resistant to VAN appeared in the literature in 2013 [19]. This study presented an in vivo model of larval infection of *Galleria mellonella* with *E. faecium*. The application of VAN with light and methylene blue (MB) increased the survival rate of infected caterpillars in comparison to treatment with only aPDI or VAN alone. Another example of successful

application of aPDI against this microorganism was described by Kang et al. in 2019. Light treatment of *E. faecium* planktonic culture in the presence of curcumin and protoporphyrin IX significantly reduced bacterial growth [20]. Moreover, it is well known that biofilm cultures are more resistant to bactericidal treatments than planktonic cultures due to the presence of a matrix that consists of polysaccharides, proteins, and nucleic acids, which constitute a mechanical barrier for antimicrobial compounds. Nevertheless, the results published by López-Jiménez et al. showed that eradication of biofilms is still possible. In their experiments, MB or toluidine blue O (TBO) excited with 670 or 628 nm wavelength light led to severe damage to biofilm cells and even increased the roughness of the biofilm surface [21].

The second representative of the genus *Enterococcus*, *E. faecalis*, was also eradicated by phototreatment of the biofilm cultures. For example, it was proven that aPDI can simultaneously affect biofilms via damage to bacterial cells and the extracellular matrix. Photoinactivation with MB was reported to reduce the *E. faecalis* biofilm surface by 89% in comparison to the samples incubated only with the PS. In multispecies biofilms (*E. faecalis* and *P. aeruginosa*), aPDI with MB reduced the biofilm-covered area by 59.3% [22]. Moreover, eradication of *E. faecalis* in the root canal was shown to be possible with the application of MB with red light (660 nm) [23]. The potentiation of the antimicrobial efficacy of RB and green light was proved by experiments performed by Li et al. The addition of potassium iodide (KI) (at a concentration of 100 mM) increased the effectiveness of the reduction in planktonic culture with aPDI by an additional reduction of 4 $\log_{10}$ CFU/mL. The same effect was observed when biofilm cells were treated with RB aPDI. Moreover, Shrestha et al. described the efficacy of RB-conjugated chitosan, used as a PS, which led to eradication of planktonic culture of *E. faecalis* and reduced the bacterial viability count in biofilms by approx. 3 $\log_{10}$ CFU/cm^2 [24]. These experiments confirmed that the effectiveness of RB as well as MB at very low molar concentrations against this pathogen can be potentiated.

In the current study, the differences in the response of both isolates to various PSs were demonstrated. *Enterococcus* spp. show greater sensitivity to RB than to FL. This finding may be related to the mechanism of action of both PSs. In the case of FL, it has been described that apart from the production of singlet oxygen in polar solvents, an important mode of action of this PS is the permeabilization of cell membranes. Research conducted by our team has shown that FL accumulates mainly in cell sheaths [14]. However, the mechanism of action of RB is mainly related to the production of singlet oxygen. In subsequent studies, the ability of RB to attach to the cell membranes of *E. faecalis* was demonstrated by flow cytometry [25], which may potentially explain the greater effectiveness of RB than FL against *Enterococcus* spp. The results of our experiments highlight the effectiveness of aPDI with RB or FL against two multidrug-resistant (MDR) isolates: *E. faecalis* and *E. faecium*. A high level of resistance was observed against antibiotics such as STR, DAP, and AMP, which was reduced after aPDI treatment, especially in the case of *E. faecalis*. Synergy testing between aPDI and antimicrobials was performed with multiple methods regarding the data presented in our published review paper [26]. The resistance to STR of *E. faecalis* isolate was reduced after application of aPDI (RB and FL) (the inhibition zone increased by 2.9 mm). Additionally, after application of STR with the aPDI (RB and FL) combination, a delay in bacterial growth was detected. The checkerboard assay is an excellent method to investigate the combinations of two factors; however, this method revealed synergy or even antagonism between aPDI and antimicrobials for only a few combinations. GEN and CIP exhibited synergistic effects with aPDI (RB) when applied against both *Enterococcus* species. Individual synergy in the case of *E. faecium* occurred for antibiotics DAP, IPM, or LZD with aPDI (RB and FL), and antagonism was revealed for DAP and VAN when combined with aPDI with FL. Moreover, the PAE results revealed that bacterial growth can be significantly disturbed after combined treatment application in comparison to monotherapies. For most of the combinations, the PAE was positive or partially positive. It is also worth mentioning that for each photoinactivation treatment, regarding the presence of RB and FL, MICs were determined for both strains and PSs. The concentrations or treatment doses

presented in Table 1 could not be used in experiments regarding the PAE. Such applied doses of aPDI with FL were too harsh for bacterial cells, and the regrowth effect could not have been observed. Therefore, for synergistic effect determination and the ability to observe the effect of aPDI with antimicrobials in terms of MIC values, the estimated photoinactivation conditions had to be weakened. Despite the very high resistance of the *E. faecalis* isolate to STR, resensitization and synergy with aPDI (RB) were confirmed for planktonic culture and biofilm cells. The combined treatment successfully reduced the bacterial load for biofilm culture from 7.1 to 2.7 CFU/cm^2. One could ask whether the sequence of treatments, i.e., starting with aPDI or antimicrobials, may affect the results. The sequence treatment studied within the current work included the application of aPDI as a first step of experimental procedure, nevertheless, the alternative sequence has also been studied (data not shown). The performed analysis revealed that similar synergies could be demonstrated regardless the sequence used. Obviously, when studying tetracyclines, that could also serve as standard PSs and be excited with appropriate wavelength irradiation, one could assume that starting with antibiotic application followed with light treatment should enhance the bactericidal outcome, nevertheless, using our experimental conditions, the expected increase in killing efficacy was not observed (data now shown). To investigate the mechanism of the obtained synergy, multiple fluorescent probes were used to detect the potentially increased production of singlet oxygen or other ROS. DCF revealed increased radical production in combination with aPDI (RB) and GEN, but the fluorescence level was quite low when compared with that of the APF probe. The second indicator (APF) confirmed a high fluorescence level for all tested antimicrobials when combined with aPDI (RB); however, this level was slightly lower than that for the monotherapy (aPDI RB); thus, the APF results did not confirm the increased production of ROS in the combined treatment. SOSG, which is suited to the detection of singlet oxygen, confirmed increased production of this radical when aPDI (RB) was combined with CIP. The last experiment trying to explain the occurrence of synergy employed the intracellular DNA probe SYTOX Green. This compound efficiently binds to nucleic acids after they leak out of cells through the permeabilized membrane. aPDI treatment leads to increased permeabilization of the cells which may be the most important reason of observed synergy. The increased membrane permeabilization may result in increased antibiotic uptake and lead to enhanced killing efficacy.

Despite demonstrating that aPDI leads to significant membrane permeabilization which could partially explain the observed synergy, the mechanism of synergistic effect remains poorly understood. Resensitization of microbes to a particular antibiotics after exposure to sub-lethal aPDI could primarily result from the following reasons: (i) aPDI inactivation of the microbial agents responsible for drug resistance mechanisms; (ii) aPDI caused increased cell envelopes permeabilization leading to increased diffusion of antibiotic into the microbial cell; (iii) aPDI mediated disruption of membrane components leading to the change in membrane potential which may further affect PS uptake or its binding to cell envelope; and (iv) increased ROS production resulting from antimicrobial ROS generation.

aPDI leads to inactivation of multiple cellular components, i.e., proteins, lipids or genetic material, thus, it exerts deleterious effects against numerous virulence factors and enzymes responsible for antimicrobial resistance mechanisms. *Enterococcus* spp. display a variety of enzymes and proteins being key factors of drug resistance mechanisms, i.e., acetyl-, phospho-, and adenyltransferases, transpeptidases, or proteins building efflux pumps [27–30]. Possible aPDI mediated inactivation of these factors could result in microbial resensitization to particular antibiotics. In case of increased membrane permeabilization, the current study provides clear evidence supporting this thesis, and indeed, this aPDI caused membrane permeabilization could be the most important reason for observed synergistic effect. Finally, we hypothesize that aPDI may lead to the disruption of cell envelope components affecting membrane potential, i.e., lipoteichoic acid (LTA) present in Gram-positive microbes. It has been evidenced that inactivation of LTA may lead to significant increase in antibiotic diffusion resulting in enhanced killing efficacy

of antibiotic treatment [31]. In addition, numerous studies demonstrate that antibiotic lethality is accompanied by ROS generation [31–33]; thus, the overall oxidative stress could be significantly enhanced when combined aPDI/antimicrobial treatment is applied. This effect could also be the reason of the observed synergistic effect.

The most intriguing aspect of the observed synergy is providing explanation why the synergy could be demonstrated only for few antibiotics and what factors determine that specific antimicrobials may exert its increased efficacy upon sub-lethal aPDI treatment. Nevertheless, this explanation is still being undiscovered and worthy further investigations. We have made an effort to identify some chemical features of tested antimicrobials regarding its molecular weight, polar surface area, formal and physiological charge, complexity, water solubility, pKa, or mechanism of action that could potentially group studied antibiotics according their synergistic cooperation with aPDI; however, none of tested feature was demonstrated to be corelated with the observed synergy.

The results of the synergy testing experiments confirm the effectiveness of aPDI in sensitizing bacteria to antibiotics. This modality holds great potential for treating infections caused by multidrug-resistant strains that are mainly acquired in hospitals. A great advantage of aPDI is the nonspecific mechanism of action allowing comprehensive cell destruction. This approach prevents bacteria from developing resistance against this type of treatment, representing a significant advantage of aPDI treatment despite the risk of increased tolerance development, as presented by our team in two recently published articles [34,35]. However, the results of these studies may be clinically applicable, especially in the fields of dentistry or wound management. The ability of biofilm eradication in combined treatment, as presented here, is of great importance and indicates that this method is efficient despite obvious limitations.

4. Materials and Methods

4.1. Bacterial Strains and Culture Conditions

In this study there were two clinical isolates used: *E. faecium* EU87 and *E. faecalis* EU92. Strains were kindly provided with dr Valentina Ebani (Pisa, Italy). Tryptic Soy Broth (bioMérieux, Craponne, France) with 1.5% agar (BTL, Warsaw, Poland) plates were used for colony forming unit (CFU) enumeration and tryptic soy broth (TSB) (bioMérieux, Craponne, France) was used for overnight planktonic cultures and batch and flow phase of biofilm culture.

4.2. Photosensitizers

4,5,6,7-Tetrachloro-2′,4′,5′,7′-tetraiodofluorescein disodium salt (RB) powder was purchased from Sigma Aldrich (Munich, Germany). The stock solution was prepared in double-distilled water (ddH_2O) and kept in the dark at 4 °C. Fullerenopyrrolidine (N-methylpyrrolidinium fullerene iodide salt) was purchased from ProChimia (Sopot, Poland). A stock solution of the compound was prepared in dimethylsulfoxide (DMSO)/ddH_2O solution (1:9, v/v) and kept in the dark at 4 °C.

4.3. Antibiotics

Gentamycin (GEN), doxycycline (DOX), streptomycin (STR), ciprofloxacin (CIP), imipenem (IPM), vancomycin (VAN), and ampicillin (AMP) were purchased from Sigma Aldrich. Daptomycin (DAP), linezolid (LZD), and tigecycline (TGC) were purchased from Cayman Chemicals (Ann Arbor, MI, USA). Stock solutions at concentrations of 10 mg/mL were prepared in the recommended solvent and stored at −20 °C.

4.4. Light Sources

The custom constructed LED-based light source was used: emitting λ_{max} 522 nm light with a radiosity of 10.6 mW/cm^2 (FWDH (full width half maximum) 34 nm) (Cezos, Gdynia, Poland).

4.5. Photodynamic Inactivation of Planktonic Cultures

Overnight culture (1 colony transferred into 5 mL of tryptic soy broth (TSB) and incubated for 18 h at 37 °C with shaking at 150 rpm) of *E. faecium* and *E. faecalis* were adjusted to 0.5 McFarland (McF) units (Densi-La-Meter II, ERBA) in phosphate-buffered saline (PBS) (Sigma Aldrich, Inc, Munich, Germany), which corresponds to a cell density of approx. 10^7 CFU/mL. Working solutions of RB were prepared in ddH_2O or in the case of FL in a mixture of distilled water:DMSO (9:1 *v*/*v*). The bacterial suspension and PS solution were mixed and incubated in the dark at room temperature (RT) for 15 min. Then, the samples with PSs (100 µL) were illuminated. Afterwards, the samples were serially diluted in PBS and transferred onto tryptic soy agar (TSA) plates. After 18–20 h of incubation at 37 °C, colonies were counted, and the CFU/mL values were determined. Samples with RB and FL were illuminated with 522 nm light.

4.6. Determination of Sublethal and Lethal Doses of aPDI for Planktonic Cultures

Bacterial overnight cultures were suspended to obtain an optical density of 0.5 McF. Next, probes for the green light were mixed with PS solutions in 96-well plates and incubated for 15 min in the dark. Bacteria were irradiated with various light doses and then serially diluted, streaked on TSA plates and incubated at 37 °C for 16 h. After 16 h, colonies were counted, and the CFU/mL values were estimated. In addition, two control samples were prepared: 1, with no PS and with light to check bacterial growth; and 2, with PS and incubation in the dark to check the possible toxicity of PS. Sublethal doses (which reduce bacterial viability from 0.5 to 2 $\log_{10}$ CFU/mL) were calculated based on the survival rate of bacteria treated with aPDI in comparison to untreated bacteria. The lethal dose was determined as a ≥ 3 $\log_{10}$ CFU/mL reduction in viability.

4.7. Determination of MIC Doses of aPDI

Overnight cultures of both strains were diluted to obtain 0.5 McF in brain–heart infusion broth (BHI media; BioMerieux, France) and then diluted 10-fold. The experiment was not performed in Mueller-Hinton medium (MHE) due to the very weak growth of *Enterococcus* species. In the next step, samples were mixed with a solution of PS at the tested concentrations in 96-well plates. Suspensions were then incubated in the dark for 15 min and exposed to various light doses. Subsequently, the plates were incubated at 37 °C for 16–20 h, and bacterial growth was assessed optically in microtiter wells. The experiment was conducted in three independent replicates.

4.8. MIC Determination of Tested Antibiotics

Overnight cultures of both strains were adjusted to 0.5 McF in BHI and then diluted 10-fold. Next, probes were administered with antibiotics to reach the tested range of concentrations (from 1024 to 0.03125 µg/mL) in 96-well plates. Afterwards, the plates were incubated at 37 °C for 16–20 h. Bacterial growth was assessed optically in microtiter wells. The experiment was conducted in three independent replicates.

4.9. Synergy Testing

4.9.1. Antimicrobial Susceptibility Testing (Disk Diffusion Method and E-Tests)

Overnight cultures were diluted in PBS to obtain 0.5 McF. For the light-treated probes, sublethal doses of PSs were added. Next, the probes were incubated in the dark for 15 min and then exposed to sublethal doses of light. The next steps were the same for the treated and untreated probes. Then, 15 min after preparing the 0.5 McF suspension for untreated probes or immediately after light exposure for treated probes, the suspensions were streaked on MH agar plates (MHE, BioMerieux, France). After another 15 min, E-tests and disks with the tested antibiotics were placed on the plates. After 15 min of incubation at RT, the plates were placed in an incubator for 16–20 h at 37 °C. For antibiotics in disks, a synergistic effect was identified when the difference between the untreated and treated inhibition zones was greater than or equal to 2 mm. In the case of E-tests, synergy was

confirmed if the minimum inhibitory concentration (MIC) of the treated probe was at least 2-fold lower than of the untreated probes (control).

4.9.2. Checkerboard Assay

Overnight cultures of both strains were diluted to obtain 0.5 McF in BHI and then diluted 10-fold. Bacterial suspensions were placed in 96-well plates combined with different concentrations of antibiotics: 2 MIC, MIC, 1/2 MIC, 1/4 MIC, 1/8 MIC, 1/16 MIC, 1/32 MIC, and 0 MIC. Next, the wells in columns were diluted 2-fold with PS to obtain final PS concentrations with MICs as follows: MIC, 1/2 MIC, 1/4 MIC, 1/8 MIC, 1/16 MIC, 1/32, 1/64 MIC, 1/128 MIC, 1/256 MIC, 1/512 MIC, and 0 MIC. All cells were incubated in the dark for 15 min and then exposed to irradiation at MIC doses. Next, the plates were incubated for 16–20 h at 37 °C. Bacterial growth was assessed, and the fractional inhibitory concentration index (FICI) coefficient was calculated (FICI = FIC_A + FIC_B). $FIC_{A/B}$ = MIC of factor A/B in combination/MIC of factor A/B alone. Synergistic effects were observed when FICI $\leq$ 0.5, and antagonism was observed when FICI > 4; 4 < FICI > 0.5 means no interaction.

4.9.3. Postantibiotic Effect

Overnight cultures of both strains were diluted in BHI (1:20). A few combinations of agents were prepared: A, 1/2 MIC aPDI; B, MIC of antibiotic; C, 1/2 MIC of antibiotic; D, MIC of antibiotic + 1/2 MIC aPDI; and E, 1/2 MIC of antibiotic + 1/2 MIC aPDI. All probes were incubated in the dark for 2 h in an orbital incubator at 150 rpm. Next, the agents were removed by two washing steps, and bacteria were finally suspended in fresh BHI. Probes A, D, and E were exposed to irradiation in 1/2 MIC aPDI. Control samples were not exposed to any agents. Next, all samples were transferred to 96-well plates and placed in an EnVision multilabel plate reader (PerkinElmer, Waltham, MA, USA) for 16 h, which monitored the optical density (λ 600 nm) of cultures every 0.5 h. All data were normalized, and the postantibiotic effect (PAE) was calculated on the basis of the formula PAE = T – C (T, time required to reach OD_{600} = 0.5 after removal of the investigated agent; C, time required to reach OD_{600} = 0.5 of untreated bacteria). PAE $\geq$ 3 h indicates a synergistic effect, and 1.5 h $\leq$ PAE < 3 h indicates partial synergy.

4.10. Determination of Singlet Oxygen Production

An experiment was conducted for *E. faecalis* and RB with TGC or CIP. Overnight cultures were diluted in PBS to 0.5 McF. Additionally, 500 μM solutions of singlet oxygen sensor green probe (SOSG) purchased from Thermo Fisher Scientific (Waltham, MA, USA), was prepared according to the manufacturer's guidelines. Bacteria were mixed with PS and antibiotics (MIC) in different combinations and transferred to black sterile 96-well plates. To 100 μL of total volume, 1 μL of SOSG solution was added to estimate the final concentration of 5 μM. Then, the probes with PS were incubated for 15 min in the dark and exposed to light at MIC and 1/2 MIC doses. Next, fluorescence was measured using an EnVision plate reader at excitation/emission wavelengths of 488/525 nm. The experiment was performed in three independent replicates.

4.11. Determination of Production of ROS/Radicals

3′-(*p*-Aminophenyl) fluorescein (APF) is a specific probe for hydroxyl radicals (•OH) and 2′,7′-dichlorodihydrofluorescein diacetate (DCF) is specific also for (•OH), but also for other oxygen radicals. Experiments were conducted for *E. faecalis* and RB with TGC, GEN, or CIP. Overnight cultures were diluted in PBS to 0.5 McF. Bacteria were mixed with PS and antibiotics (MIC) in different combinations and transferred to black and sterile 96-well plates. To 100 μL of full volume, 1 μL APF solution or 5 μL of DCF solution (Thermo Fisher Scientific, Waltham, MA, USA) was added. Then, probes with PS were incubated for 15 min in the dark and exposed to light at MIC and 1/2 MIC doses. Next, fluorescence was measured using an EnVision plate reader at excitation/emission wavelengths of

490/515 nm for APF and 492–495/517–527 nm for DCF. The experiment was performed in three independent replicates.

4.12. Cell Membrane Integrity Assay

SYTOX Green has high affinity for DNA released from cells with permeabilized membranes. An experiment was conducted for the *E. faecalis* isolate and RB aPDI with TGC, GEN, or CIP. Overnight cultures were diluted in PBS to 0.5 McF. Bacteria were mixed with PS and antibiotics (MIC) and transferred to 96-well plates. To 100 µL of full volume, 1 µL of SYTOX Green (Molecular Probes, Eugene, OR, USA) solution was added. Then, the probes with PS were incubated for 15 min in the dark and exposed to light at MIC and 1/2 MIC doses. Next, fluorescence was measured using an EnVision plate reader at excitation/emission wavelengths of 488/523 nm. The experiment was performed in three independent replicates.

4.13. Materials and Methods Referring to Biofilm Culture

4.13.1. Biofilm Culture Conditions

For biofilm culture, a CDC biofilm reactor (BioSurface Technologies, Bozeman, MT, USA), presented in Figure 8, was used with coupons made of porous polycarbonate. Before each culture, the coupons were sonicated for 10 min in 1% sodium dodecyl sulfate (SDS), washed in distilled water, sonicated for 10 min in distilled water, washed, incubated for 2 h in 2 M hydrochloric acid and finally washed in distilled water. Then, the coupons were placed in polypropylene rods, which were placed into reactors containing 500 mL of distilled water. The whole setup was autoclaved for 60 min at 10.3 psi. Water in the reactor was then replaced with 500 mL of sterile TSB (30 g/L + 100 g/L glucose) inoculated with 1 mL of 3.5 McF adjusted overnight culture of *E. faecalis*. The reactor was placed onto a magnetic stirrer with a heater set at 80 rpm and 37 °C for 24 h, referring to a batch phase. Before starting the flow phase, 1 L of 20× concentrated sterile TSB was added to a 20 L carboy containing 19 L of distilled water autoclaved for 2 h at 14.7 psi. The final concentration of broth was 30 g/L TSB with 10 g/L glucose. The carboy was connected to the reactor by silicone tubing and connected to a peristaltic pump (Watson-Marlow Fluid Technology Group, Falmouth, UK). The flow rate was set to 12.9 mL/min, and the reactor volume was 335 mL, which resulted in a residence time of 26 min, consistent with the *E. faecalis* generation time. The time of the flow phase was 24 h.

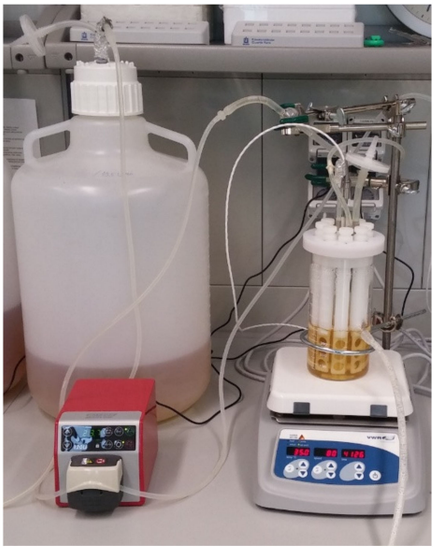

Figure 8. CDC biofilm reactor (BioSurface Technologies, Bozeman, MT, USA).

4.13.2. Biofilm Treatment

Coupons with biofilm layers were incubated with RB (5 μM) and STR (3× MIC, 768 μg/mL) or CIP (5× MIC, 10 μg/mL) in PBS for 15 min and then exposed to aPDI. The coupons were irradiated for 12.5 min, turned around and irradiated again. Four control groups without irradiation were prepared: (1) with no factor; (2) only with RB in the dark; (3) with CIP; and (4) with STR. After treatment, the coupons were placed in Falcon tubes with 10 mL of PBS. Then, biofilm layers were dispersed by sonication with 40% amplitude. Each probe was sonicated for 1 min, vortexed for 1 min and incubated on ice for 1 min. The procedure was repeated three times. After this procedure, the samples were vortexed again, and 100 μL of each sample was serially diluted in PBS, streaked on TSA plates and then incubated at 37 °C for 16 h. The CFU/cm^2 values of the coupon were calculated. The experiment was conducted in three replicates.

4.13.3. Biofilm Visualization

Biofilm growth on coupons was also visualized using confocal microscopy. Visualization of biofilms was performed with a BacLight Live/Dead viability kit. Coupons without or after aPDI/antibiotic treatment were transferred to a 12-well glass-bottom plate and incubated in the presence of SYTO 9 and propidium iodide (PI) dissolved in PBS for 15 min in the dark at RT, according to the protocol described previously [36]. Specimens were imaged using a confocal laser scanning microscope (Leica SP8X) with a 10× lens (Leica, Germany). During observation, the excitation were 488 and emission wavelengths used for detecting SYTO 9 were 501–548 nm, and for detecting PI 603–649 nm. Photographs were obtained and then analyzed with Leica LAS X software.

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Chapter V

Priming effect with photoinactivation against extensively drug-resistant *Enterobacter cloacae* and *Klebsiella pneumoniae*

1. Summary of the publication

Enterobacter cloacae and *Klebsiella pneumoniae* belong to the Enterobacteriaceae family and are part of the ESKAPE group. The term "priming effect" in the publication title can be explained as the pretreatment of cells (prokaryotic or eukaryotic) with photoinactivation, which leads to changes in the environment, making the cells more susceptible to subsequent treatments (e.g., immunotherapy, chemotherapy)[81]. In comparison to Gram-positive species, Gram-negative species are much more resistant to photoinactivation as well as to other treatment possibilities (e.g., antibiotics), which has been many times evidenced in the literature[82,83]. Therefore, pretreating microorganisms with photoinactivation conditions could lead to sensitization and a better response to other treatments.

Within **publication no. 4,** four carbapenem-resistant clinical isolates of those pathogens were examined according to the possibility of sensitization to antimicrobial agents, i.e., "cell priming". Cell priming doses were investigated based on the survival rate of the tested microorganisms after aBL/aPDI treatments. If the bacterial cells did not respond to photoinactivation conditions, the cell priming effects were not investigated. This was performed with cells that were suspended in the following environments: tryptic soy broth medium (TSB) and phosphate-buffered saline (PBS). **One of the major conclusions drawn from this experimental outcome involves the response of cells to photoinactivation with aBL/aPDI, which depends on the environmental conditions.** Therefore, the priming effect can vary depending on the cell medium/environment.

The next part of **publication no. 4** is focused on verifying the effectiveness of the priming effect; thus, the changes in the resistance profile of microorganisms were investigated as described in publications **no. 1–3** in the methods of synergy testing. Most tested isolates exhibited a synergistic effect when aBL or aPDI was combined with CST, FOF, CHL or CAZ. The postantibiotic effect did not reveal any synergies; however, it can be related to a high growth rate of *Enterobacteriaceae.* Thus, I was unable to observe significant differences with this approach.

Another critical issue is related to the presence of antibiotics in the bacterial environment and the production of ROS upon photoinactivation exposure and without an arrangement of this element. First, isolate no. D680 exhibited synergy with chloramphenicol (CHL) in most of the experiments in **publication no. 4**, and in the presence of the fluorescent probes, **increased production of ROS was detected for CHL after aBL exposure**. This observation could explain the increased synergies observed for this antimicrobial agent when aBL was administered; however, this was not confirmed for aPDI with RB. Second, antibiotics have the ability to produce ROS by activating metabolic pathways in microorganisms. Thus, the lethality of antibiotics can also be related to the toxic effect of ROS. Therefore, I investigated whether antibiotics, which in *in vitro* assays exert a synergistic impact with photoinactivation, can produce various oxygen radicals without an arrangement of light. Experiment I showed that colistin (CST) and ceftazidime (CAZ) can **produce ROS in *K. pneumoniae* cells. Therefore, CST and CAZ could be responsible for the obtained synergies.**

The last conclusion from **publication no. 4** involves the increased permeabilization of bacterial cells (*K. pneumoniae*) isolate, which occured when CST was used in combination with aPDI. The mechanism of action of colistin is related to LPS disruption and induction of changes in the cell membrane permeability[84,85]. **The level of permeabilization for CST in comparison to that of other antimicrobials (e.g., CHL, CAZ) increased when aBL was present,** and this was confirmed by implementing the SYTOX green label.

The Editors letter confirming the acceptance of **publication no. 4** is included in the attachments at the end of the doctoral dissertation.

2. Publication

Priming effect with photoinactivation against extensively drug-resistant *Enterobacter cloacae* and *Klebsiella pneumoniae*

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ABSTRACT

In this study, we present antimicrobial blue light (aBL) and antimicrobial photoinactivation with green light in the presence of Rose Bengal (aPDI) to modulate the susceptibility of extensively drug-resistant (XDR) *Enterobacter cloacae* and *Klebsiella pneumoniae* clinical isolates to antimicrobials. This process can be considered a photodynamic priming tool that influences other therapeutic options, such as antibiotics. The current study evaluated the different environments to estimate the most effective priming conditions by testing a broad spectrum of antimicrobials (including antimicrobials with different targets and mechanisms of action). The susceptibility of the *E. cloacae* and *K. pneumoniae* clinical isolates to various antibiotics after aBL and green light (with rose bengal) as aPDI treatment was examined with multiple methods of synergy testing (e.g., diffusion methods, checkerboard assay, postantibiotic effect), and most effective photoinactivation conditions were implemented for each environment. When *Enterobacteriaceae* were exposed to aBL, the most efficient reduction in survival rate under TSB conditions was observed. Similar results were observed when rose bengal, as a photosensitizer, was present during the exposure to green light in PBS. aBL and aPDI led to an increased susceptibility of *K. pneumoniae* and *E. cloacae* isolates to chloramphenicol and colistin or fosfomycin and colistin antibiotics, respectively. However, among the 4 tested isolates, we observed synergies between different antimicrobial agents and photoinactivation conditions. Thus, it may suggest that the sensitization process may be considered a strain dependent priming tool.

KEYWORDS

Antimicrobial blue light, *Enterobacteriaceae*, photoinactivation, priming effect, Rose Bengal, synergy

1. INTRODUCTION

According to the data published by the World Health Organization (WHO), antimicrobial resistance (AMR) is one of humanity's top 10 global public health threat. According to the Centers for Disease Control and Prevention (CDC), common *Enterobacteriaceae* cause healthcare-associated infections, including *Enterobacter* spp., *Klebsiella* spp., and *E. coli*. Increased resistance of *Klebsiella pneumoniae* isolates to carbapenems or cephalosporins is occurring due to the mutations of porins OmpK35, OmpK36 or production of carbapenemase (KPC), an enzymes leading to degradation of β-lactam antibiotics [1,2]. Similar resistance occurs in *Enterobacter cloacae* isolates due to decreased outer membrane protein expression (OmpF). These strains demonstrate resistance to carbapenems [3]. According to the reports by the China Antimicrobial Surveillance Network (CHINET), the resistance of *E. cloacae* to carbapenems was estimated to be approx. < 1.0% in 2007 and rapidly increased to about 10% in 2019 [4]. *E. cloacae* is responsible for mortality in more than 40% of bloodstream infections, and in carbapenem-resistant *K. pneumoniae* the mortality rate of bloodstream infections is estimated to 40-70% [5]. It should be emphasised that resistance to carbapenems and other antibiotics makes treating infections very difficult. Moreover, the increased or inappropriate consumption of antibiotics lead to further increase in AMR development. To overcome this problem, many alternative approaches are intensively studied to compromise the "superbugs activity", for example, bacteriophage therapy, antimicrobial peptides, silver nanoparticles or antimicrobial photoinactivation presented within the current study [6].

The most common phototherapies include antimicrobial photodynamic inactivation (aPDI) requiring exogenous photosensitizer or antimicrobial blue light (aBL) that involves endogenous bacterial chromophores. Exposure to visible light wavelengths ranging from 400 to 760 nm leads to the absorption of photons by exo- and endogenous photosensitizing compounds in the presence of oxygen, leading to the production of reactive oxygen species (ROS) [7,8]. Depending on the type of reaction, superoxide ($\cdot O^-_2$), hydroxyl radicals ($\cdot OH$), and hydrogen peroxide (H_2O_2) can be produced via type I mechanism, or mainly singlet oxygen via type II reaction [9,10]. Produced ROS non-specifically leads to disturbance of various cell components (e.g., lipid peroxidation, proteins oxidation), DNA damage, and overall cell death [10]. Photoinactivation as a single treatment (monotherapy), both for aBL and aPDI, was presented in literature data as an effective approach to eradicate ESKAPE pathogens and

other groups of microbial species. Antimicrobial blue light inactivation (aBL) for example efficiently reduced viability of *Streptococcus pyogenes* (by 8 $\log_{10}$ CFU; 36 J/cm^2), *Cronobacter sakazakii* (by > 8 $\log_{10}$ CFU; 240.48 J/cm^2) or MDR *Escherichia coli* (by > 5 $\log_{10}$ CFU; 206.25 J/cm^2) [8,11,12]. On other hand, aPDI with the implementation of various exogenous photosensitizing agents, e.g., methylene blue, Rose Bengal, Tri-Py+-Me-PF porphyrin, or cationic riboflavin derivative (FLASH-01a), was present as an efficient approach in eradication of *Enterococcus faecalis* (by 9.98 $\log_{10}$ CFU), *Staphylococcus aureus* (by 6 $\log_{10}$ CFU/ml), *E. coli* (by 7 $\log_{10}$ CFU/ml) or *Acinetobacter baumannii* (by 6.6 $\log_{10}$ CFU/ml) [13–16]. The examples given above are only a fraction of the results that demonstrate the effectiveness of aBL or aPDI in eradicating pathogens. Literature reviews published by our team and other scientific groups clearly and precisely present the enormous possibilities of aBL or aPDI as an effective approach against MDR pathogens, fungi and even viruses [10,17–21].

Up to this date, numerous comprehensive reviews and original studies provide multiple supportive investigations of combining antibiotics and photoinactivation [22–24]. The current study investigates whether the photoinactivation as a monotherapy can be combined with antibiotics to eradicate the two most prevalent *Enterobacteriaceae* representatives: *E. cloacae* and *K. pneumoniae.* Moreover, the current study evaluates the effectiveness of aPDI and aBL in the sensitization process with the involvement of carbapenem-resistant extensively drug-resistant strains (XDR). It is also worth mentioning that, instead of testing a single antimicrobial agent, we made an evaluation of the possible synergies including various classes of antibiotics, covering various mechanisms of action. Within this study, we investigate if aBL and aPDI can be used as a pre-treatment for *Enterobacteriaceae* representatives as a priming tool. Pre-treating cells with ROS as a priming effect can lead to a decrease in the use of antimicrobials due to decreased concentrations used; therefore, this method could address the antimicrobial resistance crisis. The second important part of this study concerned the investigation of the possible mechanisms of photodynamic inactivation using the clinical isolate of *K. pneumoniae* as a representative of *Enterobacteriaceae*. Understanding the photodynamic process of aBL and aPDI in *Enterobacteriaceae* representatives can bring us closer to identify the mechanism of the synergy between photoinactivation (aBL/aPDI) and antibiotics. Investigation of cell membrane permeabilization and the production of ROS was performed upon photoinactivation treatment (aBL and aPDI) with the implementation of XDR carbapenem-resistant *K. pneumoniae* isolate D680 which demonstrated the largest number of synergies between photoinactivation and antibiotics.

Finally, as the increased production of oxygen radicals can be also detected upon treatment of microorganisms with specific antimicrobials, i.e., aminoglycosides, fluoroquinolones or β-lactam antibiotics [25][26], the last hypothesis examined within this study concerned the involvement of antibiotic-mediated ROS production, exemplified with colistin, ceftazidime and chloramphenicol, to synergy with aPDI and aBL.

2. Materials and Methods

2.1 Bacterial Strains

We used two XDR clinical isolates of *Enterobacter cloacae* no. 2640/13, 4986/12 and two XDR *Klebsiella pneumoniae* isolates no. D479 and D680. *K. pneumoniae* isolates were kindly provided by Prof. Nico T. Mutters from the Institute for Hygiene and Public Health, Bonn University Hospital, Germany. *K. pneumoniae* isolates were isolated from catheter urine and blood sample (D680 and D479, respectively), whereas both *E. cloacae* isolates from blood samples (2640/13 and 4986/12, respectively). All isolates were cultivated in tryptic soy broth (TSB, bioMérieux, France) for 16–20 h under aerobic conditions in an orbital incubator (Innova 40, Brunswick, Germany) at 150 rpm. A solid medium (TSA) containing TSB medium with 1.5% agar (BTL, Warsaw, Poland) plates were used for colony-forming unit (CFU) enumeration.

2.2 Photosensitizer

Rose bengal (RB) 4,5,6,7-tetrachloro-2,4,5,7-tetraiodofluorescein disodium salt powder was purchased from Sigma Aldrich (Munich, Germany). The stock solution was prepared in double-distilled water (ddH_2O) and was maintained in the dark at 4°C.

2.3 Light Sources

Custom constructed LED-based light sources were used as follows: emitting λ_{max} 522 nm light with radiosity of 10.6 mW/cm^2 and a second light source emitting λ_{max} 415 nm with an irradiance of 24 mW/cm^2.

2.4 Antibiotics

Chloramphenicol (CHL), gentamycin (GEN), ceftazidime (CAZ), doxycycline (DOX), imipenem (IPM), ciprofloxacin (CIP), fosfomycin (FOF), colistin (CST), piperacillin (PIP), cefuroxime (CXM), sulbactam (SUL) and ampicillin (AMP) were purchased from Sigma-Merck (Germany), and aztreonam (ATM), tigecycline (TGC) and

tazobactam (TZB) were purchased from Cayman Chemicals (Ann Arbor, MI, USA). Stock solutions at 10 mg/mL concentrations were prepared as the producer solvent recommended and stored at −20°C.

2.5 Photoinactivation of planktonic cultures of *Enterobacteriaceae* with blue light (aBL) and antimicrobial photoinactivation (aPDI) with Rose Bengal.

Overnight bacterial cultures (16–20 h) were prepared by inoculating a single colony in 5 mL of TSB medium (BioMérieux, France) at 37°C with shaking at 150 rpm in an orbital incubator (Innova 40, Brunswick, Germany). Then, overnight bacterial cultures were adjusted to 0.5 MacFarland (McF) units (Densi-La-Meter II, ERBA) in PBS (phosphate-buffered saline, Sigma, Germany) or TSB (approx. 0.5-1 · 10^8 CFU/ml) and were transferred to a 96-well plate alone or in combination with RB. The cells diluted in TSB were incubated in the dark with PS for 15 min and were immediately exposed to a green light dose up to 57.2 J/cm^2, while the cells prepared in PBS were irradiated with the same light source up to 28.6 J/cm^2. The aBL samples prepared in TSB and PBS without RB were illuminated immediately with different blue light doses, and the highest value was 84.6 J/cm^2. Afterwards, the cells were serially diluted and streaked on standard TSA plates, which were kept at 37°C for 16 h in an incubator (Termax, Norway). In the next step, the cells were counted, and the CFU/ml was determined. The experiment was performed for three biologically independent repetitions, and control groups (including cells incubated with RB or without) were also included.

2.6 Determination of the sublethal and lethal doses of antimicrobial blue light (aBL) and antimicrobial photoinactivation (aPDI) with Rose Bengal.

According to our previously published protocols, a sublethal dose that reduces bacterial viability from 0.5 to 2 $\log_{10}$ CFU/mL was calculated based on the survival rate of the bacteria that were treated in PBS/TSB with aBL/aPDI (RB) in comparison to bacterial cells in the untreated control. The lethal dose was determined as a ≥ 3 $\log_{10}$ CFU/mL reduction in viability [27,28].

2.7 Identification of the Minimal Inhibitory Concentrations (MIC) of antimicrobial blue light (aBL) and antimicrobial photoinactivation (aPDI) with Rose Bengal.

Overnight bacterial cultures (16–20 h) were diluted in TSB medium (BioMérieux, France) to obtain 0.5 McF unit (Densi-La-Meter II, ERBA) suspension. They were then diluted 10-fold in Mueller Hinton Broth (MHB) (Roth, Germany). The cells were transferred to a 96-well plate and immediately irradiated with various blue light doses (up to 108 J/cm^2). To establish the MIC value of aPDI, Rose Bengal was added to the diluted cultures, and after incubation for 15 min in the dark the green light was applied up to a dose of 57.2 J/cm^2. Higher amounts of blue and green light could not be implemented due to the risk of the photothermal effect occurring rather than the photoinactivation effect. After exposure to photoinactivation (aBL and aPDI), plates containing the irradiated cells were protected with parafilm and incubated at 37°C for 16–20 h in an incubator (Termax, Norway). Then, inhibition of bacterial growth after aBL/aPDI was optically assessed in microtiter wells. Although MIC value is known to be a parameter for testing the inhibitory concentration of antimicrobial agents/antibiotics, it was necessary to determine this parameter in the context of the aBL/aPDI conditions due to the further implementations of this parameter in the synergy assays, i.e., checkerboard assay and post-antibiotic effect. The experiment was conducted with three independent biological replicates.

2.8 Identification of the Minimal Inhibitory Concentrations (MIC) of tested antibiotics

Overnight cultures (16–20 h) of clinical isolates were adjusted in TSB medium (BioMérieux, France) to obtain 0.5 McF units (Densi-La-Meter II, ERBA) and were then diluted 10-fold in MHB (Roth, Germany). Next, the diluted cultures were administered antibiotics to reach the tested range of concentrations (from 1024 to 0.03125 µg/mL) in 96-well plates. Afterwards, the plates were protected with parafilm and incubated at 37°C for 16–20 h in an incubator (Termax, Norway). Bacterial growth was assessed optically in microtiter wells, and the lowest antibiotic concentration that confirmed the lack of bacterial growth was established as the MIC value. The experiment was conducted in three independent biological replicates.

2.9 Determination of the interactions between aBL/aPDI (RB) and antibiotics

To assess the influence of photoinactivation conditions, the effect of photodynamic priming on the profiles of resistance of *Enterobacteriaceae* was examined through various methods of synergy testing. The most pronounced sub-lethal and lethal doses of aBL/aPDI (RB) that were established in the TBS and PBS environments were further applied to maximize the effectiveness of the sensitization process within the synergy examination.

2.9.1 Antimicrobial susceptibility testing (E-test and disc-diffusion assay)

Overnight cultures (16–20 h) of clinical isolates were adjusted in TSB medium (BioMérieux, France) to obtain 0.5 McF units (Densi-La-Meter II, ERBA) and were then diluted 10-fold in PBS. The cells were immediately exposed to a sublethal dose of aBL. For second photoinactivation conditions (aPDI), cells were combined with a Rose Bengal in sublethal conditions, incubated for 15 min in the dark, and then irradiated with a sublethal dose of green light. The following steps were the same for the treated and untreated probes. The suspensions were streaked on MHA plates (Muller Hinton Agar 2, Sigma, Germany) 15 min after the 0.5 McF suspension for untreated probes was prepared or immediately after light exposure for treated probes). After another 15 min, E-tests and disks were placed on the plates with the tested antibiotics. After another 15 min of incubation at room temperature, the plates were placed in an incubator (Thermax, Norway) for 16–20 h at 37°C. Our previously published data identified a synergistic effect for the disk-diffusion method when the difference between the untreated and treated inhibition zones was equal or greater than 2 mm. In the case of the E-test method, if the MIC of the treated probe was at least 2-fold lower than that of the untreated probes (control), then the synergy was confirmed [27,28].

2.9.2 Checkerboard assay

The bacterial cultures (16–20 h) that were inoculated in a 5 mL TSB medium (BioMérieux, France) were diluted to 0.5 McF units (Densi-La-Meter II, ERBA) in a sterile TSB medium and then an additional 10-fold in MHB. For the experiments that involved a green light source and Rose Bengal (aPDI), the bacterial suspensions were placed in 96-well plates that were combined with different 2-fold concentrations of antibiotics as follows: 2 MIC, MIC, ½ MIC, ¼ MIC, ⅛ MIC, and 0 MIC. Moreover, the wells in the columns were diluted 2-fold with RB to obtain final concentrations: 2 MIC, MIC, ½ MIC, ¼ MIC, ⅛ MIC and 0 MIC. The cells were incubated in the dark for 15 minutes and then exposed to MIC green light doses. For the experiments that involved blue light (aBL), bacterial suspensions were placed in 96-well plates, which were combined with different 2-fold concentrations of antibiotics (2 MIC, MIC, ½ MIC, ¼ MIC, ⅛ MIC, and 0 MIC) and were immediately exposed to various doses of MIC aBL light (2 MIC, MIC, ½ MIC, ¼ MIC, ⅛ MIC and 0 MIC). After exposure to aBL/aPDI, all irradiated samples were placed in an incubator (Thermax, Norway) for 16–20 h at 37°C. Bacterial growth was assessed to determine the synergistic effect between the tested factors, and the fractional inhibitory concentration index (FIC_i) coefficient was calculated ($FIC_i = FIC_A + FIC_B$). $FIC_{A/B}$ = MIC of factor A/B in combination/MIC of factor A/B alone. Synergy was observed when $FIC_I \leq 0.5$, and antagonism was observed when $FIC_i > 4$; $4 < FIC_i > 0.5$ indicates no interaction between the tested factors [29,30].

2.9.3 Time–kill curve assay

The overnight cultures that were incubated in 5 mL sterile TSB medium (BioMérieux, France) were diluted (1:20) in fresh TSB and were mixed with antibiotics and/or Rose Bengal in the following combinations: (A) ½MIC aPDI/aBL; (B), MIC of antibiotic; (C) ½ MIC of antibiotic; (D) MIC of antibiotic + ½ MIC aPDI/aBL; and (E) ½ MIC of antibiotic + 1/2 MIC aPDI/aBL. All combinations of cells and tested agents were incubated at 37°C for 2 h with shaking at 150 rpm in an orbital incubator (Innova 40, Brunswick, Germany). After incubation, the samples were washed twice with a sterile TSB medium and resuspended in a fresh portion of TSB. Combinations of samples (A), (D), and (E) were next exposed to ½ MIC of aPDI/aBL conditions. Control samples (B) and (C) were not exposed to any light conditions. After being exposed to the photoinactivation treatment, 100 µL of each sample was transferred to a 96-well plate and was placed in an EnVision multilabel plate reader (PerkinElmer, Waltham, MA, USA) for 16 h, which monitored the optical density (λ 600 nm) of cultures every 30 min. All the obtained results were normalized, and based on the growth curves, the postantibiotic effect (PAE) was calculated based on the formula PAE = T – C (T: the time required to reach an OD_{600} value of 0.5 after the investigated agent was removed, C: the time necessary to reach an OD_{600} value of 0.5 in the untreated cells). A PAE value ≥ 3 h indicates a synergistic effect, and values 1.5 h ≤ PAE < 3 h indicate partial synergy [27,31].

2.10 Detection of $OH^{\bullet}$ radicals with 3′-p-hydroxyphenyl-fluorescein (HPF) with photoinactivation approaches

The bacterial cultures for strain no. D680 (16–20 h) were inoculated in 5 mL of TSB medium (BioMérieux, France) and were diluted to 0.5 McF units (Densi-La-Meter II, ERBA) in sterile PBS. The HPF probe was purchased from Sigma–Aldrich (Germany), prepared according to the manufacturer's instructions and diluted to obtain a concentration of 50 µM. The bacterial cells diluted in PBS were mixed with the antibiotics at MIC and ½ MIC concentrations both for aBL and aPDI treatments. In case of aPDI the Rose Bengal was applied to the tested samples. The HPF probe was added to all samples to obtain a final concentration of 5µM. Then, the probes were incubated in the dark for 10 min and exposed to aBL/aPDI (RB) conditions. After phototreatment, the fluorescence of HPF was observed using wavelengths of 515 nm (emission) and 490 nm (excitation) with an EnVision multilabel plate reader (PerkinElmer, Waltham, MA, USA). The values are the mean of three independent experiments.

2.11 Detection of H_2O_2, $OH^{\bullet}$, and $ROO^{\bullet}$ radicals with HPF, H_2DCFDA and $O_2^{\bullet}$ singlet oxygen with singlet oxygen sensor green (SOSG) in bacterial cells as an antibiotic stress response signal

The experiment was performed in accordance with the protocol published by Dwyer et al. with modifications [26]. The overnight culture (16–20 h) with strain no. D680 was diluted 500-fold in fresh Luria/Miller Broth (LB) (Roth, Germany). Then, the cells were mixed with the probes to obtain final concentrations of 10 μM (HPF), 5 μM (H_2DCFDA), or 10 μM (SOSG) and were incubated in 96-deep wells at 37°C for approx. 2.5 h with shaking at 150 rpm in an orbital incubator (Innova 40, Brunswick, Germany) to obtain an optical density of ~ 0.2 in the cultures. After establishing OD_{600} equal to 0.2, the fluorescent probe samples were combined with antibiotics at 2 MIC and MIC concentrations: ceftazidime, chloramphenicol and colistin. Immediately after preparation, radical identification was performed for the HPF probe every 30 min for 3 h using wavelengths of 515 nm (emission) and 490 nm (excitation) with an EnVision multilabel plate reader (PerkinElmer, Waltham, MA, USA). For the H_2DCFDA and SOSG probes, the measurements were performed under the same conditions except for different fluorescence parameters (Ex/Em: 495/517 nm and 504/525 nm, respectively). Ten microliters of each measured sample were serially diluted in PBS and seeded on agar plates before and after fluorescence measurements. Then, the samples were incubated for 16 h at 28°C, and after incubation, the colonies were counted, and a CFU/ml number was established. The values are the mean of three independent experiments.

2.12 Detection of outer membrane permeability upon aBL/aPDI with SYTOX green labelling

The experiment was performed in accordance with the protocol published by Grinholc et al. with modifications [32]. The bacterial cultures with strain no. D680 (16–20 h) were inoculated in 5 mL of TSB medium (BioMérieux, France) and were diluted to 0.5 McF units (Densi-La-Meter II, ERBA) in sterile PBS. The bacterial cells were mixed with the antibiotics at MIC and ½ MIC concentrations for aBL treatment. For aPDI exposure, an additional photosensitizer Rose Bengal was applied to the tested samples. The cells were then exposed to photoinactivation conditions, and immediately after irradiation, SYTOX was added to obtain a final concentration of 5 μM. The fluorescence of SYTOX was measured in samples after 10 min of incubation at room temperature using an EnVision plate reader at excitation/emission wavelengths of 488/523 nm. The experiment was performed in three independent replicates.

2.13 Statistical analysis

Statistical analysis was performed using the GraphPad Prism version 9.0 (https://www.graphpad.com/) (accessed on 19.08.2022). The statistical differences between groups was performed with one-way ANOVA with the significance level $p<0.05$.

3. Results

3.1 The response of planktonic cultures of *Enterobacter cloacae* and *Klebsiella pneumoniae* clinical isolates to aPDI and aBL treatment depends on the environmental conditions

Investigation of photoinactivation with aBL and aPDI (RB) was performed under two different environmental conditions. In all synergy testing experiments, the bacterial cells were diluted under these conditions. To maximise the effectiveness of phototreatment regarding the method used, four *Enterobacteriaceae* isolates were irradiated with various doses of aBL and green light with two rose bengal concentrations (10 and 20 μM) as aPDI (RB). Fig. 1A represents the susceptibility profile of the following *E. cloacae* isolates to aBL: no. 2640/13 and no. 4986/12 in PBS and TSB medium, respectively. For *K. pneumoniae* isolates, no. D680 and D479, the effectiveness of aBL under the same conditions are presented in Fig. 1B. The grey frames in both figures indicate the sublethal doses of phototreatments, and the pink frames present the range of lethal doses (Fig. 1 and 2). The sublethal and lethal doses determined for planktonic cultures in *in vitro* conditions were used in further experiments in the present study. As for photoinactivations we could identify few conditions that exerted lethal and sublethal effects, the conditions with the lowest light dose and minimal photosensitizer concentration were implemented in futher studies.

The established sublethal doses of aBL/aPDI (RB) for all of the isolates in PBS were further used in the diffusion assays (disk-diffusion and E-test) for those strains in which the sublethal dose of photoinactivation was not detected in the experiment regarding the following light conditions (e.g., a sublethal dose of aBL for isolate no. 2640/13). In the TSB medium, photoinactivation with blue light was more promoted (Fig. 1A, B) in comparison to photoinactivation with a rose bengal and green light as aPDI (RB) against the tested *Enterobacteriaceae* in the same environment. The opposite effect was observed when cells were diluted in PBS; thus, compared to aBL treatment, aPDI (RB) inactivation was more efficient, which was observed for all tested *Enterobacteriaceae*.

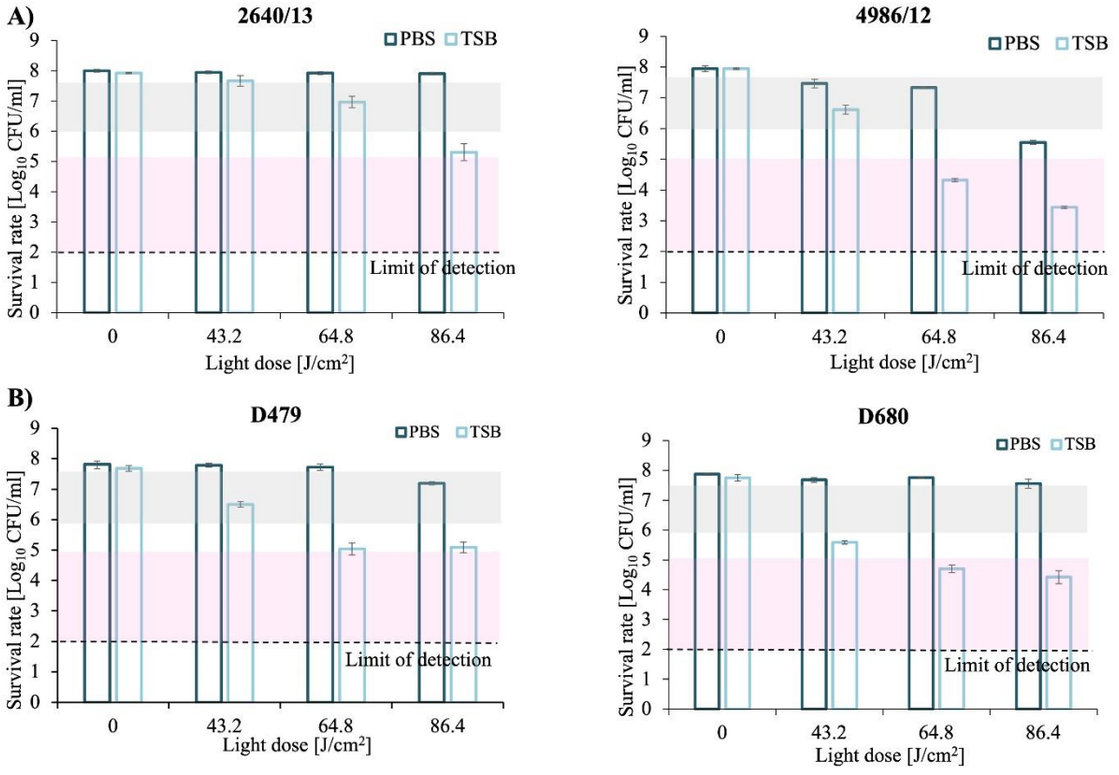

Fig 1. Antimicrobial blue light treatment (aBL) against *E. cloacae* (no. 2640/13, 4986/12) (A) and *K. pneumoniae* (no. D479, D680) (B) with various doses of blue light in PBS and TSB. The experiment was performed in three biological replicates. The grey frames in the graphs indicate sublethal photoinactivation conditions, and the pink frames show lethal doses of aBL. The detection limit was 100 CFU/mL.

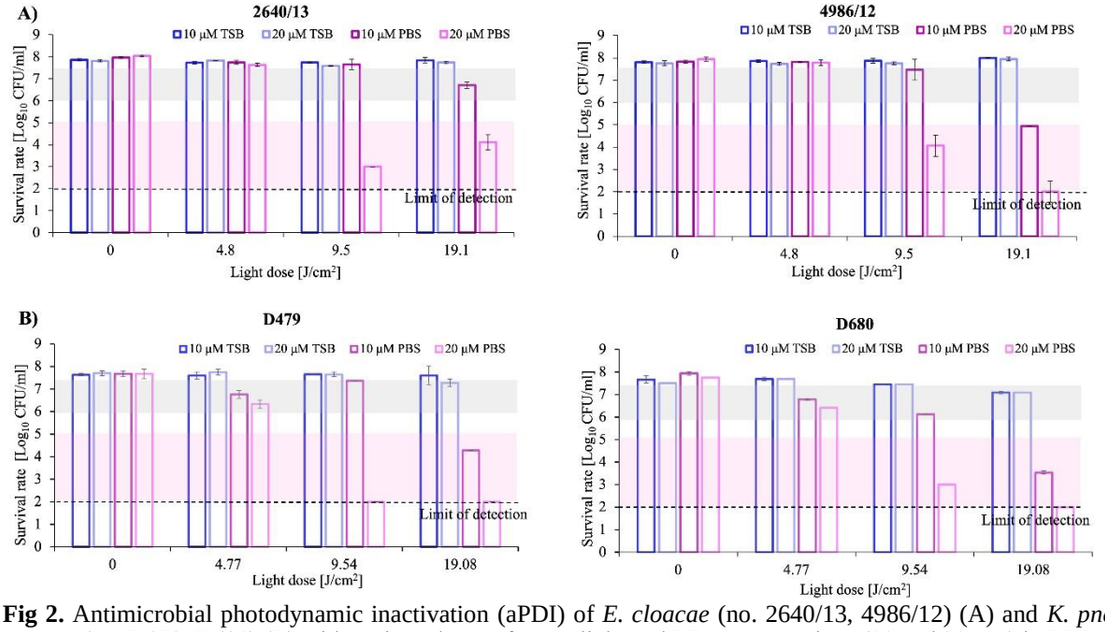

Fig 2. Antimicrobial photodynamic inactivation (aPDI) of *E. cloacae* (no. 2640/13, 4986/12) (A) and *K. pneumoniae* (no. D479, D680) (B) with various doses of green light and RB concentrations (10 and 20 µM) in PBS and TSB. The experiment was performed with three biological replicates. The grey frames in the graphs indicate sublethal aPDI (RB) conditions, and the pink frames show the lethal doses of aPDI (RB). The detection limit was 100 CFU/mL.

3.2. Identification of the treatment MIC values

Establishing MIC values for antimicrobial agents is necessary to estimate the resistance profile of the tested microorganisms in the present study and evaluate the effectiveness of combined treatments in checkerboard assay and postantibiotic effect assays. Table 1 represents the MIC values for all tested antibiotics and phototherapies. Based on the data published by Magiorakos et al., all of the tested isolates have an XDR resistance profile [33]. The MIC of antimicrobial agents was interpreted according to the breakpoint tables for interpretation of MICs and zone diameters version 12.0, valid from 01.01.2022 posted on the EUCAST website (https://www.eucast.org/clinical_breakpoints/). For isolate no. 2640/13, the MIC value for the aBL treatment was not established. Increased irradiation with blue light could have a thermal effect instead of the effect of the photooxygenation process; therefore, higher light doses were not implemented in experiments.

Table 1
Minimal Inhibitory Concentrations for antimicrobials and phototherapy conditions

			Klebsiella pneumoniae		***Enterobacter cloacae***	
Antibiotic target	Antimicrobial category	Antibiotic	**D680**	**D479**	**2640/13**	**4986/12**
			MIC[1]	MIC	MIC	MIC
Protein synthesis (50S)	Phenicols	Chloramphenicol	1024 (R)	512 (R)	1024 (R)	1024 (R)
Protein synthesis (30S)	Aminoglycosides	Gentamycin	0.5 (S)	128 (R)	1024 (R)	16 (R)
	Tetracyclines	Doxycycline	16*	32*	4*	32*
	Glycylcyclines	Tigecycline#	32*	1024*	1*	2*
DNA gyrase	Fluoroquinolones	Ciprofloxacin	4 (R)	128 (R)	2 (R)	2 (R)
Folic acid metabolism	Folate pathway inhibitors	Trimethoprim-sulfamethoxazole#	1024 (R)	1024 (S)	1024 (R)	1024 (R)
Cell-wall synthesis	Carbapenems	Imipenem	64/128 (R)	16 (R)	256 (R)	64 (R)
	Extended-spectrum cephalosporins	Ceftazidime	1024 (R)	512 (R)	1024 (R)	1024 (R)
	Penicillins	Ampicillin	1024 (R)	1024 (R)	1024 (R)	1024 (R)
	Phosphonic acid	Fosfomycin	1024 (R)	1024 (R)	1024 (R)	1024 (R)
	Monobactam	Aztreonam	1024 (R)	128 (R)	512 (R)	1024 (R)
	Antipseudomonal penicillins + β-lactamase inhibitor	Piperacillin-Tazobactam#	512/1024 (R)	256 (R)	1024 (R)	512 (R)
	Non-extended-spectrum cephalosporins	Cefuroxime#	1024 (R)	1024 (R)	1024 (R)	1024 (R)
	Penicillins + β-lactamase inhibitor	Ampicillin-Sulbactam#	1024 (R)	512 (R)	1024 (R)	512 (R)
	Penicillins	Ampicillin#	1024 (R)	1024 (R)	1024 (R)	1024 (R)
Cell membrane	Polymyxins	Colistin	2 (S)	8/4 (S)	4 (S)	32 (S)
	Light dose [J/cm^2]					
Phototherapy	**aBL** (λ 411 nm)		86.4 J/cm^2	108 J/cm^2	n.d	93.6 J/cm^2
	aPDI (λ 522 nm) + RB		38.2 J/cm^2 (10 μM RB)	44.5 J/cm^2 (10 μM RB)	28.6 J/cm^2 (20 μM RB)	28.6 J/cm^2 (20 μM RB)

[1]Estimated in μg/ml; nd – not defined. Abbreviations in brackets: (S)-susceptible; (R)-resistant, [*]resistance category not estimated due to the lack of clinical breakpoint value, [#]antibiotics not involved in the synergy testing assays.

3.3. Diffusion-based assays confirm that there is synergy between aBL/aPDI and antimicrobials

To investigate the interaction between the tested factors, e.g., antibiotics, under *in vitro* conditions, diffusion assays, such as disk diffusion and E-test assay, were used. We implemented both diffusion methods in the current study to validate the interactions between antibiotics and photoinactivation conditions (aBL/aPDI). The application of two of these monotherapies resulted in the increased sensitisation effect to the antibiotics, which can also be described as the priming effect. Exposure of *Enterobacteriaceae* to a single sublethal dose increased sensitivity to certain antibiotics, confirming synergy. As presented in Table 2, synergies between aBL/aPDI (RB) and antibiotics resulted in an increase in the inhibition zone by 2 mm for the disk-diffusion method or a 2-fold decrease in the MIC value in the E-test method. Moreover, Fig. 3 presents the visual changes in MIC distribution among the *K. pneumoniae* D680 isolate before and after exposure to aPDI with RB and green light indicated with the E-test method. Table 3 presents the synergies among *E. cloacae* isolates.

Table 2
Antimicrobial susceptibility profile of *K. pneumoniae* D479 and D680 before and after exposure to aBL/aPDI (RB).

Antibiotic	D479						D680					
	Control		aBL (64.8 J/cm^2)		aPDI (4.8 J/cm^2 + RB 10 μM)		Control		aBL (64.8 J/cm^2)		aPDI (4.8 J/cm^2 + RB 10 μM)	
	DF[1]	E -test[2]	DF	E-test	DF	E-test	DF	E-test	DF	E-test	DF	E-test
CHL	22.9	4	**24.9**	2.3	23.4	2.3	6.2	202.0	**10.5**	**96**	**10.8**	**32**
GEN	6.3	40	6.0	29.3	6.4	**16.0**	19.8	0.88	19.3	**0.038**	19.5	0.63
DOX	10.2	24	11.1	14.4	11.6	**6.0**	13.9	8.0	13.4	6	14.7	**2.7**
CIP	6.0	>32	6.0	>32	6.0	>32	10.0	3.0	**12.9**	3	**14.0**	**1.2**
IPM	20.6	0.44	22.1	2.3	20.0	0.5	26.5	0.38	**23.9**	0.625	24.7	0.25
CAZ	8.5	16	7.2	9.3	7.9	**8.0**	6.4	24.0	6.0	20	7.7	24.0
ATM	10.7	24	10.3	37.3	11.0	28.0	8.5	64.0	7.4	80	8.9	68.7
AMP	8.7	>256	6.0	>256	6.0	>256	6.0	> 256	6.0	>256	6.0	>256
FOF	16.4	64	**19.5**	85.3	16.7	56.0	13.6	18.7	**19.8**	14.7	**22.1**	21.3
CST	11.0	0.125	11.9	0.094	12.3	0.094	13.3	0.167	13.0	**0.064**	12.5	**0.084**

[1] Disk diffusion (DF) expressed in mm; [2] Expressed in μg/mL; Abbreviations: CHL, chloramphenicol, GEN, gentamycin; DOX, doxycycline; CIP, ciprofloxacin; IMP, imipenem; CAZ, ceftazidime, AMP, ampicillin; FOF, fosfomycin; CST, colistin; RB, rose bengal. Bold font indicates confirmed synergies.

Table 3
Antimicrobial susceptibility profile of the changes in *E. cloacae* 2640/13 and 4986/12 before and after exposure to aBL/aPDI (RB).

Antibiotic	2640/13				4986/12					
	Control		aPDI (19.1 J/cm^2 + RB 10 µM)		Control		aBL (64.8 J/cm^2)		aPDI (9.5 J/cm^2 + RB 10 µM)	
	DF[1]	E -test[2]	DF	E-test	DF	E -test	DF	E-test	DF	E-test
CHL	6.0	>256	6.0	>256	6.0	>256	6.0	>256	**8.0**	>256
GEN	6.0	>256	6.0	>256	8.7	25.3	8.7	21.3	**12.1**	15.3
DOX	13.0	4.0	14.9	3.0	8.8	14.7	**10.8**	12.0	**12.7**	6.7
CIP	17.7	0.92	**19.8**	1.25	20.3	0.61	22.0	0.9	**24.2**	1.2
IPM	20.4	0.75	**23.2**	0.46	14.7	9.0	15.4	6.7	15.8	8.0
CAZ	6.0	26.7	6.0	**13.3**	6.0	>256	6.0	>256	6.0	>256
ATM	6.0	112	7.1	58.7	6.0	>256	6.0	>256	6.0	>256
AMP	6.0	>256	6.0	>256	6.0	>256	6.0	>256	6.0	>256
FOF	18.4	21	**21.7**	13.3	18.3	42.7	**20.5**	**10.0**	20.8	**12.0**
CST	11.7	0.38	11.7	**0.17**	12.4	0.38	12.8	**0.178**	13.5	0.32

[1] Disk diffusion (DF) expressed in mm; [2] Expressed in µg/mL; Abbreviations: CHL, chloramphenicol, GEN, gentamycin; DOX, doxycycline; CIP, ciprofloxacin; IMP, imipenem; CAZ, ceftazidime, AMP, ampicillin; FOF, fosfomycin; CST, colistin; RB, rose bengal. Bold font indicates confirmed synergies.

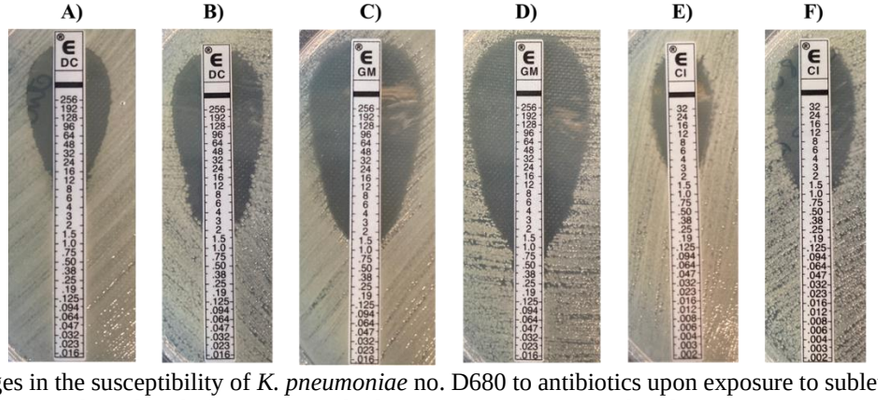

Fig. 3 Changes in the susceptibility of *K. pneumoniae* no. D680 to antibiotics upon exposure to sublethal doses of aPDI (RB) were evaluated with the E-test method. A), C), E) MIC control isolate no. D680 (DC, doxycycline; GM, gentamycin; CI, ciprofloxacin), B), D), F) figures present MIC values of isolate no. D680 treated with 10 µM RB + 4.9 J/cm^2 green light (λ_{max} 522 nm).

3.4 Checkerboard assay indicates that there is synergy between aBL/aPDI and antimicrobials

Another important *in vitro* method for evaluating the combination of light and antimicrobials is the microtiter fractional inhibitory concentration method, known as a checkerboard assay due to the process of performing this assay. The checkerboard assay must have the MIC values of antibiotics and phototherapies investigated in previous experiments; thus, the MIC values for phototreatments were established to perform this assay. For the isolate no. 2640/13, the interaction between aBL and antibiotics was not examined due to the lack of MIC value of aBL for this strain. Checkerboard assays performed with ten antibiotics in combination with aBL/aPDI (RB) can present synergy or other interactions (e.g., indifference, antagonism) between two tested factors defined by the FIC_i index. Table 4 presents the interactions between the tested monotherapies (antibiotics, aBL/aPDI (RB))

for the tested *Enterobacteriaceae*; the bold font indicates the synergies that are described in the FIC_i values that are equal to or below 0.5.

Table 4
Checkerboard FIC_i calculation *K. pneumoniae* no. D479, D680, and *E. cloacae* no. 2640/13, 4986/12

	Klebsiella pneumoniae				***Enterobacter cloacae***		
	D479		D680		2640/13	4986/12	
Antibiotic	**aBL**	**aPDI**	**aBL**	**aPDI**	**aPDI**	**aBL**	**aPDI**
CHL	**0.375**[1]	**0.312**	**0.406**	**0.4375**	**0.49**	>0.5	>0.5
GEN	>0.5	**0.4375**	>0.5	>0.5	**0.50**	>0.5	**0.1875**
DOX	**0.4375**	>0.5	>0.5	>0.5	>0.5	**0.5**	>0.5
CIP	>0.5	>0.5	>0.5	>0.5	>0.5	>0.5	**0.5**
IPM	>0.5	>0.5	**0.5**	>0.5	>0.5	>0.5	**0.5**
CAZ	**0.458**	**0.458**	**0.437**	>0.5	**0.375**	>0.5	>0.5
ATM	>0.5	>0.5	**0.375**	>0.5	>0.5	>0.5	>0.5
AMP	>0.5	>0.5	>0.5	>0.5	>0.5	>0.5	>0.5
FOF	>0.5	>0.5	>0.5	>0.5	>0.5	**0.5**	>0.5
CST	**0.4375**	**0.5**	**0.4375**	**0.375**	**0.280**	**0.375**	**0.458**

[1]FIC index; $FIC_i = FIC_A + FIC_B$; Abbreviations: CHL, chloramphenicol, GEN, gentamycin; DOX, doxycycline; CIP, ciprofloxacin; IMP, imipenem; CAZ, ceftazidime, AMP, ampicillin; FOF, fosfomycin; CST, colistin; RB, rose bengal. Experiments were performed with 3 independent biological replicates, and the values represent the mean of 3 FIC_i values that were calculated for 3 biological repetitions. Bold font indicates possible synergistic interactions.

3.5 The postantibiotic effect confirms the effectiveness of aBL/aPDI with antimicrobials

The synergies or another interaction between tested factors can also be investigated in regard to the bacterial growth rate. The last method that was used to verify the influence of the growth rate after exposure to light conditions (after pretreatment with RB and/or antibiotic) was the postantibiotic effect (PAE). Characteristic shifts between growth curves and the time of bacterial recovery are determinants for the synergy in this method. Partial synergies are estimated when the shift between curves is in the range of 90 min ≤ 180 min. The values of time-shift below 90 min and above 180 min indicate that there was no effect and a synergistic effect, respectively. The results presented in Fig. 4 A and C show the synergistic effect between aBL and DOX among *Enterobacteriaceae* isolates. Data presented in Fig. 4 B and D indicate the lack of synergy (combination of aPDI and CHL). Complete data from the PAE experiment are summarized in Table 5.

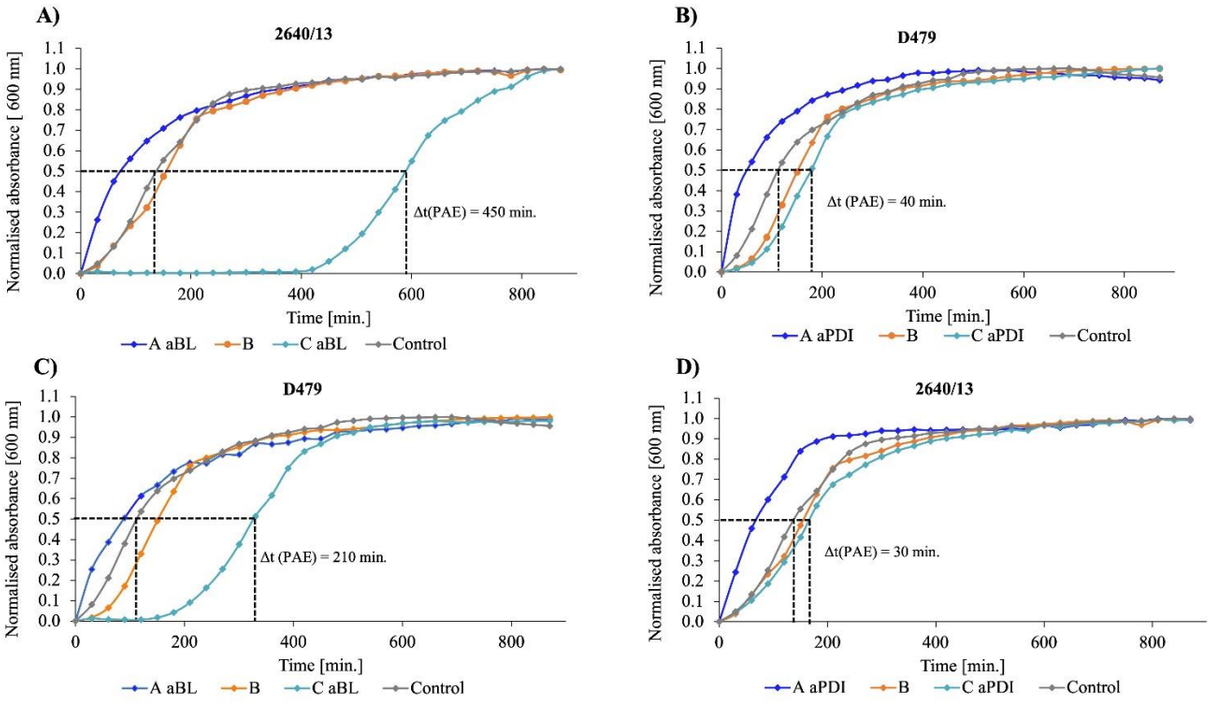

Fig. 4 Postantibiotic effect testing. Growth curve analysis of combined treatment for *E. cloacae* isolate no. 2640/13 (Fig. 4A, 4D) and *K. pneumoniae* isolate no. D479 (Fig. 4B, 4C). Monotreatments with aBL and aPDI in ½ MIC dose are presented on the graphs with symbols A. Antibiotics (DOX, CHL) were administered at the MIC concentrations and are represented in the figure by symbol B. The combination of light and antibiotics is presented as symbol C (½ MIC aBL/aPDI + MIC antibiotic). Moreover, growth curves corresponding to aBL experiments were marked as a figures A) and C), thus the curves involving aPDI experiments are presented as B) and D) curves.

Table 5
Summarized results of PAE testing for the clinical isolates of *Enterobacteriaceae*

Antibiotic	*Klebsiella pneumoniae*				*Enterobacter cloacae*		
	D680		D479		2640/13	4986/12	
	aBL	**aPDI**	**aBL**	**aPDI**	**aPDI**	**aBL**	**aPDI**
CHL	+	+/-	+	-	-	-	-
GEN	-	+/-	-	-	-	-	-
DOX	+	-	-	-	-	+	-
CIP	+	-	-	-	+/-	+	-
IPM	-	-	-	-	+	-	-
CAZ	+	-	-	-	-	-	-
ATM	-	-	-	-	-	+	-
AMP	-	-	-	-	-	+/-	-
FOF	+/-	-	+	-	-	+	+
CST	+	+	+	+	+	+	-

Abbreviations: CHL, chloramphenicol, GEN, gentamycin; DOX, doxycycline; CIP, ciprofloxacin; IMP, imipenem; CAZ, ceftazidime, AMP, ampicillin; FOF, fosfomycin; CST, colistin; RB, rose bengal. Experiments were performed in three independent biological replicates; (+), synergy; (+/-), partial synergy; (-) no synergistic effect.

3.6 When combined with antimicrobials, aBL/aPDI exerts numerous synergies for *Enterobacteriaceae*

All performed tests that combined the aBL/aPDI with RB and antibiotics for the 4 clinical isolates of *Enterobacteriaceae* indicated that despite the common category of resistance (XDR) the various synergies were observed between antibiotics and various photoinactivation conditions (Table 6). It can be seen that there is a prevalence of synergies between light and CHL, FOF, and CST, and any synergistic effect was confirmed for AMP and various tested photoinactivation conditions among the 4 tested *Enterobacteriaceae* isolates. As a method, the postantibiotic effect provided little evidence of synergy between aBL/aPDI and antibiotics, and the E-test method implicated a lesser extent of synergies in comparison to that of the checkerboard assay or disk-diffusion method. To better visualize the pattern of interactions between aBL/aPDI and antibiotics the data was summarized in Table 6 (the colour blue was used as a synergy indicator, white indicates a lack of effect, and red indicates antagonism in the combined treatment).

Table 6

Summarized results of synergy testing for *K. pneumoniae* isolate no. D680 and D479 and *E. cloacae* no. 2460/13 and no. 4986/12

	Klebsiella pneumoniae																***Enterobacter cloacae***											
	D680								D479								2640/13				4986/12							
Antibiotic	aBL				aPDI				aBL				aPDI				aPDI				aBL				aPDI			
	DD	ET	CA	PE	DD	ET	CA	PE	DD	ET	CA	PE	DD	ET	CA	PE	DD	ET	CA	PE	DD	ET	CA	PE	DD	ET	CA	PE
CHL	■	■	■		■	■	■		■		■				■				■						■			
GEN		■												■	■				■						■		■	
DOX			■			■					■			■									■		■	■		
CIP	■				■	■											■											
IPM									■	■			■				■										■	
CAZ			■								■			■	■			■	■								■	
ATM			■																									
AMP									■				■															
FOF					■				■	■				■			■				■	■			■	■		
CST		■	■			■	■				■				■			■	■			■	■				■	

Abbreviations: CHL, chloramphenicol, GEN, gentamycin; DOX, doxycycline; CIP, ciprofloxacin; IMP, imipenem; CAZ, ceftazidime, AMP, ampicillin; FOF, fosfomycin; CST, colistin; RB, rose bengal; DD – disk diffusion, ET – E-test; CA checkerboard assay; PE – postantibiotic effect; blue color - synergy, red - antagonism, white – no synergy.

3.7 Photoinactivation with aBL/aPDI (RB) leads to $OH^{\bullet}$ production (type I radicals)

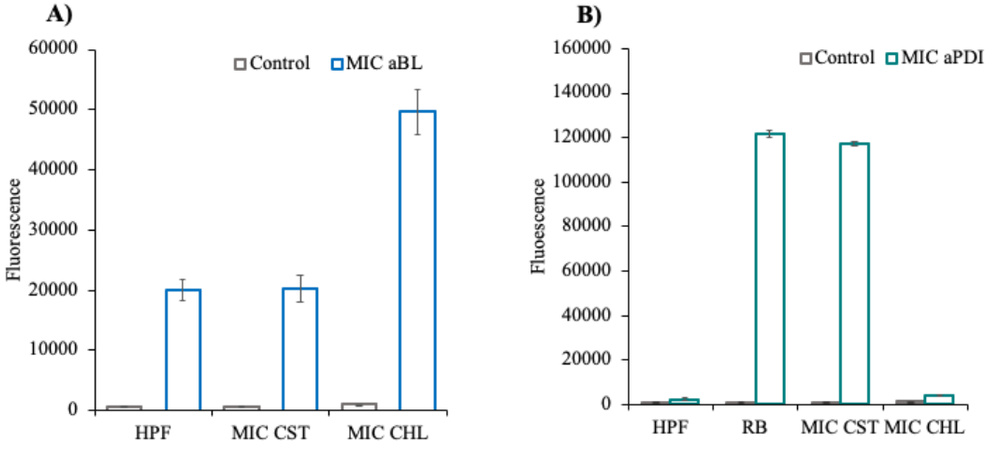

Fig. 5 Evaluation of the production of hydroxyl radicals $OH^{\cdot}$ during aBL (A) and aPDI (RB) (B) treatment of isolate no. D680 with 3′-p-hydroxyphenyl-fluorescein (HPF).

To verify whether the obtained synergies between aBL/aPDI (RB) and antibiotics, such as gentamycin or chloramphenicol, can result from the increased production of ROS, the fluorescent probe HPF was used. From the results presented in Fig. 5A, the following conclusions were drawn. First, the exposure of isolate no. D680 leads to the increased production of hydroxyl radicals during aBL exposure. Second, the presence of CST did not result in increased ROS production during aBL irradiation; however, for CHL combined with aBL, the fluorescence signal was higher than that of aBL alone. This result suggests that the synergies between aBL and CHL could be the result of increased hydroxyl radical $OH^{\cdot}$ production or other radicals, such as peroxynitrite $ONOO^{-}$. On the other hand, increased production of ROS was observed after exposure of isolate no. D680 to aPDI conditions (green light and Rose Bengal) as well as in combination with CST however, the fluorescence level for these both conditions had a similar intensity (Fig. 5B).

3.8 ROS production upon antimicrobial administration may explain the emergence of their synergies with photo-therapies

The establishment of synergy is not always possible for various antibiotics when photoinactivation is combined with them. To investigate this phenomenon, we investigated the hypothesis of whether the synergy could be explained by ROS production in antimicrobials with no light treatment. As CST, CAZ and CHL were shown to have numerous synergistic effects in the current study, we focused on the analysis of whether these antimicrobial agents at various concentrations can produce different ROS with the protocol adapted from Dwyer et al., [26]. Incubation of hydroxyphenyl-fluorescein with CST and CHL resulted in an increased fluorescence level, which can indicate the production of hydroxyl radicals $OH^{\cdot}$ and/or peroxynitrite $ONOO^{-}$ via antibiotic activity independent of light treatment (Fig. 6A). When bacterial cells were incubated with antibiotics without light, signals from another fluorescent probe, H_2DCFDA, suggested that peroxynitrite ($ONOO^{-}$), alkyl peroxyl ($ROO^{\bullet}$) radical, and/or hydrogen peroxide (H_2O_2) were produced when cells were exposed to CAZ and CST (Fig. 6B). The third fluorescent probe, Singlet Oxygen Sensor Green (SOSG), was applied to detect singlet oxygen ($^1O_2{}^{\bullet}$), and presence of this probe revealed an increased fluorescence only in the presence of CST, which can suggest that toxic oxygen radicals are produced (Fig. 6C).

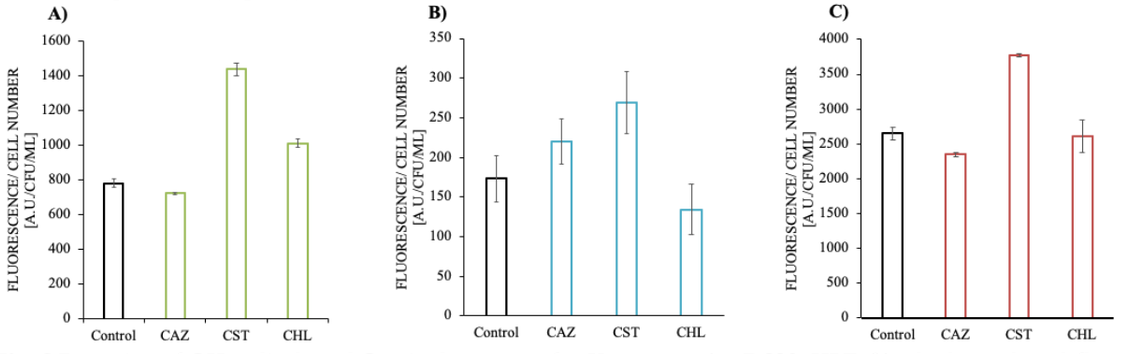

Fig. 6 Detection of $OH^{\bullet}$ radicals and $O_2{}^{\bullet}$ singlet oxygen for *K. pneumoniae* D680. HPF, 3′-p-hydroxyphenyl-fluorescein (A); H_2DCFDA, 2',7'-dichlorodihydrofluorescein diacetate (B); SOSG, singlet oxygen sensor green (C).

3.8 Evaluation of outer membrane permeability upon aBL/aPDI treatment with antimicrobials – SYTOX green labeling.

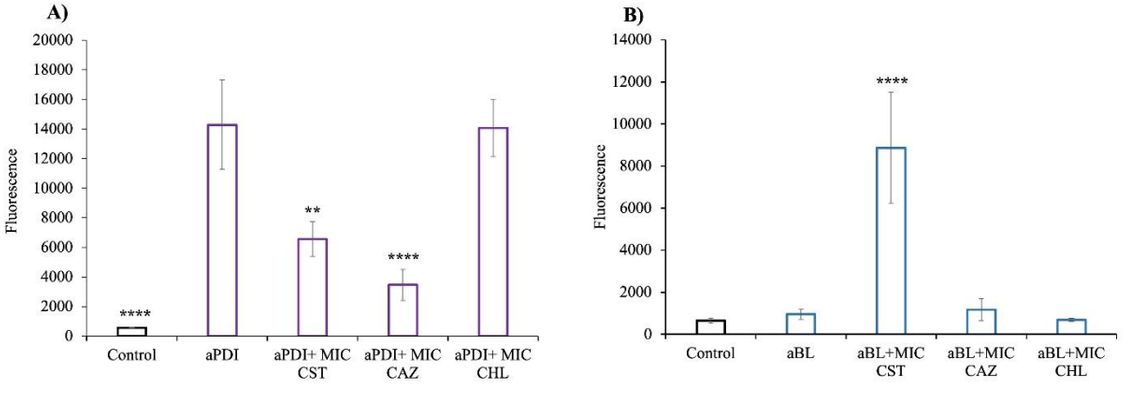

Fig. 7 Outer membrane permeability of *K. pneumoniae* no. D680. Samples were treated with aPDI (A) and aBL (B) combined with antibiotics at MIC concentrations and were exposed to the SYTOX Green label. Additionally, the control for the cells and labels was prepared. The absorbance was measured with an EnVision multiplate reader (PerkinElmer) with 504/523 nm excitation/emission filters. The experiment was performed in three independent repetitions. Statistical significance (**p<0.01; ****p<0.0001) in comparison to samples not treated with aBL or aPDI (Control).

In our previously published study, the exposure of *Enterococcus* spp. to aPDI (RB) led to increased permeability of bacterial envelopes, which resulted in increased fluorescence of the SYTOX green label [27]. Within the present study, we used the same protocol to investigate the cell membrane permeabilization of clinical isolate no. D680 upon aBL and aPDI (RB) treatment. Exposing the *K. pneumoniae* isolate to aPDI with RB resulted in an increased fluorescence level, indicating a high level of envelope permeabilization occurred upon exposure to aPDI as well as after exposure to combined aPDI and MIC CHL (Fig. 7A). However, there were no significant differences between these two groups, thus the CHL presence did not influence the permeabilization of cells. In contrast, the aBL treatment did not affect membrane permeabilization as a monotherapy; however, in the presence of CST, the fluorescence signal was increased, indicating that upon combined therapy, the cell permeabilization occurred (Fig. 7B). For aBL as a monotherapy, there is a lack of cell permeabilization after its application; thus, the mechanism of synergy between aBL and antibiotics (e.g., CAZ, CHL or CST) can be independent of the cell permeabilization process.

4. Discussion

Enterobacteriaceae that are resistant to at least one of the carbapenem antibiotics (ertapenem, meropenem, doripenem, or imipenem) or produce a carbapenemases are defined as Carbapenem-Resistant Enterobacterales (CRE) [34]. Due to multiple transmitted genetic mechanisms of resistance (e.g., New Delhi Metallo-beta-lactamase (NDM)), *Klebsiella pneumoniae* and *Enterobacter cloacae* are severe threats to human death due to a spreading resistance to carbapenem antibiotics. In 2017, approximately one thousand deaths were caused by CRE strains in the USA, and approx. a total of 13 000 CRE infections among hospitalised patients were registered [34]. An increased resistance to widely used antimicrobials, e.g., CRE and ESBL strains, has forced the creation of alternative approaches to eradicate such MDR pathogens. Photoinactivation with monotherapy, e.g., visible blue light (aBL) or with the addition of photosensitizing agents, e.g., rose bengal (aPDI), fits into a group of such alternative possibilities. Studies performed by our team indicated that multiple exposures to sublethal doses of photoinactivation do not lead to resistance development in Gram-negative and Gram-positive species, which is very beneficial in the era of widespread resistance [35–37].

Different XDR isolates of *Enterobacteriaceae* respond variously to antimicrobial blue light conditions (aBL) but similarly respond to green light and rose bengal treatment (aPDI). Moreover, with the present study, we observed that the effectiveness of photoinactivation (especially aPDI) is dependent on the cell environment. Thus, viability of *Enterobacteriaceae* isolates was different when cells were suspended in TSB and PBS solution. The increased efficacy of aPDI in PBS environment can be explained with no interaction of the photosensitizer with any of the compounds present in the growth medium (TSB), for example, proteins or sugars. The suspending medium consistency can influence photoinactivation effectiveness, which was confirmed in few studies. In example, study by Lambrechts et al. determining the minimal inhibitory concentrations (MIC) of antibiotics indicated that when *P. aeruginosa*, *S. aureus* and *Candida albicans* were suspended in PBS, the photoinactivation with photosensitizer TriP[4] was more effective in comparison to irradiation of cells suspended in human blood plasma [38]. Next, study performed by Nitzan et al. evidenced that irradiation of *A. baumannii* with tetra-methylpyridyl porphine (TMPyP) in a BHI medium was ineffective when compared to photoinactivation treatment in nutrient broth or suspended in saline [39]. It can not be excluded that the effect of suspension medium on the cell viability after aBL and aPDI treatments can influence the interactions after combining photoinactivation and antibiotics summarised below.

Investigation of the influence of aBL and aPDI on the antibiotic resistance profile of *Enterobacteriaceae* isolates, thus identifying synergies between photoinactivation and antibiotics, was another primary goal of this study. The combination of antibiotics with photoinactivation was often the object of researchers due to the effectiveness of the variety of these two monotherapies [24,27,28,40,41]. Within this study, we attempt as a first to present the results of aBL and aPDI treatment on resistance profile changes of 4 clinical isolates of *Enterobacteriaceae*, demonstrating the XDR and carbapenem-resistance profile. The combination of antibiotics with aBL or aPDI improved the bactericidal effectiveness, resulting in the synergistic effects, which are presented in Table 6. The synergistic effects for individual antibiotics differed between species and genera despite belonging to the same *Enterobacteriaceae* family.

On the other hand, our previously published studies presented that *A. baumannii* and *Enterococcus* spp. can be sensitized to various antibiotics, e.g., colistin, tigecycline, and gentamicin, due to photoinactivation treatment [27,28]. Literature data present few studies regarding the influence of photoinactivation of *K. pneumonia*e and *E. cloacae* in synergy testing or sensitization of these microorganisms to antimicrobials. For example, experiments presented by Liu et al. implementing PpIX−peptide conjugates with white light against *K. pneumoniae* ATCC

700603, demonstrated the effective reduction of the MIC by approx. 16 times after photoinactivation [42]. In another experiment conducted by Soledad Ramírez et al., blue light applied simultaneously with minocycline or tigecycline against *K. pneumoniae* and *E. cloacae* did not improve combined treatment when the disk-diffusion assay was performed [43]. Within the current study, we also observed for isolate D479 the antagonistic interactions for IMP and AMP after phototreatment. Differences in the obtained synergies relate to the use of aBL and aPDI as a sensitization tool; moreover, each of the validation methods represented different ways of obtaining synergy, so the discrepancies in synergistic effects in the used methods may result from the order of using photoinactivation or application of the antibiotic and its form (solution, discs or strip with gradient concentration) or the type cell solvent in a particular method. Above mentioned issues indicate that the methodology, used to test synergy interactions, may significantly affect the obtained results, thus, the current study including the variety of available synergy testing methods as well as variety of antimicrobials seems to present extraordinary value. It cannot be ruled out that the priming effect – sensitization of isolates to individual antibiotics may be a strain-dependent feature. However, confirmation of this hypothesis requires more detailed research.

Nevertheless, up to this date, the mechanism of synergies between aBL/aPDI and antibiotics is still unknown despite the enormous number of published data. Our previously published study suggested the impact of antimicrobials on ROS production during photoinactivation, which was confirmed by studies performed by Dai et al. [28,44]. Increased permeability of bacterial cells after exposure to photoinactivation, thus the inactivation of the enzymes, proteins (responsible for resistance mechanisms) and other bacterial elements, e.g., LPS, can lead to increased susceptibility of bacteria to antibiotics.

In a comprehensive literature review prepared by Feng et al., the explanation of synergy between light and antibiotics was assigned to the destabilisation of the cell membrane via the action of light and antibiotics that target external structures, inactivation of the enzymes that are responsible for resistance, decreasing the expression of resistance genes or enhancing the antibacterial activity via produced ROS [23]. The effectiveness of ROS action can influence the environment of bacterial cells, enabling the better action of antibiotics, and this process is defined as PDT priming. This novel approach is especially recognised in tumour therapies, e.g., pancreatic cancer, due to improved drug delivery via increased vascular permeability and lower dose photoinactivation [45]. Within the present study, we pretreated *Enterobacteriaceae* clinical isolates with two different photoinactivation conditions, "priming" the cells and environment to antibiotic action. Another explanation of the effectiveness of combining photoinactivation with antibiotics can be supported by the theory from experiments conducted by Kohanski et al., and these experiments indicated that bacteriostatic antibiotics, such as tetracycline or chloramphenicol, did not lead to the production of hydroxyl radicals in contrast to ampicillin and norfloxacin in *Escherichia coli* cells [46]. The authors suggested that applying bactericidal antibiotics at lethal doses results in changes in the intracellular environment. These changes affect the creation of genetic and biochemical changes that promote oxidative radical species [46]. The experiments performed for the present study with the modified protocol by Dwyer et al. also demonstrated that bactericidal antibiotics (colistin, ceftazidime) led to ROS production in *K. pneumoniae* clinical isolate D680. It is worth mentioning that the DNA damage response system (SOS response) initiation occurs upon treatment with norfloxacin, which was also evidenced in the photoinactivation studies performed and published by Rapacka-Zdonczyk et al. [35,46]. The synergy between light antibiotics seems to be a strain-dependent feature; thus, each particular strain represents various patterns of resistance to antibiotics. Bacterial resistance to a particular, a specific bactericidal antibiotic, cannot lead to the production of ROS via antibiotic action. Still, it could explain the lack of adequate sensitization to this bactericidal antibiotic upon aPDI/aBL treatment. Within the tested study, we implemented bactericidal antibiotics, such as CST, CIP, AMP, FOF, ATM, IPM, CAZ, and GEN, and bacteriostatic agents, DOX and CHL (CHL at higher concentrations is bactericidal). *K. pneumoniae* isolate (D680) was chosen for cell analysis: cell membrane permeability, ROS production upon antibiotic stress conditions and after photoinactivation treatment, due to increased synergies observed for this isolate between aBL/aPDI and antimicrobials (2.10, 2.11 and 2.12). Upon the experiment with fluorescent probes, we indicated that CST, CAZ and CHL can produce various ROS. This phenomenon could suggest the reason for the synergy between light and antibiotics if it occurred.

This study presents the first broad effectiveness of combining aBL or aPDI with multiple antibiotics against carbapenem-resistant XDR clinical isolates of *Enterobacteriaceae* - *Klebsiella pneumoniae* and *Enterobacter cloacae*. Obtained data suggest that cell envelope permeabilization driven by phototherapy, antibiotic-mediated increased ROS production and overall increased ROS level occurred due to combined aPDI/aBL and antibiotic approach may explain possible mechanism of observed synergies. The ability to sensitize XDR isolates to the antibiotics presented in this study is evidence that photoinactivation can be used as a priming tool to decrease the antibiotic concentration used for pathogens eradication.

Author Contributions

AW performed studies concerning *Klebsiella pneumoniae* isolates, prepared all figures, wrote the manuscript, and participated in the conception of the study. NB and IZ performed studies concerning *Enterobacter cloacae* isolates, JE participated in the data interpretation and critical manuscript review, and MG was involved in the coordination, conception, and design of the study and wrote the manuscript. All of the authors have read and approved the final manuscript.

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Data Availability Statement

The data presented in this study are available from the corresponding authors upon reasonable request.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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Chapter VI

Combined antimicrobial blue light and antibiotics as a tool for eradicating multidrug-resistant isolates of *Pseudomonas aeruginosa* and *Staphylococcus aureus*: *in vitro* and *in vivo* studies

1. Summary of the publication

In 2019, *Pseudomonas aeruginosa* and *Staphylococcus aureus* were labeled by the World Health Organization as pathogens with a critical (carbapenem-resistant) and high (vancomycin-resistant, methicillin-resistant) priority, respectively. These nosocomial pathogens are responsible for chronic wound infections. As a part of the ESKAPE group, these pathogens can easily gain mechanisms that protect them from the biocidal action of antibiotics [86,87].

These two species, which were isolated from patients and characterized by multidrug profiles of resistance to antibiotics (MDR), were examined in **publication no. 5** in *in vitro* and *in vivo* experiments. Likewise, in previously mentioned publications, the first examination stage focused on estimating the response to antimicrobial blue light photoinactivation (aBL). The tested isolates were susceptible to various aBL conditions in PBS and TSB environments; thus, the changes in their susceptibility profiles were examined with 4 methods of synergy testing. The experimental outcome indicated that *S. aureus* can be sensitized to antimicrobials such as CHL, LZD, and FOF in diffusion assays, whereas *P. aeruginosa* (especially isolate no. 802) was sensitized to CST or GEN. The results obtained from the checkerboard assay show the apparent tendency of synergistic effects between aBL and FA, CHL, FA, and CIP for both *S. aureus* isolates. However, in Gram-negative isolate no. 805, synergies were prevalent after exposure to aBL and CST, CAZ or CIP in the same methodology. Differences between strains due to various combinations of aBL and antimicrobial agents were also observed for the last experimental method, PAE; thus, the differences between the observed synergies were indicated between *S. aureus* isolates and *P. aeruginosa*. **All the results from synergy testing experiments showed that, depending on the method used, synergistic effects were observed for different antibiotics, and the tested isolates showed different responses to the combined treatment (even if the isolates belonged to the same**

genus). This conclusion indicates that the synergistic effects and sensitization to an antibiotic can be strain dependent.

Another important issue presented in **publication no. 5** was that **photoinactivation with aBL at low doses is safe for eukaryotic (HaCaT cell line) and prokaryotic cells (*E. coli* and *S.* Typhimurium mutants)**. This conclusion is crucial regarding the light doses used in *in vivo* experiments; thus, aBL doses are safe for animals. However, it cannot be excluded that aBL in high doses can influence human keratinocytes, which should be considered for the clinical application of aBL, e.g., in the treatment of skin infections in humans.

Another goal when examining the combination of blue light and antibiotics was to investigate whether the presence or absence of antimicrobial agents leads to the increased production of ROS upon irradiation. **It was shown that aBL leads to the production of ROS in the cell environment and chloramphenicol (in a cell-free environment);** this conclusion suggests the possible mechanisms of synergy between these two factors in *in vitro* conditions. The presence of ROS upon cell irradiation results from the occurrence of flavins/endogenous porphyrins in bacterial cells. Moreover, the ability to produce porphyrins by *S. aureus* cells is linked with **sensitization to antimicrobials**. This conclusion was drawn based on experiments involving the wild-type strain and its isogenic mutant, which did not possess the ability to synthesize porphyrin.

One of the most crucial results gained from the *in vivo* experiments was the confirmation that the *S. aureus* bioluminescent strain was successfully sensitized to an antibiotic (CHL), which caused the infection to be eliminated. The results obtained for the mouse model of a wound infected with Xen31 are consistent with those from *in vitro* experiments.

2. Publication

antioxidants

MDPI

Article

Combined Antimicrobial Blue Light and Antibiotics as a Tool for Eradication of Multidrug-Resistant Isolates of *Pseudomonas aeruginosa* and *Staphylococcus aureus*: In Vitro and In Vivo Studies

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Abstract: Increased development of resistance to antibiotics among microorganisms promotes the evaluation of alternative approaches. Within this study, we examined the efficacy of antimicrobial blue light (aBL) with routinely used antibiotics against multidrug-resistant isolates of *Pseudomonas aeruginosa* and *Staphylococcus aureus* as combined alternative treatment. In vitro results of this study confirm that both *S. aureus* and *P. aeruginosa* can be sensitized to antibiotics, such as chloramphenicol, linezolid, fusidic acid or colistin, fosfomycin and ciprofloxacin, respectively. The assessment of increased ROS production upon aBL exposure and the changes in cell envelopes permeability were also goals that were completed within the current study. Moreover, the in vivo experiment revealed that, indeed, the synergy between aBL and antibiotic (chloramphenicol) occurs, and the results in the reduced bioluminescence signal of the *S. aureus* Xen31 strain used to infect the animal wounds. To conclude, we are the first to present the possible mechanism explaining the observed synergies among photoinactivation with blue light and antibiotics in the term of Gram-positive and Gram-negative representatives.

Keywords: blue light; mouse model; photoinactivation; porphyrins; *Pseudomonas aeruginosa*; rose bengal; *Staphylococcus aureus*; synergy

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1. Introduction

Increased consumption and inappropriate applications of antibiotics in the medical sector and agricultural industry led to the development of multiple resistance mechanisms in microorganisms. Among these, the increased attention is nowadays directed toward two crucial pathogens: *Staphylococcus aureus* and *Pseudomonas aeruginosa*. These pathogens are responsible for hospital-acquired infections, especially in immunocompromised patients, due to their very high resistance level and the production of a broad spectrum of virulence factors [1]. Therefore, there is an urgent need to develop new therapeutic options to tackle drug resistance [2]. Alternative approaches against drug-resistant pathogens include bacteriophage therapy, antimicrobial peptides, lysins, antibodies or antimicrobial light therapy [3,4]. Light treatments involve the spectrum of visible light from 380 to 740 nm. Antimicrobial blue light (aBL) presented within the current study is one of the most attractive approaches. aBL refers to the light spectrum between 400 and 470 nm. It is an accepted hypothesis that application of this visible blue light leads to the excitation of the bacterial endogenous chromophores (e.g., flavins, iron-free porphyrins), which undergo the photochemical reaction [5,6]. Overall, this process results in the production of intracellular reactive oxygen species (ROS), which can cause lethal effects in bacterial cells as well as DNA cleavage, lipid and protein oxidation or cell membrane damage [7,8]. The overarching feature of aBL is that this method does not lead to the development of resistance in Gram-positive and Gram-negative bacteria, which has been thoroughly verified by our team [9,10].

aBL as a monotherapy is an effective tool for the eradication of pathogens and inactivation of their virulence factors [1,11]. Due to the non-specific mechanism of the action and the lack of resistance development, aBL may serve as an ideal component for combined treatments with other antimicrobial agents like antibiotics and lead to microbial resensitization to the action of routinely used antimicrobials [1,12].

Within the current study, we attempted to investigate which antimicrobial agents routinely used for *S. aureus* and *P. aeruginosa* treatment demonstrate the best activity with aBL revealing the synergistic effect. Moreover, the influence of endogenous chromophores was examined in the context of obtained synergy. Next, the study was aimed to investigate the safety of aBL toward eukaryotic cells and to assess ROS production and cell damage upon treatment (also in the presence of antibiotics). Finally, the last examined issue concerned the verification of observed synergy between aBL and antibiotics in in vivo experiments using a mouse model of infected wounds.

2. Materials and Methods

2.1. Characterisation of Clinical Isolates and Used Strains

In in vitro experiments, clinical *S. aureus* (4046/13, 1814/06) and *P. aeruginosa* (802, 805) strains isolated from blood samples were used. *S. aureus* were kindly provided by Joanna Empel from National Medicine Institute and *P. aeruginosa* by Nico T. Mutters from the Institute for Hygiene and Public Health at Bonn University Hospital. In assays investigating the influence of the porphyrin composition on the synergistic effect with aBL, two *S. aureus* strains were used: wild type (NCTC 8325-4) and its isogenic mutant (ΔhemB) with hemin biosynthesis gene interruption (hemB). Both strains (hemB and WT) were kindly provided by Karsten Becker from University Hospital Münster Institute of Medical Microbiology in Münster, Germany; moreover, construction of the mutant (hemB) was performed by C. von Eiff et al. [13]. In in vivo experiments, the bioluminescent strains, *S. aureus* Xen 31 and *P. aeruginosa* PAK were used.

2.2. Media and Culture Conditions

For all of the experiments, clinical isolates, wild-type strain NCTC 8325-4 and bioluminescent isolate Xen31 were cultivated at 37 °C in an orbital incubator for 16–10 h in Tryptic-Soy-Broth (TSB, Biomerieux, Craponne, France). For the cultivation of the ΔhemB mutant, erythromycin (ERY) at the final concentration of 2.5 μg/mL was added to the TSB broth and incubation was performed under the same conditions. The enumeration of bacterial colonies was performed on the solid plates containing the TSB medium with an addition of 1.5% agar (TSA) and ERY for hemB isolate.

2.3. Light Conditions

A light-emitting diode (LED) lamp (Figure 1A,B) manufactured by Cezos (Gdynia, Poland) and emitting 411 nm light (with an irradiance of 24 mW/cm^2) was used in experiments. Irradiance measurements of the LED lamp were performed using the PM100D power/energy meter (Thorlabs, Ann Arbor, MI, USA).

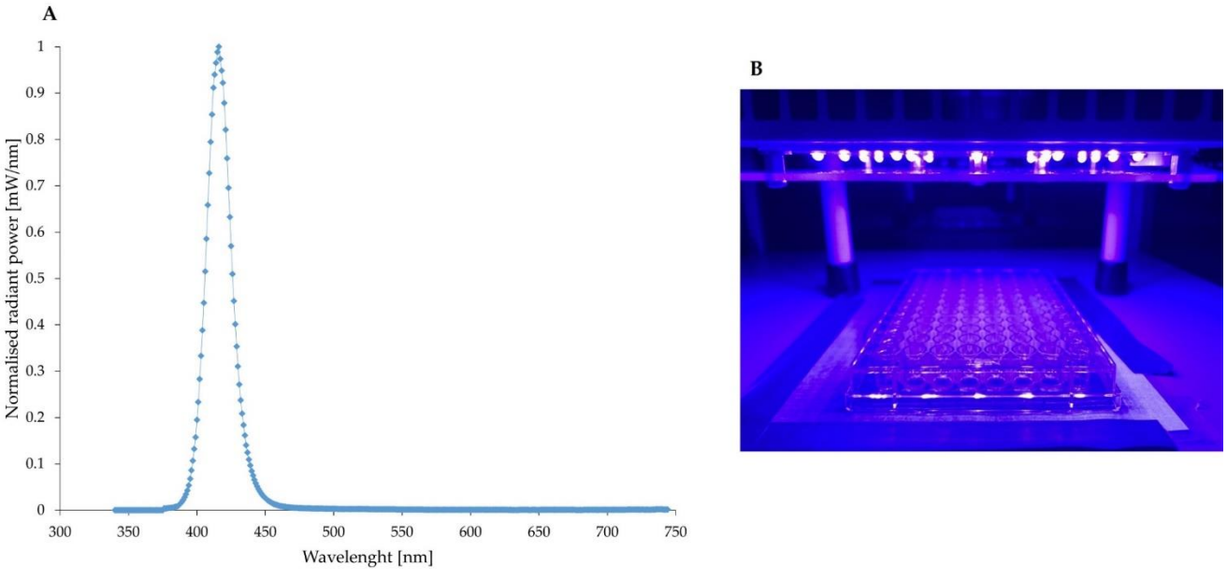

Figure 1. Normalised emission spectrum of light-emitting diode (LED) (**A**); Photograph of LED source used in experiments (**B**).

2.4. Antibiotics

Chloramphenicol (CHL), Erythromycin (ERY), Ciprofloxacin (CIP), Doxycycline (DOX), Rifampicin (RIF), Fusidic acid (FA), Fosfomycin (FOF), Aztreonam (ATM), Clindamycin (CLI), Tigecycline (TGC), Imipenem (IPM), Colistin (CST), Gentamycin (GEN), Piperacillin (PIP), Tazobactam (TZB), Trimethoprim (TMP), Sulfamethoxazole (ST), Daptomycin (DAP), Linezolid (LZD), Oxacillin (OXA), Vancomycin (VAN), and Ceftazidime (CAZ) were purchased from Sigma (Darmstadt, Germany) and Cayman Chemicals (Ann Arbor, MI, USA). All stock solutions of 10 mg/mL were prepared in recommended solvents in non-transparent Eppendorf tubes and kept in −20 °C until use.

2.5. Photoinactivation

Stationary growth-phase overnight cultures of clinical isolates were diluted to obtain the optical density of 0.5 McFarland in fresh TSB medium or PBS (Phosphate Buffered Saline), which corresponds to approx. 5×10^7 colony-forming units per millilitre (CFU/mL). Then, for the experiments involving the antimicrobial blue light (aBL), the cells were immediately transferred to 96-well plates and irradiated with various doses of visible blue light. Afterwards, samples were diluted serially in PBS, seeded on agar plates (TSA) and incubated for 16–20 h at 37 °C in the incubator (Thermax, Dreieich, Germany). The enumeration of grown colonies was performed after 16–20 h of incubation, and the level of CFU/mL for each sample was estimated. Control samples without the addition of light were also involved in the experiment. All of the experiments were performed in three independent biological replicates.

2.6. Determination of Sub-Lethal Doses of Photoinactivation

According to our previous published data, the sub-lethal dose reduced bacterial viability by 0.5 to 2 $\log_{10}$ CFU/mL, and the lethal dose by more than 3 $\log_{10}$ CFU/mL.

2.7. Identification of the Minimal Inhibitory Concentrations (MIC) of Antibiotics and Lethal MIC Values for Photoinactivation Conditions

The Minimal Inhibitory Concentrations of tested antimicrobials were determined according to EUCAST (European Committee on Antimicrobial Susceptibility Testing) guidelines. Overnight cultures in a stationary phase-growth were prepared to obtain 0.5 McFarland suspension and diluted 10-fold in Mueller Hinton Broth (MHB) (Roth,

Karlsruhe, Germany) to assess the number of cells approx. 5×10^6 CFU/mL. In the next step, diluted cells were transferred to a 96-well plate with antibiotics to obtain the 2-fold range of agent concentrations ranging from 1024 to 0.00312 µg/mL. For the establishment of aBL lethality, diluted bacterial suspensions were transferred to the 96-well plate and immediately exposed to blue light. Afterwards, all plates were protected from evaporation with parafilm and incubated from 16–20 h at 37 °C in the incubator (Thermax, Dreieich, Germany). On the following day of the experiment, the turbidity of cell suspension was estimated, and the lowest concentration of the antimicrobial agent which inhibited the bacterial growth was defined as a MIC. The blue light dose that led to complete inhibition of bacterial growth was determined as a MIC of phototreatment. All the experiments were performed in three independent biological replicates.

2.8. Identification of the Interactions between Testes Phototherapies and Antibiotics—Recommended Methods for Synergy Testing

2.8.1. Diffusion Assays

E-test strips containing the gradient concentration of antibiotics and disks containing one specific concentration of the antimicrobial agent were used in the diffusion methods (E-test and disk diffusion assay). Experiments were performed in accordance with the current guidelines for AST (antimicrobial susceptibility testing) recommendations provided by the EUCAST and presented within our previous publications [1–3]. To perform the experiment, overnight culture in a stationary growth phase was diluted to 0.5 McFarland in sterile PBS. For samples non-treated with photoinactivation (control), bacteria were spread with a cotton swab on the solid plates containing the Mueller Hinton Agar (MHA) (Sigma Aldrich, Darmstadt, Germany). For photoinactivation experiments with aBL, 1 mL diluted in PBS cells were transferred to a 12-well plate. Cells were then exposed to sub-lethal doses of aBL estimated in a PBS environment. After irradiation, phototreated cell suspension and control sample were spread on MHA plates, and after 15 min of incubation at room temperature (RT), discs and E-Tests were applied. An examination of diffusion experiments was performed after incubating plates at 37 °C in the incubator (Thermax, Dreieich, Germany) for 16–20 h. The inhibition zones were measured with the electronic caliper for disk diffusion assay, and the MIC values were determined. The synergistic effect was confirmed based on our previous published guidelines; thus, if changes in the zone of inhibition for photoinactivation-treated cells compared to the control samples are equal or more than 2 mm, then it confirms synergy. The difference in inhibition zone smaller than 4 mm indicates the antagonistic effect. For the E-test method, synergy is assessed when the MIC value of treated samples is 2-fold lower than the MIC value indicated for control samples. All the diffusion experiments were performed in three biological repetitions.

2.8.2. Checkerboard Assay

This method involves the MIC values established for antibiotics and the aBL treatments. Briefly, bacterial cell suspensions were prepared the same as for the MIC establishment and then transferred into a 96-well plate. Antibiotics were added to the wells vertically (to obtain 2× MIC concentration) and then 2-fold dilutions of each tested compound were performed. After 15 min of incubation, plates were exposed to different doses of blue light (2× MIC, MIC, 1/2 MIC, 1/4 MIC and 1/8 MIC). After exposure to photoinactivation, plates were protected with parafilm and incubated at 37 °C in the incubator (Thermax, Dreieich, Germany) for 16–20 h. The next day, the bacterial growth assessment indicated if the synergistic or another effect (antagonism or indifference) effect occurred. The interpretation of the checkerboard result was based on the Fractional Index (FICI). (FICI = FIC_A + FIC_B). $FIC_{A/B}$ = MIC of factor A/B in combination/MIC of factor A/B alone. The synergistic effect is confirmed when FICI $\leq$ 0.5; antagonism was observed when FICI > 4; 4 < FICI > 0.5 means no interaction between tested factors.

2.8.3. Postantibiotic Effect

This experiment was performed in accordance with our previous published studies; thus, the overnight culture of microorganisms was diluted in fresh TSB (1:20), and then the bacterial suspensions were mixed with MIC of antibiotic. All samples were then covered with aluminium foil and incubated for 2 h at 37 °C in an orbital shaker Innova40 (Brunswick, Hessen, Germany). Immediately after incubation, samples were centrifuged (3.5 min, 4500 rpm) and washed with a fresh TSB medium. After this step, cells were transferred in the amount of 100 µL to a 96-well plate and exposed to $\frac{1}{2}$ MIC dose of blue light for aBL. In the next step, the optical density (λ 600 nm) of samples was measured for 15 h in multiplate reader Envision (PerkinElmer, Waltham, MA, USA) every 30 min. Obtained data were normalised and the postantibiotic effect (PAE) was determined based on the following equation: PAE = T − C (T, the time required to reach the optical density to value 0.5 (OD_{600}) after removal of an agent; C, the time required to achieve the optical density (OD_{600}) of untreated control samples). A postantibiotic effect value $\geq$ 3 h indicates synergy, whereas the 1.5 h $\leq$ PAE < 3 h confirms the partial synergistic effect.

2.9. Experiments Involving the Assessment of Mutagenic and Toxic Effects of aBL

Experiments concerning the determination of mutagenic and cytophototoxic effects of aBL were performed.

2.9.1. Phototoxicity Assay

To perform this experiment, all procedures were performed in accordance with the protocol published by Michalska et al.; thus, HaCaT cells (CLS 300493, CLS Cell Lines Service GmbH, Baden-Württemberg, Germany) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 mM sodium pyruvate, 1 mM non-essential amino acids, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM glutamine and 10% fetal bovine serum (all reagents were purchased from Life Technologies/Thermo Scientific, Darmstadt, Germany) [14]. The day before the experiment, cells were seeded in a 96-well plate in the number of 1×10^4 cells/well in four repetitions for all tested conditions (photoinactivation and control). HaCaT cells were grown in a standard humified incubator (5% CO_2) for 24 h and then exposed to various doses of aBL or the non-treated (control). After 24 h of irradiation, 10µL (12 mM) MTT reagent (1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan) purchased from Sigma (Darmstadt, Germany) was added to each well and kept for 4 h at 37 °C in the incubator. In the next step of this assay, cells were lysed with DMSO, and the absorbance of formazan was established at 550 nm with plate reader Envision (PerkinElmer, Waltham, MA, USA).

2.9.2. Mutagenicity Assay

This experiment was performed using the commercial kit Ames Penta 2 (Xenometrix, Allschwil, Switzerland). The day before the experiment, three independent biological cultures of each tested strain were prepared: *Escherichia coli* [uvrA], *Salmonella* Typhimurium [TA98, TA1535], 25 mL of growth medium. After 12–14 h incubation at 37 °C in the orbital shaker Innova40 (Brunswick, Hessen, Germany), cultures were diluted in an exposure medium and exposed to the various doses of aBL. Positive controls were also included in the experiment, thus, the 2-Nitrofluorene (for TA98 and 1535) and 4-Nitroquinoline-*N*-oxide (for uvrA) were added to the cultures to induce the mutations of the cells. Moreover, the negative control (without any treatment) was prepared. All of the cells, including negative control, were incubated after adding mutagen and/or aBL for 90 min in an orbital shaker at 37 °C Innova40 (Brunswick, Hessen, Germany). Afterwards, the exposure medium was added to the incubated cultures, and samples in the amount of 120 µL were partitioned into the 384 well plates (each sample was distributed to 48 wells separately in 3 technical repetitions). In the next step, all microplates were covered with sterile foil, placed in a plastic bag, and kept for 48 h at 37 °C in the incubator. The assessment of revertants was performed after 48 h. Thus, the number of grown colonies (in each well) was determined.

2.9.3. Analysis of Eukaryotic Cell Growth Dynamic

To investigate the effect of visible blue light (aBL) on the growth rate of HaCaT (CLS 300493) cells, the day before the experiment, cells were seeded in the amount of 1×10^4 cells/well in seven technical repetitions on E-plate PET plates (ACEA Biosciences Inc., San Diego, CA, USA) according to the protocol published by Michalska et al. [14]. Cells were cultured in the same conditions as described above in Section 2.9.1. and kept in the standard humified incubator with 5% CO_2 for 24 h in the xCELLigence RTCA instrument (ACEA Biosciences Inc., San Diego, CA, USA). The next day, cells in the exponential growth rate (Cell index (CI) $\approx$ 2) were removed from the RTCA instrument, exposed to the various blue light doses and after the medium exchange, the plates were returned to the device. The CI was measured for each repetition every 10 min until the cells reached the plateau phase under tested conditions or if the cells did not survive post-irradiation.

2.10. Cell Permeabilisation

S. aureus isolate 4046/13 was cultivated in 25 mL of TSB medium for 4 h to obtain the logarithmic phase of growth, and then cells were centrifuged for 5 min/5000 rpm and resuspended in sterile PBS. Then, 1 mL of cells were exposed to aBL conditions in a 24-well plate and afterwards, SYTOX green was added to 100 µL of each sample to obtain a final concentration of 5 µM. To perform the cell membrane permeabilisation assay with propidium iodide (PI), the residual volume of the treated sample was mixed with PI to obtain a final concentration of 5 µg/mL according to the protocol published by Grinholc et al. [15]. The samples exposed to SYTOX green label were incubated for 15 min in the dark and the fluorescence, indicating the DNA leakage, was measured with multiplate reader Envision at 488/523 nm (excitation/emission wavelengths). Moreover, samples treated with PI were incubated in the dark for 30 min, then centrifuged and resuspended in a fresh portion of PBS in the amount of 200 µL. Immediately, the fluorescence signal was measured with Envision multiplate reader (PerkinElmer, Waltham, MA, USA) at 504/523 nm excitation and emission filters.

2.11. ROS Measurement

Hydroxyphenyl Fluoresceine; HPF (Thermofisher Scientific, Darmstadt, Germany) in the final concentration of 5µM was used with the cell suspension to assess the ROS production by endogenous chromophores, or in PBS with antibiotic—CHL was used to assess the increased ROS production by this agent upon aBL. All of the samples were incubated for 15 min in the dark and exposed to blue light doses. Immediately after exposure, the fluorescence signal was measured at (excitation/emission maxima) 490 nm/515 nm. Control samples containing the fluorescent probes but not exposed to visible light were also prepared. The experiment was performed in three technical and biological repetitions.

2.12. Investigation of the Porphyrin Composition Impact on the Synergistic Effect between aBL and Antibiotics

The overnight culture of the wild-type (WT) strain (NCTC 8325-4) was diluted in PBS and adjusted to the optical density of 0.5 MacFarland. Then, cells were transferred to a 96-well plate and exposed to various doses of aBL. Next, samples were serially diluted, spread on TSA plates, incubated overnight at 37 °C and afterwards, the sub-lethal dose of aBL was assessed. In the second part of the experiment, overnight cultures of the WT strain and ΔhemB mutant were in the amount of 1 mL exposed to the sub-lethal doses of aBL estimated for the WT. Irradiated samples were spread on MHA plates, incubated for 15 min in RT and the discs containing the antibiotic for susceptibility testing (the same as for the clinical isolates) were applied. In the next step, antibiograms were incubated overnight and the inhibition zones were measured. A similar experiment was performed for non-irradiated WT culture suspended in PBS in the amount of 0.5 McFarland. The differences in inhibition zones for wild type and the mutant lacking the possibility of heme synthesis were compared.

2.13. In Vivo Model of Mouse Wound Infected with Staphylococcus aureus/Pseudomonas aeruginosa—Verification of In Vitro Synergy

The 1st Local Ethical Committee for Animal Experiments in Krakow at the Institute of Pharmacology of the Polish Academy of Sciences (Warsaw, Poland) approved all experiments involving the procedures on animals. Twenty adult Balb/c mice aged 7–8 weeks were purchased from Charles River Laboratories (Wilmington, NC, USA). Animals were housed (five per cage) and maintained on a 12 h light-dark cycle with access to water and food ad libitum. The day before the experiment, mice were shaved on the dorsal surfaces, depilated with depilatory lotion, and the immunosuppressant—endoxan (150 mg/kg)—was injected intraperitoneal into each animal. The next day, overnight cultures of *S. aureus* (Xen31) or *P. aeruginosa* (PAK) cultured in a TSB medium were adjusted to 0.5 McFarland. Cells were centrifuged and resuspended in the physiological salt to obtain each 10 µL of culture 10^7 CFU/mL. The wounds were created by making a 1 cm incision on the skin with a sterile needle, and immediately 10 µL of Xen31/PAK cells were applied to the damaged skin. Thirty minutes after infection of wound, mice were given: (i) antibiotic (1/2 MIC); (ii) aBL (MIC); (iii) antibiotic (1/2 MIC) + aBL (MIC). For experiments with Xen31 and PAK, chloramphenicol and piperacillin-tazobactam were used as antibiotics, respectively. The control group (iv) of mice were not given any treatment. Immediately after irradiation, the bioluminescence imaging of infected wounds was performed with the IVIS Spectrum imaging system (Caliper Life Sciences). During the bioluminescence, quantification mice were anaesthetised with isoflurane, and the luminescence was measured daily for up to 5 days. The quantification of the treatments was measured by the changes in bioluminescent signal, defined as an average radiance, and by observing the visual changes during the experiment.

2.14. Statistical Analysis

Statistical analysis was performed using the GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA) (https://www.graphpad.com/) (accessed on 19 August 2022). The statistical differences between groups were performed with one-way ANOVA with the significance level $p < 0.05$.

3. Results

3.1. Clinical Isolates of Pseudomonas aeruginosa and Staphylococcus aureus Respond Differentially to Photoinactivation in Various Environmental Conditions

Photoinactivation of clinical isolates of Gram-positive representatives was more efficient in the TSB medium than in PBS (Figure 2A,B). Thus, the reduction in survival rate was comparatively lower when cells were distributed in PBS. The same observation can be drawn for Gram-negative isolates (Figure 2C,D) when exposed to various ranges of blue light in the TSB medium. Interestingly, isolates 802 and 805 respond better to photoinactivation than Gram-positive isolates 1814/06 and 4046/13 (lower doses of aBL were used to reduce the survival rate—up to 43.2 J/cm^2 for isolates 802, 805). For *P. aeruginosa* isolate 802, the detection limit was reached after exposure to a blue light dose of 43.2 J/cm^2 and a similar observation, but with no eradication to detection limit, can be made for the second isolate (805).

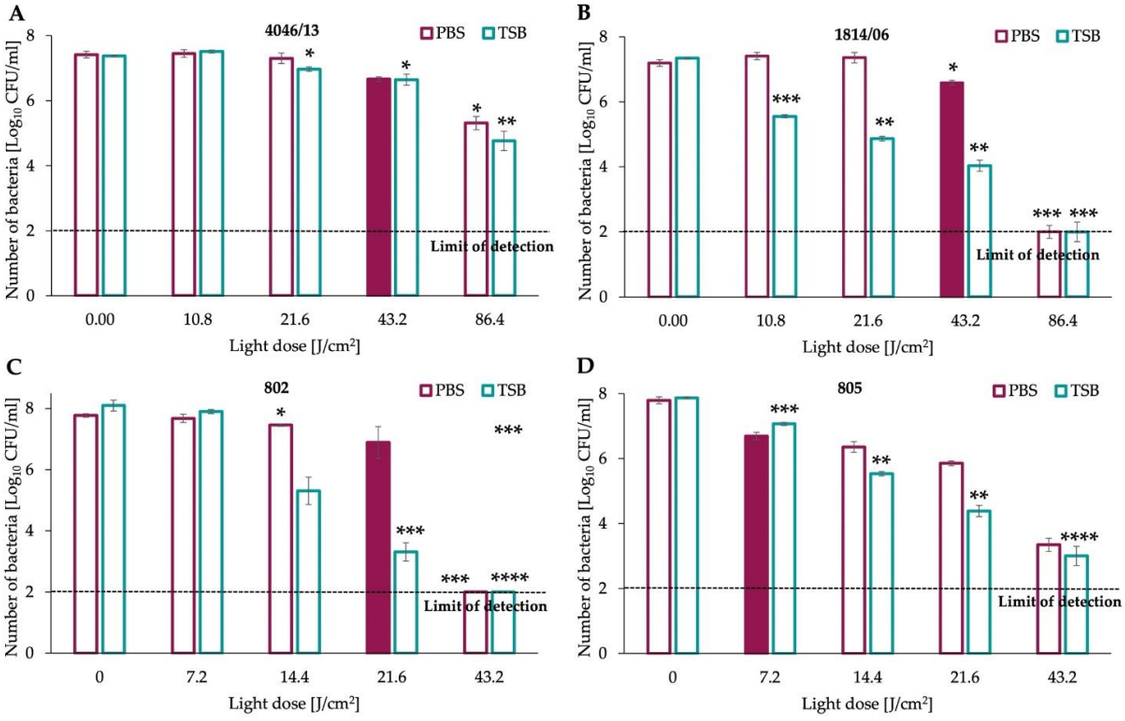

Figure 2. Antimicrobial blue light photoinactivation (aBL) of clinical isolates of *S. aureus:* (**A**) no. 4046/13; (**B**) no. 1814/06; (**C**) *P. aeruginosa* no. 802; (**D**) no. 805. Stationary growth-phase overnight cultures of clinical isolates were diluted to obtain the optical density of 5×10^7 colony-forming units per millilitre (CFU/mL) in fresh TSB medium or PBS. Then, cells were transferred to 96-well plates and irradiated with various doses of visible blue light. Afterwards, samples were diluted serially in PBS, seeded on agar plates (TSA) and incubated for 16–20 h at 37 °C. The enumeration of grown colonies was performed after 16–20 h of incubation, and the level of CFU/mL for each sample was estimated. The experiment was performed in three independent biological replicates with 100 CFU/mL detection limit. Statistical significance (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$) in comparison to samples not treated with aBL (0 J/cm^2).

3.2. Sub-Lethal Doses of Photoinactivation in PBS Differs among Gram-Positive and Gram-Negative Species

Sub-lethal doses of aBL were demonstrated (based on the data presented in Figure 2A,B) as filled purple bars and these doses were implemented in the study involving the diffusion methods in the assessment of changes in resistance profile. For both isolates of *S. aureus,* the sub-lethal dose was evidenced as 43.2 J/cm^2 and for *P. aeruginosa,* the aBL sub-lethal dose in PBS was estimated as 7.2 J/cm^2 and 21.6 J/cm^2, for strain no. 802 and 805, respectively (Figure 2C,D).

3.3. Examined Clinical Isolates Revealed XDR and MDR Categories of Resistance

The microdilution method was used to estimate the Minimal Inhibitory Concentrations (MIC) for tested isolates, thus a separate set of antimicrobials was used for Gram-negative and Gram-positive species. Table 1 represents MIC values that enabled the assignment of microorganisms to the resistance category according to the Magiorakos et al. [16]. Determination of susceptibility for each antimicrobial agent was performed with the clinical breakpoints published by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (https://www.eucast.org/clinical_breakpoints/ (accessed on 10 October 2021)). Based on the data published by the EUCAST, isolates 4046/13 and 1814/06 belong to the group of multidrug-resistant microorganisms (MDR) (Table 1). However, isolates 805 and 802 represent the XDR (extensively drug-resistance) profile of resistance (Table 1).

Moreover, although the MIC parameter is mainly defined for antibiotics, for experimental purposes (checkerboard assay and postantibiotic effect), we also determined the alternative MIC values for phototherapy. Photoinactivation doses that lead to complete inhibition of bacterial growth were assigned as MIC for aBL (Table 1).

Table 1. Minimal inhibitory concentrations (MIC) of antibiotics and photoinactivation conditions.

Antibiotic Target	Antimicrobial Category	Antibiotic	*S. aureus*		*P. aeruginosa*	
			4046/13	**1814/06**	**802**	**805**
			MIC [μg/mL]			
Protein synthesis (50S)	Lincosamides	Clindamycin	0.25 (S)	0.01 (S)	ND	
	Macrolides	Erythromycin	256 ®	1024 (R)	ND	
	Phenicols	Chloramphenicol	64 (R)	128 (R)	ND	
	Streptogamins	Quinupristin-dalfopristin	ND			
Protein synthesis (30S)	Aminoglycosides	Gentamycin	1024 (R)	1024 (R)	16 (-)	1024 (-)
	Fucidanes	Fusidic acid	0.5 (S)	8 (R)	ND	
	Tetracyclines	Doxycycline	16 (R)	16 (R)	ND	
	Glycylcyclines	Tigecycline	2 (R)	16 (R)	ND	
70S initiation complex	Oxazolidinones	Linezolid	0.25 (S)	2 (S)	ND	
Folic acid metabolism	Folate pathway inhibitors	Trimethoprim-sulfamethoxazole	1024 (S)	16 (R)	ND	
DNA-directed RNA polymerase	Ansamycins	Rifampicin	0.03125 (S)	1024 (R)	ND	
DNA gyrase	Fluoroquinolones	Ciprofloxacin	32 (R)	32 (R)	2 (R)	128 (R)
Cell-wall synthesis	Anti-MRSA cephalosporins	Ceftaroline	ND			
	Carbapenems	Imipenem	ND		32 (R)	32 (R)
	Extended spectrum cephalosporins	Ceftazidime	ND		32 (R)	1024 (R)
	Antipseudomonal penicillins + β-lactamase inhibitor	Piperacillin-tazobactam	ND		64 (R)	1024 (R)
	Anti-staphylococcal β-lactams	Oxacillin	512 (-)	512 (-)	ND	
	Glycopeptides	Vancomycin	2 (S)	4 (R)	ND	
	Phosphonic acid	Fosfomycin (NR)	512 (R)	256 (R)	1024 (-)	1024 (-)
	Monobactam	Aztreonam	ND		32 (R)	32 (R)
Cell membrane	Lipopeptides	Daptomycin	64/32 (R)	32 (R)	ND	
	Polymyxins	Colistin	ND		1 (S)	1 (S)
			Light dose [J/cm^2]			
Phototherapy	**aBL**	Blue light (411 nm)	86.4	86.4	21.6	15.8

Abbreviations: ND—not defined; R—resistant; S—susceptible, (-) category of resistance not defined according to clinical breakpoints.

3.4. Recommended Methods for Synergy Testing Indicate the Increased Effectiveness of the Combination of aBL with Antibiotics for Clinical Isolates of S. aureus and P. aeruginosa

3.4.1. Diffusion Methods Confirm Synergy after Pre-Treatment of Bacterial Cells with Blue Light

E-test and disk diffusion assay presented in Figure 3A,B performed for clinical isolates of *S. aureus* (4046/13, 1814/06) indicate that the most pronounced synergy may be evidenced for CHL, LZD and FOF in the disk-diffusion method. Synergies with the E-test method were observed for the *S. aureus* isolate 1814/06 after exposure to aBL in the case of CHL, DOX, OXA and FOF (Figure 3B). On the other hand, a decrease in the susceptibility

upon aBL treatment was indicated for CIP and Q-D. For Gram-negative isolates no. 802 and 805, it can be clearly seen that the isolate was more resistant to sensitization. The second isolate, no. 802, after exposure to aBL had a synergistic effect with ATM, GEN and CST confirmed with disk diffusion method and for FOF confirmed with E-Test (Figure 3C,D). For two antibiotics, CAZ and FOF, after exposure of isolate no. 802 to aBL, the decrease in the inhibition zones was identified.

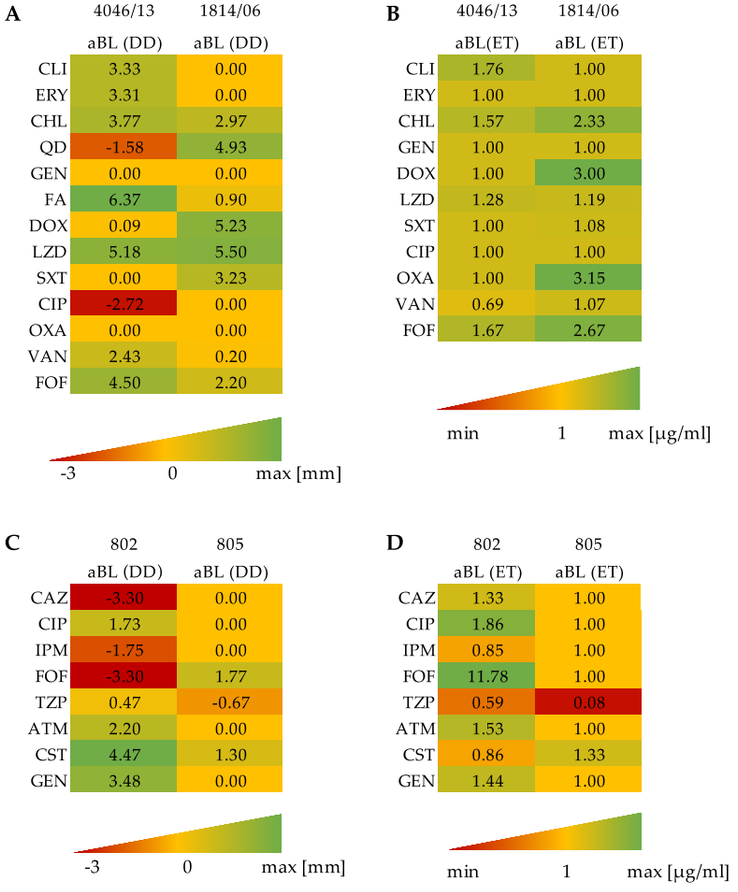

Figure 3. Graphical presentation of disk diffusion assay and E-test method. Figures present the differences in zones (in mm) of inhibition (in comparison to control) after exposure of isolates no. 4046/13 and 1814/06 to aBL (**A**); the ratio of control MIC values to MIC after exposure to aBL (**B**); (**C**,**D**) present the changes for isolates no. 802 and 805 from disk diffusion method and E-test, respectively, as it was present for *S. aureus* isolates. Each experiment was performed in three independent biological repetitions. Abbreviations: (DD) disk-diffusion; (ET) E-Test; (CLI) clindamycin; (ERY) erythromycin; (CHL) chloramphenicol; (QD) quinupristin-dalfopristin; (GEN) gentamycin; (FA) fusidic acid; (DOX) doxycycline; (LZD) linezolid; (SXT) trimethoprim-sulfamethoxazole; (CIP) ciprofloxacin; (OXA) oxacillin; (VAN) vancomycin; (FOF) fosfomycin; (CAZ) ceftazidime; (IPM) imipenem; (TZP) piperacillin-tazobactam; (ATM) aztreonam; (CST) colistin. MIN is defining the lowest relative MIC value from E-test; MAX defines the highest zone of inhibition (mm) and relative MIC value for the E-test.

3.4.2. Simultaneous Blue Light Irradiation and Antibiotic Treatment Confirms Synergy for Multiple Antibiotics in Checkerboard Assay

Results from the checkerboard assay present the FIC_i index for the combined phototreatment of aBL and antibiotics. Table 2 presents the results obtained for two clinical isolates of *S. aureus*, and for isolate no. 1814/06, the synergy between aBL and antimicrobials was observed in the cases of CHL, FA, LZD, SXT and CIP. The second isolate,

no. 4046/13, had synergy with aBL for CLI, CHL, FA, LZD and CIP (Table 2). Similarly to *S. aureus*, the synergy for clinical strains of *P. aeruginosa* were investigated with aBL and antibiotics (Table 3). Significantly less synergies were confirmed for *P. aeruginosa* isolates. In the case of strain no. 802, the synergy was confirmed for TZP, and for isolate no. 805, it was evidenced for CIP, CAZ, CST and FOF.

Table 2. Checkerboard FIC_I calculation for *S. aureus* isolates.

Antibiotic	CLI	ERY	CHL	GEN	FA	DOX	LZD	SXT	CIP	OXA	VAN	FOF
1814/06	>0.5	>0.5	**0.312**	>0.5	**0.375**	>0.5	**0.5**	**0.25**	**0.5**	>0.5	>0.5	>0.5
4046/13	**0.437**	>0.5	**0.417**	>0.5	**0.4375**	>0.5	**0.5**	>0.5	**0.5**	>0.5	>0.5	>0.5

Bold font indicates possible synergistic interactions; (CLI) clindamycin; (ERY) erythromycin; (CHL) chloramphenicol; (GEN) gentamycin; (FA) fusidic acid; (DOX) doxycycline; (LZD) linezolid; (SXT) trimethoprim-sulfamethoxazole; (OXA) oxacillin; (VAN) vancomycin; (FOF) fosfomycin.

Table 3. Checkerboard FIC_I calculation for *P. aeruginosa* isolates.

Antibiotic	GEN	CIP	IPM	TZP	CAZ	ATM	CST	FOF
802	>0.5	>0.5	>0.5	**0.5**	>0.5	>0.5	>0.5	>0.5
805	>0.5	**0.5**	>0.5	>0.5	**0.5**	>0.5	**0.375**	**0.5**

Bold font indicates possible synergistic interactions; (GEN) gentamycin; (CIP) ciprofloxacin; (IMP) imipenem; (TZP) piperacillin-tazobactam; (CAZ) ceftazidime; (ATM) aztreonam; (CST) colistin; (FOF) fosfomycin.

3.4.3. Postantibiotic Effect Presents the Synergistic Effect for aBL and Antibiotics as a Significant Delay in Bacterial Growth of Tested Clinical Isolates

Assessment of the antimicrobial effect of the combined treatment (antibiotics and blue light) was established based on the growth curves for tested isolates. In the first step of the experiment, cells were exposed to an MIC dose of antibiotic which after 2 h incubation was removed. Then, the sub-lethal dose of aBL was applied. This method is different from those previously used due to the sequential application of the treatments, i.e., (1) antibiotic, (2) aBL. Figure 4 summarizes the results obtained for all studied strains and antimicrobials, and Figure 5 presents the synergistic effect via two representative curves, i.e., for ciprofloxacin obtained for *S. aureus* isolate no. 4046/13 and colistin obtained for *P. aeruginosa* isolate no. 805. The time required for reaching the growth point 0.5 OD_{600} for the combined treatment curve (1/2 MIC aBL + MIC A) was approx. 175 min in comparison to the control curve (Figure 4A). A similar time was required for *P. aeruginosa* isolate no. 805 to reach the OD_{600} value 0.5 for the growth curve 1/2 MIC aBL+ MIC A, when CST was used in the experiment (Figure 4B) in comparison to the control.

A

	4046/13	1814/06
	Δt= (PAE) [min.]	
CLI	210.0	0.0
ERY	55.0	0.0
CHL	200.0	0.0
GEN	100.0	75.0
FA	0.0	0.0
DOX	100.0	0.0
LZD	120.0	0.0
SXT	75.0	175.0
CIP	175.0	225.0
OXA	0.0	180.0
VAN	0.0	0.0
FOF	0.0	200.0

B

	802	805
	Δt= (PAE) [min.]	
CAZ	185.0	130.0
CIP	260.0	0.0
IPM	110.0	150.0
FOF	0	200.0
TZP	150	0.0
ATM	80.0	140.0
CST	50.0	200.0
GEN	300.0	0.0

Figure 4. Graphical presentation of postantibiotic effect results (**A**) for isolate 4046/13 and 1814/06; (**B**) for isolate 802 and 805. Values of time below 90 min indicate the lack of synergy, whereas the unit of time between 90 min to 180 min confirms the partial synergy. All of the results above 180 min are recognized as a synergy.

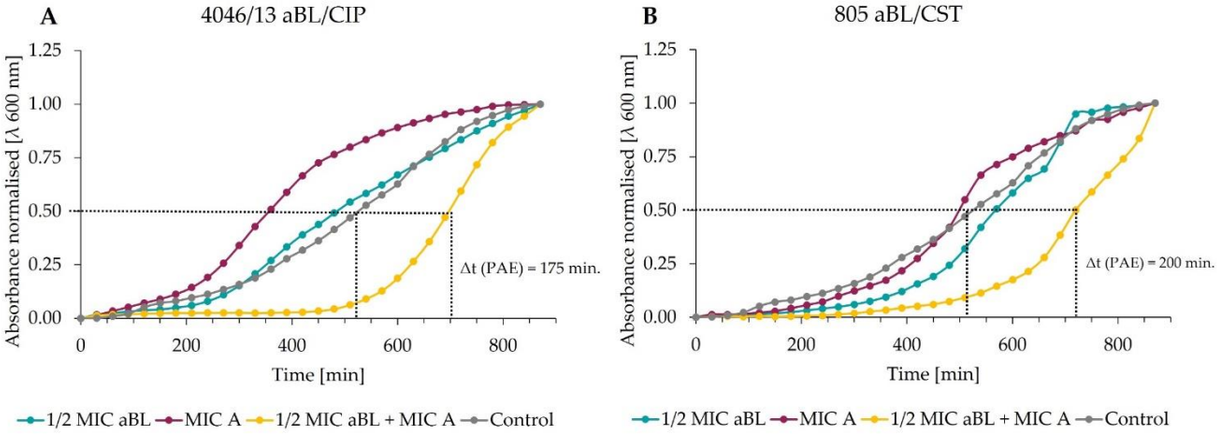

Figure 5. Postantibiotic effect growth curves. (**A**) Growth curve analysis of aBL/CIP combined treatment for *S. aureus* isolate 4046/13; (**B**) Growth curve analysis of aBL/CST combined treatment for the isolate of *P. aeruginosa* no. 805. Only one representative curve is presented. The overnight culture of microorganisms was diluted in fresh TSB (1:20), and then the bacterial suspensions were mixed with MIC of antibiotic. All samples were then covered with aluminium foil and incubated for 2 h at 37 °C. After incubation, samples were centrifuged and washed with a fresh TSB medium. Next, cells were transferred to a 96-well plate and exposed to $\frac{1}{2}$ MIC dose of blue light. In the next step, the optical density (λ 600 nm) of samples was measured for 15 h every 30 min. Obtained data were normalised and the postantibiotic effect (PAE) was determined. Postantibiotic effect value $\geq$3 h indicates synergy, whereas the 1.5 h $\leq$ PAE < 3 h confirms the partial synergistic effect.

3.5. Antimicrobial Blue Light in Low Doses Did Not Cause the Mutagenic and Toxic Effect

Figure 6A presents the results of whether aBL can cause the phototoxic effect on eukaryotic cells. Therefore, we examined three aBL doses. The survival rate of HaCaT cells decreased by approx. 80% only for the highest dose of aBL (43.2 J/cm^2). Two light doses, 9.4 J/cm^2 and 21.6 J/cm^2, were safe for eukaryotic cells (Figure 6A). We also attempted with the commercial Ames test to investigate the mutagenic effect of aBL, thus upon two implemented light doses, 4.32 J/cm^2 and 43.2 J/cm^2, we did not observe any increased number of revertants for the three tested mutants (Figure 6B). This observation is indicating that aBL is not a mutagenic treatment within the studied range of light doses.

Moreover, the last examination was focused on the analysis of the growth dynamics of HaCaT cells upon the aBL treatment. Figure 6C presents the growth dynamic upon exposure to a low dose of aBL, 4.32 J/cm^2, and it evidences that the growth dynamic presented as the Cell Index is not significantly affected in comparison to control cells. However, an increased aBL dose (43.2 J/cm^2) leads to a decrease in growth dynamic; thus, the CI decreased since the cells were exposed to blue light. Moreover, the cells did not reach the plateau phase; therefore, the applied aBL dose was lethal (Figure 6D).

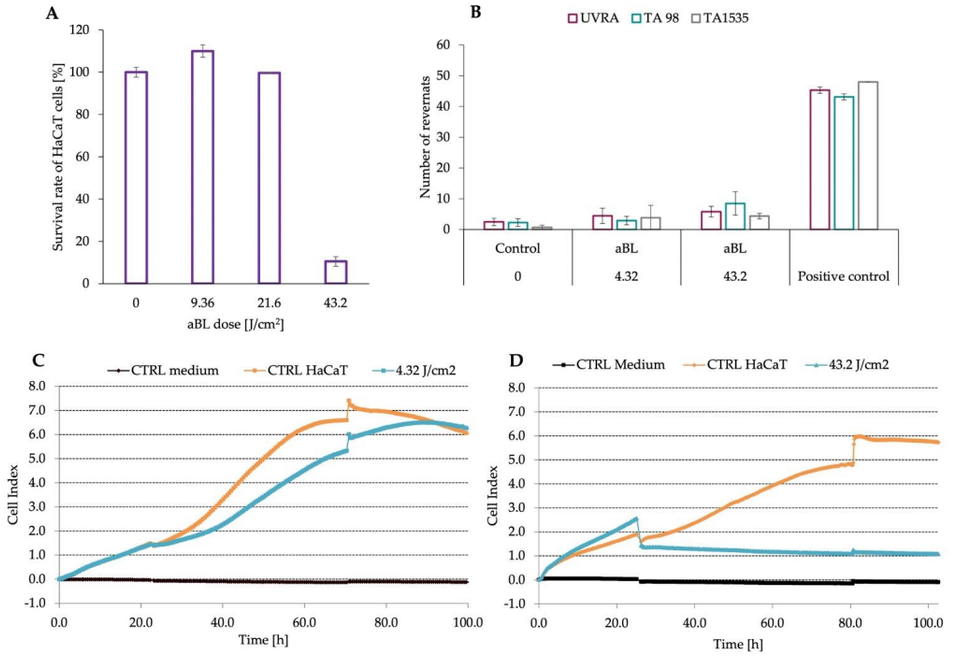

Figure 6. Assessment of phototoxicity, mutagenicity and influence on growth dynamic of eukaryotic cells upon aBL exposure to various light conditions. (**A**) Phototoxicity was assessed on the HaCaT cell line with the MTT assay; thus, eukaryotic cells were exposed to aBL doses and control–reference cells were included in the experiment. The day before the experiment, cells were seeded in a 96-well plate in the number of 1×10^4 cells/well. After 24 h cultivation, the cells were exposed to various doses of aBL or non-treated (control). A total of 24 h postirradiation, 10µL (12 mM) MTT reagent (1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan) was added to each well and kept for 4 h at 37 °C. Next, cells were lysed with DMSO, and the absorbance of formazan was established at 550 nm. (**B**) The mutagenic effect was assessed in Ames test with three different mutants (*E. coli* uvrA, *S.* Typhimurium TA 98 and TA1535). The assay included two blue light doses (4.32 and 43.2 J/cm2) and a positive and negative control (non-treated cells). The experiment was performed in three biological replicates, each replicating in three technical repetitions. *Escherichia coli* [uvrA] and *Salmonella* Typhimurium [TA98, TA1535] were diluted in an exposure medium and exposed to the various doses of aBL. Positive controls, i.e., the 2-Nitrofluorene (for TA98 and 1535) and 4-Nitroquinoline-N-oxide (for uvrA), were also included and added to the cultures to induce the mutations. The negative control (without any treatment) was also prepared. All of the cells were incubated after adding mutagen and/or aBL for 90 min at 37 °C. Afterwards, the exposure medium was added to the incubated cultures, and samples in the amount of 120 µL were partitioned into the 384 well plates (each sample was distributed to 48 wells separately in 3 technical repetitions). In the next step, all microplates were covered with sterile foil, placed in a plastic bag, and kept for 48 h at 37 °C. The assessment of revertants was performed after 48 h. Thus, the number of grown colonies (in each well) was determined. (**C**,**D**) The influence of the blue light on the growth dynamic of HaCaT cells was examined with two aBL doses (4.32 J/cm^2 and 43.2 J/cm^2), and the control cells (HaCaT CTRL) and medium control (CTRL medium) were included in the assay. The experiment was performed in 14 technical replicates. The day before the experiment, cells were seeded in the amount of 1×10^4 cells/well on E-plate PET plates. Cells were cultured in the standard humified incubator with 5% CO_2 for 24 h in the xCELLigence RTCA instrument. The next day, cells in the exponential growth rate (Cell index (CI) ≈ 2) were removed from the RTCA instrument, exposed to the various blue light doses and after the medium exchange, the plates were returned to the device. The CI was measured for each repetition every 10 min until the cells reached the plateau phase under tested conditions or if the cells did not survive post-irradiation.

3.6. aBL Leads to the Production of Various ROS and Increased Cell Permeability upon the Antibiotic Presence

To establish the ability of *S. aureus* cells to produce ROS, fluorescent probe HPF was used, and the autofluorescence of the probe was also taken into consideration during the experiment. Data present in Figure 7A clearly indicate that after exposure to aBL dose 43.2 J/cm^2, the level of fluorescence increased for the cells compared to the value estimated for the HPF probe alone. This confirms that the production of ROS occurred upon aBL exposure even at the lower dose of aBL (4.32 J/cm^2). Next, we aimed at the investigation of whether antibiotics upon aBL exposure can lead to increased ROS production. Therefore, the bacteria cells were not included in the experiment, despite the application of the HPF probe. As CHL was evidenced to give strong synergy using various synergy testing methods, this antimicrobial was chosen for ROS measurement and cell envelopes permeabilization studies. Data present in Figure 7B suggest that the CHL present in MIC and 1/2 MIC leads to the increased production of ROS upon two aBL doses in comparison to the HPF probe alone (control). On the other hand, we also evaluated whether the aBL can lead to increased cell permeabilization and, in consequence, increase the antibiotic influx into microbial cells. Figure 7C presents the data performed for the experiment with the involvement of propidium iodide, which plays a role as a cell membrane permeabilization indicator. Upon application of the blue light dose of 43.2 J/cm^2, the permeabilization of the cells did not increase in comparison to the positive and negative control indicating no cell envelope permeabilization upon aBL. The last experiment was also focused on cell permeabilization; however, this was investigated in the presence of antibiotic (CHL). Data present in Figure 7D indicate that upon aBL treatment alone, even in the highest dose of 43.2 J/cm^2, the DNA leakage of intracellular DNA was not observed, as the level of fluorescence of complex DNA-SYTOX was not higher in comparison to the control. No increase in the fluorescence signal from complex DNA-SYTOX was observed, which correlates with the PI experiment and indicates that no significant membrane permeabilization occurs upon aBL and aBL/CHL combined treatment.

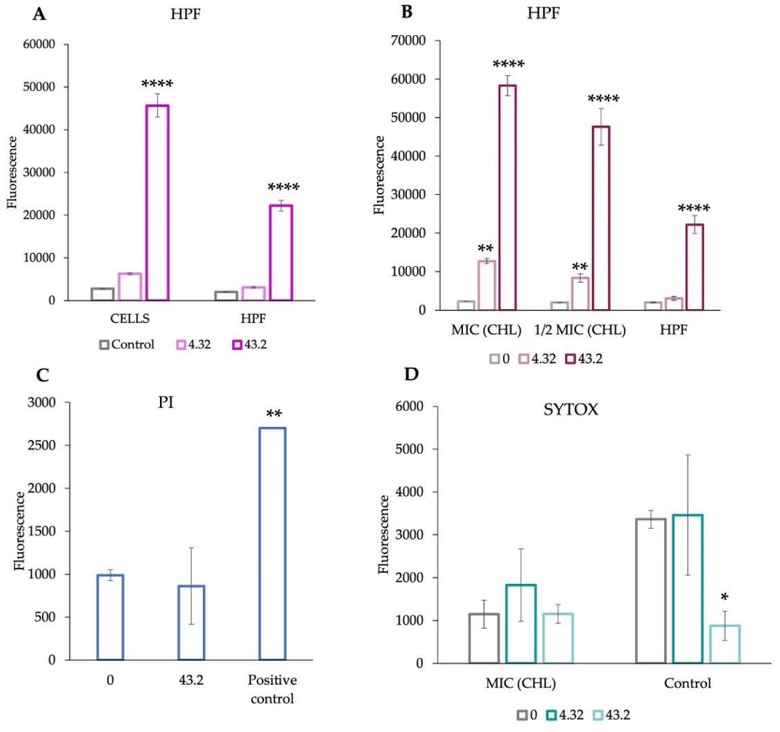

Figure 7. Assessment of the combination of CHL and/or aBL alone (in two doses) was performed with the implementation of (**A**) HPF probe (aBL); (**B**) HPF (aBL combined with CHL); (**C**) propidium

iodide (aBL); (**D**) SYTOX green label (aBL combined with CHL); Each experiment was performed in three repetitions. All of the samples were incubated for 15 min in the dark and exposed to blue light doses. Immediately after exposure, the fluorescence signal was measured at (excitation/ emission maxima) 490 nm/515 nm. Control samples containing the fluorescent probes but not exposed to visible light were also prepared. Statistical significance (* $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$) in comparison to samples not treated with the aBL (0 J/cm^2) or control.

3.7. Endogenous Porphyrins Present in Bacterial Cells Are Involved in the Synergistic Effect of aBL and Antibiotics in Staphylococcus aureus

There is a common hypothesis, that endogenous porphyrins and flavins present in bacterial cells are a crucial element in the mechanism of aBL. With application of the disc diffusion method, we investigated whether the isogenic mutant lacking heme production, and in consequence endogenous porphyrins production, can be sensitized to antibiotics as it is evidenced for the wild type (WT) strain. Table 4 presents the differences in the zone of inhibition for the WT and hemB isogenic mutant after exposure to a blue light dose of 64.8 J/cm^2. This aBL treatment led to the reduction in bacterial viability for WT by 0.5–2 $\log_{10}$. For almost all antibiotics, the wild type *S. aureus* became sensitized after exposure to a sub-lethal aBL, with the exception of FOF. Contrary, in the case of the ΔhemB mutant no significant changes in zones of inhibition were observed, indicating the crucial role of endogenous porphyrins in the aBL mechanism which, in consequence, demonstrated synergies.

Table 4. Changes in susceptibility profile of *S. aureus* WT (NCTC 8325-4) and isogenic mutant ΔhemB after aBL exposure.

Antibiotic	Control (WT) [mm]	aBL-64.8 J/cm^2 (WT) [mm]	Control (ΔhemB) [mm]	aBL-64.8 J/cm^2 (ΔhemB) [mm]
CLI	26.6	**33.2**	6.0 *	6.0 *
ERY	27.6	**32.0**	6.0 *	6.0 *
CHL	30.5	**33.5**	31.2	30.5
Q-D	26.8	**30.3**	31.8	32.2
GEN	21.1	23.9	14.7	15.4
FA	33.4	**38.6**	33.2	34.3
DOX	31.8	**37.1**	34.7	34.5
LZD	30.6	**32.3**	33.3	32.1
SXT	26.0	**29.4**	20.7	21.0
CIP	22.7	**31.3**	32.2	28.4
OXA	27.4	**30.8**	19.8	20.6
VAN	11.9	13.1	12.7	12.5
FOF	49.2	33.4	30.1	31.0

* ΔhemB mutant is resistant to the ERY and CLI, thus the zones of inhibition even after the aBL exposure are equal to 6 mm. Abbreviations: (CLI) clindamycin; (ERY) erythromycin; (CHL) chloramphenicol; (QD) quinupristin-dalfopristin; (GEN) gentamycin; (FA) fusidic acid; (DOX) doxycycline; (LZD) linezolid; (SXT) trimethoprim-sulfamethoxazole; (CIP) ciprofloxacin; (OXA) oxacillin; (VAN) vancomycin; (FOF) fosfomycin. Bold font indicates a difference min. 2 mm in comparison to control, thus it confirms the synergy after exposure to aBL.

3.8. Synergistic Effect of aBL and CHL Rescues Mice from Wound Infection

In vivo experiments were performed with the bioluminescent derivative of MRSA strain Xen31 and bioluminescent *P. aeruginosa* strain PAK. aBL was applied for wounds infected with Xen31 in a dosage of 8.6 J/cm^2 and for wounds infected with PAK in a dose of 14.4 J/cm^2. Both doses of aBL applied in in vivo experiments were the sub-MIC doses established for strain Xen31 and PAK, respectively. Moreover, based on the MTT experiment, both doses were identified as safe for eukaryotic cells (Data Not Shown). Antibiotics were used in MIC doses, for wounds infected with *S. aureus* CHL was used, and for *P. aeruginosa* TZP was applied. Though TZP was not evidenced with in vitro assays to give strong synergies for studied *P. aeruginosa* clinical isolates, it was chosen for in vivo

studies as giving strong synergy for PAK isolate (Data Not Shown). The results presented in Figure 8I,II indicate that combined treatment led to the extinction of the infection in comparison to monotreatment (aBL or Antibiotic). Moreover, the decrease in the bioluminescent signal was observed even on day 3 of the experiment (Figure 8II). The application of monotherapies, i.e., aBL and Antibiotic (chloramphenicol), was also effective for wounds infected with Xen31; however, the best results were observed when these two therapeutic options were combined. On the other hand, wounds infected with bioluminescent PAK strain did not respond to combined aBL and antibiotic treatment. The infection in this group was not extinct even on the 5th day of the experiment. Monotreatments, i.e., aBL and antibiotic, were also ineffective in treatments of wounds (Figure 8III). Bioluminescent images of wounds infected with PAK presented in Figure 8IV indicate that the signals of bioluminescence were present in all the experimental groups till the end of the experiment (5th day).

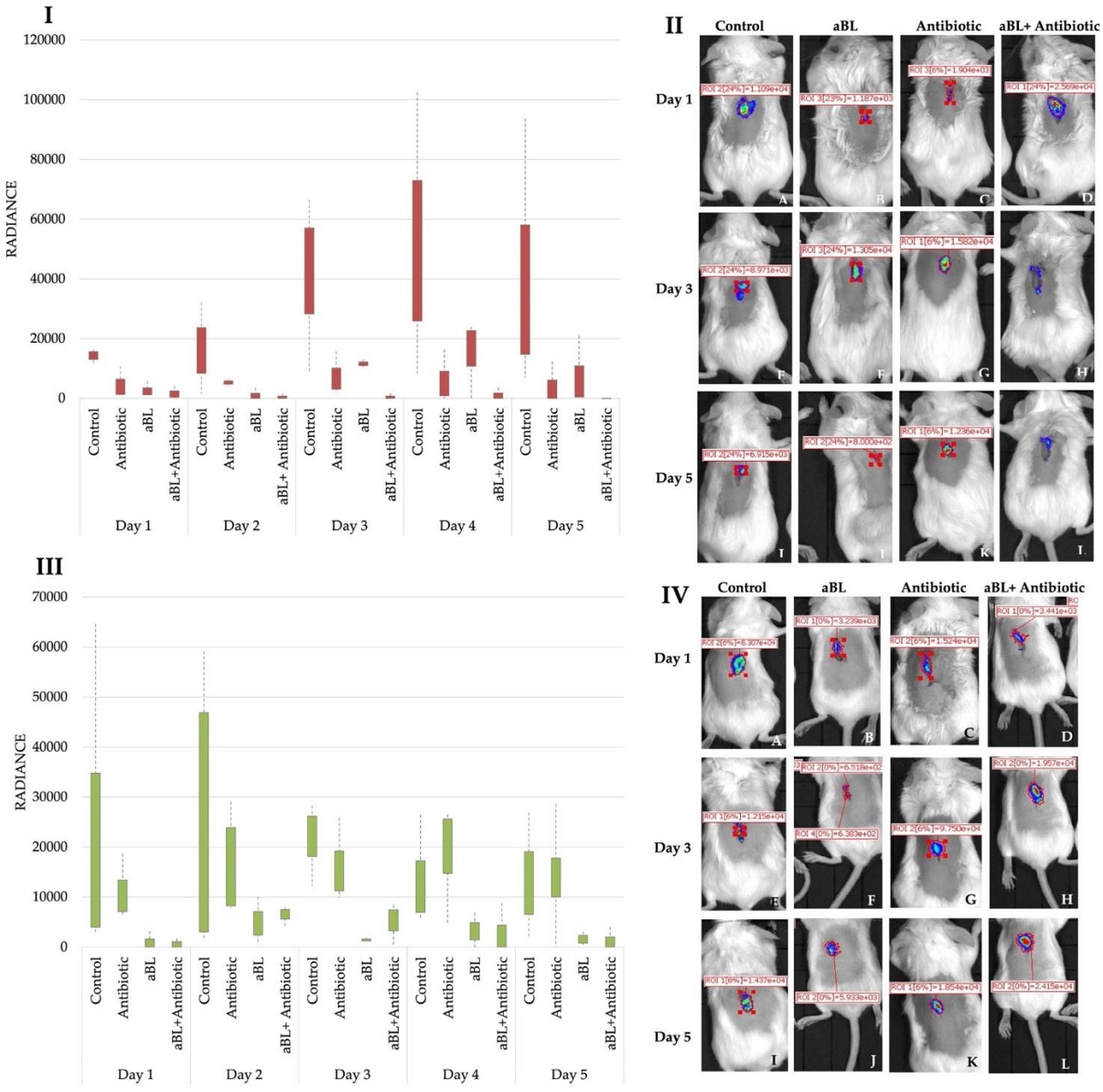

Figure 8. The combined therapy of wound infected with bioluminescent *S. aureus* Xen31 and *P. aeruginosa* PAK strains. Bioluminescence signals presented as radiance for all the experimental

groups for wounds infected with Xen31 (**I**) and for wounds infected with PAK (**III**)**.** Bioluminescent images of infected wounds with Xen31 (**II**) and PAK (**IV**). The day before the experiment, mice were shaved on the dorsal surfaces, depilated with depilatory lotion, and the immunosuppressant—endoxan (150 mg/kg)—was injected intraperitoneal into each animal. The next day, overnight cultures of *S. aureus* (Xen31) or *P. aeruginosa* (PAK) were centrifuged and resuspended in the physiological salt to obtain each 10 µL of culture 10^7 CFU/mL. The wounds were created by making a 1 cm incision on the skin with a sterile needle, and immediately 10 µL of Xen31/PAK cells were applied to the damaged skin. A total of 30 min after infection of wound, mice were given: (i) antibiotic (1/2 MIC); (ii) aBL (MIC); (iii) antibiotic (1/2 MIC) + aBL (MIC). For experiments with Xen31 and PAK, chloramphenicol and piperacillin-tazobactam were used as antibiotics, respectively. The control group (iv) of mice were not given any treatment. Immediately after irradiation, the bioluminescence imaging of infected wounds was performed with the IVIS Spectrum imaging system. The luminescence was measured daily for up to 5 days. The quantification of the treatments was measured by the changes in bioluminescent signal, defined as an average radiance, and by observing the visual changes during the experiment.

4. Discussion

Photoinactivation as a monotreatment has been presented multiple times in literature as an efficient tool for the eradication of Gram-positive and Gram-negative microorganisms, viruses, parasites and fungi [17]. Moreover, the enhancement of biocidal action of fungicides, antibiotics or natural plant extracts against resistant-to-treatment organisms could be possible with the implementation of photoinactivation [12,18–20]. Presented within this study, two important pathogens, *Staphylococcus aureus* (SA) and *Pseudomonas aeruginosa* (PA), are nosocomial microorganisms responsible for approx. 1400 deaths in the USA due to pneumonia (PA) and more than 119,000 of deaths also in the USA were recorded for SA bloodstream infections [21,22]. Increased resistance to antibiotics forces the development of findings and solutions to fight with the lack of new antibiotics or to re-sensitize microorganisms to its action. Within this study, we investigate the possibility of using routine antibiotics against *P. aeruginosa* and *S. aureus* with antimicrobial blue light inactivation (aBL) in in vitro and in vivo studies.

Firstly, we investigated the effectiveness of aBL photoinactivation in two different environmental conditions: cells suspended in Phosphate Buffered Saline (PBS) and Tryptic Soy Broth (TSB) used for the cultivation of bacteria. *S. aureus* clinical isolates were less susceptible to aBL when diluted in PBS, and this observation was also drawn for *P. aeruginosa*; however, Gram-negative strains were more susceptible overall to aBL treatment. The differences in the effectiveness of photoinactivation depending on the culture medium were multiple times examined. For example, an experiment performed by dos Anjos et al. evidenced that the inactivation of various Gram-negative and Gram-positive microorganisms with blue light (λ 413 nm) was more effective in PBS in comparison to cells diluted in the suspension contaminated with milk [23]. In the current study, aBL efficacy was increased when using TSB medium instead of PBS. One must be aware that the photosensitizing compounds that play a crucial role in aBL are endogenous chromophores that could probably be washed out when TSB medium was replaced with PBS. It could lead to decreased concentration of PSs in an extracellular environment and result in decreased aBL effectiveness. Assessment of the sub-lethal and lethal doses for tested pathogens in these two conditions (TSB, PBS) was crucial due to the further implementations of obtained doses in the experiments concerning the evaluation of interactions between light and antimicrobials, therefore in Figure 1, only the sub-lethal doses of aBL were marked. Secondly, we characterized the antimicrobial resistance profile of clinical isolates, which confirmed that all of four isolates (4046/13; 1814/06; 802; 805) belong to the category of multidrug-resistant microorganisms, according to the outlines published by Magiorakos et al. [16].

According to the latest published review focused on combining the photoinactivation with antibiotics against these two pathogens, many of the published results are investigating

the effectiveness of combined treatment in in vitro conditions only with a few antimicrobial agents. Within the current study, we examined the entire panel of antibiotics for each of the pathogen. The first method which allowed us to investigate the effectiveness of aBL with antibiotics was the disk diffusion method and E-Test. In both methods, microorganisms were at first exposed to aBL and afterwards, the antibiotics were applied as a disk or strip. *S. aureus* isolates had more synergistic effects than clinical isolates of *P. aeruginosa*; however, due to divergent peptidoglycan depth between these microorganisms and cell membrane structure, the differences in obtained results can be explained by these facts. The second method of synergy testing—checkerboard assay—also confirmed the success of combining aBL and antimicrobials, though light and antibiotics were applied simultaneously to bacterial cells. The results obtained for both *S. aureus* clinical isolates indicate that CHL, FA, LZD and CIP act synergistically with aBL. On the other hand, for *P. aeruginosa* clinical isolates, a lower number of synergies were confirmed. Single synergies were obtained for TZP, CIP, CST and FOF. The last method of synergy testing was based on the temporary treatment of cells with antibiotics in MIC concentration and subsequent treatment with aBL. The changes in the bacterial growth were the determinant of synergy for samples treated by combined treatment in comparison to the control groups in a postantibiotic effect method.

Table 5 presents all of the interactions detected within the current study for all combinations of aBL and antibiotics against two *S. aureus* clinical isolates. The green colour indicates the synergy. Moreover, the synergies that were already demonstrated in other published studies were marked as (+). A similar table was prepared for results obtained for clinical isolates of *P. aeruginosa* (Table 6). It is clearly visible that there is limited number of research concerning the aBL combination with antimicrobials. We as a first aimed to investigate the broad spectrum of antimicrobial agents against Gram-negative and Gram-positive representatives in combination with photoinactivation. One may see that aBL exhibits different potentiation effects for various antimicrobials representing various mode of action. We have demonstrated the synergies between aBL and antimicrobials targeted at protein as well as nucleic acids or cell envelopes synthesis (Tables 5 and 6).

Table 5. Summarized results of synergy testing for *S. aureus* clinical isolates and synergies presented in a literature data for representative antimicrobial agents.

Antibiotic Target		4046/13				1814/06				Synergies Confirmed in Other Studies
		DD	E-T	CA	PAE	DD	E-T	CA	PAE	
Protein synthesis	CLI									
	ERY									
	CHL									
	QD									
	GEN									
	FA									
	DOX									(+) [24] (+) [25]
	LZD									(+) [26]
Nucleic acids	SXT									
	CIP									(+) [26] (+)[22]
Cell wall	OXA									(+) [27]
	VAN									
	FOF									

Abbreviations: (DD) disk-diffusion; (E-T) E-Test; (CA) checkerboard assay; (PAE) postantibiotic effect; (CLI) clindamycin; (ERY) erythromycin; (CHL) chloramphenicol; (QD) quinupristin-dalfopristin; (GEN) gentamycin; (FA) fusidic acid; (DOX) doxycycline; (LZD) linezolid; (SXT) trimethoprim-sulfamethoxazole; (CIP) ciprofloxacin; (OXA) oxacillin; (VAN) vancomycin; (FOF) fosfomycin. (+) indicate the positive effect of combination of aBL and antibiotic in other study. The green colour indicates the synergy; red colour indicates antagonism.

Table 6. Summarized results of synergy testing for *P. aeruginosa* clinical isolates and synergies presented in a literature data for representative antimicrobial agents.

Antibiotic Target		802				805				Synergies Confirmed in Other Studies
		DD	E-T	CA	PAE	DD	E-T	CA	PAE	
Protein synthesis	GEN									(+) [1]
Nucleic acids	CIP									
Cell wall/ Cell membrane	IPM									(+) [1]
	CAZ									
	TZP									
	FOF									
	ATM									
	CST									

Abbreviations: (DD) disk-diffusion; (E-T) E-Test; (CA) checkerboard assay; (PAE) postantibiotic effect; (GEN) gentamycin; (CIP) ciprofloxacin; (IMP) imipenem; (TZP) piperacillin-tazobactam; (CAZ) ceftazidime; (ATM) aztreonam; (CST) colistin; (FOF) fosfomycin. (+) indicate the positive effect of combination of aBL and antibiotic in other study. The green colour indicates the synergy; red colour indicates antagonism.

As mentioned above in literature, there are only a few articles which attempt to investigate the combination of aBL with antibiotics to eradicate ESKAPE representatives, *P. aeruginosa* and *S. aureus*. The experiment performed by Fila et al. proved that gentamycin, ceftazidime, or meropenem result in a synergistic effect against *P. aeruginosa* [1,28]. In contrast, another study indicates the lack of effectiveness of combining tigecycline, minocycline and aBL against this pathogen [29]. On the other hand, the combination of aBL and the antimicrobial agent was also examined for *S. aureus* within the experiments performed by Reznick et al., who demonstrated no effectiveness of aBL combined with minocycline and tigecycline. Up to this date, only two studies have confirmed the successful combination of ciprofloxacin and aBL against *S. aureus* [20]. Within our study, we observed various synergies for *S. aureus* and *P. aeruginosa* in different methods, therefore one may conclude that the order of the factors administration (blue light, antibiotics) may play an important role. The synergies observed, i.e., via disk diffusion method, differed from those obtained via checkerboard assay. Moreover, the investigation of the effectiveness of combined treatments was not evidenced in most of the mentioned above studies due to inappropriate methodology applied; therefore, we attempted to use the appropriate experimental protocol that was precisely described and critically analysed in our literature review [30]. The mechanisms of synergy between blue light and antibiotics are not revealed yet, however there are few potential explanations of this phenomenon. First of all, antibiotics upon exposure to the photons can undergo the photochemical reactions and play the role of producers of ROS. Experiments performed by He et al. imply that the presence of tetracyclines (e.g., demeclocycline) can potentiate the effect of photoinactivation with blue light resulting in the decrease in MIC value after aBL exposure [25]. Secondly, photoinactivation can lead to the inactivation of the enzymes or decreased expression of their coding genes that are responsible for the resistance to particular antibiotics. For example, the experiments performed by Boluki et al. evidenced the decreased level of expression of genes responsible for resistance to colistin in pan-drug-resistant strains upon photoinactivation [31]. However, this investigation was performed applying irradiation with red light (λ 630 nm) with the presence of exogenous PS, i.e., toluidine blue O. On the other hand, certain antibiotics (especially aminoglycoside, fluoroquinolones and β-lactam antibiotics) activate the tricarboxylic acid cycle, resulting in the metabolic changes which generate ROS [32]. Moreover, another possible explanation is linked with increased permeabilization and breakage of cell walls upon photoinactivation treatment, resulting in an increased antibiotic uptake and availability to cells [33]. Treatment with ROS-generating photoinactivation and subsequent treatment with the mentioned antibiotics may explain the synergy as an effect of the action of two ROS sources. Due to these facts, the action of antibiotics was potentiated upon aBL exposure (in checkerboard assay); after microbial culture exposure to aBL (diffusion methods); or after pre-treatment of

bacterial culture with antibiotics in MIC concentration (in postantibiotic assay). It cannot be excluded that potentiation effect can also result from the mechanism of action of antibiotics.

An important issue discussed within the current work is the assessment of the toxicity of blue light in the context of in vivo experiments. The aBL doses implemented in animal experiments were lower than those used for clinical isolates in vitro. Despite this fact, aBL doses used in our investigations up to 21.6 J/cm^2 did not influence the survival rate of HaCaT cell line. Implemented in our study, visible blue light is characterised by having a short wavelength (λ_{max} 411 nm) but the highest energy from the visible light spectrum [34]. The obtained results for the highest aBL dose (43.2 J/cm^2) tested in MTT assay and used in the evaluation of cell growth dynamic with xCELLigence indicating significant cell inactivation are not surprising. Moreover, the research published by Liebman et al. also evidenced that blue light in higher fluences has a negative impact on human keratinocytes [35]. The experimental outcome from the Ames test excluded a mutagenic effect of aBL on the tested *E. coli* and *S.* Typhimurium mutants; however, it could not be excluded that the higher aBL doses may result in increased mutagenicity due to the cytotoxic effect of aBL in higher doses. Similar conclusions were drawn in research performed by Grinholc et al. who demonstrated the cytotoxic effect of New Methylene Blue and Toluidine Blue to *Salmonella* Typhimurium (TA98) upon treatment [36].

Investigation into the possible mechanism of aBL which is responsible for the synergistic interactions was another crucial part of the current study and it was investigated with the use of a Gram-positive representative. As a first, we confirmed that aBL leads to the production of various ROS with HPF probe in the presence of bacterial cells in irradiated suspension. The fluorescence level was higher after exposure of cells to aBL than the HPF probe (without cells) exposed to the same light conditions. Next, we confirmed that in the presence of CHL in MIC and 1/2 MIC, the ROS level increased also after exposure to aBL in two doses of light (4.32 J/cm^2 and 43.2 J/cm^2) when compared to the absence of antibiotic. This observation could explain the fact that in both *S. aureus* clinical isolates we observed synergistic effects for this antibiotic. In the case of cell envelopes permeabilization, propidium iodide and SYTOX green labelling were used. PI can easily uptake into the damaged bacterial cells, thus it may serve as a marker for membrane permeabilization upon aBL treatment. Upon the exposure of microbes to the highest aBL dose 43.2 J/cm^2, the level of permeabilization was not higher when compared to the control. Applying the SYTOX green-fluorescent probe, which is able to bind to DNA released from damaged cells, did not result in the increased cell envelopes permeabilization upon aBL treatment nor when the highest aBL dose was administered. In other conditions, including antibiotic presence, the membrane permeabilization was not evidenced which correlates with the PI experiment. According to the literature, aBL (λ_{max} 405 nm) may lead to the *E. coli* cell permeabilization, however, this was not detected for *S. aureus* strain when SYTOX green was used [37].

Despite the investigation of ROS production via the aBL photoinactivation and its synergy with antimicrobials, we also took a closer look into the endogenous chromophores present in *S. aureus*. It is a first report that investigates whether the lack of porphyrin production can influence the synergies between light and antimicrobials. Therefore, we implemented the wild-type strain with the ability of porphyrins production and its isogenic mutant ΔhemB with impaired endogenous porphyrin production, for studies with aBL impact on their drug susceptibility profile using the disk diffusion method. We observed that inhibition zones for antimicrobials for mutant ΔhemB upon aBL exposure did not change in contrast to the wild-type strain. It clearly indicates that the endogenous porphyrins serve as an endogenous photosensitizing compound and play a crucial role for the aBL activity as well as its synergy with antimicrobials.

The culmination of in vitro research was the verification of the obtained results using in vivo models. With a mouse model of a wound infected with bioluminescent *S. aureus* strain Xen31, we confirmed that chloramphenicol with blue light is effective in wound healing in comparison to monotherapies alone (antibiotic, aBL). Furthermore, in the in vivo

model, mice wounds infected with the Gram-negative PAK strain did not confirm the effectiveness of the combination of light and antimicrobials. The infection was not significantly inhibited up to the 5th day of the experiment. The obtained results could obviously be affected with the antimicrobial used for in vivo studies, i.e., piperacillin-tazobactam or the aBL dose. We expect that using other antimicrobial or increased aBL doses, the successful wound healing would be observed. Unfortunately, there is a lack of publications which confirm the effectiveness of aBL and antibiotics combined treatment with the use of in vivo models; thus, the current one is of high importance. Most of the in vivo studies are studying various photosensitizers, various visible light wavelengths or various agents which can potentiate the action of aBL as a monotherapy [7,38].

5. Conclusions

The results described in the current paper clearly demonstrate the enormous potential of this alternative treatment option resulting from combining blue light and antimicrobials. Obviously, further studies including the different blue light wavelengths, various light power and doses, as well as another microbial species or isolates representing different drug resistance profiles are required to support the accurate performance of in vivo studies, and to finally demonstrate the rationale for using this combined approach in the fight against drug-resistant pathogens.

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Summary

Within the doctoral dissertation all of the goals maintained in the section "Hypothesis and aims of the work" were established.

Investigation of the efficacy of antimicrobial photodynamic inactivation as a tool for sensitizing multidrug resistant pathogens to antimicrobials was examined with created protocol of synergy testing. Creation of appropriate set of experimental methods which I used in my research is one of the greatest achievements. Confirmation of biocidal action of aBL/ aPDI with photosensitizing agents against 12 ESKAPE clinical isolates representing multidrug resistance profile, was another important goal of this dissertation. Despite the differences in sensitization of clinical isolates among the used methods and the variability in synergistic effect, photoinactivation was evidenced as an efficient tool to sensitize human pathogens to antimicrobials in planktonic as well as in biofilm culture. On the other hand, this process is strain-dependent and additional examination should be carried out to understand the mechanisms of this phenomenon. Another important goal achieved in my research was associated with the confirmation of safety of blue light with its implementation towards eukaryotic and prokaryotic cells. Moreover, I confirmed that the ROS production occurs in photoinactivation process and it is a crucial factor influencing the sensitization of the bacterial cells via the permeabilization of cell membranes. Another achievement of this dissertation was indication of key role of endogenous chromophores in the process of synergy with implementation of aBL. Finally, within the current study I demonstrated the bactericidal effectiveness of the use of combined photoinactivation and antibiotic treatment in the *in vivo* model.

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Attachments

1. Statements of contribution

1.1. Publication no. 1

Wozniak, A. & Grinholc, M. Combined antimicrobial activity of photodynamic inactivation and antimicrobials-state of the art. Front. Microbiol. 9, 196–203 (2018).

Gdańsk, 06.07.2022 r.

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I hereby declare my contribution to the publication:

Wozniak, Agata, and Mariusz Grinholc. "*Combined antimicrobial activity of photodynamic inactivation and antimicrobials–state of the art.*" Frontiers in Microbiology 9 (2018): 930.

As follows:

	Ideas	**Writing**	**Stewardship**	**Adjusted Authorship contribution**
Co-author	**10%**	**80%**	**10%**	**%**
A. Woźniak	30	70	0	**60**
M. Grinholc	70	30	100	**40**

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Co-author	**10%**	**80%**	**10%**	**%**
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M. Grinholc	70	30	100	**40**

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Signature

1.2. Publication no. 2

Wozniak, A., Rapacka-Zdonczyk, A., Mutters, N. T. N. T. & Grinholc, M. Antimicrobials Are a Photodynamic Inactivation Adjuvant for the Eradication of Extensively Drug-Resistant Acinetobacter baumannii. Front. Microbiol. 10, 229 (2019).

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A. Rapacka- Zdończyk	20	25	25	20	25
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M. Grinholc	35	0	45	50	20
A. Rapacka- Zdończyk	20	25	25	20	25
N. T. Mutters	10	0	5	10	5

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1.3. Publication no. 3

Woźniak, A., Kruszewska, B., Pierański, M. K., Rychłowski, M. & Grinholc, M. Antimicrobial Photodynamic Inactivation Affects the Antibiotic Susceptibility of Enterococcus spp. Clinical Isolates in Biofilm and Planktonic Cultures. Biomolecules 11, 693 (2021).

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Co-author	**10%**	**40%**	**40%**	**10%**	**%**
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M. Grinholc	20	0	25	40	**10**
M.K. Pierański	20	25	15	20	**25**
B. Kruszewska	20	25	15	20	**25**
M. Rychłowski	5.0	5.0	5.0	0.0	**5**

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	Ideas	**Work**	**Writing**	**Stewardship**	**Adjusted Authorship contribution**
Co-author	**10%**	**40%**	**40%**	**10%**	**%**
A. Woźniak	35	45	40	20	**35**
M. Grinholc	20	0	25	40	**10**
M.K. Pierański	20	25	15	20	**25**
B. Kruszewska	20	25	15	20	**25**
M. Rychłowski	5.0	5.0	5.0	0.0	**5**

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Woźniak, A., Kruszewska, B., Pierański, M. K., Rychłowski, M., & Grinholc, M. (2021). *Antimicrobial photodynamic inactivation affects the antibiotic susceptibility of Enterococcus spp. clinical isolates in biofilm and planktonic cultures.* Biomolecules, 11(5), 693.

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Co-author	**10%**	**40%**	**40%**	**10%**	**%**
A. Woźniak	35	45	40	20	**35**
M. Grinholc	20	0	25	40	**10**
M.K. Pierański	20	25	15	20	**25**
B. Kruszewska	20	25	15	20	**25**
M. Rychłowski	5.0	5.0	5.0	0.0	**5**

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Woźniak, A., Kruszewska, B., Pierański, M. K., Rychłowski, M., & Grinholc, M. (2021). *Antimicrobial photodynamic inactivation affects the antibiotic susceptibility of Enterococcus spp. clinical isolates in biofilm and planktonic cultures*. Biomolecules, 11(5), 693

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Co-author	**10%**	**40%**	**40%**	**10%**	**%**
A. Woźniak	35	45	40	20	**35**
M. Grinholc	20	0	25	40	**10**
M.K. Pierański	20	25	15	20	**25**
B. Kruszewska	20	25	15	20	**25**
M. Rychłowski	5.0	5.0	5.0	0.0	**5**

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Woźniak, A., Kruszewska, B., Pierański, M. K., Rychłowski, M., & Grinholc, M. (2021). *Antimicrobial photodynamic inactivation affects the antibiotic susceptibility of Enterococcus spp. clinical isolates in biofilms and planktonic cultures.* Biomolecules, 11(5), 693

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A. Woźniak	35	45	40	20	**35**
M. Grinholc	20	0	25	40	**10**
M.K. Pierański	20	25	15	20	**25**
B. Kruszewska	20	25	15	20	**25**
M. Rychłowski	5.0	5.0	5.0	0.0	**5**

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1.4. Publication no. 4

Priming effect with photoinactivation against extensively drug-resistant Enterobacter cloacae and Klebsiella pneumoniae
A. Woźniak, N. Burzyńska, I. Zybała, J. Empel & M. Grinholc
Journal of Photochemistry and Photobiology B: Biology
(Article in press)1471

Gdańsk, 31.08.2022 r.

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I hereby declare my contribution to the publication:

Wozniak Agata, Natalia Burzyńska, Izabela Zybała, Joanna Empel and Mariusz Grinholc. "*Priming effect with photoinactivation against extensively drug-resistant Enterobacter cloacae and Klebsiella pneumoniae*". Journal of Photochemistry and Photobiology B: Biology.

As follows:

Co-author	Ideas 10%	Work 40%	Writing 40%	Stewardship 10%	Adjusted Authorship contribution %
A. Woźniak	30	40	60	20	**45**
N. Burzyńska	10	25	0	0	**12**
I. Zybała	10	25	0	0	**12**
J. Empel	10	0	10	0	**5**
M. Grinholc	40	5	30	80	**26**

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A. Woźniak	30	40	60	20	**45**
N. Burzyńska	10	25	0	0	**12**
I. Zybała	10	25	0	0	**12**
J. Empel	10	0	10	0	**5**
M. Grinholc	40	5	30	80	**26**

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Co-author	**10%**	**40%**	**40%**	**10%**	**%**
A. Woźniak	30	40	60	20	**45**
N. Burzyńska	10	25	0	0	**12**
I. Zybała	10	25	0	0	**12**
J. Empel	10	0	10	0	**5**
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A. Woźniak	30	40	60	20	**45**
N. Burzyńska	10	25	0	0	**12**
I. Zybała	10	25	0	0	**12**
J. Empel	10	0	10	0	**5**
M. Grinholc	40	5	30	80	**26**

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Co-author	10%	40%	40%	10%	%
A. Woźniak	30	40	60	20	**45**
N. Burzyńska	10	25	0	0	**12**
I. Zybała	10	25	0	0	**12**
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M. Grinholc	40	5	30	80	**26**

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1.5. Publication no. 5

Woźniak, A. & Grinholc, M. Combined Antimicrobial Blue Light and Antibiotics as a Tool for Eradication of Multidrug-Resistant Isolates of Pseudomonas aeruginosa and Staphylococcus aureus: In Vitro and In Vivo Studies. Antioxidants 11, 1660 (2022)

Gdańsk, 31.08.2022 r.

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Wozniak Agata, and Mariusz Grinholc. "*Combined Antimicrobial Blue Light and Antibiotics as a Tool for Eradication of Multidrug-Resistant Isolates of Pseudomonas aeruginosa and Staphylococcus aureus: In Vitro and In Vivo Studies*". Antioxidants 11, no. 9: 1660.

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As follows:

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Co-author	**10%**	**40%**	**40%**	**10%**	**%**
A. Woźniak	30	80	70	20	**65**
M. Grinholc	70	20	30	80	**35**

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2. Letter of Confirmation of Acceptance of the Manuscript no. 4

Od: "Journal of Photochemistry & Photobiology, B: Biology"

<em@editorialmanager.com>

Data: 31 sie 2022 13:10

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Do: Mariusz Grinholc <mariusz.grinholc@biotech.ug.edu.pl>

Manuscript Number: JPHOTOBIOL-D-22-00333R2

Priming effect with photoinactivation against extensively drug-resistant Enterobacter cloacae and Klebsiella pneumoniae

Dear Dr Grinholc,

Thank you for submitting your manuscript to Journal of Photochemistry & Photobiology, B: Biology.

I am pleased to inform you that your manuscript has been accepted for publication. Your accepted manuscript will now be transferred to our production department. We will create a proof which you will be asked to check, and you will also be asked to complete a number of online forms required for publication. If we need additional information from you during the production process, we will contact you directly.

We appreciate you submitting your manuscript to Journal of Photochemistry & Photobiology, B: Biology and hope you will consider us again for future submissions.

Kind regards,
Antonio Pinheiro
Editor

Journal of Photochemistry & Photobiology, B: Biology