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Membrane interactions and biological activity of antimicrobial peptides from Australian scorpion ☆

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ABSTRACT

UyCT peptides are antimicrobial peptides isolated from the venom of the Australian scorpion. The activity of the UyCT peptides against Gram positive and Gram negative bacteria and red blood cells was determined. The membrane interactions of these peptides were evaluated by dye release (DR) of the fluorophore calcein from liposomes and isothermal titration calorimetry (ITC); and their secondary structure was determined by circular dichroism (CD). Three different lipid systems were used to mimic red blood cells, *Escherichia coli* and *Staphylococcus aureus* membranes. UyCT peptides exhibited broad spectrum antimicrobial activity with low MIC for *S. aureus* and multi-drug resistant Gram negative strains. Peptide combinations showed some synergy enhancing their potency but not hemolytic activity. The UyCT peptides adopted a helical structure in lipid environments and DR results confirmed that the mechanism of action is by disrupting the membrane. ITC data indicated that UyCT peptides preferred prokaryotic rather than eukaryotic membranes. The overall results suggest that UyCT peptides could be pharmaceutical leads for the treatment of Gram negative multiresistant bacterial infections, especially against *Acinetobacter baumanni*, and candidates for peptidomimetics to enhance their potency and minimize hemolysis. This article is part of a Special Issue entitled: Interfacially Active Peptides and Proteins. Guest Editors: William C. Wimley and Kalina Hristova.

1. Introduction

The widespread use of antibiotics has contributed to the emergence of multi-resistant bacteria as a major public health problem, leading to untreatable infections and nosocomial infections becoming a critical problem [1]. The evolution and spread of new resistant strains to conventional antibiotics (*e.g.* MRSA, *Enterococcus* spp., *Acinetobacter baumannii*) is of particular interest. Focus is on Gram-negative bacteria resistant to oral antibiotics and those forming biofilms. A means of managing these is to develop novel drugs to which resistance mechanisms are less likely to evolve. In the past decades, naturally occurring antimicrobial peptides (AMP) from insects, arachnids and some vertebrates have shown promising activities, representing both an opportunity and a challenge for pharmacological development.

AMP usually consist of 13 to 50 amino acids and generally α-helical, amphipathic, positively charged membrane-acting molecules [2]. They are ribosomally synthesized and often have post-translational modifications. AMP are key components of innate immunity and, therefore, show promise as antibacterial agents. AMP are active against a broad spectrum of pathogens (bacteria, fungi, parasites and viruses) and, generally, inhibit pathogens quickly, although their mode of action presents a problem — toxicity. Nevertheless, there is potential since their mechanism of action is to disrupt the bacterial membrane so that bacteria are less likely to evolve or gain resistance.

The Antimicrobial Peptide Database (http://aps.unmc.edu/AP/main.php accessed 24 Aug 2013) contains more than 2200 AMP from different sources [3]. Despite this growing library, the relationship between their amino-acid sequences and bactericidal activity remains to be elucidated. AMP are classified into three major groups: (i) linear cysteine-free peptides with an α-helical conformation (insect cecropins, magainins,

Abbreviations: AMP, antimicrobial peptides; Chol, cholesterol; CD, circular dichroism; DR, dye release; ITC, isothermal titration calorimetry; LPC, lipopolysaccharide; LUV, large unilamellar vesicles; MDR, multi-drug resistant; MIC, minimum inhibitory concentration; MRE, mean-residue ellipticity; POPC, palmitoyloleoyl-phosphatidylcholine; POPE, palmitoyloleoyl-phosphatidylethanolamine; POPG, palmitoyloleoyl-phosphatidylglycerol; RBC, red blood cell; TOCL, tetraoeloyl-cardiolipin

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etc.) [4], (ii) cyclic and open-ended cyclic peptides with one to four disulfide bridges (defensins, protegrin, *etc.*), and (iii) peptides with an over-representation of some amino acids (proline rich, glycine rich, histidine rich, *etc.*) [5]. This report focuses on the first group of AMP.

Linear amphipathic α-helical AMP are present in invertebrates (insects and tunicates) and vertebrates, including humans [6]. Generally, they are potent broad-spectrum antimicrobials with a hemolytic tendency. Cecropin, from the moth *Hyalophora cecropia*, was one of the first α-helical AMP to be discovered [7]. Subsequently, cecropins from tunicate and nematodes were also identified. Mature cecropin peptides are cysteine-free AMP of 35–39 amino acids forming two linear α-helices connected by a hinge, which integrate into the anionic cell membranes of bacteria leading to their disruption [8]. Cecropins have two major characteristics: a tryptophan residue in position 1 or 2, and an amidated C-terminus.

AMP isolated from the venom gland of arthropods are well represented by melittin, a major component of bee venom found to be bactericidal by Schmidt-Lange in 1941 [9], and ponericins isolated from the predatory ant *Pachycondylas goeldii* [10]. Ponericins are highly similar to cecropins (60% sequence similarity) and have the tryptophan signature, but lack the C-terminal amidation.

AMP from spiders and scorpions have also been identified. Oxyopinins and cupiennins from the wolf spider *Oxyopes kitabensis* and hunting spider *Cupiennius salei*, respectively, possess anti-bacterial, hemolytic and insecticidal properties [11,12]. Oxyopinins share sequence similarities to ponericins and to the dermaseptins, α-helical AMP from amphibian skin [13]. Cupiennins are characterized by a hydrophobic N-terminal region, a C-terminus composed of polar and charged residues, and an amidated glutamic acid residue [14].

Only a few AMP from scorpion venom have been described. So far, only 27 AMP have been reported from 14 different scorpion species [15–19]. Antimicrobial peptides from scorpions are a class of scorpion peptides recently classified as non-disulfide bridges peptides (NDBP), very different from the neurotoxic peptides isolated from this species. AMP from scorpions can inhibit the growth of a wide-range of microorganisms including viruses, Gram-positive and Gram-negative bacteria, protozoa, yeast and fungi; these AMP may also be hemolytic and cytotoxic to cancer cells [18]. Their mode of action is not yet clear but they appear to disturb membranes by forming transient pores, enhancing membrane permeability, which leads to leakage of cell contents and death [20].

In an effort to investigate novel antibiotics, AMP found in the venom of the Australian scorpion *Urodacus yaschenkoi* [21] have been studied. The peptides described herein, UyCT1, UyCT3 and UyCT5, are short naturally occurring cationic peptides that are active against Gram-positive and Gram-negative bacteria. UyCT2 is an analog of UyCT1.

These four peptides and binary mixtures were studied to understand the relationship between the amino acid sequence (activity) and membrane lipid composition. *In vitro* bioassays against multidrug resistant (MDR) bacteria from clinical isolates were performed to determine the minimum inhibitory concentration (MIC) and to identify a preferential bacterial target. Also, the hemolytic activity of these peptides was determined to assess their interaction with eukaryotic cells. Furthermore, their secondary structure was determined by CD, their capacity to permeate membranes by dye release (DR) of the fluorophore calcein from liposomes, and their affinity for specific lipid membranes was assessed by isothermal titration calorimetry (ITC). These experiments were designed to shed light on the molecular mechanism by which these peptides trigger antibacterial activity. This study was carried out using phospholipid bilayers with large unilamellar vesicles (LUV, 100 nm diameter) mimicking human red blood cells (POPC/Chol), *Escherichia coli* (POPE/POPG) and *Staphylococcus aureus* (POPG/TOCL) membranes. To confirm membrane targeting by these peptides, D-isomers of UyCT peptides were synthesized and assayed.

2. Materials and methods

2.1. Materials

Peptides UyCT1, UyCT2, UyCT3 and UyCT5 (Genbank JX274240.1, JX274241.1, JX274242.1) were synthesized by Biomatik Corporation (Ontario, Canada) and delivered 98% pure (after TFA removal). The molecular weights of the pure peptides were confirmed by mass spectrometry. Palmitoyloleoyl-phosphatidylcholine (POPC), palmitoyloleoyl-phosphatidylethanolamine (POPE), palmitoyloleoyl-phosphatidylglycerol (POPG) and tetraoeloyl-cardiolipin (TOCL) phospholipids were purchased from Avanti Polar Lipids (Alabaster, USA) and were used without further purification. Calcein, Aprotonin (A3886), Triton-X100 and Sephadex G-100 gel filtration media were purchased from Sigma (St Louis, USA).

Multi-drug resistant (MDR) clinical isolates used herein were obtained from the Center for Research on Infectious Diseases bacterial collection of the National Institute of Public Health, Cuernavaca (Morelos, Mexico) and included: *E. coli*, 170 [22], 09-280, 5509, 09-240 [23]; *Klebsiella pneumoniae* 01-239, 01-252 [23,24]; *Enterobacter cloacae* 14-262, 06-26 [23]; *A. baumannii* 5821; *Pseudomona aeruginosa* 5106, 3599 [25]; and *S. aureus* 01-001, 06-051. These clinical isolates were collected from different patients from 14 hospitals in nine major cities in Mexico during June 2002 to November 2009. In all cases, the species of the organisms and susceptibility patterns were determined with a Dade Micro-Scan combo PC-20 assay for Gram-positive and NC-33 for Gram-negative bacteria (Siemens Healthcare Diagnostics Inc., West Sacramento, USA).

2.2. Susceptibility tests

2.2.1. Minimum Inhibitory Concentration (MIC) assays

The determination of the MIC for different peptides was performed using the broth microdilution method following the Clinical and Laboratory Standards Institute (CSLI) guidelines. MIC was determined as the lowest concentration of the compound that completely inhibited bacterial growth. Briefly, the bacterial cultures were grown in Mueller-Hinton broth medium (100 μL) in 96-well ELISA plates. Each well contained 5 μL of bacterial culture with 5×10^4 CFU/mL and the appropriate amount of the AMP to be tested. Dilutions of the peptides based on two-fold dilution, typically 2–64 μM, were prepared by dissolving the dried peptides in distilled water and tested in duplicate. Microbial growth inhibition was observed after incubation for 16–18 h at 35 °C. As reference strains, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 29213 were used. Experiments were performed with different clinical isolates causing nosocomial infections: *E. coli* (4 isolates), *K. pneumoniae* (2 isolates), *E. cloacae* (3 isolates), *P. aeruginosa* (2 isolates), *A. baumannii* (1 isolate) and *S. aureus* (2 isolates). Subsequent experiments were performed with intermediate concentrations to determine the MIC of each peptide and their combinations.

2.2.2. Hemolytic assays

The hemolytic activity was assessed by incubating (at 37 °C for 1 h) a suspension of human erythrocytes (7×10^7 cells/mL) in phosphate buffered saline (PBS) from a healthy donor with increasing concentrations of each peptide or combination of peptides. The samples were centrifuged for 5 min at 2000*g* and the release of hemoglobin was monitored by measuring the absorbance of the supernatant at 570 nm in a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). [For details see [21,26]]. All peptide concentrations were tested in quadruplicate and the data expressed as mean ± SD. Percentage of hemolysis was calculated using the following formula: % hemolysis = 100 $(A_{peptide} - A_{PBS})/(A_{triton} - A_{PBS})$. The zero and 100% hemolysis values were determined in PBS and 10% Triton X100, respectively. The peptide concentrations that cause 50% hemolysis of human erythrocytes (HC_{50}) were obtained using a non-linear