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Research paper

A novel synthetic peptide inspired on Lys49 phospholipase A_2 from *Crotalus oreganus abyssus* snake venom active against multidrug-resistant clinical isolates

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ABSTRACT

Currently, the evolving and complex mechanisms of bacterial resistance to conventional antibiotics are increasing, while alternative medicines are drying up, which urges the need to discover novel agents able to kill antibiotic-resistant bacteria. Lys49 phospholipase A_2s (PLA_2s) from snake venoms are multifunctional toxins able to induce a huge variety of therapeutic effects and consequently serve as templates for new drug leads. Hence, the present study was aimed at the synthesis of oligopeptides mimicking regions of the antibacterial Lys49 PLA_2 toxin (CoaTx-II), recently isolated from *Crotalus oreganus abyssus* snake venom, to identify small peptides able to reproduce the therapeutic action of the toxin. Five peptides, representing major regions of interest within CoaTx-II, were synthesized and screened for their antibacterial properties. The 13-mer peptide pC-CoaTxII, corresponding to residues 115–129 of CoaTx-II, was able to reproduce the promising bactericidal effect of the toxin against multi-resistant clinical isolates. Peptide pC-CoaTxII is mainly composed by positively charged and hydrophobic amino acids, a typical trait in most antimicrobial peptides, and presented no defined secondary structure in aqueous environment. The physicochemical properties of pC-CoaTxII are favorable towards a strong interaction with anionic lipid membranes as those in bacteria. Additional *in silico* studies suggest formation of a water channel across the membrane upon peptide insertion, eventually leading to bacterial cell disruption and death. Overall, our findings confirm the valuable potential of snake venom toxins towards design and synthesis of novel antimicrobials, thus representing key insights towards development of alternative efficient antimicrobials to fight bacterial resistance to current antibiotics.

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1. Introduction

The rapidly evolving and diversifying resistance mechanisms to conventional antibiotics acquired by Gram-positive and Gram-negative bacteria, such as, e.g., *Staphylococcus aureus* or *Pseudomonas aeruginosa*, respectively, are quickly exhausting therapeutic options available, and becoming a relevant global public health concern [1–3]. Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most significant and prevalent human pathogens able to cause nosocomial and community infections in many countries, including highly populated ones like Brazil, according to data from SENTRY Antimicrobial Surveillance Program [4,5]. The Brazilian epidemic clone (BEC) of MRSA is one of the main global clones, and has demonstrated resistance to many antibiotics used in clinical practice, such as aminoglycosides, macrolides, chloramphenicol, quinolones, sulphamethoxazole-trimethoprim, lincosamides, and tetracycline [4,6]. Antibiotic-resistant *Pseudomonas aeruginosa* strains of clinical relevance have also been isolated and associated

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to severe epidemiologic and health problems, which has prompted the search and development of new bioactive compounds able to treat and control infections caused by these resistant bacteria [7–9].

Snake venom toxins were successfully proven as interesting pharmacologically-active compounds with a huge chemical and functional variability, from small peptides to proteins, which inspired innovative discoveries and development of research tools and drugs, including antibacterial compounds [10–12]. Currently, the biotechnological and scientific exploration of venom toxins as structural templates for the design and synthesis of novel and efficient therapeutic agents is an important pharmaceutical and biomedical challenge.

Lys49 phospholipase A_2 (PLA_2) homologues comprise a wide family of intriguing proteins [13], that despite lacking catalytic activity, share high sequence identities, structural uniformity and have presented an exciting variety of effects, from toxicological or lethal to pharmacological activities of potential interest of pharmaceutic industry [14,15]. These non-catalytic toxins, found in *Viperidae* venoms, represent one of the main and more promising proteinaceous components of bioactive snake libraries with antimicrobial properties [16,17]. Based on the primary structure of Lys49 PLA_2 homologues, small cationic peptides already have been earlier designed, synthesized and proven to kill several Gram-positive and Gram-negative bacteria, which opens a new and large window of opportunity in the combat against antibiotic resistance [18,19]. Furthermore, the use of synthetic peptides inspired on snake venom proteins presents some advantages over the use of traditional drugs, such as their high specificity, potent action at low dose, low immunogenicity, high diffusion to tissues, straightforward and reasonably affordable chemical synthesis, which enables easy introduction of structural modifications, such as use of D-amino acids or peptoid mimics, in order to increase their stability and half-life in the organism [17,20,21].

Recently, our research group achieved the isolation, purification and, functional and structural characterization of a Lys49 PLA_2 homologue, CoaTx-II, from the venom of *Crotalus oreganus abyssus*, a venomous pit viper belonging to the *Viperidae* family [22]. We elucidated the primary, tertiary and quaternary structure of CoaTx-II, and reported its antimicrobial proprieties, which showed a high potential to draw novel strategies to fight antibiotic-resistant bacteria. In this context, the present study aimed at the exploration of the potential of the primary sequence of CoaTx-II as a template to design and synthesize small antibacterial peptides, active against bacterial strains of well-known resistance to current antibiotics. To this end, five peptides mimicking putative regions of CoaTx-II were chemically synthesized and screened *in vitro* against clinical isolates of relevant bacterial strains. We have further investigated *in silico* the physicochemical properties, tridimensional structure and mode of interaction with membrane lipids of the cationic peptide that displayed the highest antibacterial potential.

2. Results

The five short peptides that spanned major regions of interest within the primary structure of CoaTx-II (N-terminal, active site and C-terminal regions - Table 1) were successfully synthetized by means of standard solid-phase peptide synthesis (SPPS) protocols employing the Fmoc/^{t}Bu orthogonal protection scheme [23]. The peptides were obtained in satisfactory yields (ca. 15–50%) and high purity degrees (ca. 93–100%), and presented correct mass spectral data (Supporting Information, Table S1, Figs. S1 and S2).

The antibacterial screening of the synthetic peptides, which was performed by standard agar well diffusion assay [24], revealed that the short peptide corresponding to residues 115–129 (pC-CoaTxII) of the parent toxin was the only one able to mimic the antibacterial effects of the entire protein template Lys49 PLA_2 against the five bacterial strains tested (Fig. 1A). This 13-mer cationic peptide showed promising activity against both Gram-positive and Gram-negative bacteria, including multidrug resistant isolates, as demonstrated by the bacterial inhibition zone halo and their measures (Fig. 1A and B). Interestingly, by shortening pC-CoaTxII to its sub-sequence pC1-CoaTxII, led to loss of activity against all bacterial strains assayed.

The analysis of bacterial sensitivity to pC-CoaTxII peptide determined by standardized CLSI broth microdilution method [25] also highlighted the bactericidal potential of this short region of CoaTx-II toxin. Despite the remarkable antibacterial effect in all strains tested, most efficient inhibitory activity was observed against both strains of the Gram-negative bacteria *P. aeruginosa* tested. pC-CoaTxII is more active against ATCC and multidrug-resistant clinical isolates than the intact protein (Fig. 2).

As shown by data in Table 2, pC-CoaTxII differs from the other four biomimetic peptides synthesized, mainly in charge and pI, which were substantially higher for the bioactive peptide, putting into display the well-known importance of a largely positive net charge in antimicrobial peptides. Likewise, estimated hydrophobicity values show that peptide pC-CoaTxII was the most hydrophobic of the cationic peptides tested, again in agreement with the widely recognized role of hydrophobic amino acids for antimicrobial action of cationic amphipathic peptides. Still, it is interesting to notice that the decrease in net charge and hydrophobicity when going from pC-CoaTxII to its shorter sub-structure pC1-CoaTxII, may explain why the latter was inactive against the bacterial strains tested.

Peptide pC-CoaTxII shares some structural similarity with other synthetic antibacterial short peptides that were equally based on the primary structure of snake venom PLA_2s (Fig. 3), in particular regarding their high content in hydrophobic and positively charged amino acids.

Prediction of the secondary structure of pC-CoaTxII was performed through combination of a very long Molecular Dynamics (MD) simulation and a clustering analysis. The MD simulation, together with the small size of the peptide, allowed for a relevant exploration of the conformational space, while clustering analysis contributed to a better understanding of which are the dominant structures. From the final 1 μs of the trajectory, 50.000 microstates were retrieved for the clustering analysis. Seven clusters were obtained (Fig. 4) with the following representation; cluster A: 32%, cluster B: 18%, cluster C: 16%, cluster D: 14%, cluster E: 10%, cluster

Table 1
Primary structures and biochemical characteristics of synthetic peptides inspired on CoaTx-II toxin isolated from *Crotalus oreganus abyssus*.

Peptides	Amino acid sequence	Molecular Weight (g.mol^{-1})	Molecular region
pN1-CoaTxII	SLVELGK	743.45411	N-terminal CoaTx-II (0–8)
pN2-CoaTxII	MILQETGK	918.48444	N-terminal CoaTx-II (7–16)
pAS-CoaTxII	DATDRCCFVHK	1293.55941	Active site CoaTx-II (38–50)
pC1-CoaTxII	YPKFLCKKP	1122.44201	C-terminal CoaTx-II (120–130)
pC-CoaTxII	KKYRIYPKFLCKK	1713.02759	C-terminal CoaTx-II (115–129)

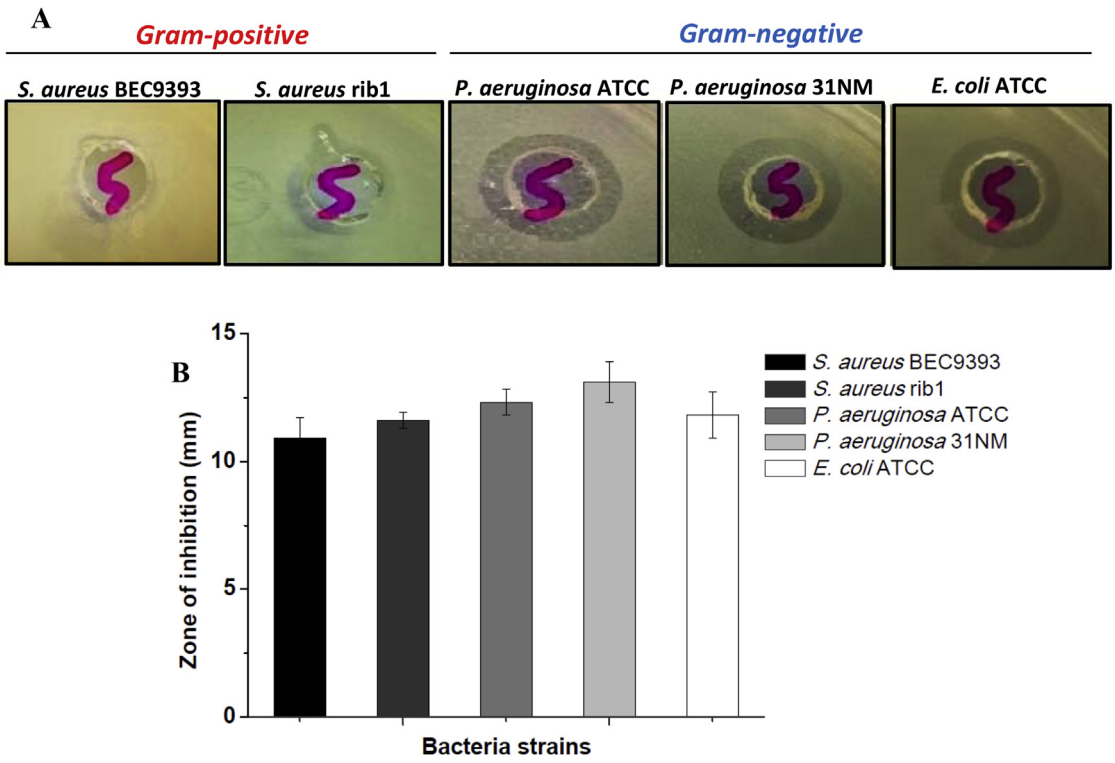

Fig. 1. Antibacterial effect of synthetic peptide, pC-CoaTxII in agar diffusion assay on Gram-positive and Gram-negative bacteria. In each antibacterial assay, 100 μl of synthetic peptide (at 575 μM) was added to agar media, which was incubated at 37 °C **(A)**. After 24 h, zones of bacterial growth inhibition were measured and reported **(B)**.

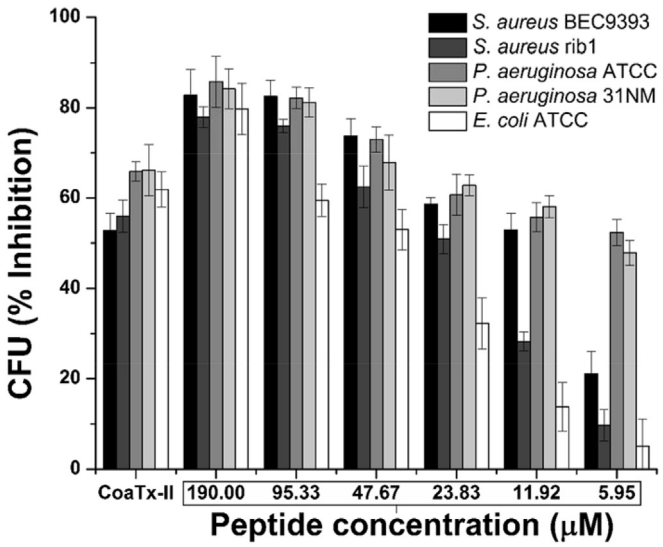

Fig. 2. Analysis of bacterial sensitivity to CoaTx-II (190 μM) and synthetic peptide (pC-CoaTxII) mimicking the C-terminal region (115–129) of CoaTx-II by broth microdilution assay. CoaTx-II (190 μM) and different amounts (5.95–190 μM) of biomimetic peptide, pC-CoaTxII were incubated at 37 °C with 1×10^8 CFU (colony-forming units)/ml of each Gram-positive and Gram-negative bacterial strain, and then viable cells after 24 h were spectrophotometrically quantified at 595 nm. The ability of the peptide to inhibit bacterial growth was expressed as percentage (%).

F: 8% and cluster G: 2%. After the subsequent seven 50 ns classic MD simulations, starting from a structure taken from each of the seven clusters, all peptides maintained the initial conformations and no aggregation was detected (data not shown). The analyses of these structures suggest that no secondary structure elements (alpha-helix or beta-sheets) are formed, despite the fact that clusters D, E, F and G present a single helix turn on the PKFLC region. The structures taken from all clusters conserve a near linear shape with a bent on the Proline residue that spans a wide range of values, from 27° (cluster A) to 176° (cluster G). There is a clear tendency for the peptides with smaller bents to be dominant in terms of population. This bent is the main conformational difference among the structures of each cluster and might be important for peptide insertion into bacterial membranes.

MD was further used to simulate peptide interactions with a 1-palmitoyl-2-*cis*-9,10-methylene-hexadecanoic-acid-sn-glycero-3-phosphoethanolamine (PMPE) membrane. Interesting and relevant observations were made, as follows:

(i) during the 100 ns MD run of the water-membrane system (peptide absent), no water molecules diffused into the membrane; membrane boundaries and integrity were preserved as well;
(ii) in the simulation of the first membrane-peptide system (peptide fully protonated, having a net charge of +6), after ~7 ns, a few water molecules started to diffuse into the intramembrane hydrophobic region of the complex; these water molecules remained close to the peptide till the end of the simulation, most of the time shifting between the inside and the outside of the membrane hydrophobic core (Fig. 5);
(iii) in the course of the MD simulation mentioned in (ii), a few water molecules have actually crossed the membrane bilayer keeping a very close contact to the peptide; this migration was observed in both directions (Supporting Information, Animation 1);
(iv) in the second membrane-peptide simulation, the monoprotonated peptide with a net charge of +1 (protonation only at the N-terminus, all Lys residues kept neutral) was used, and for its full length, practically no water molecules were

Table 2
Physicochemical parameters for the peptides addressed in this study.

Peptides	Estimated pI	Estimated Hydrophobicity (kcal/mol)	Net Charge
pN1-CoaTxII	5.72	+12.98	0
pN2-CoaTxII	5.75	+13.46	0
pAS-CoaTxII	6.73	+20.66	0
pC1-CoaTxII	9.63	+12.89	+3
pC-CoaTxII	10.14	+18.33	+6

Peptides	Primary structure	Snake venom	References
pC-CoaTxII	KKYRIYPKFLCKK	*C.o.abyssus*	-
myotoxin II-peptide	KKYRYYLKPLCKK	*B. asper*	[16]
pepMTX-II	KKYRYHLKPLCKK	*B. brazili*	[18]
pBthTx -I	KKYRYHLKPKCKK	*B. jararacussu*	[20]
pEM-2	KKWRWWLKALAKK	*B. asper*	[26]
	:* * .		

Fig. 3. Multiple sequence alignment of pC-CoaTxII with related antibacterial synthetic peptides also inspired by PLA_2 toxins from snake venoms. pC-CoaTxII shares structural similarity with other antibacterial synthetic peptides inspired by Lys49 PLA_2s from snake venoms. Asterisks represent identical amino acids, double dots (colon mark) indicate highly similar conserved groups, and dots (full stop mark) show slightly similar conserved groups.

found in the intra-membrane hydrophobic region; water molecules were almost exclusively bound to the hydrophilic phospholipid heads;

(v) the peptide appears to have kept its geometry, length (crossing the membrane leaf to leaf, distance plot available in (Supporting Information Fig. S3) and mobility (XY-axes movement along the membrane plane) for the entire 100 ns run, irrespectively of its net charge.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.ejmech.2018.02.055.

Overall, these results highlight the significant role of the positively charged side chains of Lys residues in promoting water penetration and diffusion into and across the membrane, which agrees with the well-known relevance of positively charged amino acids for peptide antimicrobial action. In other words, MD simulations undertaken provide a plausible explanation, at the molecular level, for the antibacterial activity of pC-CoaTxII.

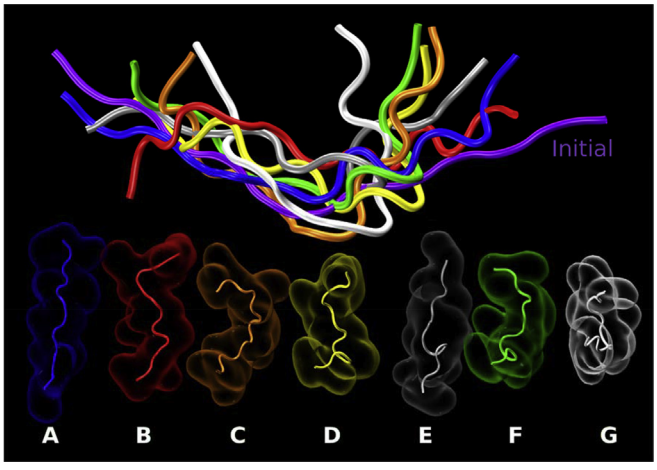

Fig. 4. Structures of the antibacterial synthetic peptide, pC-CoaTxII. Top: A representative structure of each of the seven clusters obtained from the MD simulations (A to G, from the most to the least populated) and the initial modelled structure of pC-CoaTxII, all superimposed and represented as tubes. Residue 1 is at the left and residue 13 at the right. Bottom: Individual clusters represented as tubes and volume. Residue 1 is at the top. The color code is the same in top and bottom images. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3. Discussion

According to Cardozo et al. [4], methicillin-resistant *Staphylococcus aureus, Escherichia coli 0157:H7* and *Pseudomonas aeruginosa* are some of the most virulent pathogens of clinical significance. These intriguing Gram-positive and Gram-negative bacteria have promptly developed diversified antibiotic-resistance mechanisms, alerting researchers to the urgent need for new therapeutic alternatives to fight this public health threat. Snake venom toxins, mainly Lys49 PLA_2s have been proven as promising templates for rational design of engineered antibacterial strategies with practical applications [7,17,27,28]. For instance, our research group recently isolated and characterized an antibacterial and basic Lys49 PLA_2 from *Crotalus oreganus abyssus* venom, named CoaTx-II. However, despite the promising antibacterial properties of CoaTx-II, the molecular basis for their bactericidal action is not fully understood, as this and other interesting PLA_2s are unable to hydrolyze membrane phospholipids. This suggests that some molecular regions of the toxins primary structure may act as key effectors in the induction of bacterial cell death independently of catalytic activity [29]. A more detailed understanding of the potential and mode of action of such molecular effectors is therefore relevant for future development of potent antimicrobial approaches not prone to elicit pathogens resistance. In this connection, the present study aimed at the identification of antibacterial segments within the sequence of toxin CoaTx-II. This allowed for establishment of the cationic peptide pC-CoaTxII, which mimics the C-terminal region of CoaTx-II (residues 115–129) as a highly promising antibacterial lead active against all five bacterial strains tested, which included clinically relevant isolates. The C-terminal peptide was more active against all bacterial strains than CoaTx-II. In agreement with this finding, small peptides inspired on *B. asper* myotoxin II also showed higher antibacterial effect than intact toxin [19,26]. The higher antibacterial activity of peptide pC-CoaTxII can be associated with higher membrane permeability, diffusion, number of membrane interactions and smaller size, but new experiments should be conducted to explain this.

Peptide pC-CoaTxII was more efficient against Gram-negative *P. aeruginosa* strains ATCC and 31NM, as, even at the lowest concentration tested, 5.95 μM, it was still capable of inhibiting growth of those bacterial strains in approximately 52% and 48%, respectively. Notwithstanding, one cannot say that pC-CoaTxII is selective for Gram-negative bacteria, as excellent results were also obtained

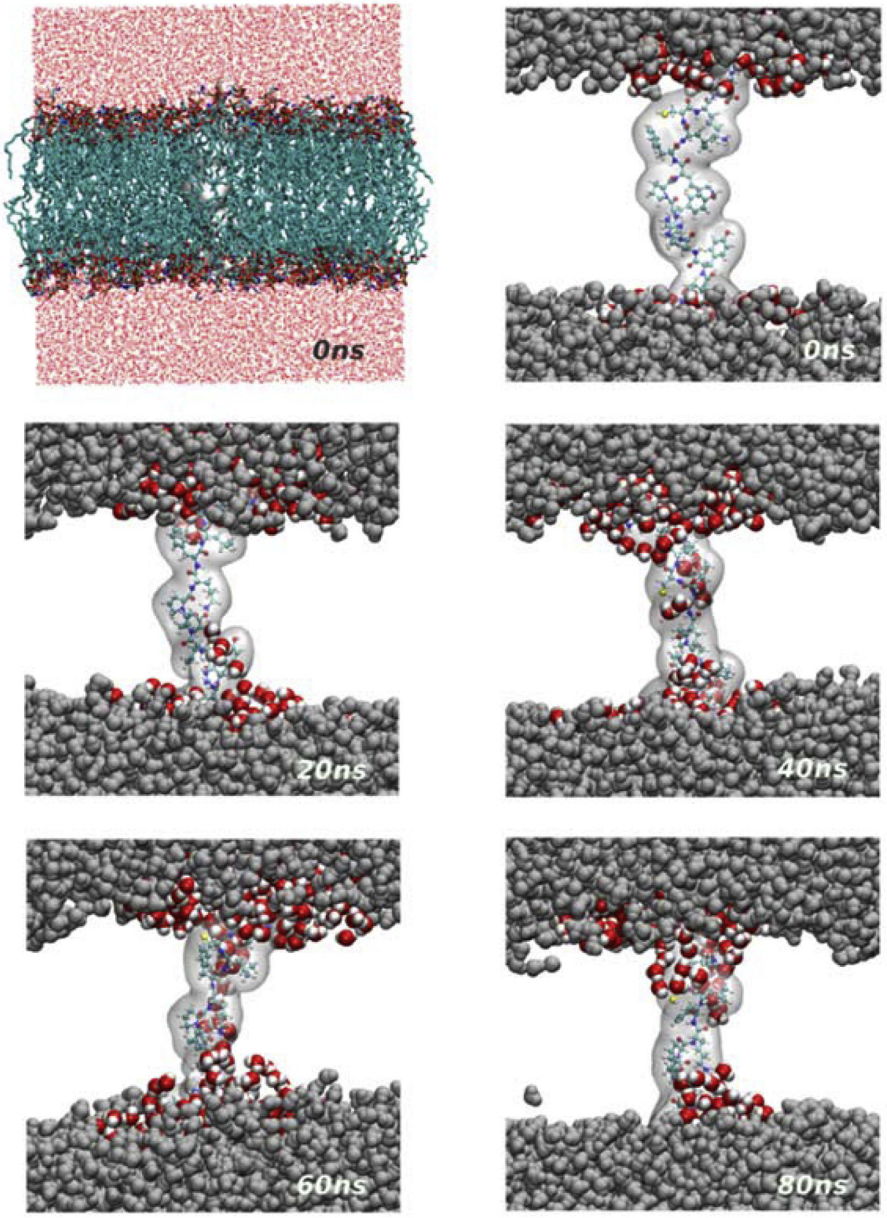

Fig. 5. Membrane-peptide complex (PMPE − pC-CoaTxII). Representative simulation snapshots from first model (peptide net charge of +6) at different time points. The top left corner shows the initial complex structure; the water molecules are represented as dots, phospholipids are shown in licorice and the peptide as surface area. The other five frames show a close up of the peptide region at different moments in time, with the water molecules represented in vdW and colored gray, and the peptide shown as a transparent surface. All water molecules within a radius of 17 Å centered in residue 1 or in residue 13 are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

against Gram-positive *S. aureus* BEC9393 and *S. aureus* rib1. As such, peptide pC-CoaTxII emerges as a most valuable wide-spectrum antibacterial lead, of extreme medical, pharmacological and epidemiological importance, given the severe pathogenicity and antibiotic-resistance of the clinical isolates included in this study. Currently, multi-drug resistant *S. aureus* are the Gram-positive bacterial strains responsible for nosocomial infections with higher rates of morbidity and mortality; likewise, antibiotic-resistant *E. coli* and *P. aeruginosa* strains are some of the major Gram-negative bacteria underlying severe nosocomial infections, which develop resistance to multiple classes of conventional drugs even during infection treatments [6,30].

Our findings further suggest that the antibacterial properties of the CoaTx-II toxin are probably related to its C-terminal region, which agrees with other studies on related toxins, where similar C-terminal short regions were shown to trigger antibacterial effects similar to that of the parent toxin [26,29]. In fact, Costa and collaborators [18] previously reported that a synthetic peptide based on the C-terminal region (residues 115–129) of a catalytically inactive PLA_2 MTX-II, from *Bothrops brazili* snake venom, presented an antibacterial activity similar to that of the parent protein. Actually, although antibacterial peptide pC-CoaTxII herein reported is novel, it resembles other C-terminal 13-mers retrieved from sequences of other snake venom toxins that also display antibiotic properties [18,31], such as peptides myotoxin I-(115–129) and pep-MTXII, from PLA_2 toxins isolated from *Bothrops asper* [31] and *Bothrops brazili* [18], respectively. Hence, it becomes clear that the C-terminal region from related PLA_2 toxins is an important source

for novel potent antibacterial peptides valuable for biomedicine and biotechnology, thus deserving deeper and wider attention from researchers, towards optimization and generation of viable, efficient and economic applications [17].

It is widely recognized that the biological activities expressed by short peptides are a consequence of both their primary and three-dimensional structures that, in turn, determine their physicochemical traits. Of all peptides herein reported, pC-CoaTxII (KKYRIYPKFLCKK) showed the highest basicity and net charge at physiological pH (estimated pI of 10.14; +6 net charge), and the highest hydrophobicity as well (estimated +18.33 kcal/mol), which is undeniably correlated with its antibacterial properties. Relevantly, its shorter analogue, pC1-CoaTxII (YPKFLCKKP), was unable to reproduce its antibacterial effects, probably due to its lower basicity (estimated pI of 9.63), net charge at physiological pH (+3) and hydrophobicity (estimated +12.89 kcal/mol). This advocates the essential role of peptide net charge and hydrophobicity as key elements for the display of antibacterial activity. The examination of the antibacterial property of new synthetic peptides with the same size (13-mer), similar hydrophobicity and net charge of pC-CoaTxII can be useful to solve this puzzle and confirm the importance of physiochemical parameters. Indeed, most reported antibacterial peptides are cationic and suggested to owe their action by targeting bacterial membranes; in this connection, basic amino acids, such as Lys and Arg, have been considered key residues to promote electrostatic binding of peptides to anionic bacterial membranes, facilitating hydrogen bonding and permeabilization and/or disruption of the phospholipid bilayer, causing bacterial cell death [32,33]. As such, the different content in basic residues of peptides pC-CoaTxII and pC1-CoaTxII, may account for their different antibacterial performance. Also, differences in the antibacterial potency of pC-CoaTxII against the different bacteria tested can arise from distinct lipid membrane compositions for each bacterial strain, which as cited above are the most probable molecular targets of cationic peptides. Although bacterial cell membranes are mainly formed by negatively charged lipids, which underlies selective binding/interaction with positively charged antibacterial peptides, phospholipid membrane composition varies amongst different species, and even amongst different strains of a given species [34].

From the above, it stems that pC-CoaTxII most likely owes its antibacterial properties to its ability to destabilize bacterial membranes. To test this hypothesis, we carried out *in silico* studies which suggest that, when inserted into such membranes, the fully protonated pC-CoaTxII promotes water diffusion into the membrane hydrophobic core. This apparently opens an artificial and unregulated water channel that might create an osmotic shock between the cytoplasm and the extracellular medium, eventually leading to collapse of the bacterial cell. Another possibility could be that water molecules that are using the peptide as an anchor/entrance point would disrupt the non-polar interactions between neighboring phopholipids tails, destabilizing the membrane and causing its disruption. Interestingly, the importance of the positive net charge for antibacterial activity can be equally correlated with *in silico* observations: when simulating peptide-membrane interactions using pC-CoaTxII with a net charge of only +1 (all Lys neutral), no water penetration into the membrane was seen.

Based on data obtained, one cannot rule out other modes of antibacterial action occurring for pC-CoaTxII, either as part of an eventual multimodal action or, less likely, as the sole mechanism. Several complex aspects that were not addressed in the present work still need to be elucidated. The energetics of peptide insertion into the membrane, the protonation state distribution for every residue inside the membrane (only extreme cases were studied), the role of the central Pro-induced bent, and a thorough exploration of the possible orientation of peptide insertion into the membrane are matters to be explored in the future.

4. Concluding remarks

In summary, it was demonstrated that the biomimetic peptide pC-CoaTxII is able to reproduce the ability of CoaTx-II Lys49 PLA_2 to kill Gram-positive and Gram-negative bacteria, including multi-resistant clinical isolates, thus suggesting a molecular mechanism for the antibacterial activity of CoaTx-II. This confirms the role of snake venom toxins as remarkable sources to get inspiration towards development of novel wide-spectrum and potent drugs against antibiotic-resistant bacteria. Insights into pC-CoaTxII, regarding its structural, physicochemical, antibacterial and membrane-interaction properties, open a window of opportunity in the field of peptide-based antibacterial drugs. In the last years, innovative peptide therapeutics have gained market and pharmaceutical relevance [35,36]. Currently, it is estimated that there are approximately 140 peptide drugs under clinical trials [37,38]. The main drawbacks and challenges for pharmaceutical exploration of peptides as drugs mainly concern their susceptibility to proteolysis and low oral bioavailability [10,38]. However, peptide-focused medicinal chemists have been developing innovative strategies to bypass such constraints, namely by use of peptidomimetics with increased bioavailability [38]. Future studies can be conducted to increase efficiency and selectivity of pC-CoaTxII, either by producing mimetics or by conjugation for targeted delivery, as recently reported by Lainson, Daly, Triplett, Johnston, Hall and Diehnelt [39].

5. Experimental section

5.1. Peptide design and synthesis

Five small peptides mimicking some molecular regions of the primary structure of CoaTx-II toxin were selected for the peptide synthesis and evaluation of antibacterial effect. Table 1 summarizes the 5 peptides selected, their molecular mass (calculated *in silico* using bioinformatics tolls available in ExPASy World Wide Web server, such as Peptide Mass Calculator and Amino Acid Calculator), amino acid sequence, charge and region of the protein used as template for the peptide design. The molecular region number of parent protein mimicked is according to numeration proposed by Renetseder et al. [40] The synthesis of small peptides selected was manually carried out according to method of solid phase peptide synthesis (SPPS) [23] using the Fmoc/^{t}Bu technology on Rink Amide AM resin (0.60 mmol/g), and also used recently by our research group [41]. All synthetic peptides of this work were prepared as C-terminal carboxamides, and the chain elongated through coupling and deprotection cycles until the full amino acid sequence was assembled. Initially, the resin was pre-swelled for 15 min with *N*,*N*-dimethylformamide (DMF), followed for 15 min with dichloromethane (DCM) and then transferred into the syringes, which were used as reaction vessel. The Fmoc deprotection step was performed using 20% piperidine in DMF. The first Fmoc-protected C-terminal amino acid was coupled to the resin, using 5 M equiv of the Fmoc-protected amino acid in DMF (0.2 M), 5 equiv of 0.5 M HBTU (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) in DMF, and 10 equiv of 2 M DIEA (*N*,*N*-Diisopropylethylamine); the coupling step was carried out for 60 min at room temperature. After this, the reaction vessel was washed with DMF (15 min) and DCM (15 min) to remove excess reagents. Each coupling and deprotection step was monitored using the ninhydrin test (primary amines) [42] or isatin assay (secondary amines) [43]. The other Fmoc amino acids were sequentially coupled in the C → N direction using the same deprotection and coupling reaction cycles

described above until obtain the desired sequence. After the completion of synthesis, the peptide was separated from the resin using a cleavage cocktail formed by 95% trifluoroacetic acid (TFA), and two scavengers: 2.5% water, and 2.5% triisopropylsilane (TIS) for 3 h at room temperature. Simultaneously, the side-chain protecting groups of amino acid residues were removed by this acid treatment. The synthetic products were precipitated using cold diethyl ether, washed, and dissolved in a buffer for the purification process.

5.2. Peptide purification and homogeneity

The synthetic peptides were separated from the crude product by reverse phase medium pressure liquid chromatography (RP-MPLC) using a gradient of 15–30% acetronitrile in water (0.05% TFA) and its homogeneity analyzed on a reverse phase high pressure liquid chromatography (RP-HPLC) Accela (Thermo Fischer Scientific, Bremen, Germany) using a C18 Nucleodur gravity column (Macherey-Nagel, USA) 5 μm particle size and dimensions 4 mm ID x 125 mm. The synthetic molecules were eluted over a gradient of 100% solvent A (100% H_2O with 0.1% (v/v) formic acid) to reach 100% Solvent B (ACN with 0.1% (v/v) formic acid) at 30 min at a flow rate of 0.5 mL/min. The peptide purity degree was quantified based on UV-absorption spectroscopy at 220 nm and determined by peak chromatographic integration using a chromatography data systems software.

5.3. Identification of synthetic product by mass spectrometry

The molecular weight (exact value m/z) of synthetic peptides were confirmed by mass spectrometry (MS) analysis, which were performed on an LTQ OrbitrapTM XL hybrid mass spectrometer (Thermo Fischer Scientific, Bremen, Germany) controlled by LTQ Tune Plus 2.5.5 and Xcalibur 2.1.0. The electrospray ionization source settings (ESI) were as follows: source voltage, 3.1 kV; the capillary temperature was 275 °C with a sheath gas flow rate ate 40 and auxiliary gas flow rate at 10 (arbitrary unit as provided by the software settings). The capillary voltage was 36 V and the tube lens voltage 110 V. MS data handling software (Xcalibur QualBrowser software, Thermo Fischer Scientific) [41].

5.4. Antibacterial activity

5.4.1. Bacterial strains

The following five bacteria strains (ATCC cells or antibiotic-resistant bacteria) were used in our antibacterial studies: *Pseudomonas aeruginosa* 31NM [44] *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Staphyloccocus aureus* BEC9393 [45] and *Staphyloccocus aureus* rib1 [5]. All microorganisms accessed were provided by Dr. Marcelo Lancellotti - Biotechnology Laboratory (UNICAMP). The bacteria strains were maintained in Mueller Hinton Broth (MHB) plus 20% glycerol and frozen at −20 °C until use.

5.4.2. Preparations of the bacterial suspensions

For the agar diffusion and broth microdilution assays, the bacteria strains were subcultured in 2 mL of MHB and growth in Mueler Hinton Agar, followed by incubation at 37 °C for a day. After this, the bacteria were resuspend in 5 mL of sterile PBS saline and the turbidity was adjusted for 10^8 UFCs (colony-forming units)/mL according to 0.5 McFarland scale. The bacterial concentrations were quantified and confirmed spectrophotometrically at 620 nm and cell counting with Neubauer chamber.

5.4.3. Agar well diffusion assay

Firstly, the evaluation of antimicrobial activity of synthetic peptides was performed by the agar diffusion method according to Bauer and collaborators [24] and Clinical and Laboratory Standards Institute (CSLI) with minimal modifications. Briefly, a hole was punched aseptically with a sterile tip, and the small peptides were dissolved in a PBS solution. After this, 100 μl of each synthetic peptide (575 μM) was added to bacterial agar media (in the holes), and incubated at 37 °C for 24 h. Commercial antibiotics were used as positive control and the diameters of inhibitory halo induced by synthetic peptides were measured using a low-pressure digital caliper (Truper). The peptide that presented promising antibacterial effect was selected for analysis by dilution method.

5.4.4. Broth microdilution method

The analysis of bacterial sensitivity to pC-CoaTxII peptide was performed by broth microdilution assays using 96-well microtitration plate in line the technique proposed in the Clinical and Laboratory Standards Institute. For this *in vitro* assay, 50 μL of MHB were added to wells, following by the addition of 50 μL of synthetic peptide solution (serial dilutions, different concentrations). The peptide solution was prepared by dissolving the peptide in medium. Posteriorly, 20 μL of Gram-positive and Gram-negative bacteria suspensions previously prepared as in 2.5.2. were added to each well of microplate. Clinical antibiotics were used as positive control, while the medium as negative control. The protein intact, CoaTx-II (190 μM), also was used for quantitative comparison. The microplate was incubated at 37 °C for 24 h and the inhibition of bacterial growth determined by spectrophotometry reading at 595 nm. All *in vitro* antibacterial assays were run using 3 replicates.

5.5. Determination of phsyco-chemical proprieties

The physico-chemical parameters (pI, hydrophobicity and net charge) of synthetic peptides derived from CoaTx-II were determined *in silico* by bioinformatic tools, such as ProtParam and PepDraw available at Expasy plataform.

5.6. Tridimensional structure of pC-CoaTxII

In silico studies were carried out in the most active peptide. The modelling of the 13-mer peptide KKYRIYPKFLCKK was carried out using the DS Visualizer software [46]. From this structure, a two-stage protocol was applied, both using AMBER12 software [47]. In the first stage an accelerated Molecular Dynamics (MD) simulations was performed [48]. The total simulation time was 1.25 μs, in the NVT ensemble with a 2 fs timestep; the temperature was regulated by the Langevin thermostat and kept at 300 K; the water model used was TIP4P with a 10.5 Å rectangular box; 6 Cl^- counter ions were added to neutralize the system; the cutoff for the long-range and van der Waals interactions was set to 10 Å; the 12SB amber force field was used for the peptide. In the second stage a clustering analysis was run in the 50.000 microstates saved from the trajectory. A total number of seven clusters was obtained. From each cluster, the most prevalent structure was used. A model system containing six solvated peptide molecules with that structure, in 3×2 parallel disposition (Supporting Information Fig. S4). A classic Molecular Dynamic simulation was performed in every model system with the following features: total simulation time was 50 ns, in the NPT ensemble; the timestep was set to 2 fs; the temperature was regulated by Langevin thermostat and set to 300 K; the pressure was regulated by Berendsen barostat and set to 1 atm; the water model used was TIP4P within a 12 Å rectangular box with 36 Cl^- counter ions neutralizing the system. The cutoff was set to 10.5 Å and the 12SB amber force field was used for the peptide.

5.7. Complex pC-CoaTxII in E. coli membrane

A mimic of *E. coli's* bacterial membrane was built. Only the lipids were included in the model. A heterogeneous bilayer containing 120 PMPE phospholipids, 2 myristic acid, 6 palmitic acid, 6 palmitoleic acid and 6 oleic acid molecules per layer was modelled using the Charmm-gui online membrane builder interface [49]. This lipidic mixture and respective proportions corresponds to the known composition of the membrane of *E. coli*. Using the same software interface, the most prevalent peptide structure among all clusters identified in the previous step, was inserted into the membrane. The peptide geometry was kept unchanged, even for the hydrogen atoms; only the orientation was altered using the interface options to align the peptide in a parallel and centered disposition relatively to the phospholipids (Supporting Information Fig. S5). As the protonation state of membrane-inserted peptides is very difficult to be determined we decided to model the two extreme situations. In a first model we kept all Lys/Arg side chains positively charged (peptide total charge +6). In a second molecular model, the protonation state of the Lys side chains was changed to neutral (the peptide total charge was changed to +1 due to a single Arg residue). For these two models a classic Molecular Dynamics simulation using the AMBER12 software was performed. The total simulation time was 100 ns in the NPT ensemble and a 2 fs time-step; the temperature was regulated by the Langevin thermostat at 300 K; the pressure was regulated by Berendsen barostat and set to 1 atm; the TIP4P water model was used, with a 30 Å rectangular box restricting solvation to the Z-axis keeping all waters above or under the membrane plane; Na^+ counter ions were added to both models; 34 ions to the +6 charge model and 39 ions to the +1 charge model; the cutoff for intermolecular interactions was set to 10.5 Å. The Coulomb interactions were calculated with the PME algorithm; the charmmff36 force field was used for phospholipids, fatty acids and the peptide [50].

5.8. Statistical analyses

All assays were carried out at least three times and the experimental results were represented as the means ± standard deviations (SD). The statistical significance was evaluated using Student's t-test and ANOVA tests, followed by Tukey's Post Hoc in the Origin Pro 8 Software (OriginLab Corporation). Differences were considered statistically significant if $p < 0.05$.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ejmech.2018.02.055.

Conflicts of interest

There is no interest conflict in this paper.

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