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A C-terminal cationic fragment derived from an arginine-rich peptide exhibits *in vitro* antibacterial and anti-plasmodial activities governed by its secondary structure properties

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ABSTRACT

The differential *in vitro* antimicrobial activity of a 12-residue-long arginine-rich peptide derived from protamine was examined against bacterial and parasite microbes. A design of discrete peptide fragments based on the thermolysin-digestion map allowed us to propose three peptide fragments to be further assessed regarding their biological and secondary structural properties. Peptide structure allowed designing three arginine-rich fragments. All peptide fragments were assessed regarding their antimicrobial activity against Gram-positive and Gram-negative bacteria and a human malaria strain. Qualitative and quantitative assays carried out for determining all peptides' antibacterial activity at different concentration levels included radial diffusion and a time-controlled technique. Tests demonstrated that all assessed molecules inhibited invasion of *Plasmodium falciparum* parasites to human red blood cells. Cytolytic activity of the parent protamine peptide was completely abolished by strategically fragmenting its aminoacid sequence. Remarkably, the cationic C-fragment exhibited stronger biological activity than its parent peptide. Interestingly, the peptide fragment denoted as 2077 displays a typical α-helix profile according to its CD spectrum. The results support proposing the protamine C-terminal fragment as a potential new antimicrobial peptide.

1. Introduction

Cationic antimicrobial peptides comprise a group of polypeptides with highly variable sequences and secondary structures, all sharing a few common characteristics like having a length varying from 12 to 50 aminoacids and a molecular size smaller than 10,000 Da [17]. It is well known that most of these molecules possess amphipathic properties, which allow them to interact simultaneously with lipid-like and negatively charged molecules through their cationic regions, so as to attach themselves to microbial membranes [14].

So far, about 800 members of the antimicrobial peptide family have been identified and characterized, among them being antibacterial, anti-viral [1], anti-fungal [10,36] anti-tumoral [30] as well as immunomodulating peptides [21,27].

Different ways of classifying the wide repertoire of antimicrobial peptides have been proposed. One of them considers the chemical characteristics of their aminoacid composition, based on the fact that peptides containing certain aminoacids such as tryptophan (Trp) and arginine (Arg) have been demonstrated to have antimicrobial activity [8].

Important factors such as the increasing microbial resistance to antibiotic and the limited number of therapeutic strategies available to tackle pathogens evidence the need of developing novel compounds that can be used as molecular tools for controlling the spread of infectious diseases.

Efforts for finding new potentially therapeutic molecules include studying the function–structure relationship as a rationale for explaining possible antimicrobial mechanisms and therefore enable a better molecule design. However, most antimicrobial mechanisms of these molecules are still unknown.

In this work, the anti-bacterial and anti-parasitic *in vitro* activities of the C-terminal fragment of protamine were examined, and a possible correlation with its secondary structure pattern was also considered.

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2. Materials and methods

2.1. Bioinformatics analysis

The aminoacid sequences of protamine and its three thermolysin cleaved derived fragments were aligned against different homologous proteins, including other protamines from different sources as well as a 756-residue-long peptidylprolyl isomerase (ISS), a representative of the serine carboxypeptidase superfamily and the hypothetical protein [*Plasmodium vivax* Sal-1] pvx_089044, all reported in the NCBI database (http://www.ncbi.nlm.nih.gov/blast.cgi). Sequences were aligned using the TBLASTN tool and the Clustal W multiple sequence alignment method (www.personal.rhul.ac.uk/ujba/110/bioinfo/clustalE.html).

2.2. Solid-phase peptide synthesis

Peptide design considered the thermolysin-digestion sites of protamine to generate three peptide segments: ^{1}Pro-^{10}Pro, ^{11}Val-^{18}Arg and ^{19}Val-^{32}Arg. Thermolysin (a metalloprotease) is produced by the Gram-positive bacteria *Bacillus thermoproteolyticus* [2]. Thermolysin specifically catalyzes the hydrolysis of peptide bonds containing hydrophobic aminoacids such as ^{10}Pro-^{11}Val (P-V), ^{18}Arg-^{19}Val (R-V) on the protamine aminoacid sequence. Protamine sulphate from salmon sperm used at 1 mg/mL was obtained from Calbiochem Corporation, USA. All synthetic peptides were produced in the chemistry-synthesis functional group of the *Fundación Instituto de Inmunología de Colombia-FIDIC* and were numbered as 2075 (fragment 1–10), 2076 (fragment 11–18) and 2077 (fragment 19–32), according to our institute's numbering system. Peptides were synthesized manually using Merrifield's protocol for *t*-Boc-based solid-phase peptide synthesis (SPPS) [28], later modified by Houghten for multiple-peptide synthesis [20]. Simultaneously, a set of previously reported antimicrobial peptides were synthesized and physicochemically characterized. Those were Maganine-1, Magainine-2, PGLa, Cecropin B, Cecropin P1, Cecropin A, Melittin, Andropin, Bombinin, TAP and the following mastoparans: MP-3, MP-7, MP-8, MP-17, MP-X and Polistes.

2.2.1. Solvents and reagents

N-Boc-α-L-protected aminoacids were obtained from Bachem (Bachem, CA, USA); benzotriazolyl *N*-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP) from Richelieu Biotechnologies Inc. (Hamon, Montreal, Canada); dicyclohexylcarbodiimide (DCC) and tetrahydrofuran (THE) from Fischer (USA); trifluoroacetic acid (TFA), hydrogen fluoride (HF), ethyl ether (Et_2O), dichloromethane (DCM), *N*,*N*'-dimethylformamide (DMF) and diisopropylethylamine (DIEA) from Merck (Bogotá, Colombia). All other solvents and reagents were of analytical grade, obtained from commercial sources and used without further purification.

2.3. Liquid chromatography

Analytical RP-HPLC was run on a Hitachi-Merck instrument model L17400 (Merck, Darmstadt, Germany) provided with an analytical Vydac C18 silica (5 μm) column (4.5 mm × 30 cm). A Vydac preparative C18 column was used for preparative RP-HPLC by linear-gradient elution from 0 to 100% B using the following solvent system: A: H_2O 0.05% TFA, B: CH_3CN, 0.05% TFA for 45 min (100 min for preparative process) at a flow rate of 1.0 mL/min (4.5 mL/min for preparative process). The eluate was monitored at 220 nm on a UV–DAD detector.

2.4. Mass spectrometry analysis

Mass spectra were recorded using a Bruker Protein TOF mass spectrometer in reflectron mode (Billerica, MA). Matrix-assisted laser desorption ionization (MALDI) experiments were performed using the time of flight (TOF) technique. This instrument uses an N_2 laser radiating at 337-nm wavelength with 3-ns pulses. Acceleration voltage was +17.5 kV and reflectron voltage +20 kV. All spectra were obtained by a respective series of 10 laser pulses to ensure comparable conditions. Laser power was as minimal as possible for each measurement. The matrix used in this work was α-cyano-4-hydroxycinnamic acid (CCA) (Sigma Chemical Co., Saint Louis, MO). The CCA matrix was prepared as a saturated solution in 1 mL TA (40% acetonitrile in 0.1% trifluoroacetic acid). Samples were dissolved in TA to give a 100 pmol/pL concentration. Samples were prepared for MALDI–TOF analysis by diluting the sample solution in the matrix-saturated solution to a 10 pmol/μL concentration. Then, 0.5 μL aliquots of the mixture sample–matrix were poured onto the target plate, left to air dried and analyzed.

2.5. Circular dichroism

Circular dichroism (CD) assays were performed at room temperature on nitrogen-flushed cells using a Jasco J-810 spectropolarimeter (Madrid, Spain). Spectra were recorded within a 190–260 nm wavelength interval using a 1-mm path length rectangular quartz cell. Each spectrum was obtained from averaging three scans taken at a scan rate of 20 nm/min with 1-nm spectral bandwidth and corrected for baseline deviation using Jasco software. CD profile of each molecule was obtained by dissolving lyophilized purified peptides in (i) 50 mM sodium phosphate buffer (PBS), pH 7.0, or (ii) 0–30% aqueous 2,2,2-trifluoroethanol (TFE) in a final volume of 500 μL. A typical 0.2 mM peptide concentration in TFE–water mixture is stabilized but does not induce secondary structure in peptides, as described elsewhere [33]. The results were expressed as mean residue ellipticity (θ), the units being degrees cm^2 dmol^{-1} according to the $(\theta) = \theta_\lambda/(100lcn)$ function where θ_λ is the measured ellipticity, l the optical path length, c the peptide concentration, and n is the number of aminoacid residues in the sequence.

2.6. Acidic gel electrophoresis

Peptide separation was performed at pH 4.3 in 20% polyacrylamide gels (acidic PAGE) having no staking gel. The electrophoresis buffer contained 0.035 M β-alanine with 0.07 M acetic acid. The system was run at a constant potential of 150 V for 45 min. Subsequently, gels were stained with a Coomassie blue dye solution prepared in 50% aqueous methanol. Water was employed as the distaining solution. In all cases, peptides migrated due to their specific charge in agreement to their dipolar moment.

2.7. Microbiological tests

2.7.1. Bacterial strains

Gram-positive bacteria strains were *Staphylococcus aureus* (ATCC 6538), *Enterococcus faecalis* (ATCC 29212); Gram-negative strains were *Escherichia coli* (ATCC 25922), a wild type *Salmonella typhimurium* (14028s) and its genetically modified *phoP S. typhimurium* (MS7953) and *pcgL S. typhimurium* (EG10627) mutant strains respectively. *S. typhimurium* strains were kindly provided by Professor E.A. Groisman from the Molecular Microbiology Department of Washington University in St. Lois MO, USA.

2.7.2. Bacterial culture media

Gram-positive bacterial strains were cultured and subcultured in trypticase soy broth (TSB, Difco Laboratories, Detroit, MI, USA).

Gram-negative strains were grown employing a Luria broth base (LB; Gibco, Cerdanyola del valle, Barcelona, Spain).

To perform radial diffusion assays, bacteria were poured on a low nutrient medium prepared with 10 g of low electroendoosmosis agarose (Sigma Chem. Co, St. Louis, MO, USA), 0.02% (v/v) Tween-20 (Merck, Darmstadt, Germany) 0.3 g TSB (Difco Laboratories, Detroit, MI, USA) to final volume of 1 L.

An overlay of highly nutritive medium was employed for assessing bacterial growth in radial diffusion assays and hence determining the antibacterial activity of antibiotics and peptides. This overlay medium was composed of 20 g agar–agar (Difco Laboratories, Detroit, MI, USA), 0.02% (v/v) Tween-20 (Merck, Darmstadt, Germany) and 10 g TSB (Difco Laboratories, Detroit, MI, USA). Phosphate buffered saline (PBS), pH 7.2–7.4, was prepared by mixing 0.2 g KH_2PO_4, 32 g NaCl, 0.8 g KCl, 4.6 g Na_2HPO_4 to a final volume of 1 L.

Standard antibiotics were employed as positive antibacterial compounds. These consisted in stock solutions of 50.8 mg/mL ampicillin (Binotal; Bayer®, Germany), 10.25 mg/mL kanamycin sulphate (Gibco, Cerdanyola del valle, Barcelona, Spain), 5.78 mg/mL tetracycline monohydrate (Sigma Chem. Co, St. Louis, MO, USA).

2.7.3. Bacterial radial diffusion assay

Pure cultures of the above listed Gram-negative and Gram-positive bacteria were cultured over night at 37 °C under constant shaking. Subsequently, a subculture of each bacterial strain was obtained by inoculating an aliquot into approximately 20 mL of the corresponding medium. Subcultures were then incubated for 5 additional hours at 37 °C under constant shaking. Once this time had elapsed, bacteria were harvested by centrifuging subcultures at 2400 × *g* for 10 min, washed twice with PBS, pH 7.2 at 4 °C, resuspended in fresh PBS and stored at 4 °C until used. The sample's optical density (OD) was read at 620 nm and precise amounts of bacteria were measured accordingly to a previously reported ratio of $OD_{620} = 0.2$ arbitrary absorbance units (au) = 5×10^7 CFU/mL) [32], as needed.

A total of 4×10^7 CFU was carefully dispersed into 15 mL of low nutrient medium previously heated at 45 °C and subsequently poured into Petri dishes. Once solidified, symmetrical wells onto which amounts of 2–10 μL could be loaded were made using a 3-mm diameter sterile cylindrical pouncher. Wells were filled with 8 μL aliquots of ampicillin, kanamycin sulphate and tetracycline, which corresponded to 0.508, 1.025 and 0.578 mg/mL concentrations, respectively. Simultaneously, 2, 5 and 8 μL aliquots of 1 mg/mL peptide stocks were poured to be analyzed. Sample diffusion was permitted by incubating Petri dishes for 30 min at 37 °C. Plates were then coated with 15 mL of nutrient-rich medium previously heated at 45–50 °C. Finally, plates were incubated overnight at 37 °C.

Antibacterial activity was observed as discrete clear rings caused by the radial diffusion of active compounds. The diameters of clear zones were carefully measured and expressed as activity units (AU) according to the relation 0.1 mm = 1 AU.

2.7.4. Quantitative anti-bacterial activity of cationic peptides

A test of bacterial growth was performed to determine the amount of CFU necessary for quantitative assays. In brief, amount of 1, 2, 5 and 10×10^6 CFU suspended in PBS, pH 7.2, were poured into 96-well culture plates. Subsequently, 150 μL of growth medium was loaded per well in a 1:1 ratio. Plates were then incubated at 37 °C under constant shaking. Optical density at 620 nm was read at different times (0, 30, 60, 120, 180, 240, 300 and 1080 min). Accordingly, an amount of 1×10^6 CFU of every bacterial strain was established to be optimal for all quantitative assays.

For quantitative assays, serial dilutions of the antibiotics and peptides were prepared in 96-well culture plates. The starting concentrations were 6.733 mg/mL ampicillin, 2.796 mg/mL kanamycin, 3.851 mg/mL tetracycline, 496 μg/mL protamine and 383 μg/mL of peptide 2075, 270 μg/mL of peptide 2076 and 381 μg/mL of peptide 2077. A final volume of 150 μL was completed by adding nutritive broth medium of each well, maintaining a bacterial suspension: media ratio of 1 to 1. Plates were incubated at 37 °C under constant shaking and antimicrobial activity was quantified by measuring OD_{620} at the same times mentioned above.

2.8. Plasmodium falciparum strains and invasion inhibition assays

Target peptides were tested for their ability to inhibit *P. falciparum* invasion of human red blood cells (RBCs) *in vitro*. In brief, *P. falciparum* parasites from the FCB-2 strain, which are intrinsically resistant to chloroquine [11], were maintained in continuous culture and synchronized at the ring stage using the sorbitol technique [24]. The so obtained ring-stage infected RBCs (parasitemia >5%) were incubated in complete RPMI 1640 media supplemented with 25 mM HEPES buffer, 1 mg/mL hypoxanthine, 4 mg/mL gentamicin, 5U/mL penicillin, 2 g/L glucose, 5% $NaHCO_3$. and 10% O+ plasma. When parasites had reached the schizont stage, 96-well plates containing the peptide samples to be tested were seeded with infected RBCs and additional non-infected erythrocytes (final volume per well was 100 μL, containing 1.5% hematocrit and 0.3% parasitemia). All peptides were assessed in triplicate, incubating plates for 18 h at 37 °C under a 5% O_2, 5% CO_2 and 90% N_2 atmosphere. Cells were then harvested by centrifugation at 150 × *g* for 10 min, and 50 μL of the supernatant was replaced for 100 μL of 15 μg/mL hydroethidine [37]. After further incubation for 30 min at 37 °C and washes with PBS, the pellet was resuspended in 300 μL PBS, poured into polystyrene tubes and the parasitemia was quantified using FACScan flow cytometer (Becton Dickinson, San Jose, CA). The sequence of events was recorded and analyzed using Cell Quest software. An FSC × FL2 profile was used to establish an inclusion gate for non-infected RBCs. Parasitized RBCs were then quantified by quadrant analysis. Normal RBCs and parasitized RBCs incubated in the presence of 15 μg/mL EGTA were used as controls. Invasion inhibition was calculated as 100 × (% parasitemia in the control − % parasitemia the in test)/(% parasitemia in the control).

2.9. Stability of protamine related peptides against endoproteases

This test was performed to evaluate the relative stability of the protamine native sequence and its three derived peptides against severe enzymatic attack by endoproteases. For such aim, the reaction time of different enzymatic extracts versus protamine peptide-derived fragments at 37 °C and pH 7.3 were determined. Enzyme enriched extracts tested were normal human serum (NHS) and a cytosolic extract from the virulent *Mycobacterium tuberculosis* H37Rv strain (ATCC 27294), which contains a variety of endoproteases. Cell fractionation of the mycobacteria was performed according to a procedure adapted from a protocol reported elsewhere [19,25]. In all cases, total protein concentration was determined by using the Pierce micro bicinchoninic acid (BCA) assay and samples were stored at −120 °C until use. In order to evaluate the sensitivity of the protamine peptide and its surrogates to proteolysis, representative amounts of each molecule were incubated with either *M. tuberculosis* cytosol or NHS at 37 °C and pH 7.3 adjusted with PBS over established point times. All reactions were stopped by adding 0.1% of trifluoroacetic acid (TFA) and subsequently an aliquot containing 5 mg of the analyzed molecule was resolved by electrophoresis on 20% acidic

acrylamide–bisacrylamide gels, as described above. After Coomassie blue dye staining, bands corresponding to intact molecule were analyzed respect to an appropriate control as reported before [26].

3. Results

3.1. Antimicrobial peptide characterization

To identify each synthesized peptide, mass spectrometry analyses were performed. Whenever necessary, antimicrobial peptides and protamine-derived peptides were purified by RP-HPLC on a Vydack C18 column, the purity of the final products were higher than 98%, as described in Experimental Procedures. As it can be seen in the MALDI-TOF analysis summarized in Table 1, the experimental *m*/*z* (mass units) obtained for each peptide agreed with their expected molecular masses. According to our experimental design, protamine-derived sequences were completely characterized and identified. Peptides were further characterized by comparing their acidic gel electrophoretical profiles with the profiles of a set of previously reported antimicrobial peptide. As observed in Fig. 1, protamine migrated slower than the three synthesized protamine fragments, which might have probably migrated faster due to their relatively high arginine content, as can be evidenced in the protein alignment shown in Fig. 2.

3.2. Anti-bacterial diffusion and quantitative assays

The results of radial diffusion assays show that the antibacterial activity of protamine and its three derived peptides varied depending on the strain being assessed. Protamine had antibacterial activity against *E. faecalis* 29212 and *S. aureus* 6538, whereas its derived fragments lacked of any activity (Fig. 3A and B, respectively). These findings indicate that the entire aminoacid sequence is required to produce antibacterial activity against these two Gram-positive bacterial strains. On the contrary, all protamine-derived peptides were active against the Gram-negative *E. coli* 25922 strain, while the complete protamine showed a poor anti-bacterial activity (Fig. 3C).

With regard to *S. typhimurium* strains, protamine and its three derived fragments were differentially active. In the *S. typhimurium* EG10627 *pcgL* mutant, both the protamine and the C-terminal fragment corresponding to peptide 2077 inhibited bacterial growth, whereas peptides 2075 and 2076 lack of any

Table 1
Physicochemical characteristics of antimicrobial peptides.

Peptide	Aminoacid sequence	MW (Da)	*m*/*z*	Retention time (min)
Protamine	[1]PRRRRSSSRPVRRRRRPRVSRRRRRRGGRRRR[32]	4332.30	4312.26	15.85
2075	PRRRRSSSRP	1236.50	1250.92	10.68
2076	VRRRRRPR	1133.40	1153.51	10.29
2077	VSRRRRRRGGRRRR	1862.00	1877.25	8.58
Maganin-1	GIGKFLHSAGKFGKAFVGEIMKS	2410.00	2409.60	28.80
PGLa	GMASKAGAIAGKIAKVALKAL	1952.00	1968.70	30.88
Dermaseptin	ALWKTMLKKLGTMALHAGKAALGAAADTISQGTQ	3438.00	3454.30	30.72
Bombinin	GIGGALLSAGKSALKGLAKGLAEHFAN	2904.00	2920.10	34.72
Andropin	VFIDILDKVENAIHNAAQVGIGFAKPFEKLINPK	3733.00	3747.70	40.85
TAP	NPVSCVRNKGICVPIRCPGSMKQIGTCVGRAVKCCRKK	4073.00	4116.00	29.28
Maganin-2	GIGKFLHSAKKFGKAFVGEIMNS	2449.00	2465.90	27.52
Criptidin	LRDLVCYCRSRGCKGRERMNGTCRKEHLLYTLCCR	2105.00	ND	30.93
Cecropin B	KWKVFKKIEKNGRNIRNGIVKAGPAIAVLGEAKAL	3818.00	3832.80	28.74
Cecropin P1	SWLSKTAKKLENSAKKRISEGIAIAIQGGPR	3321.00	3336.80	30.72
Cecropin A	KWKLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAK	4000.00	4002.60	28.80
Polistes MA	VDWKKIGQHILSVL	1618.00	1635.50	30.72
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ	2830.00	2845.50	40.90
Taquilepsin 1	KWCFRVCYRGICYRRCR	2251.00	2254.40	26.29
Apamine	CNCKPEPALCARRCQQH	2014.00	ND	ND
MP-3	LKALAALAKKIL	1235.00	1251.80	27.46
MP-7	INLKALAALAKALL	1405.00	1422.00	35.30
MP-8	INLKALAALAKRLL	1490.00	1507.60	33.81
MP-17	INLKAKAALAKKLL	1477.00	1494.40	23.09
MP-X	INWKGIAAMAKKLL	1539.00	1556.40	33.49
Polistes	VDWKKIGQHILSVL	1618.00	1635.40	30.82

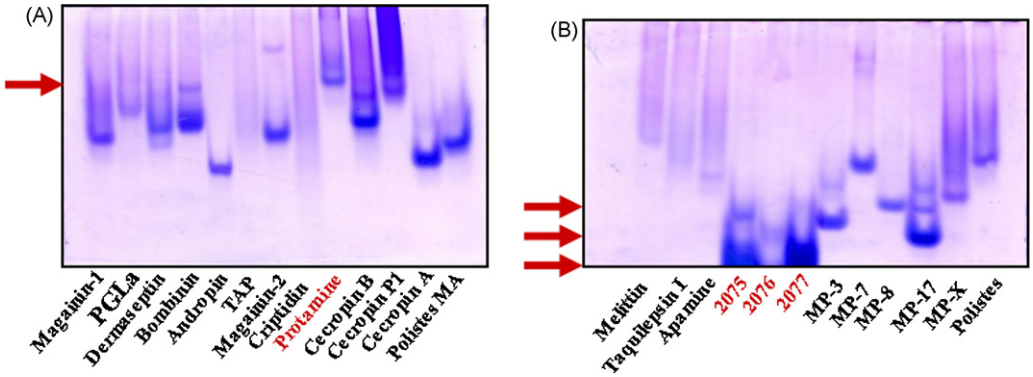

Fig. 1. Acidic PAGE peptide characterization. Reported antimicrobial peptides resolved under acidic conditions, pH 4.3, 150 V and subsequently stained with Coomassie blue dye. Peptide mobility is associated to each molecule dipolar moment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

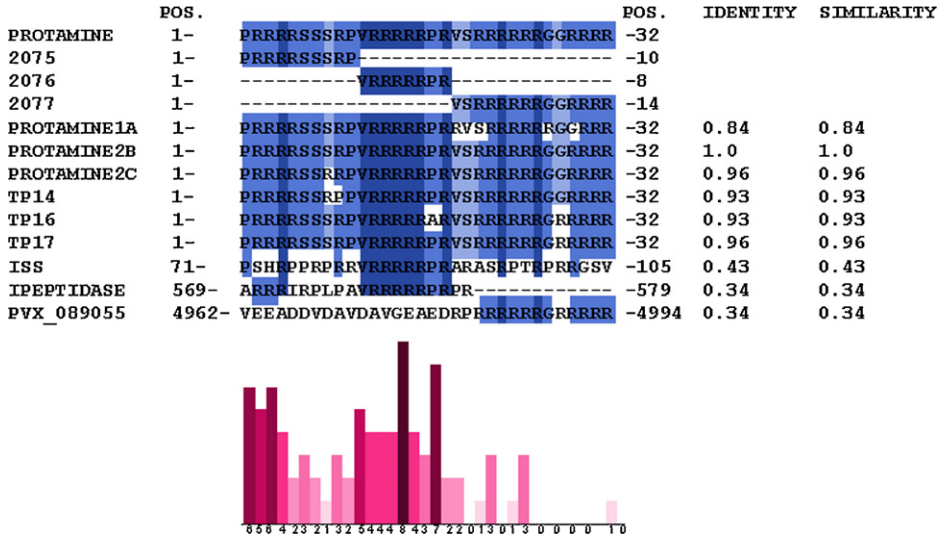

Fig. 2. Protamine aminoacid sequence comparison. Aminoacid alignment between protamine and its derived designed fragments and different related sequences as reported in the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/). Amino acids' identity degree is represented by colors, being 80% mild blue, higher than 60% light blue, higher than 40% light gray and lower than 40% as white. Conservation degree for the sequences was analyzed by Jalview-EBI, 11* score represents identical aminoacids, 8 and 7 conserved aminoacids and 6 and 5 for semi-conserved aminoacids. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

antimicrobial activity (Fig. 3F). Similar activity profiles were observed in the *S. typhimurium* MS7953 (*phoP* mutant) and in the wild type *S. typhimurium* 14028s strain (Fig. 3E and D, respectively), being peptide 2077 more active than the parent protein in both cases.

In agreement with the above-mentioned, data presented in Table 3 show that protamine inhibits growth of *E. faecalis* 29212 at 10 μg/mL and 5 μg/mL concentrations which corresponded to 60 and 40 AU, respectively. When such activity was assessed quantitatively (Fig. 4), it was observed that protamine activity against *E. faecalis* resembled the one shown by Kanamycin (used as a standard control for the test).

Synthetic peptides 2075, 2076 and 2077 exhibited lower activities than the parent protamine against this microbe. All three peptides inhibited bacterial growth after 5 h of treatment in a similar fashion, while at 18 h peptides 2075 and 2076 displayed higher activity than peptide 2077. Quantitative assays with *E. faecalis* showed antibacterial activity even at high dilutions, which were associated to concentrations of 3.80 μg/mL for protamine, 0.29 μg/mL for 2075, 2.08 μg/mL for 2076 and 2.98 μg/mL for 2077. Therefore, the data gathered in this study presents for the first time evidence of the antibacterial activity of cationic peptides designed based on the sequence of protamine, thus indicating that these peptides can be considered as new antibacterial molecules.

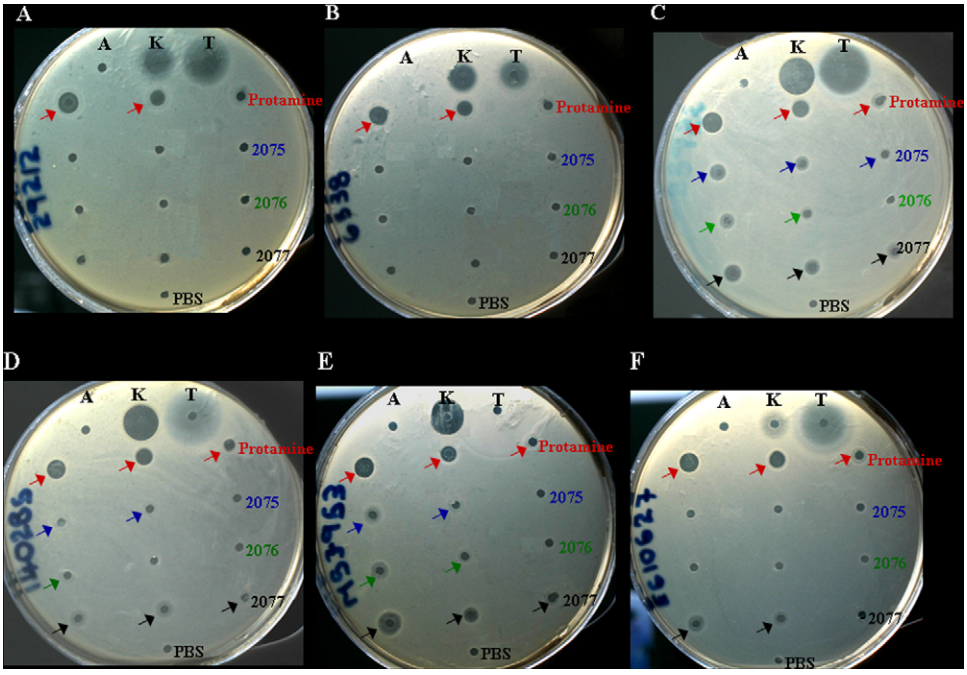

Fig. 3. Antibacterial diffusion assay. Peptides and standard antibiotics were tested against Gram-positive and Gram-negative bacteria as follows. A. *E. faecalis* 29212. B. *S. aureus 6538*. C. *E. coli 25922*. D. Wild type *S. typhimurium* 14028s. E. *S. typhimurium* MS 7953 *phoP* mutant. F. *S. typhimurium* EG 10627 mutant.

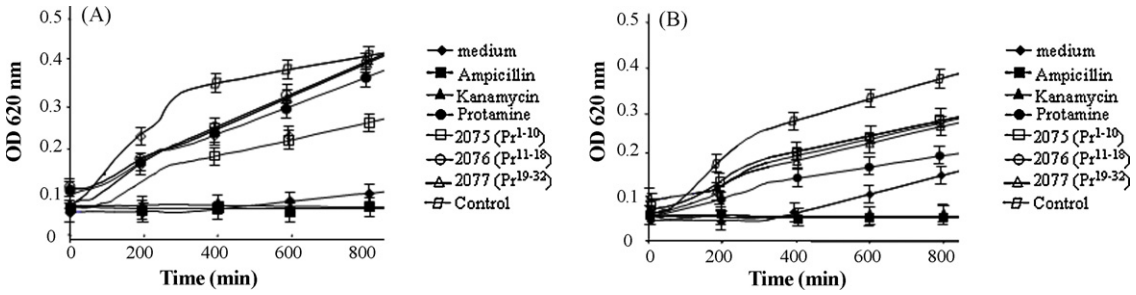

Fig. 4. Quantitative antibacterial activity of cationic peptides. Time-dependent experiments of protamine and its peptide fragments 2075, 2076 and 2077. A. *Escherichia coli* 25922. B. *Salmonella typhimurium* MS7953 *phoP* mutant.

In contrast to quantitative tests, protamine-derived fragments did not display any activity in radial diffusion assays against *E. faecalis*, probably because this bacterium is naturally resistant to protamine as elsewhere reported [13]. Such argument allows explaining the low antibacterial activity profile shown by the protamine-derived fragments herein evaluated. According to our results, protamine possesses growth inhibitory activity against *E. faecalis* which is comparable to the one shown by Kanamycin. Interestingly, only a few cationic peptides, namely *CP10A* and *Bac2A* have been reported to be active against this bacterial pathogen [19], which allows associating protamine's growth inhibitory activity to its high content of arginine (Arg) residues since peptides CP10A and Bac2A are also rich in arginine residues [8].

E. faecalis is a human pathogen of high clinical relevance. Studies performed on different nosocomial infections have shown that *E. faecalis* is the most frequently isolated microbe, being found in 7.13% of samples from patients being treated in intensive care units and 17.98% of intrahospital urinary tract infections. Furthermore, it has been described that in 26% of nosocomial infections, this bacterial pathogen abolish the antibiotic activity of aminoglycosides such as gentamicin by modifying their chemical structure [34], thus stressing the importance of finding new active molecules against *E. faecalis*.

Regarding the antibacterial activity of protamine and the three cationic peptides against *Staphylococcus aureus*, the results of this study showed that this pathogen is resistant to all four molecules (data not shown). In contrast, other studies have regarded protamine as an active anti-staphylococcal peptide [4], whereas other authors have shown that it induces permeabilization of *S. aureus* membrane in a concentration-dependent manner [23]. In such studies, when protamine was tested at 0.5 to 2 mg/mL against *S. aureus* ISP479VC and ISP479R, it did not induce membrane permeabilization [4], but did induce pore formation in other strains such as *S. aureus* 25923 at a 0.625 mg/mL concentration [23]. This later fact could indicate that the protamine concentration used in the present study (0.498 mg/mL) was smaller than the one required for inhibiting staphylococcal growth. In the radial diffusion assay, *S. aureus* displayed sensitivity to protamine at concentrations ranging from 10 μg/mL to 5 μg/mL, associated to 60–40 AU, respectively (Table 3), while no growth inhibitory activity was detected for with peptides 2075, 2076 and 2077 (see Fig. 3B).

Infections caused by *S. aureus* often lead to life-threatening conditions such as bacteremia, endocarditis and pneumonia, all of which are usually restricted to hospital settings. Nevertheless, since *S. aureus* resistance to methicillin has become evident worldwide and is rapidly spreading in the population [15]; this microbe has been considered by the WHO as a pathogen responsible for an emerging disease [18]. Based on this definition, staphylococcal infections are considered emerging diseases due to the increasing number of clinical cases reported over the last few years and the difficulty for determining their lethality, which is normally high at the beginning of the infection as a consequence of the pathogen's rapid dissemination combined with an inadequate clinical assistance.

The antibacterial activity of protamine and its derived fragments was also assessed in *E. coli* 25922. Protamine showed antibacterial activities of 60, 50 and 30 AU at concentrations of 10, 5 and 2 μg/mL, respectively; while peptide 2075 displayed antibacterial activities of 50, 40 and 30 AU, peptide 2076 of 40 and 30 AU and peptide 2077 of 50, 40 and 40 AU at the same concentrations (see Table 3). It can be clearly observed from Fig. 3C that the antibacterial activity of peptides 2075 and 2077 was similar to the one shown by protamine against this bacterial strain.

In the quantitative assays, protamine inhibited bacterial growth at 469 μg/mL, peptide 2075 at 383 μg/mL, peptide 2076 at 270 μg/mL, and peptide 2077 at 381 μg/mL, respectively (see Fig. 4A). Such results indicate that protamine has higher activity than its derived peptides, since protamine-derived fragments showed an activity comparable to the one shown by protamine after 5 h of treatment. This behavior agrees with previous studies reporting that a protamine concentration of 625 μg/mL is capable of inhibiting bacterial growth. Even though the concentration used in this study was lower, a significant activity was observed in quantitative assays up to the seventh dilution, which corresponded to 3.8 μg/mL of protamine, while being 0.29 μg/mL for 2075, 2.08 μg/mL for 2076 and 2.98 μg/mL for 2077.

It is know that *E. coli* expresses a variety of extracellular trypsin-like proteases able to cleave the protamine. Consequently, the antibacterial activity herein reported is significant keeping in mind that the protein concentration range employed was lower than the one used in previous reports [22].

In the clinical scene, *E. coli* is associated to 80% of clinical diseases cases for which a therapeutic treatment has not yet been established and has become a cause of large concern due to the presence of highly virulent strains such as *E. coli* O:157H7, the etiological agent of bloody diarrhea and the Hemolytic uremic syndrome (HUS). The primary reservoir for this bacterial strain is the bovine cattle and is transmitted via ingestion of contaminated meat, water and other foods or by direct person-to-person contact. Other highly virulent strains include shiga toxigenic *E. coli* (STEC) strains which have been implicated as the cause of severe diarrhea in people of all ages, but affect mainly children younger than 5 years of age and can cause serious clinical complications like renal failure, thrombocytopenia and anemia [5].

On the other hand, experiments regarding protamine-peptides' antibacterial activity against the *S. typhimurium* EG10627 (*pcgL* mutant) showed that protamine caused the largest bacterial growth decrease after 5 h of exposure to this molecule, followed by peptide 2075 in second place while peptides 2076 and 2077 caused comparable growth decreases up to the seventh dilution. It can be observed from Table 3 that protamine activity at 10, 5 and 2 μg/mL was associated to 60, 50 and 30 AU, respectively, while peptide 2077 exerted antibacterial activity at 10, 5 μg/mL, respectively associated to 40 and 30 AU. No activity was observed

for peptides 2075 and 2076, as indicated by the lack of growth inhibition zones in the radial diffusion assay with this peptides (Fig. 3F).

Protamine and its derived peptide fragments showed comparable activities against the *S. typhimurium* MS7953 *phoP* mutant, being higher for the parent sequence, as can be observed in Fig. 4B. Table 3 shows that protamine and its derived peptide fragments exerted growth inhibitory activities of 60, 50 and 30 AU at 10, 5 and 2 μg/mL, respectively. Peptide 2075 produced 40 and 30 AU at 10 μg/mL and 5 μg/mL, respectively. Active concentrations for peptide 2076 were 10 and 5 μg/mL associated to 50 and 40 AU, respectively; and 10, 5 and 2 μg/mL for peptide 2077 associated to 70, 50 and 30 AU.

Regarding antibacterial activity against the wild type *S. typhimurium* 14028s, a small growth decrease was observed due to protamine when tested at a concentration of 496 μg/mL, which is smaller than the protamine concentration of 1250 μg/mL reported by other authors [22]. The protamine peptide 2077 showed the higher growth inhibitory activity at the same concentration, followed by 2075 and 2076. For comparison purposes, previously reported antimicrobial peptides' antibacterial activity was also tested as shown in Table 2.

Protamine at 10, 5 and 2 μg/mL had 50, 50 and 30 AU, respectively, as can be observed in Table 3. Peptide 2075 at concentrations of 10 and 5 μg/mL displayed an activity of 50 and 30 AU. Peptide 2076 at a concentration of 10 μg/mL had 40 AU.

Table 2
Radial diffusion assay for cationic peptides against Gram-positive and Gram-negative bacteria.

Antibiotic/sample	Total μg	Gram-positive bacteria		Gram-negative bacteria			
		29212 AU	65380 AU	25922 AU	14028s AU	MS7953 AU	EG10627 AU
Ampicillin	0.81	0	0	0	0	40	0
Kanamycin	0.82	80	80	100	100	100	90
Tetracycline	0.46	150	80	150	200	0	190
Maganin-1	8	50	50	80	70	80	80
	4	40	40	70	60	70	70
	2	30	30	60	50	60	60
PYLa	8	50	70	100	80	80	90
	4	40	60	90	70	70	80
	2	30	50	80	60	60	70
Dermaseptin	8	50	50	70	60	70	70
	4	40	50	60	50	60	60
	2	30	40	50	50	50	50
Bombinin	8	70	70	90	90	70	90
	4	60	60	80	80	60	70
	2	50	50	70	60	50	60
Andropin	8	0	0	20	0	30	0
	4	0	0	0	0	0	0
	2	0	0	0	0	0	0
TAP	8	40	40	50	50	50	50
	4	30	30	50	40	40	40
	2	0	0	40	30	30	30
Maganin-2	8	60	80	70	60	60	70
	4	50	60	60	50	50	60
	2	40	50	50	40	40	5
Criptidin	8	40	30	50	40	40	30
	4	30	0	40	40	30	0
	2	0	0	30	0	0	0
Cecropin B	8	50	50	70	50	70	70
	4	40	40	60	40	60	60
	2	40	30	40	30	40	50
Cecropin P1	8	50	40	60	50	60	60
	4	30	0	50	40	50	50
	2	0	0	40	30	30	40
Cecropin A	8	30	40	70	60	60	70
	4	30	40	60	50	50	60
	2	20	0	40	40	30	50
Polistes MA	8	70	70	60	60	70	60
	4	60	60	50	50	60	50
	2	60	50	40	50	50	40
Melittin	8	50	50	60	30	40	80
	4	40	40	60	30	30	70
	2	30	30	60	30	20	60
Taquilepsin 1	8	60	70	80	50	50	60
	4	40	50	70	40	40	50
	2	0	0	60	30	30	40
Apamine	8	0	0	50	30	40	60
	4	0	0	40	0	30	50
	2	0	0	30	0	20	40

Table 2 (*Continued*)

Antibiotic/sample	Total μg	Gram-positive bacteria		Gram-negative bacteria			
		29212 AU	65380 AU	25922 AU	14028s AU	MS7953 AU	EG10627 AU
MP-3	8	60	50	60	70	70	60
	4	50	40	50	70	50	50
	2	40	30	40	60	50	40
MP-7	8	70	70	80	50	60	60
	4	60	50	70	50	50	50
	2	50	40	60	50	40	40
MP-8	8	70	70	60	60	60	70
	4	60	50	60	50	50	50
	2	50	40	50	40	40	30
MP-17	8	0	30	60	90	70	60
	4	0	30	60	60	60	60
	2	0	0	50	50	30	50
MP-X	8	60	60	70	40	60	60
	4	50	60	60	40	50	60
	2	40	0	50	40	40	50
Polistes	8	60	60	70	50	50	50
	4	50	50	60	50	40	40
	2	40	30	50	50	30	30

Peptide 2077 inhibited bacterial growth at 10, 5 and 2 μg/mL associated to 50, 50 and 30 AU, as can be observed in Fig. 3D.

Serial dilutions of peptides were assessed against the whole panel of *S. typhimurium* strains showed that the growth inhibitory activity diminished as the concentration of the tested peptide decreased antibacterial activity was observed up to the seventh dilution, which corresponded to concentrations of 0.29 μg/mL for peptide 2075, 2.08 μg/mL for 2076 and 2.98 μg/mL for peptide 2077.

Elsewhere reported data regarding several *S. typhimurium* mutants and *E. coli* have shown that the cell envelope lipopolysacharides present in K+ channel systems as well as the presence of proteolytic enzymes diminish the bacterial sensibility to protamine [3,16,35].

Salmonella typhimurium is a cause of public concern for health authorities due to since it causes an increasing number of human salmonellosis clinical cases, including bacterial DT104 strain which causes a 3% mortality and is resistant to an important number of antibiotics such as ampicillin, chloramphenicol, streptomycin and sulfonamides. Recently, treatment of salmonellosis treatment has been hampered the emergence of bacterial strains resistant to trimetropin and quinolinic antibiotics like ciprofloxacin, commonly used its treatment [5,12].

The results obtained in the present study suggest that peptide fragments derived from protamine posses a differential activity in comparison to the parent sequence despite having smaller molecular sizes. These derived peptides conserve an arginine (Arg)-rich domain RRRR which can be associated to the antimicrobial activity of cationic peptides especially against Gram-negative strains.

3.3. Sensitivity of protamine-derived fragments to proteases

The current study was designed to determine whether the entire protamine sequence or its three derived fragments can resist the proteolytic degradation without losing their antimicrobial effect. Table 1 provides an overview of the aminoacid sequences of the parental peptide and its truncated derivatives. As observed in Table 4 showing the half-life degradation times of each molecule (t_{50}), the parental protamine peptide was 8 times less stable in human sera and 12 times less stable in *M. tuberculosis* cytosol proteases, compared to its derived *N*-(2075), central (2076) and C-terminal (2077) peptide fragments. On the other hand, the specific activity (SA) of all exo- and endo-proteases present in both enzyme sources was quantified. The enzymes' specific activity for the C-terminal sequence was higher than for the remaining sequences as

Table 3
Radial diffusion assay for previously reported antimicrobial peptides against Gram-positive and Gram-negative bacteria.

Antibiotic/sample	Total μg	Gram-positive bacteria		Gram-negative bacteria			
		29212 AU	65380 AU	25922 AU	14028s AU	MS7953 AU	EG10627 AU
Ampicillin	0.81	0	0	0	0	40	0
Kanamycin	0.82	80	80	100	100	100	90
Tetracycline	0.46	150	80	150	200	0	190
Protamine	10	50	60	60	50	60	60
	5	40	50	50	50	50	50
	2	0	0	30	30	30	30
2075	10	0	0	50	50	40	0
	5	0	0	40	30	30	0
	2	0	0	30	0	0	0
2076	10	0	0	40	40	50	0
	5	0	0	30	0	40	0
	2	0	0	0	0	0	0
2077	10	0	0	50	50	70	40
	5	0	0	40	50	50	30
	2	0	0	40	30	30	0

Table 4
Stability of protamine and its derived fragments.

Peptide code	t_{50} (min) for a 50% of processing			
	Human normal sera		*M. tuberculosis* H37Rv strain (cytosolic extract)	
	t_{50} (min)	S.A. ($\times 10^{-3}$) (nmole μg^{-1} min^{-1})	t_{50} (min)	S.A. ($\times 10^{-3}$) (nmole μg^{-1} min^{-1})
Protamine	30	4.11	30	3.80
2075	60	7.10	360	1.10
2076	90	5.10	60	7.10
2077	240	1.20	180	1.50

t_{50} values were determined by inoculation reaction between each molecule and human normal sera as well as a mycobacterial cytosolic extract. S.A. for specific activity.

well as its stability. The larger stability of peptide 2077 (VSRRRRRRGGRRRR) to a severe proteolytic attack can be attributed to its well-shaped secondary structure, which might favor a better interaction of this molecule with negatively charged membranes of either bacteria and infected red blood cells as discussed further herein. In consequence, the likelihood of this C-terminal protamine peptide to establish specific contact with folded receptors would be probably increased by this peptide's constrained thermodynamic properties and tri-dimensional structure.

3.4. Protamine-derived fragments in vitro anti-plasmodial activity

Similarly, protamine and its derived peptides were tested for their ability to inhibit invasion of the human malaria *P. falciparum* (FCB-2 strain) to human red blood cells (RBCs), by using flow-cytometry-based assay. According to the results of this assay, protamine inhibits parasite invasion to RBCs by 87% at 2.25 mg/mL. This peptide inhibited invasion up to fourth and eighth dilution regarding its starting concentration, as can be observed in Table 5. On the other hand, peptide 2075 inhibited the RBC invasion by 62% at 1.96 mg/mL, peptide 2076 by 57% at 2.35 mg/mL and peptide 2077 inhibited invasion by 79% at 2.02 mg/mL. All tested peptides inhibited the *P. falciparum* invasion to RBCs in a dose-dependent manner.

Table 5
Anti-plasmodial activity of cationic peptides against *Plasmodium falciparum* FCB-2 strain.

Sample in test	% Invasion ± SD	% Invasion inhibition ± SD
Normal invasion (RBCs + parasites)	100 ± 0.3	0 ± 0.3
Chloroquine		
1.85 mg/mL	24 ± 0.5	76 ± 0.5
0.93 mg/mL	19 ± 0.6	81 ± 0.6
EGTA	28 ± 0.3	72 ± 0.3
Protamine		
2.25 mg/mL	Hemolytic	Hemolytic
1.10 mg/mL	13 ± 0.7	87 ± 0.7
0.55 mg/mL	14 ± 0.3	86 ± 0.3
2075		
1.96 mg/mL	43 ± 0.2	57 ± 0.2
0.98 mg/mL	29 ± 0.1	71 ± 0.1
0.49 mg/mL	19 ± 0.4	81 ± 0.4
2076		
2.35 mg/mL	38 ± 0.5	62 ± 0.5
1.17 mg/mL	34 ± 0.3	66 ± 0.3
0.59 mg/mL	31 ± 0.4	69 ± 0.4
2077		
2.02 mg/mL	21 ± 0.6	79 ± 0.6
1.01 mg/mL	22 ± 0.3	78 ± 0.3
0.55 mg/mL	25 ± 0.3	75 ± 0.3
PBS		
1:2 (fold diluted)	83 ± 0.4	7 ± 0.4
1:4	90 ± 0.1	10 ± 0.1
1:8	95 ± 0.2	5 ± 0.2

The higher activity of protamine's C-terminal fragment can be associated to its secondary structure pattern, according to CD analysis. CD spectra of previously reported antimicrobial peptides shown in Fig. 5A indicates that Magainin-2, Cecropins and all mastoparans display typical α-helical profiles whereas the profile shown for Magainin-1 correspond to β-strands. Both the protamine parent sequence (red line) and its N-terminal derived fragment (blue line) do not have a clear secondary structure pattern as indicated by their random coil patterns. Remarkably, the central fragment (green line) and the C-terminal fragment (black line) display a clear α-helical tendency, being even stronger for the latter peptide since it induced two minimal signals at 208 nm and 222 nm. This structural behavior can provide a possible explanation of the stronger antimicrobial activity shown by the C-terminal fragment. Further studies with this 12-arginine-rich fragment are needed in order understand the possible antimicrobial mechanism used by this peptide, as shown in Fig. 5B.

4. Discussion

Host defense peptides are an evolutionarily conserved component of the innate immune response and are widely distributed in the animal kingdom. These peptides are potent, broad spectrum antibiotics which have been shown to present potential as novel therapeutic agents. Antimicrobial peptides have been demonstrated to kill Gram-negative and Gram-positive bacteria (including strains that are resistant to first line antibiotics), mycobacteria (including *M. tuberculosis*), enveloped viruses, fungi and even transformed or cancerous cells. Unlike the majority of commonly used antibiotics, it appears as though antimicrobial peptides may also have the ability to enhance immunity by functioning as immunomodulators. However in recent research, bacterial resistance to some of these molecules has become evident.

Bacteria employ various resistance strategies to evade being killed by antimicrobial peptides [6]. Some microorganisms alter net surface charges. For instance, *Staphylococcus aureus* transports D-alanine from the cytoplasm to the cellular surface where becomes attached to teichoic acid as to reduce the net negative charge by introducing basic amino groups [31]. *S. aureus* also modifies its anionic membrane via MprF blocking by L-lysine, increasing the membrane's positive net charge. The interaction of antimicrobial peptides with membrane targets can be limited by the capsule polysaccharides of *Klebsiella pneumoniae* [7]. Alterations occur in Lipid A, such as in *Salmonella* species which are able to reduce the fluidity of their outer membrane by increasing hydrophobic interactions between myristate and Lipid A, thereby forming hepta-acylated Lipid A. The increased hydrophobic moment is though to delay or abolish the insertion of antimicrobial peptides and pore formation. Residues membrane proteins undergo alterations, as in some Gram-negative bacteria, in which such alterations in the composition of outer membrane proteins correlate with resistance to antimicrobial peptides [9]. ATP-binding cassette

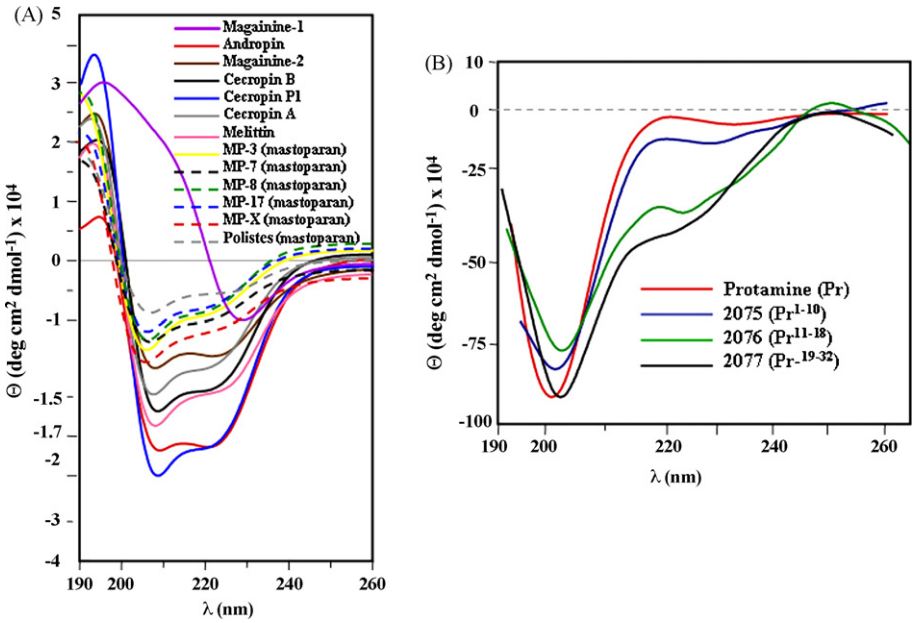

Fig. 5. Secondary structure profile for antimicrobial peptides. Circular dichroism experiments were performed to analyze possible secondary patterns present in a variety of cationic antimicrobial peptides. A. Literature reported antimicrobial peptides. B. CD profiles for protamine (red line), 2075 (Pr^{1-10}), blue line, 2076 (Pr^{11-18}), green line, 2077 (Pr^{19-32}) black line. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

transporters import antimicrobial peptides and the resistance-modulation cell-division efflux pump exports antimicrobial peptides [29]. Both transporters have been associated with antimicrobial peptide resistance. Bacteria produce proteolytic enzymes, which may degrade antimicrobial peptides, thus rendering them resistant [38].

According to our experimental aim, protamine-derived sequences were completely characterized and identified. Peptides were further characterized by comparing their acidic gel electrophoretical profiles with those of a set of previously reported antimicrobial peptides. Thus, all protamine-derived sequences migrate faster than other tested sequences including the parent entire protamine sequence. This can be associated to the high Arginine content of these small peptide fragments.

The results of radial diffusion assays show that the antibacterial activity of protamine and its three derived peptides varied depending on the strain being assessed. Protamine had antibacterial activity against *E. faecalis* 29212 and *S. aureus* 6538 strains, whereas its derived fragments lacked any activity. These findings indicate that the entire aminoacid sequence is required to produce antibacterial activity against these two Gram-positive bacterial strains. Moreover, all protamine-derived peptides were active against the Gram-negative *E. coli* 25922 strain, while the complete protamine showed a poor antibacterial activity. Therefore, the data gathered in this study presents for the first time evidence of the antibacterial activity of cationic peptides designed based on the sequence of protamine, thus indicating that these peptides can be considered as new antibacterial molecules.

In the radial diffusion assay, *S. aureus* displayed sensitivity to protamine, while no growth inhibitory activity was detected for its three derived fragments.

The results obtained in the present study suggest that peptide fragments derived from protamine posses a differential activity in comparison to the parent sequence, despite having smaller molecular sizes. These derived peptides conserve an arginine (Arg)-rich domain RRRR which can be associated to the antimicrobial activity of cationic peptides especially against Gram-negative strains, thus becoming a potential specific chemical moiety for a specific biological activity.

Our results also demonstrated that all protamine-derived peptides possess anti-plasmodial activity since they were able to inhibit parasite invasion to RBCs in a dose-dependent manner while the protamine parent sequence had hemolytic activity, reflecting its toxicity.

Interestingly, the higher antimicrobial activity of the protamine's C-terminal fragment can be associated to its secondary structure pattern, according to CD analysis. CD spectra of previously reported antimicrobial peptides indicate that Magainin-2, Cecropins and all mastoparans display typical α-helical profiles whereas the profile shown for Magainin-1 corresponds to β-strands. Both the protamine parent sequence and its N-terminal-derived fragment do not have a clear secondary structure pattern, as indicated by their random coil patterns. Remarkably, both the central fragment and the C-terminal fragment derived from protamine display a clear α-helical tendency, which was even stronger for the latter peptide. This structural behavior can provide a possible explanation to the stronger antimicrobial activity shown by the C-terminal fragment. Further studies with this 12-arginine-rich fragment are needed in order understand intrinsically possible antimicrobial mechanisms used by this peptide.

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