An Acad Bras Cienc (2021) 93(Suppl. 4): e20210362 DOI 10.1590/0001-3765202120210362
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

BIOMEDICAL SCIENCES

De novo design of short antimicrobial lipopeptides

VANESSA POSADA, BLANCA FABIOLA ESPEJO & SERGIO ORDUZ

Abstract: The increase in bacterial resistance to antibiotics available leads to the search for new compounds with antimicrobial potential, such as peptides and lipopeptides. In this work, eight short lipopeptides with the structural pattern Cn-X_1 X_2 X_3-NH_2 were *de novo* designed, synthesized by Fmoc solid phase and characterized by instrumental techniques. The results of the *in vitro* tests indicated that two of them, LIP 4 and LIP 12 display antibacterial activity against 4 pathogenic bacteria with minimum inhibitory concentrations (MIC) between 9.50 and 100 μM and between 8.50 and 10.0 μM, respectively; they did not displayed toxicity to human erythrocytes at concentrations between 3.13 and 50.0 μM. The antibacterial mechanism of action observed by scanning electron microscopy indicate that the cell membrane was the target, causing the formation of blisters and vesicles, with size ranging from 100 to 120 nm. The lipopeptide LIP 12, with higher activity, was stable to proteases of human blood serum.

Key words: antimicrobial activity, *de novo* design, Fmoc synthesis, protease stability, short lipopeptides

INTRODUCTION

Infectious diseases caused by bacteria represent a public health problem worldwide, despite the availability of antibiotics. The number of cases of bacterial infections has increased in the last decade due to the emergence of resistance which has led to an increase in the mortality rate (WHO 2014), mainly due to infections caused by bacteria resistant to antibiotics such as *Klebsiella pneumoniae*, *Escherichia coli* and *Staphylococcus aureus*, among others (WHO 2017). This situation indicates the need to search for new antimicrobial compounds with alternative mechanisms of action.

Previous studies show that antimicrobial peptides (AMPs) display biological activity, including antimicrobial, anticancer (Hoskin & Ramamoorthy 2008, Gaspar et al. 2013), antiviral and immunomodulatory, among others (Conlon et al. 2014). Recent research indicates that AMPs are a promising alternative for the treatments of resistant bacteria; for instance, peptides WR12 and D-IK18 were active against methicillin (MRSA) and vancomycin resistant (VRSA) *S. aureus* (Mohamed et al. 2016), while peptides temporin A, citropin 1.1, CA(1-7)M(2-9)NH_2 and Pal-KGK-NH_2 showed antimicrobial activity against MRSA *S. aureus* biofilms formed in polystyrene and medical devices when used in synergistic combinations (Ciandrini et al. 2020).

Most AMPs are small, positively charged, amphipathic molecules that are part of the innate immunity of all living organisms and interact electrostatically and hydrophobically with the pathogen's cell membrane, destabilizing it and causing its death (Peters et al. 2010) and the microorganisms have not developed effective mechanisms of resistance in comparison with the antibiotics available in

the market (Yu et al. 2018). On the other hand, the design of modified peptides has great potential in the search for new drugs (Fosgerau & Hoffmann 2015, Reinhardt & Neundorf 2016), which can be done by modifying a precursor peptide, changing amino acids in the sequence or alternating their order, eliminating amino acids or changing some or all to the D form, in order to increase the antimicrobial activity and decrease the susceptibility to proteases; other modifications include the addition of sugars or lipids, such as lipopeptides, potentiating their activity (Nasompag et al. 2015), or by *de novo* design, simulating the main characteristics of AMPs such as cationicity, amphipathicity and/or formation of secondary structures (Ong et al. 2014, Khara et al. 2017). The lipopeptides have a fatty acid conjugate at the N-terminal of a cyclic or linear peptide sequence by means of a covalent bond (Laverty et al. 2010, Etchegaray & Machini 2013); and may be of natural or synthetic origin. Recent studies indicate that some lipopeptides are antimicrobials (Cochrane et al. 2016), antifungals (Mnif et al. 2015), insecticides (Yang et al. 2017) and antitumor (Domalaon et al. 2016). Synthetic lipopeptides generally have a short linear peptide sequence, with 5 amino acids or less, linked to a fatty acid with a chain length between 8 and 16 carbon atoms, which provides the amphipathic characteristics, similar to the antimicrobial peptides (Makovitzki et al. 2006, Domalaon et al. 2014, Nasompag et al. 2015).

The lipopeptides of natural origin have no selective activity and can cause high toxicity in mammalian cells; therefore, synthetic lipopeptides generate high expectations as an alternative to AMPs and lipopeptides of natural origin, since they show low cytotoxicity, high retention time and greater stability to proteases (Laverty et al. 2010, Domalaon et al. 2014, Nasompag et al. 2015), and have demonstrated to be promising molecules against MRSA strains. Recently, it was shown that short dialkyl lipopeptides display antibacterial activity by generating changes in cell morphology, probably caused by cell membrane perturbations, in MRSA strains (Greber et al. 2020). Some lipopeptides in solution could behave in a similar manner to antimicrobial peptides, since they can form micelles to reach high local concentrations with bactericidal effect (Chongsiriwatana et al. 2011). The use of a few amino acids including Ornithine linked to the fatty acids provide stability, maintaining the antimicrobial activity at low concentrations (Meir et al. 2017, Pan et al. 2020).

In this work, we de novo designed, synthesized and evaluated 8 lipopeptides, some of which showed activity against Gram-positive and Gram-negative bacterial species at low micromolar concentrations and were stable in human blood serum.

MATERIALS AND METHODS

Materials

The Rink amide AM resin and the amino acids Fmoc-Gly-OH, Fmoc-Orn(Boc)-OH, Fmoc-Lys(Boc)-OH and Fmoc-Leu-OH were obtained from Novabiochem. The lauric and myristic acids were obtained from Merck. All reagents and solvents used were of high purity grade such as N,N-dimethylformamide (DMF), dichloromethane (DCM), isopropanol (IPA), 4-methyl-piperidine, dicyclohexylcarbodiimide (DCC), *O*-hydroxybenzotriazole (HOBt), trifluoroacetic acid (TFA) and acetonitrile (ACN) from Merck.

Lipopeptides design and synthesis

The lipopeptides sequences were *de novo* designed with the following structural pattern, C_n-X_1-X_2-X_3-NH_2, where C_n is a fatty acid, lauric

acid (C_{12}) or myristic acid (C_{14}). X_1, corresponds to one or two molecules of glycine (Gly), as a linker. X_2, corresponds to the amino acids that provides hydrophilicity and net positive charge by the incorporation of ornithine and/or lysine and X_3 represents, for some of the lipopeptides, 2 leucine residues.

The lipopeptides were manually synthesized by Fmoc solid phase using the Rink amide AM resin (100-200 mesh) with a degree of substitution of 0.74 mmol/g. The coupling reaction of the amino acids protected with the Fmoc group was made with the activators DCC and HOBt with five times molar excess of each amino acid and activator, dissolved in DMF. Deprotection was carried out with 25% (v/v) of 4-methyl-piperidine in DMF and the coupling reaction was repeated as many times as necessary until the peptide sequence was formed. The traditional Fmoc synthesis for antimicrobial peptides was adapted to the synthesis of lipopeptides through the binding of a fatty acid to the N-terminus of the peptide sequence, using the DCC and HOBt activators with a five times molar excess of each reagent, followed by lipopeptide de-anchoring with TFA/TIS/H_2O in a volume ratio (95: 2.5: 2.5).

Lipopeptides purification and characterization

Purity of the lipopeptides was analyzed by reverse phase high performance liquid chromatography (RP-HPLC) in an Agilent Technologies 1200 Series chromatograph with a UV-VIS detector at 220 nm fitted with a Zorbax Eclipse RP-18 XDBC18 analytical column (150 x 4.6 mm, 5 µm pore diameter). Lipopeptides were eluted with a ACN : TFA (0.1%) and water : TFA (0.1%) linear gradient for 21 minutes at room temperature. Injection volume was 20 µL at 1 mg/mL. Lipopeptides composition was confirmed by ESI-MS (electrospray ionization mass spectrometry) in a Shimadzu model 2020 mass spectrometer. The secondary structure of the lipopeptides were determined by circular dichroism (CD) on a Jasco spectropolarimeter model J-810, with a quartz cell and optical length of 1.0 mm. The lipopeptides were prepared at a concentration of 5.0 µM in a 30% (v/v) solution of 2,2,2-trifluoroethanol (TFE) and measurements were taken in a range of 190 to 260 nm at room temperature, with a bandwidth of 0.5 nm and a scanning speed of 50 nm/min.

Antibacterial activity

Bacterial strains used were *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853. Bacterial strains were seeded on nutrient agar and incubated at 37 °C overnight and the colonies were harvested and grown in Luria Bertani broth at 37 ºC with constant agitation at 100 rpm until obtaining an inoculum in the logarithmic growth phase.

The antimicrobial activity of the lipopeptides was determined by turbidimetry by microdilution in 96-well plates using the protocol described by Wiegand et al. (2008). One hundred µL of the bacterial suspension containing $1x10^6$ CFU/mL was added to 100 µL of lipopeptide solution in each well to obtain final concentrations of 100, 50, 25, 12.5 and 6.25 µM and a cell concentration of $1x10^5$ CFU/mL. Absorbance readings were performed for 20 hours at hour intervals on a MultiSkan Go plate reader (Thermo Scientific) at 600 nm with incubation at 37 °C and shaking for 10 seconds every hour. Ampicillin (0.25 mg/L) and a bacterial culture without treatment were used as controls. The tests were performed on 2 different days with 2 replicates of each lipopeptide concentration used.

Minimal inhibitory concentration

The lipopeptides that inhibited bacterial growth at a concentration less than or equal to 25.0 µM were evaluated using the same microdilution method in 96-well plates as described above to obtain the minimal inhibitory concentration (MIC). The tests were performed on 2 different days with 2 replicates each. The MIC is the lowest lipopeptide concentration that totally inhibits the growth of a bacterium. To determine the bacteriostatic or bactericidal effect of the active lipopeptides, 100 µl of the MIC wells were plated on LB agar and incubated for 24 hours at 37°C.

Hemolysis assay

Human erythrocytes were obtained from a healthy voluntary donor with EDTA. The blood sample was centrifuged at 1000 x g at room temperature for 7 minutes (Evans et al. 2013, Zhang et al. 2017). The pellet was washed three times with sterile 0.9% saline solution by centrifuging at 1000 x *g* and discarding the supernatants. A 1:10 suspension of erythrocytes in saline solution was prepared and 90 µL of the diluted suspension of erythrocytes and 10 µL of each lipopeptide were added to obtain final concentrations of 200, 100, 50, 25, 12.5, 6.25 and 3.13 µM. The suspensions were incubated at 37 °C for 3 hours at 90 rpm and centrifuged at 1000 x *g* at room temperature for 5 minutes. Fifty microliters of the supernatant were taken and transferred to a 96-well plate and the absorbance was read at 545 nm. Ten microliters of a 0.1% solution of Triton X-100 in the erythrocyte suspension were used as a positive control, which corresponds to 100% hemolysis and as a negative control 10 µL of a 0.9% sterile saline solution were used (Evans et al. 2013). The assays were performed by triplicate for each evaluated lipopeptide and concentration. Percent hemolysis was calculated for a given lipopeptide concentration with the following equation:

% hemolysis = (OD LIP - OD 0.9% NaCl / OD 0.1% Triton X-100 - OD 0.9% NaCl)

Bacterial cell morphology by Scanning Electron Microscopy

The morphological changes of the bacterial cell membrane were observed at different times after treatment with the lipopeptides. Four hundred microliters of the bacterial cultures containing $1x10^6$ CFU/mL were added to a 24-well plate and 400 µL of the solution of each lipopeptide to obtain a final concentration of 2 x MIC and a cell concentration of $1x10^5$ CFU/mL. Treatments were incubated at 37 °C with constant agitation of 90 rpm and then filtered with 1.3 mm diameter cellulose membranes with 0.2 µm pore size at 30 min, 120 min and 20 hours after adding each lipopeptide. A bacterial suspension without treatment was used as growth control and filtered after 20 hours. Membranes were treated with 2.5% glutaraldehyde overnight at 4 °C, followed by dehydration with increasing ethanol concentration and membranes were air dried for 1 day at room temperature according to the protocols reported by Marcellini et al. (2010) and O'Driscoll et al. (2013). Samples were coated with a gold layer in a Quorum Technologies coating model Q150 and observed in a Carl Zeiss EVO MA10 scanning electron microscope (SEM).

Enzymatic treatment of lipopeptides

1.5 mg of LIP 12 were added to 1000 µL of RPMI (Roswell Park Memorial Institute) supplemented with 25% (v/v) of human blood serum and incubated at 37 °C for 15 minutes. A 100 µL aliquot of the solution was taken and transferred to eppendorf tubes after 0, 1, 2, 3, 4, 8 and 24 hours of treatment and 200 µL of 96% ethanol were added. Samples were left at 4 °C for 15 minutes and centrifuged at 18,000 x g for 2 minutes. The supernatant was analyzed by reverse phase HPLC on an Agilent Technologies 1200 Series

chromatograph with a UV-VIS detector and fitted with a Zorbax Eclipse RP-18 XDBC18 150 mm x 4.6 mm column with a pore diameter of 5 µm (Jenssen & Aspmo 2008), using ACN: TFA (0.1%) and H_2O: TFA (0.1%) as the mobile phase.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (Universidad Nacional de Colombia sede Medellin, Ethical Committee authorization CEMED – 071) and with the Helsinki declaration of 1975 revised in 2000. Informed consent was obtained from the blood donor for being included in the study.

RESULTS AND DISCUSSION

Lipopeptides design

A set of eight short linear lipopeptides were designed based on the structural pattern C_n-X_1 X_2 X_3-NH_2, where C_n is lauric or myristic acid and X_n corresponds to a set of 3 to 5 amino acids, including Gly, Orn, Lys or Leu (Fig. 1). The fatty acids were conjugated to the N-terminal end of the peptide sequences (Laverty et al. 2010, Lohan et al. 2014, Nasompag et al. 2015). The designed lipopeptides contain one or two glycine molecules as a linker (X_1) between the fatty acid and the amino acid sequence (He et al. 2009), separating the hydrophilic and charged region from the hydrophobic region, increasing the affinity of the lipopeptide for the surface of the bacterial cell membrane (Chu-Kung et al. 2010).

Most antimicrobial peptides have less than 30 amino acids and are cationic and amphipathic, but the presence of Lys and Arg turn them susceptible to serine proteases (Wadhwani et al. 2017). Therefore, in this work Orn was included in some of the lipopeptides in the X_2 region to provide positive charge and to avoid proteolytic degradation (Bisht et al. 2007, Berthold et al. 2013); finally, some of the lipopeptides have two molecules of leucine (X_3) to increase the hydrophobicity.

Synthesis and structural characterization of the designed lipopeptides

The purity of the eight lipopeptides synthesized was greater than 90%. The identity of each lipopeptide was confirmed by ESI-MS, in which the calculated molecular weight corresponds to the observed molecular weight + H^+, indicating that the synthesis was performed correctly (Table I). Circular dichroism analysis carried out by diluting the lipopeptides in 30% trifuloroethanol (TFE) to stabilize secondary structures formation having a high dipole moment (Naumenkova et al. 2010, Tiburu et al. 2017) indicated that, in general, the designed lipopeptides showed a probable random coil secondary structure according to the CD spectra, with two negative bands at 200 nm and 220 nm (Fig. 2).

Antibacterial activity

In general, lipopeptides containing lauric acid (LIP 1, LIP 2, LIP 3 and LIP 4) were less active than the lipopeptides containing myristic acid (LIP 5, LIP 6, LIP 11 and LIP 12). It should be noted that the lipopeptides LIP 4, LIP 5, LIP 6, LIP 11 and LIP 12 were active against Gram-positive bacteria, possibly by being more hydrophobic than LIP 1, LIP 2 and LIP 3 (Table II), similar to that of the lipopeptide Daptomycin and the glycopeptide Vancomycin, who are selective for Gram-positive bacteria (Mascio et al. 2007, Azmi et al. 2016). The bactericidal/bacteriostatic activity of the lipopeptides was determined by plating the results of the MIC´s. The lipopeptides showed a differential behavior depending on the bacterial species tested. LIP4 was bactericidal in *S. aureus* and *E. faecalis* but it was bacteriostatic in *P. aeruginosa*; on the other hand, LIP12 showed a bactericidal effect on all the Gram-positive and

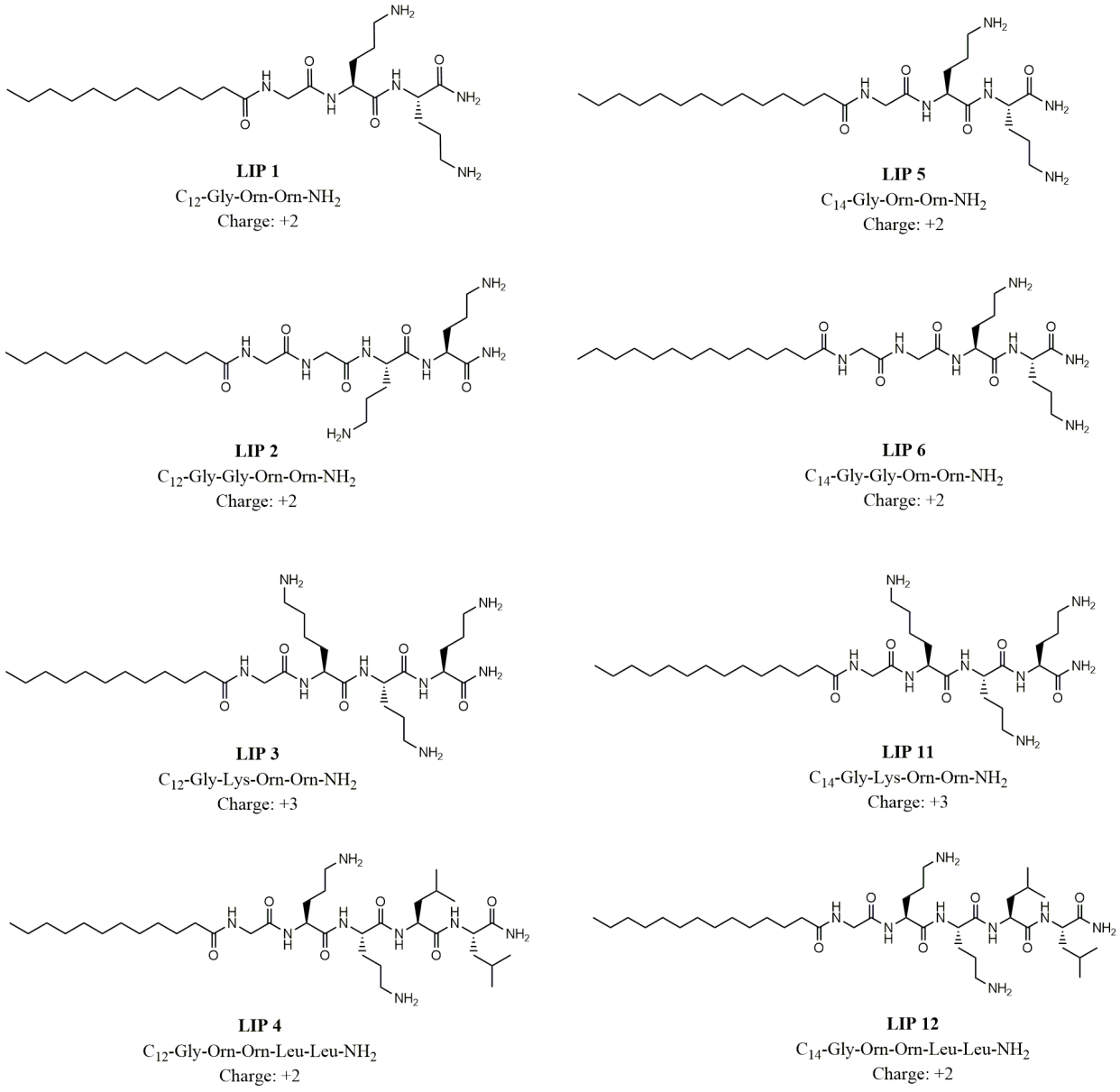

Figure 1. Molecular structures of the eight de novo designed lipopeptides.

Gram-negative bacteria between 8.5 and 10 μM (Table II) and similar to the modified lipopeptide C_{14}Lys-LysC_{12}Lys (Meir et al. 2017).

The presence of one or two molecules of glycine as a linker between the fatty acid and the peptide sequence did not cause significant differences in the antimicrobial activity between LIP 1 and LIP 2 nor between LIP 5 and LIP 6. On the other hand, LIP 4 and LIP 12 were designed with increased hydrophobicity with two leucine residues at the C-terminal region of the peptide sequence, and as a result, not only were active at lower concentrations, but these two lipopeptides were active against Gram-positive and Gram-negative bacteria, so they could be considered as broad-spectrum antimicrobial compounds.

It was also observed that the antimicrobial activity was also influenced by the number of carbon atoms of the fatty acid conjugated to

Table I. Sequence and physicochemical characterization of designed and synthesized short lipopetides.

Lipopeptide	Sequence	Net charge	HI [1] (% ACN)	Purity [2] (%)	M [3] (Da)	M+H [4] (Da)
LIP 1	C_{12}-Gly-Orn-Orn-NH_2	+2	43.6	93.63	484.4	485.4
LIP 2	C_{12}-Gly-Gly-Orn-Orn-NH_2	+2	43.7	92.30	541.7	542.4
LIP 3	C_{12}-Gly-Lys-Orn-Orn-NH_2	+3	40.9	93.92	612.5	613.5
LIP 4	C_{12}-Gly-Orn-Orn-Leu-Leu-NH_2	+2	49.7	97.13	710.5	711.5
LIP 5	C_{14}- Gly-OrnOrn -NH_2	+2	49.6	96.21	512.4	513.4
LIP 6	C_{14}- Gly-Gly-Orn-Orn -NH_2	+2	49.9	90.47	569.8	570.5
LIP 11	C_{14}- Gly-Lys-Orn-Orn -NH_2	+3	46.6	96.39	640.5	641.5
LIP 12	C_{14}- Gly-Orn-Orn-Leu-Leu -NH_2	+2	55.6	91.93	738.6	739.6

[1]hydrophobicity index calculated with the percentage of ACN. [2]percent of purity calculated with the percent of relative area in the chromatograms. [3]expected mass. [4]observed mass by LC-MS.

the N-terminal of the peptide sequence and the increase in hydrophobicity of the peptide chain by addition of two molecules of leucine, significantly increased the activity of LIP 4 and LIP 12 compared to lipopeptides synthesized without leucine (LIP 1 and LIP 5). In general, it was observed that the 4 lipopeptides that have myristic acid displayed activity against Gram-positive bacteria, unlike those that contain lauric acid, with the exception of LIP 4, that containing the 2 leucines, became more hydrophobic and its antimicrobial activity increased.

Lipopeptides LIP 4 and LIP 12 have a molecular weight of 710.5 Da and 738.5 Da respectively, being smaller than the natural lipopeptides found on the market such as Polymyxin B (1301.5 Da) and Daptomycin (1619.7 Da), but similar in size to the synthetic lipopeptides C_{12}-Orn-Orn-Trp-Trp-NH_2 with 799.5 Da (Laverty et al. 2010), C_{14}-Lys-Tyr-Arg-NH_2 with 674.9 Da (Nasompag et al. 2015) and others designed and synthesized by Lohan et al. (C_{14}-Orn-Orn-Orn-NH_2) having a molecular weight around 700 Da (Lohan et al. 2014) or between 440 and 767 Da as those designed by Greber et al. (2017).

Hemolytic activity

Lipopeptides LIP 4 and LIP 12 showed the highest antimicrobial activity but their hemolytic activity was less than 5% in a range of concentrations between 3.13 and 50 µM; however, their hemolytic activity increased when LIP 4 and LIP 12 were tested at 100 and 200 µM reaching 38 and 58% for LIP 4 and LIP 12 respectively (Fig. 3), indicating that LIP 4 and LIP 12 at lower concentrations have high selectivity towards bacterial cells. Triton X-100, 0.1 % produced 100% hemolysis.

Effect of lipopeptides on bacterial cell morphology

The effect of LIP 4 and LIP 12 on the bacterial morphology at 2x their MICs was observed by

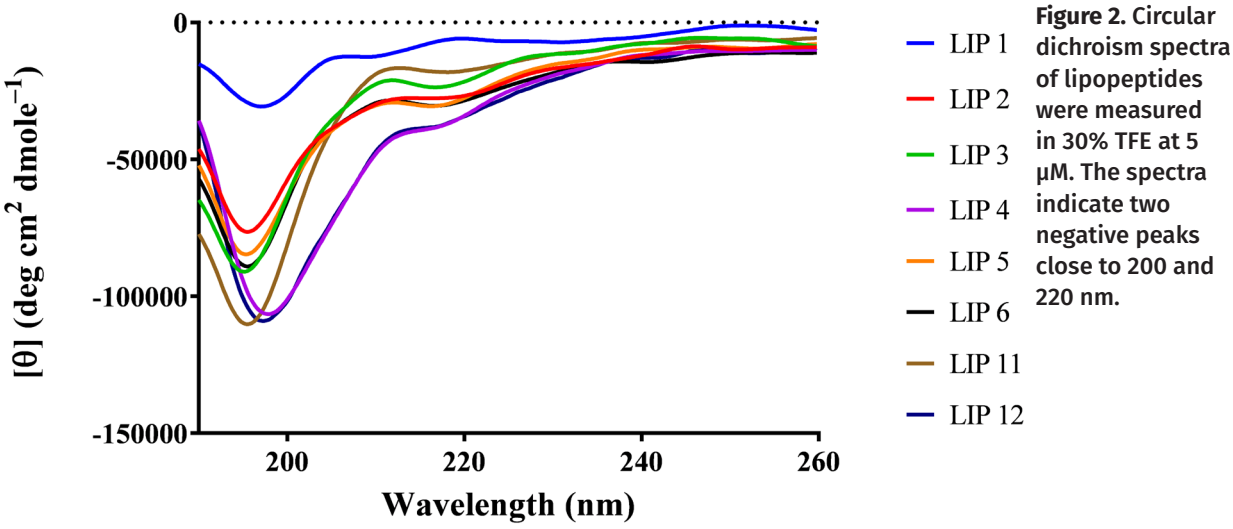

Figure 2. Circular dichroism spectra of lipopeptides were measured in 30% TFE at 5 µM. The spectra indicate two negative peaks close to 200 and 220 nm.

SEM. The growth controls of *P. aeruginosa, S aureus, E. faecalis* and *E. coli* indicate that after growth at 37 °C for 20 hours, the surface of the cells is complete, smooth and the cells remain turgid (Fig. 4). After treating *P. aeruginosa* cells for 30 minutes with LIP 4 at 19 µM, little microbial growth was observed and cells showed slight deformation of the cell membrane, whereas in the treatment with LIP 12 at 17 µM, wrinkles in the cell membrane were observed after 30 minutes; additionally, vesicle formation with diameter between 100 and 200 nm were also observed; and finally, in bacterial cells treated with LIP 4 for 2 and 20 hours, not complete cells were observed, as well as in *P. aeruginosa* treated with LIP 12 for 20 hours, indicating that cell lysis occurred and remains of cytoplasmic material were observed on the cellulose membrane.

Cells of *S. aureus* treated with LIP 12 at 20 µM after 30 minutes of exposure were scarce, and remains forming vesicles of 100 to 120 nm were observed; while after 2 and 20 hours of exposure to LIP 12, complete destruction of the cells were observed (Fig. 4).

Damage to the membrane of *E. faecalis* caused by treatment with 19 µM of LIP 12 was similar to that observed in *S. aureus*. During the first 30 minutes, vesicles and deformation of the cell surface was observed, after 2 hours of exposure to LIP 12 an increase of vesicles formation was observed, with size between 100 and 120 nm; and finally, after 20 hours, only vesicles and cytoplasmic material was observed (Fig. 4).

E. coli cells treated with 17 µM of LIP 12 show blister formation after 30 minutes of treatment and a large number of aggregates of vesicles of approximately 80 nm in diameter that increases after 2 hours of exposure. At this time, the surface of cells became rough with dents and residues of cytoplasmic material were observed on the cellulose membrane. Finally, after 20 hours of treatment with LIP 12, a few cells were observed with vesicles and dents.

According to the SEM images of *S. aureus*, *E. faecalis*, *P. aeruginosa* and *E. coli*, treated with lipopeptides LIP 4 and LIP 12 a surfactant effect on the cell membrane was observed. Recent studies have shown SEM and TEM micrographs that indicate that the damage in the bacterial membrane of *E. coli* caused by the synthetic lipopeptide C_{14}-Orn-Orn-Orn-NH_2 (Lohan et al. 2014), has a degree of similarity with the damage caused by LIP 12 in *E. coli*, which is also similar to the damage caused by peptide P11-5 in *S. aureus* (Qi et al. 2010); additionally, a comparison of

Table II. Antimicrobial activity of *de novo* designed short cationic lipopeptides. Minimum inhibitory concentrations (MICs) in micromolar concentrations and µg/mL and bactericidal/bacteriostatic effects.

Lipopeptide	Minimum Inhibitory Concentration *µM* / µg/mL			
	***S. aureus* ATCC 25923**	***E. faecalis* ATCC 29212**	***P. aeruginosa* ATCC 27853**	***E. coli* ATCC 25922**
LIP 1	> 100 / > 48.4	100 / 48.4**	> 100 / > 48.4	> 100 / > 48.4
LIP 2	> 100 / > 54.2	> 100 / > 54.2	> 100 / > 54.2	> 100 / > 54.2
LIP 3	> 100 / > 61.3	> 100 / > 61.3	> 100 / > 61.3	> 100 / > 61.3
LIP 4	30.0 / 21.3*	50.0 / 35.5*	9.50 / 6.75**	100 / 71.1
LIP 5	50.0 / 25.6*	12.5 / 6.41*	> 100 / > 51.2	100 / 51.2*
LIP 6	50.0 / 28.5*	20.0 / 11.4**	100 / 57.0**	> 100 / > 57.0
LIP 11	50.0 / 32.0*	25.0 / 16.0*	>100 / > 64.1	>100 / > 64.1
LIP 12	10.0 / 7.39*	9.50 / 7.02*	8.50 / 6.28*	8.50 / 6.28*

***Bactericidal effect. **Bacteriostatic effect.**

the membrane damage caused by lipopeptides LIP 4 and LIP 12 show similarities with the damage attributed to the natural lipopeptide Polymyxin B, in which disturbance in the outer membrane and escape of cytoplasmic material was observed in 3 different studies by SEM in *K. pneumoniae* cells (Rahim et al. 2015, Scavuzzi et al. 2016, Sharma et al. 2017).

The micrographs of this study show the morphological changes in the membranes of bacteria, mainly by the formation of vesicles or blisters and dents produced at different times of exposure to the lipopeptides, in a similar manner as Vancomycin in Gram-positive bacteria (Liu et al. 2009, Azmi et al. 2016). Cell membrane damage in Gram-negative bacteria is possibly due to initial electrostatic interaction with phospholipids head groups and in Gram-positive bacteria to electrostatic interactions of lipopeptides with teichoic and lipoteichoic acids and with peptidoglycans, which are formed by alternating polymers of N- acetylglucosamine and N-acetylmuramic acid linked by β-1,4 bond (Liu et al. 2009). Therefore, the damage caused by the designed lipopeptides in the bacterial membrane occurs in the initial 120 minutes and subsequently cause structural disruption and cell death, confirming the mode of action of lipopeptides according to their amphiphilic properties (Qi et al. 2010, Chen et al. 2012, Lohan et al. 2014, Nasompag et al. 2015, Azmi et al. 2016).

Lipopeptide stability to proteases

To determine the blood serum stability of the lipopeptides attributed to the presence of the amino acid Ornithine (Lohan et al. 2014), LIP 12, the lipopeptide with greater antibacterial activity was treated with human blood serum. It was observed that LIP 12 shows no signs of degradation when incubated with blood serum at 37 °C for 24 hours, since the peak areas corresponding to the lipopeptide in the chromatograms (t_R: 15.9 minutes approximately), do not have significant changes during the test, and the formation of new peaks corresponding to degradation products was not observed.

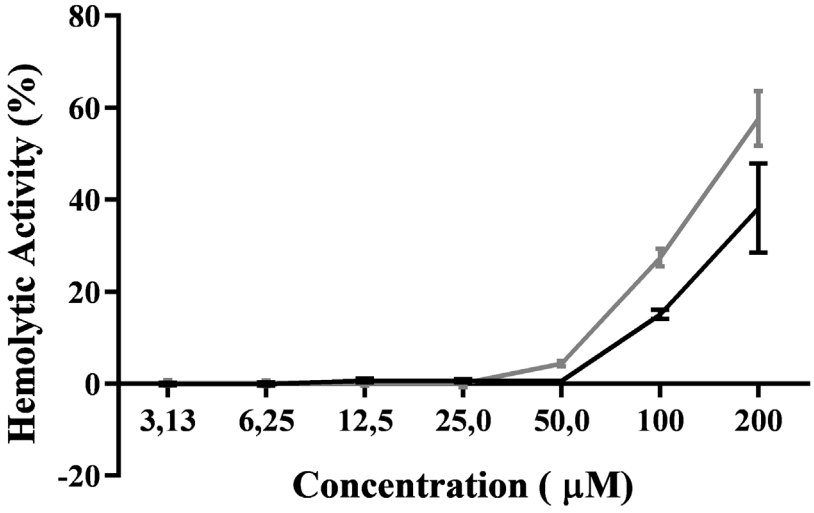

Figure 3. Hemolytic activity of LIP 4 and LIP 12. The lipopeptides were evaluated in a concentration range between 3.13 and 200 µM in human erythrocytes suspended in 0,9% saline solution. The hemoglobin released by cell lysis was measured in a microplate reader at 545 nm.

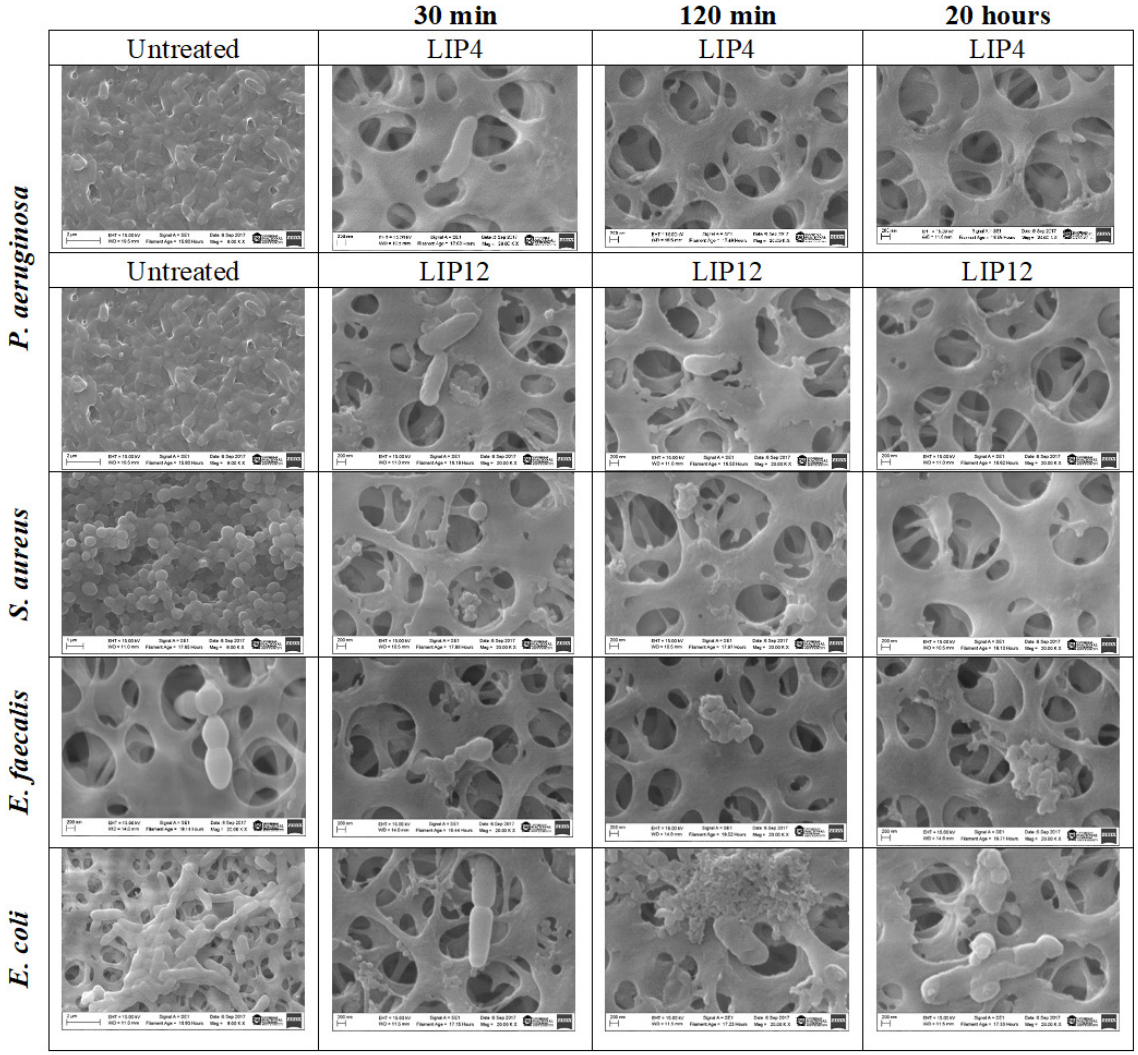

Figure 4. Scanning electron micrographs of *E. coli*, *E. faecalis*, *S. aureus* and *P. aeruginosa* cells untreated and treated with LIP 4 and LIP12 for 30 minutes, 120 minutes and 2 hours at 2 times their MICs.

CONCLUSIONS

The *de novo* design of short antimicrobial lipopeptides proved to be a valuable alternative in the search for new antibiotics in the age of increasing bacterial resistance worldwide. The active lipopeptides of this study have low molecular weight (710.5 Da and 738.5 Da), compared to Daptomycin or Polymyxin B. This study shows that lipopeptides LIP 4 and LIP 12 display antimicrobial activity against Gram positive and Gram-negative bacteria at low micromolar concentrations and cause damage to the bacterial cell membrane. Additionally, LIP 12 demonstrated *in vitro* stability to blood serum proteases. It should be noted that the hydrophobicity of the fatty acids in the sequences of these lipopeptides is determinant in the activity, presenting a mechanism of action similar to the AMPs that cause disruption of the cell membrane, inducing formation of vesicles and blebs, leakage of cytoplasmic material, lysis and therefore cell death.

Acknowledgments

This work was funded by Universidad Nacional de Colombia sede Medellín, Hermes Projects 35058, 39323 and 49011. Authors declare they have no conflict of interest with the information contained in this manuscript.

REFERENCES

AZMI F, ELLIOTT AG, MARASINI N, RAMU S, ZIORA Z, KAVANAGH AM, BLASKOVICH MAT, COOPER MA, SKWARCZYNSKI M & TOTH I. 2016. Short cationic lipopeptides as effective antibacterial agents: Design, physicochemical properties and biological evaluation. Bioorganic Med Chem 24: 2235-2241.

BERTHOLD N, CZIHAL P, FRITSCHE S, SAUER U, SCHIFFER G, KNAPPE D & ALBER G. 2013. Novel Apidaecin 1b analogs with superior serum stabilities for treatment of infections by Gram-Negative pathogens. Antimicrob Agents Chemother 57: 402-409.

BISHT GS, RAWAT DS, KUMAR A, KUMAR R & PASHA S. 2007. Antimicrobial activity of rationally designed amino terminal modified peptides. Bioorganic Med Chem Lett 17: 4343-4346.

CHEN C, HU J, ZHANG S, ZHOU P, ZHAO X, XU H, ZHAO X, YASEEN M & LU JR. 2012. Molecular mechanisms of antibacterial and antitumor actions of designed surfactant-like peptides. Biomaterials 33: 592-603.

CHONGSIRIWATANA NP, MILLER TM, WETZLER M, VAKULENKO S, KARLSSON AJ, PALECEK SP, MOBASHERY S & BARRON AE. 2011. Short alkylated peptoid mimics of antimicrobial lipopeptides. Antimicrob Agents Chemother 55: 417-420.

CHU-KUNG AF, NGUYEN R, BOZZELLI KN & TIRRELL M. 2010. Chain length dependence of antimicrobial peptide-fatty acid conjugate activity. J Colloid Interface Sci 345: 160-167.

CIANDRINI E, MORRONI G, CIRIONI O, KAMYSZ W, KAMYSZ E, BRESCINI L, BAFFONE W & CAMPANA R. 2020. Resistance synergistic combinations of antimicrobial peptides against biofilms of methicillin-resistant *Staphylococcus aureus* (MRSA) on polystyrene and medical devices. J Glob Antimicrob Resist 21: 203-210.

COCHRANE SA, FINDLAY B, BAKHTIARY A, ACEDO JZ, RODRIGUEZ-LOPEZ EM, MERCIER P & VEDERAS JC. 2016. Antimicrobial lipopeptide tridecaptin A 1 selectively binds to Gram-negative lipid II. Proc Natl Acad Sci 113: 11561-11566.

CONLON JM, MECHKARSKA M, LUKIC ML & FLATT PR. 2014. Potential therapeutic applications of multifunctional host-defense peptides from frog skin as anti-cancer, anti-viral, immunomodulatory, and anti-diabetic agents. Peptides 57: 67-77.

DOMALAON R, FINDLAY B, OGUNSINA M, ARTHUR G & SCHWEIZER F. 2016. Ultrashort cationic lipopeptides and lipopeptoids: Evaluation and mechanistic insights against epithelial cancer cells. Peptides 84: 58-67.

DOMALAON R, YANG X, NEIL JO, ZHANEL GG & MOOKHERJEE N. 2014. Structure - activity relationships in ultrashort cationic lipopeptides : the effects of amino acid ring constraint on antibacterial activity. Amino Acids 46: 2517-2530.

ETCHEGARAY A & MACHINI MT. 2013. Antimicrobial lipopeptides: in vivo and in vitro synthesis. In: Méndez-Vilas et al. (Eds), Microbial pathogens and strategies for combating them: Science, technology and education. Formatex Research Center S. L. Badajoz, España, p. 951-959.

EVANS BC, NELSON CE, YU SS, BEAVERS KR, KIM AJ, LI H, NELSON HM, GIORGIO TD & DUVALL CL. 2013. Ex vivo red blood cell hemolysis assay for the evaluation of pH-responsive

endosomolytic agents for cytosolic delivery of biomacromolecular drugs. J Vis Exp 73: e50166.

FOSGERAU K & HOFFMANN T. 2015. Peptide therapeutics: Current status and future directions. Drug Discov Today 20: 122-128.

GASPAR D, SALOMÉ VEIGA A & CASTANHO MARB. 2013. From antimicrobial to anticancer peptides. A review. Front Microbiol 4: 294.

GREBER KE, DAWGUL M, KAMYSZ W & SAWICKI W. 2017. Cationic net charge and counter ion type as antimicrobial activity determinant factors of short lipopeptides. Front Microbiol 8: 123.

GREBER KE, ROCH M, ROSATO MA, MARTINEZ MP, ROSATO AE & ROCH M. 2020. Efficacy of newly generated short antimicrobial cationic lipopeptides against methicillin-resistant *Staphylococcus aureus* (MRSA). Int J Antimicrob Agents 55: 105827.

HE J, ANDERSON MH, SHI W & ECKERT R. 2009. Design and activity of a "dual-targeted" antimicrobial peptide. Int J Antimicrob Agents 33: 532-537.

HOSKIN DW & RAMAMOORTHY A. 2008. Studies on anticancer activities of antimicrobial peptides. Biochim Biophys Acta 1778: 357-375.

JENSSEN H & ASPMO SI. 2008. Serum stability of peptides. In: Otvos L (Ed), Peptide-Based Drug Design. Totowa, NJ, p. 177-186.

KHARA JS, OBUOBI S, WANG Y, HAMILTON MS, ROBERTSON BD, NEWTON SM, YANG YY, LANGFORD PR & EE PLR. 2017. Disruption of drug-resistant biofilms using de novo designed short α-helical antimicrobial peptides with idealized facial amphiphilicity. Acta Biomater 57:103-114.

LAVERTY G, MCLAUGHLIN M, SHAW C, GORMAN SP & GILMORE BF. 2010. Antimicrobial activity of short, synthetic cationic lipopeptides. Chem Biol Drug Des 75: 563-569.

LIU L, XU K, WANG H, JEREMY TAN PK, FAN W, VENKATRAMAN SS, LI L & YANG YY. 2009. Self-assembled cationic peptide nanoparticles as an efficient antimicrobial agent. Nat Nanotechnol 4: 457-463.

LOHAN S, MONGA J, CAMEOTRA SS & BISHT GS. 2014. *In vitro* and *in vivo* antibacterial evaluation and mechanistic study of ornithine based small cationic lipopeptides against antibiotic resistant clinical isolates. Eur J Med Chem 88: 19-27.

MAKOVITZKI A, AVRAHAMI D & SHAI Y. 2006. Ultrashort antibacterial and antifungal lipopeptides. Proc Natl Acad Sci USA 103: 15997-16002.

MARCELLINI L, GIAMMATTEO M, AIMOLA P & MANGONI ML. 2010. Fluorescence and electron microscopy methods for exploring antimicrobial peptides mode(s) of action. Methods Mol Biol 618: 249-266

MASCIO CTM, ALDER JD & SILVERMAN JA. 2007. Bactericidal action of Daptomycin against stationary-phase and nondividing *Staphylococcus aureus* cells. Antimicrob Agents Chemother 51: 4255-4260.

MEIR O, ZAKNOON F, COGAN U & MOR A. 2017. A broad-spectrum bactericidal lipopeptide with anti-biofilm properties. Sci Rep 7: 1-11.

MNIF I, GRAU-CAMPISTANY A, CORONEL-LEÓN J, HAMMAMI I, TRIKI MA, MANRESA A & GHRIBI D. 2015. Purification and identification of *Bacillus subtilis* SPB1 lipopeptide biosurfactant exhibiting antifungal activity against *Rhizoctonia bataticola* and *Rhizoctonia solani*. Environ Sci Pollut Res 23: 6690-6699.

MOHAMED MF, ABDELKHALEK A & SELEEM MN. 2016. Evaluation of short synthetic antimicrobial peptides for treatment of drug-resistant and intracellular *Staphylococcus aureus*. Sci Rep 6: 2-15.

NASOMPAG S, DECHSIRI P, HONGSING N, PHONIMDAENG P, DADUANG S, CAMESANO SKTA & PATRAMANON R. 2015. Effect of acyl chain length on therapeutic activity and mode of action of the CX-KYR-NH2 antimicrobial lipopeptide. Biochim Biophys Acta 1848: 2351-2364.

NAUMENKOVA TV, LEVTSOVA OV, NIKOLAEV IN & SHAITAN KV. 2010. Comparative molecular dynamics study of the structural properties of melittin in water and trifluoroethanol/water. Biophysics (Oxf) 55: 32-38.

O'DRISCOLL NH, LABOVITIADI O, CUSHNIE TPT, MATTHEWS KH, MERCER DK & LAMB AJ. 2013. Production and evaluation of an antimicrobial peptide-containing wafer formulation for topical application. Curr Microbiol 66: 271-278.

ONG ZY, WIRADHARMA N & YANG YY. 2014. Strategies employed in the design and optimization of synthetic antimicrobial peptide amphiphiles with enhanced therapeutic potentials. Adv Drug Deliv Rev 78: 28-45.

PAN M, LU C, ZHENG M, ZHOU W, SONG F, CHEN W, YAO F, LIU D & CAI J. 2020. Unnatural amino-acid-based star-shaped poly(l-Ornithine)s as emerging long-term and biofilm-disrupting antimicrobial peptides to treat *Pseudomonas aeruginosa*- infected burn wounds. Adv Healthc Mater 9: e2000647.

PETERS BM, SHIRTLIFF ME & JABRA-RIZK MA. 2010. Antimicrobial peptides: primeval molecules or future drugs? PLoS Pathog 6: e1001067.

QI X ET AL. 2010. Novel short antibacterial and antifungal peptides with low cytotoxicity: Efficacy and action mechanisms. Biochem Biophys Res Commun 398: 594-600.

RAHIM NA ET AL. 2015. Synergistic killing of NDM-producing MDR *Klebsiella pneumoniae* by two "old" antibiotics-polymyxin B and chloramphenicol. J Antimicrob Chemother 70: 2589-2597.

REINHARDT A & NEUNDORF I. 2016. Design and application of antimicrobial peptide conjugates. Int J Mol Sci 17: 701.

SCAVUZZI AML, ALVES LC, VERAS DL, BRAYNER FA & LOPES ACS. 2016. Ultrastructural changes caused by polymyxin B and meropenem in multiresistant *Klebsiella pneumoniae* carrying blaKPC-2 gene. J Med Microbiol 65: 1370-1377.

SHARMA R, PATEL S, ABBOUD C, DIEP J, LY NS, POGUE JM, KAYE KS, LI J & RAO GG. 2017. Polymyxin B in combination with meropenem against carbapenemase-producing *Klebsiella pneumoniae*: pharmacodynamics and morphological changes. Int J Antimicrob Agents 49: 224-232.

TIBURU EK, ZHUANG J, FLEISCHER HNA, ARTHUR PK & AWANDARE GA. 2017. Expression, purification, and monitoring of conformational changes of hCB2 TMH67H8 in different membrane-mimetic lipid mixtures using circular dichroism and NMR techniques. Membranes (Basel) 7: 10.

WADHWANI P, HEIDENREICH N, PODEYN B, BÜRCK J & ULRICH AS. 2017. Antibiotic gold: Tethering of antimicrobial peptides to gold nanoparticles maintains conformational flexibility of peptides and improves trypsin susceptibility. Biomater Sci 5: 817-827.

WIEGAND I, HILPERT K & HANCOCK R. 2008. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protoc 3:163-175.

WHO. 2014. Antimicrobial resistance: global report on surveillance.

WHO. 2017. Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics.

YANG SY, LIM DJ, NOH MY, KIM JC, KIM YC & KIM IS. 2017. Characterization of biosurfactants as insecticidal metabolites produced by *Bacillus subtilis* Y9. Entomol Res 47: 55-59.

YU G, BAEDER D, REGOES R & ROLFF J. 2018. Predicting drug resistance evolution: antimicrobial peptides vs. antibiotics. Proc R Soc B Biol Sci 285: 1-9.

ZHANG B, SHI W, LI J, LIAO C, LI M, HUANG W & QIAN H. 2017. Synthesis and biological evaluation of novel peptides as potential agents with antitumor and multidrug resistance- reversing activities. Chem Biol Drug Des 90: 972-980.

How to cite

POSADA V, ESPEJO BF & ORDUZ S. 2021. *De novo* design of short antimicrobial lipopeptides. An Acad Bras Cienc 93: e20210362. DOI 10.1590/0001-3765202120210362.

Manuscript received on March 9, 2021;
accepted for publication on August 5, 2021

VANESSA POSADA[1]
https://orcid.org/0000-0003-1347-4929

BLANCA FABIOLA ESPEJO[1]
https://orcid.org/0000-0001-7188-2539

SERGIO ORDUZ[2]
https://orcid.org/0000-0001-7587-3816

[1]Universidad Nacional de Colombia, Sede Medellin, Escuela de Química, Facultad de Ciencias, Carrera 65, No. 59A – 110, 050034, Medellin, Colombia

[2]Universidad Nacional de Colombia, Sede Medellin, Escuela de Biociencias, Facultad de Ciencias, Carrera 65, No. 59A – 110, 050034, Medellin, Colombia

Correspondence to: **Sergio Orduz**
E-mail sorduzp@unal.edu.co

Author contributions

VP, BFE and SO conceived the study. VP was involved in aspects of the study related with data collection, data analysis and the in vitro assays. VP, BFE and SO were involved in drafting and editing the manuscript. All authors read and approved the final manuscript.