SUPPLEMENTARY MATERIAL

Violacein Antimicrobial Activity on Staphylococcus epidermidis Biofilm

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The aim of this study was to evaluate the antimicrobial potential of violacein (VIO) on *Staphylococcus epidermidis* biofilm. The minimum biofilm inhibition concentration (MBIC) and minimum biofilm eradication concentration (MBEC) were determined, as well as the effect of VIO exposure time on microbial viability in mature biofilm. Violacein showed good antibiofilm action, inhibiting biofilm formation and eradicating mature biofilm of *S. epidermidis* at concentrations of 20 μg.mL⁻¹ and 160 μg.mL⁻¹, respectively. At concentrations equal to MBEC and 2x MBEC, the biofilm was eradicated in 3h and 2h30min of incubation, respectively. When evaluating VIO modulating effect on the action of clinically-used drugs (vancomycin, cefepime, ciprofloxacin and meropenem), especial synergism was observed in the violacein-ciprofloxacin association, it can completely eradicated the mature biofilm at the concentration of 1/2xMBEC and 1/4xMBEC, respectively. VIO shows good antimicrobial action on *S. epidermidis* biofilm and has the potential to synergistically modulate the activity of clinically-used antimicrobials.

**Keywords:** violacein; *Staphylococcus epidermidis* biofilm; antibiofilm action; antibiotic synergism; antimicrobial activity.
Experimental section

Violacein (VIO)

The VIO used in the study was extracted from *Chromobacterium violaceum* ATCC 12472. Purification and characterization was adapted from Rettori and Duran (1998) as previously published by Dodou et al. (2017).

Bacterial strains

The tested strain was *Staphylococcus epidermidis* (ATCC 35984), obtained from the Collection of Reference Microorganisms in Sanitary Surveillance (*Coleção de Microrganismos de Referência em Vigilância Sanitária – CMRV5*), FIOCRUZ-INCQS, Rio de Janeiro, Brazil.

Evaluation of the strain regarding bacterial adhesion and biofilm formation

Initially the bacterial adhesion and biofilm formation capacity of the strain were analyzed, as described by Stepanovic et al. (2000). The microbial inoculum, containing $10^6$ CFU.mL$^{-1}$, was prepared in Trypsine Soy broth (TSB; KASVI) supplemented with 1% (w/v) glucose. 200 μL of the inoculum were added to 96-well, sterile microplates (KASVI) and incubated for 24 h at 37 °C.

The biofilm biomass was quantified by the crystal violet technique: the wells were washed with 0.85% sterile saline solution. Methanol 99% was used for the fixation of the adherent cells. After 15 minutes, the methanol was removed and the plate dried at room temperature. Aliquots of 200 μL of 2% (v/v) crystal violet (CV) solution were added and after 15 minutes the excess was removed. The CV was resolubilized by adding 160 μL of 33% acetic acid. The optical density (OD) reading was performed after 15 min in an Elisa Bio-Tek reader at a wavelength of 570 nm (Stepanovic et al. 2000).
The microorganism was classified according to Stepanovic et al. (2000), as: OD \leq OD_C (non-ADHERENT); OD_C < OD \leq 2 \times OD_C (poorly ADHERENT); 2 \times OD_C < OD \leq 4 \times OD_C (moderately ADHERENT); 4 \times OD_C < OD (strongly ADHERENT). OD corresponds to the optical density of the tested strains and OD_C corresponds to the three standard deviations above the mean OD of the negative control (containing only TSB culture medium).

**Determination of the Minimum Biofilm Inhibition Concentration (MBIC)**

The determination of the MBIC was performed according to the methodology of Nostro et al. (2004). 100 μL aliquots of the inoculum (10^6 CFU.mL^-1) and 100 μL of each concentration of VIO (0.156 μg.mL^-1 to 320 μg.mL^-1) were added to the 96-well microplate. After 24 h of incubation at 37 °C, the biofilm biomass was quantified by the previously described crystal violet technique. MBIC was considered the lowest concentration capable of inhibiting biofilm formation. Wells containing TSB culture medium and microorganism were used as control (Petitt et al. 2005).

**Determination of the Minimum Biofilm Eradication Concentration (MBEC)**

The determination of VIO MBEC was performed as described by Kwieciński et al. (2009). A 100-μL aliquot of the microbial inoculum (10^6 CFU.mL^-1) was transferred to microplates and incubated for 24 h at 37 °C. The biofilm was washed with 0.85% saline solution. For each well containing biofilm, 50 μL of TSB and 50 μL of VIO were added at different concentrations (20 μg.mL^-1 to 320 μg.mL^-1). The microplates were incubated for 24 h at 37 °C and, after that period, the biofilms from each well were resuspended in 0.85% saline solution. Aliquots of 5 μL of the serial dilutions of the biofilm suspensions were seeded on Plate-Count agar and, after incubation for 24 hours at 37 °C, the colonies were counted. The MBEC was considered the VIO concentration capable of
eradicating the mature biofilm. 24-hour mature biofilm was used as control (Kwiecinski et al. 2009).

**Determination of the effect of VIO exposure time on microbial viability in mature biofilm**

20μL aliquots of VIO, at concentrations equal to 1/2x MBEC, MBEC and 2x MBEC, were added to the wells of microplates containing 100μL of TSB broth and mature biofilm, formed according previously described. Wells with TSB broth and mature biofilm, without violacein, were used as control.

The microplates were incubated at 37 °C and at time zero and at time intervals of 30 minutes up to a period of 6 hours, 5μL aliquots were withdrawn and diluted in 0.85% sterile saline solution, which were seeded on Plate-Count agar. The colonies were counted after 24 hours of incubation at 37 °C and the results were expressed as CFU.mL⁻¹ (Kwiecinski et al. 2009).

**Modulatory activity of VIO on clinically-used antibiotics (ATB) on mature biofilm**

This assay was performed to evaluate whether VIO can positively modulate the antimicrobial activity of different antibiotics on *S. epidermidis* biofilm. The biofilm was formed as described above and washed with 0.85% saline solution.

100 μL of TSB, 50 μL of VIO (1/2x MBEC and 1/4x MBEC) and 50 μL of ATB (1/2x MBEC and 1/4x MBEC), isolated and in association, were added to each well containing biofilm. Wells without VIO and ATB were used as control. The microplates were incubated for 24 h at 37 °C, and the biofilm from each well was washed and resuspended in 0.85% saline solution. 5 μL aliquots of the serial biofilm suspension
dilutions were seeded on Plate-Count agar. After incubation for 24 hours at 37 °C, the colonies were counted (Gomes et al. 2012).

**Statistical Analysis**

Statistical analysis was performed using ANOVA with Dunnett's post-test or Tukey's post-test. All assays were performed in triplicate and repeated three times. The results were expressed as mean ± standard error and were considered significant when p <0.05. The software used was GraphPad Prism 6.

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Figure S1. Antimicrobial activity of VIO on the biofilm of *S. epidermidis* ATCC 35984. 
(a) Evaluation of the strain *S. epidermidis* ATCC 35984 regarding bacterial adhesion and biofilm formation. (b) Violacein Minimum Biofilm Inhibition Concentration on *S. epidermidis* ATCC 35984 biofilm. (c) Violacein Minimum Biofilm Eradication Concentration on *S. epidermidis* ATCC 35984 mature biofilm. (d) Effect of violacein exposure time on microbial viability in mature biofilm.
Figure S2. Effect of VIO-ATB (vancomycin, cefepime, ciprofloxacin and meropenem) associations on the cell viability of *S. epidermidis* ATCC 35984 biofilm.

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\begin{align*}
1/2 \text{ VIO} & = 1/2 \times \text{VIO MBEC}; \\
1/4 \text{ VIO} & = 1/4 \times \text{VIO MBEC}; \\
1/2 \text{ ATB} & = 1/2 \times \text{ATB MBEC}; \\
1/4 \text{ ATB} & = 1/4 \times \text{ATB MBEC}; \\
1/2 + 1/2 & = 1/2 \times \text{VIO MBEC} + 1/2 \times \text{ATB MBEC}; \\
1/2 + 1/4 & = 1/2 \times \text{VIO MBEC} + 1/4 \times \text{ATB MBEC};
\end{align*}
\]
1/4 + 1/2 = 1/4 x VIO MBEC + 1/2 x ATB MBEC;
1/4 + 1/4 = 1/4 x VIO MBEC + 1/4 x ATB MBEC;

* p<0,05 when compared to control;

a p<0,05 when compared to 1/2 VIO; b p<0,05 when compared to 1/4 VIO;

c p<0,05 when compared to 1/2 ATB; d p<0,05 when compared to 1/4 ATB;