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Design of perfectly symmetric Trp-rich peptides with potent and broad-spectrum antimicrobial activities

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Abstract

Tritrpticin, a member of the cathelicidin family, is a Trp-rich or Pro/Arg-rich peptide. Since the Trp, Pro and Arg residues are important in membrane disruption and/or cell entry, tritrpticin is a particularly attractive template around which to design novel antimicrobial peptides. Although tritrpticin is effective against a broad spectrum of microorganisms, it also has relatively strong haemolytic activity, which may compromise its therapeutic effects. To identify antimicrobial analogues of tritrpticin that lack cytotoxicity, we have designed and synthesised several molecules based on the amphipathic turn structure of tritrpticin. C-terminal amidation of tritrpticin enhanced its antimicrobial activity, comparable with indolicidin, another Trp-rich peptide. In contrast, the additional insertion of positively-charged amino acids resulted in only small variations in antibiotic activity, suggesting that a total of five positive charges is sufficient for high antimicrobial activity. We found that perfectly symmetric analogues of tritrpticin with C-terminal amidation showed two- to eight-fold improved antimicrobial activity compared with tritrpticin, as well as significantly reduced haemolytic activity. This reduction in cytotoxicity was correlated with decreased permeabilization of the zwitterionic phosphatidylcholine membrane, the major component of the outer leaflet of red blood cells. In addition, we designed a symmetric indolicidin analogue that possessed antimicrobial potency and selectivity. Moreover, we found that these analogues of tritrpticin and indolicidin were effective against several antibiotic-resistant clinical bacterial isolates. Circular dichroism spectroscopy suggested that the structure of these symmetric analogues resembled that of tritrpticin or indolicidin in a membrane mimetic environment. Overall, our findings suggest that these symmetric peptides with an amphipathic turn structure may serve as useful templates for pharmaceutical compounds that may be effective against increasingly antibiotic-resistant microbes.

Keywords: Tritrpticin; Symmetric peptides; Amphipathic turn structure; Selectivity

1. Introduction

Numerous antimicrobial peptides that act as components of the innate immune system have been isolated from living organisms [1–4]. Among these are members of the cathelicidin family, which are synthesised in the granules of mammalian myeloid cells [5,6]. The cathelicidins have a highly conserved N-terminal domain called cathelin and highly variable C-terminal domains that possess antimicrobial activity. Cathelicidin-derived antimicrobial peptides can be classified into three groups: amphipathic α-helical peptides such as CRAMP and PMAP-23; Pro/Arg-rich peptides (e.g. PR-39) and Trp-rich peptides (e.g. tritrpticin and indolicidin); and Cys-containing β-sheet peptides (e.g. protegrins) [7–10]. Despite their remarkable diversity in primary structure, cathelicidins share two common features: a net positive charge and an amphipathic secondary structure [11]. These properties enable these peptides to interact with negatively-charged bacterial membranes by electrostatic interaction and to exert a wide spectrum of antimicrobial activity by permeabilizing cell membranes and/or inhibiting intracellular macromolecule synthesis after penetration across cell membranes [12,13].

To overcome the emergence of widespread antibiotic resistance, considerable attention has been focused on the design

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of peptide analogues that have more potent antimicrobial activity than natural peptides but that lack cytotoxicity against mammalian cells. Tritrpticin is a Trp-rich member of the cathelicidin family whose sequence was deduced from that of porcine myeloid mRNA [14]. Owing to its small size and broad spectrum of antimicrobial activity, tritrpticin has potential in the therapeutic control of pathogenic bacteria. Natural tritrpticin, however, has potent membrane permeabilization activity, which is associated with high toxicity towards mammalian cells such as human erythrocytes. This undesirable feature may compromise its therapeutic use and should therefore be reduced or eliminated. A recent nuclear magnetic resonance study of tritrpticin showed that this molecule was comprised of a hydrophobic core flanked by two dispersed, positively-charged regions [15]. Based on this amphipathic turn structure, we have designed several analogues of tritrpticin and characterised their antimicrobial potency and selectivity. We identified perfectly symmetric Trp-rich peptides with strong antibacterial activity against several antibiotic-resistant bacteria but without haemolytic activity.

2. Experimental procedures

2.1. Peptide synthesis, purification and characterisation

Peptides were synthesised using solid-phase methodology with Fmoc-protected amino acids. Fmoc-protected peptides were de-protected and cleaved using a mixture of trifluoroacetic acid, phenol, H_2O, thioanisole and 1,2-ethandithiol (82.5:5:5:5:2.5, v/v) for 3 h at room temperature. High-performance liquid chromatography (HPLC) analysis was performed using an LC-6AD or a LC-10Avp system (Shimadzu, Tokyo, Japan) with an ODS column (4.6 mm × 250 mm). Purification by preparative reversed phase (RP)-HPLC gave final products that were >98% pure as determined by analytical RP-HPLC. Peptides were characterised by Kratos Kompact matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) (Shimadzu).

2.2. Microorganisms and antimicrobial activity

Escherichia coli KCTC 1682, *Salmonella typhimurium* KCTC 1926, *Bacillus subtilis* KCTC 3068 and *Staphylococcus aureus* KCTC 1621 were purchased from the Korean Collection for Type Cultures (KCTC), Korea Research Institute of Bioscience & Biotechnology, Daejon, Korea. Clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecium* (VRE) were supplied by the Research Institute of Bacterial Resistance, Yonsei University, College of Medicine, Seoul, Korea.

Peptide antimicrobial activity against Gram-positive and Gram-negative bacteria, including MRSA and VRE, was measured using the broth microdilution assay. Briefly, single colonies of bacteria were inoculated into Lauria–Bertani broth and cultured overnight at 37 °C. An aliquot of each was transferred to 10 mL of fresh culture medium and incubated for an additional 3–5 h at 37 °C to obtain mid-logarithmic phase organisms. Then, 100 μL of a set of two-fold serial dilutions of peptides in 1% peptone was added to 100 μL of bacteria (2×10^6 colony-forming units/mL) in 96-well microtitre plates and the plates were incubated at 37 °C for 18 h. The minimal inhibitory concentration (MIC) of each peptide was defined as the lowest peptide concentration that completely inhibited growth. Each MIC was determined from two independent experiments performed in triplicate.

2.3. Haemolytic activity

Human red blood cells (hRBCs) were centrifuged, washed three times with phosphate-buffered saline (PBS; 35 mM phosphate, pH 7.0, 150 mM NaCl) and re-suspended to 4% (v/v) in PBS. Then, 100 μL of peptide solution was added to 100 μL of 4% (v/v) hRBCs in sterile 96-well plates, which were incubated for 1 h at 37 °C and centrifuged at $1000 \times g$ for 5 min. Aliquots (100 μL) of supernatant were transferred to fresh 96-well plates and haemoglobin release was measured using an enzyme-linked immunosorbent assay (ELISA) plate reader (Molecular Devices, Sunnyvale, CA) by absorbance at 414 nm. Zero and 100% haemolysis were determined in PBS and 0.1% Triton X-100, respectively. Percent haemolysis was calculated as $[(\mathrm{Abs}_{414\,nm}$ in the peptide solution $- \mathrm{Abs}_{414\,nm}$ in PBS)/($\mathrm{Abs}_{414\,nm}$ in 0.1% Triton X-100 $- \mathrm{Abs}_{414\,nm}$in PBS)] × 100.

2.4. Calcein release from large unilamellar liposomes

A dried lipid film composed of 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) or POPC/1-palmitoyl-2-oleoylphosphatidyl-DL-glycerol (POPG) (2:1) was hydrated with Tris–HCl buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1 mM EDTA) or 70 mM calcein solution and vortexed. Each suspension was freeze–thawed five times and extruded 20 times through polycarbonate filters (LiposoFast, pore diameter 100 nm; Sigma–Aldrich, St Louis, MO). Untrapped dye was removed by gel filtration on a Sephadex G-50 column (Pharmacia, Uppsala, Sweden). The fluorescence intensities of calcein released from the liposomes were monitored at 520 nm, after excitation at 490 nm, on a Shimadzu RF-5301 spectrofluorometer. To measure the maximum fluorescence intensity for 100% dye leakage, 20 μL of Triton X-100 (20% in Tris buffer) was added to dissolve the liposomes. The percentage of dye leakage caused by the peptides was calculated as $100 \times (F - F_0)/(F_t - F_0)$, where F_0 is the fluorescence intensity in the absence of peptide, F_t is the initial fluorescence intensity after Triton X-100 treatment and F is the fluorescence intensity after treatment with peptide.