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Cationic Peptides Harboring Antibiotic Capacity are Selective for *Leishmania panamensis* and *Leishmania major*

José Manuel Lozano[1,2]*, Bernarda Soraya Cuadrado[3], Gabriela Delgado[4] and Manuel Elkin Patarroyo[1,2]

[1]*Universidad Nacional de Colombia, Bogotá, Colombia*
[2]*Biocatalysis Department, Fundación Instituto de Inmunología de Colombia (FIDIC) / Universidad del Rosario, Bogotá, Colombia*
[3]*Pharmacy Department, Science Faculty, Universidad de Cartagena, Cartagena, Colombia*
[4]*Immunotoxicology Research Group, Pharmacy Department, Science Faculty, Universidad Nacional de Colombia, Bogotá, Colombia*

Abstract

The fairly recent appearance of Leishmania resistance to currently-used therapy has led to the search for new therapeutic strategies. This work was thus aimed at evaluating the *in vitro* effect of 18 cationic synthetic antimicrobial peptides for antileishmanial and cytotoxic and haemolytic activity. The viability of murine (J774) and human (U937), peripheral blood monocytes, HeLa and HepG2 cells and *L. (V) panamensis* and *L. (L) major* promastigotes was then ascertained using the aforementioned peptides. All antimicrobial peptides were synthesised and each cell and parasite line was treated with different peptide concentrations. Melittin, bombinin, mastoparan 8 (MP-8), MP-X and dermaseptin-S1 reduced human and murine host cells' viability at greater concentrations than pentamidine isethionate, human peripheral blood and U937 monocytes being the most sensitive to peptide action. Melittin had a toxic effect on all the cells evaluated in this study and *L. (L) major* was more sensitive than *L. (V) panamensis* to peptide effect. As MP-8, bombinin, dermaseptin-S1 and tracheal antimicrobial peptide (TAP) were active against both parasite species, and tachyplesin 1 and polystes MA selectively so for *L. (L) major*, they were selected as being promising as they had a >1 selectivity index (SI) and greater than 50 µg/mL haemolytic concentration (HC50), suggesting that they should continue to be studied in *in vitro* and *in vivo* infection assays as there have been no previous reports of MP-8, bombinin, TAP and Polystes MA activity regarding *L. (V) panamensis* and *L. (L) major*.

Keywords: Antimicrobial Peptide; Leishmaniasis; Leishmania ssp; Cytotoxicity; Microbial Resistance to Antibiotics

Introduction

Leishmaniasis covers a group of diseases characterised by their clinical and epidemiological diversity; it is caused by around 22 species of protozoa from the genus Leishmania, belonging to the family Trypanosomatidae. They are grouped into two subgenera: Leishmania (L), mainly in Afro-Eurasia, and Viannia (V) on the American continent. They are transmitted by a haematophagous insect's bite (most being zoonotic where carnivorous animals are concerned) and some rodents, acting as the main reservoirs [1,2].

The number of people becoming infected with leishmaniasis around the world exceeds 20 million each year, around two million new cases being reported annually; 1.5 million have cutaneous manifestations but only 20% of the cases are accurately accounted for. Mortality has been estimated at around 60,000 people per year (mainly associated with the visceral form) and almost 350 million people are at risk of contracting the disease [3].

Leishmaniasis is endemic in almost all of Colombia, around 10 million people being at risk in rural areas. Around 98% of such cases involve the cutaneous clinical form which is mainly caused by L. Viannia panamensis; however, it is attributed to *L. (L) major* and *L. (L) tropica* [4] in the Old World (Europe, Asia and Africa).

Leishmaniasis leads to a 2.35 million disability-adjusted life years (DALY) burden of disease, 2.3% of them occurring in the Americas. Both cutaneous and mucocutaneous manifestations deform and disfigure to such a degree that such patients may become socially-isolated and their quality of life becomes significantly reduced as cutaneous leishmaniasis (CL) is not fatal [3].

The drugs usually used in treatment are hepatotoxic and cardiotoxic (i.e. pentavalent antimony salts, bis-amidines, pentamidine and amphotericin B), thereby leading to clinical resistance following a few weeks' treatment. The cost of treatment and the need for parenteral administration hampers treatment and leads to its failure. A lack of worldwide interest in investing in research and development regarding new drugs and the diversity of clinical manifestations, the parasite's species, difficulty in controlling vectors and reservoirs and a lack of an effective vaccine [3,5] all make it imperative for a search to be made for therapeutic strategies for replacing or complementing existing ones.

Antimicrobial peptides are key effector molecules in an organism's innate immunity [6]; they are isolated from different sources [7], about 2,000 sequences having been reported to date. They share some chemical features; such families of active molecules represent the main mechanism of action regarding the plasmatic membrane causing direct destabilisation through pore formation on the target cell, followed by cell lysis. This is why their potential use is being studied as a therapeutic strategy in infectious diseases such as leishmaniasis so that target cells resistance in not induced due to their action on membrane phospholipids and an organism's difficulty in modifying its own structure. Positive properties may be found among this group of molecules exceeding the disadvantages involved in using currently-available drugs for leishmaniasis.

***Corresponding author:** José Manuel Lozano, Head of the Biocatalysis Department, Fundación Instituto de Inmunología de Colombia (FIDIC), Carrera 50 No. 26-20, ZIP Code 020304, Bogotá DC, Colombia, Tel: +57-1-481-5219; Fax: +57-1-481-5269; E-mail: jm_lozano@fidic.org.co/jmlozanom@unal.edu.co

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Peptide anti-leishmanial activity against promastigotes from the subgenera Leishmania has so far involved testing dermaseptin, bombinin, magainin, cathelicidin, defensin, temporin, decoralin, Pr-2 and Pr-3 (protamine derivatives), a cathelicidin (bovine myeloid antimicrobial peptide-28 or BMAP-28) and two stereoisomers (D-BMAP-28 and RI-BMAP-28) [11] against *L. (L) major* [8-11]. Likewise, the following peptides have been evaluated against the subgenera Viannia: tachyplesin, magainin, clavanin, penadein, mytilinin against *L. (V) braziliensis* and dermaseptin and a DM1 analogue and Pr-2 and Pr-3 protamine derivatives against *L. (V) panamensis* [8,12-14]. A model of *L. (V) panamensis* infection of dendritic cells has recently been reported demonstrating potencial antileishmanial activity of andropin and cecropin. Relatively few studies have been published evaluating antimicrobial peptide activity against *L. (V) panamensis* that is why we decide to assess some antimicrobial peptides and their potential against leishmania strains in controlled tests.

This study was aimed at screening the *in vitro* antileishmanial activity of 18 synthetic antimicrobial peptides regarding extracellular forms of *L.(V) panamensis* and *L.(L) major* and the effect they induced in human and murine erythrocytes and cells, thereby leading to ascertaining promising peptides having selective activity against the target parasite.

Materials and Methods

Bioinformatics and in silico molecular analyses

The amino acid sequences of all antimicrobial peptides selected for this research were aligned against different homologous proteins, including other human and non-human leishmanial parasites, all reported in the NCBI database (http://www.ncbi.nlm.nih.gov/blast.cgi). Sequences were aligned using the TBLASTN tool and the ClustalW multiple sequence alignment method (www.personal.rhul.ac.uk/ujba/110/bioinfo/clustalE.html). Also PDB stored coordinates for most antrimicrobial peptides were downloaded from the Protein data bank web site (http://www.rcsb.org/pdb/home/home.do) regarding peptides 3D structure determined either by NMR or X-ray crystallography and then those were molecularly modelled. Molecular models were obtained using Accelrys Inc. Viewer Lite 4.2 software (www.accelrys.com).

Solid phase synthesis of cationic antimicrobial peptides

Eighteen previously-reported natural antimicrobial peptides as well as a human endogenous MHC-II restricted peptide so-named CLIP (Class II-associated invariant chain peptide) which lack any antimicrobial property (control peptide), were selected and synthesized for this study in line with standard protocols. In brief, all molecules were manually synthesized through tert-butyloxycarbonyl (t-Boc)-based solid-phase peptide synthesis (SPPS), following a protocol first reported by Merrifield [15] and later modified for multiple peptide synthesis [16]. Solid supports of 0.4 meq/g and 0.7 meq/g methylbenzhydrilamine (MBHA) resin substitutions were used in agreement to each peptide length for producing an average of 100 mg of each peptide product. A controlled step-wise synthesis was performed by systematically coupling each building block (amino acid) accordingly with designed sequences. Amino acids were t-Boc protected at their N-terminus and a low-high hydrogen fluoride (HF) concentration procedure for peptide-resin cleavage was employed when finalizing all amino acid couplings for releasing the synthesised peptide and so obtaining all crude peptide products in an average of 70% in yield. Total coupling was checked by the Ninhydrin test and, repeating the coupling reaction when necessary to ensure right couplings. Coupling was allowed to proceed for one to two hours under constant shaking, followed by washes with N,N'-dimethylformamide (DMF), isopropanol and dichloromethane. Standard solid phase peptide synthesis was carried out to introduce all required t-Boc amino acids to the last N-terminal residue. Protected peptide-resin batches were treated with trifluoroacetic acid (TFA) to release those amino acids' side-chain protectin groups and the product peptide was cleaved from the resin by treatment with low concentrations of anhydrous hydrogen fluoride (HF) containing 10% anisole at 0°C for 60 min. After HF evaporation in an N_2 stream, each peptide-resin product was washed with cold diethyl ether, then extracted with 5% acetic acid (HOAc) and lyophilized.

Peptide identity and purity were analysed by mass spectrometry matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) and reverse phase high resolution liquid chromatography (RP-HPLC), respectively. The secondary structure profile of every synthesised peptide was then identified by circular dichroism (CD) experiments [17].

An analytical RP-HPLC was run (Hitachi-Merck model L17400, Merck, Darmstadt, DE) using a silica analytical column (Vydac C-18) (5 μm, 4.5 mm × 30 cm). A Vydac C-18 column was used in preparative RP-HPLC for 0% to 100% B gradient-lineal elution using the following solvent system: a) H_2O, 0.05% trifluoroacetic acid (TFA), b) CH_3CN, 0.05% TFA for 45 min (100 min for preparation) at 1.0 mL/min flow speed (4.5 mL/min for preparation). The eluate was monitored on a UV-DAD detector at 220 nm.

Mass spectra were recorded on a Bruker Protein TOF mass spectrometer (Billerica, MA) in reflectron mode. MALDI experiments involved TOF using a 337 nm long N_2 radiant laser with 3-ns pulses. Acceleration voltage was +17.5 kv with +20 kv reflectron voltages; all spectra were obtained by a series of 10 laser pulses to ensure comparable conditions and that laser intensity was the minimum possible for each measurement. α-cyano-4-hydroxycinamic acid (CCA) (Sigma Chemical Co., Saint Louis, MO) was used as matrix in this work; it was prepared with a saturated solution in 1 mL TA (40% acetonitrile in 0.1% trifluoroacetic acid).

The samples were dissolved in TA to obtain a 100 pmol/pL concentration and prepared for MALDI-TOF analysis by diluting the sample in the matrix-saturated solution at 10 pmol/μL concentration. 0.5 μL aliquots of the matrix-sample mixture were placed on a plate to become air-dried for later analysis (i.e. identifying secondary structure tendency by circular dichroism).

The peptides having greater than 95% purity which were included in the study were apamin, andropin, tracheal antimicrobial peptide (TAP), polystes MA (MA), mastoparan 8 (MP-8), mastoparan 17 (MP-17), mastoparan X (MP-X), a protamine derivative or Pr-2, mangainin-1, dermaseptin S1, melittin, bombinin, tachyplesin I and cecropins A, B and P1. All were 10- to 40-amino acid long, had less than 3 kDa molecular weight; seven of them had previously-described antileishmanial activity and nine lacked reports concerning their activity (Table 1). A lineal non-related peptide from the major histocompatibility complex class II-associated invariant chain peptide (or CLIP) sequence was included as negative control (i.e. lacking α-helix structure), being non-microbicidal or immunogenic and naturally produced by cells (Table 1).

Lyophilised antimicrobial peptides were suspended in 3% (v/v) dimethylsulfoxide or DMSO (Panreac, Barcelona, Spain) and 1 mg/mL concentration (stock solution) incomplete Roswell Park Memorial Institute-1640 (RPMI-1640) culture medium (Gibco, Scotland, UK) and stored until use at -20°C.

***L. (V.) panamensis* Antimicrobial peptide**	**Sequence**	**Molecular weight (Da)**	**Natural source**	**Secondary structure tendency**	**Antileishmanial activity**
Manganin-1	GIGKFLHSAGKFGKAFVGEIMKS	2410.4	*Xenopus laevis*	β-sheet	Manganin-2*: L. (L.) donovani* [14] and analogues: *L. (V.) braziliensis* [13]
Dermaseptin S1	ALWKTMLKKLGTMALHAGKAALGAAADTISQGTQ	3437.6	*Phyllomedusa bicolor*	α-helix	*L.(L.) amazonensis* [31] *L. (V.) panamensis* and *L.(L.) major* [18]
Bombinin	GIGGALLSAGKSALKGLAKGLAEHFAN	2904.4	*Bombina maxima*	α-helix	*L. (L.) donovani* [10,12]
Andropin	VFIDILDKVENAIHNAAQVGIGFAKPFEKLINPK	3732.8	*Drosophila melanogaster*	α-helix	*L. (V.) panamensis*[8]
TAP	NPVSCVRNKGICVPIRCPGSMKQIGTCVGRAVKCCRKK	4073.6	*Bos taurus*	α-helix	Not described
Cecropin B	KWKVFKKIEKNGRNIRNGIVKAGPAIAVLGEAKAL	3818.3	*Hyalophour cecropia*	α-helix	Not described
Cecropin P1	SWLSKTAKKLENSAKKRISEGIAIAIQGGPR	3321.4	*Ascaris suum*	α-helix	No activity in *L. (V.) panamensis* and *L.(L.) major* [8]
Cecropin A	KWKLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAK	4003.8	*Hyalophour cecropia*	α-helix	*L. (V.) panamensis* [8]
Polystes MA	VDWKKIGQHILSVL	1618.1	*Polystes jadwigae*	Non-structured	Not described
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ	2829.7	*Polystes sp.* HQL-2001	α-helix	*L. (L.) major* [8]
Tachyplesin I	KWCFRVCYRGICYRRCR	2251.2	*Tachypleus tridentatus*	β-sheet	*L.(V.) braziliensis*, *guyanensis* and *panamensis* [13]
Apamin	CNCKPEPALCARRCQQH	2014.5	*Apis cerana cerana*	α-helix	Not described
Pr-2	VRRRRRPR	1133.4	*Oncorhynchus mykiss gairdneri*	α-helix	*L. (V.) panamensis* [8]
MP-8	INLKALAALAKRLL	1490.3	*Vespa magnifica*	α-helix	Not described
MP- 17	INLKAKAALAKKLL	1477.3	*Vespa magnifica*	α-helix	Not described
MP-X	INWKGIAAMAKKLL	1539.3	*Vespa simillina xanyhophera*	α-helix	Not described
CLIP	LPKPPKPVSKMRMATPLLMQALPM	2674.5	MHC class II CD74 (invariant chain) molecule [*Homo sapiens*]	polyprolin-II (PPII) helix	Endogenous peptide No antimicrobial or cytotoxic activity

Table 1: Antimicrobial peptides' physical and antimicrobial profiles CLIP was used as a non-related peptide.

Circular dichroism experiments

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 190–250 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 0–30% aqueous 2,2,2-trifluoroethanol (TFE) in a final volume of 500 mL. A typical 0.2 mM peptide concentration in TFE–water mixture is stabilized but does not induce secondary structure in peptides, as described elsewhere [18].

Parasite culturing

Leishmania (V) panamensis (MHOM/PA/71/LS94) and *Leishmania (L) major* Friedlin 6 clone (MHOM/IL/80/Friedlin) promastigotes cultured at 27°C in complete RPMI 1640 medium: RPMI medium (Gibco, Scotland, UK) buffered with 2 mM $NaHCO_3$, pH 7.2 and supplemented with 5% (v/v) fetal calf serum (FCS) (Microgen, Bogotá, CO), 2 mM L-glutamine (Gibco, Grand Island, NY, USA), 100 U/mL penicillin and 100 mg/mL streptomycin (complete medium) were used for the assays.

Cells lines

Human (U937) and murine (J774) monocyte/macrophage cell lines, human HepG2 lines (representing hepatocytes) [19] and HeLa (representing immortal cells) (ATCC, Rockville, USA), peripheral blood monocytes from healthy donors and fresh O Rh+ red blood cells (RBC) were used for evaluating the peptides' antibiotic effect. Monocytes/macrophages and HepG2 and HeLa cells were cultured in complete RPMI-1640 medium at 37°C with 95% humidity and 5% CO_2 until forming a monolayer at 80% confluence.

Human peripheral blood monocytes (with Buffy coat or white cell layer) and erythrocytes were obtained from healthy donors using blood bags (Marly Clínic Blood Bank, Bogotá, Colombia), complying with Colombian Ministry of Social Protection resolution 8430/1993 concerning research involving human tissue. The samples were processed using a previously-described technique, with some modifications [20]. The monocytes were separated by Ficoll-Paque 1077 density gradient (Lympho separation medium, MP Biomedicals LLC, Ohio, USA) and spun at 1.077 x g for 30 minutes; the cells were then washed and suspended in incomplete RPMI medium and 1:1 saline solution supplemented with 5% (v/v) foetal calf serum (FCS) and sown on 150 cm^2 plastic Petri dishes (NalgeNunc International Rochester, New York, USA). They were then incubated at 37°C with 5% CO_2 for two hours to allow them to adhere to the plates. The adhered cells were then separated, their number and percentage viability (greater than 90%) determined by 0.4% Trypan blue dye exclusion assay (Gibco, NY) and then sown on flat-bottomed 96-well plates (TPP, Techno Plastic Products AG, Trasadingen, CH) at $2x10^5$ cells/mL complete RPMI-1640 concentration, until being used in the respective assays.

Antimicrobial peptide effect on the cells

The fluorometric resazurin reduction test was used, following a previously-described methodology [21]; this led to identifying peptides which could cause a possibly harmful effect on eukaryotic cells, particularly on potential host cells. All were different to the Leishmania parasite (safety and efficacy criteria). Four series of peptide dilutions in duplicate and two antileishmanial drugs (paromomycin sulphate and pentamidine isethionate) were added to the cell cultures, including controls for the diluent used dimethylsulfoxide (DMSO) and peptide-free cells. Resazurin reduction to rezorufin (fluorescent compound) was detected on Tecan Genios Spectra Fluor Plus equipment (Tecan Austria GmbH, Untersbergstrasse Grödig, Salzburg, Austria), at 535 nm excitation and 590 nm emission wavelength using Magellan RFU-4 software (Tecan, UK). Viability was calculated then estimated.

Haemolytic activity of antimicrobial peptides

Human O+ RBC from heparinised blood obtained by venopuncture was studied as it was considered part of the safety criteria. The RBC were diluted at 2% haematocrite and incubated with 1:1 series of dilutions of the peptides and the controls (50 µL peptide dilutions with 50 µL O+ erythrocyte solutions) and haemolysis % was calculated as recommended [13].

The effect of antimicrobial peptides on *L. (V) panamensis* and *L. (L) major* promastigote forms

Pentamidine isethionate (Pentacarinat Rhone Poulenc Rorer, Dagenham, UK) and paromomycin sulphate (Paromomycin, PhytoTechonology Laboratories, Shawnee Mission, Kansas, USA) were used as control drugs having antileishmanial activity. The parasites were monitored by *L. (V) panamensis* promastigote morphology and motility at 5, 24 and 72 hours exposure and their viability was evaluated following the methodology described in [22]. Rezorufin production was monitored as recommended [23] and the data was normalised for calculating the percentage of live parasites in the samples, as described in [24].

Statistical analysis

The normalised values were expressed as the arithmetic mean ± standard deviation of the mean (95% confidence limit), the 50% of inhibitory concentration (IC50) being calculated in µg/mL and the 50% of effective concentration (EC50) in µg/mL by a non-linear regression method in dose-response ratio using GraphPad Prism (version 5.0) demo software (GraphPad Software, Inc, the Jolla, CA, USA) [25]. Two-way analysis of variance (ANOVA test) was followed by Bonferroni post-test and establishing whether a significant difference could be found in treatment, bearing associated error in mind.

Results

Bioinfomatics and secondary structure elements in antimicrobial peptides

Blast analysis were performed to each peptide sequence in order to verify existance of homologous or orthologous genes coding for them in different species such as invertebrates, anphibians, fish, mammals, fungi, bacteria, virus and parasites including those belonging to the leismania genus amongst others. Also multiple alignments of peptide sequences by ClustalW were performed in order to examining possible identity and homology degrees. As a result most sequences do not share specific amino acid motifs responsible for their antimicrobial activity as well as structural differences at individual level between them became evident as further discussed and few of them have been previously

Analyte	J774	Adherent U937	Non-adherent U937	HPBM	*L. (V.) panamensis*	*L (L.) major*	*L. (V.) panamensis* SI = (IC_{50} / EC_{50} promastigotes)				*L. (I.) major* SI = (IC_{50} / EC_{50} promastigotes)			
	IC_{50} (µg/mL)	IC_{50} (µg/mL)	IC_{50} (µg/mL)	IC_{50} (µg/mL)	EC_{50} (µg/mL)	EC_{50} (µg/mL)	J774	Adherent U937 e	Non adherent U937	HPBM	J774	Adherent U937	Non-adherent U937	HPBM
Mangainin 1	25.3	> 50.0	> 50.0	> 50.0	> 50.0	> 50.0	0.5	1.0	1.0	1.0	0.5	1.0	1.0	1.0
Dermaseptin S1	36.7	> 50.0	46.3	49.9	24.6	25.4	1.5	2.0	1.9	2.0	1.4	2.0	1.8	2.0
Bombinin	27.4	> 50.0	25.6	30.1	26.4	< 6.3	1.0	1.9	1.0	1.1	4.4	8.0	4.1	4.8
Andropin	> 50.0	> 50.0	> 50.0	> 50.0	> 50.0	> 50.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
TAP	> 50.0	> 50.0	> 50.0	> 50.0	46.3	23.2	1.1	1.1	1.1	1.1	2.2	2.2	2.2	2.2
Cecropin B	> 50.0	> 50.0	> 50.0	> 50.0	> 50.0	> 50.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Cecropin P1	> 50.0	> 50.0	> 50.0	> 50.0	> 50.0	> 50.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Cecropin A	> 50.0	> 50.0	> 50.0	> 50.0	> 50.0	> 50.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Polystes MA	> 50.0	> 50.0	> 50.0	> 50.0	> 50.0	40.7	1.0	1.0	1.0	1.0	1.2	1.2	1.2	1.2
Melittin	43.8	> 50.0	> 50.0	22.3	7.6	< 6.3	5.8	6.6	6.6	2.9	7.0	8.0	8.0	3.6
Tachyplesin I	> 50.0	> 50.0	> 50.0	> 50.0	> 50.0	42.1	1.0	1.0	1.0	1.0	1.2	1.2	1.2	1.2
Apamin	> 50.0	> 50.0	> 50.0	> 50.0	> 50.0	> 50.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Pr-2	> 50.0	> 50.0	> 50.0	> 50.0	> 50.0	> 50.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
MP-8	34.4	> 50.0	20.9	49.6	27.0	15.9	1.3	1.9	0.8	1.8	2.2	3.1	1.3	3.1
MP-17	> 50.0	> 50.0	> 50.0	> 50.0	> 50.0	> 50.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
MP-X	35.4	> 50.0	25.1	> 50.0	49.9	> 50.0	0.7	1.0	0.5	1.0	0.7	1.0	0.5	1.0
Pentamidin isothionate	8.5	> 3.0	20.5	<3.4	<3.1	<1.5	2.7	16.1	6.6	1.1	5.7	33.3	13.7	2.3
Paromomycin sulphate	> 50.0	> 3.0	> 50.0	> 50.0	22.8	<1.5	2.2	2.2	2.2	2.2	33.3	33.3	33.3	33.3
CLIP	> 50.0	> 50.0	> 50.0	> 50.0	> 50.0	> 50.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

Table 2: The effect of antimicrobial peptides on cell viability. Promastigote forms of *L. (V) panamensis* and *L. (L) major* used for determining (IC50), (EC50) and selectivity index (SI) after 72 hours exposure to peptides.

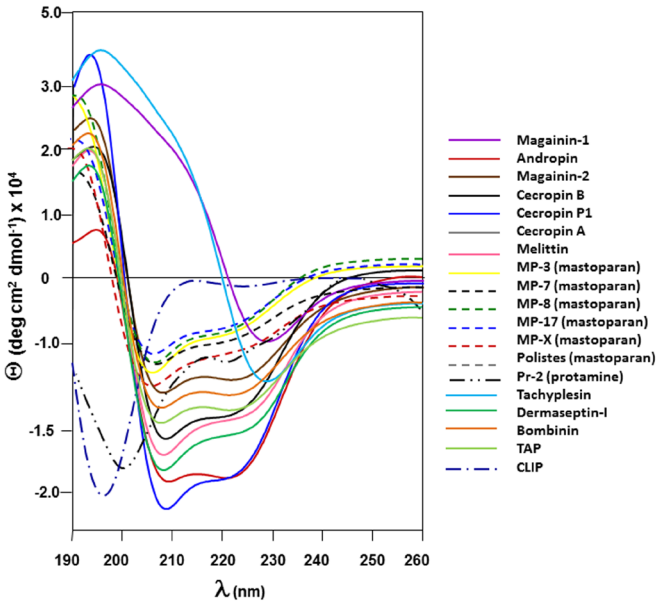

Figure 1: Secondary structure patterns for antimicrobial peptides. Circular Dichroism (CD) experiments were carried-out to analyse secondary profiles present in selected cationic antimicrobial peptides. Resulting patterns are expressed as mean residue ellipticity (θ), the units being degrees·cm²·dmol⁻¹ according to the (θ) = θλ/(100lcn) function where θλ is the measured ellipticity, l the optical path length, c the peptide concentration, and n is the number of amino acid residues in the sequence.

tested for its potencial antileismanial activity. However some peptide features can be extrapolated form these preliminary studies, most of them are amphipathic and cationic residues such as Lys, Arg and His, are frequently found in their primary structure as well as some small amino acid stretches constituted by non-polar residues such as Ala, Val, Leu and Gly seem to play an important role for their antimicrobial activity, and so Cys residues strategicaly located in some members of this peptide kingdom, led formation of domains key for peptidase resistance to degradation and so preserving their biological activity. In consequence, eighteen antimicrobial peptide sequences were selected for their assessment as potencial antileishmanial activity, and the human endogenous expressed peptide so-called CLIP, a non-relevant sequence was also included as the control.

All synthesised antimicrobial peptides and peptide controls were analysed by CD experiments. As observed in Figure 1 sixteenth peptides displayed strong structure patterns resembling α-helix as the predominant secondary structure profile, amongst them the six-member mastoparan family, andropin, cecropins A, B and P1, magainin-2, melittin, dermaseptin S1, TAP and bombinin. On the other hand only two antimicrobial peptides displayed β-strand profiles, these were magainin-1 and tachyplesin, one displayed random structure fetarures (Pr-2, a protamine derivative) and a classical polyproline-II structure profile was evidenced by CLIP. All peptide CD profiles were deconvoluted for studying each structure element proportion (data not shown but available).

The effect of antimicrobial peptides on nucleated eukaryotic cells and erythrocyte

The antimicrobial peptides had a negative concentration-dependent relationship regarding viability in the cells evaluated here. Only magainin-1, dermaseptin-S1, bombinin, melittin, MP-8 and MP-X at maximum 50 μg/mL concentration ($p>0.05$) caused a reduction of more than 70% live cells (Figure 2A) in J774 cells at the concentrations used here (6.25 to 50μg/mL). These six peptides IC50 were close to 25 μg/mL compared to 8.5 μg/mL IC50 for pentamidine isethionate (Table 2).

Differences were observed between the peptides' effect on both U937 cell line phenotypes in such a way that whilst bombinin and melittin induced a slight loss of macrophage viability (20%) at the maximum concentration used (50μg/mL), bombinin, MP-8, MP-X and pentamidine isethionate inhibited monocyte growth by 50% at a lower concentration (25μg/mL) (Figure 2B and 2C). Melittin was not evaluated in non-adherent U937 cells.

Melittin and bombinin reduced the viability of 50% of the human peripheral-blood monocyte (HPBM) population at 22.3 μg/mL and 30.1 μg/mL concentration, respectively, and around 90% of the cells at 50 μg/mL. On the other hand, MP-8, dermaseptin-S1, Pr-2 and TAP had their maximum effect on 50% of the population at concentrations around 50 μg/mL (Figure 2D); these cells, together with U937 monocytes, were the most sensitive cells to the selected peptides' action. Melittin was the only antimicrobial peptide (like pentamidine isethionate) which reduced the cell viability of around 60% of the population at 50 μg/mL maximum concentration.

HeLa and HepG2 cells were the most resistant to antimicrobial peptide action; it was observed that only melittin reduced the viability of 60% to 70% of the cell population at 50 μg/mL and, like all previously evaluated cells (J774, non-adherent U937 phenotype, HPBM and HeLa cells), melittin's effect on cell viability was less than that of pentamidine isethionate (Figure 2F and 2G).

Melittin lysed 96.8% of the erythrocytes at 12.5 μg/mL, as previously described [26], having absorbance greater than that of distilled water (100% haemolysis as control). Other haemolytic peptides at maximum 50 μg/mL concentration were MP-X (52.1%), dermaseptin-S1 (32.1%), MP-8 (29.6%), bombinin (17.4%) and Polystes MA (14.9%) (Figure 2G).

The influence of antimicrobial peptides on *Leishmania panamensis* and *Leishmania major* Promastigote form

Melittin and pentamidine isethionate inhibited 98% and 100% of cell viability at 25 and 50μg/mL concentrations, respectively, whilst MP-8, bombinin, dermaseptin-S1 and paromomycin sulphate only had an effect on 50% and 80% of the population at the same concentrations ($p<0.05$). Polystes MA, MP-X, TAP and Pr-2 had little activity at 50 μg/mL, only reducing 50% of the population. Magainin-1, andropin, cecropins A, B and P1, tachyplesin, apamin and MP-17 had no effect on promastigote viability following 72 hours incubation at the maximum concentration used (50 μg/mL) (Figure 3A).

It was observed that 13 of the antimicrobial peptides (76.5%) reduced promastigote motility from 25% to 100% after five hours' exposure at 50 μg/mL concentration, having no effect after 72 hours' incubation on parasites treated with cecropin P1, apamin and/or MP-17.

Only parasites exposed to TAP and cecropin A acquired a rounded form, no alterations to the rest of the 14 peptides occurring after five hours. Abundant cell debris was produced in the wells containing parasites treated with melittin, MP-8 and MP-X and pentamidine isethionate at 24 hours and just at 72 hours in those exposed to Polystes MA, tachyplesin I and paromomycin sulphate.

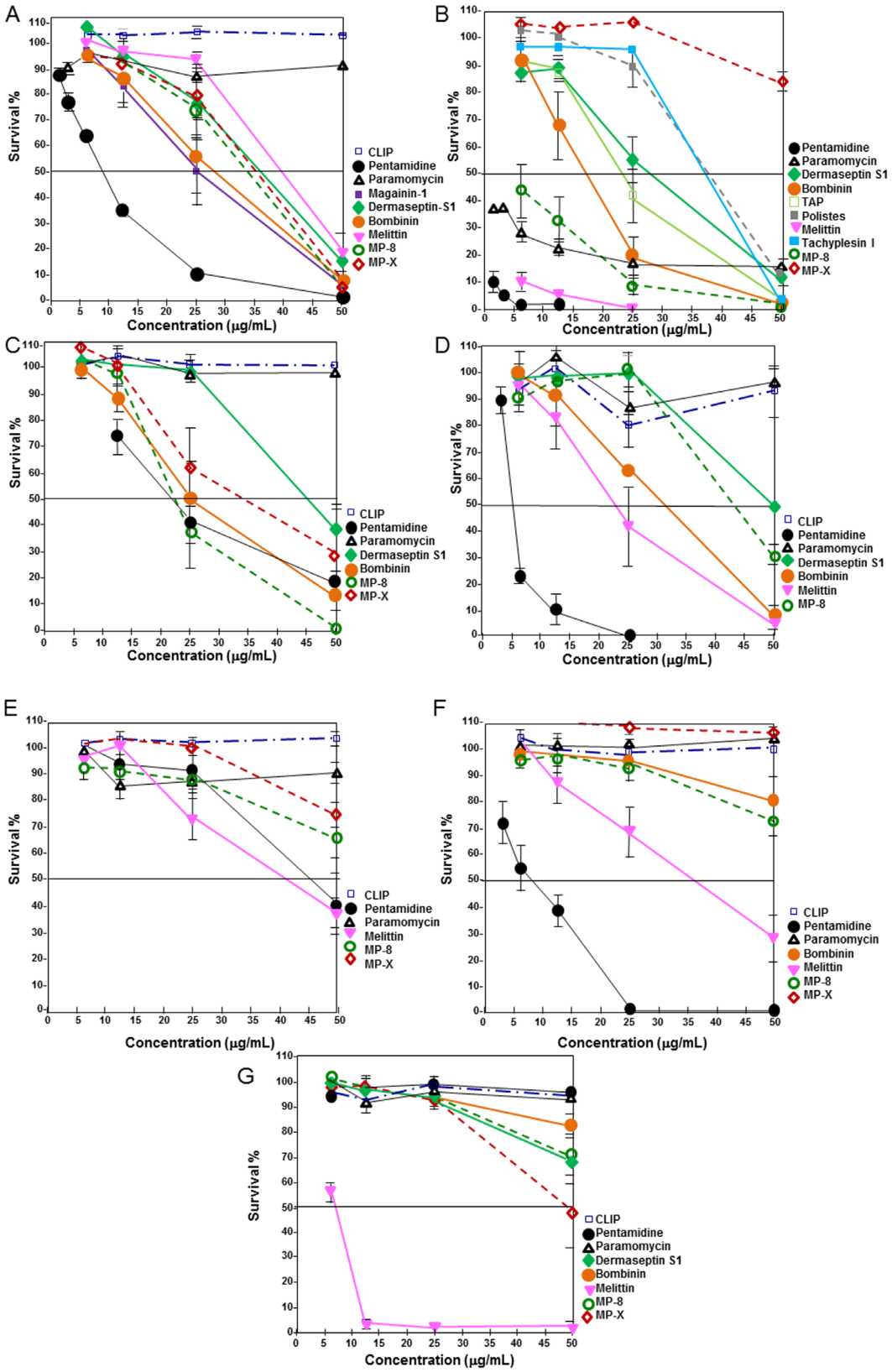

Figure 2: Antimicrobial peptides' effect on potential Leishmania parasite host cells, HeLa and HepG2 cells and O+ erythrocytes. A) J774, B) U937 adherent phenotype (macrophage), C) U937 non-adherent phenotype (monocyte) and D) human peripheral-blood monocytes (HPBM), E) HeLa cells, F) HepG2 cells and G) O+ erythrocytes.

Antimicrobial peptides dermaseptin-S1, bombinin, melittin, tachyplesin I, MP-8 and MP-X caused a 100% reduction of *L. (V) panamensis* promastigote motility at 72 hours, accompanied by apparent parasite destruction (debris), in turn reducing parasite viability in the assay with resazurin.

Melittin and pentamidine isethionate reduced *L. (L) major* promastigote viability for more than 90% of promastigotes at concentrations below 6.3 and 1.5 µg/mL, respectively, followed by bombinin, MP-8 and paromomycin sulphate having an effect on 80% of the population at close to 25 µg/mL concentration ($p>0.05$). Other peptides having activity on this species were TAP, dermaseptin-S1, maganin-1 and MP-X, but at greater concentrations (Figure 3B) (Table 2).

Thus, the most active antimicrobial peptides against *L.(V) panamensis* promastigote forms were melittin, dermaseptin-S1, bombinin and MP-8, TAP, MP-X, Polystes MA and Pr-2 and melittin. Bombinin, MP-8, TAP and dermaseptin-S1 on *L. (L) major*, followed by tachyplesin I, Polystes MA, MP-X and magainin-1. Differences were identified regarding the sensitivity of these two parasite species in promastigote form to the action of TAP, bombinin, Polystes MA and paromomycin sulphate, *L. (L) major* being sensitive to the action of the peptides (Table 2).

The selectivity index (SI) was obtained by dividing the IC50 of a substance in the host cell into the EC50 in promastigotes; if this were greater than one it would have indicated that a substance being evaluated was more selective on the promastigote form and if it were equal to or less than 1 it would have been more selective for the potential host cell, based on that fact that promastigotes are more resistant than amastigotes to the action of substances having antileishmanial activity [27]. Melittin, dermaseptin-S1, MP-8, bombinin and TAP were more selective (SI>1) on *L. (V) panamensis*, whilst this was true for bombinin, melittin, MP-8, TAP, dermaseptin-S1, Polystes MA and tachyplesin I on *L. (L) major* (Table 2).

Discussion

As mentioned above, some features displayed by the analysed antimicrobial peptides became evident form this study. They do not share either a consensus secondary structure pattern, or a specific antimicrobial motif composed by given amino acid stretchs or a given peptide-length that can be associated to an active antimicrobial peptide. However some of these molecules are neither toxic nor haemolytic but dispaly amphipathic helices and cationic amino acid residues such as Lys, Arg and His, can be found in their primary structure as well as some small amino acid stretches constituted by non-polar residues such as Ala, Val, Leu combined with Gly seem to play an important role for their antimicrobial activity associated to their tridimenssional conformation which paradoxically can be randomly organised in some of these peptides, and finally some of them contain Cys residues strategicaly located led the formation of active domains as occurs in complex active polypeptides fond in nature.

As there is a direct correlation between resazurin reduction in culture medium and the proliferation/amount of live organisms in the resazurin reduction test [28], it was considered appropriate for evaluating antimicrobial peptides' effect on nucleated cells and parasites. Dermaseptin-S1, bombinin, melittin, MP-8 and MP-X reduced the cell viability of the four types of potential Leishmania host cells whilst only melittin had an effect on immortal HeLa cell line and HepG2 cells. The resistance shown by HeLa and HepG2 cells suggested that the peptides evaluated here (except for melittin) might not have any harmful effect on HeLa cells and hepatocytes, thereby favouring the desired topical use.

HPBM cells had the greatest variability of the four cell groups. The lot-by-lot differences of the cells having a Buffy coat obtained from the bags of donors' blood may have influenced the quality of the monocytes obtained and thus the results, especially the varity of cells from different individuals [29]. On the other hand, both types of response in U937 cells could be explained by a mechanism in which cells being free in culture have greater surface and longer contact time with different compounds, especially because all the medium consumed during the 72 hours' exposure cannot be removed (such action probably having taken place during the four hours required for rezorufin production). However, a potential protease effect cannot be discarded, particularly metalloproteases produced by activated U937 macrophages [30].

Natural peptides have a short half-life in the blood, possibly due to the presence of peptidases. It thus becomes indispensable that their protease resistance or sensitivity profile be evaluated (after studying promising peptides in *in vitro* and *in vivo* infection models in hamsters), especially when considering their future use in different formulations by different administration routes to the topical one and because Leishmania also produces proteases able to damage the peptides and thus inhibit their action.

Pentamidine isethionate was not haemolytic in spite of having a greater inhibitory effect regarding viability on most types of cell analysed here (4.0 to 20.5 µg/mL IC50) (Table 2). This led to considering haemolytic activity as a safety parameter when identifying promising peptides, as it was hoped that the erythrocytes would be less susceptible to rupture due to the presence of cholesterol on the membrane, such molecule stabilising the lipid bilayer and increasing resistance to antimicrobial peptide entry [31].

The fact that MP-8 and dermaseptin-S1, together with another 11 peptides, reduced *L. (V) panamensis* promastigote motility after 5 hours' incubation and did not produce substantial changes in morphology (rounded cells) led to considering possible action at membrane level due to the short time in which activity appeared and the peptides' secondary structure tendency to form an α-helix (Table 1).

Members of the subgenera *Leishmania* Viannia express lipophosphoglycan (LPG) levels from 5% to 10%, as well as high levels of glycoinositol phospholipids in *L. (V) panamensis* and braziliensis parasite membranes have been reported [32]. As antimicrobial peptides have an affinity for phospholipids, then such lower LPG percentage

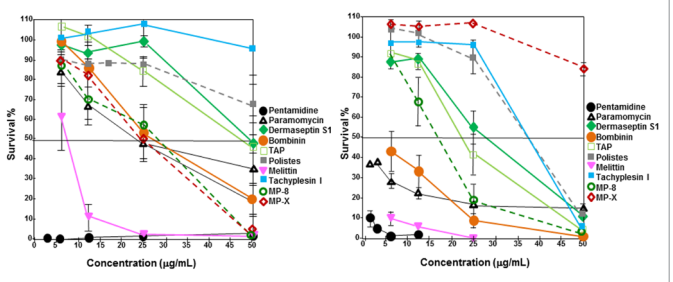

Figure 3: Antimicrobial peptides' effect on Leishmania promastigotes. A. Leishmania Viannia panamensis and B. Leishmania *Leishmania Major.*

at membrane level in the Viannia subgenera could give place to less interaction with the membrane and explain the difference in activity regarding *L. (V) panamensis* and *L. (L) major*.

The *Leishmania* membrane has some particular characteristics. It has a strong negative charge due to the high levels of LPG anionic polysaccharide, covering more than 60% of the surface, it is associated with high proteolytic activity due to the presence of Gp63 (more than 5 x 10^5 copies/parasite) and its lipid composition is characterised by having a higher percentage of anionic phospholipids than standard mammal membranes (30% ergosterol in place of cholesterol). This contributed towards increasing membrane fluidity, thereby explaining melittin, MP-8, dermaseptin-S1, bombinin, TAP, Polystes MA and tachyplesin I selectivity regarding promastigotes (SI>1).

Antimicrobial peptides were selected as promising if they were seen to be effective on *Leishmania* promastigotes (SI>1) and haemolysis percentage-inducing (safety criterion) at concentrations greater than that inhibiting human nucleated cell viability (Table 2). This led to MP-8, bombinin, dermaseptin-S1 and TAP for both parasite species and tachyplesin I and Polystes MA being selected for *L. (L) major*; their effectiveness should continue to be evaluated through studies regarding the intracellular or amastigote form in an *in vivo* model in hamsters. Even though melittin had a high SI, its highly haemolytic and cytotoxic behaviour disqualified it from being considered as promising [26].

The EC50 found by other authors for tachyplesin I, dermaseptin-S1 and melittin regarding Leishmania promastigotes were similar to those reported in the present study [8,9,13,14]. Slight differences could have been due to species' membrane characteristics or the technique used for evaluating antileishmanial activity.

Andropin, cecropins A, B and P1, apamin, Pr-2 and MP-17 and MP-X were not very effective on the Leishmania promastigote form at the maximum concentrations used (50 μg/mL); however, their antileishmanial activity should not be disregarded as they may need intracellular mechanisms to become activated, as shown by andropin and Pr-2 not being effective on promastigotes but, conversely, being so on intracellular amastigotes in an infection model with dendritic cells [8].

As all antimicrobial peptides had been tested for their *in vitro* activity against two representative Leishmania species and their individual conformational profiles had been previously obtained by circular dichroism (CD) experiments [17], it was decided to analyse the most active ones by in silico molecular modelling. Coordinates and data for each peptide were thus downloaded from the Protein Data Bank (PDB) (http://www.rcsb.org/pdb/home/home.do) regarding bombinin (PBD code 2AP8), dermaseptin-S1 (PBD code 2DD6), mastoparan (PDB 1D7N) and tachyplesin (PDB 1WO0) 3D structure determined by NMR or X-ray crystallography and molecularly modelled. Molecular models were obtained using Accelrys Inc. Viewer Lite 4.2 software (www.accelrys.com).

Figure 4 shows the backbone disposition of bombinin (in A) and mastoparan (in B), both having high α–helix content (90% and 80%, respectively), whilst dermaseptin-S1 had a small α–helical stretch conserving high energy content (C) and, remarkably, tachyplesin (D) was strongly β-stranded, facilitating antiparallel β–sheet features. Antileishmanial *in vitro* activity could provide amphipathic structural profiles present in these peptides related to their primary structure's high cationic and aliphatic amino-acid content, lysine, arginine and alanine being the most representative. As mentioned above, in spite of their high antimicrobial activity, strong differences at both structural and 3D conformation levels can be observed amongst all studied molecules.

It is well known that most mechanisms shown by antimicrobial peptides for being active against pathogen microbes depend on many aspects such as their capacity of stabilizing α-helical conformations when in contact with negatively charged lipid bilayers, secondly this structure stabilisation can be the result of a voltage dissipation from the pathogen cell membrane, third, high hydrophobic environments would led specific peptide structures become better organized, that is the case of highly compact α-helices in which a intra-catenary hydrogen bridge network allows amphipathic faces of the molecule to be properly organized. In consequence, carpet-shaped peptide agregates and pore formation will be the final step of mechanisms able to disrupt and destabilise the pathogens' outer membranes due to well organised peptide molecules and agregation so provoking cell damaging by the consequent uptake of bivalent cations and water to the cell cytosol. However, β-stranded and disulfide domains present on active peptides allow a sort of different antimicrobial mechanisms in which the peptide backbone integrity is crucial for interaction with the pathogen's membranes. Also, random conformations of a wide-range of antimicrobial peptides such as protamine and its derivatives facilitate these molecules to adopt specific bioactive conformations able to a better interaction with pathogen's membranes and even these peptides high content of Arginine residues stimulate the peptide travel to the cell cytosol and crossing the nucleus membrane and so mechanisms directed towards interactions with DNA, will govern further pathogen destabilization and death. When thinking in novel antimicrobial peptides, independently of their secondary structure and amino acid composition, potent antimicrobial peptides would need to be neither toxic nor haemolytic, to posses high stability to peptidase and protease degradation and be formulated in suitable delivery systems to be used for topic or even parentally administratation in an infected subject to be trated. The next challenge will be consistent with site-directed modifications of selected antimicrobial peptides to afford a novel generation of antimicrobials, antitumoral, vaccine candidates

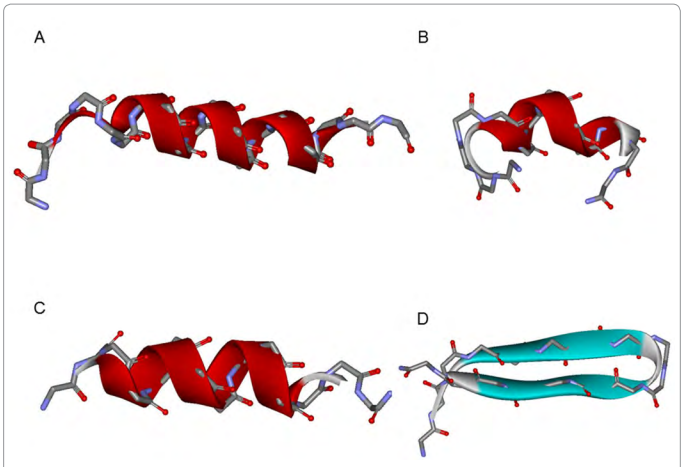

Figure 4: Conformational properties of antileishmanial active antimicrobial peptides. The most representative structures for peptide backbones were analyzed regarding their 3D structure. A) bombinin (α–helical of approximately 4.5 turns), B) dermaseptin S1 (α–helical of approximately 3 turns), C) mastoparan (α–helical of approximately 3.5 turns), and D) tachyplesin (antiparallel β–strand). Red ribbons represent α–helixes and green arrows β–strands. Side-chain hydrogens were omitted in this analysis and peptide backbones are N to C orientated.

and immunomodulating molecules. To revise latest advances in these interesting field some literature is recommended [33-36].

Taken together, the results presented herein demonstrate the potential use of these four molecules (or their modified versions) in appropriate formulations as possible antileishmanial agents. This should be explored further in *in vivo* assays thereby providing new insights as an alternative strategy for dealing with transmissible diseases in which causative pathogens are resistant to traditional antibiotics.

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