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RESEARCH ARTICLE

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The activity and action mechanism of novel short selective LL-37-derived anticancer peptides against clinical isolates of *Escherichia coli*

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Abstract

Human cathelicidin LL-37 has recently attracted interest as a potential therapeutic agent, mostly because of its ability to kill a wide variety of pathogens and cancer cells. In this study, we aimed to investigate the antibacterial activity and cytotoxicity of previously designed LL-37 anticancer derivatives (i.e., P7, P22, and P38). Calcein release assay and field emission-scanning electron microscopy (FE-SEM) were performed to elucidate the possible mechanism of action of P38, the peptide with the highest bactericidal activity. In silico analysis demonstrated the amphipathic alpha-helical structure for three peptides. Antibacterial activity of P38 against multidrug-resistant (MDR) clinical isolates of *Escherichia coli* was higher than that of P7 and P22. P38 caused no hemolysis or cytotoxicity. Treating calcein-loaded *E. coli* with 4× MIC of P38 resulted in more than 96% leakage of calcein. Noticeably, FE-SEM revealed that P38 killed *E. coli* by disrupting the bacterial membrane. Molecular docking studies showed that P38 had a much higher affinity for the outer membrane of Gram-negative bacteria compared with both P22 and P7. Owing to the bactericidal activity of P38 against MDR *E. coli* isolates and its negligible cytotoxicity, P38 has the potential for further studies in a mouse model of infectious disease.

KEYWORDS

activity, antimicrobial peptides, LL-37, mechanism, molecular docking, toxicity

1 | INTRODUCTION

The ever-escalating problem of antibiotic resistance, coupled with dwindling antimicrobial armamentarium, has necessitated the exploration of efficient antimicrobials toward which bacteria would not be easily able to develop resistance.[1] Among the very few novel anti-infective agents in the pipeline, antimicrobial peptides (AMPs) were widely proposed as promising alternatives to conventional antibiotics owing to their broad-spectrum and rapid antimicrobial properties, as well as the perceived low likelihood of inducing drug resistance.[2,3] Ubiquitous in nature, AMPs play a prominent role in initial line of defense against invading pathogens.[4] These fascinating peptides usually maintain common physicochemical characteristics. In general, AMPs consist of less than 50 amino acid residues, exhibit a positive net charge, and display an amphipathic nature.[3,5] These characteristics are believed to be crucial for antimicrobial activity of AMPs.[5] In contrast to conventional antibiotics targeting specific biosynthetic pathways such as protein and cell wall synthesis, AMPs can destabilize the physical integrity of the bacterial membrane, thereby circumventing classical mechanisms of drug resistance.[6]

In addition to microbicidal activity, several cationic AMPs have been shown to exert cytotoxic effects on cancerous cells. The anticancer activity of AMPs may usually occur either by membranolytic or nonmembranolytic mechanisms.[7] In fact, the presence of anionic components (e.g., phosphatidylserine, *O*-glycosylated mucins, sialylated gangliosides, and heparin sulfate in the membrane of cancerous cells) allows cationic anticancer peptides to target and bind these cells with increased efficacy compared to normal mammalian cells, which are overall neutrally charged as a result of zwitterionic phosphatidylcholine and sphingomyelin.[7–9] Based on targets of cells, these peptides can be classified into two groups: The first group encompasses peptides that possess toxicity against microbial and cancerous cells while not being cytotoxic against normal mammalian cells, such as magainins and cecropins. The second group is composed of peptides that are toxic against all three types of cells including microbial, cancerous, and normal mammalian cells.[10] Some instances of the second group include the bee venom melittin and human cathelicidin LL-37.[11]

Cathelicidins are a family of antimicrobial and endotoxin-binding proteins characterized by a highly conserved N-terminal prosequence, named the cathelin-like domain, and a variable C-terminal antimicrobial domain.[12] Although some mammals harbor multiple cathelicidin genes, only one human cathelicidin gene, *CAMP*, has been identified as a coding region for the preproprotein human CAP18 (18-kDa cationic antimicrobial protein).[13] The C-terminus end of the human CAP18 protein consists of a 37-amino acid peptide named LL-37 that starts with a pair of leucines.[14] This cathelicidin is found on the skin, as well as in neutrophils, numerous epithelial linings, and salivary glands.[15] As a linear cationic alpha-helical AMP, human LL-37 has recently attracted great interest by virtue of its capability to kill a wide variety of pathogens including bacteria, fungi, human immunodeficiency virus (HIV)-1, and parasites.[16] Furthermore, LL-37 can play an important role in inducing or killing cancer cells and can exert immunomodulatory effects.[16] Thus, human cathelicidin LL-37 is a promising candidate for developing novel therapeutic agents.

Very recently, our research group designed several short peptides based on the human cathelicidin LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES). After a molecular docking study between LL-37 and a tumor-specific antigen named carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), 16 amino acid residues of LL-37 were found to be involved in CEACAM1 binding. These amino acid residues were merged into a 16-mer peptide named LL-16 (LLSKEKERIVQRVRTS), which was selected as a template for designing new analogues in a stepwise manner. LL-16 had a much higher affinity for CEACAM1 compared to LL-37. At first, a new analogue of LL-16 was designed by amino acid residue substitution in a single position and the binding affinity of the analogue with CEACAM1 was evaluated by molecular docking. If the analogue had a higher binding affinity for CEACAM1 compared to LL-16, it was subjected to another round of amino acid residue substitution in different position, followed by evaluation via molecular docking. Hence, the analogue showing higher binding affinity for CEACAM1 than its parental peptide served as an input for further modification. Using this approach, a total of sixty 16-mer peptide analogues were designed, of which three peptides (named P7, P22, and P38) had the highest binding affinity for CEACAM1 in molecular docking studies. The three peptides were chemically synthetized and cytotoxically evaluated against a panel of human cancer cell lines (data not shown). Here, we set out to explore whether our designed LL-37-derived anticancer peptides can eradicate clinically isolated multidrug-resistant (MDR) bacteria in vitro. The in silico molecular docking was also used to investigate binding properties of designed peptides with the outer membrane of Gram-negative bacteria. Furthermore, calcein release assay and field emission-scanning electron microscopy (FE-SEM) were performed to elucidate the mechanism of action of the peptide with the highest bactericidal activity.

2 | METHODS AND MATERIALS

2.1 | Peptide analysis and synthesis

The ProtParam tool on the ExPASy server (http://web.expasy.org/protparam/) was used to determine the physicochemical properties of LL-37-derived peptides. Hydrophobic moment was obtained by EMBOSS explorer (http://www.bioinformatics.nl/emboss-explorer/). The secondary and tertiary structures of the peptides were predicted by the PSIPRED[17] and the I-TASSER servers,[18] respectively. Schiffer–Edmundson wheel representations of the peptides were obtained using RZLab (http://rzlab.ucr.edu/scripts/wheel/wheel.cgi).

All the peptides were amidated at the C-terminal and synthesized at Mimotopes Company (Clayton, Victoria, Australia) using the standard Fmoc (9-fluorenyl-methoxycarbonyl) procedure.[19] The final purity of the peptides was ≥95%, as assessed by C18 reversed-phase high-performance liquid chromatography (RP-HPLC). Furthermore, the identity of each peptide was confirmed by mass spectrometry analysis on a Sciex API100 LC/MS instrument (Perkin Elmer Co., Norwalk, CT, USA) in positive ion mode.

2.2 | Bacterial strains

Escherichia coli ATCC 25922, *Staphylococcus aureus* ATCC 29213, and MDR clinical isolates of bacteria including five

E. coli and five *S. aureus* strains were used in this study. Most of the *E. coli* strains were isolated from urinary tracts of patients hospitalized in Firoozgar Hospital, Iran, while *S. aureus* strains were obtained from burn wounds of patients admitted to Shahid Motahari Hospital, Iran. All the isolates were stored in tryptic soy broth (TSB) containing 25% (*v/v*) glycerol at −70°C until further study.

2.3 | Reagents and media

Müeller-Hinton broth (MHB), Müeller-Hinton agar (MHA), and TSB were purchased from Merck (Darmstadt, Germany), while the other materials such as those used for cell culture were all procured from Sigma (St. Louis, MO, USA).

Except for the calcein release assay, which was conducted in duplicate, all the experiments were performed in triplicate and data were expressed by using the mean ± standard deviations (*SD*).

2.4 | Antimicrobial assays

The antibacterial activity of each peptide or antibiotic against a panel of bacteria was examined by broth microdilution protocol of the Clinical and Laboratory Standards Institute.[20] The minimum inhibitory concentration (MIC) was taken as the minimum concentration at which no bacterial growth was observed at 600 nm using a microplate spectrophotometer (BioTek, Winooski, VT, USA). The minimum bactericidal concentration (MBC) was considered as the lowest concentration of each peptide or antibiotic from which no colonies were observed on the MHA plate.

2.5 | Time-kill assay

The time-kill assay follows a previous protocol using a MDR clinical isolate of *E. coli* (MDR-EC3).[21] To this end, 1.5×10^5 colony-forming units (CFUs)/ml of exponentially growing bacteria in fresh MHB were added to 96-well microtiter plates containing different concentrations of P38 and gentamicin. After 60, 180, 300, and 480 min of incubation, viability expressed as CFUs/ml was determined by plating serially diluted sample onto MHA plates, followed by incubation at 37°C for 24 hr. Compared to the growth control curve, a ≥ 3 $\log_{10}$ reduction in CFUs was regarded as bactericidal activity.[22]

2.6 | In vitro toxicity assays

2.6.1 | Hemolytic activity assay

Human red blood cells (hRBCs) were obtained from an individual participant, who signed the informed consent for venepuncture procedure. Whole blood was taken from the antecubital vein of a healthy volunteer, who signed the informed consent for venepuncture procedure, into glass tubes containing EDTA (2 mg/ml) and harvested by centrifugation at $800 \times g$ for 10 min. The fresh hRBCs were rinsed thrice and resuspended in phosphate-buffered saline (PBS, pH 7.4) to yield a 2% (*v/v*) erythrocytes/PBS suspension. Next, 50 µl of the resultant suspension was incubated with 50 µl of twofold serially diluted peptides for 1 hr at 37°C, after which the samples were centrifuged at $800 \times g$ for 10 min. The hemolytic activity of peptides against hRBCs was then monitored by measuring the absorbance of the supernatant at 540 nm, as previously outlined.[21] Triton X-100 and 0.9% (*w/v*) NaCl served as positive and negative controls, respectively.

The percentage hemolysis was calculated according to the following equation:

$$\text{Percentage hemolysis} = \left[\frac{\text{OD sample}-\text{OD negative control}}{\text{OD positive control}-\text{OD negative control}}\right] \times 100$$

2.6.2 | Cytotoxicity assay

To provide further insight into potential cell toxicity, we assessed the cytotoxic activity of the peptides against human embryonic kidney 293 (HEK-293) cells by the colorimetric MTT viability assay for 24 hr, as described earlier.[23] HEK-293 cells were cultured in RPMI 1640 (supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin, 2 mM/L glutamine, and 1 mM sodium pyruvate) in the presence of 5% CO_2 at 37°C. The percentage of viable cells was calculated by using the following equation:

$$\text{Viability}\,(\%) = \left[\frac{(A_s - A_b)}{(A_c - A_b)}\right] \times 100,$$

where A_s, A_c, and A_b correspond to the absorbance of the sample, the control (no peptide added), and the background, respectively.

2.7 | Mechanism of action

2.7.1 | Calcein release assay

The mechanism of action of P38 toward *E. coli* ATCC 25922 was examined by measuring its membrane-permeabilizing activity by calcein release assay.[24] A nonfluorescent, lipid-soluble derivative of calcein named calcein acetoxymethylester (calcein AM) is able to diffuse across the bacterial membrane and enter the cytoplasm; wherein, it is cleaved by bacterial intracellular esterases to produce fluorescent calcein.[24] Bacterial cells were grown to midlogarithmic phase in MHB at 37°C. After removing media, bacteria were washed three times with PBS and diluted in the same buffer. Next, aliquots (100 µl) of bacterial suspension were added to a calcein solution (final concentration of 10 µg/

ml). After 1 hr of incubation in darkness at 25°C, 100 µl of calcein-loaded *E. coli* (1.5×10^5 CFUs/ml) was added to each well of a 96-well black microplate (SPL, South Korea) containing different concentrations of P38. Following incubation for 30, 60, 120, 180, and 300 min, calcein retention was quantified using a fluorescence spectrometer (Synergy 4, BioTek Co., USA) at 490 and 517 nm for excitation and emission, respectively. Furthermore, calcein-loaded bacteria without treatment served as the negative control. A known membrane-disrupting peptide, melittin was also used as positive control. The experiments were performed in duplicate. The percentage of calcein leakage was calculated according to the following equation:

$$\text{Calcein leakage}\,(\%) = \left[\frac{(F - F_c)}{(F_t - F_c)}\right] \times 100,$$

where F corresponds to the fluorescence intensity of P38-treated bacteria, F_c is the fluorescence intensity of intact bacteria (control), and F_t represents the fluorescence intensity after the addition of Triton X-100 (0.5% [*v*/*v*]).

2.7.2 | FE-SEM

FE-SEM was used to evaluate bacterial membrane damage following treatment with P38. Midlogarithmic phase *E. coli* MDR-EC3 was adjusted to 1.5×10^6 CFUs/ml and incubated with 1× MIC (12.5 µg/ml) and 4× MIC (50 µg/ml) of peptide P38 at 37°C. After 30 or 60 min of incubation, each sample was pelleted down at 1,000 *g* for 5 min, rinsed twice with PBS, fixed for 1 hr in 2% (*v*/*v*) gluteraldehyde, and dehydrated via graded series of ethyl alcohols. The cells were then air-dried, sputter coated with gold nanoparticles, and visualized using a FE-SEM instrument (TESCAN MIRA3, Brno, Czech Republic).

2.8 | In silico molecular docking study

To address the atomistic details of peptide–membrane interaction and also to determine the binding affinity of the peptides against the outer membrane of Gram-negative bacteria, docking-based interaction analysis was performed by AutoDock Vina (ADV) v.1.1.2.[25] CHARMM-GUI server was employed for construction of the membrane.[26] The membrane composition was in accordance with Lohner et al.[27] Dipalmitoyl-phosphatidylethanolamine (DPPE) and dipalmitoyl-phosphatidylglycerol (DPPG) at a ratio of 9:1 was used, as described earlier.[27,28] Each leaflet of the membrane contained 45 DPPE lipids and five DPPG lipids. Using AutoDock tools (ADT) v.1.5.6,[29] the membrane and each peptide were treated as the receptor and the ligand, respectively. All the hydrogen atoms were added, and all the side chains of each peptide were defined as flexible. The molecular docking study was performed with a grid box of 60 × 48 × 30 points, grid spacing of 1.0 Å centered on the membrane surface, allowing interaction with all head groups exposed. For each peptide, at least 10 docking runs were performed, after which the best output model with minimum binding energy was selected for analysis.

3 | RESULTS

3.1 | Peptide analysis

As evidenced in Table 1, P22 had the highest net positive charge among all peptides. In addition, the hydrophobic moment of the peptides was according to the following order: P7 > P22 > P38. The theoretical molecular weights of P7, P22, and P38 (Table 1) were in accordance with their observed values (Supporting Information Figure S1). The purity of the peptides was confirmed as higher than 95% using RP-HPLC (Supporting Information Figure S1). The prediction of secondary and tertiary structures of the peptides revealed that they adopted a typical alpha-helix with a helical content of 81.2%. The hydrophobic amino acid residues and positively charged amino acid residues of the peptides were dispersed, as shown in helical wheel representations (Supporting Information Figure S1).

3.2 | Antimicrobial assays

P38 had higher antibacterial activity against MDR *E. coli* isolates with MIC and MBC values in the range of 12.5–50 µg/

TABLE 1 Secondary structure predictions and physicochemical properties of LL-37-derived peptides

AMP[a]	Amino acid sequences	Secondary structure (N → C)[b]	H[c] (%)	TMW[d] (Da)	OMW[e] (Da)	NC[f]	P[g] (%)	<µH>[h]
P38	TSVRQRWRWRQRVRTS—NH_2	CHHHHHHHHHHHHHCC—NH_2	81.2	2157.4	2157.5	+7	95.56	0.368
P22	KRSKRKRRIHQRVRIS—NH_2	CHHHHHHHHHHHHHCC—NH_2	81.2	2103.5	2103.2	+10	99.27	0.648
P7	TLSKEKERIVQRVRTS—NH_2	CCHHHHHHHHHHHHHC—NH_2	81.2	1929.2	1929.2	+4	95.04	0.708

Notes. C-terminus was amidated for the peptides.

[a]AMP: Antimicrobial peptide; [b]Secondary structure: secondary structures of AMPs predicted by PSIPRED server. N → C showed the N-terminal to C-terminal direction of peptides. C and H denote random coils and helix, respectively. [c]H (%): Percentage of alpha-helix, obtained from http://bioinf.cs.ucl.ac.uk/psipred/. [d]TMW (Da): Theoretical molecular weight (Dalton). [e]OMW (Da): Observed molecular weight (Dalton), obtained from mass spectrometry experiment. [f]NC: Net charge, obtained from http://web.expasy.org/protparam/. Net charges were calculated at pH 7. [g]P: Percentage of the peptide purity. [h]<µH>: hydrophobic moment.

ml (Table 2). However, P7 and P22 exhibited poor efficacy against all bacterial strains tested (MICs and MBCs of >50 μg/ml). Furthermore, no antimicrobial activity against all *S. aureus* isolates (MICs and MBCs of >50 μg/ml) was observed for P38. Compared to meropenem, all the peptides had higher MIC and MBC values against *E. coli* isolates (Table 2).

3.3 | Time-kill assay

The MIC value was 12.5 μg/ml for an MDR clinical isolate of *E. coli* (MDR-EC3). At 1× MIC, P38 required 180 min to reduce >3log10 the initial inoculum, whereas gentamicin required longer exposure times (Figure 1A). At 4× MIC (50 μg/ml), P38 completely eliminated the initial inoculum within 300 min, while gentamicin at its 2× MIC (50 μg/ml) failed to eradicate the initial inoculum, even after 480 min of incubation, highlighting faster bactericidal kinetics of the peptide compared with the antibiotic.

3.4 | In vitro toxicity assays

As for hemolytic activity, P7 showed negligible hemolysis (>0.5%) at all peptide concentrations tested. Neither P22 nor P38 caused obvious hemolysis at 50 μg/ml. These results suggested that all of the peptides were nonhemolytic to hRBCs at the maximal concentration tested. A dose-dependent reduction in cell survival rates was observed for P7, particularly in the concentration range of 12.5 to 50 μg/ml (Figure 1B). In this regard, the cell survival rates seen with P7 exceeded 85% at the highest concentration tested (50 μg/ml). By contrast, both P22 and P38 did not show any cytotoxicity to HEK-293 cells even at 50 μg/ml (Figure 1B).

3.5 | Mechanism of action

P38 at its 1× MIC and 4× MIC permeabilized the bacterial membrane (Figure 1C), resulting in release of preloaded calcein in both a time- and dose-dependent manner. Of note, P38 at its 1× MIC and 4× MIC caused more than 77% and 96% leakage, respectively, within 300 min of incubation.

According to the FE-SEM results, control *E. coli* (Figure 2A) showed regular, smooth, and unbroken surface, while P38 treatment induced a significant membrane-damaging effect (Figure 2B–E). The bacteria treated with 1× MIC of P38 for 30 min shrank and holes appeared on the cell surface (Figure 2B). When treated with 1× MIC of the peptide for 60 min, cytoplasmic outpouring was obvious (Figure 2C), demonstrating that the peptide killed bacterial cells by disrupting the cell membrane. As shown in Figure 2D,E, treatment with 4× MIC of peptide induced significant membrane damage in *E. coli*, resulting in cell lysis.

3.6 | In silico molecular docking study

Using molecular docking study, the binding interaction of each peptide with outer membrane of Gram-negative bacteria was investigated (Figure 3A–C). The calculated binding affinity for P38, P22, and P7 were -6.7 ± 0.1, -4.2 ± 0.3, and -3.7 ± 0.1 kcal/mol, respectively. This finding demonstrated that P38 had a much higher affinity for bacterial membrane compared with both P22 and P7, reinforcing the above-mentioned data regarding antimicrobial activity in vitro. The in silico interaction between each peptide and outer membrane of Gram-negative bacteria are summarized in Supporting Information Tables S1, S2, and S3. P38 penetrated deeper into the membrane compared to both P7 and P22, as can be seen in Figure 3A–C. Docking analysis indicated that R4, Q5, R6, W7, R8, R10, Q11, R12, and T15 of the P38 were strongly involved in binding with Gram-negative membrane through electrostatic, van der Waals, and hydrogen bondings (Supporting Information Tables S1).

4 | DISCUSSION

During the past years, AMPs have gained substantial attention as potential therapeutic agents, not only against invading pathogens but also against cancerous cells.[30] These activities primarily arose from higher levels of negatively charged lipids in both bacterial and cancerous cells compared to

TABLE 2 Antibacterial activity of LL-37-derived peptides and meropenem against *E. coli* isolates

Escherichia coli isolates	Isolation period	Hospital	Specimen site	MIC (MBC)				
				P38	P22	P7	LL-37	Meropenem
ATCC 25922	—	—	—	25 (50)	>50 (>50)	>50 (>50)	30 (60)	0.39 (0.39)
MDR-EC1	2014, Q3	Motahari	Burn wound	50 (50)	>50 (>50)	>50 (>50)	30 (60)	12.5 (12.5)
MDR-EC2	2010, Q1	Firoozgar	Urinary tract	50 (50)	>50 (>50)	>50 (>50)	15 (30)	0.78 (0.78)
MDR-EC3	2010, Q2	Firoozgar	Urinary tract	12.5 (50)	>50 (>50)	>50 (>50)	15 (60)	1.56 (3.12)
MDR-EC4	2010, Q3	Firoozgar	Urinary tract	12.5 (50)	>50 (>50)	>50 (>50)	30 (60)	0.78 (0.78)
MDR-EC5	2015, Q2	Firoozgar	Urinary tract	12.5 (50)	>50 (>50)	>50 (>50)	30 (60)	3.12 (3.12)

Note. MIC: Minimum inhibitory concentration (μg/ml); MBC: Minimum bactericidal concentration (μg/ml); Q: Quarter.

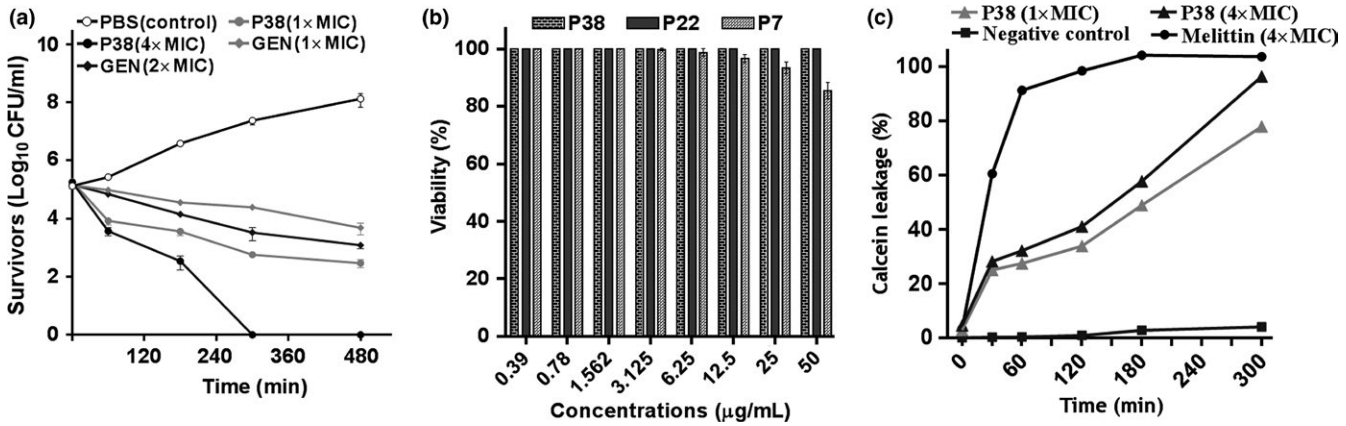

FIGURE 1 Time-kill analysis of antimicrobials toward MDR *E. coli* (a), cytotoxicity of P38, P22, and P7 on HEK-293 cell line using MTT assay (b), and calcein release assay for evaluating membrane-damaging activity of P38 against *E. coli* ATCC 25922 (c). In panel A, GEN corresponds to gentamicin. Except for calcein release assay, conducted in duplicate, the mean and standard deviation (*SD*) of triplicate values are shown

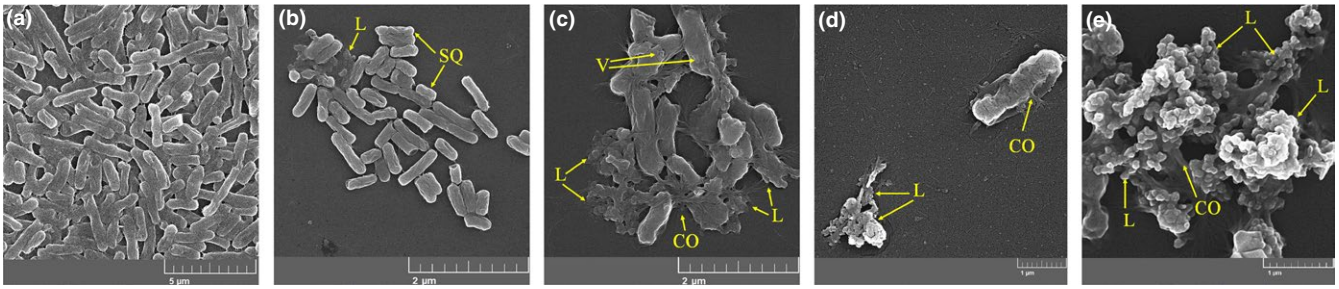

FIGURE 2 FE-SEM images of *E. coli* (MDR-EC3) after exposure to P38. Negative control (a) was *E. coli* in the absence of P38. 1.5×10^6 CFUs/ml (100 μl) were exposed to 1× MIC (100 μl) (b, 30 min; c, 60 min) and 4× MIC (100 μl) of peptide P38 (d, 30 min; E, 60 min). Labels V, CO, L, and SQ indicate vesicle, cytoplasmic outpouring, lysis, and squeezing, respectively

healthy mammalian cells, resulting in stronger electrostatic interaction and membrane lytic activity. In this respect, there is an escalating body of evidence regarding the dual activity (i.e., microbicidal and anticancer effects) of some AMPs, perhaps in consequence of the multifunctional nature of host-defense peptides in multicellular organisms.[30,31] These features prompted us to investigate the antibacterial efficiency of LL-37-derived anticancer peptides and possible mechanisms behind it.

In the present study, P38 had higher antibacterial activity against MDR *E. coli* isolates compared to both P7 and P22. Comparison of the antibacterial activity of LL-37 and P38 against *E. coli* ATCC 25922 demonstrated that P38 had a slight greater antimicrobial activity than that of LL-37. In the case of P38, the net charge of +7 appears to be sufficient for the initial electrostatic interactions between the peptide and the negatively charged lipids in the bacterial membrane. A possible reason why P22 did not exhibit antibacterial effect is its high cationic charge (+10) and its low number of hydrophobic amino acid residues. In the case of positive charge density on a given alpha-helical AMP, there is a critical threshold which governs antibacterial effects.[32] In the case of P22, the number of cationic amino acid residues in the peptide may exceed the threshold and compromise its antimicrobial activity. Furthermore, a low content of hydrophobic amino acid residues of P22 can reduce the insertion ability of the peptide into lipid bilayers of the bacterial membrane, resulting in weaker antibacterial activity. In the case of P7, negative charge of the glutamic acid residues may disturb initial electrostatic interaction between the cationic peptide and the anionic lipids of the bacterial membrane, thereby reducing its antibacterial effectiveness. Given that all the peptides presented a helical amphipathic structure and exhibited similar helical content based on the prediction of secondary structure, it seems that helicity alone does not determine the activity of peptides.

Previous studies demonstrated that truncated analogues of LL-37 had higher antibacterial activities against *E. coli*, *S. aureus*, and pan-drug-resistant *Acinetobacter baumannii* isolates compared with those of LL-37.[33,34] P38 exhibited a slightly stronger antimicrobial activity against *E. coli* ATCC 25922 compared to LL-37. Given the shorter length of P38, maintaining its antibacterial activity is a considerable issue. As for the time-kill assay, our results revealed that P38 had a

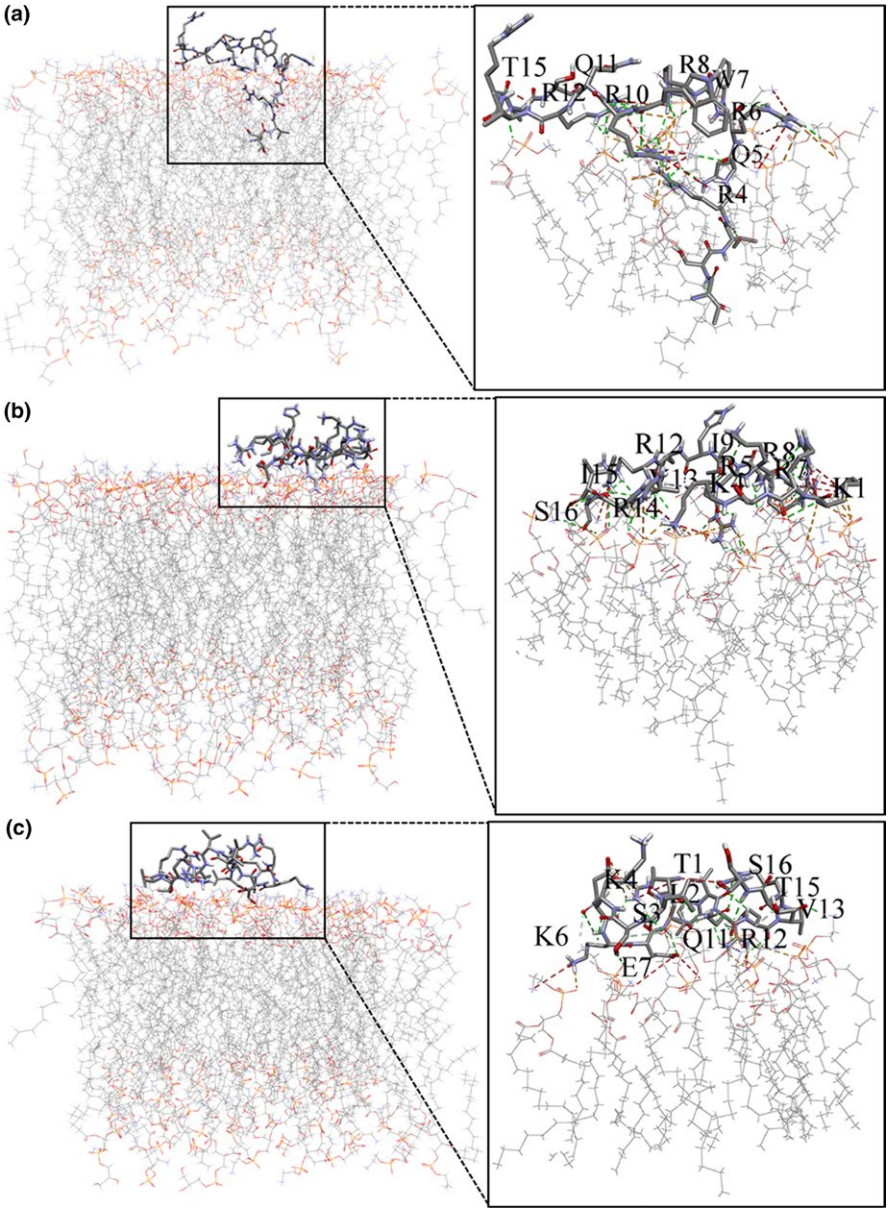

FIGURE 3 Interaction of P38 (a), P22 (b), and P7 (c) with outer membrane of Gram-negative bacteria. Single-letter code and position of each amino acid residue involved in binding process are shown. Orange, green, and red dashed lines represented the electrostatic, hydrogen, and van der Waals bonding interactions in binding site region, respectively

faster bactericidal rate against MDR-EC3 compared to gentamicin. In agreement with the current study, previous reports revealed rapid bactericidal activities of synthetic AMPs over tested antibiotics.[3,35] It is worthwhile noting that a faster killing action of AMPs has unique advantages over traditional antibiotics such as limiting spread of infection, diminishing the likelihood of developing drug resistance, and reducing treatment duration.[5]

Concerning toxicity, neither P22 nor P38 showed hemolytic and cytotoxic activities. In the case of truncated derivatives of LL-37, Ren et al.[36] showed that FK-16, a fragment of LL-37 corresponding to amino acid residues 17 to 32, had a minimal toxic effect on the viability of human normal colon mucosal epithelial NCM460 cells. In a recent study conducted by Kim et al.,[37] LL-37-derived short AMPs showed weak hemolytic effects on human erythrocytes. Using truncated LL-37 derivatives, Jacob et al.[38] found that the peptides had significantly lower hemolytic activities compared to LL-37. In fact, there are several factors that influence the selectivity of cationic AMPs against bacteria but not against mammalian cells. The reason for such selectivity on bacterial membrane includes the presence of anionic lipids in the bacterial membrane,[21] the existence of cholesterol inside the mammalian cell membrane,[39] and the significant difference between transmembrane potential of mammalian and bacteria cells.[40]

Regarding the action mechanism of P38, calcein release assay revealed that the peptide permeabilized the bacterial membrane in a time- and dose-dependent manner. In this respect, calcein release was increased during 60 min of exposure to the peptide, most likely as a result of rapid accumulation of P38 on *E. coli* cell membrane. This accumulation caused membrane damage and subsequent cell lysis.

To gain additional insights into the effect of P38 on *E. coli* membrane, FE-SEM was used. Treatment of bacterial cells with P38 resulted in vesicle formation, shrinkage of cells, cytoplasmic outpouring, lysis, decomposition, and squeezing in cells, demonstrating that the bacterial membrane is the chief target for P38. In agreement with our findings, it was shown that an embedded-hybrid peptide named R-FV-I16 (RFRRLFRIRVRVLKKI) induced roughness and blebbing on the membrane surface of *E. coli* ATCC 25922 during 30 min and resulted in release of intracellular contents within 120 min.[41] It should be noted that rapid killing of bacteria by membrane-disrupting mode of action can reduce the probability of emerging AMP-resistant mutants, since significant alteration of lipid composition would affect the viability of bacteria.[6]

Based on molecular docking results, P38 had the highest binding affinity for bacterial membrane among tested peptides, supporting the in vitro experiments. Noticeably, arginine residues (i.e., R4, R6, R8, R10, and R12) are involved in interaction with negatively charged lipids of the bacterial membrane. In good agreement with our results, previous investigations indicated that positively charged amino acids play a prominent role in the interactions of cationic AMPs and bacterial membranes.[42,43] It is known that some amino acid residues including arginine and tryptophan help to improve antimicrobial activity.[44] In this study, it seems that W7 on the nonpolar face of P38 plays an important role in peptide–membrane interaction. In fact, tryptophan has some crucial chemical features that make it suitable component of AMPs.[45] For instance, tryptophan residues partition more favorably into the membrane interface and have a higher affinity for bulky hydrophobic phases than other aromatic amino acid residues.[46] Noticeably, tryptophan and arginine can participate in cation–π interactions, thereby facilitating enhanced peptide–membrane interactions.[45] In addition, it is thought that the cation–π interaction between peptide side chains stabilizes the structure of small AMPs such as indolicidin.[47] Taken together, these data strongly suggest that tryptophan and arginine are interesting amino acid residues for designing short AMPs.

5 | CONCLUSIONS

In this study, all the peptides showed a high helix-forming propensity according to the in silico analysis. Among three peptides, P38 not only had the greatest binding affinity for the outer membrane of Gram-negative bacteria but also exhibited the highest antimicrobial activity. Noticeably, P38 possessed a greater selectivity for the bacterial cells compared to the mammalian cells, suggesting that the peptide is a promising candidate for developing peptide-based antibiotics. As evidenced by calcein release assay and FE-SEM, P38 killed *E. coli* by destroying bacterial membrane. This mode of action, together with rapid bactericidal kinetics of P38, can reduce the ability of bacteria to acquire resistance to the peptide. Although the results reported here reflect the in silico and in vitro efficacy of P38, in vivo effects and stability of the peptide in animal models of infection must be further assessed to better understand its possible therapeutic applications.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHORS' CONTRIBUTION

H.A. contributed to peptide designing, all bioinformatics analyses, experimental sections, and revision of the manuscript. H.M. contributed to writing and revision of the manuscript and also partially to bioinformatics analysis. R.R. served as advisor. K.P.B. supervised the project, contributed to peptide designing, and also to redaction of the manuscript.

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