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RESEARCH ARTICLE

Design of Bactericidal Peptides Against *Escherichia coli* O157:H7, *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus*

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Abstract: ***Background*:** Antimicrobial peptides are on the first line of defense against pathogenic microorganisms of many living beings. These compounds are considered natural antibiotics that can overcome bacterial resistance to conventional antibiotics. Due to this characteristic, new peptides with improved properties are quite appealing for designing new strategies for fighting pathogenic bacteria.

***Methods*:** Sixteen designed peptides were synthesized using Fmoc chemistry; five of them are new cationic antimicrobial peptides (CAMPs) designed using a genetic algorithm that optimizes the antibacterial activity based on selected physicochemical descriptors and 11 analog peptides derived from these five peptides were designed and constructed by single amino acid substitutions. These 16 peptides were structurally characterized and their biological activity was determined against *Escherichia coli* O157:H7 (*E. coli* O157:H7), and methicillin-resistant strains of *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa (P. aeruginosa)* were determined.

***Results*:** These 16 peptides were folded into an α-helix structure in membrane-mimicking environment. Among these 16 peptides, GIBIM-P5S9K (ATKKCGLF**K**ILKGVGKI) showed the highest antimicrobial activity against *E. coli* O157:H7 (MIC=10μM), methicillin-resistant *Staphylococcus aureus* (MRSA) (MIC=25μM) and *Pseudomonas aeruginosa* (MIC=10 μM). Peptide GIBIM-P5S9K caused permeabilization of the bacterial membrane at 25 μM as determined by the Sytox Green uptake assay and the labelling of these bacteria by using the fluoresceinated peptide. GIBIM-P5S9K seems to be specific for these bacteria because at 50 μM, it provoked lower than 40% of erythrocyte hemolysis.

***Conclusion*:** New CAMPs have been designed using a genetic algorithm based on selected physicochemical descriptors and single amino acid substitution. These CAMPs interacted quite specifically with the bacterial cell membrane, GIBIM-P5S9K exhibiting high antibacterial activity on *Escherichia coli* O157:H7, methicillin-resistant strains of *Staphylococcus aureus* and *P. aeruginosa*.

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1. INTRODUCTION

The worldwide spread of antibiotic-resistant pathogenic bacteria is a severe threat to global public health [1].

Furthermore, infections with resistant bacteria lead to a high economic burden due to the extended treatments and expensive drugs required [2]. To tackle this threat, new and more potent antibacterial molecules with a different mechanism of action are urgently required [3-6]. In this context, antimicrobial peptides (AMPs) are appealing candidates because their broad spectrum of their antibacterial activity,

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low propensity to develop resistance, rapid clearance of pathogens, different mechanisms of action and synergism with classical antibiotics [7-9].

It has been demonstrated that it is possible to improve the antibacterial activity of natural AMPs by modifying the AMP amino acid sequence by taking into account the reduced length of amino acid chain and the scarcity of post-translational modification. In fact, improvement of AMP antimicrobicidal activity relies mostly on physicochemical characteristic modifications of the entire AMPs rather than the modification of specific sequential motifs. This is associated with the evidence that most of the natural AMPs execute their lethal activity by interacting with the charge of membrane phospholipids [10–14]. Frequently, the discovery of the minimal active sequence is the first step in the optimization process; which decreases the AMP production cost and immunogenicity of the active peptide. This is the case of the peptide hLF1-11(GRRRRSVQWCA) encompassing the N-terminal undecapeptide of human lactoferrin (LF), which showed 3 to 6-fold higher antimicrobial activity than its parental protein (MW = 80 KDa), being active against methicillin-resistant strains of *Staphylococcus aureus* (MRSA), multidrug resistant *Acinetobacter baumannii* (MDR Ab) and invasive fluconazole-resistant *Candida albicans*. The potent antimicrobial effect of hLF1-11 is attributed to the first two arginine residues at the N-terminus of human lactoferrin [15].

Antimicrobial activity optimization is frequently carried out by an iterative process, as was shown in the design of three Lactoferrampin (LFampin) analogs, another LF-derived peptide. Initially the peptide design was based on modifications of descriptors, such as charge distribution, hydrophobicity, size and peptide sequence; then a second peptide generation is generated by adding basic residues at the N terminus of these first analogs looking for increasing the activity against enterohemorrhagic *E. coli* O157: H7 [16]. Sometimes enhancement of peptide antimicrobial activity can be achieved through a single substitution, as was demonstrated with the amphipathic peptide RI16, derived from the N-terminal region of the porcine AMP PMAP-36. This peptide exhibited an increased antimicrobial activity against *P. aeruginosa* when substituting the Thr located in the center of the hydrophobic region of the helix by Trp (T9W), which disrupted the amphipathicity in this sequence stretch [17].

The multiple modifications carried out on the amphibian AMP magainin-2 resulted in MSI-78, a peptide developed by Genaera Corporation that reached phase III clinical trials. MSI-78 displayed increased antimicrobial activity than magainin-2 on bacteria, even on a multiresistant strain [18].

Among the most important physicochemical characteristics of peptides involved in AMP-phospholipid interaction are: high isoelectric point (Ip), associated to the content of basic amino acids that interact with membrane anionic phospholipids [7,19]; the presence of hydrophobic residues which facilitates the interactions with the fatty acyl chains of the membrane; Instability Index, that estimates the stability of the peptide; and the Grand Average of Hydropathy (GRAVY) for finding the hydrophobic peptide stretch segment for insertion into the membrane [20].

In this work, antimicrobial peptides were designed based on the aforementioned parameters used as descriptors of their antimicrobial potential. The main goal of the genetic algorithm used was to find multiple local minima antimicrobial function instead of finding the best overall minimum. Examples of peptide optimization through computational methods can be found in several publications [21-23]. In this study, the genetic algorithm allowed designing five new potential antibacterial peptides and the prediction was validated through biological assays.

2. MATERIALS AND METHODS

2.1. Materials

Fmoc-L-amino acids were purchased from IRIS Biotech GmbH. Rink amide resin 4 MBHA was obtained from **Merck** Novabiochem. Lysogeny agar and broth were purchased from Sigma-Aldrich. All reagents and organic solvents for peptide synthesis were of HPLC grade. The three bacterial strains, *E. coli* O157:H7, *P. aeruginosa* and MRSA were donated by the School of Microbiology at Pontificia Universidad Javeriana, an institution belonging to the World Federation of Culture Collection, and were kept in BHI until analysis. The A549 human lung carcinoma epithelial-like cell line was donated by "Biological and cell culture oxidation laboratory" at Universidade Federal do Rio de Janeiro (Brazil).

2.2. Peptides Design

The design of antibacterial peptides was performed through genetic algorithm optimization strategy. This provides antibacterial peptide candidates in short computational time. This algorithm designed peptides fulfilling the selected physicochemical descriptors and their established range values of charge, hydrophobicity, isoelectric point, and instability index. The optimization was performed using the Equation 1, where x_1, x_2, x_3 and x_4 are the charge, isoelectric point, hydrophobicity and instability index, respectively.

$$min \quad f(x_1,x_2,x_3,x_4) = (x_1-4)^2 + (x_2-9.5)^2 + x_3^2 + x_4$$
$$s.t. \quad 0 < x_1 < 8;\ 7 < x_2 < 12;\ -1.5 < x_3 < 1.5;\ x4 < 40 \quad (1)$$

The ranges of physicochemical properties were established using the average and standard deviation of the CAMP antibacterial peptide database [24]. The number of physicochemical descriptors was kept as low as possible for minimizing the fitness evaluation time for each candidate peptide, *i.e.*, the computational time required for obtaining the result of *fitness* in Equation 2.

$$fitness = -\left((x_1-4)^2 + (x_2-9.5)^2 + x_3^2 + x_4 + r * (p_1^2 + p_2^2 + p_3^2 + p_4^2 + p_5^2 + p_6^2 + p_7^2)\right) (2)$$

Where x_1 is the charge, x_2 is the mean hydrophobicity, x_3 is the isoelectric point, x_4 is the instability index and r is set to 100. p_1 is zero if $x_1>0$. Otherwise $p_1=-x_1$. p_2 is zero if $x_1-8<0$, otherwise $p_2=x_1-8$. p_3 is zero if $7-x_2<0$, if not $p_3=7-x_2$. If $x_2-12<0$, then $p_4=0$. In other case, $p_4=x_2-12$. p_5 was set to zero if $-1.5-x_3<0$, otherwise $p_5=-1.5-x_3$. p_6 was zero if $x_3-1.5<0$,

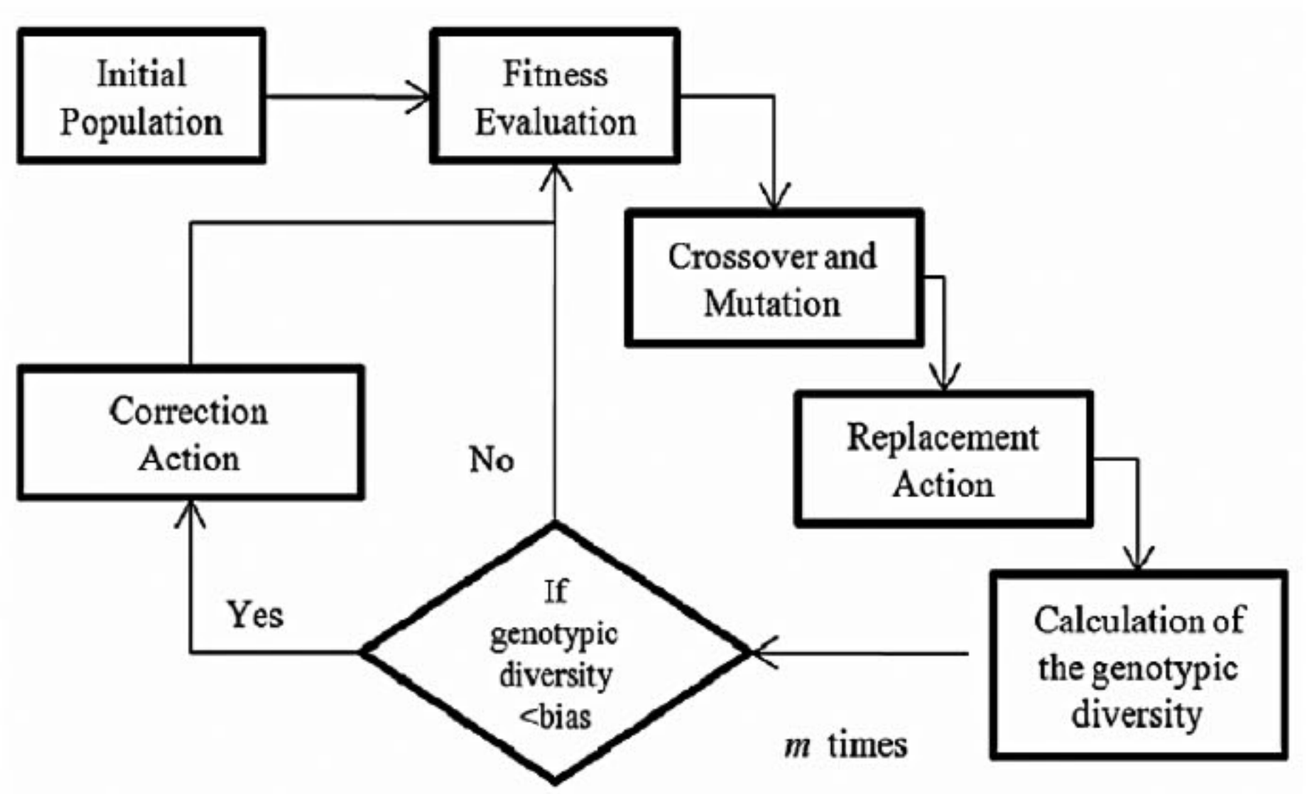

Fig. (1). The genetic algorithm diagram.

otherwise, $p_6=x_3$-1.5. Finally, p_7 was zero if x_4-40<0, or $p_7=x_4$-40 in other cases.

The inputs for the genetic algorithm basic operation (Fig. **1**) were: peptide length, the size of the peptides population, number of generations used by the genetic algorithm, crossover percentage, (the ratio of how many couples of peptides will be selected to create the new individuals), the "mutation" percentage that is the probability of a given peptide to undergo a random mutation in one amino acid, the population replacement percentage per generation (the percentage of peptides that will be replaced with new peptides in each generation), and the population genotype diversity bias (the desired value of genotypic diversity among the peptide population). The algorithm used the antibacterial peptide sequences compiled at the CAMP database to create the initial population. Firstly, all antibacterial peptides are joined in one sequence; then, a random number between 1 and the length of the new sequence minus the candidate peptide length is generated. At that position, the algorithm starts creating the individuals of the population by cutting the long sequence in fragments of the desired length. When the end of the long sequence is reached, a new random number is generated, and this process is performed as many times as required to generate the initial population. The crossover is performed using the single-point operator for obtaining two offsprings from the information exchange coming from the amino acid sequences between the parents. The parent selection for crossover uses the roulette method. The mutation operator modifies a candidate peptide character in a random way by generating two random numbers: *j=rand(1, ...,n)*, which is the amino acid that will be mutated, being *n* the desired length of the peptides and *c=rand(1,..., 20),* which is the new amino acid. In each generation, the genetic algorithm replaces a population percentage with the worst fitness values. The genetic algorithm calculates the population genotypic diversity per generation; if lower than the established threshold, 40% of the population is replaced with new individuals until the number of generation stop criterion is reached. The correction action allows the algorithm to find a list of 50 different peptides per simulation. In this sense, the main purpose of the proposed genetic algorithm is to find multiple local minima instead of finding the best local minimum. Our approach is different than that used for the *in silico* design of new antimicrobial peptides in multiple studies [21-23]. For example, in the study of Fjell and co-workers [21], a genetic algorithm was designed to generate candidate antibacterial peptide sequences. In this approach, the results were dependent on the starting population, and this fact is considered as a limitation. On the contrary, in our approach this is taken as an advantage due to the goal of the algorithm, which is the generation of a library of new potential antibacterial peptides and not the specific improvement of a given antibacterial peptide.

Five peptides with the higher antimicrobial score were designed with our genetic algorithm and their predicted secondary structure were determined by the CPH model tool (http://www.cbs.dtu.dk/services/CPHmodels/Raouf Ghavami MC MS.docx) and visualized using Pymol v3.0. m.

2.3. Design of Peptide Analogues

Eleven analogue peptides were derived from these five designed peptides by changing their non-conserved amino acid in order to keep the peptides' positive charges >3, having a high score as AMPs and exhibiting a theoretical α-helix structure. To find the conserved amino acid in the original five peptides, their sequences were compared with the amino acid sequences of proteins compiled in the NCBI database (https://blast.ncbi.nlm.nih.gov/), as well as in the CAMP antimicrobial peptide database by BLASTP (https://blast.ncbi.nlm.nih.gov/) [25, 26]. The alignment of the peptide sequences having homology <87% were performed with Clustal W [27-29].

2.4. Peptide Synthesis and Characterization

Peptides were synthesized by solid phase peptide synthesis (SPPS) [30, 31] using the tea-bag procedure reported by Houghten for multiple peptide synthesis [32, 33], in accordance with standard Fmoc chemistry and using a Rink amide 4MBHA resin (100-200 mesh; Loading: 0.63 mmol/g) as solid support and Fmoc amino acids [34-36].

The coupling reactions were performed by activating the carboxylic group of each amino acid with equivalent moles of 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hex-

afluorophosphate (HBTU) in *N, N*-dimethylformamide using *N, N*-diisopropylethylamine (DIPEA) as a neutralizer and the reaction efficiency was verified with the bromophenol blue assay. The removal of the protecting group (Fmoc-), from the NH_2-terminal, was performed with 20% piperidine. A portion of peptide-resin was used for rhodamine B coupling to the N-terminus.

These peptides were cleaved from the resin by treatment with trifluoroacetic acid (TFA)/triisopropylsilane (TIS)/ethanedithiol/H_2O (92.5/2.5/2.5:2.5) for 2 hours and then precipitated with cold diethyl ether [37]. The peptides were desalted by gel exclusion chromatography using G-10 columns (Amersham, USA).

Peptides were purified by reverse phase-high Performance liquid chromatography (RP-HPLC) (Jasco Corporation, Tokyo, Japan) in a Vydac C-18 preparative column using a 30 min mobile phase gradient of (A) H_2O with TFA 0.1% (v/v) and (B) acetonitrile (ACN) containing TFA 0.1% (v/v) between 5 and 70% of B, at 1 mL/min and detection at 220 nm. Molar mass of purified peptides was determined by MALDI-TOF [38, 39].

2.5. Secondary Peptide Structure Determination by Circular Dichroism (CD) and Structure Modeling

CD spectra of these peptides were determined at 25°C in a 1 mm path length cuvette over 190-260 nm in a CD spectrometer (J-815 Jasco Corporation, Japan) using 0.2 mM peptide solution in 70% (v/v) 50 mM sodium phosphate buffer (pH 7.4)/ 30% (v/v) 2,2,2-trifluoroetanol (TFE). Each spectrum was recorded as an average of four scan repetitions in continuous scanning mode at 50 nm/min scanning speed and at a response time of 1 s. The solvent contribution blank was subtracted from each sample spectrum. Molar ellipticity [θ] was calculated for the next equation for each peptide as:

$[\theta] = 3298.2\,\Delta\varepsilon$; where ε is the molar extinction coefficient

In addition, the secondary structure of the peptides was simulated by the PEP-FOLD 3.5 software (http://mobyle.rpbs.univ-paris-diderot.fr/cgi bin/portal.py#forms::PEP-FOLD3) and was visualized in Pymol.

2.6. Determination of Peptide Antibacterial Activity

Antibacterial activity of these peptides was assessed as described previously [17]. In brief, 7.0 $x10^6$ CFU per mL of *E. coli* O157: H7, MRSA and *P. aeruginosa* were grown in 96-well microplates (Corning) containing Luria-Bertani (LB) agar for Gram-negative bacteria and Mueller-Hinton (MH) agar for Gram-positive bacteria in the presence and absence of peptides, at a range of peptide concentrations between 0.5 and 100 μM. The commercial Ofloxacin was used as a control for growth inhibition. These bacterial cultures were incubated at 37°C with constant shaking. Bacterial growth was monitored every hour for 8 hours by quantifying the optical density at 595 nm in a microplate reader.

2.7. Peptide Bacterial Killing Assays

To evaluate the bactericidal effect of the peptides at the end of the 8 hours of bacterial growth, a 100 μL aliquot of the bacterial culture was inoculated into brain heart infusion (BHI) culture medium in Eppendorf tubes at 37°C, and incubated for 24 h. Then, 10 μL of these cultures were poured into BHI agar petri dishes, and the minimal bactericidal concentration (peptide concentration inhibiting 100% of bacterial growth, MBC) was determined. Control experiments were performed in the absence of peptides.

2.8. Peptide Hemolytic Activity on Sheep Erythrocytes

The peptide cytotoxic activity on the mammalian cell was determined by measuring hemolysis of sheep erythrocyte induced by these peptides [40]. One mL of defibrinated sheep blood was centrifuged at 1000×g for 10 min, at 4ºC; and the erythrocytes were subsequently washed four times with Hanks + glucose balanced salt solution (HBSS + Glc). Aliquots of 100 μL of $2x10^7$ erythrocytes/mL in HBSS + Glc were transferred to Eppendorf tubes and incubated at 37°C for 4 h with peptides at different concentrations. At the end of incubation, the erythrocyte suspension was centrifuged at 13,000xg for 5 min and 80 μL aliquots of the supernatant were transferred to a 96-well microplate. Absorbance at 550 nm, accounting for the release of hemoglobin, was measured in a Bio-Read 640 microplate reader. As a control, 100% hemolysis was obtained by treatment of erythrocytes with 0.1% Triton X-100. The percentage of hemolysis was calculated as: hemolysis (%) = $(A_s - A_o) / (A_{100} - A_0)$ x100, where A_s is the absorbance of the sample, A_{100} is the absorbance of the completely lysed erythrocytes in 0.1% Triton X-100 and A_0 is the absorbance in the absence of hemolysis.

2.9. Peptide Permeabilization of the Bacterial Membrane

The vital fluorescent probe SYTOX green was used to determine permeabilization of the bacterial membrane. Aliquots of 100 μL of the bacterial culture ($1x10^8$ CFU/mL) having 1 μM of SYTOX green in HBSS + Glc were transferred to black 96-well microplate and incubated at 37°C, fluorescence being measured in a microplate fluorescence reader Polarstar Galaxy (λexc = 504nm, λem = 523nm). Once the baseline fluorescence (in the absence of peptide) was stable, peptides were added at different concentrations and fluorescence monitored for 40 min or until reaching a plateau. Maximum permeabilization of Gram-negative pathogenic bacteria (100%), was obtained by addition of 4 μM Peptide Cecropin A (1-8)-melittin (1-10) (CAME) plus 0.1% Triton X -100; for MRSA, cells were treated with Lysostaphin (0.07μg/μL, final concentration) plus 0.1% TX-100. All assays were performed in triplicate [41].

2.10. Cell Culture and *in vitro* Cytotoxicity Assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay was performed according to a previously described method [42]. Briefly, cells from human lung carcinoma *cell line A549* were cultured in modified Eagle's medium (EMEM) plus 10% FBS (complete media). Cells were seeded into a 96-microwell plate at 7500 cells/well and 0.2 mL/well in complete medium and incubated under 5% CO_2 at 37°C. After 24 h incubation, cells were treated with GIBIM-P5S9K (5 to 100 μM final concentration) and incubated for 48 h. Then, 200 μL of MTT (0.5 mg/mL) was added and incubated for 3

h. The supernatant was removed, and 200 µL DMSO was added to the cells in order to solubilize the formed formazan. Cytotoxicity was determined, measuring the absorbance at 550 nm using a microplate reader (Thermo) with respect to untreated controls.

2.11. Confocal Laser Scanning Microscopy

E. coli O157:H7, *P. aeruginosa* and MRSA cells incubated in the presence of rhodamine labeled peptide were analyzed by confocal laser scanning microscopy in Leica TCS SP5 II microscope (Berlin, Germany) for determining the cellular distribution of the GIBIM-P5S9K. Aliquots of bacterial cultures in the mid-logarithmic phase were centrifuged (2500 g x 5 min) and resuspended in the presence of 37 µM rhodamine labeled- GIBIM-P5F8W, in isotonic PBS buffer during 1.5 h. Then, the cell suspension was centrifuged (2500 g × 5 min), the supernatant was discarded, and the cells were washed four times in PBS, and resuspended in TO-PRO®-3 stain (Molecular Probes) solution for 2 min, centrifuged at 2,500 × g for 5 min and washed four times with PBS. Afterward, the bacterial cells were fixed with paraformaldehyde on glass slides. Finally, fluorescence mounting medium was added. The images were analyzed by Leica Application Suite Advanced Fluorescence (LAS AF) software in a 512×512 pixel format [35].

2.12. Statistical Analysis

All experiments were carried out by triplicate and repeated at least three times in independent assays. The results were reported as mean ± SD. The results are reported as mean ± SD Results were subject to the t-test analysis of variance using R, and statistical significance of differences ($p < 0.05$) was evaluated by the least significant difference (LSD) procedure.

3. RESULTS

3.1. Peptide Design

Five peptides (GIBIM-P1 to P5) were selected from a collection of twelve peptides designed by the genetic algorithm (Fig. **1**, Table **1**), and eleven analogues were obtained by single amino acid substitution of these five peptides. The peptide positive charge, which is directly related to its antimicrobial activity, was increased by replacing the residues Ala6 in the analogue peptide GIBIM-P3A6K, Thr2 in the analogue peptide GIBIM-P5T2K and Ser9 in the analogue peptide GIBIM-P5S9K by Lys [43]. In order to increase hydrophobicity, Gly17 were replaced by Ile and Leu in peptides GIBIM-P2G17I and GIBIM-P2G17L, respectively. Ala1 was replaced by Ile and Val in the analogue peptides GIBIM-P4A1I, and GIBIM-P4A1V, respectively. Asn2 was replaced by Phe in the analogue peptide GIBIM-P4N2F. All these 16 peptides had a probability of being an antimicrobial peptide (PAP) higher than 96% according to the prediction tool of CAMP database and an instability index lower than 40, except for GIBIM-P3A6K (Table **1**).

These 16 peptides were synthesized, purified by RP-HPLC (purity<95%) (See Supplementary Information, fig. **S1**) and their molecular weight verified by MALDI-TOF mass spectrometry (Table **1** and Supplementary Information figure S2). Their α-helical conformation was characterized in 30% TFE by CD and identified by two minima at 202 and 210 nm and one maximum at 190 nm (Fig. **2**). The CD structural data agreed with those provided by Pepfold (Fig. **2**).

3.2. Antibacterial Activity of GIBIM Peptides on Pathogenic Bacteria

The designed cationic peptides inhibited the growth of the Gram-positive bacterium MRSA and the Gram-negative bacteria *E. coli* O157: H7 and *P. aeruginosa* in a concentration-dependent manner (Table **2**). Overall, 14, 15 and 11 out of 16 peptides exhibited MIC_{99} (peptide concentration inhibiting 99% of bacterial growth) equal or lower than 100 µM against *E. coli* O157: H7, MRSA and *P. aeruginosa,* respectively.

In the five peptides designed with the genetic algorithm GIBIM-P1 and GIBIM-P3 showed the highest antibacterial activity against *E. coli* O157:H7 (MIC_{99} 50 µM) and MRSA (MIC_{99} 25 µM), and GIBIM-P5 against *P. aeruginosa* (MIC_{99} 10 µM). GIBIM-P4 had MIC_{99s} between 50 and 100 µM against *E. coli* O157: H7, MRSA and *P. aeruginosa*. Some of the analogue peptides showed higher antibacterial activity than the genetic algorithm designed peptides; *i.e.* the analogue peptide GIBIM-P5S9K, derived from GIBIM-P5 by S5K substitution showed a MIC value 10 µM against *E. coli* O157: H7 and *P. aeruginosa,* and 25 µM against MRSA (Fig. **3**). This peptide exhibited the highest activity and was close to the activity of ofloxacin against *E. coli* O157: H7 (Table **2**). Peptides GIBIM-P4A1I and GIBIM- P4A1V showed increased antibacterial activity against *P. aeruginosa* with respect to GIBIM-P4, having MIC_{99}s of 25 µM.

Additionally, some of these peptides showed antibacterial effect; GIBIM-P3, GIBIM-P4 and GIBIM-P5S9K against *E. coli* O157: H7 and MRSA, with MBCs between 50 and 100 µM. GIBIM-P3A6K against *E. coli* O157: H7 with a MBC of 100 µM. GIBIM-P2G17L, GIBIM-P4A1I and GIBIM-P5T2K against MRSA with the MBC between 75 and 100 µM. Eleven out of the 16 peptides were bacteriostatic against *P. aeruginosa*, but none of them were bactericidal.

3.3. GIBIM-P5S9K Induced the Bacterial Membrane Permeability

One of the most frequent mechanisms of action of AMPs is cell membrane permeabilization in which they interact with membrane phospholipids, distorting the bilayer structure and resulting in the loss of membrane functionality [44, 45]. SYTOX green is a cationic probe (Mw = 600 Da), unable to access the intracellular space in unscathed cells, but not in permeabilized ones. Once inside the bacteria, this probe binds to nucleic acids increasing its fluorescence. In the presence of SYTOX green, *E. coli* O157: H7, MRSA and *P. aeruginosa* incubated with GIBIM-P5S9K at concentrations between 1.0 and 10.0 µM increased SYTOX fluorescence over time to a final value dependent on peptide concentration (Fig. **4**).

3.4. Cytotoxicity of Peptides Against Mammalian Cells

Plasma membrane permeabilization of mammalian cells was monitored by hemolysis of sheep erythrocytes and the A549 cell proliferation. None of these peptides at 50 µM,

Table 1. Physicochemical properties of designed and analogs peptides.

Peptide	Sequence	Net Charge	MW(Da)	m/z	PAP(%)[a]	pI	Instab. Index[b]	GRAVY[c]
GIBIM-P1	QVKGAKLWLSLMKWGGI	3	1915.37	1915.15	96.5	10.3	5.65	0.28
GIBIM-P2	GFLGKGLFSVVKGVLKG	3	1706.1	1706.06	99.3	10.3	-17.25	0.89
GIBIM-P2G17I	GFLGKGLFSVVKGVLK**I**	3	1762.21	1762.04	99.5	10.3	-17.25	1.18
GIBIM-P2G17L	GFLGKGLFSVVKGVLK**L**	3	1762.21	1762.04	99.5	10.3	-17.25	1.14
GIBIM-P2G13N	GFLGKGLFSVVK**N**VLKG	3	1763.16	1763.17	99.3	10.3	-12.26	0.71
GIBIM-P3	NVAATARGWLKKLGKRI	5	1882.29	1882.45	96.7	12.02	23.13	-0.29
GIBIM-P3A3G	NV**G**ATARGWLKKLGKRI	5	1868.26	1868.20	96.4	12.02	13.14	0.42
GIBIM-P3T5S	NVAA**S**ARGWLKKLGKRI	5	1868.26	1868.41	97.2	12.02	23.13	-0.29
GIBIM-P3A6K	NVAAT**K**RGWLKKLGKRI	6	1939.38	1938.38	98.0	12.03	42.31	-0.62
GIBIM-P4	ANVAATARGWLKKIGKK	5	1812.19	1811.96	98.7	11.33	3.95	-0.37
GIBIM-P4A1I	**I**NVAATARGWLKKIGKK	5	1854.27	1854.09	99.3	11.33	3.95	-0.21
GIBIM-P4A1V	**V**NVAATARGWLKKIGKK	5	1840.25	1840.10	98.9	11.33	3.95	-0.23
GIBIM-P4N2F	A**F**VAATARGWLKKIGKK	5	1845.26	1844.97	98,5	11.33	3.95	0.00
GIBIM-P5	ATKKCGLFSILKGVGKI	4	1763.22	1763.02	97.0	10.04	-4.23	0.56
GIBIM-P5T2K	A**K**KKCGLFSILKGVGKI	5	1790.28	1790.04	96.6	10.2	-4.23	0.37
GIBIM-P5S9K	ATKKCGLF**K**ILKGVGKI	5	1804.31	1804.03	96.0	10.2	-18.06	0.38

[a] Percentage probability of being an antimicrobial peptide
[b] Instability Index
[c] Grand Average of Hydropathicity

which was the highest concentration assayed (Fig. **5**). Also, peptides GIBIM-P3, GIBIM-P4, GIBIM-P5 and GIBIM-P5S9K at 100 μM displayed no detectable cytotoxicity againt A549 cells. However, the viability of A549 cells incubated with peptide GIBIM-P5S9K at 100 μM (which is four times higher than the MIC_{99}) was 50% of the viability of untreated cells.

3.5. GIBIM-P5S9K-peptide Interaction with Bacteria Assessed by Fluorescence Microscopy

Confocal fluorescence microscopy was employed to determine the intracellular location in *E. coli* and *P. aeruginosa* of the rhodaminated version GIBIM-P5S9K. After 1.5 h of incubation, the peptide was localized at the surface of the bacteria, but not inside the cytoplasm (Fig. **6A** and **B**). Interestingly, in the Gram-positive bacteria MRSA, the peptide was located in both in the membrane and in the cytoplasm (Fig. **6C**).

4. DISCUSSION

New potent AMPs were designed using a combination of genetic algorithm named DEsign and PRediction of AntiMicrobial Peptides (DEPRAMPs) and the rational modification of the sequences generated by this program. In the design of analogues non-conserved amino acids were substituted by other natural amino acids predicted to increase the effectiveness of an AMP by reducing the instability index (<40%), while maintaining both its pI between 7 and 12 and its amphipathicity. Our approach is different than reported by others for the *in silico* design of new AMPs [21-23].

The amphipathic structure of AMPs is key to their antimicrobial activity by ensuring their interaction with the cell membrane, their further insertion and disruption of the phospholipid membrane matrix [46]. Additionally, Arg was substituted by Lys to avoid peptide degradation by trypsin-like enzymes, whose hydrolytic activity is higher close to Arg residues than to Lys residues [47].

The differences in antimicrobial activity of the AMPs against different bacteria could be due to a fine tuning of peptide-bacteria interaction, occurring mostly, but not exclusively, at the level of membrane structure (Table **2**). For instance, GIBIM-P3A3G is more active than GIBIM-P3 against *P. aeruginosa* but less active against *E. coli* O157:H7 and MRSA, which could be due to the fact that *P. aeruginosa* possesses an extremely low outer membrane permeability compared with other bacteria [48]. This peptide specificity underlies the importance of designing peptides targeting pathogenic bacterial strains but hardly noxious to eukaryotic cells. In fact, the set of peptides assayed fulfilled this criterion.

GIBIM-P1, GIBIM-P3 and GIBIM-P5S9K presented, antibacterial activity against the Gram-positive bacteria MRSA comparable to other amphiphilic cyclic peptides and their analogues; specifically, the cyclic peptide [R4W4], the most potent AMP against MRSA described to date (MIC_{99} of 2.67 μg/mL or 2 μM) [49].

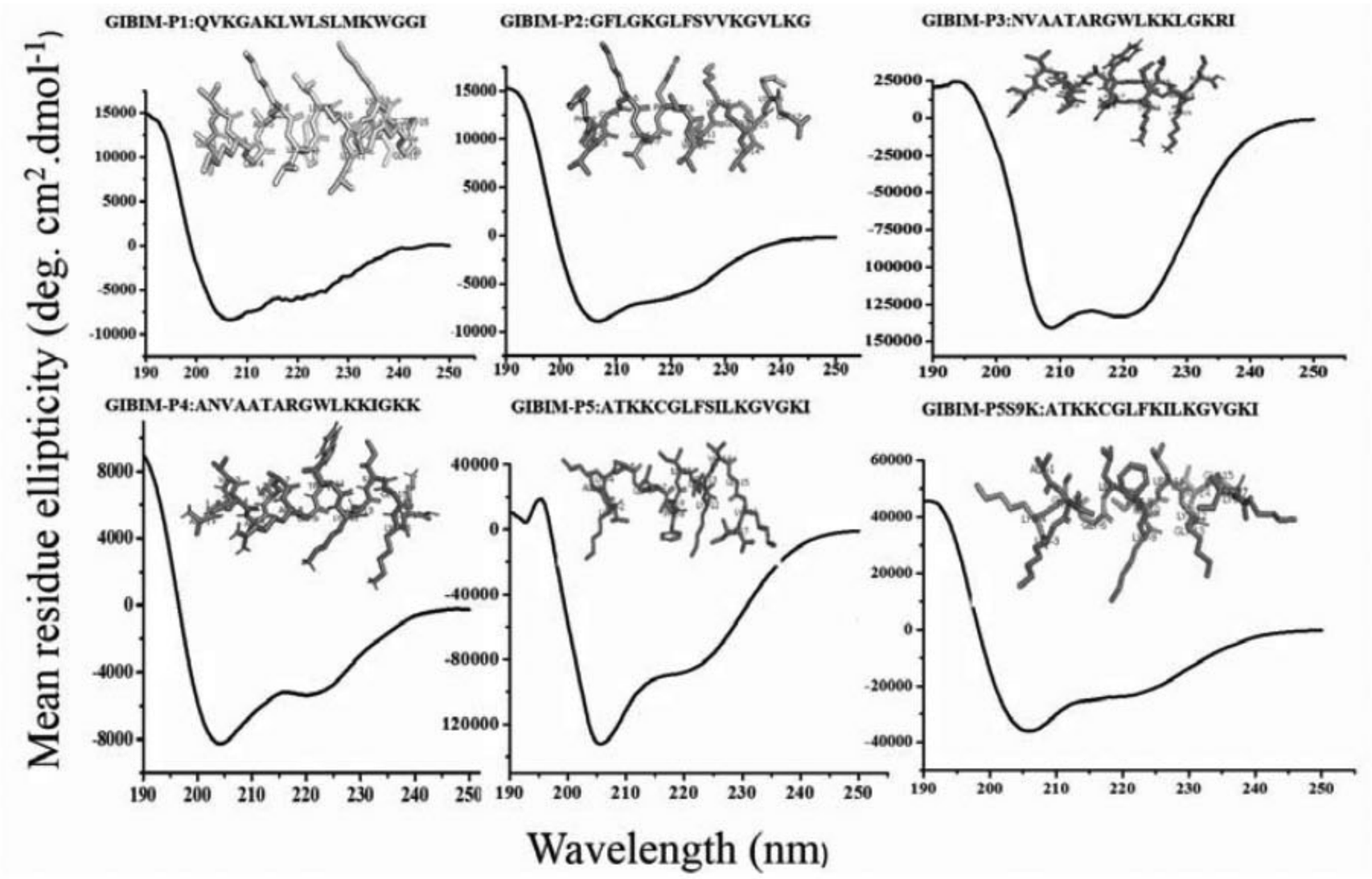

Fig. (2). Predicted secondary structures of the peptides GIBIM-P1, GIBIM-P2, GIBIM-P3, GIBIM-P4, GIBIM-P5 and GIBIM-P6 and their corresponding CD spectra. The peptides were dissolved in 30% TFE at 100 μM. The mean residue ellipticity was plotted against wavelength. The values from three scans per sample were averaged.

Table 2. Antibacterial activity of designed peptides and their analogs.

Peptide	*E. coli* O157:H7		MRSA		*P. aeruginosa*	
	MIC_{99} (μM)	MBC (μM)	MIC_{99} (μM)	MBC (μM)	MIC_{99} (μM)	MBC (μM)
GIBIM-P1	**50**	**>100**	**25**	**>100**	**>100**	**>100**
GIBIM-P2	**100**	**>100**	**75**	**>100**	**>100**	**>100**
GIBIM-P2G17I	**50**	**>100**	**75**	**>100**	**>100**	**>100**
GIBIM-P2G17L	**>100**	**>100**	**50**	**100**	**>100**	**>100**
GIBIM-P2G13N	**100**	**>100**	**100**	**>100**	**100**	**>100**
GIBIM-P3	**50**	**100**	**25**	**75**	**>100**	**>100**
GIBIM-P3A3G	**75**	**>100**	**100**	**>100**	**50**	**>100**
GIBIM-P3T5S	**100**	**>100**	**75**	**>100**	**75**	**>100**
GIBIM-P3A6K	**75**	**100**	**>100**	**>100**	**100**	**>100**
GIBIM-P4	**50**	**75**	**50**	**75**	**100**	**>100**
GIBIM-P4A1I	**>100**	**>100**	**50**	**100**	**25**	**>100**
GIBIM-P4A1V	**50**	**>100**	**75**	**>100**	**25**	**>100**
GIBIM-P4N2F	**75**	**>100**	**100**	**>100**	**50**	**>100**
GIBIM-P5	**100**	**>100**	**100**	**>100**	**10**	**>100**
GIBIM-P5T2K	**50**	**>100**	**75**	**100**	**25**	**>100**
GIBIM-P5S9K	**10**	**50**	**25**	**75**	**10**	**>100**
Ofloxacin	**8.8**	**>8.8**	**3.6**	**>5.5**	**2.8**	**5.5**

Each MIC value is the average of at least three independent experiments.

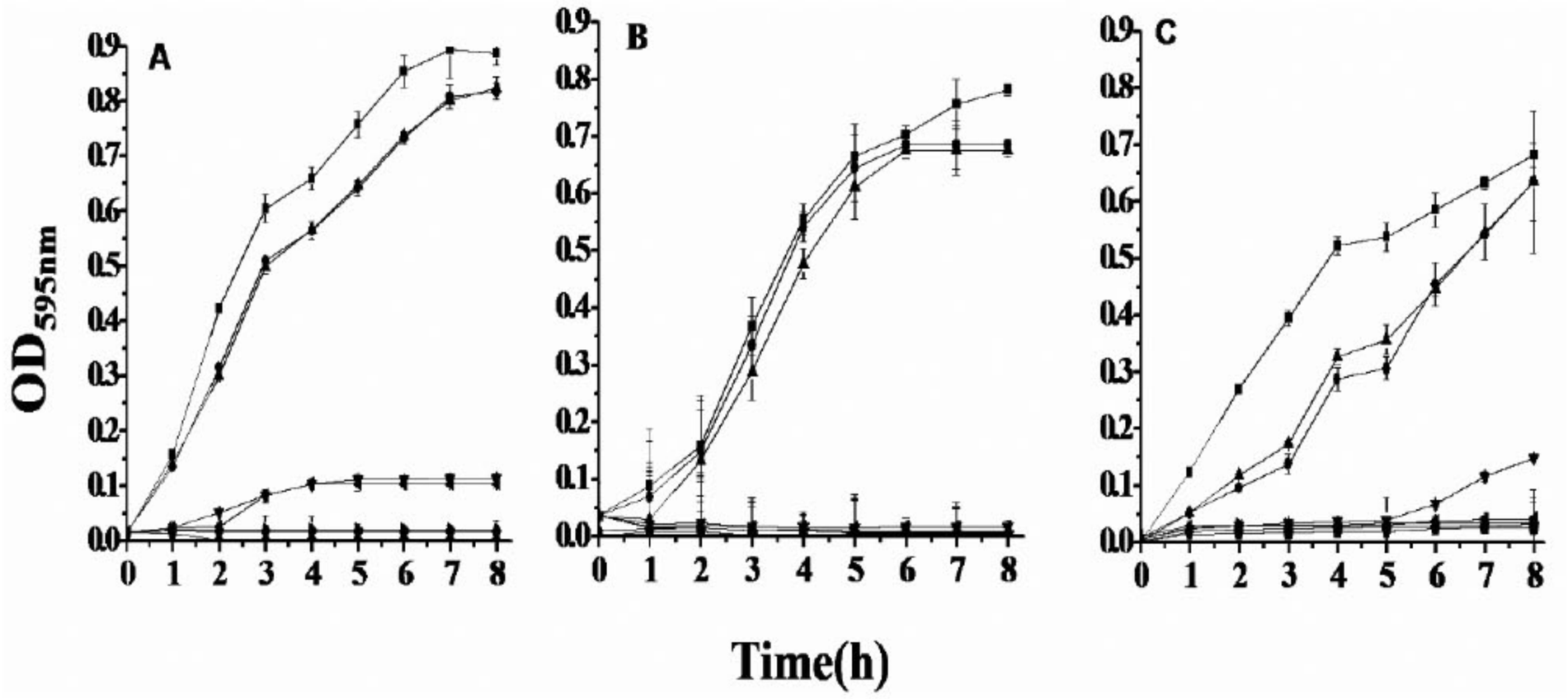

Fig. (3). Antimicrobial activity of the GIBIM-P5S9k peptide. A) *E. coli* O157:H7, B) *P. aeruginosa* and C) MRSA.Legend (●) Control, (▲) 0.5μM, (▼) 1.0μM, (◀) 10.0 μM, (▶) 25μM, (◆) 50μM, (⬟) 75μM and (■) 100μM. Error bars stand for SD.

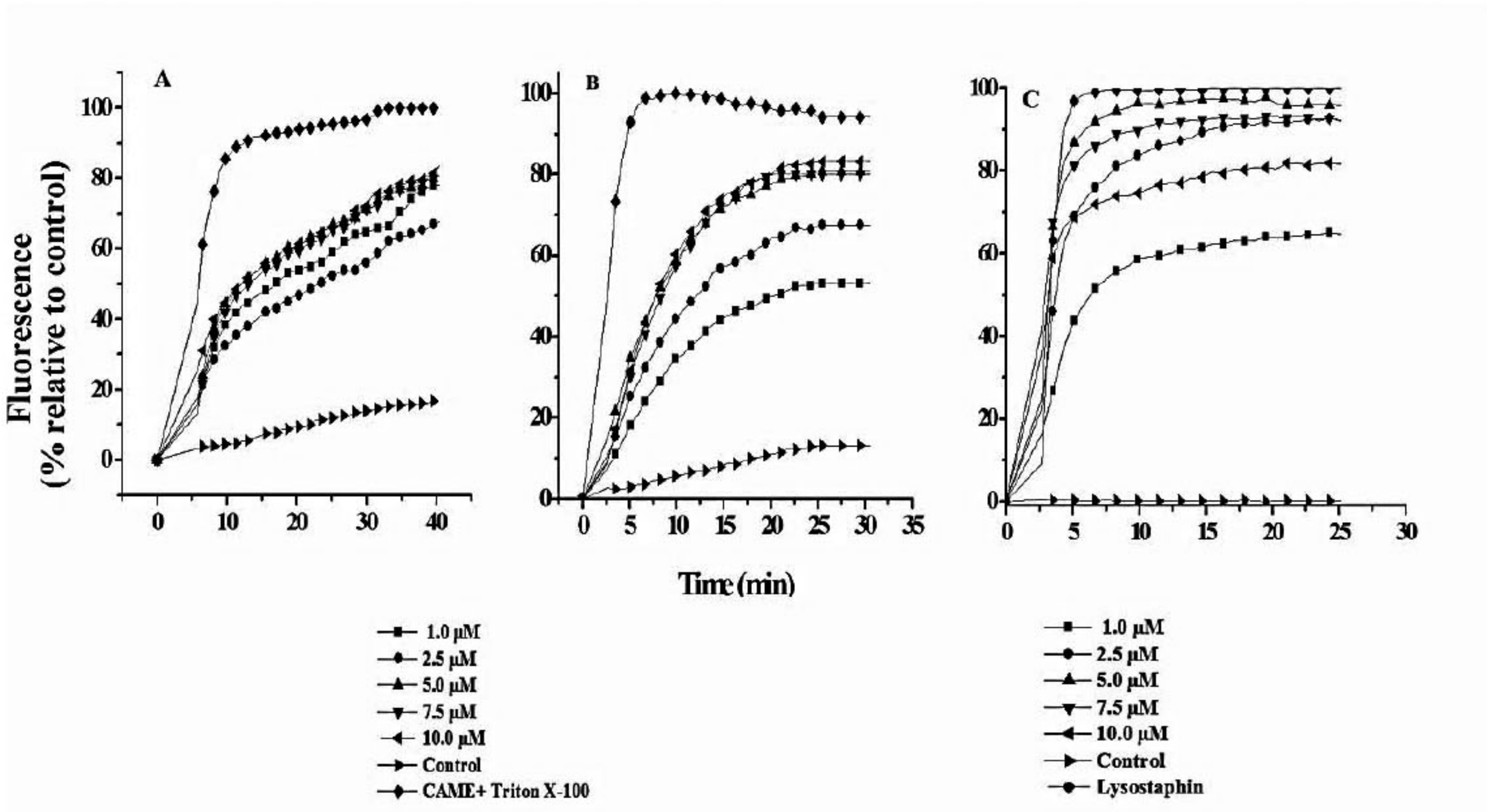

Fig. (4). Membrane permeabilization assay Sytox-green assay: A) *E. coli* O157: H7, B) *P. aeruginosa* and C) MRSA. Cecropin A (1-8)-melittin (1-10) (CAME) hybrid peptide was selected as a model of antimicrobial peptide against Gram-negative bacteria and lysostaphin against Gram-positive bacteria.

The potential of the proposed strategy for designing and optimizing AMPs was demonstrated. As a proof-of-concept, GIBIM-P5S9K increased ten times the antimicrobial activity of its parental GIBIM-P5 against *E. coli* and four times against MRSA, and was more active than GIBIM-P5T2K on these bacteria. The modifications in GIBIM-P5T2K and in GIBIM-P5S9K were made at the hydrophobic face of the α-helix. In both cases the replacement was similar: Thr and Ser were changed by Lys. This suggests that the higher antimicrobial activity is not exclusively due to the increase of the overall peptide positive charge, but also to the distribution of positive charges throughout the peptide structure. In fact, GIBIM-P3A6K having the highest positive net charge +6, did not increase its antibacterial activity against these strains compared to GIBIM-P3. Peptide positive charge promotes binding to the anionic head of phospholipids on the bacterial membrane, but could impair its membrane insertion [50]. This fact would presumably make the peptide antibacterial activity less susceptible to the well-known strategy of resistance to AMP activity by decreasing the anionic character of lipopolysaccharides or teichoic acids in Gram negative and positive bacteria, respectively [51].

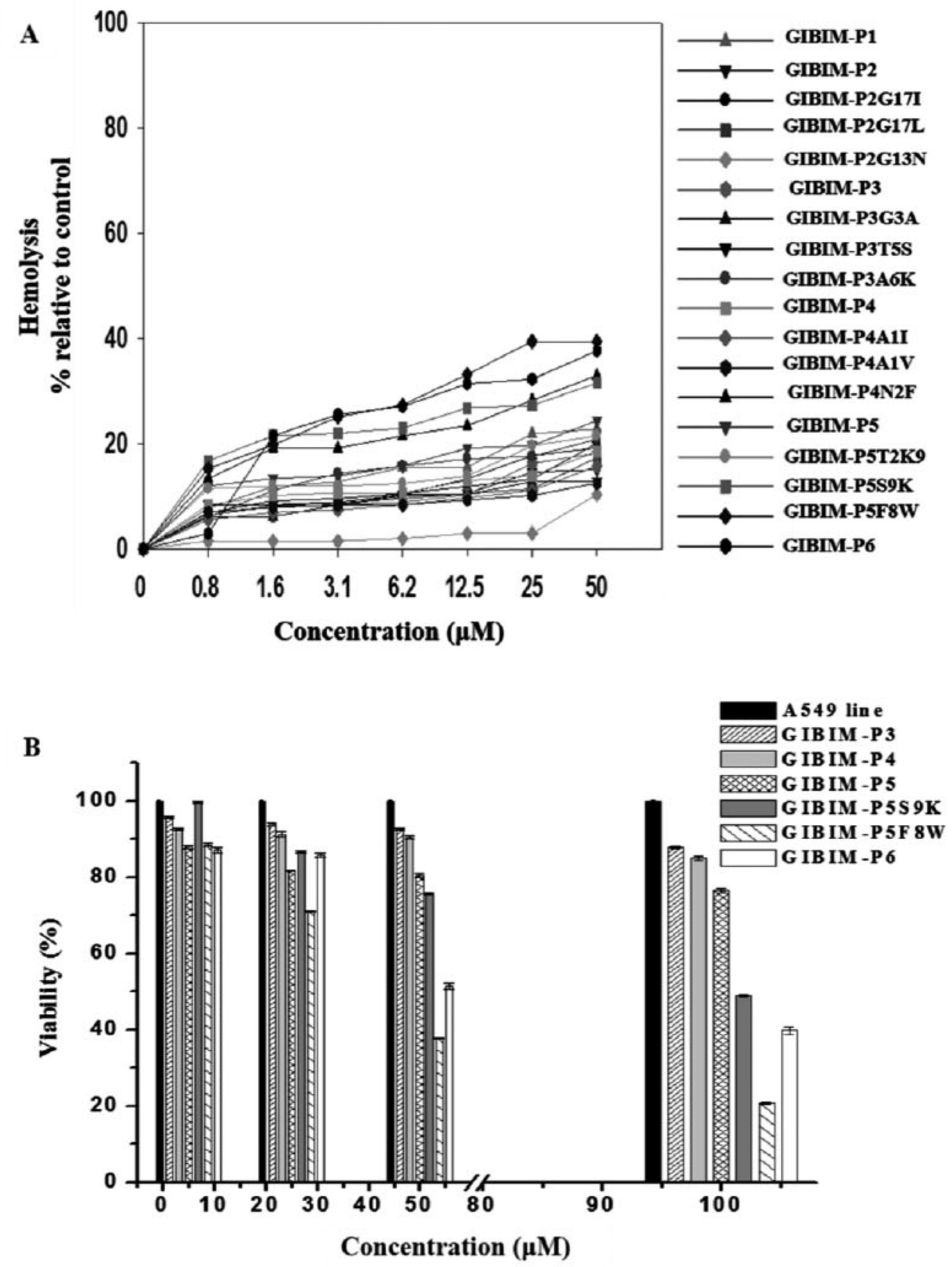

Fig. (5). Cytotoxicity on mammalian cells of the peptides having the higher antimicrobial activity. A) Hemolytic activity on sheep erythrocytes; B) *in vitro* cytotoxicity assay in A549 line cells. Error bars indicate SD. Little or no SD was observed. Cell viability is expressed as percentage of viability respect to 0.1% TX-100.

GIBIM-P1, GIBIM-P3 to GIBIM-P4N2F, presented lower antibacterial activity than other peptides having similar composition [18], possibly because the location of Trp residues does not allow the a π-cation interaction, with a concomitant stabilization of the indole aromatic ring and a higher antibacterial activity [52-54]. Moreover, the presence of the indole aromatic ring might produce a depolarization of the bacterial membrane by the presence of this conjugation system.

The differences in the antimicrobial activity cannot be ascribed to differences in the secondary structure. In fact, no significant differences were observed among the peptide circular dichroism spectra (See Fig. **2**). These differences could be due to other physicochemical characteristics, for example, some antimicrobial peptides can form aggregates in solution that compete for an effective insertion of monomers or peptide aggregates into the membrane. These peptide aggregates have been described as inactive because they do not effectively insert in the membrane and thus do not promote the cell depolarization [55]. Another possibility is peptide degradation by bacterial enzymes.

Some of the new peptides produced in this paper have antibacterial activity comparable or higher than peptides such as Cecropin B, Cecropin P1 and Hepcidin [56, 57].

Interestingly, GIBIM-P5S9K has 92% homology with the Amphibian AMP esculentin (http://www.ebi.ac.uk/pdbe/entry/pdb/2n6m). In some way, the algorithm may mimic the evolution driven by nature on some AMPs.

Therefore, we have developed a method using a computational model for correlating sequence-derived physicochemical peptide properties with the antimicrobial activity

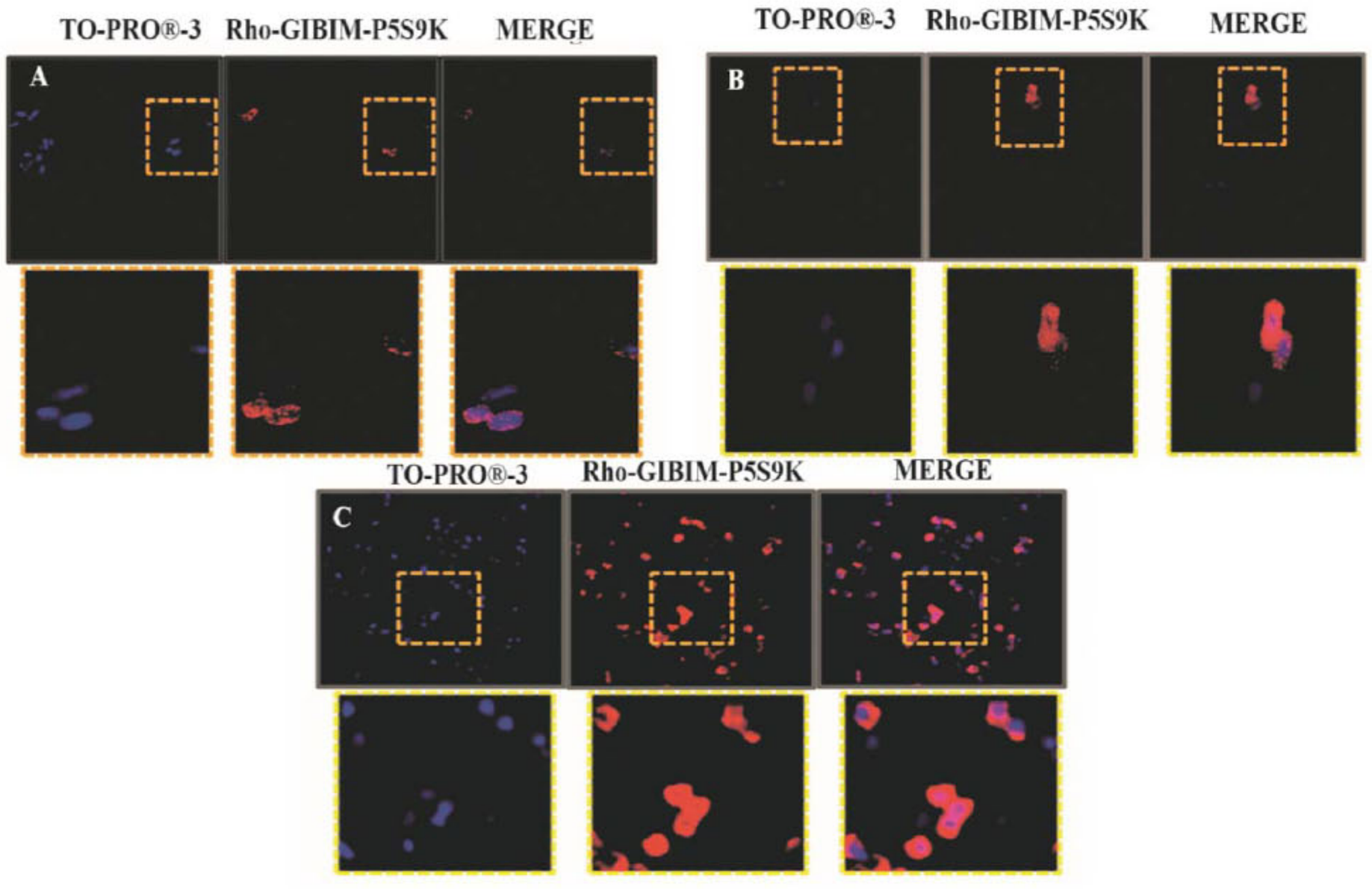

Fig. (6). Cellular location of GIBIM-P5S9K in bacteria. (**A**) *E. coli* O157:H7; (**B**) *P. aeruginosa*; (**C**) MRSA. Confocal laser scanning microscopy images of *E. coli* O157:H7 cells treated with GIBIM-P5F8W rhodamine coupled (Rho-GIBIM-P5S9K) and TO-PRO®-3 fluorophore was used as DNA stain. Wavelengths used were λ_{EXC} = 642 nm and λ_{EM} = 661 nm for TO-PRO®-3 and λ_{EXC} = 554 nm and λ_{EM} = 579 nm for the rhodaminated peptide.

that resulted in the design of efficient AMPs, as illustrated by GIBIM-P5.

CONCLUSION

New AMPs were designed using a novel genetic algorithm and rational modifications of their sequences based on optimization of some peptide physicochemical characteristics. Additionally, tryptophan and lysine amino acid substitutions in the middle of the sequences were found to improve the antimicrobial activity to a greater extent than substitutions in the C- or N-terminal region. The evaluated cytotoxicity of the new AMPs against human cell line A549 and erythrocytes demonstrated the specificity towards bacterial cells of these peptides.

The most potent AMP against the most common pathogenic bacteria (MRSA, *P. aeruginosa* and *E. coli* O157:H7), designed in this work was GIBIM-P5S9K. This peptide adopts an α-helical structure in environments designed to mimic cell membranes and is able to permeate the membrane of pathogenic bacteria in a dose-dependent manner. In addition, it can be considered a selective compound due to its low toxicity to eukaryotic cells. Overall, these data suggest that GIBIM-P5S9K is an attractive AMP and a promising candidate against infectious diseases caused by these pathogenic bacteria.

LIST OF ABBREVIATIONS

CAN	=	acetonitrile
AMPs	=	antimicrobial peptides
BHI	=	brain heart infusion
CAMPs	=	cationic antimicrobial peptides
CD	=	circular dichroism
DEPRAMPs	=	DEsign and PRediction of AntiMicrobial Peptides
DIPEA	=	*N, N*-diisopropylethylamine
DMF	=	*N, N*-Dimethylformamide
E. coli O157:H7	=	*Escherichia coli* O157:H7
EMEM	=	Eagle's modified Eagle medium
GA	=	genetic algorithm
GRAVY	=	Grand Average of Hydropathy
HBSS	=	Hanks balanced salt solution
HBTU	=	2- (1H-benzotriazol-1-yl) -1,1, 3,3-tetra-methyluronium hexafluorophosphate
Ip	=	isolelectric point
LASAF	=	Leica Application Suite Advanced Fluorescense
LF	=	Lactoferrin
LP	=	lipopolysaccharide
LSD	=	least significant difference
MBC	=	minimal bactericidal concentration

MDRAb	=	multidrug resistant *Acinetobacter baumannii*
MH	=	Mueller-Hinton
MRSA	=	methicillin-resistant *Staphylococcus aureus*
OM	=	outer membrane
P. aeruginosa	=	*Pseudomonas aeruginosa*
RP-HPLC	=	Reverse Phase-High Performance Liquid Chromatography
SPPS	=	solid phase peptide synthesis
TA	=	teichoic acids
TFA	=	trifluoroacetic acid
TFE	=	2,2,2-trifluoroetanol
TIS	=	triisopropylsilane

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article.

REFERENCES

[1] Brogan, D.M.; Mossialos, E. A critical analysis of the review on antimicrobial resistance report and the infectious disease financing facility. *Global Health,* **2016**, 12, 8-14.

[2] Melnyk, A. H.; Wong, A.; Kassen, R. The fitness costs of antibiotic resistance mutations. *Evol. Appl*., **2015**, 8, 273–283

[3] Andersson, D. I.; Hughes, D. Antibiotic resistance and its cost: is it possible to reverse resistance?. *Nat. Rev. Microbiol*., **2010**, 8 (4), 260-271.

[4] Schroeder, C. M.; Zhao, C.; Debroy, C.; Torcolini, J.; Zhao, S.; White, D. G.; Wagner, D. D.; McDermott, P. F.; Walker, R. D.; Meng, J. Antimicrobial resistance of *Escherichia coli* O157 isolated from humans, cattle, swine, and food. *Society*, **2002,** 68(2), 576-581.

[5] Turos, E.; Shim, J-Y.; Wang, Y.; Greenhalgh, K.; Reddy, G. S.; Dickey, S.; Lim, D. V. Antibiotic-conjugated polyacrylate nanoparticles: new opportunities for development of anti-MRSA agents. *Bioorg. Med. Chem. Lett*., **2007,** 17(1), 53-56.

[6] Xu, Z-Q.; Flavin, M. T.; Flavin, J. Combating multidrug-resistant Gram-negative bacterial infections. *Expert Opin. Investig. Drugs*, **2014**, 23, 163-182.

[7] Cruz, J.; Ortiz, C.; Guzmán, F.; Fernández-Lafuente, R.; Torres, R. Antimicrobial peptides: promising compounds against pathogenic microorganism. *Curr. Med. Chem*., **2014**; 21(20), 2299-2321.

[8] Batoni, G.; Maisetta, G.; Esin, S. Antimicrobial peptides and their interaction with biofilms of medically relevant bacteria. *Biochim. Biophys. Acta - Biomembr*., **2016**, 1858(5), 1044-1060.

[9] Malanovic, N.; Lohner, K. Gram-positive bacterial cell envelopes: The impact on the activity of antimicrobial peptides. *Biochim. Biophys. Acta - Biomembr*., **2016**, 1858(5), 936-946.

[10] Ahmad, A.; Ahmad, E.; Rabbani, G.; Haque, S.; Arshad, M.; Hasan Khan, R. Identification and design of antimicrobial peptides for therapeutic applications. *Curr. Protein Pept. Sci*., **2012**, 13(3), 211-223.

[11] Avitabile, C.; Netti, F.; Orefice, G.; Palmieri, M.; Nocerino, N.; Malgieri, G.; D'Andrea, L. D.; Capparelli, R.; Fattorusso, R.; Romanelli, A. Design, structural and functional characterization of a Temporin-1b analog active against Gram-negative bacteria. *Biochim. Biophys. Acta*, **2013**, 1830(6), 3767-3775.

[12] Lee, J. K.; Park, S. C.; Hahm, K. S.; Park, Y. A helix-PXXP-helix peptide with antibacterial activity without cytotoxicity against MDRPA-infected mice. *Biomaterials,* **2014**, 35, 1025-1039.

[13] Scudiero, O.; Galdiero, S.; Nigro, E.; Del Vecchio, L.; Di Noto, R.; Cantisani, M.; Colavita, I.; Galdiero, M.; Cassiman, J. J.; Daniele, A.; Pedone, C.; Salvatore, F. Chimeric Beta-Defensin Analogs, Including the Novel 3NI Analog, Display Salt-Resistant Antimicrobial Activity and Lack Toxicity in Human Epithelial Cell Lines. *Antimicrob. Agents Chemother*., **2013**, 57(4), 1701-1708.

[14] Torcato, I. M.; Huang, Y-H.; Franquelim, H.G.; Gaspar, D.; Craik, D. J.; Castanho, M. A.; Troeira Henriques, S. Design and characterization of novel antimicrobial peptides, R-BP100 and RW-BP100, with activity against Gram-negative and Gram-positive bacteria. *Biochim. Biophys. Acta*, **2013**, 1828(3), 944-955.

[15] van der Does A. M.; Bogaards, S. J. P.; Ravensbergen, B.; Beekhuizen, H.; van Dissel, J. T.; Nibbering, P. H. Antimicrobial peptide hLF1-11 directs granulocyte-macrophage colony-stimulating factor-driven monocyte differentiation toward macrophages with enhanced recognition and clearance of pathogens. *Antimicrob. Agents Chemother*. **2010**, 54(2), 811-816.

[16] Cruz, J.; Ortiz, C.; Guzmán, F.; Cárdenas, C.; Fernandez-Lafuente, R.; Torres, R. Design and activity of novel lactoferrampin analogues against O157:H7 enterohemorrhagic Escherichia coli. *Biopolymers*, **2014**, 101(4), 319-328.

[17] Zhu, X.; Ma, Z.; Wang, J.; Chou, S.; Shan, A. Importance of Tryptophan in Transforming an Amphipathic Peptide into a *Pseudomonas aeruginosa*-Targeted Antimicrobial Peptide. I *PLoS One*, **2014**, 9(12), e114605.

[18] Hallock, K. J.; Lee. D. K.; Ramamoorthy A. MSI-78, an Analogue of the magainin antimicrobial peptides, disrupts lipid bilayer structure via positive curvature strain. *Biophys. J*., **2003**, 84(5), 3052-3060.

[19] Kang, S. J.; Kim, D. H.; Mishig-Ochir, T.; Lee, B.J. Antimicrobial peptides: their physicochemical properties and therapeutic application. *Arch. Pharm. Res.,* **2012**, 35(3), 409-413.

[20] Edwards, I. A.; Elliot, A. G.; Kavanagh, A. M.; Zuegg, J.; Blaskovich, M A. T.; Cooper, M. A. Contribution of amphipathicity and hydrophobicity to the antimicrobial activity and cytotoxicity of β-hairpin peptides. *Infect. Dis.,* **2016**, 2(6), 442-450.

[21] Fjell, C. D.; Jenssen, H. V.; Cheung, W. A.; Hancock, R. E. W.; Cherkasov, A. Identification of novel antibacterial peptides by chemoinformatics and machine learning. *J. Med. Chem.*, **2009**, 52(7), 2006–2015.

[22] Maccari, G.; Di Luca, M.; Nifosí, R.; Cardarelli, F.; Signore, G.; Boccardi, C.; Bifone, A. Antimicrobial Peptides Design by Evolutionary Multiobjective Optimization. *PLoS Comput. Biol.* **2013**, 9(9), e1003212–e1003223.

[23] Juretić, D.; Vukicević, D.; , Ilić, N.; Antcheva, N.; Tossi, A. Computational design of highly selective antimicrobial peptides. *J. Chem. Inf. Model.*, **2009**, 49(12), 2873–2882.

[24] Thomas, S.; Karnik, S.; Barai, R. S.; Jayaraman, V. K.; Idicula-Thomas S. CAMP: a useful resource for research on antimicrobial peptides. *Nucleic Acids Res*. **2010**, 38, D774-80.

[25] Ye, J.; McGinnis, S.; Madden, T. L. BLAST: improvements for better sequence analysis. *Nucleic Acids Res*. **2006**, 34, W6-9.

[26] Camacho, C.; Coulouris, G.; Avagyan, V.; Ma, N.; Papadopoulos, J.; Bealer, K.; Madden, T. L. BLAST+: architecture and applications. *BMC Bioinformatics*, **2009,** 10, 421-429.

[27] Thompson, J. D.; Higgins, D. G.; Gibson, T. J. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res*., **1994**, 22(22), 4673-4680.

[28] Chenna, R.; Sugawara, H.; Koike, T, Lopez R, Gibson TJ, Higgins DG, Thompson J. D. Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Res*. **2003**, 31(13), 3497-3500.

[29] Larkin, M. A.; Blackshields, G.; Brown, N. P.; Chenna, R.; McGettigan, P. A.; McWilliam, H.; Valentin, F.; Wallace, I. M.; Wilm, A.; Lopez, R.; Thompson, J. D.; Gibson, T. J.; Higgins, D. G. Clustal W and Clustal X version 2.0. *Bioinformatics*, **2007**, 23(21), 2947-2948.

[30] Merrifield, B. R. B. Solid phase peptide synthesis. *Adv. Enzymol. Realt. Areas Mol. Biol*., **1969,** 32, 221-296.

[31] Guzman, F.; Barberis, S.; Illanes, A. Peptide synthesis: chemical or enzymatic. *Electron J. Biotechnol*., **2007**, 10(2), 279-314.

[32] Pandeya, S. N.; Thakkar, D.. Combinatorial chemistry: a novel method in drug discovery and its application. *Indian J. Chem. - Sect B Org Med Chem*. **2005**, 44B, 335-348.

[33] Houghten, R. A. General method for the rapid solid-phase synthesis of large numbers of peptides: specificity of antigen-antibody interaction at the level of individual amino acids. *Proc. Natl. Acad. Sci. U S A*. **1985**, 82(15), 5131-5135.

[34] Krchnák, V.; Vágner, J.; Lebl, M. Noninvasive continuous monitoring of solid-phase peptide synthesis by acid-base indicator. *Int. J. Pept. Protein Rex*. **1988**, 32(5), 415-416.

[35] Fields, G. B.; Noble, R. L. Solid phase peptide synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids. *Int. J. Pept. Protein Res*. **1990**, 35(3), 161-214.

[36] Alvarez, C. A.; Guzmán, F.; Cárdenas, C.; Marshall, S. H.; Mercado, L. Antimicrobial activity of trout hepcidin. *Fish Shellfish Immunol*., **2014**, 41(1), 93-101.

[37] Jofré, C.; Guzmán, F.; Cárdenas, C.; Albericio, F.; Marshall, S. H. A natural peptide and its variants derived from the processing of infectious pancreatic necrosis virus (IPNV) displaying enhanced antimicrobial activity: a novel alternative for the control of bacterial diseases. *Peptides*, **2011**, 32(5), 852-858.

[38] Santoveña, A.; Alexis, O.; Guzmán, F.;Patarroyo M. E.; Llabrés, M.; Fariña, J. B. Chromatographic characterization of synthetic peptides: SPf66 malaria vaccine. *J Chromatogr B*. **2001**, 766(1), 3-12.

[39] Schaiberger, A. M.; Moos, J. A. Optimized sample preparation for MALDI mass spectrometry analysis of protected synthetic peptides. *J. Am. Soc. Mass Spectrom*. **2008**, 19(4), 614-619.

[40] Liu, B.; Huang, H.; Yang, Z.; Liu, B.; Gou, S.; Zhong, C.; Han, X.; Zhang, Y.; Ni, J.; Wang, R. Design of novel antimicrobial peptide dimer analogues with enhanced antimicrobial activity in vitro and in vivo by intermolecular triazole bridge strategy. *Peptides*, **2016**, 88, 115-125.

[41] Fernández-Reyes, M.; Díaz, D.; De La Torre, B. G.; Cabrales-Rico, A.; Vallès Miret, M.; Jiménez-Barbero, J.; Andreu, D.; Rivas L. Lysine N(epsilon)-trimethylation, a tool for improving the selectivity of antimicrobial peptides. *J . Med. Chem*., **2010**, 53(15), 5587-5596.

[42] Sang, M.; Zhang, J.; Zhuge, Q. Selective citotoxicity of the antibacteriL peptide ABP-dHC-Cecropin A and its analog towards lukemis cells. *Eur. J. Pharmacol*., **2017,** 803, 138-147.

[43] Kim, M. S.; Yeon, J. H.; Park, J. K. A microfluidic platform for 3-dimensional cell culture and cell-based assays. *Biomed. Microdevices*, **2007**, 9(1), 25-34.

[44] Kosikowska, P.; Lesner, A. Antimicrobial peptides (AMPs) as drug candidates: a patent review (2003-2015). *Expert Opin. Ther. Pat*., **2016**, 26(6), 689-702.

[45] Brogden, K. A. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria?. *Nat. Rev. Microbiol*., **2005**, 3(3), 238-250.

[46] Craik, C. S.; Largman, C.; Fletcher, T.; Roczniak, S.; Barr, P. J.; Fletterick, R.; Rutter, W. J. Redesigning trypsin: alteration of substrate specificity. *Science,* **1985**, 228(4697), 291-297.

[47] Lee, S. H., Kim, S. J.; Lee, Y. S.; Song, M. D.; Kim I. H.; Won, H. S. De novo generation of short antimicrobial peptides with simple amino acid composition. *Regul. Pept*., **2011,** 166(1-3), 36-41.

[48] Rieg, S.; Huth, A.; Kalbacher, H.; Kern, W. V. Resistance against antimicrobial peptides is independent of *Escherichia coli* AcrAB, *Pseudomonas aeruginosa* MexAB and *Staphylococcus aureus* NorA efflux pumps. *Int. J. Antimicrob. Agents*. **2009**, 33(2), 174-176.

[49] Oh, D.; Sun, J.; Nasrolahi Shirazi, A.; LaPlante, K. L.; Rowley, D. C.; Parang, K. Antibacterial activities of amphiphilic cyclic cell-penetrating peptides against multidrug-resistant pathogens. *Mol. Pharm*., **2014**, 11(10), 3528-3536.

[50] Shai, Y. Mode of action of membrane active antimicrobial peptides *Biopolymers,* **2002**, 66(4), 236-248.

[51] Joo H. S.; Fu, C. I.; Otto, M. Bacterial strategies of resistance to antimicrobial peptides. *Philos Trans R. Soc. Lond B. Biol. Sci.*, **2016**, 371(1695), pii: 20150292.

[52] Chan, D. I.; Prenner, E. J.; Vogel H. J. Tryptophan- and arginine-rich antimicrobial peptides: structures and mechanisms of action. *Biochim. Biophys. Acta*, **2006**, 1758(9), 1184-1202

[53] Schibli, D. J.; Epand, R. F.; Vogel, H. J.; Epand, R. M. Tryptophan-rich antimicrobial peptides: comparative properties and membrane interactions. *Biochem. Cell Biol*., **2002**, 80(5), 667-677.

[54] Epand, R. M.; Vogel, H. J. Diversity of antimicrobial peptides and their mechanisms of action. *Biochim. Biophys. Acta*, **1999**, 1462(1-2),11-28.

[55] Torrent, M.; Andreu, D.; Nogués, V. M.; Boix, E. Connecting peptide physicochemical and antimicrobial properties by a rational prediction model. *PLoS One*, **2011,** 6(2),e16968.

[56] Moore, A. J.; Beazley, W. D.; Bibby, M. C.; Devine, D. A. Antimicrobial activity of cecropins. *J. Antimicrob. Chemoth.*, **1996**; 37(6), 1077-1089.

[57] Park, C. H.; Valore, E. V.; Waring, A. J.; Ganz, T. Hepcidin, a urinary antimicrobial peptide synthesized in the liver. *J. Biol. Chem*., **2001**, 276(11), 7806-7810.

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