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Enhancement of the antimicrobial activity and selectivity of GNU7 against Gram-negative bacteria by fusion with LPS-targeting peptide

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ABSTRACT

Antimicrobial peptides (AMPs) provide a potential source of new antimicrobial therapeutics for the treatment of multidrug-resistant pathogens. To develop Gram-negative selective AMPs that can inhibit the effects of lipopolysaccharide (LPS)-induced sepsis, we added various rationally designed LPS-targeting peptides [amino acids 28–34 of lactoferrin (Lf28–34), amino acids 84–99 of bactericidal/permeability increasing protein (BPI84–99), and de novo peptide (Syn)] to the potent AMP, GNU7 (RLLRPLLQLLKQKLR). Compared to our original starting peptide GNU7, hybrid peptides had an 8- to 32-fold improvement in antimicrobial activity against Gram-negative bacteria, such as *Escherichia coli* and *Salmonella typhimurium*. Among them, Syn-GNU7 showed the strongest LPS-binding and -neutralizing activities, thus allowing it to selectively eliminate Gram-negative bacteria from within mixed cultures. Our results suggest that LPS-targeting peptides would be useful to increase the antimicrobial activity and selectivity of other AMPs against Gram-negative bacteria.

1. Introduction

Multidrug-resistant (MDR) bacterial infections have emerged as one of the world's greatest health threats. Infections caused by MDR pathogens are a major burden to modern healthcare as a result of high morbidity/mortality rates and the higher treatment cost of MDR infection [2,9]. Among all of the bacterial resistance problems, Gram-negative pathogens are particularly worrisome because they are becoming resistant to most currently available antibacterial treatments. The development of novel antibiotics to treat MDR pathogens, however, has stagnated over the last half century. In particular, the pharmaceutical pipeline of antibiotics active against MDR Gram-negative bacteria is very limited [4,42]. Therefore, more antimicrobial research is required to overcome this critical deficit.

Antimicrobial peptides (AMPs) isolated from organisms across the phylogenetic spectrum form part of the innate immune system and serve as the first line of defense against invading microbes [6,44]. Despite the diversity in their structure and amino acids sequences, AMPs can be defined as short (less than 50 amino acids) peptide possessing overall positive charge (in general, +2 to +9) and large percentage (≥30%) of hydrophobic amino acids [16]. These properties permit the peptides to fold into amphipathic conformation upon contact with negatively charged bacterial membranes. Unlike conventional antibiotics which operate on specific targets, many AMPs physically and rapidly permeate and destroy the bacterial membrane or disrupting essential components inside the cells, thus making it difficult for the bacteria to develop resistance [25,26]. AMPs are effective at low micromolar concentrations against a broad range of microorganisms, including in many cases those resistant to conventional antibiotics [29]. Because of these properties, AMPs are considered to be potential alternatives to conventional antibiotics [24,35]. We previously demonstrated that GNU7 (RLLRPLLQLLKQKLR), a de novo generated short AMP, display broad activities against bacteria and fungi, including clinically isolated drug-resistant strains under physiological salt and serum conditions without causing human cell cytotoxicity [20]. Importantly, because GNU7 did not contain either non-natural or chemically modified amino acids, GNU7 can be produced in a cost-effective manner in biological expression systems. These features make GNU7 a promising candidate to be developed as novel antimicrobial agents. However, preliminary study showed that GNU7 had relatively low activity against Gram-negative bacteria compare

Abbreviations: MDR, multidrug-resistant; AMP, antimicrobial peptide; LPS, lipopolysaccharide; MIC, minimal inhibitory concentration; LAL, limulus amebocyte lysate.

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to Gram-positive bacteria. Therefore, the antimicrobial activity of GNU7 against Gram-negative bacteria should be enhanced to fully exploit its therapeutic potential as anti-Gram-negative agents.

In this study, we used a specific targeted AMP (STAMP) strategy to enhance the antimicrobial activity of GNU7 against Gram-negative bacteria. STAMP strategy is based on the construction of a fusion peptide by combining two functionally independent components, namely, a targeting domain and a killing domain, using a short flexible linker [11,12,17]. The targeting domain gives selectivity to the killing domain by binding to the pathogen using specific determinants on pathogen surface, such as membrane hydrophobicity, charge, pheromone receptors, cell wall components, or characteristic virulent attributes. Lipopolysaccharide (LPS) is the major constituent of the outer membrane of Gram-negative bacteria. LPS is essential for bacterial survival through establishing an efficient permeability barrier against a variety of antimicrobial compounds including hydrophobic antibiotics, detergents, and AMPs [32,38]. Apart from its role as the permeability barrier function, LPS is also known as an endotoxin, a potent inducer of innate immune system. Intrinsically, LPS-induced activation of innate immune system is essential to counteract invading pathogens. However, under severe Gram-negative infections or extensive antibiotic therapies, deregulated immune response promotes the release of large excess of cytokines [7,30]. Uncontrolled production of these cytokines may cause septic shock, characterized by hypotension, coagulopathy, and multiple organ failure, often resulting in death [27]. Thus, LPS is one of the most promising targets for the development of anti-Gram-negative agents. We hypothesized that addition of LPS-targeting peptide to GNU7 would substantially potentiate both its selectivity against Gram-negative bacteria and LPS-neutralizing activity. To address this prediction, we designed hybrid peptides via addition of various LPS-binding peptides to GNU7, and analyzed the antimicrobial, LPS-binding, and LPS-neutralizing activities of the hybrid peptides. The selectivity of the hybrid peptides against Gram-negative bacteria was also assessed by time-kill experiments in dual-species cultures.

2. Materials and methods

2.1. Microorganisms

The bacterial strains used in this study were obtained from the American Type Culture Collection (ATCC) and the Korean Collection for Type Cultures (KCTC), and included: *Micrococcus luteus* ATCC 10240, *Staphylococcus aureus* ATCC 15752, *Escherichia coli* KCTC 2223, and *Salmonella typhimurium* KCTC 2370.

2.2. Peptide preparation

Peptides used in this work were synthesized by the standard Fmoc-solid phase peptide synthesis protocol at Peptron (Daejeon, Korea). Synthesized peptides were purified to over 95% by reversed-phase high-pressure liquid chromatography (HPLC) on a capcell pak C18 column (Shiseido, Tokyo, Japan), and their molecular weights were further confirmed by liquid chromatography/mass spectrometry (HP 1100 series LC/MSD, Hewlett-Packard, Palo Alto, CA, USA). The peptide content of lyophilized samples was determined by quantitative amino acid analysis with a Pico-tag system on a Beckman 121 MB amino acid analyzer (Beckman Coulter, Fullerton, CA, USA).

2.3. Antimicrobial activity assay

The antimicrobial activity of each peptide was determined using the broth microdilution assay as described by Park et al. with a slight modification [28]. Briefly, mid-logarithmic phase cells were diluted to 1×10^6 cfu/ml in phosphate buffered saline (PBS) to mimic physiological salt concentration. Each well of 96-well propylene microtiter plates (Costar, Cambridge, MA, USA) was filled with 50 μl of the diluted cell suspension and 50 μl of serially diluted peptide samples, and incubated at 37 °C for 3 h to minimize interference with the peptide's biological activity. Then, fresh medium [tryptic soy broth (TSB)] was added to the mixture and incubated for an additional 18 h at 37 °C. Controls were done without peptides. The inhibition of growth was determined by measuring absorbance at 595 nm with a Model 680 Microplate Reader (Bio-Rad, Hercules, CA, USA). The lowest concentration of peptide that completely inhibited growth was defined as the 'minimal inhibitory concentration' (MIC). The MIC values were calculated as the average of three independent experiments performed in triplicate.

2.4. Hemolysis assay

Hemolytic activity was assayed as described by Jang et al. with a slight modification [19]. Briefly, three milliliters of freshly prepared human red blood cells (RBCs) were washed with PBS, pH 7.4, until the color of the supernatant turned clear. The washed RBCs were then diluted to a final volume of 20 ml with the same buffer. Peptide samples (5 μl), serially diluted in PBS, were added to 95 μl of the cell suspension in microfuge tubes. Following gentle mixing, the tubes were incubated at 37 °C for 30 min and then centrifuged at 4000*g* for 5 min. Fifty microliters of supernatant were taken, diluted to 1 ml with PBS, and absorbance at 567 nm was measured to monitor the release of hemoglobin that indicated RBC membrane damage. Zero hemolysis and 100% hemolysis consisted of RBC suspended in PBS and 0.2% Triton X-100, respectively. The percentage of hemolysis was calculated using the following equation: Hemolysis (%) = $(A_s - A_0)/(A_{100} - A_0) \times 100$, where A_s is the absorbance of the sample, A_{100} is the absorbance of completely lysed RBC in 0.2% Triton X-100, and A_0 is the absorbance of zero hemolysis.

2.5. Cell culture and in vitro cytotoxicity assay

HaCaT keratinocytes [34] were cultured in a complete medium (DMEM supplemented with 10% FBS and 0.1% penicillin-streptomycin) in a humidified atmosphere of 5% CO_2 at 37 °C. Trypsin-EDTA (0.05%) was used to detach cells in subculturing. All the cell culture media and reagents were purchased from Lonza (Basel, Switzerland). Cells were seeded onto 96-well plates at a density of 8000 cells/well in 0.1 ml of complete medium. After 16 h of incubation, cells were treated with serially diluted peptide samples and incubated for another 24 h. Cell viability was measured with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay using the CellTiter 96-cell proliferation assay kit (Promega, Madison, WI, USA). The percentage of cell viability was determined using the following equation: Viability (%) = $(A_s - A_0)/(A_c - A_0) \times 100$, where A_s is the absorbance of the sample, A_c is the absorbance of the control (no peptide added) and A_0 is the background absorbance. Each experiment was performed in triplicate, and repeated three times independently.

2.6. Measurement of LPS binding

The ability of the peptides to bind LPS was assessed by using a quantitative chromogenic limulus amebocyte lysate (LAL) with QCL-1000 kit (Lonza). Stock solutions of the peptides were prepared in pyrogen-free water provided with the kit. Peptides at concentrations of 5 and 10 μM were incubated with LPS from *E. coli* O111:B4 (0.5 U/ml final concentration, Sigma-Aldrich, St. Louis, MO, USA) in a flat-bottom nonpyrogenic 96-well tissue culture plate (SPL, Pocheon, Korea) at 37 °C for 30 min to allow peptide binding to LPS. A total 50 μl of this mixture was then added to equal volume of LAL