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Conformational analysis of the broad-spectrum antibacterial peptide, ranatuerin-2CSa: Identification of a full length helix–turn–helix motif

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ABSTRACT

Design of clinically valuable antibacterial agents based upon naturally occurring peptides requires the use of spectroscopic methods, particularly NMR, to determine the three-dimensional structure of the native peptide so that analogues with improved therapeutic properties can be made. Ranatuerin-2CSa (GILSSFKGVAKGVAKDLAG KLLETLKCKITGC), first isolated from skin secretions of the Cascades frog, *Rana cascadae*, represents a promising candidate for drug development. The peptide shows potent growth inhibitory activity against *Escherichia coli* (MIC = 5 μM) and *Staphylococcus aureus* (MIC = 10 μM) but displays haemolytic activity against human erythrocytes (LC_{50} = 160 μM). The solution structure of ranatuerin-2CSa was investigated by proton NMR spectroscopy and molecular modelling. In aqueous solution, the peptide lacks secondary structure but, in a 2,2,2-trifluoroethanol (TFE-d_3)-H_2O solvent mixture, the structure is characterised by a full length helix–turn–helix conformation between residues I^2–L^{21}, L^{22}–L^{25} and K^{26}–T^{30} respectively. This structural information will facilitate the design of novel therapeutic agents based upon the ranatuerin-2CSa structure with improved antimicrobial potencies but decreased cytolytic activities against mammalian cells.

1. Introduction

The emergence in all regions of the world of strains of pathogenic bacteria and fungi with resistance to commonly used antibiotics constitutes a potentially serious threat to public health [1]. This situation has necessitated a search for novel types of antimicrobial agent to which the micro organisms have not been exposed [2]. Cationic α-helical peptides with broad-spectrum antibacterial and antifungal activities are synthesized in the skins of many species of frogs and are released into skin secretions, often in very high concentrations, in response to infection or stress [3,4]. Frogs belonging to the genus *Rana* represent a particularly rich source of peptides with diverse structures and specificities against micro organisms and these compounds are being considered as anti-infective agents with therapeutic potential [5].

The antimicrobial peptide ranatuerin-2 was first isolated from an extract of the skin of the American bullfrog *Rana catesbeiana* (now reclassified as *Lithobates catesbeianus*) [6] but subsequently orthologs of the peptide have been identified in skin extracts and/or skin secretions of a range of North American and Eurasian frogs belonging to the genus *Rana* [5]. Members of the ranatuerin-2 family comprise between 27 and 35 amino acid residues and are characterized by a cyclic disulfide-bridged domain comprising six amino acid residues at, or near, the C-terminal region of the peptide. The primary structure of ranatuerin-2 has been relatively poorly conserved and it has been used in cladistic analyses to infer phylogenetic relationships between evolutionarily related species of ranid frogs [7]. Ranatuerin-2CSa (GILSSFKGVA^{10} KGVAKDLAGK^{20}LL ETLKCKIT^{30}GC) represents one member of the family that was isolated in high yield from norepinephrine-stimulated skin secretions from the Cascades frog *R. cascadae* [7].

This study uses NMR spectroscopy and molecular modelling to determine the three-dimensional solution structure of ranatuerin-2CSa in aqueous solution and in a membrane-mimetic solvent mixture (2,2,2-trifluoroethanol (TFE-d_3)-H_2O). Our data provide insight into the structure of ranatuerin-2CSa that will be useful in the development of analogues of the naturally occurring peptide with improved therapeutic properties.

2. Materials and methods

2.1. Synthesis of the peptide

Ranatuerin-2CSa was supplied in crude form by GL Biochem Shanghai (China) and purified to near homogeneity by reverse-phase HPLC on a (2.2×25-cm) Vydac 218TP1022 (C-18) column (Separations group. Hesperia, CA, USA) equilibrated with acetonitrile/water/trifluoroacetic acid (21.0/78.9/0.1) at a flow rate of 6 mL/min. The concentration of acetonitrile was raised to 56% over 60 min using a linear gradient. The

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Abbreviations: CD, circular dichroism; DQF-COSY, double-quantum filtered correlation spectroscopy; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; TFE, trifluoroethanol; TOCSY, total correlation spectroscopy; RMSD, root mean square deviation

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final purity of the synthetic peptide was >98% and its identity was confirmed by electrospray mass spectrometry (observed molecular mass 3246.0 a.m.u., calculated molecular mass 3245.8 a.m.u.).

2.2. Antibacterial and haemolytic activities

Minimum inhibitory concentration (MIC) of synthetic ranatuerin-2CSa against reference strains of *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25726) was measured in duplicate in three independent experiments by a standard microdilution method [8] as previously described [7] and was taken as the lowest concentration of peptide where no visible growth was observed. In order to monitor the validity and reproducibility of the assays, incubations were carried out in parallel with increasing concentrations of ampicillin. The haemolytic activity of the peptides against washed human erythrocytes from a healthy donor was determined as previously described [7]. The LC_{50} value was taken as the mean concentration of peptide producing 50% haemolysis in four independent experiments.

2.3. NMR spectroscopy

NMR experiments were performed using the 5 mm inverse probe head on Bruker DRX 500 and 800 NMR spectrometers (Bruker BioSpin, Germany) operating at a ^{1}H resonance frequency of 500.13 MHz and 800.13 MHz respectively, at 298 K. 2 mM peptide sample was dissolved in 500 μl of 1:1 mixture of TFE-d_3 and H_2O (pH 3.2 uncorrected). One-dimensional (1D) proton NMR spectra were acquired with a relaxation delay of 2.5 s and acquisition time of 2.73 s. 512 scans were acquired over a 6 kHz spectral width and 32768 data points were collected. The data sets were zero-filled to 65536 data points and was multiplied by 0.2 Hz line broadening. Two-dimensional (2D) phase-sensitive double-quantum filtered correlation spectroscopy (DQF-COSY) [9], total correlation spectroscopy (TOCSY) [10], and nuclear Overhauser effect spectroscopy (NOESY) [11] data sets were acquired with a relaxation delay of 1.5 s, acquisition time of 0.146 s, and a spectral width of 10 kHz. The TOCSY data were acquired with 40 ms and 80 ms mixing times, whereas the NOESY data were acquired with a 200 ms mixing time. The 2D experiments were performed with 32, 16 and 32 number of scans with 8192 (F2) and 2048 (F1) complex data points for the DQF-COSY, TOCSY and NOESY data respectively. 2D data were zero-filled to 4096 data points in F_1 prior to transformation. All data sets were apodised in both dimensions using a shifted squared sinebell window function. All spectra were referenced internally to the residual ^{1}H signal of $F_3CCDHOD$, resonating at 3.98 ppm. Data were processed using the Bruker XWINNMR program version 3.5 (Bruker BioSpin, Germany).

2.4. Structure calculations

2D NMR spectra were analysed and the spectral integration of the NOESY peaks were done using the SPARKY software version 3.110 [12]. NOESY peak volume integrals were converted into distance constraints by calibrating of nuclear Overhauser effect (nOe) intensity versus distance bonds routine of backbone, flexible side chain and methyl proton classes with CALIBA [13]. Pseudoatoms were introduced for the protons which could not be stereospecifically assigned. Distance constraints involving fixed distances as well as those limits that could not be violated were regarded as irrelevant by the CYANA program [14]. A set of constraints was thus obtained with upper distance limits as input for further calculations. Torsional angle constraints in the −90° to −40° range were also included for $^3J_{HNH\alpha}$<6.0 Hz. Structure calculations were carried out using the CYANA program (version 2.0). Distance and torsional constraints were used as input to CYANA. Two hundred structures were randomly generated and energy minimised in CYANA, including 10,000 steps of simulated annealing, as well as 5000 steps of conjugate-gradient minimisation. During the CYANA calculations, distance and torsional constraints were weighted using the force constants k_{NOE}=1 kJ mol^{-1} Å^{-2} and k_{Dihed_c}=5 kJ mol^{-1} deg^{-2} respectively. Finally, the 20 structures with lowest target function values were subjected to 1000 steps of unconstrained Powell minimisation using the Tripos force field within SYBYL (version 6.8.1) [15]. The Biopolymer module of TRIPOS software and the MOLMOL (version 2K.2) graphics packages were used for final structure analysis. MOLMOL and PyMOL (version 0.99) were used for structure representation [16]. Calculated structures were analysed using the PROCHECK-NMR [17].

3. Results

3.1. Cytolytic activities of ranatuerin-2CSa

The MIC of the ranatuerin-2CSa used in this study was 5 μM against a reference strain of *E. coli* and 10 μM against a reference strain of *S. aureus*. The LC_{50} value for haemolysis of erythrocytes from a healthy donor was 160±8 μM (mean±SD).

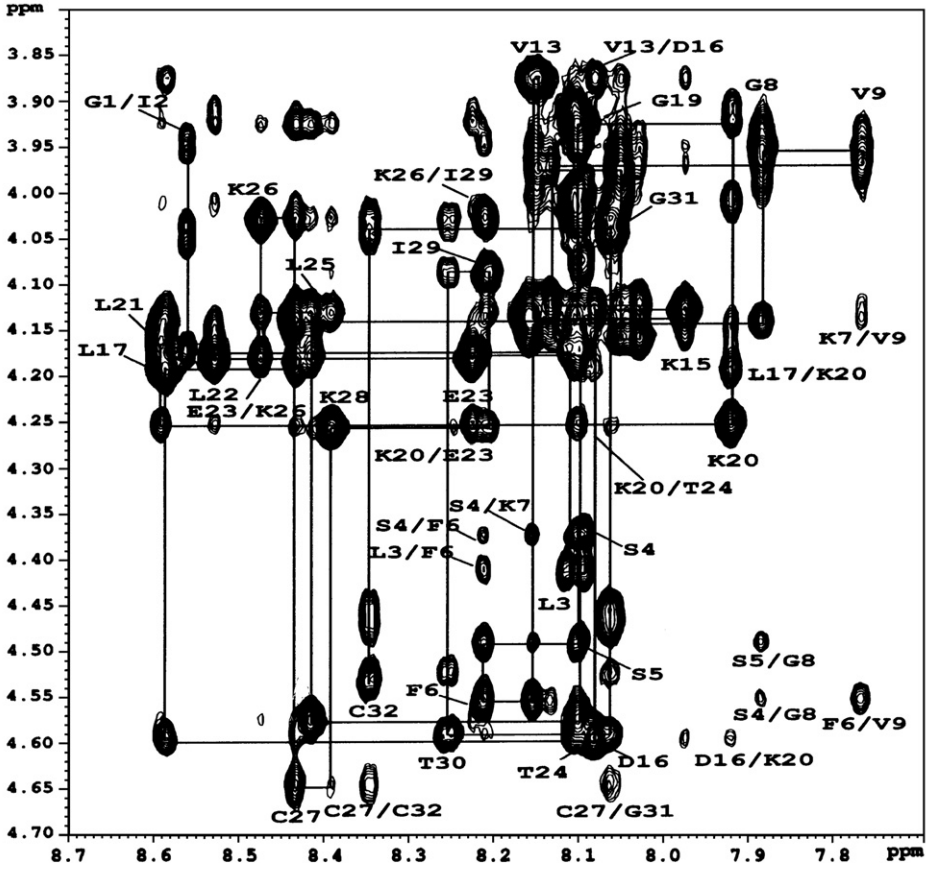

Fig. 1. Fingerprint (NH-αH) region of the 2D NOESY NMR spectrum of ranatuerin-2CSa (800 MHz). The backbone 'walk' is indicated together with residue assignments.