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RESEARCH ARTICLE

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Cationic amphipathic peptide analogs of cathelicidin LL-37 as a probe in the development of antimicrobial/anticancer agents

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Cathelicidin LL-37 belongs to the class of human defense peptides and is overexpressed in many cancers. Segments of LL-37 derived through biochemical processes have a wide range of activities. In this study, novel analogs of the 13-amino acid cathelicidin 17-29 amide segment $F^{17}KRIV^{21}QR^{23}IK^{25}DF^{27}LR\text{-}NH_2$ were prepared and examined for their antimicrobial and hemolytic activities, as well as for their cytotoxicity on cancer bronchial epithelial cells. Selected substitutions were performed on residues R^{23} and K^{25} in the hydrophilic side, $V^{21}$and F^{27} in the hydrophobic side of the interphase, and F^{17} that interacts with cell membranes. Specific motifs IIKK and LLKKL with anticancer and antimicrobial activities isolated from animals were also inserted into the 17-29 fragment to investigate how they affect activity. Substitution of the amino-terminal positive charge by acetylation and replacement of lysine by the aliphatic leucine in the peptide analog $Ac\text{-}FKRIVQRIL^{25}DFLR\text{-}NH_2$ resulted in significant cytotoxicity against A549 cancer cells with an IC_{50} value 3.90 μg/mL, with no cytotoxicity to human erythrocytes. The peptide $Ac\text{-}FKRIVQI^{23}IKK^{26}FLR\text{-}NH_2$, which incorporates the IIKK motif and the peptides $FKRIVQL^{23}L^{24}KK^{26}L^{27}LR\text{-}NH_2$ and $Ac\text{-}FKRIVQL^{23}L^{24}KK^{26}L^{27}LR\text{-}NH_2$, which incorporate the LLKKL motif, displayed potent antimicrobial activity against gram-negative bacteria (MIC 3–7.5 μg/mL) and substantial cytotoxicity against bronchial epithelial cancer cells, (IC_{50} 12.9–9.8 μg/mL), with no cytotoxic activity for human erythrocytes. The helical conformation of the synthetic peptides was confirmed by circular dichroism. Our study shows that appropriate substitutions, mainly in positions of the interphase, as well as the insertion of the motifs IIKK and LLKKL in the cathelicidin 17-29 segment, may lead to the preparation of effective biological compounds.

KEYWORDS

amphipathic α-helical peptides, antimicrobial activity, cathelicidin LL-37 analogs, cytotoxicity, hemolytic assay

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1 | INTRODUCTION

According to the Schiffer-Edmunson projection, amphipathic helical cationic peptides isolated over the last few decades from a variety of plant and animal organisms are characterized as natural antimicrobials.[1,2] They represent the first line of defense for organisms and target a wide variety of microbes that include bacteria, viruses, parasites, and fungi. Antimicrobial peptides that also inactivate a wide range of cancer cell types and act as anticancer agents have been isolated. The inappropriate and irrational use of antibiotics has induced the worldwide emergence and spreading of resistant microorganisms. As well, radiation and chemotherapy are conventional treatments for cancer, which is one of the main causes of disease-related death worldwide. These treatments suffer from low performance and serious side effects.[3–6] The development of new antimicrobial/anticancer therapeutics is of urgent priority. Amphipathic peptides or their analogs may be a potential alternative.

Many relatively small-size peptides have a number of advantages. These include favorable pharmacokinetic profiles and bioavailability, good solubility, lessened potential for development of tolerance because of the short interaction time with cells, low toxicity, and selective recognition by microbial and cancer cells.[7] Cationic antimicrobial amphipathic peptides rupture the cell membrane through membrane-disrupting mechanisms (carpet, barrel-stave, and toroidal models), do not act via receptors, and decrease tolerance. Antimicrobial peptides can be divided into those that cause cell death by direct cell lysis and those that penetrate the cell membranes and kill the cell without lysis by interacting with critical intracellular targets. Interestingly, peptides that are active in microbial and cancer cells but inactive in normal cells have also been reported.[8] Peptides that contribute to the defense of an organism are expected to show strong toxicity and selectivity to cancer cells compared with normal cells.

The detailed mechanisms of action of anticancer peptides are not fully understood, but it is accepted that in most cases, they cause necrosis and/or apoptosis as they affect the cell or the mitochondria.[3,9–14] The net negative charge of cancer and microbial cells, due to the presence of anionic compounds in the cell membrane, such as phospholipids, phosphatidylserine, glycosyl, and sialyl compounds, is an important difference from the zwitterion-neutral form of normal mammalian cells. Other important aspect is cholesterol, which modulates membrane fluidity and facilitates the destabilization of cancer cells by anticancer peptides.[15–22]

Cathelicidin is the 18 kDa precursor human protein of the active 37 amino acid form, LL-37, that results following proteolysis. LL-37 belongs to the class of human defense peptides and is secreted from bone marrow, leukocytes, epithelial tissues, and the gastrointestinal system. LL-37 is overexpressed in many cancers, including ovarian, breast, and lung cancer.[23–28] Segments of LL-37 derived from biochemical processes have diverse actions that include antiseptic, antibacterial, antimicrobial, apoptotic, metastatic, and immunological activities.[29–34]

Two- and three-dimensional nuclear magnetic resonance spectroscopy conducted in a membrane-like environment comprised of dodecylphosphocholine and sodium dodecyl sulfate (SDS) has revealed that aromatic residues F^{5}, F^{6}, F^{17}, and F^{27} on the hydrophobic nonpolar side of the helical 2-31segment of LL-37 are important sites for the interaction of the protein with cell membranes. The same is also true for residues R^{7}, R^{19}, R^{23}, R^{29}, and R^{34} on the polar hydrophilic side of the helix.[23]

With the aim of further contributing to the development of new effective antimicrobial/anticancer compounds, we report the design, synthesis, and study of cationic amphipathic peptide analogs of the cathelicidin 17-29 segment. Selected substitutions were made on residues R^{23}, K^{25}, V^{21}, and F^{27} of both sides of the interface, as well as on residue F^{17}, which is related to cell membrane interactions.[8,23] Sequences containing two consecutive lysines and the hydrophobic residues of leucine and isoleucine (e.g., IIKK, IIKKI, IKK, and LLKKL) with anticancer and antimicrobial activities have been isolated from animals.[4,5] The IIKK and LLKKL motifs were inserted into the 17-29 segment and studied.[8,35–37] Peptide-based factors as polarity/hydrophobicity, aromaticity, total charge, and side chain stereochemical volume are discussed in relation to their biological properties.

The anticancer activity of the synthesized peptide analogs was investigated by measuring the cytotoxicity of the peptides on the cancer obronchial epithelial cell line A549, using the (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) photometric assay, and concentration that causes 50% inhibition (IC_{50}) was determined.[8] The antimicrobial properties of the synthesized peptides were studied in gram-negative and gram-positive bacteria, the minimum inhibitory concentration (MIC) was estimated, and the concentration of the peptide that causes lysis of 50% of erythrocytes (EC_{50}) was established.[38–40]

The data concerning the correlation of the anticancer and antimicrobial properties of the peptide analogs will help to understand the prerequisites for the development of effective pharmaceuticals.

2 | MATERIALS AND METHODS

2.1 | Peptide synthesis

The synthesis of the peptides was performed by the stepwise solid-phase synthesis procedure on a Rink-Amide (AM) resin (0.37 mmol/g resin) using the 9-fluorenylmethyloxycarbonyl (Fmoc) methodology. Arginine was used as Fmoc-Arg (Pbf)-OH (Pbf: 2,2,4,6,7-pentamethyl-dihydrobenzofurane-5-sulfonyl), lysine as Fmoc-Lys (Boc)-OH (Boc: tert-butyloxycarbonyl), aspartic acid as Fmoc-Asp (OtBu)-OH (OtBu: Otert-butyloxy), and glutamine as Fmoc-Gln (Trt)-OH. Fmoc-groups were removed using 20% piperidine in dimethylformamide. The coupling reactions were performed using an Fmoc–amino acid/HBTU/HOBt/DIEA/resin with a molar ratio of 3/3/3/6/1 (HBTU: O-benzotriazol-1-yl-N,N,N′,N′ tetramethyluronium hexafluorophosphate, HOBt: hydroxybenzo-1.2,3-triazole, and DIEA: N, N-diisopropylethylamine). Completion of coupling reactions was ensured by the use of the ninhydrin-

based Kaiser test. The peptides were cleaved from the resin by treatment with trifluoroacetic acid/triisopropylsilane/water (TFA/TIS/H_2O, 95/2.5/2.5). The resin was removed by filtration, the filtrate was evaporated under reduced pressure, and the product was precipitated with cold diethyl ether. The crude products were purified by semipreparative reverse phase-high performance liquid chromatography (RP-HPLC) using a C18 column. Appropriate programs were applied using eluent A (H_2O/0.1% TFA) and B (acetonitrile [CH_3CN]/0.1% TFA). Yields ranged from 40% to 55%. The purity of the peptides was checked by analytical HPLC, and the correct molecular masses were confirmed by electrospray ionization-mass spectrometry (ESI-MS) (Table 1).

TABLE 1 Parameters and characterization of the synthesized peptides

Peptides	RP-HPLC gradient elution[a]	t_R (min)[b]	ESI-MS	Net charge
1 F^{17}KRIV^{21}QR^{23}I^{24}K^{25}D^{26}F^{27}LR-NH_2	A:B 90:10	12.5	Calculated $[M + 3H]^{3+}$: 573.7	+5
	A:B 30:70		Found $[M + 3H]^{3+}$: 573.96	
2 L^{17}KRIVQRIKDFLR-NH_2	A:B 90:10	12.5	Calculated $[M + 3H]^{3+}$: 562.36	+5
	A:B 30:70		Found $[M + 3H]^{3+}$: 562.68	
3 A^{17}KRIVQRIKDFLR-NH_2	A:B 90:10	13.0	Calculated $[M + 3H]^{3+}$: 548.33	+5
	A:B 30:70		Found $[M + 3H]^{3+}$: 548.97	
4 Ac-FκRIVQRIL^{25}DFLR-NH_2	A:B 80:20	17.0	Calculated $[M + 3H]^{3+}$: 582.7	+3
	A:B 20:80		Found $[M + 3H]^{3+}$: 581.61	
5 Ac-FK (SA)RIVQRIL^{25}DFLR-NH_2	A:B 90:10	17.0	Calculated $[M + 3H]^{3+}$: 704.14	+2
	A:B 0:100		Found $[M + 3H]^{3+}$: 703.36	
6 FKRIVQRIR^{25}DFLR-NH_2	A:B 90:10	13.5	Calculated $[M + 3H]^{3+}$: 583.00	+5
	A:B 30:70		Found $[M + 3H]^{3+}$: 582.04	
7 Ac-FKRIVQRIR^{25}DFLR-NH_2	A:B 90:10	17.5	Calculated $[M + 3H]^{3+}$: 59 5.05	+4
	A:B 30:70		Found $[M + 3H]^{3+}$: 595.94	
8 FKRIVQκ^{23}IKDFLR-NH_2	A:B 90:10	12.5	Calculated $[M + 3H]^{3+}$: 564.37	+5
	A:B 30:70		Found $[M + 3H]^{3+}$: 563.26	
9 FKRIVQL^{23}IKDFLR-NH_2	A:B 90:10	10.5	Calculated $[M + 3H]^{3+}$: 559.03	+4
	A:B 30:70		Found $[M + 3H]^{3+}$: 558.12	
10 Ac-FKRIVQL^{23}IKDFLR-NH_2	A:B 90:10	14.5	Calculated $[M + 3H]^{3+}$: 573.25	+3
	A:B 30:70		Found $[M + 3H]^{3+}$: 572,21	
11 FKRIVQRIKDL^{27}LR-NH_2	A:B 90:10	10.2	Calculated $[M + 3H]^{3+}$: 562.37	+5
	A:B 30:70		Found $[M + 3H]^{3+}$: 561.09	
12 Ac-FKRIVQRIKDL^{27}LR-NH_2	A:B 90:10	13.8	Calculated $[M + 3H]^{3+}$: 576.38	+4
	A:B 30:70		Found $[M + 3H]^{3+}$: 575.10	
13 FKRIVQI^{23}IKK^{26}FLR-NH_2	A:B 90:10	17.5	Calculated $[M + 2H]^{2+}$: 845.60	+5
	A:B 30:70		Found $[M + 2H]^{2+}$: 843.69	
14 Ac-FKRIVQI^{23}IKK^{26}FLR-NH_2	A:B 90:10	22.5	Calculated $[M + 2H]^{2+}$: 866.62	+4
	A:B 30:70		Found $[M + 2H]^{2+}$: 864.72	
15 FKRIVQL^{23}L^{24}KK^{26}L^{27}LR-NH_2	A:B 90:10	14.5	Calculated $[M + 3H]^{3+}$: 552.34	+5
	A:B 30:70		Found $[M + 3H]^{3+}$: 551.10	
16 Ac-FKRIVQL^{23}L^{24}KK^{26}L^{27}LR-NH_2	A:B 90:10	18.0	Calculated $[M + 3H]^{3+}$: 566.40	+4
	A:B 30:70		Found $[M + 3H]^{3+}$: 565.12	
17 FKRIL^{21}QRIKDFLR-NH_2	A:B 90:10	15.5	Calculated $[M + 3H]^{3+}$: 578.05	+5
	A:B 30:70		Found $[M + 3H]^{3+}$: 577.09	
18 Ac-FKRIL^{21}QRIKDFLR-NH_2	A:B 90:10	20.5	Calculated $[M + 2H]^{2+}$: 887.59	+4
	A:B 30:70		Found $[M + 2H]^{2+}$: 886.79	

Abbreviations: ESI-MS, electrospray ionization-mass spectrometry; RP-HPLC, reverse phase-high performance liquid chromatography.
[a]A: H_2O/0.1%TFA, B: CH_3CN/0.1%TFA.
[b]t_R: retention time; $_{SA}$: sialic acid.

2.2 | Circular dichroism

Circular dichroism (CD) spectra were recorded from 260 to 180 nm at a scan speed of 50 nm/min. The average of three scans was determined. The procedure was performed using a Jasco J-710 spectropolarimeter equipped with a 0.1-cm path-length quartz cell and peptide concentration 10^{-4} M at 25°C. Phosphate-buffered saline (PBS, p 7.4), trifluoroethanol (TFE) in PBS, and sodium dodecylsulfate (SDS) were used in all experiments. The spectra were smoothed after subtraction of the solvent contribution. The α-helical content was estimated on the basis of $[\theta]_{222}$ nm values ($[\theta]$ in deg.cm^2.dmol^{-1}) according to the Chen equation[41] as follows:

$$\%\alpha - \text{helical content} = [\theta]_{222}/[\theta]^{\infty}(1 - k/n),$$

where n is the number of residues of the peptide and $[\theta]_{222}$ the molar ellipticity at 222 nm. The estimated value $[\theta]^{\infty}$, which corresponds to a 100% helical content at 222 nm, is 39,500 and the constant k = 2.57.

2.3 | Antimicrobial activity

The peptides were tested for their antimicrobial activity against gram-negative bacteria (*Escherichia coli* DH5a, *Pseudomonas aeruginosa* PAO, and *Zymomonas mobilis* ATCC 10988), gram-positive bacteria (*Bacillus subtilis* DELTA and *Mycobacterium smegmatis* mc^2 155), and the fungus *Candida parapsilosis*. *E. coli* is a representative of potent pathogens, and *P. aeruginosa* PAO and *Z. mobilis* ATCC 10988 possess natural multidrug resistance. The selection of the gram-positive bacteria was based on differences of their membrane lipid components. Aliquots (100 μL) containing 2×10^6 logarithmic phase bacteria per milliliter obtained from microorganisms grown in the appropriate culture medium were dispensed into wells of 96-well microplates. Peptide stock solution was obtained by solubilizing the peptides in water followed by filter sterilization using a 0.22-μm pore size filter. After serial dilutions of the stock solution, 100 μL was added to each well to obtain a final concentration of 5 to 500 μg/mL. Control wells contained filter-sterilized water instead of peptide solution. The experiments were performed in triplicate. Inhibition of the growth was determined by measuring the absorbance at 620 nm after incubation for 24 h at the appropriate temperature for each microorganism. The antimicrobial activities were expressed as the minimum concentration at which 100% inhibition of growth was observed (the MIC).

2.4 | Hemolytic activity assay

The hemolytic activity of the peptides was monitored by determining hemoglobin release from a suspension of erythrocytes suspension as previously described.[42] In brief, fresh human red blood cells were washed three times in 10 mM Tris buffer containing 150 mM NaCl, pH 7.4. Cell suspensions containing 1.9×10^8 cells/mL were incubated with varying amounts of peptides solubilized in Tris buffer. Zero hemolysis and 100% hemolysis were determined by using Tris buffer and 0.5% NH_4OH, respectively. The experiments were performed in triplicate. Concentrations causing 50% hemolysis (EC_{50}) were derived from the dose-response curves.

2.5 | Cytotoxicity test

The possible anticancer activity of the peptide analogs of cathelicidin LL 17-29 was assessed using A549 cancer bronchial epithelial cells, with comparison against DLF lung fibroblasts as normal control cells. We investigated the cytotoxicity of the peptides for cancer and control cells using the MTT photometric viability assay as previously described.[43,44] The results were used to determine the IC_{50}. A549 or DLF cells (18,000 cells/well) were precultured in a sterile 96-well plate. After 24 h of incubation, cells were treated with 100 μL of peptide solutions (10-200 μg/mL) and incubated for a further 24 h. Then, 10 μL of MTT solution (5 mg/mL) was added to each well and incubated for 4 h. The supernatant was removed from each well and the formazan crystals that had formed in each well were dissolved in 100 μL dimethyl sulfoxide (DMSO). After 2 h, the absorbance intensity was measured at 570 nm using a microplate reader and compared with a standard curve to determine the number of viable cells in each sample. Wells without peptides were used as a positive control and wells without cells as a negative control (blank) of the assay. The experiments were carried out three times independently

3 | RESULTS AND DISCUSSION

3.1 | Design of the peptides

The design of the peptides was based on the Schiffer-Edmunson helical wheel projection of the cathelicidin 17-29 amide segment. The aim of the design was to preserve and improve the amphipathic character of the peptides Figure 1. Generally, the amides are more proteolytic

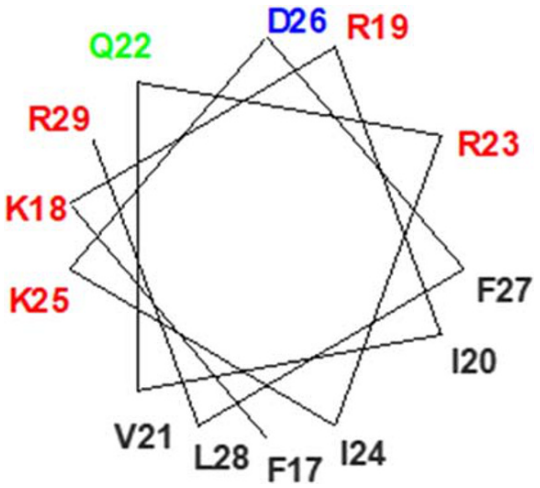

FIGURE 1 Schiffer-Edmundson α-helical wheel projection of the sequence 17-29 of LL-37, FKRIVQRIKDFLR-NH_2

stable and enhance the α-helical conformation inducing better activity. Selected substitutions were performed on residues R^{23}, K^{25}, V^{21}, and F^{27} of both sides of the interface, as well as on residue F^{17}. The specific motifs IIKK and LLKKL, which are found in animal peptides, were inserted into the 17-29 segment and their influence on antimicrobial and anticancer activities was evaluated.

Table 1 shows the sequence 17-29 amide of LL-37(1) and the synthesized analogs (2-18). The aromatic phenylalanine residue in position 17 of the hydrophobic side of the amphipathic helix was substituted by the aliphatic leucine and alanine residues in compounds 2 and 3, respectively. The positively charged lysine in position 25 of the interface was replaced by the aliphatic leucine residue, in analogs 4 and 5 and by arginine, another positively charged residue, in analogs 6 and 7. Arginine in position 23, which is also located in the interface, was substituted by lysine in analog 8 and by leucine in analogs 9 and 10. Leucine replaced phenylalanine in position 27 of the hydrophobic side in analogs 11 and 12. Leucine also replaced valine in position 21 of the hydrophobic side in analogs 17 and 18. The IIKK segment (23-26) substituted for aspartic acid 26 and arginine 23 of the hydrophilic face in analogs 13 and 14,while the LLKKL segment (23-27) substituted for arginine 23 and aspartic acid 26 of the hydrophilic face as well as F^{27} and I^{24} of the hydrophobic face in analogs 15 and 16. Finally, sialic acid, which is successfully incorporated in microbial pathogens where it assists in evasion of host immunity,[45] was inserted into the ε- amino group of lysine in position 18.

The location of the replaced residues within the amphipathic helix, their charges, and hydrophilic/hydrophobic properties, as well as their stereochemical volume, were investigated in relation to their biological activities.

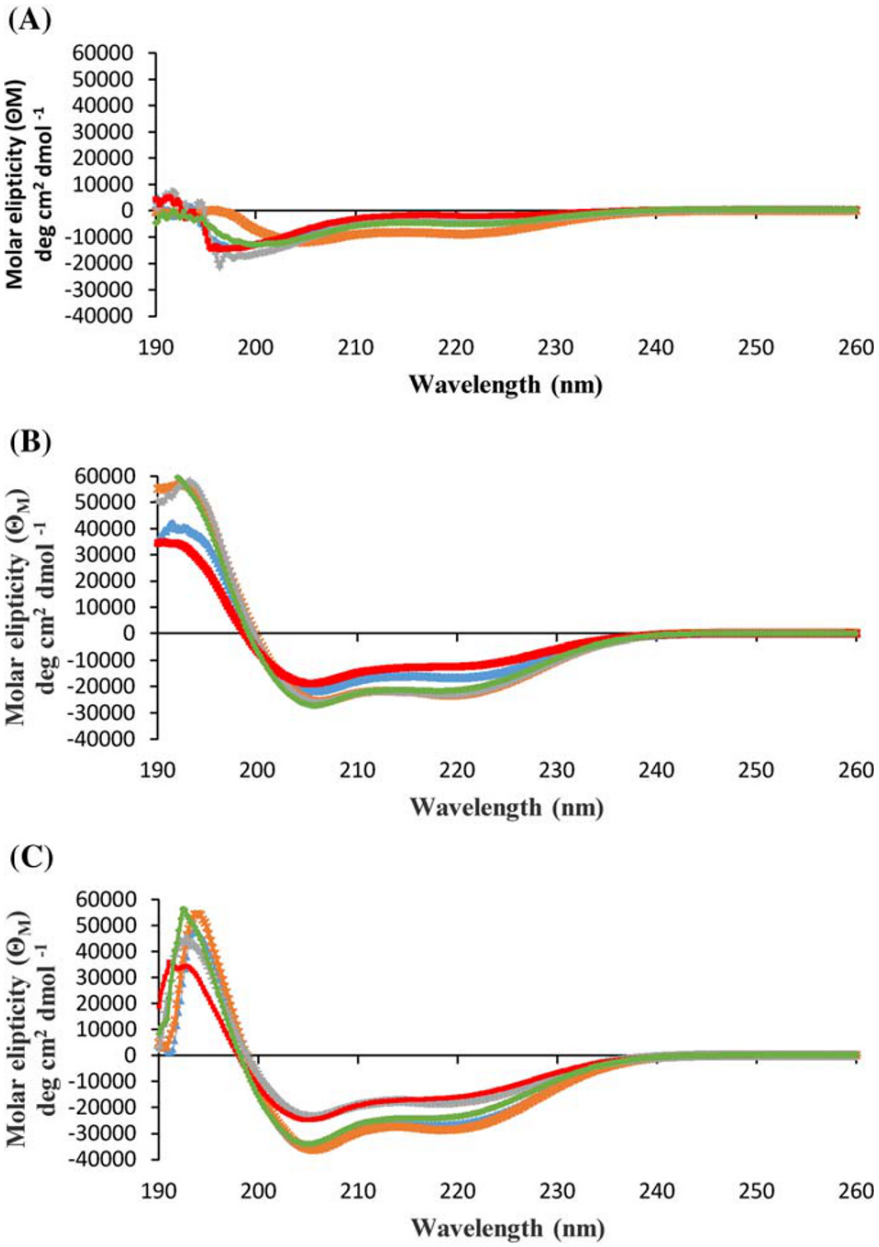

FIGURE 2 Circular dichroism (CD) spectra of the peptides 1(), 4(), 14(), 15(), and 16() (100 μM). A, Phosphate-buffered saline (PBS); B, sodium dodecyl sulfate (SDS) 8 mM; C, trifluoroethanol (TFE)/PBS (50/50, *v*/v)

3.2 | CD analysis

All the CD experiments of the peptides in this study were carried out in PBS (pH 7.4), TFE in PBS (50/50, *v*/v), and in the SDS membrane mimic at the critical micelle concentration of 8 mM[38–40] Figure 2. The percent helicity of the peptides was calculated on the basis of $[\theta]_{222}$ nm values (Table 2). The hydrophobic moment of the peptides was also calculated Table 2.[46,47] The native sequence 17-29 of LL-37 (peptide 1) indicated a percent helicity of 51% in SDS and 83% in TFE/PBS. All the synthesized analogs preserved or increased, in some cases, their alpha helical content indicating that the selected substitutions did not affect the overall helical conformation, which might be critical for their activity. The calculated hydrophobic moments of all free peptides were almost the same.

3.3 | Antimicrobial activity

The antimicrobial activity of the peptides was studied using gram-negative bacteria (*E. coli* DH5a, *P. aeruginosa* PAO, and *Z. mobilis* 10,988), gram-positive bacteria (*B. subtilis* DELTA and *M. smegmatis* mc^2 155), and the fungus *C. parapsilosis*. The MIC values of the peptides are shown in Table 3. Differences in the MIC values could be attributed to the different composition of the outer envelope of each microorganism. The native sequence 17-29 of LL-37 (peptide 1) showed weak antimicrobial activity, with an MIC of 200 μg/mL, for *E. coli* DH5a and *C. parapsilosis*, and an MIC of 50 μg/mL for *M. smegmatis* mc^2 155. Substitution of the aromatic phenylalanine in position 17 (which interacts with cell membranes) of the hydrophobic side of the amphipathic helix, by aliphatic residues leucine and alanine in peptides 2 and 3, respectively, did not substantially affect the activity of the natural sequence (peptide 1), indicating that phenylalanine may not interact with the cell membranes in this analog. Substitution of the positively charged lysine in position 25 of the interface by leucine and acetylation of the amino-terminal group increased the activity in peptide 4. Analog 5, which incorporates sialic acid into the ε-amino group of lysine in position 18, displayed reactivity only against *B. subtilis* DELTA, although the native sequence (peptide 1) was inactive to this microorganism. Substitution of lysine 25 by arginine, another positively charged residue, did not substantially change the reactivity in analog 6. The same finding was also found for the acetylated N-terminus peptide 7.

Replacement of arginine in position 23, which is also located in the interface, by lysine in peptide 8 did not enhance the activity of the peptide compared with the peptide 1. Arginine in position 23 was also substituted by leucine in peptides 9 and 10. This substitution increased the activity against *E. coli* DH5a (MIC 30 μg/mL and 100 μg/mL for peptide 9 and 10, respectively), whereas these peptides showed low reactivity (MIC 20 and 100 μg/mL, same respective order) against *P. aeruginosa* PAO. On the contrary, peptides 9 and 10 were not effective against the tested gram-positive bacteria and the fungus. These findings highlighted the role of position 23 in the interphase. Replacement of the aromatic phenylalanine in position 27 of the hydrophobic side by leucine produced comparable reactivities against all tested bacteria and fungus, for peptides 11 and 12.

Insertion of the IIKK motif (23-26) in peptides 13 and 14 and the LLKKL motif (23-27) in peptides 15 and 16 found in many defense proteins improved the antimicrobial activity. The substitutions of R^{23}

TABLE 2 Percent helicity on the basis of $[\theta]_{222}$ nm values and hydrophobic moment μH of the peptides

	Peptides	μH	PBS	SDS (8mM)	TFE/PBS (50/50 v/v)
1	FKRIVQRIKDFLR-NH_2	8.846	5.8	50.7	83.1
2	L^{17}KRIVQRIKDFLR-NH_2	0.840	7.6	51.5	90.1
3	A^{17}KRIVQRIKDFLR-NH_2	0.743	8.0	58.3	46.6
4	Ac-FKRIVQRIL^{25}DFLR-NH_2	-	25.8	70.6	88.6
5	Ac-FK (SA)RIVQRIL^{25}DFLR-NH_2	-	46.1	68.4	58.4
6	FKRIVQRIR^{25}DFLR-NH_2	0.847	8.4	87.0	55.8
7	Ac-FKRIVQRIR^{25}DFLR-NH_2	-	9.2	70.9	57.4
8	FKRIVQK^{23}IKDFLR-NH_2	0.846	6.0	53.0	62.0
9	FKRIVQL^{23}IKDFLR-NH_2	0.847	15.0	81.6	60.5
10	Ac-FKRIVQL^{23}IKDFLR-NH_2	-	9.8	79.3	52.0
11	FKRIVQRIKDL^{27}LR-NH_2	0.843	12.1	55.6	44.6
12	Ac-FKRIVQRIKDL^{27}LR-NH_2	-	7.2	77.4	76.0
13	FKRIVQI^{23}IKK^{26}FLR-NH_2	0.863	7.7	51.9	66.4
14	Ac-FKRIVQI^{23}IKK^{26}FLR-NH_2	-	9.9	70.6	56.5
15	FKRIVQL^{23}L^{24}KK^{26}L^{27}LR-NH_2	0.848	5.2	39.7	53.4
16	Ac-FKRIVQL^{23}L^{24}KK^{26}L27LR-NH_2	-	14.6	67.8	76.5
17	FKRIL^{21}QRIKDFLR-NH_2	0.864	2.8	44.7	71.6
18	Ac-FKRIL^{21}QRIKDFLR-NH_2	-	6.3	89.8	99.5

Abbreviations: PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; TFE, trifluoroethanol.

TABLE 3 Minimum inhibitory concentration (MIC, μg/mL) values of the peptides

Peptides	Gram (–) bacteria			Gram (+) bacteria		Fungus
	Escherichia coli DH5a	*Pseudomonas aeruginosa* PAO	*Zymomonas mobilis* 10,988	*Bacillus subtilis* DELTA	*Mycobacterium smegmatis* mc^2 155	*Candida parapsilosis*
1 $F^{17}KRIV^{21}QR^{23}I^{24}K^{25}D^{26}F^{27}LR\text{-}NH_2$	200	-	-	-	50	200
2 $L^{17}KRIVQRIKDFLR\text{-}NH_2$	200	-	200	-	200	-
3 $A^{17}KRIVQRIKDFLR\text{-}NH_2$	-	-	-	-	200	-
4 Ac-FκRIVQRIL^{25}DFLR-NH_2	100	-	-	100	-	-
5 Ac-FK (SA)RIVQRIL^{25}DFLR-NH_2	-	-	-	200	-	-
6 FKRIVQRIR^{25}DFLR-NH_2	100	-	-	50	20	200
7 Ac-FKRIVQRIR^{25}DFLR-NH_2	100	-	200	40	30	-
8 FKRIVQκ^{23}IKDFLR-NH_2	-	-	200	-	50	-
9 FKRIVQL^{23}IKDFLR-NH_2	30	20	-	50	200	100
10 Ac-FKRIVQL^{23}IKDFLR-NH_2	100	100	100	20	200	100
11 FKRIVQRIKDL^{27}LR-NH_2	200	200	200	200	200	200
12 Ac-FKRIVQRIKDL^{27}LR-NH_2	200	40	40	200	200	200
13 FKRIVQI^{23}IKK^{26}FLR-NH_2	100	3	5	20	20	20
14 Ac-FKRIVQI^{23}IKK^{26}FLR-NH_2	50	3	5	20	100	30
15 FKRIVQL^{23}L^{24}KK^{26}L^{27}LR-NH_2	200	3	7.5	30	30	30
16 Ac-FKRIVQL^{23}L^{24}KK^{26}L^{27}LR-NH_2	100	3	5	10	50	40
17 FKRIL^{21}QRIKDFLR-NH_2	100	30	100	100	40	100
18 Ac-FKRIL^{21}QRIKDFLR-NH_2	100	20	50	100	20	40

Note. (–) not active.

by I and D^{26} by K (peptides 13 and 14) significantly increased the activity of peptide 13 against *P. aeruginosa* PAO (MIC 3 μg/mL) and *Z. mobilis* 10,988 (MIC 5 μg/mL). Peptide 14 also was effective against *P. aeruginosa* PAO (MIC 3 μg/mL) and *Z. mobilis* 10,988 (MIC 7.5 μg/mL).

The substitutions of R^{23} and I^{24} by L, D^{26} by K ,and F^{27} by L in the peptides 15 and 16 also significantly increased the activity against *P. aeruginosa* PAO (MIC 3 μg/mL in both peptides) and *Z. mobilis* 10,988 (MIC 7.5 and 5 μg/mL for peptides 15 and 16, respectively), and against *B. subtilis* DELTA (MIC 10 μg/mL for peptide 16).

Replacement of valine by leucine in position 21 of the hydrophobic side in peptide analogs 17 and 18 increased the activity of peptide 18 against *P. aeruginosa* PAO (MIC 20 μg/mL) and *M. smegmatis* mc^2 155 (MIC 20 μg/mL).

The overall data of Table 3 demonstrated that the incorporation of the IIKK and LLKKL motifs into the native sequence 17-29 of the cathelicidin LL-37 significantly improved the antimicrobial activity against most of the tested microorganisms. Substitution of the positively charged arginine in position 23 and lysine in position 25, which are located in the interface, by aliphatic residues (leucine or isoleucine) increased the activity against almost all the tested bacteria. Replacement of phenylalanine, an aromatic residue, in positions 17 and 27 of the hydrophobic side of the amphipathic helix by aliphatic residues leucine and alanine did not enhance the activity of peptide 1.

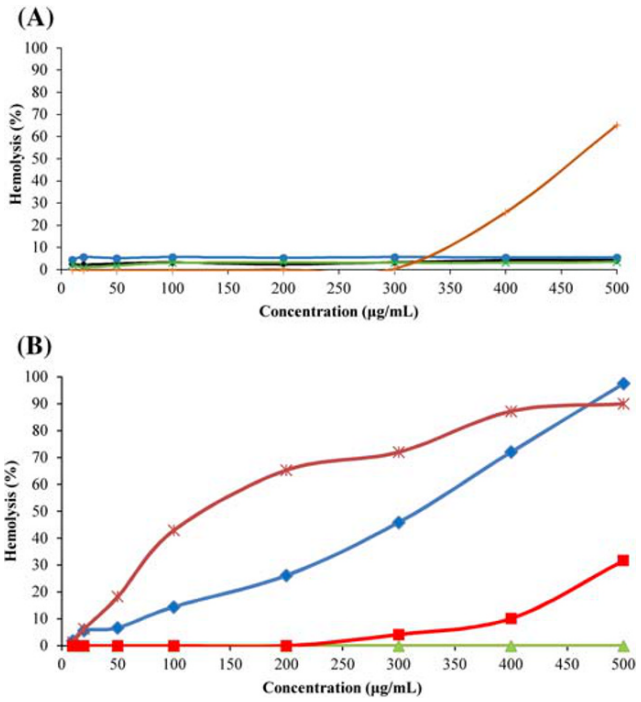

FIGURE 3 Hemolytic activity of peptides versus concentration (μg/mL), A: 1(—×—), 2(—●—), 3(—◆—), and 4(—+—); B: 6(—▲—), 7(—■—), 9(—◆—), and 10(—✱—). The experiments were performed in triplicate

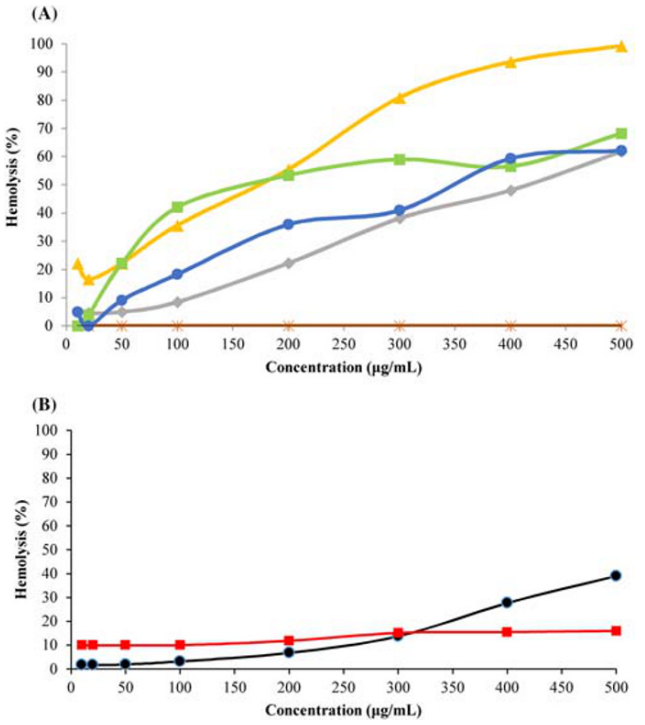

FIGURE 4 Hemolytic activity of peptides versus concentration (μg/mL), A: 12(—✱—), 13(—▲—), 14(—▲—), 15(—●—), and 16(—■—); B: 17(—●—) and 18(—■—). The experiments were performed in triplicate

TABLE 4 Hemolytic activity of the peptides

Peptides	EC_{50}, μg/mL
1 $F^{17}KRIV^{21}QR^{23}I^{24}K^{25}D^{26}F^{27}LR\text{-}NH_2$	-
2 $L^{17}KRIVQRIKDFLR\text{-}NH_2$	-
3 $A^{17}KRIVQRIKDFLR\text{-}NH_2$	-
4 Ac-FκRIVQRIL^{25}DFLR-NH_2	450
5 Ac-FK (SA)RIVQRIL^{25}DFLR-NH_2	-
6 FKRIVQRIR^{25}DFLR-NH_2	-
7 Ac-FKRIVQRIR^{25}DFLR-NH_2	-
8 FKRIVQκ^{23}IKDFLR-NH_2	-
9 FKRIVQL^{23}IKDFLR-NH_2	114
10 Ac-FKRIVQL^{23}IKDFLR-NH_2	227
11 FKRIVQRIKDL^{27}LR-NH_2	-
12 Ac-FKRIVQRIKDL^{27}LR-NH_2	-
13 FKRIVQI^{23}IKK^{26}FLR-NH_2	361
14.Ac-FKRIVQI^{23}IKK^{26}FLR-NH_2	292
15 FKRIVQL^{23}L^{24}KK^{26}L^{27}LR-NH_2	411
16 Ac-FKRIVQL^{23}L^{24}KK^{26}L^{27}LR-NH_2	185
17 FKRIL^{21}QRIKDFLR-NH_2	-
18 Ac-FKRIL^{21}QRIKDFLR-NH_2	-

Note. (–) not active.

3.4 | Hemolytic assay

The cathelicidin 17-29 segment and all its analogs were also tested for their toxicity against human erythrocytes. Peptides 1-3, 5-8, 11, 12, 17, and 18 did not exhibit any toxicity against erythrocytes, while peptides 4, 9, 10, and 13-16 displayed low toxicity at concentrations much higher than their MIC values Figures 3 and 4 and Table 4. The lack of hemolytic activity can be attributed to the different lipid composition of the bacteria and erythrocyte membranes. The cationic AMPs interact with the anionic surface of bacterial membranes, which lead to the lysis of the bacteria, whereas they cannot interact with the neutral surface of the erythrocytes.

3.5 | Cytotoxicity test

The cytotoxicity of the peptides against A549 bronchial epithelial cancer cells and the control DLF normal lung fibroblasts was investigated using the MTT photometric assay. The IC_{50} values of the peptides are shown in Table 5.

The native sequence 17-29 of LL-37 (peptide 1) was cytotoxic to A549 cells, with an IC_{50} of 20.13 μg/mL Figure 5. Substitution of phenylalanine in position 17 by the aliphatic residues leucine and alanine in compounds 2 and 3, respectively, and replacement of phenylalanine in position 27 of the interphase by leucine in peptides 11 and 12 did not enhance their cytotoxicity compared to peptide 1. Substitution of the positively charged lysine in position 25 of the interface by leucine in analogs 4 and 5 increased the cytotoxicity, as evidence by the IC_{50} values of 3.90 and 10.70 μg/mL, respectively. The incorporation of SA into the ε-amino group of lysine in position 18 (analog 5) improved the cytotoxicity compared with peptide 1. Substitution of lysine in position 25 by arginine in analog 6 did not enhance the cytotoxicity, while acetylation of analog 6 increased the cytotoxicity of analog 7 (IC_{50} 12.46 μg/mL). Replacement of arginine in position 23 by lysine in analog 8 did not enhance the cytotoxicity. Arginine in position 23 was also substituted by leucine in analogs 9 and 10. This substitution did not enhance the cytotoxicity of analog 9, but enhanced the cytotoxicity of the acetylated analog 10 (IC_{50} 13.92 μg/mL).

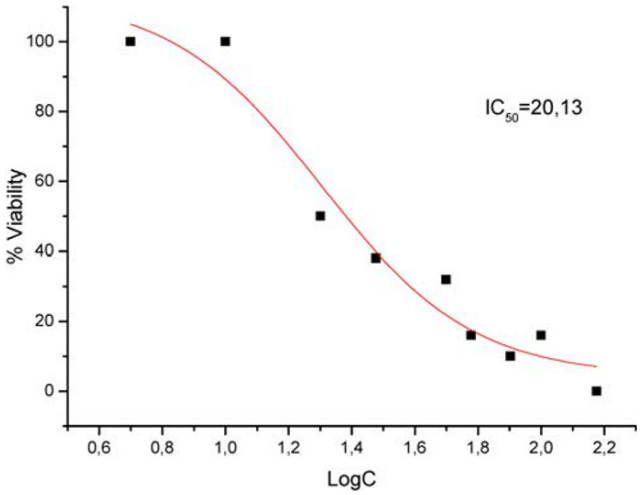

FIGURE 5 Cytotoxicity of peptide 1, $F^{17}KRIV^{21}QR^{23}I^{24}K^{25}D^{26}F^{27}LR\text{-}NH_2$; IC_{50} (μg/mL), 20.13. The experiments were carried out three times independently

TABLE 5 IC_{50} (μg/mL) of the peptides for A549 cancer cells and DLF normal cells

Peptides	A549	DLF
1 $F^{17}KRIV^{21}QR^{23}I^{24}K^{25}D^{26}F^{27}LR\text{-}NH_2$	20.1	29.3
2 $L^{17}KRIVQRIKDFLR\text{-}NH_2$	33.7	56.9
3 $A^{17}KRIVQRIKDFLR\text{-}NH_2$	29.8	-
. 4 Ac-FκRIVQRIL^{25}DFLR-NH_2	3.9	20.1
5 Ac-FK (SA)RIVQRIL^{25}DFLR-NH_2	10.7	24.3
6 FKRIVQRIR^{25}DFLR-NH_2	56.0	-
7 Ac-FKRIVQRIR^{25}DFLR-NH_2	12.5	54.1
8 FKRIVQκ^{23}IKDFLR-NH_2	145.1	-
9 FKRIVQL^{23}IKDFLR-NH_2	18.9	23.5
10 Ac-FKRIVQL^{23}IKDFLR-NH_2	13.9	22.5
11 FKRIVQRIKDL^{27}LR-NH_2	75.0	-
12 Ac-FKRIVQRIKDL^{27}LR-NH_2	45.5	-
13 FKRIVQI^{23}IKK^{26}FLR-NH_2	20.0	27.0
14 Ac-FKRIVQI^{23}IKK^{26}FLR-NH_2	12.9	15.7
15 FKRIVQL^{23}L^{24}KK^{26}L^{27}LR-NH_2	12.8	23.7
16 Ac-FKRIVQL^{23}L^{24}KK^{26}L^{27}LR-NH_2	9.8	17.3
17 FKRIL^{21}QRIKDFLR-NH_2	19.2	76.8
18 Ac-FKRIL^{21}QRIKDFLR-NH_2	29.2	160.9

Note. (–) not active.

Insertion of the IIKK motif (23-26) in peptides 13 and 14, and the LLKKL motif (23-27) in peptides 15 and 16 improved the cytotoxicity, with IC_{50} values of 12.92 μg/mL (peptide 14), 12.83 μg/mL (peptide 15), and 9.8 μg/mL (peptide 16). Substitution of valine in position 21 by leucine in analogs 17 and 18 did not enhance cytotoxicity.

All the peptides were also tested against normal DLF cells. Lower cytotoxicity was evident compared with A549 cells, as indicated the significantly higher IC_{50} values (Table 4).

The collective data of Table 5 demonstrated that the incorporation of the IIKK and LLKKL motifs, which are present in many defense peptides, into the native sequence 17-29 of the cathelicidin LL-37 significantly improved cytotoxicity against cancer cell line A549 cells as compared with the native sequence 17-29 of LL-37 (peptide 1). Substitution of the positively charged arginine in position 23 and lysine in position 25, which are located in the interface, by aliphatic residues significantly increased the cytotoxicity of the acetylated analogs compared with the native sequence 17-29 of LL-37 (peptide 1). Substitution of phenylalanine, an aromatic residue, in positions 17 and 27 of the hydrophobic side of the amphipathic helix by aliphatic residues did not enhance the cytotoxicity of peptide 1. Peptide 4, Ac-FKRIVQRIL^{25}DFLR-NH_2, displayed the best

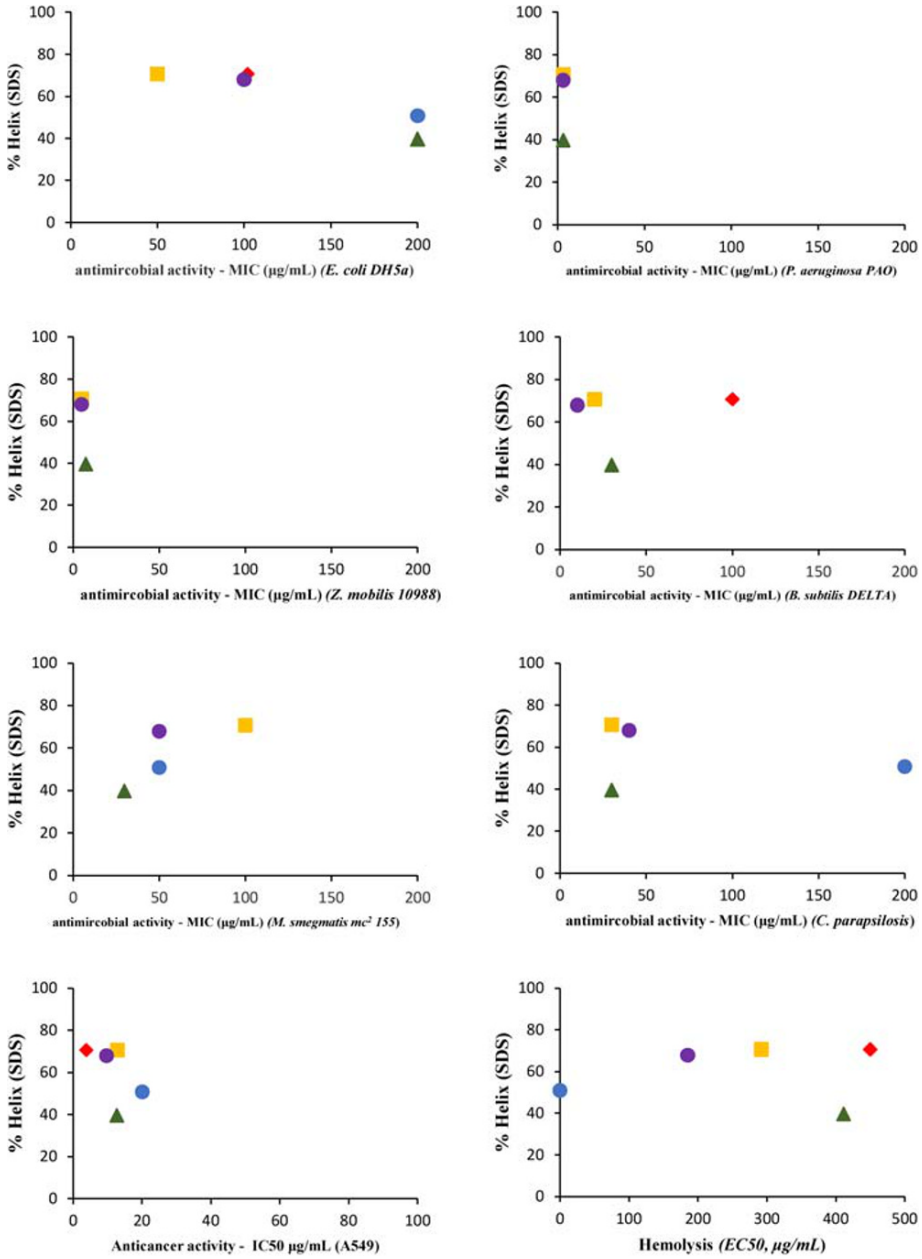

FIGURE 6 Schematic representation (SAR) of the % helical content of the peptides 1(●), 4(◆), 14(■), 15(▲), and 16(●) in sodium dodecyl sulfate (SDS) against antimicrobial and anticancer activity. The experiments were performed in triplicate

cytotoxicity (IC_{50} 3.90 μg/mL), highlighting the significance of position 25 in the interphase, as well as the importance of the N-terminus neutralization.

4 | CONCLUSIONS

In this study, we present the design, synthesis, and analysis of cationic amphipathic peptide analogs of the cathelicidin 17-29 segment. The aim of this study is to further contribute to the development of new effective antimicrobial/anticancer compounds. The native sequence 17-29 of LL-37 (peptide 1) showed weak antimicrobial activity with an MIC of 200 μg/mL against the gram-negative bacterium *E. coli* DH5a and fungus *C. parapsilosis* and an MIC of 50 μg/mL against the gram-positive bacterium *M. smegmatis* mc^2 155. Peptide 1 was not toxic for human erythrocytes but was cytotoxic to A549 cells (IC_{50} 20.13 μg/mL).

Selected substitutions were performed on residues R^{23}, K^{25}, V^{21}, and F^{27} of the interphase and the aromatic residue F^{17}. The IIKK and LLKKL motifs were inserted into the 17-29 fragment, and their antimicrobial/anticancer reactivity was studied. The substituted peptide Ac-FKRIVQRIL^{25}DFLR-NH_2 (4) exhibited the best cytotoxicity against A549 cancer cells, with an IC_{50} value of 3.90 μg/mL, probably due to its increased helical content. The peptide was not toxic for human erythrocytes and displayed relatively low antibacterial activity, with an MIC of 100 μg/mL for both gram-negative (*E. coli* DH5a) and gram-positive (*B. subtilis* DELTA) bacteria.

The peptides Ac-FKRIVQI^{23}IKK^{26}FLR-NH_2 (14), FKRIVQL^{23}L^{24}KK^{26}L^{27}LR-NH_2 (15), and Ac-FKRIVQL^{23}L^{24}KK^{26}L^{27}LR-NH_2

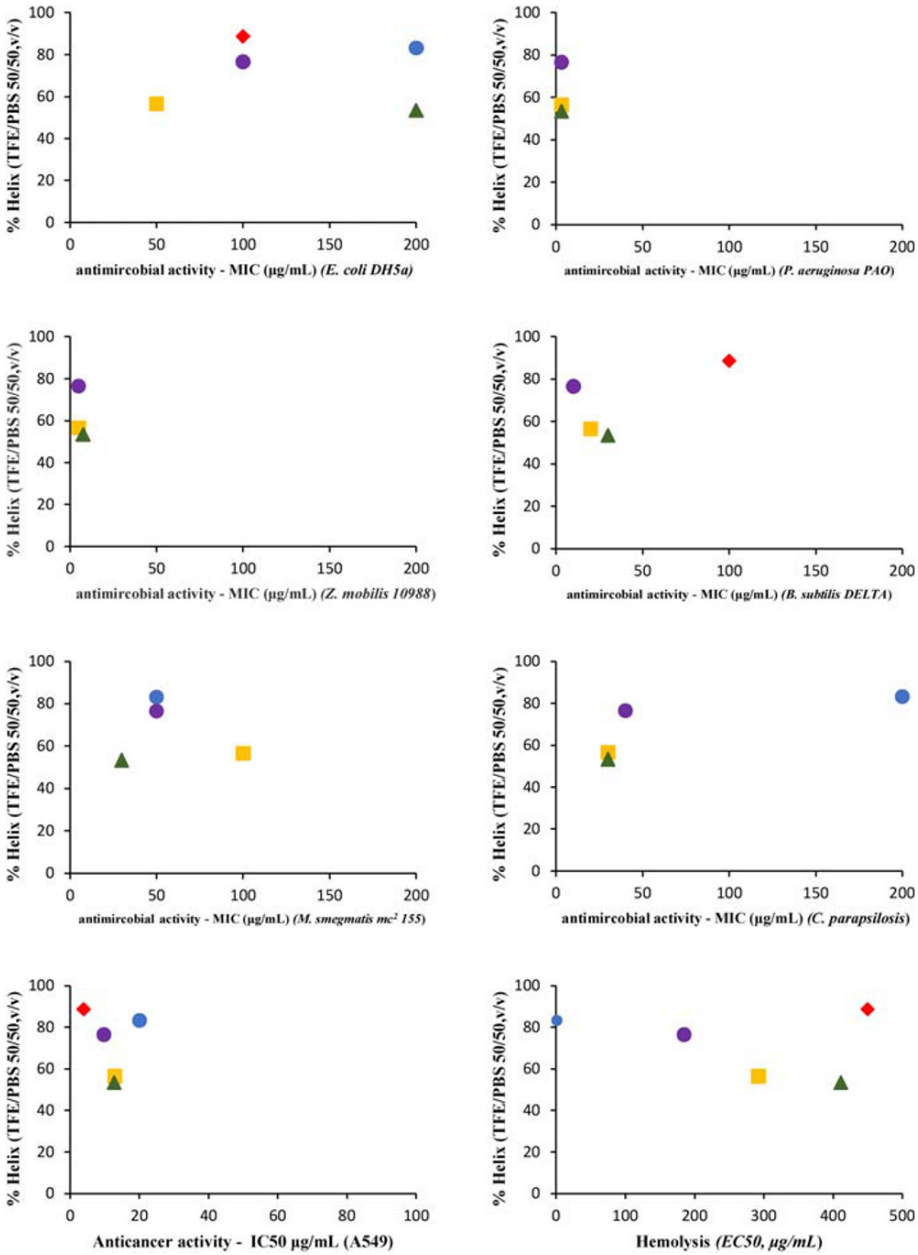

FIGURE 7 Schematic representation (SAR) of the % helical content of the peptides 1(●), 4(◆), 14(■), 15(▲), and 16(●) in trifluoroethanol (TFE)/phosphate-buffered saline (PBS) 50/50 v/v against antimicrobial and anticancer activity. The experiments were performed in triplicate

(16) displayed potent antimicrobial activity against the gram-negative species *P. aeruginosa* PAO and *Z. mobilis* 10,988, with MIC values ranging from 3 to 7.5 μg/mL, were not toxic for human erythrocytes and were cytotoxic for A549 cancer cells (IC_{50} values of 12.9, 12.8, and 9.8 μg/mL, respectively). All the peptides were also tested against normal DLF cells. Toxicity was lower as compared with A549 cancer cells, as indicated by their significantly higher IC_{50} values. Schemes were added in understanding the SAR studies and the conclusion achieved (Figures 6 and 7).

We conclude that the selected substitutions in positions located in the interphase of the amphipathic helix of the cathelicidin 17-29 segment is crucial role in the antimicrobial/anticancer activities of the peptide. Even residues within the IIKK and LLKKL motifs belong to the interphase. The net positive charges of the most active peptides, ranging from 3 to 5 (Table 1), may also contribute to their reactivity against the negatively charged cancer and microbial cells, whereas changes of the side chain stereochemical volume (Ala, Ile, Val) did not affect the overall reactivity of the peptides. N-terminal acetylation, which increased the peptide stability, increased the antibacterial and anticancer activity of most of the peptide analogs.

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REFERENCES

1. Huang HW, Charron NE. Understanding membrane-active antimicrobial peptides. *Q Rev Biophys*. 2017;50:1-17. https://doi.org/10.1017/S0033583517000087
2. Kang HK, Kim C, Seo CH, Park Y. The therapeutic applications of antimicrobial peptides (AMPs): a patent review. *J Microbiol*. 2017;55(1):1-12. https://doi.org/10.1007/s12275-017-6452-1

3. Baxter AA, Lay FT, Poon IKH, Kvansakul M, Hulett MD. Tumor cell membrane-targeting cationic antimicrobial peptides: novel insights into mechanisms of action and therapeutic prospects. *Cell Mol Life Sci*. 2017;74(20):3809-3825. https://doi.org/10.1007/s00018-017-2604-z
4. Gaspar D, Veiga AS, Castanho MA. From antimicrobial to anticancer peptides. A review. *Front Microbiol*. 2013;4:1-12. https://doi.org/10.3389/fmicb.2013.00294
5. Wu D, Gao Y, Chen L, Ma Y. Peptide-based cancer therapy: opportunity and challenge. *Cancer Lett*. 2014;351(1):13-22. https://doi.org/10.1016/j.canlet.2014.05.002
6. Evans HG, Guthrie JW, Jujjavarapu M, et al. D-amino acid analogues of the antimicrobial peptide CDT exhibit anti-cancer properties in A549, a human lung adenocarcinoma cell line. *Protein Pept Lett*. 2017;24(7):590-598. https://doi.org/10.2174/0929866524666170621093647
7. Wang G. Human antimicrobial peptides and proteins. *Pharmaceuticals*. 2014;7(5):545-594. https://doi.org/10.3390/ph7050545
8. Chen J, Hu J, Zeng P, et al. Molecular mechanisms of anticancer action and cell selectivity of short α-helical peptides. *Biomaterials*. 2014;35(5):1552-1561. https://doi.org/10.1016/j.biomaterials.2013.10.082
9. Babii O, Afonin S, Ishchenko AY, et al. Structure-activity relationships of photoswitchable diarylethene-based β-hairpin peptides as membranolytic antimicrobial and anticancer agents. *J Med Chem*. 2018;61(23):10793-10813. https://doi.org/10.1021/acs.jmedchem.8b01428
10. Chen X, Zhang L, Ma C, et al. A novel antimicrobial peptide, Ranatuerin-2PLx, showing therapeutic potential in inhibiting proliferation of cancer cells. *Biosci Rep*. 2018;38(6):1-12. https://doi.org/10.1042/BSR20180710
11. Akef HM. Anticancer, antimicrobial, and analgesic activities of spider venoms. *Toxicol Res (Camb)*. 2018;7(3):381-395. https://doi.org/10.1039/c8tx00022k
12. Roudi R, Syn NL, Roudbary M. Antimicrobial peptides as biologic and immunotherapeutic agents against cancer: a comprehensive overview. *Front Immunol*. 2017;8:1-10. https://doi.org/10.3389/fimmu.2017.01320
13. Leite ML, da Cunha NB, Costa FF. Antimicrobial peptides, nanotechnology, and natural metabolites as novel approaches for cancer treatment. *Pharmacol Ther*. 2018;183:160-176. https://doi.org/10.1016/j.pharmthera.2017.10.010
14. Deslouches B, Di YP. Antimicrobial peptides with selective antitumor mechanisms: prospect for anticancer applications. *Oncotarget*. 2017;8:46635-46651. https://doi.org/10.18632/oncotarget.16743
15. Pachon-Ibanez ME, Smani Y, Pachon J, Sanchez-Cespedes J. Perspectives for clinical use of engineered human host defense antimicrobial peptides. *FEMS Microbiol Rev*. 2017;41(3):323-342. https://doi.org/10.1093/femsre/fux012
16. Nieuwdorp M, Meuwese MC, Mooij HL, et al. Measuring endothelial glycocalyx dimensions in humans: a potential novel tool to monitor vascular vulnerability. *J Appl Physiol*. 2008;104(3):845-852. https://doi.org/10.1152/japplphysiol.00440.2007
17. Li C, Liu H, Yang Y, et al. N-myristoylation of antimicrobial peptide CM4 enhances its anticancer activity by interacting with cell membrane and targeting mitochondria in breast cancer cells. *Front Pharmacol*. 2018;9:1-15. https://doi.org/10.3389/fphar.2018.01297
18. Schaefer L, Schaefer RM. Proteoglycans: from structural compounds to signaling molecules. *Cell Tissue Res*. 2010;339(1):237-246. https://doi.org/10.1007/s00441-009-0821-y
19. Zwaal RF, Comfurius P, Bevers EM. Surface exposure of phosphatidylserine in pathological cells. *Cell Mol Life Sci*. 2005;62(9):971-988. https://doi.org/10.1007/s00018-005-4527-3
20. Harris F, Dennison SR, Singh J, Phoenix DA. On the selectivity and efficacy of defense peptides with respect to cancer cells. *Med Res Rev*. 2013;33(1):190-234. https://doi.org/10.1002/med.20252
21. Zevgiti S, Zabala JG, Darji A, Dietrich U, Panou-Pomonis E, Sakarellos-Daitsiotis M. Sialic acid and sialyl-lactose glyco-conjugates: design, synthesis and binding assays to lectins and swine influenza H1N1 virus. *J Pept Sci*. 2012;18(1):52-58. https://doi.org/10.1002/psc.1415
22. Zhou C, Wang Z, Peng X, et al. Discovery of two bombinin peptides with antimicrobial and anticancer activities from the skin secretion of oriental fire-bellied toad, Bombina orientalis. *Chem Biol Drug Des*. 2018;91(1):50-61. https://doi.org/10.1111/cbdd.13055
23. Wang G, Mishra B, Epand RF. High-quality 3D structures shine light on antibacterial, anti-biofilm and antiviral activities of human cathelicidin LL-37 and its fragments. *Biochim Biophys Acta*. 1838;2014(9):2160-2172. https://doi.org/10.1016/j.bbamem.2014.01.016
24. Ren SX, Shen J, Cheng AS, et al. FK-16 derived from the anticancer peptide LL-37 induces caspase-independent apoptosis and autophagic cell death in colon cancer cells. *PLoS ONE*. 2013;8(5):1–9. e63641. https://doi.org/10.1371/journal.pone.0063641
25. Hayashi M, Kuroda K, Ihara K, Iwaya T, Isogai E. Suppressive effect of an analog of the antimicrobial peptide of LL-37 on colon cancer cells via exosome-encapsulated miRNAs. *Int J Mol Med*. 2018;42:3009-3016. https://doi.org/10.3892/ijmm.2018.3875
26. Aghazadeh H, Memariani H, Ranjbar R, Bagheri KP. The activity and action mechanism of novel short selective LL-37-derived anticancer peptides against clinical isolates of Escherichia coli. *Chem Biol Drug Des*. 2019;93(1):75-83. https://doi.org/10.1111/cbdd.13381
27. Jia J, Zheng Y, Wang W, et al. Antimicrobial peptide LL-37 promotes YB-1 expression, and the viability, migration and invasion of malignant melanoma cells. *Mol Med Rep*. 2017;15(1):240-248. https://doi.org/10.3892/mmr.2016.5978
28. Thomas AJ, Pulsipher A, Davis BM, Jeremiah A. LL-37 causes cell death of human nasal epithelial cells, which is inhibited with a synthetic glycosaminoglycan. *PLoS ONE*. 2017;12(8):1–16. e0183542. https://doi.org/10.1371/journal.pone.0183542
29. Wang G, Narayana JL, Mishra B, et al. Design of antimicrobial peptides: progress made with human cathelicidin LL-37. *Adv Exp Med Biol*. 2019;1117:215-240. https://doi.org/10.1007/978-981-13-3588-4_12
30. Ji P, Zhou Y, Yang Y, et al. Myeloid cell-derived LL-37 promotes lung cancer growth by activating Wnt/β-catenin signaling. *Theranostics*. 2019;9(8):2209-2223. https://doi.org/10.7150/thno.30726
31. Majewski K, Kozłowska E, Żelechowska P, Brzezińska-Błaszczyk E. Serum concentrations of antimicrobial peptide cathelicidin LL-37 in patients with bacterial lung infections. *Cent Eur J Immunol*. 2018;43(4):453-457. https://doi.org/10.5114/ceji.2018.81355
32. Chen Z, Yang G, Lu S, et al. Design and antimicrobial activities of LL-37 derivatives inhibiting the formation of *Streptococcus* mutans biofilm. *Chem Biol Drug Des*. 2019;93(6):1175-1185. https://doi.org/10.1111/cbdd.13419
33. Zhu Y, Mohapatra S, Weisshaar JC. Rigidification of the *Escherichia coli* cytoplasm by the human antimicrobial peptide LL-37 revealed by super resolution fluorescence microscopy. *Proc Natl Acad Sci U S A*. 2019;116(3):1017-1026. https://doi.org/10.1073/pnas.1814924116. Epub 2018 Dec 31
34. Chena X, Zoua X, Qib G, et al. Roles and mechanisms of human cathelicidin LL-37 in cancer. *Cell Physiol Biochem*. 2018;47(3):1060-1073. https://doi.org/10.1159/000490183
35. Papo N, Seger D, Makovitzki A, et al. Inhibition of tumor growth and elimination of multiple metastases in human prostate and breast xenografts by synthetic inoculation of a host defense-like lytic peptide. *Cancer Res*. 2006;66(10):5371-5378. https://doi.org/10.1158/0008-5472

36. Wang KR, Yan JX, Zhahb BZ, Song JJ, Jia PF, Wang R. Nobel mode of action of polybia-MPI, a novel antimicrobial peptide, in multi-drug resistant leukemia cells. *Cancer Lett*. 2009;278(1):65-72. https://doi.org/10.1016/j.canlet.2008.12.027
37. Wang KR, Yan JX, Dang W, et al. Membrane active antimicrobial activity and molecular dynamics study of a novel cationic antimicrobial peptide polybia-MPI, from the venom of Polybia paulista. *Peptides*. 2013;39:80-88. https://doi.org/10.1016/j.peptides.2012.11.002
38. Chionis K, Krikorian D, Koukkou AI, Sakarellos-Daitsiotis M, Panou-Pomonis E. Synthesis and biological activity of lipophilic analogs of the cationic antimicrobial active peptide anoplin. *J Pept Sci*. 2016;22(11-12):731-736. https://doi.org/10.1002/psc.2939
39. Sakka M, Balliou A, Marianou A, et al. Synthesis and antimicrobial evaluation of amphipathic peptide models. *Lett Drug des Discov*. 2016;13(7):715-734. https://doi.org/10.2174/1570180813666160616122410
40. Zikou S, Koukkou AI, Mastora P, et al. Design and synthesis of cationic Aib-containing antimicrobial peptides: conformational and biological studies. *J Pept Sci*. 2007;13(7):481-486. https://doi.org/10.1002/psc.876
41. Chen YH, Yang JT, Chau KH. Determination of the helix and β form of proteins in aqueous solution by cicular dichroism. *Biochemistry*. 1974;13(16):3350-3359.
42. Dathe M, Schümann M, Wieprecht T, et al. Peptide helicity and membrane surface charge modulate the balance of electrostatic and hydrophobic interactions with lipid bilayers and biological membranes. *Biochemistry*. 1996;35(38):12612-12622.
43. Xu JM, Song ST, Tang ZM, et al. Predictive chemotherapy of advanced breast cancer directed by MTT assay in vitro. *Breast Cancer Res Treat*. 1999;53(1):77-85.
44. Chen C, Hu J, Zeng P, et al. Molecular mechanisms of anticancer action and cell selectivity of short α-helical peptides. *Biomaterials*. 2014;35(5):1552-1561.
45. Varki A. Sialic acids in human health and disease. *Trends Mol Med*. 2008;14(8):351-360.
46. Eisenberg D, Weiss RM, Terwilliger TC. The helical hydrophobic moment: a measure of theamphiphilicity of a helix. *Nature*. 1982;299(5881):371-374.
47. Gautier R, Douguet D, Bruno B, Drin G. Heliquest: a web server to screen sequences with specific α-helical properties. *Bioinformatics*. 2008;24(18):2101-2102.

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