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Violacein antimicrobial activity on *Staphylococcus epidermidis* and synergistic effect on commercially available antibioticsH.V. Dodou¹, A.H. de Moraes Batista¹ , G.W.P. Sales¹, S.C. de Medeiros¹, M.L. Rodrigues¹, P.C.N. Nogueira², E.R. Silveira² and N.A.P. Nogueira¹¹ Department of Clinical and Toxicological Analysis, Federal University of Ceará, Fortaleza, Brazil² Department of Organic Chemistry, Federal University of Ceará, Fortaleza, Brazil**Keywords**antibiotic synergism, antibiotics, antimicrobial activity, *Staphylococcus epidermidis*, violacein.**Correspondence**Hilania Valéria Dodou, Department of Clinical and Toxicological Analysis, Federal University of Ceará, Fortaleza, Ceará, Brazil.
E-mail: valeriadodou@gmail.com2017/0163: received 20 February 2017,
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Abstract**Aims:** The study aimed to assess whether violacein has antimicrobial activity on *Staphylococcus epidermidis* and synergistically modulates the action of commercially available antimicrobial drugs.**Methods and Results:** Violacein showed excellent antimicrobial activity on biofilm-forming and nonbiofilm-forming *S. epidermidis* strains (ATCC 35984) (ATCC 12228), with bacteriostatic (MIC = 20 µg ml⁻¹ and 10 µg ml⁻¹ respectively) and bactericidal effects (MBC = 20 µg ml⁻¹ for both strains), observed in short periods of exposure. The violacein bactericidal concentration led to *S. epidermidis* death after 2–3 h of exposure. Additionally, violacein synergistically modulated the activity of different antimicrobial classes on *S. epidermidis* ATCC 12228 (81.8%; *n* = 9) and on *S. epidermidis* ATCC 35984 (54.5%; *n* = 6), reducing the MIC of these antibiotics by up to 16-fold.**Conclusion:** Violacein shows excellent antimicrobial activity on *S. epidermidis* strains.**Significance and Impact of the Study:** Violacein shows the potential for the development of a new drug for the treatment of infections caused by *S. epidermidis*.**Introduction**

Staphylococcus epidermidis is a Gram-positive coccus, which is part of the human skin and mucosal microbiota, although this micro-organism also has pathogenic potential when related to healthcare-associated infections (HAI). It is one of the main infection-causing pathogens associated with medical devices (such as catheters and prostheses, among others) due to virulence factor expression, mainly biofilm formation (Namvar *et al.* 2014; Chessa *et al.* 2015).

The emergence of this micro-organism resistant to several antimicrobials in the last decades has become a challenge for treatment choice, as most of the strains isolated in hospital environments are resistant to beta-lactam drugs and cases of strains resistant to glycopeptides have already been described (Montúfar Andrade *et al.* 2016). Considering this scenario, studies that investigate new effective drugs against this pathogen become very important.

Violacein (VIO) is a metabolite produced by bacteria of different species, especially *Chromobacterium violaceum* and has several biological activities previously described in the literature, such as its antimicrobial action on Gram-positive bacteria (Choi *et al.* 2015). Nevertheless, regarding *S. epidermidis*, the action of this pigment has not been specifically described on this pathogen.

The aim of this study was to evaluate the antimicrobial activity of VIO on biofilm-forming and nonbiofilm-forming *S. epidermidis* and its modulating effect on commercially available antibiotic agents.

Materials and methods**Violacein (VIO)**

The VIO used in this study was extracted from *C. violaceum* ATCC 12472, purified and characterized by

ultraviolet-visible spectroscopy (modified Rettori and Durán 1998) (Fig. 1). *Chromobacterium violaceum* ATCC 12472 culture was obtained in nutrient broth and, subsequently, bacterial cells were separated from the culture medium by centrifugation. To extract the violacein, the supernatant was discarded, the bacterial pellet was washed with distilled water three times and submitted to successive extractions with methanol, until the absence of the pigment's characteristic violet colour was attained. This solution was filtered, the methanol was rotavaporated and the methanolic dry extract was obtained, which was then placed in a separation funnel to start the purification process. The separation was performed using methanol, distilled water and ethyl acetate. The hydroalcoholic fraction was collected and submitted to the separation process several times, whereas the violacein-ethyl acetate fraction was collected, dried with sodium sulphate, rotavaporated and kept at 60°C for 3 days. The yield was 48.86%.

Bacterial strains

The tested strains were *S. epidermidis* ATCC 12228 (non-biofilm forming) and *S. epidermidis* ATCC 35984 (biofilm forming), obtained from the Collection of Reference Microorganisms on Health Surveillance (Coleção de Microrganismos de Referência em Vigilância Sanitária—CMRVS), FIOCRUZ-INCQS, Rio de Janeiro.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC of VIO and the clinically used antibiotics (ATBs) were determined using the microdilution method in culture broth (CLSI 2012). In 96-well, sterile microplates, 100 µl of Brain Heart Infusion (BHI) broth (Merck Millipore Corporation), 20 µl of different concentrations of VIO (0.019 to 40 µg ml⁻¹) or ATBs (0.024 to 50 µg ml⁻¹) and 80 µl of microbial suspension (10⁶ CFU ml⁻¹) were placed.

Controls were carried out with sterile BHI broth and BHI broth with microbial inoculum. The microplates were incubated for 24 h at 37°C and the MIC was considered as the lowest concentration of VIO or ATB capable of inhibiting microbial growth.

Inoculums of 5 µl from the microplate wells used for MIC determination that showed no visible microbial growth, were plated. The plates were incubated at 37°C and the colonies were counted after 24 h. MBC was considered the lowest VIO concentration capable of causing 99.9% of cell death in relation to the initial inoculum (Shanholtzer et al. 1984). The assays were performed in triplicate.

Determination of the effect of violacein exposure time on microbial viability—Time-kill assay

Aliquots of 20 µl of VIO, at concentrations equal to ½ MIC, MIC, 2× MIC and 4× MIC, were added to

Figure 1 Absorption spectrum in the VIO infrared region.

96-well microplates containing 100 μl of BHI broth and 80 μl of the microbial suspension (10^6 CFU ml^{-1}). The microplates were incubated at 37°C and 5 μl aliquots were removed, diluted in sterile 0.85% saline solution and plated at times zero and every 30 min until 6 h of incubation. The counting of the colonies that grew on the plates was performed after 24 h of incubation at 37°C (Olajuyigbe and Afolayan 2012). Microbial cultures not treated with VIO were used as experimental controls.

Determination of VIO modulatory action on antibiotics (ATB)

The effect of VIO on the activity of the clinically used ATBs, such as piperacillin + tazobactam, amikacin, gentamicin, ciprofloxacin, cefepime, ceftazidime, vancomycin, meropenem, ceftriaxone, cephalothin and oxacillin, was evaluated by the checkerboard method (Cleeland and Squires 1991). In 96-well microplates, aliquots of 80 μl of microbial cultures (10^6 CFU ml^{-1}), 80 μl of BHI broth, 20 μl of VIO and 20 μl of ATB were added, at the final concentrations of 1/2, 1/4, 1/8 and 1/16 $\times$ MIC. The microplates were incubated for 24 h at 37°C and visual inspection of the microbial growth was performed.

To evaluate the effect of the associations, the fraction inhibitory concentration index (FICI) (Shin and Lim 2004) was calculated: $\text{FICI} = \text{FIC}_{\text{VIO}} + \text{FIC}_{\text{ATB}} = [\text{VIO}]/\text{MIC}_{\text{VIO}} + [\text{ATB}]/\text{MIC}_{\text{ATB}}$. Where, [VIO] is the lowest concentration of VIO in association with antimicrobial activity; MIC_{VIO} is the MIC of VIO alone; FIC_{VIO} is the Fraction Inhibitory Concentration of VIO; [ATB] is the lowest concentration of ATB in association with antimicrobial activity; MIC_{ATB} is the MIC of ATB; FIC_{ATB} is the Fractional Inhibitory Concentration of ATB. The FIC Index (FICI) was interpreted as: synergistic effect for $\text{FICI} \leq 0.5$, additive or indifferent effect for $\text{FICI} > 0.5$ and < 1.0 , and antagonistic effect for $\text{FICI} > 1.0$ (Eucast 2003).

Determination of the effect of VIO-ATB association exposure time on microbial viability—Time-kill assay

To perform this assay, two synergistic associations were chosen, with the lowest concentrations of VIO for each micro-organism found in the checkerboard test. Twenty-microlitre aliquots of VIO and ATB, alone and in combination, were added to the wells of the 96-well microplate containing 80 μl of BHI broth and 80 μl of the microbial suspensions (10^6 CFU ml^{-1}). The microplates were incubated at 37°C and at time intervals of 0, 2, 4, 6, 8, 10, 12 and 24 h, 5- μl aliquots were removed, diluted in 0.85% sterile saline and plated. After 24 h of incubation at

37°C, colonies that had grown on the medium surface were counted (Olajuyigbe and Afolayan 2012).

Statistical analysis

Statistical analysis was performed using ANOVA with Dunnett's post-test. The assays were performed in triplicate and the results expressed as mean $\pm$ SD and were considered significant when $P < 0.05$.

Results

VIO antimicrobial activity

VIO showed excellent antimicrobial, bacteriostatic and bactericidal activities on the tested *S. epidermidis* strains, as shown in Table 1.

In the time-kill assay, when strains were exposed to 1/2 VIO MIC, growth inhibition was observed after 5 h and 6 h for *S. epidermidis* ATCC 12228 and *S. epidermidis* ATCC 35984 respectively. When exposed to VIO MIC, growth inhibition was observed for *S. epidermidis* ATCC 12228 and loss of viability after 3 h for *S. epidermidis* ATCC 35984. When exposed to concentrations equal to 2 $\times$ MIC and 4 $\times$ MIC of VIO, there was loss of cell viability after 2 h and 1 h for *S. epidermidis* ATCC 12228, respectively, and after 2 h 30 min and 1 h 30 min for *S. epidermidis* ATCC 35984 respectively (Fig. 2).

VIO MIC (10 $\mu\text{g ml}^{-1}$) for *S. epidermidis* ATCC 12228 was capable of reducing cell growth by 33.3% in the first two hours of incubation. For *S. epidermidis* ATCC 35984, VIO MIC (20 $\mu\text{g ml}^{-1}$) reduced microbial growth by 40.7%, also after 2 h of incubation.

VIO modulatory activity

Modulatory activity assays showed a high synergistic effect between VIO and most of the tested antimicrobials used in clinical practice. For *S. epidermidis* ATCC 12228, VIO showed a synergistic and additive effect on 81.8% ($n = 9$) and 18.2% ($n = 2$) of the tested ATBs, respectively, with no antagonistic effect in any VIO-ATB

Table 1 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of VIO for *Staphylococcus epidermidis* strains

Microbial strains	MIC ($\mu\text{g ml}^{-1}$)	MBC ($\mu\text{g ml}^{-1}$)
<i>Staphylococcus epidermidis</i> ATCC 12228	10	20
<i>Staphylococcus epidermidis</i> ATCC 35984	20	20

Figure 2 Effect of exposure time at different VIO concentrations on viability of *Staphylococcus epidermidis*. (●) Control: without VIO; (○) $\frac{1}{2}$ MIC; (▲) MIC; (■) 2xMIC; (X) 4xMIC. MIC (*S. epidermidis* ATCC 12228) = $10 \mu\text{g ml}^{-1}$. MIC (*S. epidermidis* ATCC 35984) = $20 \mu\text{g ml}^{-1}$.

association. In the associations that showed a synergistic effect, VIO was capable of reducing the MIC by 16-fold of five ATBs (55.5%): gentamicin, ceftazidime, vancomycin, cephalothin and oxacillin; it also reduced the MIC by eightfold of one ATB (11.1%): meropenem; and reduced the MIC by fourfold of three ATBs (33.3%): amikacin, cefepime and ceftriaxone (Table 2).

For *S. epidermidis* ATCC 35984, there was a synergistic and additive effect in 54.5% ($n = 6$) and 18.2% ($n = 2$) respectively. There was no growth inhibition for the other associations between VIO and the ATBs piperacillin + tazobactam, amikacin and gentamicin (27.3%; $n = 3$). VIO reduced the MIC by 16-fold of the six drugs that it showed synergism with: ciprofloxacin, cefepime, ceftazidime, vancomycin, meropenem and cephalothin (Table 3).

In the cell-death assay of the VIO-ATB association, it was demonstrated that all combinations inhibited microbial growth over the entire period (24 h), when compared to the control and the substances alone (Figs 3 and 4).

Discussion

VIO showed excellent antimicrobial activity, with bacteriostatic and bactericidal actions, on biofilm-forming (ATCC 12228) and nonbiofilm-forming *S. epidermidis* (ATCC 35984), and synergistically modulated the effect of several clinically used antimicrobials, reducing up to 16-fold the concentrations required to inhibit microbial growth.

The literature reports an important *in vitro* antimicrobial activity of VIO on Gram-positive micro-organisms, especially on *Staphylococcus aureus*; however, in relation

to Gram-negative bacteria, VIO shows less effect on most species (Durán *et al.* 2012).

One study demonstrated VIO action on *S. aureus*, with a MIC of $7.8 \mu\text{g ml}^{-1}$ and $15.6 \mu\text{g ml}^{-1}$ for methicillin-sensitive and resistant strains, respectively, whereas for Gram-negative bacteria, the observed MIC was much higher, $125 \mu\text{g ml}^{-1}$ for *Pseudomonas aeruginosa* and $1000 \mu\text{g ml}^{-1}$ for *Klebsiella pneumoniae* (Aruldass *et al.* 2015).

In the present study, we verified that VIO showed excellent antimicrobial action against the Gram-positive pathogen, *S. epidermidis*, as it showed bacteriostatic and bactericidal action even at low concentrations.

The minimum concentration required to inhibit the micro-organism growth was lower for *S. epidermidis* strain ATCC 12228 (MIC = $10 \mu\text{g ml}^{-1}$) when compared to *S. epidermidis* ATCC 35984 (MIC = $20 \mu\text{g ml}^{-1}$), which can be attributed to the biofilm-forming capacity of the latter. For biofilm formation, the bacterium undergoes adaptations in its entire genome, which makes it 10- to 1000-fold more resistant, and justifies antimicrobial substance activity limitations on such micro-organisms (Aguila-Arcos *et al.* 2015).

The bactericidal and bacteriostatic activities of substances can be determined by time-kill assays, which identify the antimicrobial action velocity of the substance being tested. The cell-death assay showed that VIO has a high antimicrobial effect on both strains of *S. epidermidis*, with a reduction of at least 3 logs in microbial growth when compared to the control. Moreover, its bactericidal action on the tested strains occurred with short exposure times.

The bacteriostatic action was also observed at subinhibitory concentrations ($\frac{1}{2} \times \text{MIC}$) for periods of 5 h and 6 h for *S. epidermidis* ATCC 12228 and

Table 2 Modulatory effect of VIO on the antimicrobial activity of ATBs of clinical use on the growth of *Staphylococcus epidermidis* ATCC 12228*

Antibiotics	[VIO]§	MIC VIO†	FIC VIO**	[ATB]¶	MIC ATB‡	FIC ATB††	FICI‡‡	Effect§§
Piperacillin + Tazobactam	0.625	10	0.0625	1.5625	3.125	0.5	0.5625	Additive
Amikacin	0.625	10	0.0625	0.7812	3.125	0.25	0.3125	Synergic
Gentamicin	1.25	10	0.125	0.0244	0.3906	0.0625	0.1875	Synergic
Ciprofloxacin	2.5	10	0.25	0.0976	0.1953	0.5	0.75	Additive
Cefepime	0.625	10	0.0625	0.3906	1.5625	0.25	0.3125	Synergic
Ceftazidime	1.25	10	0.125	0.7812	12.5	0.0625	0.1875	Synergic
Vancomycin	1.25	10	0.125	0.1953	3.125	0.0625	0.1875	Synergic
Meropenem	0.625	10	0.0625	0.7812	6.25	0.125	0.1875	Synergic
Ceftriaxone	0.625	10	0.0625	1.5625	6.25	0.25	0.3125	Synergic
Cephalothin	1.25	10	0.125	0.0122	0.1953	0.0625	0.1875	Synergic
Oxacillin	1.25	10	0.125	0.0244	0.3906	0.0625	0.1875	Synergic

*The table shows only the association of the minor concentrations in which there was inhibition of microbial growth.

†MIC VIO: MIC of isolated violacein ($\mu\text{g.ml}^{-1}$).

‡MIC ATB: MIC of isolated antibiotics ($\mu\text{g.ml}^{-1}$).

§[VIO]: MIB of violacein in association($\mu\text{g.ml}^{-1}$).

¶[ATB]: MIC of antibiotics in association($\mu\text{g.ml}^{-1}$).

**FIC VIO = [VIO]/MIC VIO.

††FIC ATB = [ATB]/MIC ATB.

‡‡FICI = FIC VIO + FIC ATB.

§§FICI ≤ 0.5 : synergism; FICI > 0.5 and < 1.0 : additive or indifferent; FICI ≥ 1.0 : antagonism (EUCAST 2003).

Table 3 Modulatory effect of VIO in the antimicrobial activity of ATBs of clinical use on the growth of *Staphylococcus epidermidis* ATCC 35984*.

Antibiotics	[VIO]§	MIC VIO†	FIC VIO**	[ATB]¶	MIC ATB‡	FIC ATB††	FICI‡‡	Effect§§
Piperacillin + Tazobactam	-*	20	-*	-*	6.25	-*	-*	-*
Amikacin	-*	20	-*	-*	6.25	-*	-*	-*
Gentamicin	-*	20	-*	-*	12.5	-*	-*	-*
Ciprofloxacin	1.25	20	0.0625	0.3906	6.25	0.0625	0.125	Synergic
Cefepime	5	20	0.25	0.3906	6.25	0.0625	0.3125	Synergic
Ceftazidime	5	20	0.25	0.7812	12.5	0.0625	0.3125	Synergic
Vancomycin	5	20	0.25	0.3906	6.25	0.0625	0.3125	Synergic
Meropenem	1.25	20	0.0625	0.7812	12.5	0.0625	0.125	Synergic
Ceftriaxone	2.5	20	0.125	6.25	12.5	0.5	0.625	Additive
Cephalothin	5	20	0.25	0.0244	0.3906	0.0625	0.3125	Synergic
Oxacillin	5	20	0.25	0.7812	1.5625	0.5	0.75	Additive

*The table shows the association of the minor concentrations in which there was inhibition of the microbial growth.

†MIC VIO: MIC of isolated violacein ($\mu\text{g ml}^{-1}$).

‡MIC ATB: MIC of isolated antibiotics ($\mu\text{g ml}^{-1}$).

§[VIO]: MIC of violacein in association($\mu\text{g ml}^{-1}$).

¶[ATB]: MIC of antibiotics in association($\mu\text{g ml}^{-1}$).

**FIC VIO = [VIO]/MIC VIO.

††FIC ATB = [ATB]/MIC ATB.

‡‡FICI = FIC VIO + FIC ATB.

§§FICI ≤ 0.5 : synergism; FICI > 0.5 and < 1.0 : additive or indifferent; FICI ≥ 1.0 : antagonism (Eucast 2003).

-*There was no growth inhibition in none of the associations.

S. epidermidis ATCC 35984 respectively. Additionally, VIO bactericidal action verified at 2 h for *S. epidermidis* ATCC 12228 and at 3 h for *S. epidermidis* ATCC 35984, occurred more rapidly when the concentration increased

and, therefore, the bactericidal effect of this pigment is concentration dependent.

The increasing resistance of *S. epidermidis* to several antimicrobial drugs (Bender *et al.* 2015; O'Connor *et al.*

Figure 3 Effect of exposure time to VIO-CEF and VIO-MER associations on viability of *Staphylococcus epidermidis* ATCC 12228. (a) (●) Control: without VIO and without ATB; (○) VIO (1/16 MIC = 0,625 $\mu\text{g ml}^{-1}$); (▲) CEF (1/4 MIC = 0,3906 $\mu\text{g ml}^{-1}$); (X) VIO-CEF. (b) (●) Control: without VIO and without ATB; (○) VIO (1/16 MIC = 0,625 $\mu\text{g ml}^{-1}$); (▲) MER (1/8 MIC = 0,7812 $\mu\text{g ml}^{-1}$); (X) VIO-MER.

Figure 4 Effect of exposure time to VIO-CIP and VIO-MER associations on viability of *Staphylococcus epidermidis* ATCC 35984. (a) (●) Control: without VIO and without ATB; (○) VIO (1/16 MIC = 1,25 $\mu\text{g ml}^{-1}$); (▲) CIP (1/16 MIC = 0,3906 $\mu\text{g ml}^{-1}$); (X) VIO-CIP. (b) (●) Control: without VIO and without ATB; (○) VIO (1/16 MIC = 1,25 $\mu\text{g ml}^{-1}$); (▲) MER (1/16 MIC = 0,7812 $\mu\text{g ml}^{-1}$); (X) VIO-MER.

2015; and Montúfar Andrade *et al.* 2016) is an important factor to be considered for studies that investigate not only the antibacterial action of new molecules but also evaluate the capacity of such molecules to potentiate the action of commercially available antimicrobial drugs.

Studies show that VIO interacts synergistically with several commercially available antibiotics on *Salmonella typhi*, *Vibrio cholera*, *P. aeruginosa*, *K. pneumonia* and *S. aureus*, and with azole antifungals (ketoconazole, fluconazole, clotrimazole and itraconazole) on the dermatophyte fungus *Trichophyton rubrum* (Subramaniam *et al.* 2014; Anju *et al.* 2015).

The present study showed that VIO synergistically modulates the action of several clinically used antimicrobials on biofilm-forming and nonbiofilm-forming *S. epidermidis*,

increasing the effectiveness of antimicrobials of different classes, such as penicillins, aminoglycosides, cephalosporins, glycopeptides and carbapenems for *S. epidermidis* ATCC 12228; and of quinolones, cephalosporins, glycopeptides and carbapenems for *S. epidermidis* ATCC 35984.

The time-kill assay can also be performed to determine synergistic combinations between substances, confirming the results of the checkerboard test, as well as identifying the velocity of the associations' bactericidal action. The bacterial mortality rate is identified by counting viable cells (CFU ml^{-1}) at different times and synergism is considered when a reduction $\geq 2 \log_{10}$ CFU ml^{-1} occurs due to exposure to the association, when compared to the inhibition caused by the substances alone (Jayaraman *et al.* 2010).

When assessing the influence of time of exposure on the association activity, two associations were selected for each one of the strains (VIO-CEF and VIO-MER for *S. epidermidis* ATCC 12228; and VIO-CIP and VIO-MER for *S. epidermidis* ATCC 35984). All tested associations were capable of significantly inhibiting microbial growth for up to 24 h when compared to the inhibition resulting from the isolated use of the substances, at the same concentrations used in the association. These results demonstrate that VIO is highly capable of potentiating the effect of commercially available antibiotics.

Considering the increasing rates of bacterial resistance to multiple drugs, synergistic combinations between new molecules and commercially available drugs may be a good alternative to control such micro-organisms, due to factors such as prevention of the emergence of resistant strains and toxicity reduction, since the same effect is obtained with much lower concentrations, in addition to the fact that the action spectrum will be increased in relation to that observed for the substances alone.

The present study demonstrates the excellent antimicrobial activity of VIO on *S. epidermidis*, with a high synergistic modulating effect when associated with clinically used antimicrobials. Since there are no studies that described the action of VIO specifically on *S. epidermidis*, this is a pioneering study that can be used as the basis for new researches investigating the action mechanism of this pigment on *S. epidermidis*.

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Conflict of Interest

The authors declare no conflicts of interest.

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