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Design of potent, non-toxic antimicrobial agents based upon the structure of the frog skin peptide, pseudin-2

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Abstract

Pseudin-2, a naturally occurring 24 amino-acid-residue antimicrobial peptide first isolated from the skin of the South American paradoxical frog *Pseudis paradoxa*, has weak hemolytic and cytolytic activity but also relatively low potency against microorganisms. In a membrane-mimetic environment, the peptide exists in an amphipathic α-helical conformation. Analogs of the peptide with increased cationicity and α-helicity were chemically synthesized by progressively substituting neutral and acidic amino acid residues on the hydrophilic face of the α-helix by lysine. Analogs with up to three L-lysine substitutions showed increased potency against a range of gram-negative and gram-positive bacteria (up to 16-fold) whilst retaining low hemolytic activity. The analog [D-Lys^3, D-Lys^{10}, D-Lys^{14}]pseudin-2 showed potent activity against gram-negative bacteria (minimum inhibitory concentration, MIC=5 μM against several antibiotic-resistant strains of *Escherichia coli*) but very low hemolytic activity (HC_{50}>500 μM) and cytolytic activity against L929 fibroblasts (LC_{50}=215 μM). Increasing the number of L-lysines to four and five did not enhance antimicrobial potency further but increased hemolytic activity towards human erythrocytes. Time-kill studies demonstrated that the analog [Lys^3, Lys^{10}, Lys^{14}, Lys^{21}]pseudin-2 at a concentration of 1×MIC was bacteriocidal against *E. coli* (99.9% cell death after 96 min) but was bacteriostatic against *S. aureus.* Increasing the hydrophobicity of pseudin-2, while maintaining the amphipathic character of the molecule, by substitution of neutral amino acids on the hydrophobic face of the α-helix by L-phenylalanine, had only minor effects on antimicrobial and hemolytic activities.

Keywords: Pseudin-2; Antimicrobial; Hemolytic; Cytolytic; Amphibian

1. Introduction

The emergence in all regions of the world of strains of pathogenic bacteria and fungi with resistance to commonly used antibiotics has necessitated a search for novel types of antimicrobial agent to which the microorganisms have not been exposed. Peptide-based anti-infectives are being increasingly considered as potential therapeutic agents [1–4]. On the positive side, because of their relatively non-specific mechanism of action (either a detergent-like disruption of the bacterial cell membrane into peptide-coated vesicles or formation of transient transmembrane pores [5,6]), the peptides show broad spectrum activity and development of resistance occurs at rates that are orders of magnitude lower than those observed for conventional antibiotics [7]. The major obstacles to their development as useful drugs are their toxicities, particularly if they are to be administered systemically, and their short half-lives in the circulation. Thus, future therapeutic applications are more likely to involve topical rather than systemic administration, for example, in treatment of infected foot ulcers of diabetic patients [8].

The synthesis of peptides with antimicrobial activity in granular glands located in the skin is a feature of several anuran (frog and toad) species, particularly those belonging to the families Bombinatoridae, Hylidae, Hyperoliidae, Leptodactylidae, Myobatrachidae, Pipidae, and Ranidae

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(reviewed in [4,9–11]). These antimicrobial peptides comprise between 12 and 48 amino acid residues and are characterized by a remarkable degree of structural diversity, which is considered to be important in protecting the organism against invasion by a wide range of pathogenic microorganisms [12]. The peptides lack any consensus amino acid sequences that are associated with biological activity but, with few exceptions, they are cationic, relatively hydrophobic and have the propensity to form an amphipathic α-helix in a membrane-mimetic solvent such as trifluoroethanol [4,13].

Pseudin-2 (GLNALKKVFQ^{10}GIHEAIKLIN^{20}NHVQ) is a 24 amino-acid-residue antimicrobial peptide first isolated from an extract of the skin of the paradoxical frog, *Pseudis paradoxa* (Hylidae) [14]. The peptide shows moderately high potency against the gram-negative bacterium *Escherichia coli* (Minimum Inhibitory Concentration, MIC=20 μM) but is only very weakly active (MIC>100 μM) against the gram-positive bacterium *Staphylococcus aureus* However, in comparison with most other antimicrobial peptides from frog skin [4,9], pseudin-2 shows very low hemolytic activity against human erythrocytes (concentration producing 50% hemolysis, HC_{50}=360 μM).

A previous study using circular dichroism spectroscopy [14] has shown that, in aqueous solution, pseudin-2 exists predominantly as a random coil but in 50 % trifluoroethanol/water, a solvent that mimics the hydrophobic environment of the cell membrane, the peptide adopts an α-helical conformation. A Schiffer–Edmundson helical wheel projection [15] of the pseudin-2 structure indicates that this α-helix has considerable amphipathic character with the hydrophilic residues Lys^6, Lys^7, Glu^{14}, and Lys^{17} segregating on one face and the hydrophobic residues Leu^2, Leu^5, Val^8, Phe^9, Ile^{12}, Ile^{16}, and Val^{23} segregating on the opposite face (Fig. 1). The aim of the present study was to design analogues of pseudin-2 that maintain the amphipathic α-helical character and the low hemolytic activity of the naturally-occurring peptide but display increased potencies towards a range of pathogenic microorganisms. The strategy adopted was to increase cationicity by progressively substituting neutral and acidic residues on the hydrophilic face by either L-lysine or D-lysine.

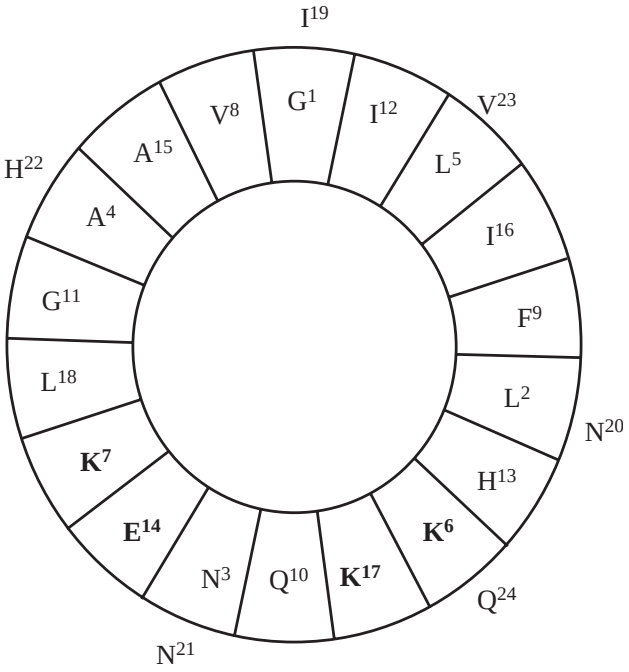

Fig. 1. A Schiffer–Edmundson helical wheel projection [15] of the pseudin-2 structure. The polar residues are shown in bold type. Amino acid residues at positions 3, 10, 14, 21 and 24 were progressively substituted by lysine.

2. Materials and methods

2.1. Peptide synthesis

All synthetic peptides were supplied in crude form either by Sigma Genosys (USA) or by GL Biochem (Shanghai) (China). The peptides were purified to near homogeneity by reverse-phase HPLC on a (25×5-cm) Vydac 218TP1022 (C-18) column (Separations Group) equilibrated with acetonitrile/water/trifluoroacetic acid (28.0/71.9/0.1) at a flow rate of 6 ml/min. The concentration of acetonitrile was raised to 56 % (v/v) over 60 min using a linear gradient. Absorbance was measured at 214 and 280 nm and the major peak in the chromatogram was collected by hand. The identities of the synthetic peptides were confirmed by MALDI mass spectrometry. For all peptides, the average molecular masses determined by mass spectrometry were consistent with the masses calculated from the proposed structures. The purity of all peptides tested was >95%.

2.2. Antimicrobial assays

Reference strains were purchased from the American Type Culture Collection (Rockville, MD), from the National Collection of Type Cultures (London, UK), and from the Hungarian National Type Culture Collection (Budapest, Hungary) and have been described previously [16]. Clinical isolates of antibiotic-resistant strains of *E. coli* were recovered from the urines of patients from Tawam Hospital (Al-Ain, U.A.E.) with urinary tract infections.

MIC testing of the peptides was determined by a standard microdilution method using 96-well microtiter cell-culture plates [17]. Serial dilutions of the peptides in Mueller-Hinton broth (50 μl) were mixed with an inoculum (50 μl of 10^6 CFU/ml) from a log-phase culture. Bacteria were incubated for 18 h at 37 ^{0}C in a humidified atmosphere of air. Incubations with *C. albicans* were carried out in RPMI 1640 medium for 48 h at 35 °C. After incubation, the absorbance at 630 nm of each well was determined using a microtiter plate reader. In order to monitor the validity of the assay, incubations with bacitracin were carried out in parallel with increasing