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Photodynamic inactivation of methicillin-resistant *Staphylococcus aureus* on skin using a porphyrinic formulation †

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Abstract: The antimicrobial photodynamic therapy (aPDT) efficacy of a porphyrinic formulation (FORM) to photoinactivate methicillin-resistant *Staphylococcus aureus* (MRSA) on skin was evaluated alone and in the presence of potassium iodide (KI) or iodopovidone (PVP-I) as possible potentiator agents. FORM was effective to inactivate MRSA *in vitro* causing a substantial reduction in the irradiation time when combined with KI or PVP-I. In the *ex vivo* assays on skin, the best achievements were obtained in the presence of FORM alone with reductions of 3.1 Log_{10} CFU mL^{-1}. So, aPDT using FORM is a promising approach for MRSA inactivation on skin.

Keywords: antimicrobial photodynamic therapy; porphyrinic formulation; potentiator agents; potassium iodide; iodopovidone; methicillin-resistant *Staphylococcus aureus*

1. Introduction

Staphylococcus aureus is responsible for serious skin and soft-tissue clinical infections that can progress to invasive and life-threatening pathologies [1]. Although these infections are usually treated with antibiotics [2], this bacterium easily acquires antibiotic resistance [3]. Community-associated and hospital-acquired infections with *S. aureus* have increased, also rising the antibiotic-resistant strains, particularly methicillin-resistant *S. aureus* (MRSA). MRSA infections impose a significant burden on healthcare with higher mortality, morbidity and financial costs [3].

Antimicrobial photodynamic therapy (aPDT) is a promising approach for localized infections in response to antibiotic resistance [4]. This therapeutic approach requires the activation of a photosensitizer (PS) by visible light in the presence of dioxygen to produce reactive oxygen species (ROS), namely singlet oxygen ($^1O_2$) [5]. The reaction of these cytotoxic species with different biomolecules leads to a rapid and irreversible microbial inactivation [6]. Despite the aPDT advantages [7], it is still possible to improve this technology to be transposed to the clinical area. An easy preparation of a PS associated to a low price are important features to consider in its design [8].

A formulation (FORM) based on a non-separated mixture of cationic porphyrins showed already its effectiveness as PS towards several microorganisms, such as Gram-positive, including *S. aureus*, and Gram-negative bacteria, fungi and viruses [9,10]. FORM lacks the laborious and time-consuming purification after the synthetic procedure and its photodynamic efficiency is similar to that of the most effective PSs also present in FORM (Tri-Py(+)-Me and Tetra-Py(+)-Me) [9].

Another strategy to improve the aPDT efficiency relies on the use of inorganic salts, such as potassium iodide (KI), as potentiator agents. KI reacts with $^1O_2$, giving free iodine, hydrogen peroxide and iodine radicals that are considered to have a potent microbicidal action [11]. Other preparations,

such as iodopovidone (PVP-I), indicated for wounds and skin disinfection before surgical interventions, can also provide microbicidal iodine [12].

Considering the importance of increasing the aPDT efficacy in clinical area, with safe and cost-effective protocols, in this study, the aPDT efficiency of FORM alone and combined with KI or PVP-I to treat human skin infections by MRSA, was assessed [13]. Porcine skin was selected as a model for human skin [14].

2. Materials and Methods

2.1. PS, KI and PVP-I solutions

The formulation (FORM) based on a non-separated mixture of 5 cationic *meso*-tetraarylporphyrins was synthetized according to the described procedure [15]. A stock solution (500 μM) was kept in the dark and prior to each assay was sonicated (30 min) at room temperature. A stock solution of KI at 5.0 M was prepared immediately before each experiment, in a buffered solution of PBS. A 100 mg/mL PVP-I cutaneous solution of Betadine® (Meda) with 10 mg of active iodine was used and prior to each assay the PVP-I solution was hand agitated and vortexed (~5 s). An aliquot of each solution (FORM, KI or PVP-I) was transferred to the corresponding beakers, to reach the required concentration for each test.

2.2. Bacterial strain and culture conditions

MRSA DSM 25693 was grown on solid medium BD Baird-Parker Agar (BPA) at 37 °C during 48 h and posteriorly kept at 4 °C. Before each assay, one isolated colony was inoculated in 30 mL of Tryptic Soy Broth (TSB) and grown aerobically at 37 °C overnight, for 18−24 h, under stirring (120 rpm). An aliquot of this culture (300 μL) was transferred into a new fresh TSB liquid medium, grew under the same growth conditions till stationary growth phase was achieved and was then used for the assay.

2.3. Light source and irradiation conditions

The aPDT effect was evaluated using white light (400−750 nm) provided by a light-emitting diode (LED) system (ELMARK – VEGA20, 20 W, 1400 lm). *In vitro* assays were performed at an irradiance of 25 mW cm^{-2} during 60 min. *Ex vivo* experiments required higher irradiance (60 mW cm^{-2}) during 180 min (PVP-I) or 270 min (KI).

2.4. aPDT with FORM, FORM + KI or FORM + PVP-I in PBS (in vitro studies)

Diluted bacterial culture [~10^8 colony-forming unit per milliliter (CFU mL^{-1})] was equally distributed in beakers and incubated in the dark (10 min) at room temperature under stirring (100 rpm) to promote FORM binding to MRSA cells. Different FORM concentrations (5.0, 1.0 or 0.5 μM) were tested but only FORM at 0.5 μM was tested with KI at 100 mM (FORM + KI) or PVP-I at 1% (FORM + PVP-I). In all experiments using FORM and FORM + KI, the following controls were included: light control (LC - bacterial suspension exposed to light to assess the light effect on microbial viability); dark control [DC- bacterial suspension treated with FORM (5.0 μM) protected from light to assess FORM cytotoxicity in the dark] and KI light control [LC + KI - bacterial suspension containing KI (100 mM) exposed to light to evaluate the effect of KI alone]. In the PVP-I tests, LC and the following controls were included: PVP-I light control (LC + PVP-I - bacterial suspension containing PVP-I 1% exposed to light to evaluate the effect of PVP-I alone) and PVP-I dark control (DC – PVP-I - bacterial suspension treated with PVP-I 1% protected from light to assess PVP-I cytotoxicity in the dark).

After dark incubation, samples and light controls were irradiated (25 mW cm^{-2}) under stirring for 60 min. Dark controls were kept under stirring but were light protected during irradiation. Aliquots were collected at different times of exposure and serially diluted in PBS. Then, two drops

(drop plate method) per dilution were plated on Tryptic Soy Agar (TSA) and incubated at 37 °C for 18–24 h. Colonies were counted on the most proper dilution and the concentration stated as Log_{10} CFU mL^{-1}. Data was graphed from three independent experiments performed with two replicates of each condition.

2.5. aPDT with FORM, FORM + KI or FORM + PVP-I in porcine skin and viability recovery evaluation (ex vivo studies)

FORM (50 μM) was tested alone and combined with KI at 100 mM (FORM + KI) or PVP-I at 1% (FORM + PVP-I). The same *in vitro* controls were also included. The disinfection of porcine skin was performed according to the procedure already reported in a previous study of our research group [16]. Thereafter, an aliquot (500 μL) of an overnight culture of MRSA diluted in PBS (1:10) was distributed over the skin pieces using an aerosol spray (~10^7 CFU mL^{-1}). After 30 min of incubation, 200 μL of FORM (50 μM) was sprayed on the skin pieces of each condition (FORM, FORM + KI, FORM + PVP-I and DC). FORM + KI and FORM + PVP-I samples were also sprayed with KI at 100 mM and PVP-I at 1% (200 μL), respectively (final volume of 900 μL). The same volume was sprayed on LC + KI and LC + PVP-I controls. For LC, LC + KI, LC + PVP-I, DC, and FORM pieces, PBS was used to complete the volume of 900 μL. Additionally, a bacterial control (not sprayed with MRSA) was, after the disinfection process, only sprayed with 900 μL of PBS and used as control to verify the efficiency of the skin disinfection.

To maintain a moist surface, the Petri plates (60 mm) with the skin samples were placed inside Petri plates (90 mm) with sterile PBS. All pieces were incubated (30 min) in the dark to promote the PS binding to MRSA cells. The samples and light controls were irradiated (60 mW cm^{-2}) for 270 min in the case of FORM and FORM + KI assays and for 180 min in the case of FORM and FORM + PVP-I assays. A sterile cotton wool swab, moistened in PBS, was used to remove the bacteria from each skin portion at different times of exposure. The bacteria present in the cotton wool swab were suspended and serially diluted in PBS. Then, the same procedure used for the *in vitro* experiments was performed.

To evaluate if the bacterium recovered its viability after aPDT treatment, all plates used in three tests with FORM and FORM + KI, were left in the incubator at 37 °C for one more week. The number of CFU was then evaluated daily to check if it remained the same.

2.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.04. The significance of bacterial concentration between and along treatments was assessed by two-way ANOVA analysis of variance. Tukey´s multiple comparison test was used for a pairwise comparison of the means. The significance of differences was evaluated comparing the results obtained in the test samples with each other and with the results obtained for the correspondent control samples, for the different times. A value of $p<0.05$ was considered significant. Three independent experiments were conducted in duplicate for each assay.

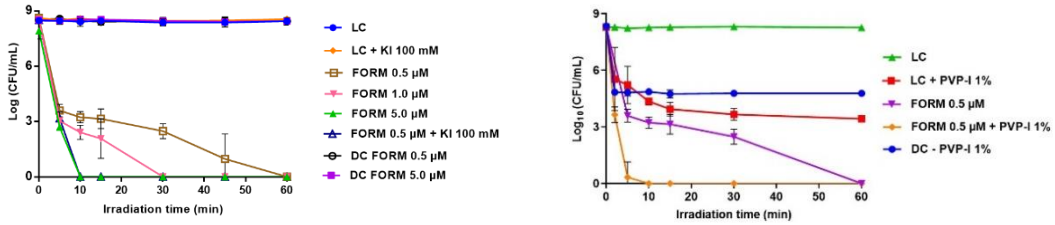
3. Results

3.1. MRSA photoinactivation with FORM, FORM + KI or FORM + PVP-I (in vitro assays)

The *in vitro* results of MRSA inactivation using FORM at 5.0, 1.0 and 0.5 μM and FORM with KI or PVP-I at 1% are presented in Figure 1. MRSA cell viability was not affected by light alone (LC), light and KI (LC + KI) or by FORM at 0.5 and 5.0 μM in the dark (DC), proving that the bacterial reduction was only due to aPDT (ANOVA, $p>0.05$). However, a significant reduction (ANOVA, $p<0.05$) in MRSA concentration was observed with PVP-I at 1% either in the presence (LC + PVP-I) or absence (DC – PVP-I) of light; no considerable differences between both PVP-I controls (ANOVA, $p>0.05$) were noted.

When MRSA was treated with FORM at 5.0 μM, an abrupt photoinactivation of 8.0 Log_{10} CFU mL^{-1} was noticed (ANOVA, $p<0.05$), reaching the detection limit of the method after 10 min of

irradiation (Figure 1a). At 1.0 μM, FORM was able to cause a reduction of 5.0 Log_{10} CFU mL^{-1} (ANOVA, $p<0.05$) in the first 5 min, however the photoinactivation until the detection limit of the method was only observed after 30 min of irradiation (Figure 1a). For FORM at 0.5 μM, it was also observed a reduction of 4.9 Log_{10} CFU mL^{-1} (ANOVA, $p<0.05$) in the first 5 min of irradiation, but the detection limit of the method was only reached after 60 min of irradiation (Figure 1a). Regarding the combination of FORM at 0.5 μM + KI at 100 mM, a more effective MRSA inactivation was obtained, reaching the detection limit after 10 min (ANOVA, $p<0.05$) (Figure 1a). A similar result was found when FORM at 0.5 μM + PVP-I at 1% were used (ANOVA, $p>0.05$; Figure 1b).



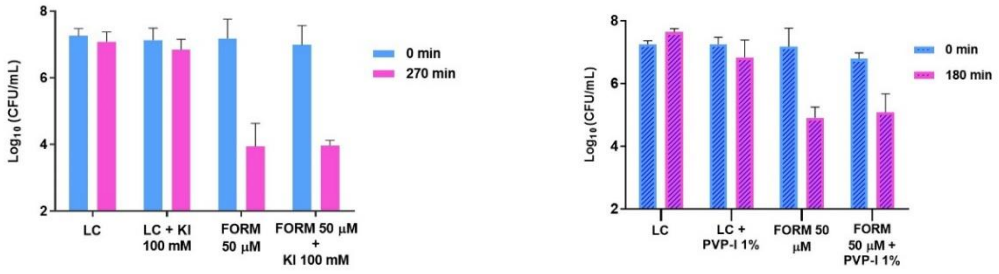
(**a**) (**b**)

Figure 1. MRSA photoinactivation with FORM at 0.5, 1.0 and 5.0 μM and FORM at 0.5 μM + KI 100 mM (**a**) and FORM at 0.5 μM and FORM at 0.5 μM + PVP-I at 1% (**b**) at 25 mW cm^{-2} in PBS. Values are shown as the mean of three independent tests with two replicates each; standard deviation is represented by error bars.

3.2. MRSA photoinactivation with FORM, FORM + KI or FORM + PVP-I and viability recovery evaluation (ex vivo assays)

The *ex vivo* assays on skin (Figure 2a) show that the MRSA cell viability was not affected by light alone (LC), light and KI (LC + KI) or FORM at 50 μM in the dark (DC) (data not shown) being the bacterial reduction only due to aPDT effect (ANOVA, $p>0.05$). Although, skin portions treated with PVP-I and exposed to light (LC + PVP-I), showed a reduction in the MRSA cell viability but this reduction was lower than that observed in PBS (ANOVA, $p<0.05$; Figure 2b).

When FORM at 50 μM was used alone or combined with KI at 100 mM (Figure 2a), a reduction of 3.1 Log_{10} CFU mL^{-1} (ANOVA, $p<0.05$) was observed after 270 min of irradiation at 60 mW cm^{-2}. A similar result was observed with FORM at 50 μM + PVP-I at 1% combination, which caused a MRSA reduction of 2.6 Log_{10} CFU mL^{-1} (ANOVA, $p<0.05$) after 180 min and was also identical to the MRSA reduction obtained when FORM was used alone after the same irradiation period (Figure 2b). These results show that neither KI nor PVP-I improved the FORM efficiency. After aPDT of MRSA in porcine skin, no regrowth was observed even after one week of incubation.



(**a**) (**b**)

Figure 2. MRSA photoinactivation at an irradiance of 60 mW cm^{-2} on porcine skin with FORM at 50 μM and FORM at 50 μM + KI at 100 mM after 270 min (**a**) and FORM at 50 μM and FORM at 50 μM

+ PVP-I at 1% after 180 min (**b**). Values are presented as the mean of three independent assays with two replicates each; standard deviation is represented by error bars.

4. Discussion

Herein, the porcine skin model was used to evaluate the aPDT efficacy of FORM alone and combined with the potentiator agents (KI or PVP-I) against MRSA [13].

In vitro, FORM alone and combined with KI or PVP-I (Figure 1) was effective in MRSA inactivation, confirming the potential of KI and PVP-I as potentiator agents. Similar results were obtained with FORM in prior studies [9,10] and for Tetra-Py(+)-Me [16]. The combination of FORM at 0.5 μM with KI at 100 mM (the most effective concentration for aPDT potentiation, without toxic effects [17]), clearly potentiated the aPDT outcome with a significant reduction in the irradiation time (6x) in comparison to FORM alone at the same concentration (Figure 1a). The inactivation achieved with FORM + KI was similar to that obtained with FORM alone at a concentration tenfold higher (5 μM) (Figure 1a). Similar results were already obtained for the same PS and bacterium [10] and for other PSs and bacterial strains of *S. aureus* [17].

Contrarily to KI alone, PVP-I at 1%, either with or without light, inactivated MRSA. These results are in line with the literature, where it was shown that PVP-I inactivated bacteria causing reduced side effects to host cells [12]. When PVP-I was combined with FORM the potentiator effect was also observed (Figure 1b).

Due to skin matrix complexity, which can reduce the aPDT efficiency [16] the *ex vivo* assays were performed using higher PS concentration (50 μM) and light irradiance (60 mW cm^{-2}). The bacterial reduction with FORM after 270 min of irradiation was 3.1 Log_{10} CFU mL^{-1} (Figure 2a), which is in line with the American Society of Microbiology criterium to consider any new approach as antimicrobial [18]. Similar results for *S. aureus* using other PSs were already noticed both *ex vivo* [16] and *in vivo* [19]. Considering the *in vitro* results and to enhance MRSA inactivation on skin, KI was tested with FORM, but no increase was perceived (Figure 2a). A higher concentration (1.0 M) of KI was also tested but no increase in aPDT efficacy was detected (data not shown). As for the FORM + KI combination, no increase in MRSA inactivation with FORM + PVP-I was observed (Figure 2b).

Given that after irradiation very short-lived bactericidal ROS are no longer produced, nothing remains to suppress bacterial regrowth [20]. KI and PVP-I can inhibit recurrence through short-lived reactive iodine species and long-term stable bactericidal species that may remain for a much longer time and inhibit regrowth. Here, no regrowth of bacteria was observed up to one week after aPDT, even when FORM was used alone.

One of the major challenges of aPDT is the lack of highly effective antimicrobial PSs with clinical approval [21]. In this study we proved that the efficient *in vitro* FORM was still efficient in the photoinactivation of *S. aureus*, causing a decrease of 3.1 Log_{10} CFU mL^{-1} on the bacterium survival on skin (*ex vivo*). This fact together with the reduction of time and production costs associated to the PS, implies that the use of FORM to treat localized skin infections, including the ones caused by MRSA strains, can be regarded as a promising alternative to antibiotics, even without potentiator agents. The FORM incorporation in an ointment or other kind of delivery devices can help to transpose this technology to clinical practice, as already happens in dermatology and dentistry [4].

Conflicts of Interest: The authors declare no conflict of interest.

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