ACCESS MICROBIOLOGY

RESEARCH ARTICLE

Hampden-Martin *et al.*, *Access Microbiology* 2021;3:000273

DOI 10.1099/acmi.0.000273

MICROBIOLOGY SOCIETY

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Photodynamic antimicrobial chemotherapy coupled with the use of the photosensitizers methylene blue and temoporfin as a potential novel treatment for *Staphylococcus aureus* in burn infections

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Abstract

Photodynamic antimicrobial chemotherapy (PACT) is a novel alternative antimicrobial therapy that elicits a broad mechanism of action and therefore has a low probability of generating resistance. Such properties make PACT ideally suited for utilization in localized applications such as burn wounds. The aim of this study was to determine the antimicrobial activity of MB and temoporfin against both a *S. aureus* isolate and a *P. aeruginosa* isolate in light (640 nm) and dark conditions at a range of time points (0–20 min). A *Staphylococcus aureus* isolate and a *Pseudomonas aeruginosa* isolate were treated *in vitro* with methylene blue (MB) and temoporfin under different conditions following exposure to light at 640 nm and in no-light (dark) conditions. Bacterial cell viability [colony-forming units (c.f.u.) ml^{-1}] was then calculated. Against *P. aeruginosa*, when MB was used as the photosensitizer, no phototoxic effect was observed in either light or dark conditions. After treatment with temoporfin, a reduction of less than one log (7.00×10^{7} c.f.u. ml^{-1}) was observed in the light after 20 min of exposure. However, temoporfin completely eradicated *S. aureus* in both light and dark conditions after 1 min (where a seven log reduction in c.f.u. ml^{-1} was observed). Methylene blue resulted in a loss of *S. aureus* viability, with a two log reduction in bacterial viability (c.f.u. ml^{-1}) reported in both light and dark conditions after 20 min exposure time. Temoporfin demonstrated greater antimicrobial efficacy than MB against both the *S. aureus* and *P. aeruginosa* isolates tested. At 12.5 µM temoporfin resulted in complete eradication of *S. aureus*. In light of this study, further research into the validity of PACT, coupled with the photosensitizers (such as temoporfin), should be conducted in order to potentially develop alternative antimicrobial treatment regimes for burn wounds.

INTRODUCTION

Widespread antibiotic misuse, coupled with an increasingly mobile global population, has facilitated an alarming increase in the rates of emerging antimicrobial-resistant (AMR) bacteria. The treatment of AMR bacteria results in both a decline in the physiological and psychological well-being of patients (including morbidity and mortality) and serious financial burdens to healthcare providers and their respective countries worldwide [1]. In Europe alone, multidrug-resistant (MDR) bacteria are estimated to be responsible for ~25000 deaths per year [2]. Furthermore, it is estimated that by 2050 mortality rates attributed to AMR bacterial infections will surpass 10 million people per annum, superseding cancer as the leading cause of global mortality [3, 4]. Commonly isolated AMR bacteria from patients include methicillin-resistant *Staphylococcus aureus* (MRSA) [5], vancomycin-resistant *Enterococcus* spp. (VRE) [6], carbapenem-resistant *Enterobacteriaceae* spp. [7] and MDR *Pseudomonas* spp. [8].

Received 13 May 2021; Accepted 11 August 2021; Published 27 October 2021

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Keywords: bacteria; burns; infection; methylene blue; photodynamic antimicrobial chemotherapy; temoporfin.

Abbreviations: AMR, antimicrobial resistant; ANOVA, two-way analysis of variance; c.f.u. ml^{-1}, colony forming units per milliliter; EPI–MB, efflux pump inhibitor–methylene blue; LB, Luria–Bertani broth; LED, light-emitting diode; L+P–, with light but with no photosensitizer; L–P–, without the light and a photosensitizer; L–P+, with no light but with a photosensitizer; MB, methylene blue; MDR, multidrug resistant; PACT, photodynamic antimicrobial chemotherapy; PDT, photodynamic therapy; TB, toluidine blue; VRE, vancomycin-resistant *Enterococcus* spp.

The main therapeutic strategies that are currently used to control AMR include antimicrobial stewardship, improved infection control and the development of new antimicrobials (including novel antibiotics) [9]. However, since the 'golden era' of antibiotic discovery (~1950–1970) [10], the development and approval of novel antibiotic classes has decreased significantly. This is mainly due to the high cost (>USD $1 billion for new molecular entities) involved in antibiotic development, the low success rate and a lengthy process time (10–15 years) [11, 12]. In addition, the limited mechanism of action of most antibiotics has indicated that resistance is likely to develop and therefore novel antibiotics potentially have a limited shelf life [9].

Burns patients are at high risk of nosocomial infection due to compromised innate host defences (in this instance damage to the epidermidis) [13]. Bacterial colonization of burns can result in invasive infection, septicaemia, multi-organ failure and ultimately death [14]. *Pseudomonas aeruginosa* is the most commonly isolated bacteria from burn wounds, followed by *S. aureus* [15].

The antimicrobial effect of photodynamic antimicrobial chemotherapy (PACT) relies on three components: the presence of oxygen (O_2), a photosensitizer and a wavelength of light that coincides with the peak absorption of the photosensitizers [16]. Methylene blue (MB) is a well-established photosensitizer that has been extensively documented throughout the past decade [17, 18]. Due to the antimicrobial efficacy of MB against a broad range of micro-organisms it is often utilized as a potent photodynamic therapy (PDT) drug for the local treatment of periodontal diseases [19, 20]. The efficiency of MB-mediated PACT has also been confirmed on antibiotic-resistant polymicrobial biofilms of *P. aeruginosa* and MRSA in a maxillary sinus model [21]. In addition, several *in vitro* studies have assessed its antimicrobial efficacy against a range of bacteria commonly isolated from burn infections [22, 23].

Temoporfin is a second-generation photosensitizer that has been utilized successfully in PDT to treat squamous cell carcinoma of the head and neck and has been investigated for use as a treatment for other cancers, such as biliary tract carcinomas [24, 25]. Temoporfin has been shown to achieve the same PDT response at lower concentrations and with lower light doses than its first-generation predecessors [26, 27]. In addition, temoporfin has a better safety profile than other photosensitizers, as it does not cause damage to underlying anatomical structures [26, 27]. Therefore, temoporfin has potential as a promising photosensitizer, although its antibacterial efficacy has not yet been thoroughly characterized in the context of burn infections.

Novel therapies to treat burn infections are urgently needed; particularly therapies that will not facilitate the development of antimicrobial resistance. One potential avenue to be explored is PACT. The current study aimed to assess the antimicrobial efficacy of methylene blue- and temoporfin-mediated PACT against both Gram-positive and Gram-negative bacterial species (namely *S. aureus* and *P. aeruginosa*) that are commonly isolated from burn infections

METHODS

Bacterial cultures

S. aureus (NCTC 6571) and *P. aeruginosa* (B9T2436) were utilized throughout this study. Both species of bacteria were cultured aerobically in Luria–Bertani broth (LB) (Fisher Scientific, USA) in a shaking incubator at 180 r.p.m. for 24 h at 37 °C. Following incubation, the bacterial cultures were normalized in LB broth to achieve an optical density ($OD_{600\ nm}$) of 0.05 (±0.01), equating to approximately 1.0×10^6 colony-forming units (c.f.u.) ml^{-1}.

Photosensitizers and light source

Methylene blue (Sigma Aldrich, UK) was dissolved in sterile water to produce a 1% stock solution (w/v) (10 mg ml^{-1}). Temoporfin (Sigma Aldrich, UK) was dissolved in absolute ethanol (≥99.8%; Sigma Aldrich, UK) at a concentration of 1 mM and stored at −20 °C prior to use. Both photosensitizers were stored in a dark environment to minimize light exposure prior to experimentation. For the MB PACT experiments, the concentration of MB used was 1 mg ml^{-1} (3.13 mM) and the concentration of temoporfin was 50 µM for *P. aeruginosa* and 12.5 µM for *S. aureus*. A portable light-emitting diode (LED) PDT light source that had a red wavelength (λ) (640 nm) was utilized throughout this study. Previous studies have determined that the maximum absorption for methylene blue and temoporfin is 668 and 650 nm, respectively [27, 28].

PACT assays

Photodynamic antimicrobial chemotherapy experiments were conducted in clear, flat-bottom, 96-well microtitration plates (Fisher Scientific, UK). *S. aureus* and *P. aeruginosa* were exposed to four different parameters in the presence of both MB and temoporfin, and red light. A maximal light exposure time of 20 min was used, due to the assumption that patients would tolerate longer treatment times poorly. All PACT experiments were conducted in triplicate alongside a LB broth (negative control) (n=3). The bacteria were tested in the presence of the light and a photosensitizer (L+P+) – methylene blue (1 mg ml^{-1}) or temoporfin (50 µM used for *P. aeruginosa* and 12.5 µM for *S. aureus*). The bacterial suspensions (~1.0×10^6 c.f.u. ml^{-1}) were incubated in the dark for 20 min by covering the sterile microtitre plates with aluminium foil. Samples were illuminated using red light (λ=640 nm) for up to 20 min. Serial dilutions were performed at intervals of 1, 10 and 20 min of light exposure and plated onto LB agar plates (Fisher Scientific, USA). The inoculated agar was incubated overnight at 37 °C in the dark. After incubation, the bacterial colonies were enumerated and the c.f.u. ml^{-1} determined. The antimicrobial efficacy testing for the light and the photosensitizer was also carried out without the light and a photosensitizer (L−P−) as a negative control, with no light but with a photosensitizer (L−P+) or with light but with no photosensitizer (L+P−).

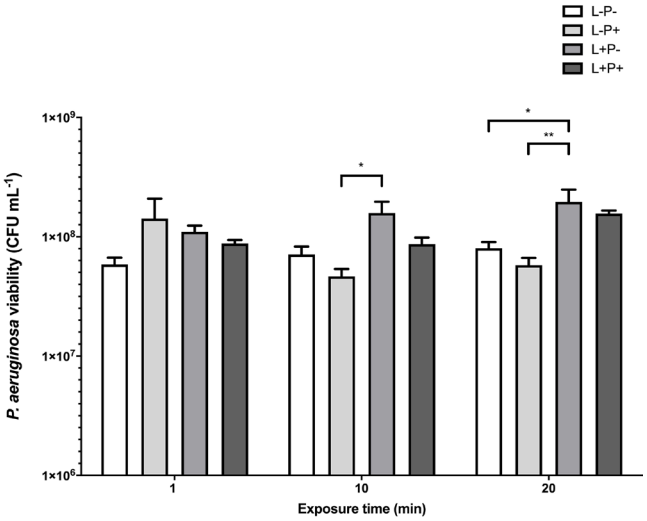

Fig. 1. Effect of MB (1 mg ml^{-1}) on *P. aeruginosa* (B9T2436) after 1, 10 and 20 min of red light exposure (λ=640 nm; *n*=3). Group L+P+, incubated with MB for 20 min, and then irradiated with red light. Group L+P−, no incubation with MB but exposed to red light. Group L−P−, no incubation with MB or exposure to red light. Group L−P+, incubated with MB, but not exposed to red light. Bars represent median value +range of three biological replicates. Two-way ANOVA tests were performed between experimental groups at different time points. Asterisks denote significance (**P*≤0.05, ***P*≤0.01).

Statistical analysis

Statistical analysis was conducted by performing two-way analysis of variance (ANOVA) coupled with Tukey's multiple comparison tests for post hoc analysis using GraphPad Prism (version 8.4.2; GraphPad Software, USA) to determine significant differences at a confidence level of 95% (*P*<0.05). Error bars represent the standard error of the mean. Asterisks denote significance, **P*≤0.05, ***P*≤0.01, ****P*≤0.001 and *****P*≤0.0001.

RESULTS

The effect of MB- and temoporfin-mediated PDT on *P. aeruginosa*

Initially, the effect of PACT using MB on *P. aeruginosa* was determined. It was demonstrated that the number of viable cells increased with increased light exposure in the untreated experimental group (L−P−) with a mean of 5.44×10^7 c.f.u. ml^{-1} at 1 min and 8.00×10^7 c.f.u. ml^{-1} by 20 min (Fig. 1). There was also a similar pattern observed with the L+P+ and L+P− groups. The L−P + group, representing the dark control and hence the antimicrobial activity of MB alone, was the only group to show a decrease in the number of viable cells with increasing time. However, no statistical difference was found between the negative control (L−P−) and (L−P+) at 20 min (*P*=0.9434) (Fig. 1).

The effect of temoporfin-mediated PACT on *P. aeruginosa* was determined. In contrast to the MB-mediated PACT experiment with *P. aeruginosa*, the L+P+ group demonstrated a decrease in cell viability from 1.49×10^8 c.f.u. ml^{-1} at 1 min to 7.00×10^7 c.f.u. ml^{-1} by 20 min. The number of bacterial colonies present at 20 min was significantly lower than for all other experimental groups. The bacterial viability (c.f.u. ml^{-1}) in the L−P− group was 2.89×10^8 c.f.u. ml^{-1} at 20 min, and the antimicrobial effect of temoporfin with 20 min of red light exposure resulted in 7.00×10^7 c.f.u. ml^{-1} (Fig. 2).

The effect of MB- and temoporfin-mediated PDT on *S. aureus*

The MB-mediated PACT experiments demonstrated that the Gram-positive bacterium, *S. aureus*, was more susceptible to MB than the Gram-negative bacterium, *P. aeruginosa*. Cell viability was determined at 2.83×10^7 c.f.u. ml^{-1} and 2.05×10^6 c.f.u. ml^{-1} between 1 and 20 min in the L−P + and L+P+ groups, respectively (Fig. 3). The viable bacterial counts were higher (with statistical significance) in the experimental controls compared to the L+P+ and L−P + groups at 1, 10 and 20 min, indicating that MB demonstrated antimicrobial efficacy under both light and dark conditions against *S. aureus*. The toxicity of MB alone when no light was applied had a greater effect on *S. aureus* than when illuminated, with the c.f.u. ml^{-1} being consistently lower at 1, 10 and 20 min in the L−P + group when compared to the L+P+ group. Relative to the control (L−P−) at 20 min (1.79×10^7 c.f.u. ml^{-1}), when MB was used without exposure to light (L−P+), a reduction in viable *S. aureus* (1.50×10^5 c.f.u. ml^{-1}) was achieved, whilst

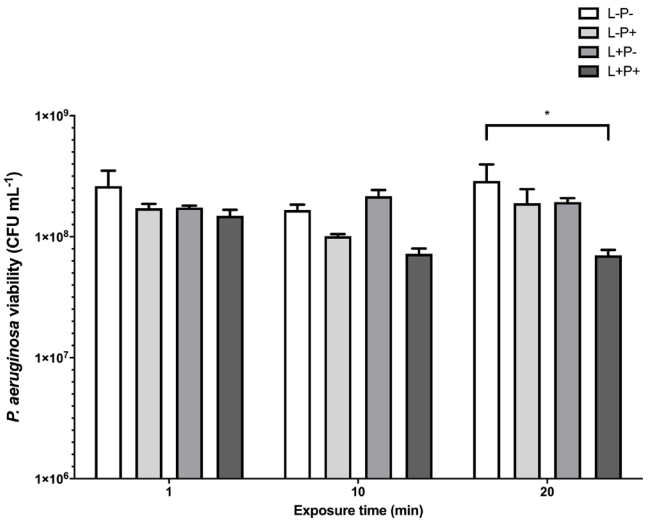

Fig. 2. Effect of temoporfin (50 µM) on *P. aeruginosa* (B9T2436) after 1, 10 and 20 min of red light exposure (λ=640 nm; *n*=3). Group L+P+, incubated with temoporfin for 20 min, and then exposed to red light. Group L+P−, not incubated with temoporfin but exposed to red light. Group L−P−, not incubated with temoporfin or exposed to red light. Group L−P+), incubated with temoporfin but not exposed to red light. Bars represent the mean of three biological replicates whilst error bars denote standard error of mean (SEM). Two-way ANOVA tests were performed between experimental groups at different time points. Asterisks denote significance (**P*≤0.05).

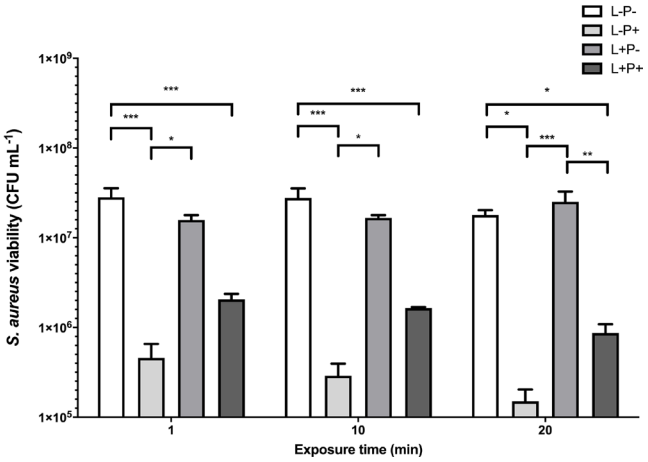

Fig. 3. Effect of MB (1 mg ml^{-1}) on *S. aureus* c.f.u. ml^{-1} after 1, 10 and 20 min of red light exposure (λ=640 nm; *n*=3). Group L+P+, incubated with MB for 20 min, and then exposed to red light. Group L+P−, no incubation with MB but exposed to red light. Group L−P−, no exposure to MB and no exposure to red light. Group L−P+, incubation with MB but no exposure to red light. Bars represent the mean of three biological replicates whilst error bars denote standard error of mean (SEM). Two-way ANOVA tests were performed between experimental groups at different time points. Asterisks denote significance (**P*≤0.05, ***P*≤0.01 and ****P*≤0.001).

the phototoxicity group, L+P+, reported 8.67×10^5 c.f.u. ml^{-1} of viable *S. aureus* (Fig. 3).

Temoporfin also demonstrated greater antimicrobial efficacy against the Gram-positive bacterium, *S. aureus* (Fig. 4). The killing effect of temoporfin at 12.5 µM was substantially greater than that of MB (which was tested at a higher concentration of 3.13 mM), with a complete eradication of *S. aureus* observed in both the L−P + and L+P+ groups after 1 min (Fig. 4). The L−P + and L+P+ groups both showed statistically significant differences from the L−P− and L+P− groups at 1, 10 and 20 min. This indicated that temoporfin had an antimicrobial effect against *S. aureus* in the dark at 12.5 µM, with complete eradication observed after 1 min of incubation (Fig. 4).

DISCUSSION

This study aimed to determine the efficacy of light-activated photosensitizers against bacterial species commonly found in burn wound infections. The results from this *in vitro* study demonstrated that *S. aureus* (a Gram-positive bacterium) was more susceptible to killing by the photosensitizers in the absence of light than *P. aeruginosa* (a Gram-negative bacterium). Temoporfin demonstrated a photodynamic effect against *P. aeruginosa* and did not demonstrate an antimicrobial effect in the absence of light against *P. aeruginosa*. Incubation of *S. aureus* with temoporfin at 12.5 µM (but no light exposure) demonstrated antimicrobial activity, with complete bacterial eradication after 1 min. Temoporfin at 12.5 µM combined with red light exposure also resulted in the complete loss of *S. aureus* viability after 1 min, and therefore exclusive phototoxicity activity could not conclusively be determined. The toxicity of MB when tested against *S. aureus* in the dark was greater than its antimicrobial activity following exposure to light. MB did not demonstrate an antimicrobial effect in the absence of light against *P. aeruginosa*

The greater sensitivity of Gram-positive bacteria to photosensitizers has been reported by other *in vitro* studies. In 2001, Usacheva *et al.* detailed the photobactericidal efficacy of the photosensitizers, MB and toluidine blue (TB), which was assessed against a range of Gram-positive and Gram-negative bacteria [20]. It was reported that the concentrations of both temoporfin and MB required to achieve complete eradication of Gram-negative bacteria with light were in general 3- to 30-fold higher than those required to kill the Gram-positive bacteria tested. Another *in vitro* study conducted by Yang *et al.* (2012) reported complete eradication of MRSA with temoporfin after a 90 min incubation period followed by continuous exposure to 100 J cm^{-2} of light (λ=652 nm) [29]. The discrepancy in sensitivity is believed to be due to differences in cell wall structure, with Gram-negative bacteria having an additional negatively charged outer membrane that impedes the diffusion of non-cationic photosensitizers [30]. However, this does not fully explain the decreased efficacy of MB, as it is a positively charged photosensitizer. An alternative explanation was provided in a study

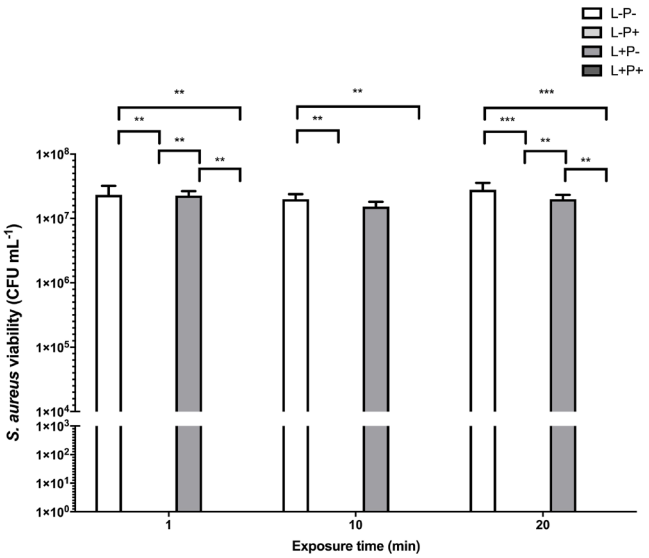

Fig. 4. Effect of temoporfin (12.5 µM) on *S. aureus* c.f.u. ml^{-1} after 1, 10 and 20 min of red light exposure (λ=640 nm; *n*=3). Group L+P+, incubated with temoporfin for 20 min, and then exposed to red light. Group L+P−, no incubation with temoporfin but exposed to red light. Group L−P−, no incubation with temoporfin or exposure to red light. Group L−P+, incubated with temoporfin but not exposed to red light. Bars represent the mean of three biological replicates whilst error bars denote standard error of mean (SEM). Two-way ANOVA tests were performed between experimental groups at different time points. Asterisks denote significance (**P*≤0.05, ***P*≤0.01 and ****P*≤0.001).

by Rineh *et al.* (2018), which reported a potential efflux mechanism against MB [31]. In this study it was shown that a NorA efflux pump inhibitor–methylene blue (EPI–MB) hybrid compound displayed a greater PDT against the Gram-negative bacteria *Escherichia coli* and *Acinetobacter baumannii* than MB alone. The antimicrobial activity against Gram-negative bacteria may therefore be enhanced by mitigating the effect of efflux pumps, through the use of shorter incubation times with photosensitizers, or by repeated doses of photosensitizers.

Another potential explanation for the poor photodynamic efficacy of MB against *P. aeruginosa* is a phenomenon called the self-shielding effect [30]. This arises when high concentrations of the photosensitizer are present in solution and absorb a significant proportion of the light, thereby reducing the light exposure to photosensitizer-loaded cells [30]. For many photosensitizers this self-shielding effect is observed when the concentration reaches ≥300 µM [30]. The concentration of MB used throughout this study was 3.13 mM and was greater than that of the temoporfin, and was selected since studies in this area use a range of MB concentrations from ≤25 µg ml^{-1} to 10 mg ml^{-1} and hence the MB concentration selected for use in this study was taken for use at a conservative mid-range [30, 32–34]. The use of this higher concentration may explain the potential shielding effect demonstrated.

The current study demonstrated that a temoporfin concentration of 50 µM enabled a photodynamic effect to be observed against *P. aeruginosa*. *P. aeruginosa* cell viability at this concentration reduced from 2.89×10^{8} to 7.00×10^{7} c.f.u. ml^{-1}. In a previous study by Yang *et al.* (2012), a similar phenomenon was observed; no overall significant reduction in *P. aeruginosa* viability was observed when temoporfin was utilized at 12.5 µM, and the authors stated that this was likely due to the neutral charge of temoporfin, which meant that penetration of the outer membrane was less probable [29]. The threshold required by the American Society of Microbiology for a treatment to be termed antimicrobial is when it can achieve at least a three log reduction in c.f.u. ml^{-1} (killing efficiency of 99.9%) [35]. It would therefore appear to be an ineffective antimicrobial treatment against antibiotic-resistant Gram-negative bacteria when used at this concentration.

Future research may involve the use of temoporfin as a photosensitizer against resistant strains of bacteria, in particular MRSA, as this species commonly colonizes burn wounds. This research has shown that temoporfin is effective in the eradication of a Gram-positive *S. aureus* species, meaning that it may result in the killing of other Gram-positive species causing burn infections, such as *Enterococcus* spp. This has been shown by Kranz *et al.* (2011), who described a six log reduction in *Enterococcus faecalis* c.f.u. ml^{-1} after treatment with 30 µM of a liposomal formulation of temoporfin, subjected to a light dose of 100 J cm^{-2}, at a wavelength of 652 nm [36].

CONCLUSIONS

Temoporfin demonstrated greater antimicrobial efficacy than MB against a *S. aureus* isolate and a *P. aeruginosa* isolate tested *in vitro*. At 12.5 µM, temoporfin resulted in complete eradication of *S. aureus*. Although the use of light and temoporfin decreased the numbers of *P. aeruginosa*, viable cells were still present following treatment. The results of this study demonstrate that the antimicrobial activity of temoporfin as a photosensitizer could be more suited to Gram-positive bacterial infections. In light of this study, further research is warranted for the development of an alternative treatment option for burn wound infections.

Funding information
This research received funding from the University of Liverpool.

Author contributions
A.H-M. carried out the laboratory work and produced the initial draft of the work. J.F. and K.S. devised the concept and supervised the project. J.F., M.El.M., L.C., A.S. and K.W. were involved in analysis of the data. All the authors were involved in the preparation and proofreading of the manuscript. All authors approved the final version of this manuscript.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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