Synergistic and antibiofilm properties of ocellatin peptides against multidrug-resistant *Pseudomonas aeruginosa*

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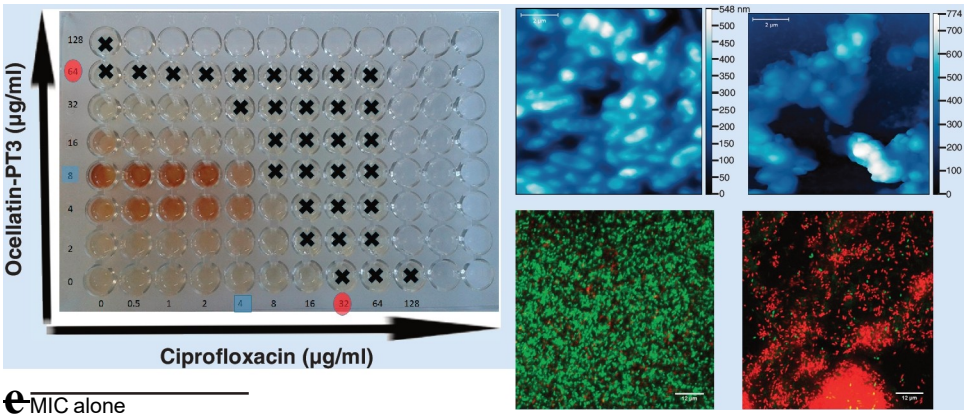
Aim: To test ocellatin peptides (ocellatins-PT2-PT6) for antibacterial and antibiofilm activities and synergy with antibiotics against *Pseudomonas aeruginosa.* **Materials & methods:** Normal- and checkerboard-broth microdilution methods were used. Biofilm studies included microtiter plate-based assays and microscopic analysis by confocal laser scanning microscopy and atomic force microscopy. **Results:** Ocellatins were more active against multidrug-resistant isolates of *P. aeruginosa* than against susceptible strains. Ocellatin-PT3 showed synergy with ciprofloxacin and ceftazidime against multidrug-resistant isolates and was capable of preventing the proliferation of 48-h mature biofilms at concentrations ranging from 4 to 8x the MIC. Treated biofilms had low viability and were slightly more disaggregated. **Conclusion:** Ocellatin-PT3 may be promising as a template for the development of novel antimicrobial peptides against *P. aeruginosa.*

Ocellatin peptides (ocellatin-PT3)

Synergy with antibiotics

Antibiofilm activity

P. aeruginosa biofilm Ocellatin-PT3-treated



MIC alone

D MIC in combination

Keywords: AMPs • antibiofilm activity • antimicrobial peptides • multidrug-resistant *Pseudomonas aeruginosa* • ocellatin peptides • ocellatin-PT3 • synergy

Antimicrobial resistance (AMR) is one of the greatest threats to health today [I,2]. It can affect anyone, at any age, in any country. It is of consensus that the threat of drug resistance can only be tackled with the right set of actions, including the development of novel treatment options and alternative antimicrobial therapies [3]. Gram-negative pathogens are of particular concern since they are becoming resistant to nearly all the antibiotic drug options available, in fact, infections by pandrug-resistant Gram-negative bacilli are rising [4]. The most worrisome Gram-negative infections are commonly caused by Enterobacteriaceae (mostly *Klebsiella pneumoniae), Pseudomonas aeruginosa* and *Acinetobacter* spp. [5].

P aeruginosa is a ubiquitous microorganism that commonly causes hospital-acquired infections, including pneumonia, bloodstream and urinary tract infections and it is well known for chronically colonizing the respiratory tract of patients with cystic fibrosis, causing severe intermittent exacerbation of the condition [6]. *P aeruginosa* infections are particularly difficult to control because of its high level of intrinsic resistance to antibiotics due primarily to a combination of the impermeable outer membrane and a number of broad-spectrum efflux pumps [7]. Some strains of *P aeruginosa* have been found to be multidrug-resistant (MDR), with resistance to nearly all antibiotics, including aminoglycosides, cephalosporins, Buoroquinolones and carbapenems [5]. Moreover, this Gram-negative bacterium is capable of forming structured aggregates known as biofilms, which definitely contribute to the increase of both pathogenicity and resistance to antibiotic treatment [8,9]. Biofilm formation by *P aeruginosa* is the result of a complex adaptation process driven by genetic variation and the qualitative composition of the polysaccharide content in the biofilm matrix is highly dependent on phenotypic features including the ability to synthesize high amounts of alginate (mucoid strains) or Psl/Pel (nonmucoid strains) [IO]. Indeed, biofilms of *P aeruginosa* are one of the bottlenecks in the treatment of such infections. Therefore, there is an urgent need of novel antimicrobial agents and treatment strategies able to effectively counteract planktonic as well as biofilm modes of growth.

Antimicrobial peptides (AMPs) historically have been called defensive molecules, and are believed to be the first line of the innate immune response system against viruses, bacteria and fungi [I I,12]. Natural AMPs can be found in every organism from prokaryotes to eukaryotes (protozoan, fungi, plants, insects and animals) [I I].

In the last few years, many AMPs have been reported as promising novel antimicrobial drugs due to their main mechanisms of action, which include disrupting membranes, interfering with metabolism, and targeting cytoplasmic components [13]. Additionally, AMPs are increasingly being considered as novel agents against biofilms by inhibiting the biofilm formation or eradicating established biofilms [14,15].

Another approach to overcome the problem of MDR bacteria is by combining different drugs. The combination of AMPs with commercially available antibiotics have also been explored as a potential alternative for combating drug-resistant infections caused by several microorganisms [16-18].

In this study, five peptides – ocellatin-PT2-PT6 – previously isolated from the skin secretion of the frog *Leptodactylus pustulatus* [19] were tested for antimicrobial activity and synergistic effects with antibiotics against *P aeruginosa*. The peptides herein studied were part of a set of eight new AMPs, called ocellatins, which had been isolated from the crude skin secretion of *L. pustulatus,* identified and tested for antimicrobial activity against *Escherichia coli, Staphylococcus aureus, K pneumoniae* and *Salmonella choleraesuis* strains by Marani *et al.* [19]. Moreover, those ocellatins were reported to present little or no hemolytic activity against human erythrocytes and no cytotoxicity against murine fibroblasts.

Recently, several frog skin-derived AMPs have been largely reported to show antibacterial properties [20-23]. There have also been reports of antibiofilm activity from some such molecules [15]. Therefore, the skin secretions from many species of anurans are a rich source of peptides with antimicrobial activities that should be explored for further research and development of novel therapeutic agents.

Table 1. Amino acid sequence and molecular weight of ocellatin-PT2-PT6.

Peptides	Sequence	MW	Ref.
Ocellatin-PT2	GVFDIIKDAGKQLVAHATGKIAEK vt	2609.0	[19]
Ocellatin-PT3	GVIDIIKGAGKDLIAHAIGKLAEKV 1	2530.0	[19]
Ocellatin-PT4	GVFDIIKGAGKQLIAHAMGKIAEKV 1	2595.1	[19]
Ocellatin-PT5	GVFDIIKDAGRQLVAHAMGKIAEKV 1	2667.1	[19]
Ocellatin-PT6	GVFDIIKGAGKQLIAHAMEKIAEKVGLNKDGN	3365.9	[19]

t c-terminus-amidated peptide.
MW: Molecular weight.

Materials & methods

Antimicrobial agents & ocellatin peptides

Standard laboratory powders of ceftazidime and ciproB.oxacin hydrochloride were purchased from Sigma-Aldrich (MO, USA). All the antibiotic discs used were from Oxoid (Basingstoke, England). The five ocellatin peptides (ocellatin-PT2-PT6), whose amino acid sequences are shown in Table 1, were manually synthesized, purified and quantified through slightly different protocols than those previously described [19]. The peptides were synthesized using the Merrifield solid phase synthesis techniques on a 24 channel multiplex Symphony® peptide synthesizer (Gyros Protein Technologies, Inc, AZ, USA) and were assembled using 0-(6-chlorobenzotriazol-l-yl)-N,N,N',N'-tetramethyluronium hexaB.uorophosphate and N,N-diisopropylethylamine-coupling conditions. The full and detailed protocol of synthesis and purification is provided as supplementary material. All peptides were dissolved in Milli-Q water to obtain stock solutions of 10 mg/ml.

Bacterial strains & growth conditions

P. aeruginosa ATCC 27853, *P. aeruginosa* PAOl and a susceptible clinical isolate, PA007, as well as MDR clinical isolates of *P. aeruginosa* (Pal-SA2, Pa4-SA2 and PA006) were used in this study. The AMR profile of MDR isolates is shown in Supplementary Table 1. These bacteria were grown on Mueller-Hinton (MH) agar (Liofilchem srl, Roseto degli Abruzzi [Te], Italy) from stock cultures. MH plates were incubated at 37°C prior to obtain fresh cultures for each *in vitro* bioassay.

MIC & MBC determination

The minimum inhibitory concentration (MIC) values of the five ocellatins, ceftazidime and ciproB.oxacin against *P. aeruginosa* isolates were determined by the broth microdilution method, following the recommendations contained in the Clinical and Laboratory Standards Institute (CLSI) guidelines [24], with the exception that MH broth was used instead of cation-adjusted MH broth. The MIC was defined as the lowest concentration that completely inhibited the growth of bacteria as detected by the naked eye. The minimum bactericidal concentration (MBC) was determined by spreading 10 µl on MH agar from the wells corresponding to/and above the MIC showing no visible growth, with further incubation for 24 h at 37°C; the lowest concentration at which no bacterial growth occurred on MH plates was defined as the MBC. These experiments were performed in three independent experiments.

Synergy testing

The disc-diffusion method on agar was used as a screening test to assess the combined effect between ocellatins and antibiotics. MDR *P. aeruginosa* isolates from fresh cultures in MH were suspended in buffered peptone water (Oxoid) in order to reach a turbidity equal to a 0.5 McFarland standard and spread on MH agar plates. CiproB.oxacin and ceftazidime discs were used as controls and were also impregnated with 15 µl of a 10-mg/ml solution of each peptide. The plates were incubated overnight at 37°C. Potential synergism was inferred when the zone of inhibition caused by the antibiotic discs impregnated with ocellatins was greater than the inhibition zone produced by the antibiotic discs or peptide-impregnated blank discs alone.

Based on the results of the previous assay, potential synergism observed between ocellatins, particularly ocellatin-PT3 and ocellatin-PT4, and antibiotics (ciproB.oxacin or ceftazidime), was then checked using a broth microdilution checkerboard method and tested against Pa4-SA2 and Pal-Sa2 as previously described [25]. Three independent experiments were carried out. The fractional inhibitory concentrations (FIC) were calculated and interpreted as stated by Gomes *et al.* [25]. BrieB.y, FIC of drug A (FIC A) = MIC of drug A in combination/MIC of drug A alone, and FIC of drug B (FIC B) = MIC of drug B in combination/MIC of drug B alone. The FIC index (I;FIC) is the

sum of each FIC and is interpreted as follows: I;FIC :S 0.5, synergy; 0.5 < I;FIC :S 4, indifference; 4 < I;FIC, antagonism.

Biofilm inhibition assay

Given the promising antibacterial activity of ocellatin-PT3 against *P. aeruginosa* isolates, its ability to inhibit the biofilm formation was assessed. Ocellatin-PT3 at concentrations equal to MIC, 1/2 x MIC, 1/4 x MIC and 1/8 x MIC was added to bacterial suspensions of 1 x 10^6 CFU/ml in tryptic soy broth. Bacterial suspensions without ocellatin-PT3 were used as controls. Each suspension was dispensed into a 96-well microtiter plate (200 µI/well) and incubated at 37°C for 48 h. After that time, biofilms were stained with 0.5% crystal violet for 5 min, rinsed with water, air dried and eluted with acetic acid 33% *(v/v)*. The optical density was measured at 595 nm.

Biofilm treatment assay

The efficacy of ocellatin-PT3 on established biofilm of *P. aeruginosa* was also assessed by obtaining the minimum biofilm inhibitory concentration (MBIC). Briefly, biofilms were allowed to form for 48 h in 96-well microtiter plates, then the planktonic phase were discarded and the biofilms were rinsed twice and further treated with different concentrations of ocellatin-PT3 ranging from the MIC value up to 12x MIC. The optical density (OD)6oo was immediately measured and measured again after 24 h of incubation at 37°C. The MBIC was defined as the lowest concentration of ocellatin inhibiting the bacterial proliferation in the planktonic phase, confirmed by no increase or :::;10% increase in the optical density compared with the initial reading [26].

Evaluation of biofilm metabolic activity

48-h biofilms of Pal-SA2, Pa4-SA2 and PA006 formed as described above in 96-well microtiter plates were subsequently treated with ocellatin-PT3 at a concentration equivalent to the respective MBIC. After 24 h of incubation at 37°C, the bacterial metabolic activity of biofilms was quantified using the 3-(4,5-dimethylthiazol-2- yl)-2,5-diphenyltetrazolium bromide – MTT (0.5 mg/ml; Sigma-Aldrich) for 3 h at 37°C in the dark. Dimethyl sulfoxide (DMSO) was used to extract the formazan dye product and then absorbance at 570 nm was measured.

Confocal laser scanning microscopy

For confocal laser scanning microscopy (CLSM) analysis, 48-h biofilms of Pal-SA2, Pa4-SA2 and PA006 were formed in µ-Dish (35 mm, high), ibidi Polymer Coverslips (ibidi GmbH, Planegg-Martinsried, Germany) from starting inocula of 1 x 10^6 CFU/ml in tryptic soy broth. After 48 h, biofilms were rinsed twice with phosphate-buffered saline and treated with a concentration of ocellatin-PT3 equal to the respective MBIC for 24 h. Biofilms were then rinsed and stained using the live/ dead staining BacLight bacterial viability kit (Molecular Probes, Thermo Fisher Scientific, MA, USA). Biofilms were examined by a widefield fluorescence microscope Zeiss Axiolmager Zl equipped with a Plan-Apochromat 63x /1.40 Oil DIC objective and a camera Axiocam MR ver.3.0 (Carl Zeiss, Oberkochen, Germany) and by a laser scanning confocal system Leica TCS SP5 II using a HC PL APO CS 63x /1.30 Glycerine 21°C objective (Leica Microsystems, Wetzlar, Germany). All experiments were performed at room temperature, and each chamber slides was used for no longer than 10 min.

As for the viability cell counts, the proportion oflive and dead cells was determined by counting six representative images taken from each biofilm visualized, using software Image analysis [27].

Atomic force microscopy imaging

48-h biofilms of Pal-SA2, Pa4-SA2 and PA006 were formed on a glass coverslip previously put inside 35-mm diameter polystyrene plates and treated with or without ocellatin-PT3 as above described for CLSM. Biofilms formed on the coverslips were rinsed with sterile phosphate-buffered saline and dried before atomic force microscopy (AFM) imaging. Samples were scanned with a TT-AFM from AFMWorkshop in air in vibrating mode. A 50-µm scanner and 300 kHz silicon cantilevers (ACT, AppNano, CA, USA) were used. Images were processed using Gwyddion 2.47 software.

Conformational analysis of ocellatin-PT3 by circular dichroism

The secondary structure content of ocellatin-PT3 was assessed by circular dichroism (CD) spectroscopy in the far UV, using a Jasco J-815 CD Spectropolarimeter QASCO) as previously reported [28]. Briefly, the measurements

Table 2. Minimum inhibitory concentration and minimum bactericidal concentration values (fig/ml) of ocellatins against susceptible and multidrug-resistant *Pseudomonas aeruginosa*.

Peptides	*P. aeruginosa* ATCC 278531 (MIC [MBC])	*P. aeruginosa* PAQ11 (MIC [MBC])	PA0071 (MIC [MBC])	Pa4-SA21 (MIC [MBC])	Pa1-SA21 (MIC [MBC])	PA0061 (MIC [MBC])
Ocellatin-PT2	1024 (>1024)	512 (>1024)	1024 (>1024)	128 (256)	256 (512)	16 (32)
Ocellatin-PT3	>512 (-)	512 (>1024)	1024 (>1024)	64 (128)	128 (256)	16 (16)
Ocellatin-PT4	>512 (-)	512 (>1024)	1024 (>1024)	256 (256)	512 (1024)	16 (32)
Ocellatin-PT5	>512 (-)	512 (>1024)	1024 (>1024)	128 (256)	256 (512)	32 (64)
Ocellatin-PT6	1024 (>1024)	512 (>1024)	1024 (>1024)	128 (256)	256 (512)	32 (64)

!Susceptible.
1Multidrug-resistant.
MBC: Minimum bactericidal concentration; MIC: Minimum inhibitory concentration.

were carried out under a nitrogen gas Bow of 8 l/h at 20°C. Spectra were obtained between 190 and 260 nm. The lipopolysaccharides (LPS) from *P. aeruginosa* were obtained from Sigma-Aldrich. Ocellatin-PT3 was used in a concentration of lOO µM and the LPS in concentrations of O, 0.50, 0.75 and 1.00% *(p/v)* in Milli-Q water. These experiments were performed at 37°C and a scan speed of 50 nm/min, a response time of 1 s and a bandwidth of 1 nm were used. The spectra were converted to molar ellipticity per residue as previously reported [28,29].

Statistical analysis

The biofilm inhibition and treatment assays as well as the biofilm metabolic activity assay were carried out in two independent experiments, being each experiment performed in triplicate. The results of the biofilm formation were expressed as mean values ± standard deviation. The statistical significance of differences between controls and experimental groups was evaluated using Student's *t-test.* Probability values (p) of <0.05 were considered statistically significant.

Results

Antibacterial activity of ocellatin peptides against *P. aeruginosa*

MIC values of ocellatins were initially determined against a *P. aeruginosa* ATCC 27853 and an MDR isolate, Pa4-SA2. Interestingly, the MIC and MBC values were lower against Pa4-SA2 than against the reference strain. Therefore, subsequently, we have determined the MIC of that ocellatin against other two non-MDR (susceptible) and two MDR *P. aeruginosa* isolates (Table 2). Ocellatin-PT3 was the most active among the five peptides with lower MIC and MBC values. As shown, the activity against the clinical isolate PA006 was particularly strong, the highlight being the bactericidal activity of ocellatin-PT3 at only 16 µg/ml.

Synergy between ocellatins & antibiotics

The screening for potential synergy between ocellatins and antibiotics against MDR *P. aeruginosa* isolates revealed that the combinations of ocellatin-PT3/ceftazidime and ocellatin-PT3/ciproBoxacin increased (by 3-4 mm) the zones of inhibition in comparison to the zones caused by each compound alone. Equally, the combinations ocellatin-PT4/ceftazidime and ocellatin-PT4 also increased growth inhibition (by 2-3 mm) compared with single components. Photos of the combined effect between ocellatins and antibiotics against Pa4-SA2 can be seen in Supplementary Figure 1. Those combinations were further tested using a checkerboard method. Only the synergies between ocellatin-PT3/ceftazidime and ocellatin-PT3/ciproBoxacin were confirmed (FIC index :::;0.5; Table 3).

Antibiofilm activities of ocellatin-PT3

The ability of ocellatin-PT3 to inhibit the biofilm formation by Pa4-SA2 and PA006 isolates was examined (Figure 1). In presence of concentrations equal to the MIC and 1/2 x MIC, less biofilm biomass was quantified. However, at lower subinhibitory concentrations more biofilm was formed compared with the control.

The MBIC of ocellatin-PT3 against 48-h established biofilms formed by the three MDR isolates are shown in Table 4. The concentrations of ocellatin-PT3 that could inhibit the proliferation of mature biofilms ranged from 4 to lO x the respective MIC values. The relative MBIC of ciproBoxacin was considerably higher than that recorded for this peptide. For instance, when a 48-h biofilm of Pa4-SA2 was treated with a concentration 32 x MIC of

Table 3. MIC values of ocellatins-PT3 and -PT4 in combination with antibiotics and respective fractional inhibitory concentration index values obtained from the checkerboard method.

MOR isolate	MIC (µgL ml)				l:FIC
	Alone		In combination		
	PT3	CIP	PT3	CIP	
Pa4-SA2	64	32	8	4	0.25
Pa1-SA2	128	16	16	4	0.38
	PT3	*CAZ.*	PT3	*CAZ.*	
Pa4-SA2	64	32	16	4	0.38
Pa1-SA2	128	16	32	4	0.5
	PT4	CIP	PT4	CIP	
Pa4-SA2	256	32	128	8	0.75
	PT4	*CAZ.*	PT4	*CAZ.*	
Pa4-SA2	256	32	128	16	1.00

1FIC :s0.5, synergy; 0.5 < 1FIC :s 4, indifference; 4 < 1FIC, antagonism.
CAZ: Ceftazidime; CIP: Ciprofloxacin; FIC: Fractional inhibitory concentration; MDR: Multidrug-resistant; MIC: Minimum inhibitory concentration; PT3: Ocellatin-PT3; PT4: Ocellatin-PT4.

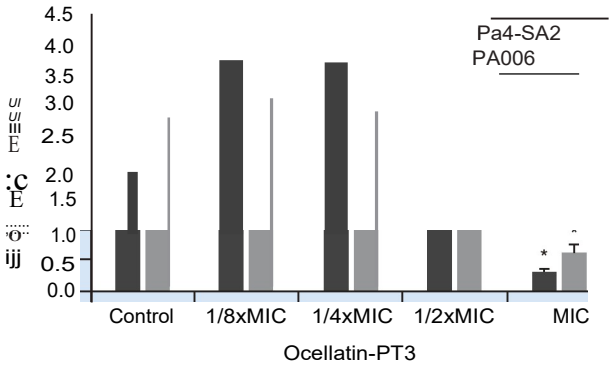


Figure 1. Biomass quantification of biofilms of *Pseudomonas aeruginosa* Pa4-Sa2 and PAOOG. Biofilms were formed in the presence of different concentrations of ocellatin-PT3 (ranging from $1/B\,x$ MIC to MIC). Differences between the experimental group and the control were statistically significant for a *p < 0.05.
MIC: Minimum inhibitory concentration.

Table 4. Minimum biofilm inhibitory concentration (fig/ml) values against 48-h preformed biofilms of multidrug-resistant *Pseudomonas aeruginosa* isolates.

	Pa4-SA2	Pa1-SA2	PA006
Ocellatin-PT3	512 (8x MIC)	512 (4x MIC)	160 (10x MIC)
Ciprofloxacin	>1024 (>32x MIC)	>512 (>32x MIC)	>64 (>32x MIC)

MIC: Minimum inhibitory concentration.

ciproB.oxacin, there still was an increase of 48% in the optical density after 24 h. Therefore, although the amount of peptide required to inhibit biofilm growth was quite high, it compares favorably to the conventional antibiotic.

Metabolic activity of biofilms

The metabolic activity of 48-h biofilms treated for 24 h with ocellatin-PT3 at concentrations equivalent to the respective MBIC was reduced by almost 70% (Figure 2). Differences between ocellatin-PT3-treated biofilms and controls were statistically significant ($p < 0.001$).

Ocellatin-PT3 effect on biofilm after microscopic analyses

The CLSM images (Figure 3) showed, for all three isolates, a clear effect of ocellatin-PT3 in lowering the viability of

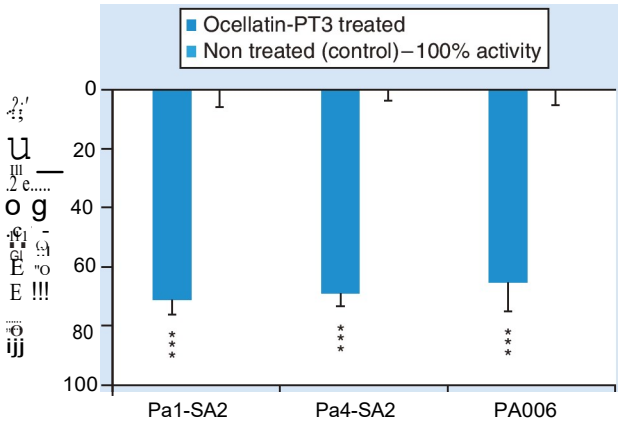


Figure 2. Metabolic activity reduction of *Pseudomonas aeruginosa* biofilms after treatment with ocellatin-PT3. 48-h preformed biofilms of Pa1-SA2, Pa4-SA2 and PA006 were treated for 24 h with a concentration of ocellatin-PT3 corresponding to the respective minimum biofilm inhibitory concentration. The metabolic activity was assessed through the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Differences were statistically significant in relation to the control for a ***p < 0.001.

the bacterial cells within the biofilm. A slight disaggregating effect in the biofilm's structure could also be perceived. Additionally, AFM images (Figure 4) of the biofilms formed and treated in the same conditions as for CLSM analysis reflect also a disaggregating effect on the biofilm caused by the ocellatin-PT3 as well as a direct effect on bacterial cells, which became more wrinkled and seem hollow. In some cases, for example, in the left image in Figure 4A, showing the Pal-SA2 strain under control conditions, single cells were hard to distinguish from each other. This is normal, since AFM only shows the upper surface of the biofilm, which for mature biofilms typically consist more of the polymeric matrix than individual cells. However, after treatment, this biofilm also showed exposed cells, which would further reduce the viability of the biofilm.

Secondary structural studies

Ocellatin-PT3 secondary structures were studied using CD spectroscopy in both the absence and presence of increasing amounts of LPS (Figure 5). This study not only evaluates the structure of the AMP in the solution but also shows its activity against *P. aeruginosa* by interacting with the LPS. The first peak exhibited a λ_{max} at approximately 198 nm and double λ_{min} at approximately 210 and 225 nm. CD measurements of ocellatin-PT3 in water and 0.5% of LPS indicate a random conformation with a minimum close to 198 nm. Nevertheless, when the LPS concentration increases, the shape of CD spectra suggests the predisposition to form defined secondary structures. The CD spectrum at 1.00% of LPS presents one maximum at 192 nm and two minima around 207 and 222 nm, which are characteristic of a-helix structures. The estimated helicities indicated 39% for ocellatin-PT3 in the presence of LPS membrane lipid.

Discussion

AMPs have been largely explored in order to assess their possible use as alternatives to conventional antimicrobial agents. In particular, AMPs derived from frog skin secretions have been the focus of many studies lately, being isolated from frogs belonging to different families, genus and species, and most of them endemic of a particular region in the world [30-32]. Skin secretions of the frog *L. pustulatus* (found in the central Brazilian cerrado biome) furnished a set of AMPs, named ocellatins that differ only in a few amino acid substitutions [19]. Although those ocellatins had shown low antimicrobial activity against *E. coli* ATCC 25922, *S. aureus* ATCC 29313, *K pneumoniae* ATCC 700603, *S. choleraesuis* ATCC 14028, five of them (ocellatin-PT2-PT6) have now shown to be more active against *P. aeruginosa,* especially against MDR isolates of that bacterial species. Others have also described a particular AMP, T9W, to have strong and specific activity against *P. aeruginosa* and low or no activity against other Gram-negative and Gram-positive bacteria [33]. The explanation for the higher activity of ocellatins against drug-resistant isolates than against susceptible strains may be related to membrane permeability/impermeability. It is known that

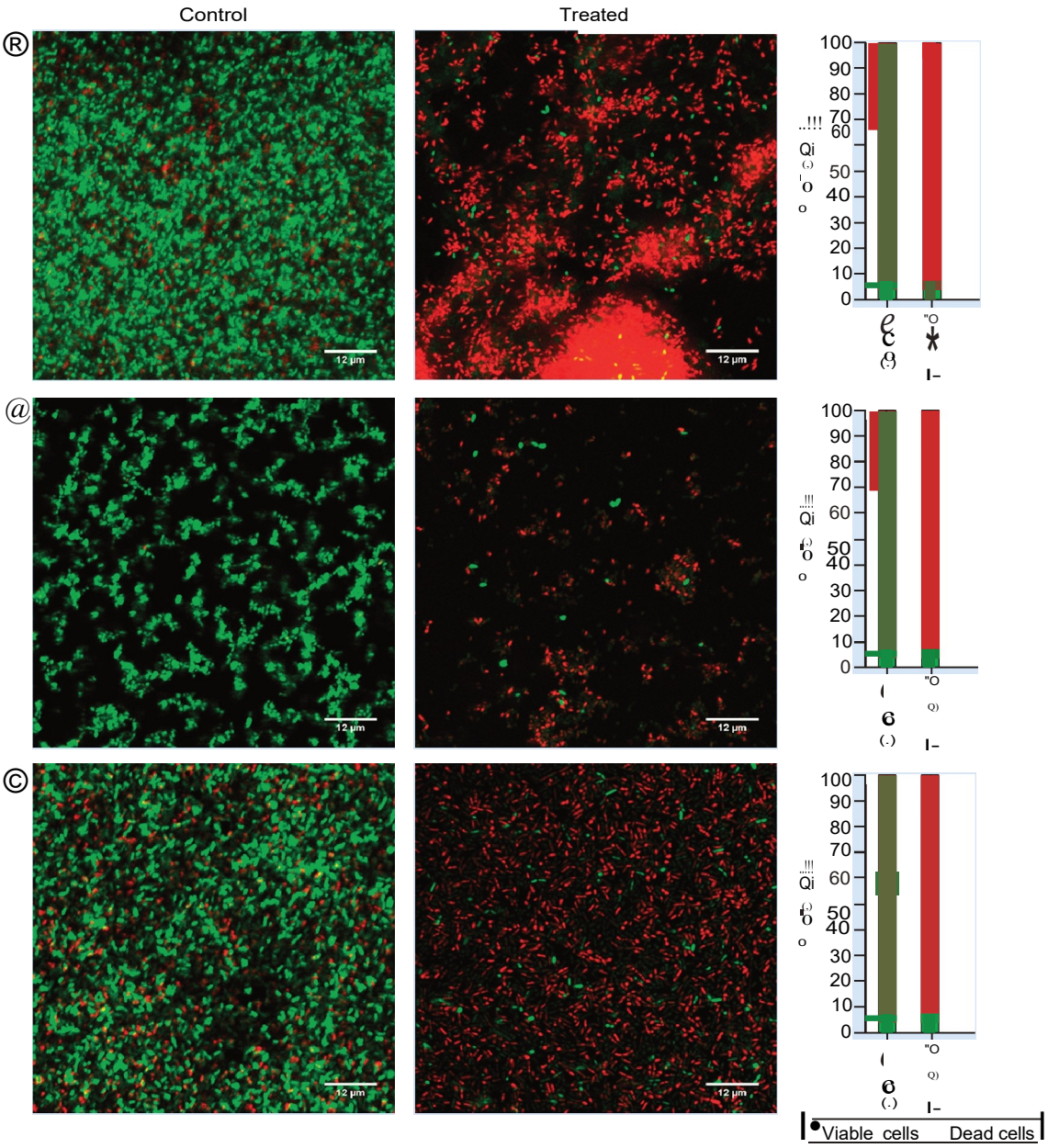


Figure 3. Confocal laser scanning microscopy qualitative evaluation of *Pseudomonas aeruginosa* biofilms not treated (controls) and treated with ocellatin-PT3. 48-h preformed biofilms of Pa1-SA2 (A), Pa4-SA2 (B) and PA006 (C) were treated for 24 h with a concentration of ocellatin-PT3 corresponding to the respective minimum biofilm inhibitory concentration. All biofilms, after live/dead staining, show viable (green fluorescence) and dead (red fluorescence) cells. Percentages of viable and dead cells are also shown; the values are the means ± standard deviation obtained from the counts of six representative images taken from each biofilm that was visualized.

permeability mutations are responsible for increasing the resistance to many classes of antimicrobials (-lactams, Buoroquinolones, aminoglycosides) in *P. aeruginosa* [34], therefore, those mutations, present in the MDR isolates, may revert a natural impermeability to ocellatins, suggesting that the development of resistance to known antibiotics and to ocellatins may be mechanistically independent. Such hypothesis may also explain why the MIC values of ocellatin-PT3 were a little variable depending on the MDR isolate.

The proximity of MBC values to those of MIC (MBC values were equally or twofold higher than the MIC) may foreshadow the bactericidal effect of ocellatins.

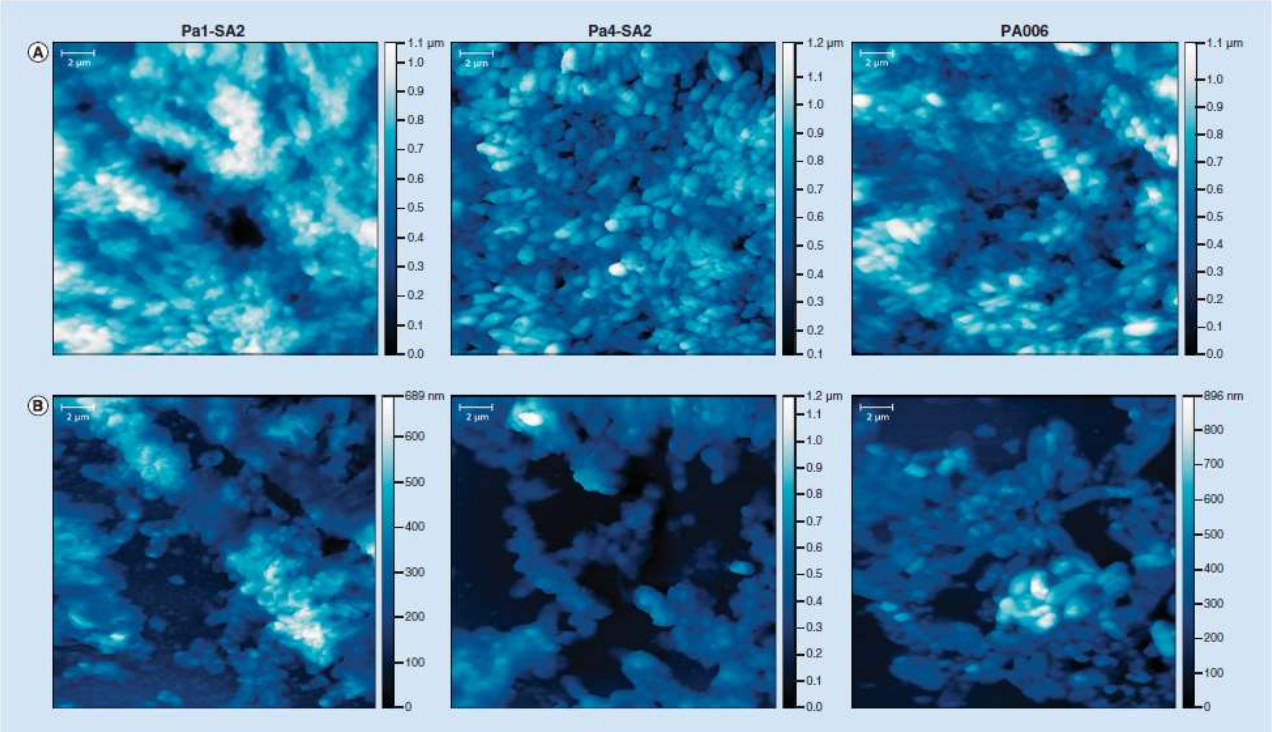


Figure 4. Atomic force microscopy images of *Pseudomonas aeruginosa* biofilms not treated (A) and treated (B) with ocellatin-PT3. 48-h preformed biofilms of Pa1-SA2, Pa4-SA2 and PA006 were treated for 24 h with a concentration of ocellatin-PT3 corresponding to the respective minimum biofilm inhibitory concentration.

A plausible and accepted strategy to treat drug-resistant-associated infections is using a combination of antimicrobial agents, particularly with different mechanisms of action, which would hamper the emergence of resistance. It was therefore appropriate to test ocellatins for potential synergy with different classes of conventional antibiotics against *P. aeruginosa.* Since the treatment of *P. aeruginosa* infections are very challenging, due to their intrinsic resistance to multiple antimicrobials [7], a new agent that can revert the installed resistance to a particular antibiotic might hold promise for novel antipseudomonal therapies. Among the five ocellatin peptides tested, ocellatin-PT3 acted synergistically with ceftazidime and ciproBoxacin against MDR isolates. The mechanisms behind these synergies must be further explored, however, hypothetically, we can suppose ocellatin-PT3 can increase the membrane permeability allowing the entrance of the antibiotic into the cell. Moreover, since the MIC of ocellatin-PT3, when in combination, was lowered itself, it may indicate that the antibiotic may also potentiate other mechanism of action of ocellatin-PT3, probably at the cytoplasmic level.

The growing interest in biofilm treatment by AMPs has been notorious in the recent years. In fact, a database gathering AMPs active on biofilms was created in 2015 [14,35]. Many AMPs have been reported to prevent biofilm formation and/or to eradicate established biofilms, and in some cases the mechanisms beyond those antibiofilm effects have been disclosed or hypothesized [9,15,36]. Herein, we have demonstrated the antibiofilm activity of ocelatin-PT3.

The ability ofocellatin-PT3 to prevent the biofilm formation by MDR P. *aeruginosa* isolates was not marked, since in the presence of 1/2 x MIC a biofilm could be formed even if its biomass was not as abundant as in the respective control biofilm. Moreover, for lower concentrations (l/4 x MIC and 1/8x MIC) even more biofilm biomass was quantified in comparison to the control biofilm. Therefore, ocellatin-PT3 may not present an interesting potential to hamper *P. aeruginosa* biofilm formation.

Nevertheless, its effects on established biofilms were more promising. Ocellatin-PT3 was able to inhibit the proliferation of 48-h mature biofilms in concentrations up to lO x MIC. In the same conditions, ciproBoxacin could not hamper the biofilm proliferation even when present in a concentration of 32 x MIC (at this concentration,

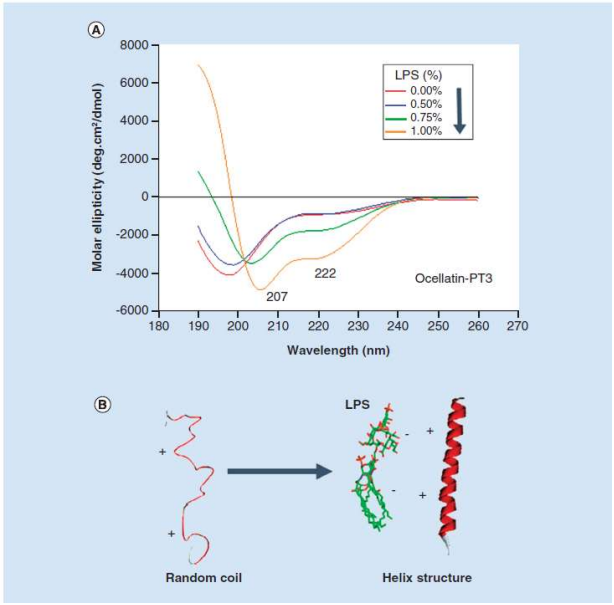


Figure 5. Conformational analysis of ocellatin-PT3 by circular dichroism. (A) Circular dichroism spectra of ocellatin-PT3 in aqueous solution (red line) as well as in the presence of LPS in many concentrations. **(B)** Schematic representation of the behavior of ocellatin-PT3 in the presence of LPS corroborating with the circular dichroism experiment.
LPS: Lipopolysaccharide.

it only reduced in about 50% the proliferation of the biofilm). Indeed, it is commonly accepted that biofilms are more tolerant to antibiotics than are planktonic cells [37].

We could also observe that biofilms treated with ocellatin-PT3 in a concentration equal to the MBIC had a much lower metabolic activity (around 70% less activity in respect to controls). One could suppose that the reduction of metabolic activity may be due to cell death, nevertheless, in the case of biofilms, that correlation cannot be so straightforward, since biofilm-associated bacterial cells can enter a reversible dormant status, in which cells are metabolically inactive [38]. Therefore, in order to explore the effects of ocellatin-PT3 on the biofilms viability, CLSM was used in conjunction with the live/dead-staining technique, revealing in fact a marked decrease in the viability of bacterial cells within the ocellatin-PT3-treated biofilms. Indeed, it has been suggested that AMPs that can reduce or eradicate mature biofilms at concentrations equal or higher than their MIC against the corresponding planktonic cells, are very likely to act by a classical bactericidal effect [9].

The effects of AMPs on the surface structure of microorganisms can be related to their affinities to the lipid composition of the membrane: prokaryotic and eukaryotic membranes vary considerably in lipid contents [28]. Anionic lipids are exposed at the surface of many bacterial membranes. Importantly, *P. aeruginosa* colonization of host tissues is triggered by an initial attachment of the bacterium to epithelial cells, via a variety of surface appendages (e.g., flagella, pili). This is then followed by cell internalization, presumably mediated by the bacterial

LPS (i.e., the major component of the outer membrane in Gram-negative bacteria) [39]. In the presence of LPS isolated from *P. aeruginosa,* the CD spectra of ocellatin-PT3 exhibit helical-like features. Recent studies concluded that cationic peptides can act on Gram-negative bacteria through interaction with LPS [40]. In brief, ocellatin-PT peptides present a solvent (e.g., 2,2,2-triBuoroethanol [TFE]) and/or LPS-dependent helix structure adoption that suggests a change in confirmation upon interaction with microorganisms [28]. Hence, the CD results suggest an interaction between ocellatin-PT3 and LPS isolated from *P. aeruginosa* that may represent the beginning of the mechanism of bactericidal action.

Apart from CLSM, biofilms formed and treated in similar conditions were also visualized by AFM. Noteworthy, the only difference in the preparation of the biofilms for these two techniques regarded the supporting material used for the formation of the biofilms that was ibidi Polymer Coverslips for the CLSM and glass coverslips for AFM. As shown in Figures 3 and 4, a bigger biofilm was formed by Pa4-SA2 on the glass coverslip (AFM image) in comparison to the one formed in the ibidi coverslip. Therefore, it was evident that the type of material had influence on the adhesion of Pa4-SA2 cells, consequently affecting the quantity and type of biofilm formed. While for the other two isolates, the type of supporting material apparently did not account for substantial differences in the biofilms formed.

CLSM and AFM are microscopic techniques widely used in biofilm research as they offer the unique possibility to obtain valuable information on the biofilm structure and organization. In fact, the microscopic analyses showed that ocellatin-PT3 could also affect the biofilms structure, by causing a slight disaggregation. The mechanism beyond this effect can only be speculated at this point; for instance, it may be due to interference with regulatory signals or interference with the accumulation of matrix components [9].

Conclusion & future perspective

Over the last years, AMPs have gained increasing attention as potential novel antimicrobial drugs alternatives for combating infections caused by antibiotic-resistant bacteria and/or associated to biofilms. Thus, new AMPs with such potential must be reported and further explored to create advances in the overall goal to overcome the problem of AMR.

Ocellatin-PT3 may be promising as a lead molecule for the design and development of novel AMPs with significant activity and selectivity against MDR *P. aeruginosa* biofilms.

Summary points

- In this study, five ocellatin peptides, originally isolated from the skin secretion of *Leptodactylus pustulatus,* were explored for their potential use against the opportunistic pathogen *Pseudomonas aeruginosa*.
- The ocellatins tested were more active against multidrug-resistant *P. aeruginosa* isolates than against susceptible strains.
- Ocellatin-PT3 showed synergistic effects with ciprofloxacin and ceftazidime, which might hold promise for developing future combinatorial therapies after uncovering the mechanisms triggering such synergy.
- Ocellatin-PT3 could inhibit the proliferation of established biofilms at concentrations from 4 to 10x the MIC, which was mostly due to a direct killing effect on the bacterial cells within the biofilm as shown by confocal laser scanning microscopy after live/dead staining.
- Atomic force microscopy images of ocellatin-PT3-treated biofilms also reveal membrane alteration of *P. aeruginosa* cells.
- Conformational analysis by circular dichroism of ocellatin-PT3 in the presence of lipopolysaccharide (LPS) let foresee that such peptide interacts with the LPS isolated from *P. aeruginosa*.
- Interaction with lipopolysaccharides may constitutes the the initial step of its main mechanism of action that is likely to be the disruption of the cell membrane integrity.
- Ocellatin-PT3 may be explored for the design and development of novel antimicrobial peptides.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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