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Synergism of Leu–Lys rich antimicrobial peptides and chloramphenicol against bacterial cells

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Abstract

To investigate the antibiotic activity and synergistic effect, analogues were designed to increase not only net positive charge by Lys-substitution but also hydrophobic helix region by Leu-substitution from CA (1–8)-MA (1–12) hybrid peptide (CA-MA). In particular, CA-MA analogue P5 (P5), designed by flexible region (GIG→P)-substitution, Lys- (positions 4, 8, 14, 15) and Leu- (positions 5, 6, 12, 13, 16, 17, 20) substitutions, showed potent antibacterial activity in minimal inhibition concentration (MIC) and minimal bactericidal concentration (MBC) without having hemolytic activity. In addition, P5 and chloramphenicol has potent synergistic effect against tested cell lines. As determined by propidium iodide (PI) staining, flow cytometry showed that P5 plus chloramphenicol-treated cells had higher fluorescence intensity than untreated, P5- and chloramphenicol-treated cells. The effect on plasma membrane was examined by investigating the transmembrane potential depolarizing experiments of *S. aureus* with P5 and chloramphenicol. The result showed that the peptide exerts its antibacterial activity by acting on the plasma membrane. Furthermore, P5 caused significant morphological alterations of *S. aureus*, as shown by scanning electron microscopy. Our results suggest that peptide P5 is an excellent candidate as a lead compound for the development of novel antiinfective agents and synergistic effects with conventional antibiotic agents but lack hemolytic activity.

Keywords: Synergistic effect; Positive charge; CA (1–8)-MA (1–12) hybrid peptide; CA-MA analogue P5; Minimal bactericidal concentration; Scanning electron microscopy

1. Introduction

The emergence of clinical bacterial strains exhibiting resistance against conventional antibiotics has urged the search for novel antibiotic agents. Among the compounds that are currently under investigation for their therapeutic potential are a number of antimicrobial peptides of the innate immune system, and their synthetic derivatives. Widely distributed in nature, antimicrobial peptides are an essential defense component of invertebrates and vertebrates, destined to control cell proliferation and invading pathogens [1,2]. Among the more potent of these compounds are small bioactive peptides such as cecropin A (CA), magainin 2 (MA), melittin (ME) and plant defensin [3].

CA, a cationic 37-amino acid antimicrobial peptide was isolated from *Hyalaphora cecropia* pupae [4] and MA, a cationic 23-amino acid antimicrobial peptide was discovered from the skin of the African clawed frog, *Xenopus laevis* [5]. Both CA and MA exhibit strong antibacterial activity but no cytotoxicity against normal mammalian cells. One of the main targets for these peptides is the lipid bilayer of the cytoplasmic membrane [6]. Permeabilization of the membrane leads to dissipation of the transmembrane potential, allowing the cell contents to be released, finally resulting in cell death [7]. Cell lysis by these peptides requires two steps: initial binding to the cell surface, followed by membrane permeabilization [8].

Numerous studies using synthetic peptides has been focused on designing analogue peptides having more potent antimicrobial activity than that of natural peptides without damaging against mammalian cells [9,10]. Several attempts have been made to improve antimicrobial activity against bacterial cells while eliminating the cytotoxicity against mammalian cells such as red blood cells by flexible region [11], chain length

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[12], chaining the net charge [13,14], hydrophobicity [15] and/or α-helicity [16].

In our previous study, novel analogue peptides by chain length deletion and increasing net positive charge and hydrophobicity were designed and synthesized from the sequence of CA-MA [17]. In the present study, we tested the antibacterial effect of these synthetic peptides against Gram-positive and Gram-negative bacteria, as well as their synergism with the antibiotic agent, chloramphenicol. Finally, the antibacterial effect of these peptides on bacterial cells was examined by fluorescence activated flow cytometric analysis and scanning electron microscopy (SEM).

2. Materials and methods

2.1. Peptide synthesis

Peptides (Table 1) were synthesized by the solid phase method using Fmoc(9-fluorenyl-methoxycarbonyl) chemistry [18]. Peptide concentrations were determined by amino acid analysis. The molecular weights of the synthetic peptides were determined using a matrix-assisted laser desorption ionization MALDI-mass spectrometer (data not shown) [19].

2.2. Microbial strains

Streptococcus aureus (KCTC 1621), *Bacillus subtilis* (KCTC 1918), *Streptococcus epidermidis* (KCTC 3096), *Proteus vulgaris* (KCTC 2433), *Pseudomonas aeruginosa* (KCTC 1637) and *Salmonella typhymurium* (KCTC 1926) were obtained from the Korean Collection for Type Cultures (KCTC), Korea Research Institute of Bioscience and Biotechnology (KRIBB), Taejon, Korea.

2.3. Antibiotic activity

Microdilution assays to establish minimal inhibition concentration (MIC) and minimal bactericidal concentration (MBC) values of synthetic peptides were performed. Cells were grown to mid-phase in 10 g/l bactotryptone, 5 g/l yeast extract, 10 g/l NaCl, pH 7.0. The peptides were filtered through a 0.22-μm filter and stepwise-diluted in a medium of 1% bactopeptone. Each organism to be tested was suspended at 2×10^6 colony formation units (CFU)/ml in growth medium, and 100 μl was mixed with 100 μl of each two-fold serial solution of peptide in a microtiter plate well; there were three replicates for each test sample. The plates were incubated for 18 h at 37 °C. The MIC was defined as the lowest concentration of peptide that gave no visible growth on the plate [17]. MBC was evaluated from the same test by viable counting assay and defined as the lowest concentration of peptide that killed 99.9% of the test inoculum [20].

Table 1
Amino acid sequence and molecular masses determined by MALDI-MS of CA-MA hybrid peptide and its analogues

Peptides	Amino acid sequence
CA-MA	KWKLFKKIGIGKFLHSAKKF-NH_2
P1	KWKLFKKIGIGKFL-NH_2
P2	KKKKWKLFKKIGIGKFL-NH_2
P3	KWKLFKKIGIGKFLKKK-NH_2
P4	KWKKKKKKPKFL-NH_2
P5	KWKKLLKKPLLKKLLKKL-NH_2
P6	KWKLKPLLKKLLKKL-NH_2
P7	KWKKLLKKPLKLKL-NH_2
P8	KPLLKKLLKKL-NH_2
P9	KLLKKPLKLKL-NH_2
P10	KWKKLLKKPLLKK-NH_2
P11	KWKKPLLKKLLKKL-NH_2

2.4. RBC preparation and hemolytic activity

Human red blood cells were centrifuged and washed three times with phosphate-buffered saline solution, which is 35 mM phosphate buffer with 0.15 M NaCl at pH 7.0. The hemolytic activities of the peptides were evaluated by determining the hemoglobin release of 8% suspensions of fresh human erythrocytes at 414 nm. One hundred μl aliquots of the 8% suspended red blood cells were transferred to the 96-well plates. Hemolysis was measured by absorbance at 414 nm with an ELISA plate reader (Molecular Devices Emax, Sunnyvale, CA, USA). Zero percent hemolysis and 100% hemolysis were determined in PBS and 0.1% Triton-X 100, respectively. The hemolysis percentage was calculated using the following equation: % hemolysis = [($Abs_{414\ nm}$ in the peptide solution − $Abs_{414\ nm}$ in PBS)/($Abs_{414\ nm}$ in 0.1% Triton-X 100 − $Abs_{414\ nm}$ in PBS)] × 100.

2.5. Hemolytic effect of the peptide under RBC morphology

RBCs were incubated at 37 °C for 1 h with 50% MIC of P5 and melittin. Negative controls were run without peptides. The RBCs were fixed with equal volumes of 4% glutaraldehyde and 1% paraformaldehyde in 0.05 M cacodylate buffer (pH 7.2). After lyophilization and gold coating, the samples were examined on a HITACHI S-2400 (Tokyo, Japan).

2.6. Effects of two-agent combinations on cell growth

The effects of two-agent combinations were analyzed as described previously [21]. For each peptide–drug combination tested, a 96-well plate was filled with Mueller–Hinton broth. Test peptide was diluted twofold from column 1 to column 8 of each deep-well. Two fold dilutions of drugs were then added to deep-well plate containing a specific test peptide. The final concentration of each strain to be tested was approximately 5×10^5 CFU/ml. The plates were incubated at 37 °C for 18 h. The MIC of each drug was determined as the lowest concentration that inhibited the growth of the organism, FICs were determined by the following formula: (MIC of peptide in combination/MIC of peptide alone) + (MIC of drug × in combination/MIC of drug × alone), where x is any of the drugs used in combination with peptide. Synergism was defined as FIC index <0.5, antagonism was defined as FIC index >4.0, and indifference was defined as an average FIC index from >0.5 to 4.0.

2.7. FACScan analysis

For analysis of membrane integrity after peptide treatment, 2×10^6 cells harvested at log phase were mixed with P5 and incubated for 30 min at 28 °C with constant shaking (140 rpm). The cells were harvested by centrifugation, washed three times with PBS, and incubated with propidium iodide (PI, 50 μg/ml final concentration) at 4 °C for 30 min, followed by removal of the unbound dye through washing with an excess of PBS. Flow cytometry was performed using a FACScan (Becton Dickinson, San Jose, CA).

2.8. Transmembrane potential depolarization assay with S. aureus

S. aureus was grown at 37 °C with agitation to mid-log phase. The cells were washed once with buffer A (20 mM glucose, 5 mM HEPES, pH 7.3) and resuspended to an OD600 of 0.05 in a buffer A containing 0.1 M KCl. The cells were incubated with 1 μM DiS-C_3-5 until a stable baseline was achieved. Experiments were performed in sterile 96-well plates in a final volume of 200 μl. The peptides were dissolved in the buffer A and added to achieve the desired concentration. Membrane depolarization was monitored by an increase in the fluorescence of DiS-C_3-5 (excitation wavelength: 622 nm, emission wavelength: 670 nm) after the addition of different concentrations of peptides.

2.9. Scanning electron microscopy (SEM)

Bacteria cells were suspended at 108 CFU/ml in Na-phosphate buffer, pH 7.4, supplemented with 100 mM NaCl (buffer A), and incubated at 37 °C with peptides and chloramphenicol. Controls were run in the absence of peptide and