Physicochemical-guided design of cathelicidin-derived peptides generates membrane active variants with therapeutic potential

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The spread of multi-drug resistance and the slow pace at which antibiotics come onto the market are undermining our ability to treat human infections, leading to high mortality rates. Aiming to overcome this global crisis, antimicrobial peptides are considered promising alternatives to counter bacterial infections with multi-drug resistant bacteria. The cathelicidins comprise a well-studied class of AMPs whose members have been used as model molecules for sequence modifications, aiming at enhanced biological activities and stability, along with reduced toxic effects on mammalian cells. Here, we describe the antimicrobial activities, modes of action and structural characterization of two novel cathelicidin-like peptides, named BotrAMP14 and CrotAMP14, which were re-designed from snake batroxicidin and crotalicidin, respectively. BotrAMP14 and CrotAMP14 showed broad-spectrum antibacterial activity against susceptible microorganisms and clinical isolates with minimal inhibitory concentrations ranging from 2–35.1 μM. Moreover, both peptides had low cytotoxicity against Caco-2 cells in vitro. In addition, in vivo toxicity against Galleria mellonella moth larvae revealed that both peptides led to >76% larval survival after 144 h. Microscopy studies suggest that BotrAMP14 and CrotAMP14 destabilize E. coli membranes. Furthermore, circular dichroism and molecular dynamics simulations indicate that, in a membrane-like environment, both peptides adopt α-helical structures that interact with bilayer phospholipids through hydrogen bonds and electrostatic interaction. Thus, we concluded that BotrAMP14 and CrotAMP14 are helical membrane active peptides, with similar antibacterial properties but lower cytotoxicity than the larger parent peptides batroxicidin and crotalicidin, having advantages for drug development strategies.

The discovery of penicillin and streptomycin during the golden age of antibiotics heralded a revolution in medical treatment of infections. Nevertheless, the spread of antibiotic resistance has made these antibiotics ineffective against many common pathogens¹. The overuse and/or misuse of antibiotics results in microbial resistance through a variety of mechanisms¹. Horizontal transfer of resistance determinants within and between species, and/or DNA mutations, has led to the emergence of pathogens resistant to multiple drugs², reducing our capacity to treat multi-drug resistant bacterial infections³. Consequently, research on the development of new strategies to control antibiotic-resistance has intensified, including the usage of antimicrobial peptides (AMPs), which represent one of the oldest innate defense mechanisms in living organisms³–⁵. The composition and biological effects...
of AMPs found in nature are diverse. AMPs can range in length from 2–50 amino acid residues, and have a great diversity of amino acid composition, structure and size. These compounds were initially studied because of their antimicrobial activity, and it was discovered that they possess a rich spectrum of other biological activities, including immunomodulatory actions, wound healing and anticancer properties.

AMPs are being considered as therapeutic agents but have a number of limitations, including serum stability, bioavailability, cytotoxicity and size, as well as the cost of peptide synthesis. The rational (re)design of AMPs provides further possibilities to enhance their activity, increase stability, as well as specificity, and reduce their size. For example, sequence modifications of natural peptides can increase peptide helicity, hydrophobic moment and net charge, while reducing hydrophobicity11, all factors which are reported as crucial for cationic AMPs, providing further possibilities to enhance their activity, increase stability, as well as specificity, and reduce their size. For example, sequence modifications of natural peptides can increase peptide helicity, hydrophobic moment and net charge, while reducing hydrophobicity11, all factors which are reported as crucial for cationic AMPs, including cecropins, magainin, melittin and the cathelicidins. Moreover, the structural arrangements adopted by AMPs can be partially related to their amphipathic nature, which makes them able to present varied structures, including α-helices, β-sheets, coils or a mixture of all, which are important for interactions with their bacterial targets.

Among AMP classes, cathelicidin related antimicrobial peptides (CRAMPs) are produced by diverse vertebrates, including mammals and snakes, and have been widely studied. Cathelicidins are often expressed and secreted by epithelial cells lining animal glands or mucosa. The N-terminal domain is a highly conserved region composed of its signal peptide and the cathelin domain. In contrast, the CRAMP C-terminal domain, containing the mature peptide sequence, is highly variable in length and sequence, but both interspecies and intraspecies. Reptilian CRAMPs, particularly those from lizards and snakes, show broad-spectrum antimicrobial activity against a large number of bacteria, fungi and viruses. In addition to their antimicrobial effect, cathelicidins have been reported to have multiple activities, including the activation of immune cells, promotion of cell proliferation, cell migration, cell survival, cytokine release, angiogenesis, chemotaxis, and wound healing, thus clearly representing a promising class of therapeutic agents.

The cathelicidin AMPs originate from several sources, including snake venom, which has been widely studied for its antimicrobial properties. In the present study, two cathelicidin peptides (batroxocidin and crotalicidin) have been chosen for the physicochemical-guided design of improved variants. Batroxocidin and crotalicidin were identified in the South American pit vipers Bothrops atrox and Crotalus durissus terrificus. These peptides contain 34 amino acid residues and have exerted pronounced antimicrobial activity against diverse bacteria, with few haemolytic effects or proinflammatory effects on RAW 264.7 cells. These properties suggest that batroxocidin and crotalicidin are promising candidates for antibacterial therapy. Snake AMPs, such as the snake cathelicidin NA-CATH and its smaller derivative (ATRA1), have achieved similar therapeutic potential, suggesting it would be worthwhile to characterize these smaller batroxocidin and crotalicidin variants.

This paper describes two new synthetic peptides (BotrAMP14 and CrotAMP14) derived from the vipers, batroxocidin and crotalicidin, which were generated by a physicochemical-guided design strategy aiming to reduce their size, as well as preserve or improve the antibacterial properties and low toxicity of their parent peptides. In addition, the modes of action of both variants on E. coli cells were also evaluated, and computational and experimental structural studies were performed in different biomimetic conditions to investigate structure-function relationships in these two peptides.

**Material and Methods**

**Peptide re-design.** Initially, the cathelicidin template sequences (batroxocidin and crotalicidin) were collected from the Uniprot knowledgebase. The physicochemical characteristics for the templates and designed peptides, including pI, hydrophobicity, hydrophobic moment and net-charge, were obtained by submitting the sequences to the compute_pi algorithm and the Heliquest server (helixquest.ipmc.cnrs.fr) helical-wheel projections.

**Peptide synthesis and preparation.** The peptides were purchased from Peptides 2.0 (USA) at a purity of >95%. The molecular mass for the peptides was confirmed using Matrix Assisted Laser Desorption Ionization – Time of Flight (MALDI-ToF) on a mass spectrometer Ultraflex MALDI-TOF III (BrukerDaltonics). The concentration of the designed peptides was determined using measurements of absorbance at 205, 215 and 225 nm as described by Niebergall and co-workers.

**In vitro antimicrobial assays.** Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) experiments were performed for the ATCC and Clinical Isolated strains of E. coli 25922 K. pneumoniae 13822, and S. aureus 25923. The antimicrobial activities of all the peptides were tested as previously described with minor modifications. Briefly, Mueller- Hinton (Himedia) broth was used to grow the strains overnight at 37 °C. MIC measurements were performed using 1 × 10^4 CFU.mL^-1 and serial dilution of the peptides BotrAMP14 and CrotAMP14 starting at 50µM. The MIC (100% inhibitory concentration) was determined after 24 h of incubation at 37 °C. The absorbance was measured in a 96-well microplate at 600 nm. Bacteria cultured only in MHB and containing the antibiotics (chloramphenicol, gentamicin and imipenem) were used as negative and positive control, respectively. MBCs were determined by plating out 10µL of the contents of the wells where no bacterial growth was observed on MH agar plates. MBC was recorded as the lowest concentration at which no colonies were observed after 24 h incubation at 37 °C. All the measurements were performed in triplicate.

**Neutral-red (NR) in vitro toxicity assay.** Toxicity of the peptides on Caco-2 cells, at increasing peptide concentrations, was determined using a neutral-red (NR) assay as described previously. After overnight incubation of Caco-2 cells with the peptides BotrAMP14 and CrotAMP14 at concentrations from 2–35 µM, 10µL of a 33 µg.mL^-1 NR solution was added to the wells containing the peptide-incubated cells. After 3 h of incubation, the supernatant was removed, and the cells were washed with phosphate buffer saline (PBS). Next, 150µL of 1% acetic
acid-50% ethanol was added and shaken for 10 min at room temperature. Finally, the absorbance was measured at 540 nm and 690 nm (background absorbance) using a SpectraMax M5 microplate reader (Molecular Devices). Readings were expressed as NR uptake relative to the uptake of the cells exposed to the negative control (medium or DMSO). All the measurements were performed in triplicate.

**Galleria mellonella in vivo toxicity assay.** In vivo toxicity was assayed using *Galleria mellonella* larvae in their final instar stage. The larvae were purchased (UK Waxworms Ltd, Sheffield, UK), and acclimatized in the dark at 15 °C, and used within 14 days. Only larvae weighing between 0.2 and 0.3 g were used for experiments. Larvae were injected with 20 μL of BotrAMP14 and CrotAMP14 peptide solutions, or controls in the left posterior proleg using Terumo Myjector 29 G insulin syringes (VWR International). Two negative control groups were included in every experiment; one group was not injected to control for background larval mortality (no manipulation control) and the other group (uninfected control) was injected with PBS to control for the possible effect of physical trauma on mortality. After injection, larvae were acclimatized in Petri dishes in the dark at 37 °C with 5% CO₂ for up to 144 h post-inoculation and inspected every 24 h for survival. For each sample (non-manipulated control, water control, peptides) fifteen randomly chosen larvae were used. The peptide concentration was 10 mg, kg⁻¹ of body weight. The data were expressed as % larval survival of the survival of the uninjected control. All the measurements were performed in triplicate.

**Circular Dichroism.** Circular dichroism (CD) spectra were obtained using a Jasco-715 spectrophotometer equipped with a Peltier element for temperature control. CD spectra were obtained in the far-UV range, from 200 to 260 nm using a quartz cuvette with 0.1 cm optical path. An averaging of 20 single scans was obtained for the peptide concentration of 110 μM, for demi water, 10 mM potassium phosphate buffered saline at pH 7.4, and 30 mM of sodium dodecyl sulphate (SDS). All the spectra were fitted using the CONTIN algorithm as implemented in the DICROWEB²⁹–³² webserver, using the data basis set #7 for a quantitative interpretation of the spectra in terms of a percentage of α-helical structure.

**Molecular modeling.** Initially, BLASTp³¹ was performed in order to find the best template structures for the molecular modeling of BotrAMP and CrotAMP. Further, 100 theoretical three-dimensional models were constructed using Modeller v. 9.12³², based on the nuclear magnetic resonance (NMR) structure of a crotalicidin isolated from venom of the rattlesnake (*Crotalus durissus*) (PDB code: 2MWT)³³. The lowest free-energy theoretical models (DOPE score) for both peptides were then selected and used for validation procedures according to their fold (ProSA-web)³⁴ and stereochemistry (PROCHECK)³⁵. Structure visualization was performed in PyMOL (http://www.pymol.org).

**Molecular dynamics in water and SDS micelles.** Molecular dynamics (MD) simulations for BotrAMP14 and CrotAMP14 were initially carried out in contact with an SDS micelle, according to Cardoso et al.³⁶. The simulations were performed using the CHARMM36 force field from the computational package GROMACS v.5.0.4³⁷. MD simulations in SDS were carried out in dodecahedron boxes, where both peptides were put in contact with SDS micelles constituted of 100 detergents. SDS micelles were built, and their topologies generated using the CHARMM-GUI server³⁸. Chloride ions were added to neutralize the systems’ charge in both simulations. The simulations were performed under 0.15 M NaCl ionic strength. Geometry of water molecules was constrained using the SETTLE algorithm³⁹. Moreover, the LINCS algorithm³⁹ was used to link all the atom bond lengths. Particle Mesh Ewald (PME)⁴⁰ was used for electrostatic corrections with a radius cut-off of 1.4 nm to minimize the computational simulation time. The same radius cut-off was used for van der Waals interactions. The list of neighbors of each atom was updated every 10 simulation steps of 2 fs each. The steepest descent algorithm (50,000 steps) was applied for energy minimization. The systems underwent a normalization of temperature and pressure to 310 K and 1 bar using the velocity rescaling thermostat (NVT)⁴¹ and the Parrinello-Rahman barostat (NPT)⁴², respectively, for 100 ps. The systems with minimized energy and balanced temperature and pressure were submitted to MD simulations for 800 ns. MD simulations were analyzed by means of root mean square deviation (RMSD). Moreover, peptide-SDS atomic interactions were measured on PyMOL v. 1.8 (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC).

**Scanning electronic microscope.** Scanning electronic microscopy (SEM) was performed by the adherence of bacteria to Poly-L-Lysine-coated slides. Prior to SEM analysis, *E. coli* ATCC 25922 was grown in MHB overnight at 37 °C, and the concentration of 1 × 10^⁷ CFU.mL⁻¹. The bacterial cultures were centrifuged, and phosphate buffer was used to replace the MHB. The bacterial culture was increased by adding 8 μM and 2 μM for BotrAMP14 and CrotAMP14, respectively, for 5, 30 and 60 min of incubation. For control group, *E.coli* ATCC 25922 was used without peptides (time 0). Samples for SEM were prepared by leaving microscope slides coated with poly-L-lysine in 10 mL suspension of bacteria incubated with the peptides and allowing them to settle and adhere to the slides. After 60 min incubation at RT, the bacteria were fixed using 2.5% glutaraldehyde in phosphate buffer. Finally, water was removed using critical point drying: first, the samples were immersed in a graded series of increasing ethanol: 25%, 50%, 75%, and two times at 100% each for 10 min. This was followed by transferring the samples to absolute ethanol, and by critical point drying.

**Results and Discussion**

**Rational design of batroxicidin and crotalicidin variants.** The rational (re)design of AMPs to tune amphipathicity, degree of helicity, charge, etc., can be used to further improve their usefulness as future antimicrobial medicines. We therefore present two novel AMPs derived from snake cathelicidins. Previously, Falcao and colleagues reported the discovery of two vipericidins, named crotalicidin and batroxicidin, with very similar sequences and high antimicrobial capacity. Subsequently, Falcao and co-workers showed that just a small
fragment of the C-terminal region of crotalicidin (fragment 15–34), named Ctn [15–34], was highly active against diverse microorganisms; whereas the N-terminal sequence showed low antimicrobial activity. We therefore used the peptide Ctn [15–34] as template sequence for the generation of CrotAMP14. Following the same rationale used for the peptide crotalicidin, where only its C-terminal region has been described as active against microorganisms, the C-terminal region of the peptide batroxicidin (fragment 15-34), named here as Btn [15-34], was also used as a template for the generation of another peptide called BotrAMP14.

BotrAMP14 and CrotAMP14 are 14-amino acid peptides derived from the cathelicidin subsequences: Btn[15-34] (KRRKKFRRKPRVIGVTFPP–NH₂) from batroxicidin; and Ctn[15-34] (KRLKKIFKKPMIVGVTIPF–NH₂) from crotalicidin, respectively (Fig. 1A). Initially, the parent sequences were mapped into a helical wheel diagram to identify the cationic and hydrophobic faces of Btn[15-34] and Ctn[15-34] when folded into an α-helix. Based on these diagrams, a series of modifications in Btn[15-34] and Ctn[15-34] were carried out to reduce their size and enhanced helical amphipathicity, thus generating BotrAMP14 (KRWKKFFRKVIKFF–NH₂) and CrotAMP14 (KRLKKIFKKMIKIF–NH₂). The aim was to exclude amino acid residues not favorable for the electrostatic surface, while maintaining an alternation between positively charged and hydrophobic residues. The amino acid residues K1, V4, P11, R12, G15, V16, T17 and P19 in Btn[15-34] were removed from BotrAMP14, followed by the addition of tryptophan and lysine residues at position 3 and 12, respectively. Similarly, the residues K1, P11, V13, G15, V16, T17 and P19 in Ctn[15-34] were removed from CrotAMP14 and a lysine residue was added at position 12.

The resulting physicochemical changes in BotrAMP14 and CrotAMP14 in comparison to their parent sequences are summarized in Table 1. Both peptides were designed focusing on maintaining the high net charge of the cathelicidins’ parental sequences, alongside an increased hydrophobic moment and, mainly, the reduction of the parental sequence lengths. In a previous study developed by our group, we demonstrated that the cationic subsequence Ctn[15–34] can be inactivated through the insertion of a negatively charged propeptide sequence, as a potential prodrug strategy. These peptide inactivation strategies may also be used with BotrAMP14 and CrotAMP14 peptides, as their small size and cationic properties were preserved.
Cationic AMPs from several classes (cathelicidins, defensins and magainins), are commonly modified using rational design strategies, which allow a shorter motif to be obtained, decreasing the cost of producing synthetic peptides for therapeutic applications. In addition, the removal of specific residues with undesired properties may also produce peptide analogues with lower toxicity and immunostimulatory activities. In the present work, the amino acid Lys (K12) was inserted in both redesigned variants (BotrAMP14 and CrotAMP14) with the aim of boosting electrostatic interactions with bacterial membranes and promoting antibacterial activity. Furthermore, studies have shown that lysine-rich peptides have reduced toxicity against eukaryotic cells. The Trp (W9) was inserted in BotrAMP14 because of its ability to arrange itself more deeply into the bacterial membranes, presenting a distinct preference for the interfacial region of lipid bilayers. Therefore we expected BotrAMP14 to disrupt the bacterial cell membranes more efficiently. Overall, these amino acids modifications interfered at the helical wheel diagram for BotrAMP14 and CrotAMP14, resulting in clear cationic and hydrophobic residues distribution along the peptide sequence, thus favoring the amphipathicity (Table 1 and Fig. 1B).

Table 1. Physicochemical properties of BotrAMP14, CrotAMP14 and their parent peptides. aDetermined for C-terminal amidated (NH2) peptides; bDetermined by the compute_pi algorithm (http://web.expasy.org/compute_pi/); cDetermined by the Heliquest server (http://heliquest.ipmc.cnrs.fr/).

| Peptides       | Residues | Net charge (pI) | pIb | Hydrophobicity<sub>H</sub> | Hydrophilic moment<sub><sub>H</sub></sub> | Molecular mass |
|---------------|----------|----------------|-----|---------------------------|------------------------------------------|--------------|
| Batroxicidin   | 34       | +16            | 12.50 | 0.207                     | 0.473                                   | 4258.29      |
| Crotalicidin   | 34       | +16            | 12.09 | 0.263                     | 0.440                                   | 4151.33      |
| Btn[15–34]     | 20       | +9             | 12.32 | 0.317                     | 0.306                                   | 2478.88      |
| Ctn[15–34]     | 20       | +8             | 11.43 | 0.455                     | 0.311                                   | 2371.87      |
| BotrAMP14      | 14       | +8             | 12.04 | 0.390                     | 0.688                                   | 1957.46      |
| CrotAMP14      | 14       | +8             | 11.43 | 0.354                     | 0.849                                   | 1820.43      |

The efficient antibacterial activity described here for the designed peptides BotrAMP14 and CrotAMP14 is consistent with previous works regarding the precursor CRAMPs. Regarding antimicrobial activity, the higher MICs observed for the Gram-positive bacteria than for the Gram-negative bacteria tested indicate that both redesigned peptides possess a differential affinity for these types of bacterial membranes. By reducing the number of amino acid residues and increasing hydrophobic moment, the redesigned peptides had a higher MIC for the Gram-positive bacterium S. aureus than Gram-negative bacteria. This feature may be related to the increased helicity of the redesigned peptides BotrAMP14 and CrotAMP14 compared to the parental peptides. The propensity to form structures in amphipathic helices in membrane-mimicking membrane environments has been demonstrated in several studies as an essential factor for the disruptive activity of AMPs.

More recently, a smaller derivative of cathelicidin KP36, designated AMP (RN15), also demonstrated promising activity, mostly against Bacillus bacteria, and low hemolytic and cytotoxicity against dermal human dermal fibroblasts. However, for Gram-negative strains, a slight increase of BotrAMP14 over the parental Btn [16–34] was observed. This increase may be related to the tryptophan residue presence, which could lead to an increase in membrane/peptide interaction due to tryptophan insertion into the interfacial region of the phospholipid bilayer. This increase was not observed for CrotAMP14 peptide, which maintained the overall parental activity. Ultimately, the bacterial cytoplasmic membrane lipid composition seems essential for AMPs’ mechanism of action. Generally, it is constituted by zwitterionic lipid phosphatidylethanolamine (PE), and anionic lipids phosphatidylglycerol (PG) and cardiolipin (CL), which are essential for membrane organization. The lipid composition differs from one species to another. Usually Gram-positive bacteria present a high anionic lipid (PG and CL) amount, which may favor electrostatic interactions. Gram-negative bacteria show higher PE content in comparison to Gram-positive strains. Such features could explain the higher increased activity against Gram-positive bacteria for the redesigned peptides over parental.

Structural analyses. To elucidate the secondary structure of BotrAMP14 and CrotAMP14, CD spectroscopy, molecular modeling and dynamics simulations were carried out under different experimental conditions. CD spectra were acquired in demi-water, 10 mM potassium phosphate buffer at pH 7.4 and in 30 mM SDS, and they are shown in Fig. 2. A qualitative comparison with typical CD spectra illustrates that BotrAMP14 and CrotAMP14 spectra are characteristic of predominantly random coil configurations in demi-water and in 10 mM potassium phosphate buffer (Fig. 2A,B). In contrast, CD spectra of BotrAMP14 and CrotAMP14 indicated substantial α-helical configuration in a membrane-like environment (SDS) (Fig. 2C). For a more quantitative assessment, we used the CONTIN algorithm to fit the CD spectrum and extract percentages of the α-helical secondary
| Microorganisms | Gram | Bta [15-34] (μM) | Ctn [15-34] (μM) | BotrAMP14 (μM) | CrotAMP14 (μM) | Gentamicin (μM) | Imipenem (μM) | Chloramphenicol (μM) | Polimyxin B (μM) |
|---------------|------|-----------------|-----------------|----------------|---------------|----------------|-------------|---------------------|-----------------|
| E. coli ATCC 25922 | — | 6.2 (50) | 3.1 (25) | 3.1 (50) | 3.1 (25) | 0.03 (1.5) | ** | 6.2 (6.2) | 0.03 (1.5) |
| E. coli KpC1 1812446 | — | 3.1 (12.5) | 1.5 (6.2) | 1.5 (3.1) | 1.5 (3.1) | ** | 12.5 (>50) | ** | ** |
| K. pneumoniae ATCC 13822 | — | 12.5 (50) | 6.2 (12.5) | 6.2 (6.2) | 6.2 (6.2) | 0.03 (1.5) | ** | ** | ** |
| K. pneumoniae CI 1445333 | — | >50 (>50) | 6.2 (25) | 6.2 (>50) | 12.5 (>50) | 6.2 (12.5) | ** | ** | ** |
| S. aureus ATCC 25923 | + | >50 (>50) | >50 (>50) | 12.5 (50) | 12.5 (50) | 0.03 (1.5) | ** | ** | ** |
| S. aureus CI | + | 25 (50) | 25 (50) | 3.1 (50) | 3.1 (50) | 0.03 (1.5) | ** | ** | ** |

Table 2. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of the redesigned peptides, and the parental peptides against susceptible bacteria and clinical isolate strains.

**Figure 2.** CD spectra of the peptides BotrAMP14 (red) and CrotAMP14 (Blue). Residue molar ellipticity [q] in deg.cm²/dmol⁻¹ is plotted versus the wavelength l in nm. Measurements were done at a peptide concentration of 0.2 mg.mL⁻¹ in demi water (A) 10 mM K₂HPO₄, 50 mM Na₂SO₄ buffer, pH 7.4 (B) and 30 mM SDS (C).

structure of the peptides (2A-B). The results show BotrAMP14 has an estimated percentages of random coil structure in demi water and 10 mM potassium phosphate buffer of ~38% and ~61%, respectively.

For CrotAMP14, the estimated percentages of random coil were very similar in demi-water and buffer (i.e. 48% and ~66% respectively). However, the dominant α-helical content of secondary structure in 30 mM SDS was ~65% and ~69%, respectively, for BotrAMP14 and CrotAMP14. Similarly, Chen and coworkers⁵¹ also demonstrated that the smaller oligopeptide of 15 amino acid residues ('VKRKKFFRKLLKSV'⁵²) derived from the cathelicidin BF-30 (Bf-CRAM), revealing that an oligopeptide of 15 amino acid residues ('VKRKKFFRKLLKSV'⁵²) maintained its minimal α-helical structure required for an antimicrobial activity. Another study on cathelicidins focused on the use of short synthetic peptides of sequences incorporated into Ophiophagus hannah peptides (Oh-CRAMP and Oh-CATH) and distinct antimicrobial activity and hemolysis were demonstrated relative to human erythrocytes in comparison with their original parent sequences⁵².

MD simulations were performed for similar conditions as those used in the CD experiments. MD simulations in the presence of SDS micelles showed that the SDS clearly promoted α-helical conformations for both BotrAM14 and CrotAMP14 (Fig. 3C,D). Also, supporting the results of the CD experiments, the two peptides differ in overall helical chemical contents (Fig. 3A,B). According to the RMSD analysis CrotAMP14 showed ~3-fold higher deviations (~0.45 nm) in its trajectory when compared to BotrAMP14 (~0.15 nm) (Fig. 3A). This was also reflected in the fluctuation per residue observed for these peptides, where higher RMSF values were observed for CrotAMP14 (Fig. 3B).

Our SEM results suggest that both BotrAMP14 and CrotAMP14 directly attach to bacterial surfaces and trigger membrane perturbation events in Gram-negative bacteria. Interestingly, as for our simulations in the presence of SDS micelles, CrotAMP14 deviated and fluctuated more during the entire simulation (Fig. 3A). A total of 16 atomic interactions were predicted for the molecular complex BotrAMP14/SDS. These interactions involved the residues K¹, R², W³, K⁴, K⁵, E⁶, R⁸, V¹⁰, I¹¹, K¹² and F¹⁴ in BotrAMP14 (Supplementary Table 1). Furthermore, 13 interactions were predicted for the complex CrotAMP14/SDS. These interactions involve the residues K¹, R³, L⁷, K⁸, K⁹, F¹² and I¹³ in CrotAMP14 (Supplementary Table 2). These data clearly indicate the relevance of the positively charged residues of both peptides for initial electrostatic and hydrogen bond interactions with the sulfate groups of the SDS molecules.

Membrane interaction analyses. Scanning electron microscope (SEM) analysis was performed to clarify possible morphological alterations at MIC concentration for BotrAMP14 and CrotAMP14 at different incubation times. The high-resolution images showed that both peptides induced similar damage to E. coli ATCC 25922 bacterial cells (Fig. 4). At time 0h (control), no damage in the bacterial cells (Fig. 4A,B) was observed. However, after 5 min of exposure to the peptides, bacterial outer membrane damage, including holes and fissures, was clearly visible (Fig. 4C,D). The number of these “holes” increased after 30 min incubation (Fig. 4E,F). After 1 h of incubation larger-scale damage to the cells was apparent (Fig. 4G,H). The results achieved by SEM corroborate the MD
The peptide/membrane interactions using SDS micelles were used to check the interaction between the peptides under membrane-mimicking conditions, indicating that peptide/membrane interactions in both cases are driven by hydrogen bond, hydrophobic interaction, and salt bridge (electrostatic interactions) between the BotrAMP14 and CrotAMP14 peptides, and the SDS micelles groups (sulfate and acyl chain). According to Pérez-Peinado and coworkers, cationic and α-helical peptides in crotalicidin and its analogue Ctn [15-34] would accumulate and interact with the negative charges on the bacterial surface. The results of flow cytometry confirmed that both crotalicidin and Ctn [15-34] permeabilize the bacterial cell membrane in different ways, suggesting their precise mechanisms of action differ. Based on results obtained by Pérez-Peinado and coworkers, we inferred that both the redesigned peptides used in our study can act in a similar way to those described for crotalicidin and Ctn [15-34]. Indeed, the peptides BotrAMP14 and CrotAMP14 maintained many of the characteristics presented by original peptides (i.e. Btn [15-34] and Ctn [15-34]), even after their reduction in size.

Cytotoxicity of BotrAMP14 and CrotAMP14. To be used as drugs, antibacterial compounds must have high selectivity to the pathogens at therapeutic concentrations in the body and must lack cytotoxicity to the host cells. Usually, the cytotoxic properties associated with both naturally occurring and rationally designed AMPs represent a bottleneck in the treatment of infections using these molecules. In this context, the toxic effects of BotrAMP14 and CrotAMP14 on mammalian cells were initially investigated in vitro using Caco-2 cells. Neutral red uptake (NRU) assay was used to determine the viability of mammalian Caco-2 cells as a function of peptide concentration. We observed that cells incubated with BotrAMP14 showed 100% viability at the maximum concentration tested (88 μM, Fig. 5A). At this same concentration, however, the CrotAMP14 peptide was cytotoxic, reducing cell viability to 60% (Fig. 5A). Caco-2 cells can be widely used to test the intestinal permeability and toxicity for several drugs. The lack of toxicity of BotrAMP14 and CrotAMP14 against Caco-2 cells is very promising and can be used for future intestinal permeability assays, since antibiotics may lead to an imbalance of the microbiota, which is important when considering the inclusion of new drugs on the market.
Toxicity of BotrAMP14 and CrotAMP14 to Galleria mellonella larvae. Infectivity trials and toxicity testing in rodents are important requisites for the use of drug candidates in humans. However, trials in rats and mice are expensive and there are ethical considerations. *G. mellonella* (greater wax moth) larvae are a potential alternative as they have evolutionarily conserved immunity consisting of both cellular and humoral defenses\(^5\). *In vivo* experiments were performed using greater wax moth *G. mellonella* larvae for a period of 144 h, at a peptide concentration of 10 mg kg\(^{-1}\) of body weight (Fig. 5B). Survival curves indicated that both BotrAMP14 and
was demonstrated here, Wang and coworkers showed that derivative crotalicidin EVP50 presented the highest of the ATRA motif, i.e., KR(F/A)KKFFKK(L/P)K, with a trivial toxicity against erythrocytes. Contrary to what other studies performed with analogues/derivatives of cathelicidins reported that these peptides had low toxicity and hemolytic activity. In experiments performed with peptides derived from vipers, the authors observed low toxicity against zebrafish larvae in comparison to the original vipericidin sequences. On the other hand, other studies performed with analogues/derivatives of cathelicidins reported that these peptides had low toxicity and hemolytic activity.

In conclusion, it was possible to observe that the physicochemical-guided design of BotrAMP14 and CrotAMP14 preserved the antibacterial and non-toxic potential of their parent sequences despite their smaller size, suggesting the applicability of these variants as new drug candidates. We also concluded that both peptides act on Gram-negative bacteria through a membrane destabilization mechanism, which is mainly driven by electrostatic interactions and hydrogen bonding between the N-terminus region of these peptides and anionic membranes, leading to peptide anchoring and insertion. Overall, BotrAMP14 and CrotAMP14 appear as suitable candidates for further drug development, especially for the treatment of resistant Gram-negative bacteria-associated infections.

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R.d.V. and O.L.F. obtained funding. Antimicrobial activity assays were designed by N.G.J.O. and J.M.W. and carried out by N.G.J.O. Toxicity assays were designed by T.M.B.R. and N.G.J.O. and carried out by N.G.J.O. and N.V. Circular Dichroism experiments were designed, performed and analyzed by N.G.J.O. and R.d.V. Microscopy experiments were designed and carried out by M.G. and N.G.J.O. Molecular Dynamics simulations were designed and performed by N.G.J.O. and M.H.C., N.G.J.O. wrote the manuscript. All authors reviewed the manuscript.

**Competing interests**
The authors declare no competing interests.

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