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Design and Synthesis of Lipopolysaccharide-Binding Antimicrobial Peptides Based on Truncated Rabbit and Human CAP18 Peptides and Evaluation of Their Action Mechanism

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Abstract

Lipopolysaccharide (LPS) is a toxic and immunogenic agent for human. Additionally, LPS is a good target for some antimicrobial compounds, including antimicrobial peptides (AMPs). LPS-binding peptides (LBPs) can recognize and neutralize LPS. Rabbit and human cathelicidins are AMPs with LPS-binding activity. In this study, we designed and synthesized two new truncated LBPs from rabbit and human CAP18 peptides by in silico methods. After synthesis of peptides, the antimicrobial properties and LPS-binding activity of these peptides were evaluated. The parental rabbit and human CAP18 peptides were selected as positive controls. Next, the changes in the secondary structure of these peptides before and after treatment with LPS were measured by circular dichroism (CD). Human cytotoxicity of the peptides was evaluated by MTT and red blood cells (RBCs) hemolysis assays. Finally, field emission scanning electron microscopy (FE-SEM), confocal microscopy, and flow cytometry were performed to study the action mechanism of these peptides. Results indicated that the hCap18 and rCap18 had antibacterial activity (at a MIC of 4–128 μg/mL). The results of the quantitative LAL test demonstrated that LPS-binding activity of hCap18 peptide was better than rCap18, while rCap18 peptide had better antimicrobial properties. Furthermore, rCap18 had less cytotoxicity than hCap18. However, both peptides were nontoxic for normal human skin fibroblast cell in MIC range. In conclusion, rCap18 has good antibacterial properties, while hCap18 can be tested as a diagnostic molecule in our future studies.

Keywords Antimicrobial peptides · Lipopolysaccharide · Cap18 · LPS-binding peptide · In silico drug design

Introduction

Septic shock arises from a cascade of molecular and cellular events following infection by microorganisms, predominantly Gram-negative bacteria [1]. Lipopolysaccharide (LPS) is the main cause of Gram-negative bacterial sepsis [2]. LPS consists of a lipid A component, a sugar moiety forming the core, and an O-polysaccharide of variable length [3]. In addition, LPS is one of the pyogenic agents in biological pharmaceutical products. Therefore, detection and removal of LPS from these products is one of the important requirements of the quality control (QC) department of a pharmacy factory. To detect LPS, two methods are widely employed in the pharmaceutical industry. The rabbit pyrogen test is the most widely used method [4]. The method entails parenteral administration of the product to a group of healthy rabbits, with subsequent monitoring of rabbit temperature using rectal probes. Another LPS detection method is in vitro limulus amebocyte lysate (LAL) test [4, 5]. LAL test is based on the endotoxin-stimulated coagulation of the amebocyte lysate obtained from horseshoe crabs [4, 5]. Although LAL test has a high sensitivity in pharmacy industry, there are limitations in demonstrating circulating LPS [6, 7]. The presence of inhibitors in

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plasma that bind to LPS can LPS invisible to LAL reagents [7]. Therefore, the LAL test is not routinely used to detect endoxemia [7]. Furthermore, this test is relatively expensive for laboratory research. Moreover, many environmentalists and researchers maintain that the biomedical industry has a negative effect on the horseshoe crab population. Therefore, trying to reach other diagnostic molecules for LPS detection is essential. AMPs are a part of the host innate immunity and produced by living organisms of all types [8]. Some AMPs, such as LL37and Cap18, can detect LPS and attach to it [9]. Cathelicidins are small, cationic antimicrobial peptides found in humans and other species like rabbit [10]. The cationic antibacterial protein of 18 kDa (CAP18) has LPS-binding activity. Originally, this protein was isolated from rabbit granulocytes [11]. A 32-mer sequence from C-terminal of this protein (amino acid residues 106–137) is called CAP18 peptide [12]. CAP18 peptide from this rabbit protein has the most antibacterial activity. This C-terminal fragment carries 2 negative and 15 positive residues and has a high-affinity binding site for heparin [13]. Additionally, CAP18-c (amino acid residues 137–162) is a LPS-binding domain in the C-terminal of human cathelicidins [10]. In this study, the changes in native CAP18 peptides were made to shorten their sequences. To compare the effect of the new peptides on native parental peptides, native rabbit and human CAP18 peptides were synthesized as positive controls. In the following, secondary structure, LPS-binding activity, antimicrobial effects, human cell toxicity of these peptides, and their effects on bacterial morphology as well as their cell surface were evaluated by FE-SEM, confocal microscopy, and flow cytometry.

Material and Methods

In Silico Design and Bioinformatics Studies

At first, the sequences of all rabbit and human CAP18 peptides were extracted from NCBI (https://www.ncbi.nlm.nih.gov/ 2019). Then, by ligand and protein interaction comparison and analysis or LPIcom software (http://crdd.osdd.net/raghava/lpicom/ 2019), important amino acids in interaction with LPS were predicted. LPIcom is a web server developed to understand protein-ligand interaction for almost all ligands available in the Protein Data Bank [14]. In the following, 15 first amino acid residues from rabbit CAP18 sequence and 16 primary residues from human CAP18 sequence were selected and mutated in some of their amino acids based on high frequent amino acids in the LBPs obtained from the LPIcom server. In addition, to increase the hydrophobicity of these sequences, a tryptophan (W) was added to N terminal of these peptides. Afterward, the probability of binding to the LPS of these peptides was predicted by the LPIcom software. Helical wheel projection was carried out to understand the position of amino acid residues in these peptides (http://lbqp.unb.br/NetWheels/ 2019). The probability of antimicrobial activity for these peptides was evaluated by machine learning algorithms such as support vector machine (SVM), random forest (RF), artificial neural network (ANN), and discriminant analysis (DA) from the $CAMP_{R3}$ server (http://www.camp.bicnirrh.res.in/ 2019). The threshold of each algorithm is between 0.5 and 1. The peptide is AMP if its score is > 0.5. Next, the physicochemical properties of these sequences were evaluated by the Protparm (https://web.expasy.org/protparam/ 2019) and Antimicrobial Peptide Calculator and Predictor software from the APD3 server (http://aps.unmc.edu/AP/prediction/prediction_main.php/ 2019).

Peptides Synthesis

rCap18 and hCap18 peptides as well as their native forms as positive controls were synthesized in Mimotopes Company (Clayton, Victoria, Australia) by a solid-phase method using N-9-fluorenylmethoxycarbonyl (Fmoc) chemistry, obtained with 95% purity by reversed-phase high-performance liquid chromatography (RP-HPLC), and their molecular weights and sequence accuracy were confirmed by mass spectrometry (Perkin Elm Co., Norwalk, CT, USA).

Bacterial Strains, Cell Line, and Chemical Compounds

Pseudomonas aeruginosa ATCC 27853, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, and *Bacillus subtilis* ATCC 6051 were used to conduct the antibacterial assay. All bacteria strains were stored in nutrient broth (NB) supplemented with glycerol (25%) and maintained at − 70 °C. Human skin fibroblast cell line (Hu02 cell line, National Cell Bank of Iran, Pasteur Institute of Iran) was used for cytotoxicity test. These cells are cryopreserved in liquid nitrogen at − 196 °C (90% FBS + 10% DMSO at approximately1 × 10^6 cells/vial). Nutrient broth (NB), nutrient agar (NA), Mueller-Hinton broth (MHB) and Mueller-Hinton agar (MHA), ethanol, phosphate-buffered saline (PBS), penicillin, and streptomycin were purchased from the Merck Millipore Company (Merck, Darmstadt, Germany), and polymixyn B sulfate provided from the Bio Basic Company (Bio Basic, Canada). Fetal bovine serum (FBS) and RPMI (Roswell Park Memorial Institute medium) medium were obtained from the Gibco Company (Gibco, England). Cell culture antibiotics (penicillin, streptomycin, and cyclosporin), trypsin, trepan blue dye, NaOH, HCl, MTT dye (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide), Triton-X 100 and glutaraldehyde, propidium iodide (PI), acridine orange, and dimethyl sulfoxide (DMSO) were purchased from the Sigma Company (Sigma-Aldrich, St. Louis, MO, USA).

In vitro Antimicrobial Assay

The antimicrobial efficacy of the different peptides was tested using a serial dilution titration method, to determine the peptides minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) against the different bacterial strains according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [15]. The bacteria were grown overnight at 37 °C in MHB and were diluted in the same medium. Two fold serial dilutions of the peptides were added to the microtiter plates in a volume of 100 μL, followed by addition of 100 μL of the bacteria to give a final inoculum of 5×10^5 colony-forming units (CFU)/mL. The plates were incubated at 37 °C for 24 h and 48 h, and the MICs were determined. Next, 100 μL of the 24-h inhibitory concentration test sample (MIC well) and its further concentrations was plated on MHA and incubated at 37 °C overnight to determine the minimal bactericidal concentrations (MBC) [16]. All the tests were conducted in triplicate, and their mean ± SD results were obtained.

Hemolytic Assay

To evaluate the lytic effects of the peptides on human erythrocytes, a hemolysis assay was conducted. Primarily, fresh blood samples were taken from a volunteer with blood type O. Next, 20% (vol/vol) suspension of the human erythrocytes in PBS was prepared, then, the suspension was diluted 1:20 in PBS, and 100 μL was added in triplicate to 100 μL of a 2-fold serial dilution series of peptide in a 96-well plate. As a positive control for 100% lysis of RBCs, 1% Triton-X 100 was added, and sterile 0.9% NaCl was added for negative control [17]. The plates were incubated at 37 °C for 1 h and centrifuged for 10 min at 3000 rpm (revolutions per minute). Then, 150 μL of the supernatant was transferred to a new 96-well plate to measure the absorbance at 414 nm using a microplate reader (STAT FAX 2100, USA). Eventually, the percentage of hemolysis was calculated as follows:

$$\text{Hemolysis}\% = \frac{\text{Mean OD of sample} - \text{Mean OD of negative control}}{\text{Mean OD of Positive control} - \text{Mean OD of negative control}}$$

Cytotoxicity Assay

To determine the toxicity of the designed peptides, Hu02 cell line was cultured at /1×10^5 cell/well in 96-well plates for 24 h under optimal conditions (37 °C, 5% CO2 in humidified incubator). Then, the growth media (10% FBS) were removed, and the cells were washed two times with PBS. The new maintenance RPMI medium (10% FBS) containing 0.5, 5, 50, 500, and 1000 μg/mL of each peptides were added, and the cells were incubated for 24, 48, and 72 h. Quintet wells were analyzed for each concentration, and column elusion buffer was used as control. A 10 μL solution of freshly prepared 5 mg/mL MTT in PBS was added to each well and allowed to incubate for an additional 4 h. In the following, the media were removed, and isopropanol was added at 100 μL/well. Plates were then shaken gently to facilitate formazan crystal solubilization. The absorbance was measured at 545 nm using a microplate reader (STAT FAX 2100, USA) [18, 19]. Then, percentage of cell toxicity was calculated as follows:

$$\text{Toxicity}\% = \left(1 - \frac{\text{Mean OD of sample}}{\text{Mean OD of control}}\right) \times 100\%$$

$$\text{Viability}\% = 100 - \text{Toxicity}\%$$

Evaluation of LPS Neutralization by the LAL Method

Percentage of neutralization of LPS by designed peptides was estimated using a limulus amebocyte lysate (LAL) with a Thermo Scientific™ Pierce™ LAL Chromogenic Endotoxin Quantitation Kit [20]. The protocol was followed to perform this experiment by the guidance provided in the kit. First, standard stock solutions in 0.50 EU/mL (0.05 ng/mL) from *Escherichia coli* endotoxin standard (011:B4) (available in LAL kits) were prepared by endotoxin-free water (EFW). In the following, 50 μL of 0.50 EU/mL standard solutions were added to the microtiter plate wells and incubated for 5 min at 37 °C. Next, serial dilutions of the peptides were added to the microtiter plates in a volume of 50 μL and mixed with LPS standard solution and incubated for 30 min at 37 °C. Fifty microliters of EFW and 50 μL of 0.50 EU/mL standard solutions without peptide treatment were used as negative and positive controls, respectively. Also, in this test, polymyxin B was used as a control. Then, 50 μL of LAL was added to each well using a pipettor, and the plate with the lid was covered and gently shaken on a plate shaker for 10 s. The plate was incubated at 37 °C for 10 min. After 10 min, 100 μL of chromogenic substrate solution was added to each well. The plate was incubated at 37 °C for 6 min. Afterward, 50 μL of stop reagent (25% acetic acid) was added to all wells. Finally, their absorbance was measured at 405–410 nm on a plate reader (STAT FAX 2100, USA), and the percentage of LPS neutralization by peptides at certain concentrations was calculated as follows:

$$\text{LAL/LPS binding}\% = \left(\frac{\text{Mean OD of sample-mean OD of negative control}}{\text{Mean OD of positive control-mean OD of negative control}}\right) \times 100\%$$

$$\text{LPS neutralization}\% = 100 - (\text{LAL} - \text{LPS binding}\%)$$

Evaluation of the Secondary Structure of Peptides and their Changes after Interaction with LPS by Circular Dichroism

The mean residue molar ellipticities of peptides and peptide-LPS mixture were determined by circular dichroism (CD) spectroscopy, using a Jasco J-810 spectropolarimeter CD (Jasco, Tokyo, Japan) at 25 °C with 200 nm/min scanning speed. In addition, 0.2–0.5 mg/mL of the aqueous solution of the peptides (and peptide-LPS) was loaded into a 1-mm quartz cell, and its spectra were scanned from 190 to 260 nm with five scans [18]. Furthermore, to study the changes of the secondary structure of peptides after treatment with LPS, 100 μL of 0.50 EU/mL LPS standard solutions with 100 μL of inhibitory concentrations of rCap18 and hCap18 were incubated at 37 °C for 1 hour.

. Field Emission Scanning Electron Microscopy

At first, *E. coli* ATCC 25922 were grown in NB and incubated at 37 °C for 24 h. The bacteria cells were harvested by centrifugation at 5000 rpm for 2 min and washed three times with PBS (pH 7.4). *E. coli* suspension of 10^7 CFU was incubated with 0.5X MIC concentration of hCap18 and rCap18 for 2, 4, and 6 h and precipitated at 5000 rpm for 2 min [21]. Next, bacteria pellet was washed twice with PBS (pH 7.4) and fixed 1 h (temperature of 25 °C, dark chamber) with 2.5% glutaraldehyde in 0.1 M PBS (pH 7.4). The samples were precipitated at 5000 rpm for 2 min, washed three times in distilled water, put on the glass slides (1 cm^2), and subsequently dehydrated with ethanol gradient (at 10, 30, 60, 70, and 90%). Finally, the dehydrated bacteria cells were dissolved in 100% ethanol for 15 min and dried at room temperature (25 °C). The *E. coli* cells were coated with gold nanoparticles using an automatic sputter coater [21]. The samples were then observed using a FE-SEM instrument (JSM-7610F, JEOL Ltd, Japan).

Propidium Iodide Absorption Assay

E. coli cells (ATCC 25922) were cultivated in a NB medium up to the mid-logarithmic phase and pelleted at 5000 rpm for 2 min. Then, the cells were washed, and their number was adjusted to 10^6 CFU/mL in the PBS buffer. The bacteria cells were incubated with 1X MIC of hCap18 and rCap18 peptides at 37 °C for 3 and 6 h. Afterward, the cells were washed in the PBS buffer and incubated with 1 μL of PI (1.3 μg/mL) at a temperature of 37 °C for 30 min in dark. The excitation was performed at a wavelength of 544 nm, and the resulting fluorescence was measured at 620 nm using a fluorescence spectrophotometer (CyFlow, Partec Co., Görlitz, Germany). The PI-treated and untreated bacteria cells were used as negative control and blank, respectively [21].

Acridine Orange/Propidium Iodide Double Staining Assay

To investigate effects of rCap18 and rCap18 peptides on the cell wall of bacteria, we performed acridine orange/propidium iodide (AO/PI) double staining assay followed by confocal microscopy, as previously described with some modifications [22]. For this purpose, mid-logarithmic growth phase *E. coli* cells (ATCC 25922) were diluted to 10^7 CFU/mL in PBS, added to a new tube containing the 0.5X MIC concentrations of peptides, and incubated at 37 °C for 1 h. In the following, 20 μL of dye mixture (100 μg/mL AO and 100 μg/mL PI in distilled water) was mixed with 100 μL of bacterial cell suspension on a clean microscope slide. The mixture was quickly evaluated by fluorescence microscopy (Leica TCS SP5 II laser scanning confocal microscope, Leica microsystem Inc., USA).

Statistical Analysis

Statistical analysis to compare MIC, hemolysis and toxicity data, and LPS-binding activity values of new peptides with native peptides and classic antibiotics was conducted by a *t* test by the SPSS Statistics 22.0 software (SPSS Inc. Chicago, IL, USA). The p values of < 0.05 were considered statistically significant.

Results

Design of hCap18 and rCap18 Peptides

Human and rabbit CAP18 peptide sequences were obtained from the NCBI. Rabbit and human CAP18 sequences used as the study pattern are RKRLRKFRNKKKIGQKIQ and DFFRKSKEKIGKEFKRIVQRIKDFLR, respectively. The information from the LPIcom server indicated that R, L, K, and F amino acids had the greatest possibility for binding to LPS. Subsequently, 15 first residues from the rabbit CAP18 (RKRLRKFRNKKKIGQ) sequence were selected and mutated in N9 (N9:L9) and G14 (G14:L14) positions. In return, 16 primary amino acid residues from the human CAP18 (DFFRKSKEKIGKEFKR) sequence were selected and mutated in D1 (D1:K1), S6 (S6:L6), K7 (K7:L7), E8 (E8:K8), G11 (G11:R11), and E13 (E13:K13) positions. Furthermore, to increase the hydrophobicity of these sequences, a tryptophan (W) was added to the N terminal of these peptides. Afterward, the probability of binding to the LPS of these peptides was predicted by the LPIcom software. The replacement of residues in new designed peptides was done based on high frequent amino acids in LPS-binding proteins that obtained by the LPIcom server. Therefore, hCap18 and rCap18 were obtained (Fig. 1a and b). By helical wheel projection, it was found that in hCap18, nonpolar amino acids are continuous

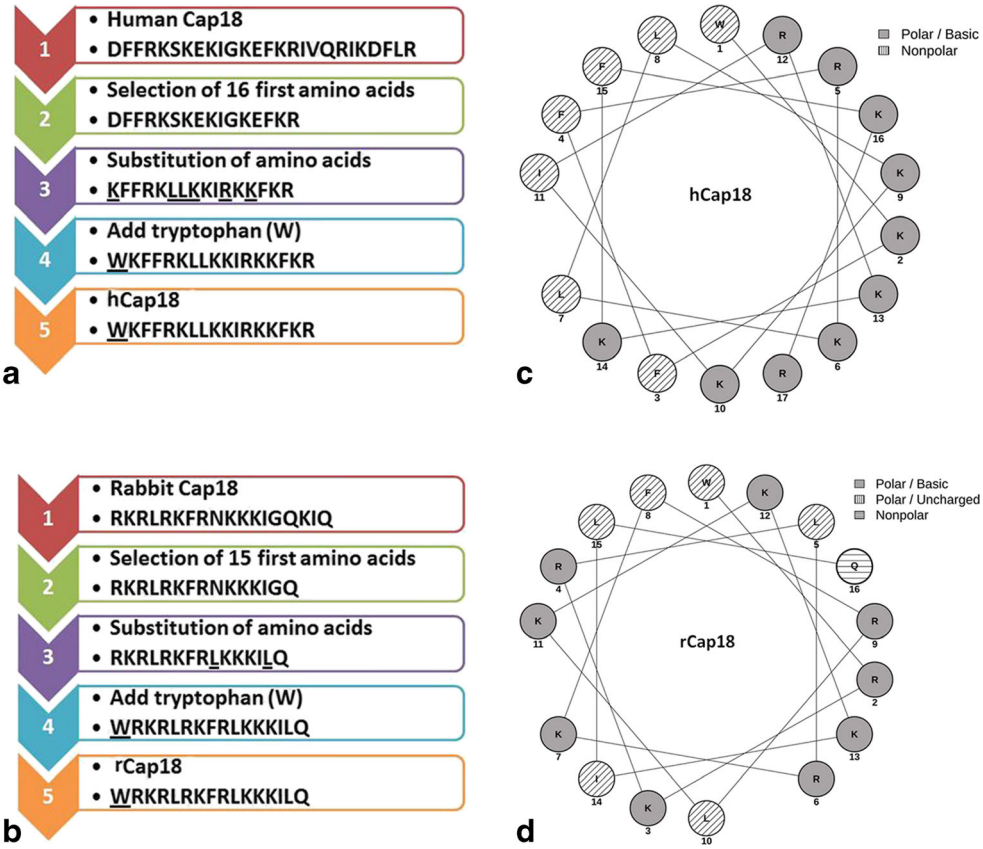

Fig. 1 Peptide design steps. This figure shows hCap18 (**a**) and rCap18 (**b**) peptides design steps as a flowchart. Also, the helical wheel projection for hCap18 (**c**) and rCap18 (**d**) has been shown at this illustration

and separated from polar amino acids. On the contrary, in rCap18, hydrophobic or nonpolar residues are dispersed between polar amino acids or hydrophilic faces. Therefore, .hydrophobic and hydrophilic faces in rCap18 were asymmetric, while in hCap18, these faces were symmetrical (Fig. 1c and d). Finally, the probability of the antimicrobial activity of hCap18, rCap18, and their parental peptide was predicted by machine learning algorithms. Table 1 reports the probability score and physicochemical properties of these peptides.

MIC and MBC Determination

The MICs and MBCs of hCap18, rCap18, and their native forms were assessed for selected bacteria (Table 2). Results indicated that native human CAP18 had no antibacterial effect against *P. aeruginosa* and *S. aureus*, while rCap18 was effective on all bacteria strain. The results of measuring these two parameters demonstrated that rCap18 had more antimicrobial properties than native Cap18R. However, rCap18 and hCap18

Table 1 The probability of antimicrobial activity and some of the peptide features

Name: sequence	Number of residues	Score of algorithms				Hyd[e] (%)	Nt[f]
		SVM[a]	RF[b]	ANN[c]	DA[d]		
Human CAP18: DFFRKSKEKIGKEFKRIVQRIKDFLR	26	0.85	0.68	AMP	0.98	34	+ 6
Rabbit CAP18: RKRLRKFRNKKKIGQKIQ	18	0.65	0.70	AMP	0.98	22	+ 10
hCap18: WKFFRKLLKKIRKKFKR	17	0.99	0.96	AMP	0.99	41	+ 10
rCap18: WRKRLRKFRLKKKILQ	16	0.98	0.82	AMP	0.96	37	+ 9

[a] Support vector machine

[b] Random forest

[c] Artificial neural network

[d] Discriminant analysis

[e] Hydrophobicity ratio

[f] Net charge

Table 2 MIC and MBC in μg/mL of new Cap18 peptides and native Cap18 peptides against the Gram-positive and Gram-negative bacteria

Agents	MIC/MBC (μg/mL)			
	B. subtilis	*S. aureus*	*E. coli*	*P. aeruginosa*
Rabbit CAP18	128 /256	512/512	64/64	> 1000
rCap18	4/4	16/32	4/8	32/32
Human CAP18	32/32	> 1000	32/32	512/512
hCap18	8 /16	32/32	16/16	16/16
Streptomycin	16/16	32/32	2/2	32/64
Penicillin	4/8	2/2	16/32	> 1000

showed good activity against *E. coli*, *B. subtilis*, *S. aureus*, *and P. aeruginosa* at a minimal inhibitory concentration (MIC) of 4–32 μg/mL. Statistical analysis indicated that the antibacterial activity of rCap18 on *E. coli* was significantly higher than native rabbit CAP18 and penicillin ($p \leq 0.001$). Furthermore, these analyses showed that antimicrobial effects of rCap18 in *S. aureus* were significantly more than those of streptomycin ($p \leq 0.01$) and rabbit CAP18 R. Only against *P. aeruginosa*, hCap18 was stronger than rCap18. Statistical analysis showed that the antibacterial activity of hCap18 in *E. coli*, *S. aureus*, *B. subtilis*, *and P. aeruginosa* was significantly higher than that of native human CAP18 ($p < 0.05$).

Hemolytic Assay

Hemolysis potency of peptides was measured by the hemolytic assay on human red blood cells (RBCs). The results showed that rCap18 had not any hemolytic effects, while hCap18 was very toxic on human RBCs. Statistical analysis showed that the hemolytic activity of rCap18 was significantly less than its native form rabbit Cap18 and hCap18 ($p < 0.001$). The hemolysis rate of hCap18 in its MIC range was more than 60% that was significantly higher than native human Cap18 ($p < 0.001$). This peptide lysed 95% of RBCs at the concentration of 1000 μg/mL. Generally, hemolytic effect of native human CAP18 was very less than hCap18. Figure 2a reports the hemolysis percentages of peptides at different concentrations. The results are the mean of 3 independent experiments.

MTT Assay Results

MTT assay was used to compare the toxicity between new designed peptides and natural peptides on the Hu02 cell line. Based on the toxicity/concentration chart, the IC_{50} of each peptide was calculated. The results showed that rCap18 was nontoxic at its MIC range, while native rabbit CAP18 peptide in this concentration had more than 10% toxicity on the Hu02 cells. Statistical analysis indicated that the toxicity effect of rCap18 was significantly less than rabbit Cap18 and hCap18 ($p < 0.001$). However, the IC_{50} of the rCap18 and rabbit CAP18 was > 1000 μg/mL. The toxicity of hCap18 at its MIC range was less than 3%. However, hCap18 is more toxic than native human CAP18 ($p < 0.05$). It can be concluded that both newly designed peptides are nontoxic at their MIC ranges. Furthermore, the IC_{50} of the rCap18 and hCap18 is > 1000 μg/mL. Figure 2b shows the toxicity percentage of peptides at different concentrations.

Result of Endotoxin Neutralization by Peptides

LAL (limulus amebocyte lysate) test is an efficient, quantitative assay to detect endotoxins. LPS catalyzes the activation of a proenzyme in the modified LAL test. In LAL test by chromogenic endotoxin quantitation kit in the presence of LPS a proenzyme is activated that catalyzes the splitting of *p*Nitroaniline (pNA) from the colorless substrate, Ac-Ile-Glu-Ala-Arg-pNA and generate a colored substrate. The experiment was conducted at the concentrations of 0.5 EU/mL (0.05 ng/mL) of LPS and 8 different peptide concentrations (from 0.78 to 100 μg/mL). The results showed that hCap18 was capable of neutralizing 100% of 0.5 EU/mL of LPS at the concentration of 25 μg/mL, while polymyxin B and native human CAP18 were neutralized 96.4% and 73% of 0.05 ng/mL of LPS at the same concentration, respectively. Statistical analysis revealed that the LPS neutralization activity of hCap18 was significantly higher than native human Cap18 ($p < 0.05$) and rCap18 ($p < 0.001$). rCap18 could neutralize a lower percentage of LPS than all of the peptides at the concentration of 25 μg/mL. rCap18 neutralized 6.75% of 0.5 EU/mL at this concentration. Figure 3 presents the results of the LPS neutralization by peptides.

Determination of Changes in Secondary Structure of rCap18 and hCap18 Peptides Before and After Interaction with LPS by CD

CD spectroscopy measurements were conducted to identify the secondary structure of hCap18 and rCap18 before and after treatment with the neutralizing concentrations of LPS to evaluate changes in the secondary structure of the peptides after interaction with LPS. The data obtained from CD were analyzed by the CAPITO web server [23]. Table 3 and Fig. 4 present the fraction ratio results of the secondary structure for pure peptides and peptides LPS complex as well as positive maximum and negative minimum peaks for each substance in curve format. As it is seen, changes in the secondary structure of rCap18 and hCap18 peptides after treatment with LPS are extremely clear. The results show that interaction of hCap18 with LPS caused secondary structure shift into helix. These changes reflect the effect of LPS on the peptides structure.

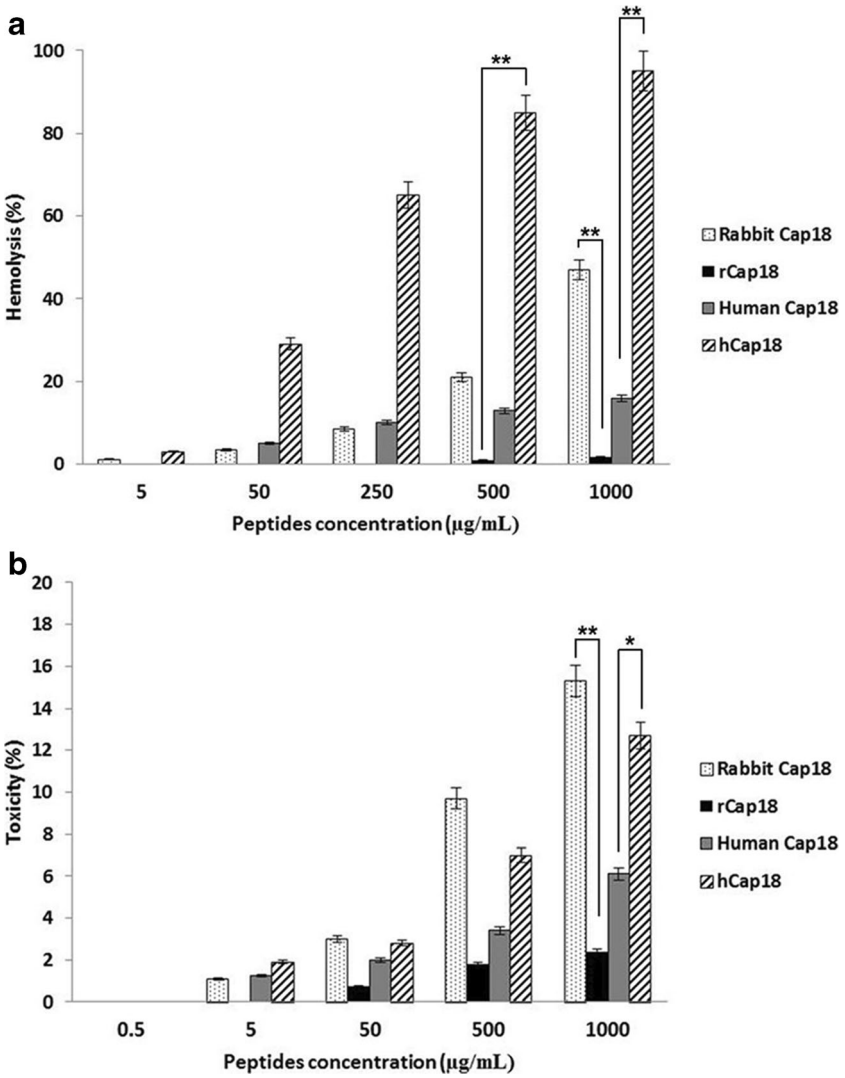

Fig. 2 Hemolytic and cytotoxic effects of peptides. These graph bars show the hemolytic percentage (**a**) and the toxicity percentage (**b**) at different concentrations. As it is clear, IC_{50} of all of the peptides are > 1000 μg/mL. The results are presented as mean ± standard deviation. Error bars indicate standard deviation. Statistical differences were shown by stars (*, $p < 0.05$; **, $p < 0.001$)

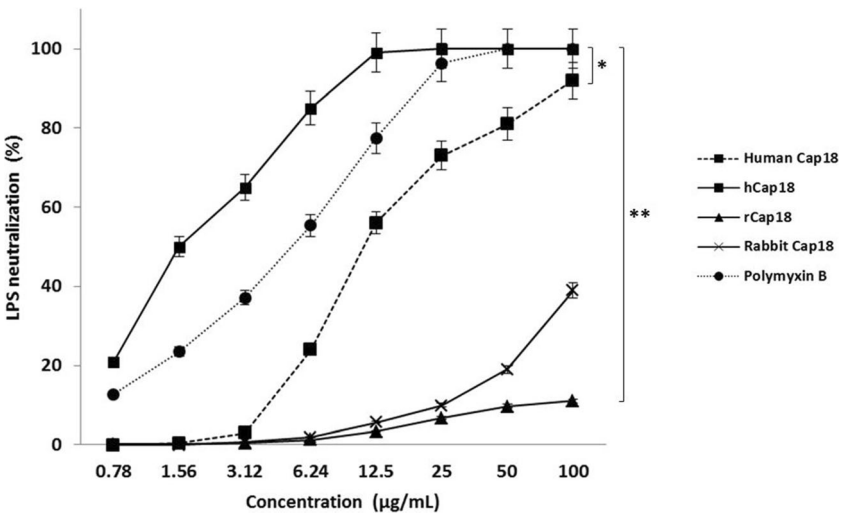

Fig. 3 LPS neutralization curve**.** This chart shows percentage of LPS neutralization by peptides for 0.5 EU/mL LPS standard solution. According to the curve, hCap18 is the most potent LPS-binding peptides. The experiment was repeated three times. Statistical differences were shown by stars (*, $p < 0.05$; **, $p < 0.001$)

Table 3 Fraction ratio of secondary structure of hCap18 and rCap18 before and after the treatment with LPS

Substances name	Fraction of ratio (%) of secondary structure		
	Helix	β-sheet	Random coil
hCap18	10.5	36.5	53
hCap18 + LPS	67	5	28
rCap18	27.4	19	53.6
rCap18 + LPS	32.8	17.5	49.7

Results of Morphology Studies by FE-SEM

FE-SEM is a high performance tool for studying the effects of antimicrobial peptides on the bacterial cell surfaces and morphology. *E. coli* ATCC 25922 without peptide treatments or negative control was intact. The result shows that both peptides at 0.5X MIC concentration affect the cell surface of the bacteria, but they are more intense and stronger in rCap18. Two hours after treatment of *E. coli* cells with hCap18 and rCap18, cell surface roughness and cell pores were created. After 4 h of treatment, the amount of cell surface shrinkage and porosity increased in *E. coli* cells. With 6-h treatment, some of the cells were completely lysed. A complete cell lysis was observed in treatment by rCap18 peptide. Figure 5 depicts the images of FE-SEM from *E. coli* cells before and after treatments by hCap18 and hCap18.

Study of Propidium Iodide Absorption by Flow Cytometry Assay

Since PI can only penetrate in to the dead cells with permeable walls and bind to their DNA, flow cytometry can be useful to investigate the action mechanism of peptides. The experiment was performed in two time intervals but was not repeated. The flow cytometry data indicated that 9.82% and 39.79% of the *E. coli* cells were shifted from quadrants 4 (Q4) to 2 (Q1) at 1X MIC (16 μg/mL) of hCap18 after 3 and 6 h, respectively. These results suggest that 39.79% of the bacteria were killed by the cell wall disruption at this intervals, while PI absorption was increased up to 61.40% at the 1X MIC (4 μg/mL) of rCap18 after 6 h. The rate of cell death in the negative control (without peptide and PI treatments) and the dye control (without peptide and with PI treatments) was 0.13% and 0.19%, respectively. Furthermore, rCap18 at 1X MIC was able to kill 36.41% of cells in 3 h. These results show that both peptides can create pores in the bacteria cells surface (Fig. 6).

Results of AO/PI Double Staining by Confocal Microscopy Studies

AO can enter both viable and nonviable cells (green fluorescence), while PI is taken up only by nonviable cells (red fluorescence). Therefore, using the AO/PI mixture staining, it is possible to stain cells with either intact or damaged cell wall or membrane simultaneously. The results of confocal microscopy studies indicated that hCap18 and rCap18 were able to damage *E. coli* cell surface coat. Therefore, like most antimicrobial peptides, the cell wall and cell membrane of the bacteria are the major targets for the action mechanism of hCap18 and rCap18 peptides (Fig. 7).

Discussion

Lipopolysaccharides (LPS), also known as endotoxins, are the integral components of the outer membrane of all Gram-negative bacteria [24]. Because LPS is responsible for pyrogenic reaction and septic shock, the tracing of LPS is very important for medical, pharmacological, and food safety [25]. Furthermore, LPS is a good target for antimicrobial agents against Gram-negative bacteria. The standard and major LPS detection method is the limulus amebocyte lysate (LAL) assay in the European Pharmacopoeia [26]. LAL test has some limitations, for example, LAL test is extremely expensive and time-consuming (it takes several hours). Moreover, other LAL-reactive materials such as β-(1–3)-D-Glucan can react with limulus amebocyte lysate, causing false positive answers [24, 27]. In addition, the massive slaughter of horseshoe crabs can create environmental problems and will even lead to extinction of their generation [7]. Therefore, trying to find new LPS-binding molecules is essential. Research has indicated that some AMPs such as LL-37, Indolicidin, and CAP18 possess antibacterial properties and LPS-binding

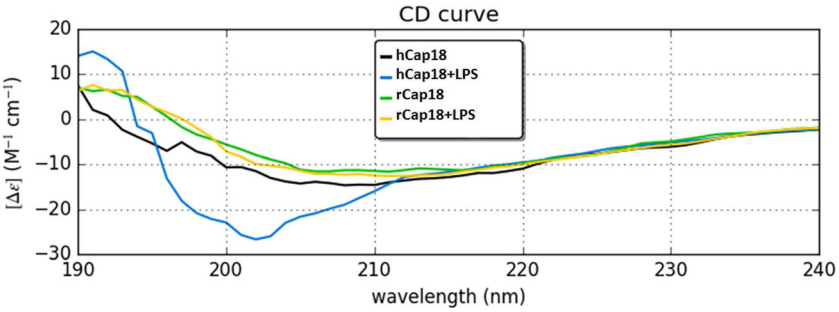

Fig. 4 CD spectra of peptides before and after interaction with LPS. The illustration indicates that the secondary structure of rCap18 and hCap18 peptides were changed after treatment with LPS

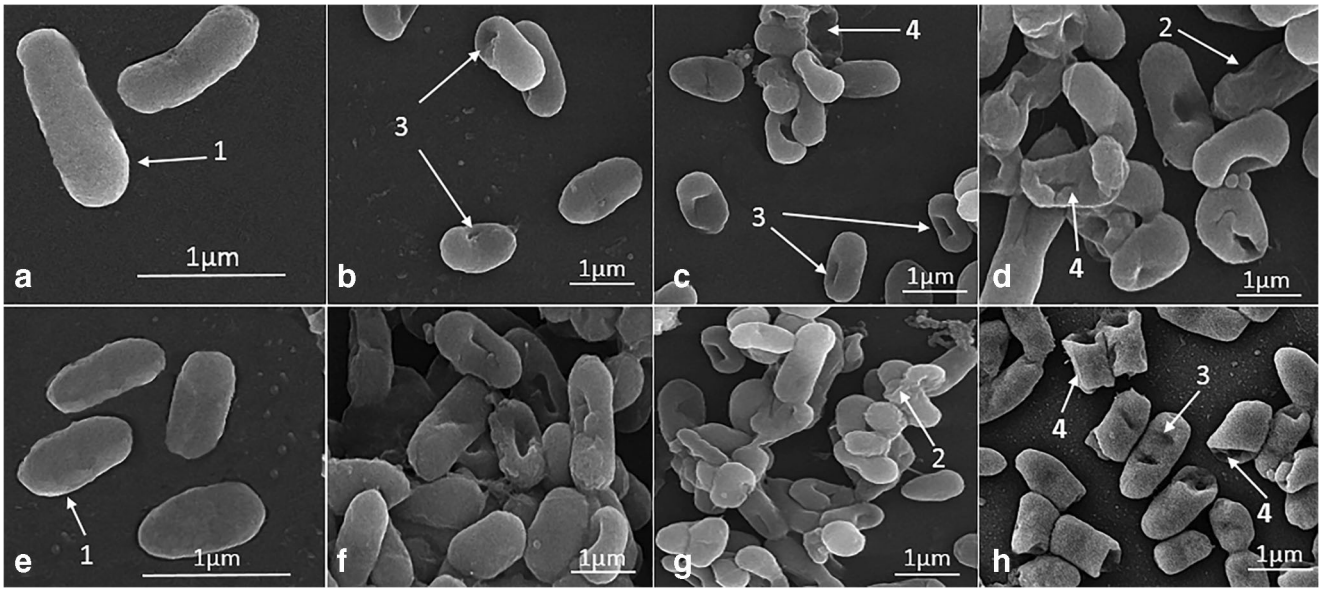

Fig. 5 FE-SEM studies. FE-SEM images (**b–d**) show *E. coli* following treatment with 0.5X MIC (8 μg/mL) of hCap18 at different intervals (**b**, 2 h after treatment; **c**, 4 h; and **d**, 6 h). **a** and **e** are as negative control (without peptide treatment). Images **f** to **h** demonstrate *E. coli* treatment with 0.5X MIC (2 μg/mL) of rCap18 after 2, 4, and 6 h, respectively. The tags include (1) intact cells, (2) cell surface shrinkage, (3) cell pores, and (4) full cell lysis

activity [28].To simply design antimicrobial peptides, computer-based methods can be used [29]. In this project, we used peptide truncation, targeted mutation, the LPIcom server data, and machine learning algorithms to predict antimicrobial activity of the designed peptides. The bioinformatics logic in the design of peptides was the use of knowledge-

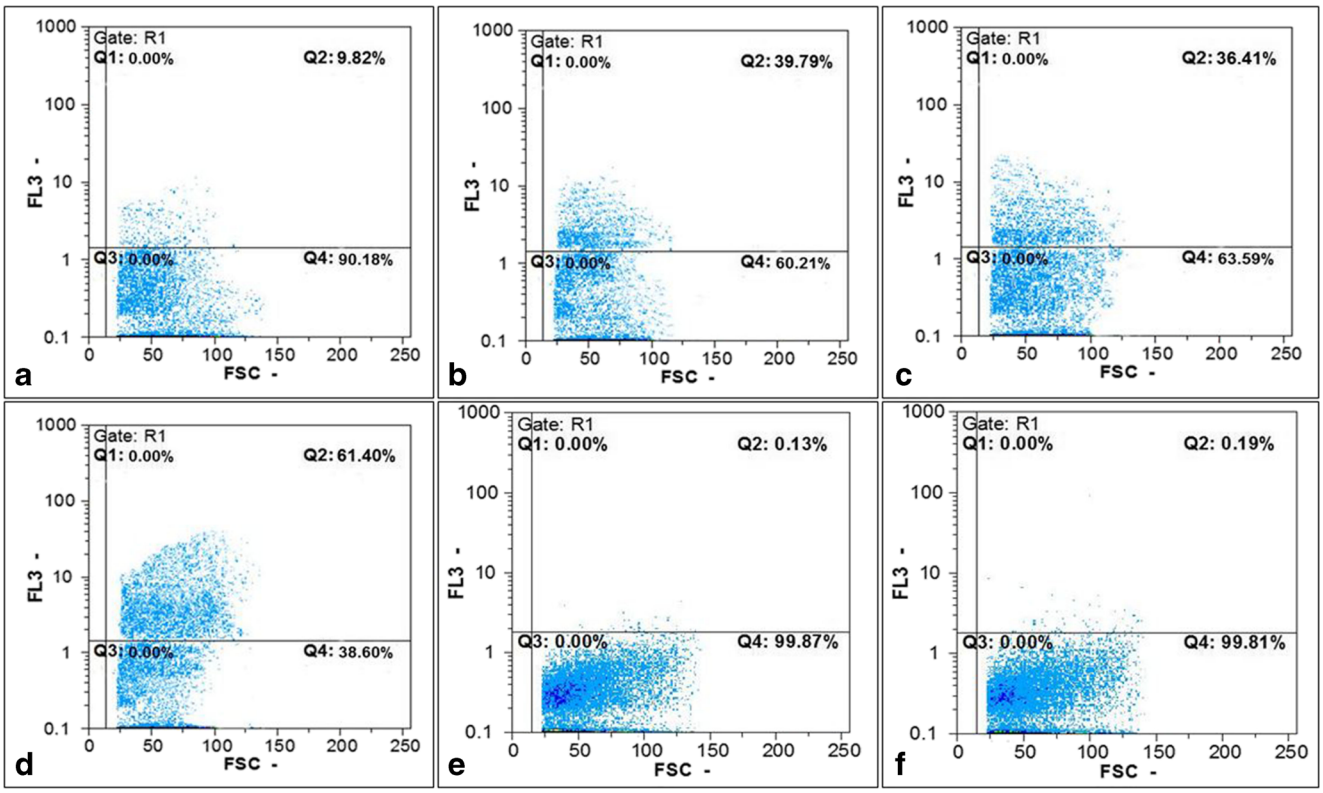

Fig. 6 Result of PI absorption assay. This illustration shows the amount of PI absorption by *E. coli* before and after treatment with peptides at the concentrations of 1X MIC by flow cytometry. Images include (**a** and **b**) 3- and 6-h treatment with hCap18, (**c** and **d**) 3- and 6-h treatment with rCap18, (**e**) negative control, and (**f**) dye control

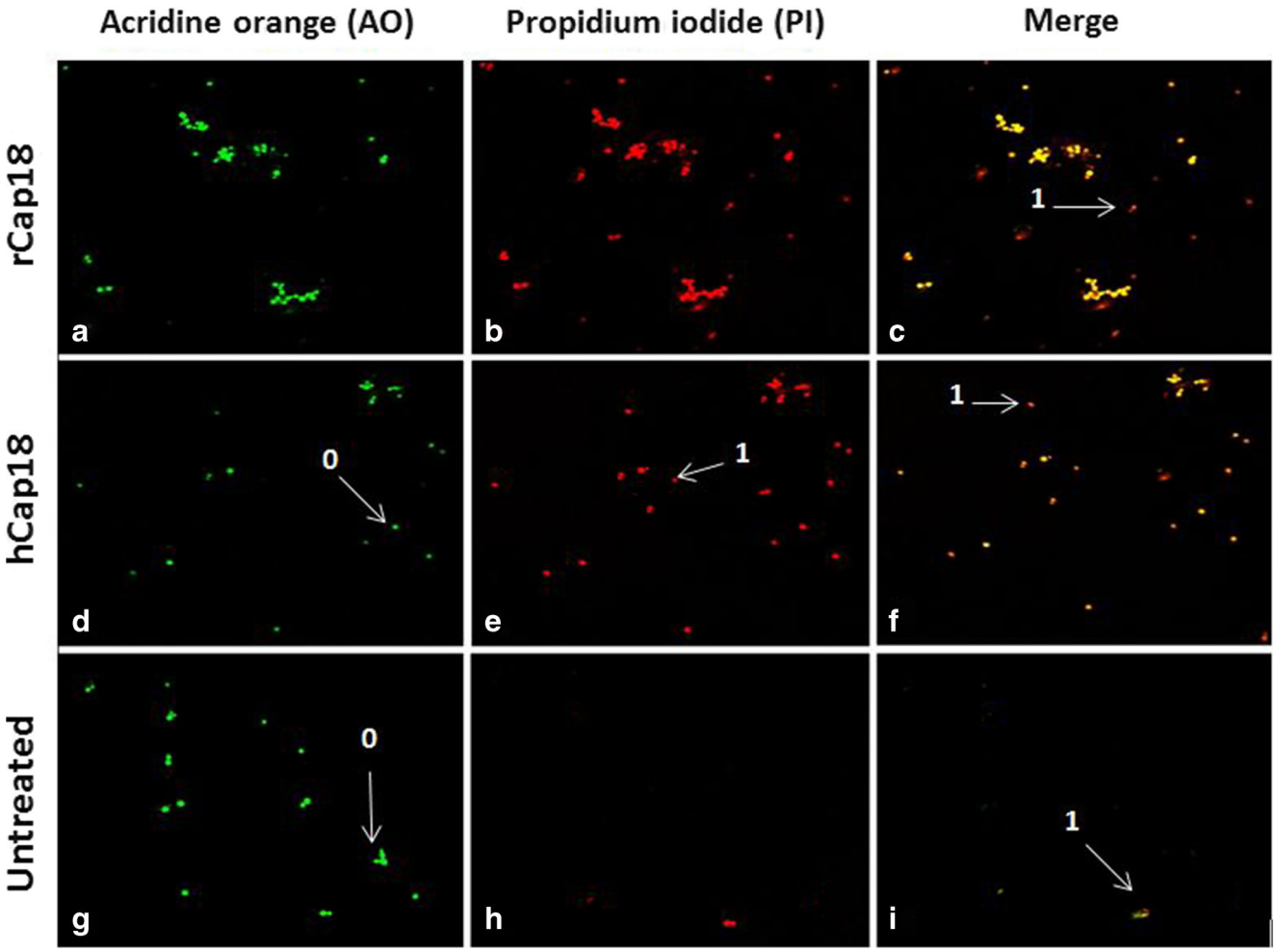

Fig. 7 AO/PI double staining assay. Confocal microscopy images show *E. coli* following treatment with the 1X MIC (16 μg/mL) of hCap18 (**a**–**c**) and rCap18 (**d**–**f**) (4 μg/mL). The images of **g**–**i** are as the negative control (without peptide treatment). The first and second columns demonstrate AO and PI stains, respectively, while the third column shows the merge state of AO and PI stains. The tags include (0) intact cells and (1) damaged cells. The experiment was repeated three times

based design strategy, and all mutations in the initial design of the peptides were created based on high frequent amino acids in LPS-binding peptides. Furthermore, according to the studies conducted by Ghos et al., to increase hydrophobicity and LPS-binding activity, tryptophan (W) was added to the N terminal of both peptides [20]. Both native rabbit CAP18 and native human CAP18 peptides are relatively long, and their synthesis is expensive, while our designed Cap18 peptides are shorter than native Cap18 peptides, and their synthesis is more economic [9, 10]. The increase of the hydrophobic ratio in the nonpolar face of the peptides to increase the symmetry in hydrophobic and hydrophilic faces can enhance their hemolytic and anticancer activity [18, 30, 31]. In our project, helical wheel projection showed that rCap18 has asymmetrical faces, while hydrophobic and hydrophilic faces in hCap18 are symmetric. Antimicrobial tests indicated that rCap18 and hCap18 had more antibacterial effects than their native forms. These results indicated that rCap18 had more antimicrobial properties than hCap18. Larrick et al. designed a truncated rabbit CAP18 peptide with 9 amino acid residues ($Cap18_{106–114}$) that was inactive to neutralize 100 μg/mL of LPS, while rCap18 inhibited more 11% of the 0.5 EU/mL of LPS at the concentration of 100 μg/mL peptide [9]. In another study conducted by Larrick et al., human CAP18 was truncated to a new derivative with 32 residues ($huCap18_{104–135}$). This derivative was more active than native $Cap18_{104–140}$ against the bacteria [32]. The minimum concentration of this derivative ($huCap18_{104–135}$) to neutralize 100 μg/mL of LPS was 1.6 μg/mL that was measured by the MAC (minimum agglutination concentration) parameter [32]. However, the minimum concentration of hCap18 to neutralize 0.05 ng/mL of LPS was 0.78 μg/mL. hCap18 was capable of neutralizing 100% of 0.05 ng/mL of LPS at the concentration of 25 μg/mL, while best peptide in Kaconis et al. studies could not neutralize 100% at the concentration of 1 ng/mL of LPS [33]. It is necessary to mention that the measurement of the MAC parameter by LPS-coated RBC is an old assay having much lower accuracy than the LAL test. Nevertheless, the

LPS-binding activity of hCap18 is better than rCap18 and native peptides. Therefore, hCap18 is a potent LPS-binding peptide. In order to investigate the LPS inhibition by peptides or other agents, the measurement of inflammatory cytokines such as TNF-α and in vivo tests can be more useful and give us more accurate information [34, 35]. MTT assay and hemolysis assay determined that although native rabbit CAP18 was very toxic, its designed derivative (rCap18) was nontoxic in its MICs range, while hCap18 was the most toxic peptide. Regarding the toxicity of hCap18 and its high binding power to LPS, it can be concluded that this peptide can only be tested as a diagnostic molecule (not as a therapeutic molecule) in next our studies, but rCap18 has the potential to be used as an antimicrobial peptide. The results of the CD spectra for Aurein1.2 antimicrobial peptide indicate that the peptides are as random coils in aqueous solution and adopt an α-helical conformation in the amphipathic solution such as 2,2,2-Trifluoroethanol (TFE) [36]. LPS has an amphipathic structure, and it can mimic the cell membrane. In our study, the results of CD demonstrate that the secondary structure of hCap18 and rCap18 changed after interaction with LPS. Our CD studies for hCap18 and rCap18 in the absence of LPS showed that there was a random coil spectrum for both peptides, but after treatment of peptides by LPS, helix content was increased in hCap18 (67%) and rCap18 (32.8%).Therefore, in solutions containing LPS that can simulate membrane, the secondary structure of hCap18 and rCap18 shifts to α-helix. The mode of action section studies such as FE-SEM, PI absorption, and AO/PI double staining suggests that like most antimicrobial peptides, hCap18 and rCap18 target the cell membrane and cell wall of bacteria. Obviously, other action mechanisms of these peptides should be considered in separate studies.

Conclusion

In this project, we designed two truncated derivatives from rabbit and human CAP18 peptides. By in silico methods, we could design and modify two CAP18 derivatives with good antibacterial activity. In addition, hCap18 showed very good LPS-binding properties. The minimum concentration of hCap18 to inhibit 0.5 EU/mL of LPS is 25 μg/mL. Although hCap18 peptide has good LPS neutralization activity, this peptide can only be tested as a diagnostic molecule, since hCap18 is very toxic. Since rCap18 is non-toxic and has good antibacterial activity, it can considered as antibacterial peptide for therapeutic goals. However, to find these goals, more studies are needed into the action mechanism, serum stability and in vivo activity of this peptide.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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