Structure–function relationships in novel peptide dodecamers with broad-spectrum bactericidal and endotoxin-neutralizing activities

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A series of designed peptide 33-mers (β pep peptides) are known to be bactericidal [Mayo, Haseman, Ilyina and Gray (1998) *Biochim. Biophys. Acta* **1425**, 81–92]. Here dodecapeptides (SC-1–SC-8), which ‘walk through’ the sequence of β pep-25, were investigated for their ability to kill Gram-negative and -positive bacteria and to neutralize endotoxin. SC-4 (KLFRHLKWKI I-NH₂; the -NH₂ at the right of each sequence indicates amidation of the C-terminal carboxylate group) is the most effective, more so than β pep-25, at killing Gram-negative bacteria with nanomolar LD₅₀ values. Against Gram-positive bacteria, SC-4 also shows good activity with submicromolar LD₅₀ values. Leakage studies indicate rapid bacterial membrane permeability, with $t_{1/2}$ values of 10–15 min. SC-4 in the micromolar range also effectively neutralizes endotoxin and is not haemolytic below 10^{−4}

M. For all SC peptides, CD and NMR data indicate the presence of both 3₁₀- and α -helix. For SC-4, nuclear-Overhauser-effect-based computational modelling yields an amphipathic helix with K1, K4, R5, and K8 arrayed on the same face (K is lysine, R is arginine). Activity differences among SC peptides and single-site variants of SC-4 allow some structure–function relationships to be deduced. Relative to other known bactericidal peptides in the linear peptide, helix-forming category, SC-4 is the most potent broad-spectrum antibacterial identified to date. The present study contributes to the development of agents involved in combating the ever-recurring problem of drug-resistant micro-organisms.

Key words: CD, conformation, dodecapeptides, NMR structure.

INTRODUCTION

A series of designed peptide 33-mers (β pep peptides) has been reported to be bactericidal and to be capable of neutralizing bacterial endotoxin lipopolysaccharide (LPS) [1]. CD and NMR conformational analyses indicate that β pep peptides form β -sheets [2], and one of these, β pep-4, folds compactly as an anti-parallel β -sheet sandwich [3]. β pep-19 is potentially bactericidal in the 100 nM range [1]. β pep peptides function like *Limulus* (horseshoe crab) anti-LPS factor (LALF) and the homologous bactericidal/permeability-increasing protein (B/PI) [4] in that all appear to express activity through an amphipathic β -sheet structural motif having a cationic β -sheet face [5–8].

Numerous studies on bactericidal peptides indicate the functional importance of a net positive charge and high hydrophobicity in the context of an amphipathic, usually helical, structure [9]. The net positive charge promotes interaction with the negatively charged surface of bacterial membranes [10], whereas structure–activity relationships demonstrate that the amphipathic conformation of the peptide promotes bacterial cell lysis [11]. Bactericidal peptides, for example cecropins [12], magainins [13], proline+arginine-rich peptides [14] and sapecin [15], like β pep peptides, all have a net positive charge and considerable hydrophobic character. The cecropins and magainins are helix-forming peptides [16,17], whereas the sapacins contain both α -helix and β -sheet segments [18]. Structures for the proline+arginine-rich peptides are unknown. Tachyplesin, a bactericidal and endotoxin-neutralizing peptide isolated from haemocytes of the horseshoe crab [19], as well as anti-bacterial peptide defensins [20,21], form dimeric β -sheets which are stabilized by three intramolecular disulphide bridges [22]. In

addition, a number of small, antibiotic peptides based on the structure of the anti-LPS cyclic peptide polymyxin B [23] have been designed as short β -hairpins constrained by a disulphide bridge [24].

In the present study, a series of dodecapeptides (SC-1–SC-8), which ‘walk through’ the amino acid sequence of β pep-25 (homologous with β pep-19) was investigated for the ability to kill bacteria and to neutralize LPS. One of these SC peptides, SC-4, has been identified as being exceptionally potent, with an LD₅₀ of 3 nM, against *Pseudomonas aeruginosa* – even more potent than parent peptide, β pep-25. To demonstrate broad-spectrum bactericidal activity, several other clinically relevant Gram-negative and Gram-positive bacteria were investigated, namely rough strain *Escherichia coli* J5, three smooth strains of *E. coli*, namely J96, H5 and IA2, and two strains of *Staphylococcus aureus* MN-8 and MNHO. Moreover, since peptides which form both β -sheet and helix conformations can be bactericidal and these dodecapeptides were derived from a β -sheet-forming β pep peptide, CD and NMR data were acquired in aqueous solution and in 30% (v/v) trifluoroethanol (TFE) to investigate into which conformational type these 12-mers fall. The present work contributes to the development of solutions to the problem of the multiple-drug-resistant opportunistic micro-organisms.

MATERIALS AND METHODS

Peptide preparation

Peptides were synthesized using a Milligen/Bioscience 9600 solid-phase peptide synthesizer using fluorenylmethoxycarbonyl (‘Fmoc’) chemistry. Freeze-dried crude peptides were purified by

Abbreviations used: B/PI, bactericidal/permeability-increasing protein; LAL, *Limulus* amoebocyte lysate; LALF, *Limulus* anti-lipopolysaccharide factor; LPS, lipopolysaccharide; PF4, platelet factor 4; NOE, nuclear Overhauser effect; rf, radio frequency; FID, free induction decay; PFG, pulsed field gradient; HOHAHA, (homonuclear Hartman–Hahn spectroscopy), homonuclear magnetization transfer; TFE, trifluoroethanol.

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preparative reversed-phase HPLC on a C_{18} column with an elution gradient of 0–60 % (v/v) acetonitrile with 0.1 % trifluoroacetic acid in water. Purity and composition of the peptides were verified by HPLC (Beckman model 6300 apparatus), amino acid analysis and MS.

Bacterial strains

P. aeruginosa type 1 is a clinical smooth-strain isolate serotyped by using the scheme of Homma [25] and maintained in the laboratory by monthly transfer on blood-agar plates. *E. coli* J96, IA2 and H5 are smooth-strain uropathogenic clinical isolates kindly maintained and provided by Dr J. R. Johnson (Department of Medicine, University of Minnesota, MN, U.S.A.) and described in Johnson and Brown [26] for J96 and IA2 and in Johnson and Brown [27] for H5. J5 is an *E. coli* rough strain initially referenced by G. R. Siber and discussed in Warren et al. [28] and is analogous to the smooth-strain *E. coli* 0111:B4 used in the BioWhittaker *Limulus* amoebocyte lysate (LAL) endotoxin detection and quantification kit described below. Gram-positive MN8 and MNHO are two patient isolates of *S. aureus*, which were kindly provided by Professor P. M. Schlievert (Department of Microbiology, University of Minnesota, MN, U.S.A.) and described in Bohach et al. [29] for MNHO and in Schlievert and Blomster [30] for MN8. All cultures were maintained on nutrient-agar plates.

Bactericidal assay

Pyrogen-free solutions were used throughout the assay. Exponential-phase bacteria were obtained by transferring an overnight culture or scraping crystals off -85°C glycerol stocks of overnight cultures. Bacteria were washed and resuspended in 0.9 % NaCl with adjustment to an attenuation (D_{650}) that yields 3×10^8 colony-forming units/ml. Bacteria were then diluted 1:10 in 0.08 M citrate/phosphate buffer, pH 7.0 (prepared by mixing 0.08 M citric acid with 0.08 M dibasic sodium phosphate). Bacteria (0.15 ml) were incubated with peptide in a final volume of 1.0 ml of buffer. The assay was done in 17 mm $\times$ 100 mm polypropylene tubes in a reciprocal water-bath shaker at 37°C for 30 min. Following this 30 min incubation, 10-fold dilutions were made in 0.9 % NaCl. Dilutions were done to 1:10000, and 20 μl of each dilution was streaked across an agar plate. Gram-positive organisms were plated on nutrient-agar plates containing 2 % agar, and Gram-negative organisms were plated on MacConkey agar (2 %). Plates were incubated overnight at 37°C and counted for colonies the next morning. The dilution containing 10–100 bacteria was counted and the number multiplied by 50 to adjust all counts to the number of bacteria killed/ml. Peptide concentrations were converted into their base-10 logarithm and plotted against bacteria killed. Bactericidal activity was determined by the dose response, where LD_{50} values were determined by best fits of a sigmoidal curve to the dose–response data.

LAL assay for LPS neutralization

The ability of synthetic peptides to neutralize endotoxin was detected using the chromogenic QCL-1000 kit from BioWhittaker, Inc. (Walkersville, MD, U.S.A.) and as described in their protocol. This method is quantitative for Gram-negative bacterial endotoxin (LPS). In this LAL assay, peptides that are active inhibit the LPS-mediated activation of a proenzyme [31] whose active form would release, from a colourless synthetic substrate (Ac-Ile-Glu-Ala-Arg *p*-nitroanilide), a yellow product, *p*-nitroaniline, whose absorbance was monitored spectrophotometrically at 405–410 nm.

The initial rate of enzyme activation is proportional to the concentration of endotoxin present. The concentration of peptide required to bind to LPS, and therefore to inhibit the LAL driven by 0.04 unit (or 0.01 ng) of *E. coli* 055:B5 LPS (from Sigma), was determined by dose response.

Leakage kinetics

Bacterial-cell-membrane integrity was measured using the LIVE/DEAD BacLight bacterial viability kit (L-7007) from Molecular Probes, Inc. (Eugene, OR, U.S.A.), which uses mixtures of red and green fluorescent stains to differentiate damaged and intact bacteria respectively. Two *E. coli* strains (H5 and J5) and one *Staphylococcus aureus* strain (MN8) were investigated, and SC peptides were used at a dose that initiates 100 % killing. Time points were taken between zero time and 60 min. Fluorescence emission (excitation at 470 nm and emission at 490–700 nm) of each cell suspension was measured in a fluorescence spectrophotometer. A 5 μl portion of each sample was placed on a microscope slide with a square coverslip and sealed to prevent movement.

Haemolytic activity

Human red blood cells were washed three times using PBS (35 mM phosphate buffer/0.15 M NaCl, pH 7.0) prior to performing the assay. A 100 μl portion of human red blood cells diluted to 0.4 % (v/v) in PBS, was placed in Eppendorf tubes, and 100 μl portions of serially diluted peptides in PBS were added (peptide concentrations ranged from 1 μM to 100 μM). Tubes were incubated for 1 h at 37°C and centrifuged at 1000 *g* for 5 min. Aliquots (100 μl) of the supernatant were then transferred to Eppendorf tubes, and haemolysis was measured by A_{414} ; 0 and 100 % haemolysis were determined in PBS and in 1 % Triton-X 100 respectively. The percentage haemolysis was calculated using the following formula:

$$\text{Percentage haemolysis} = (A_{414} \text{ in the peptide solution} - A_{414} \text{ in PBS}) / (A_{414} \text{ in 1 \% Triton-X 100} - A_{414} \text{ in PBS}) \times 100$$

CD

CD spectra were measured on a JASCO JA-710 automatic recording spectropolarimeter coupled with a data processor. Curves were recorded digitally and fed through the data processor for signal averaging and baseline subtraction. Peptides were dissolved (0.6 mg/ml) in 10 mM potassium phosphate buffer, pH 5.5, and CD spectra were recorded at 20°C over the range 190–250 nm using a 0.5-mm-path-length thermally jacketed quartz cuvette. Temperature was controlled by using a NesLab (Newington, NH, U.S.A.) waterbath. The scan speed was 20 nm/min. Spectra were signal-averaged eight times, and an equally signal-averaged solvent baseline was subtracted. CD spectra on each peptide were acquired as a function of the concentration of TFE from 0 to 80 % (v/v). CD spectra were deconvoluted as described by Sreerama and Woody [32].

NMR measurements

For NMR measurements, freeze-dried peptide was dissolved in water. The peptide concentration was usually about 5–6 mM. The pH was adjusted to pH 5.5 by adding microlitre quantities of NaOH or HCl to the peptide sample. NMR spectra were acquired on a Varian UNITY Plus-600 NMR spectrometer. The water resonance was suppressed by direct irradiation (0.8 s) at the water frequency during the relaxation delay between scans.

Two-dimensional homonuclear magnetization transfer (HOHAHA, homonuclear Hartmann–Hahn spectroscopy) spectra, obtained by spin-locking with a MLEV-17 sequence [33] with a mixing time of 60 ms, were used to identify spin systems. NOESY experiments [34] were performed for conformational analysis. All two-dimensional NMR spectra were acquired in the States-TPPI phase-sensitive mode [35,36]. The water resonance was suppressed by direct irradiation (0.8 s) at the water frequency during the relaxation delay between scans as well as during the mixing time in NOESY experiments. Two-dimensional NMR spectra were collected as 256–512 t1 experiments, each with 2 k complex data points over a spectral width of 6 kHz in both dimensions with the carrier placed on the water resonance (t1 is one of the time domains in a two-dimensional NMR data set). For HOHAHA and NOESY spectra, 16 scans were time-averaged per t1 experiment. Data were processed directly on the spectrometer or offline using VNMR (Varian, Inc., Palo Alto, CA, U.S.A) or NMRPipe [37] on an SGI workstation. Data sets were multiplied in both dimensions by a 30–60°-shifted sine-bell function and zero-filled to 1 k in the t1 dimension prior to Fourier transformation.

Since SC peptides are quite hydrophobic, and were later found to be amphipathic, pulsed-field-gradient (PFG) NMR self-diffusion measurements were performed as a check for peptide aggregation. PFG-NMR experiments were done as described by Mayo et al. [2] using a Varian Unity-Plus 500 NMR spectrometer. The maximum magnitude of the gradient was 6 mT (60 G)/cm, and the PFG longitudinal eddy-current delay pulse-sequence was used for all self-diffusion measurements, which were performed in $^2\text{H}_2\text{O}$ at temperatures of 5 and 40 °C. Peptide concentrations ranged from 0.1 mM to 15 mM. PFG NMR data were also analysed as described by Mayo et al. [2].

Structural modelling

Analysis of NOE growth curves indicated that backbone-to-backbone inter-proton NOEs were normally maximum at 300–400 ms. Inter-proton distance constraints were derived from NOEs assigned in ^1H NOESY spectra acquired with mixing times of 200 and 400 ms. NOEs were classified as strong, medium, weak or very weak, corresponding to upper bound distance constraints of 2.8, 3.3, 4.0, and 4.5 Å (1 Å = 0.1 nm) respectively. The lower bound restraint between non-bonded protons was set to 1.8 Å. Pseudo-atom corrections were added to the upper bound distance constraints where appropriate, and a 0.5 Å correction was added to the upper bound for NOEs involving methyl protons. Hydrogen-bond constraints were identified from the pattern of sequential and interstrand NOEs involving NH and C α H protons, together with evidence of slow amide proton–solvent exchange. Each hydrogen bond identified was defined using two distance constraints; $r_{\text{NH}\cdots\text{O}} = 1.8\text{--}2.5$ Å and $r_{\text{N}\cdots\text{O}} = 1.8\text{--}2.5$ Å.

Derived internuclear distance constraints were used in calculating structures for dodecapeptide SC-4 by using X-PLOR [38]. SC-4 was created using parallhdg.pro force fields. A template co-ordinate set was generated by using the *Template* routine. The *ab-initio*-simulated annealing protocol was then used. The simulated-annealing procedure ran high-temperature dynamics (3000 K for 120 ps) and then cooled down to 100 K in 50 K steps with 1.5 ps molecular dynamics at each step. Powell minimization was performed at 100 K for 1000 steps. Structure refinement was done on the basis of simulated annealing starting at 1000 K and ending at 100 K. Final structures were subjected to the X-PLOR Accept routine with the violation threshold for NOEs of 0.5 Å and dihedral angles of 5°. Angles, bond lengths or improper

were not allowed to deviate from ideal geometry by more than 5°, 0.05 Å and 5° respectively. Structures were superimposed using the BIOSYM INSIGHT viewer (Molecular Simulations, Inc., San Diego, CA, U.S.A.) and were analysed using X-PLOR analysis routines.

RESULTS

Figure 1 shows the amino acid sequences for β pep-19, β pep-25 and peptide dodecamers (SC peptides) which ‘walk through’ the sequence of β pep-25. The bactericidal activity of β pep-25 was observed to be equal to, or better than, that of β pep-19, and these ‘walk throughs’ were designed to identify segments of that amino acid sequence that contributed most to this activity. It was anticipated that bactericidal activity in these shorter SC peptides would be considerably less than that for parent peptide β pep-25. As reported below, this was not the case, and this is the basis for the present study.

Bactericidal activities

Chosen for investigation were seven bacterial strains, representing both Gram-negative and Gram-positive bacteria. For β pep peptides and β pep-derived dodecapeptides, bactericidal activities against these strains are given in Table 1 as the concentration which is effective at killing 50% of bacteria, i.e., LD₅₀. LD₅₀ values were determined from dose–response curves like those shown in Figure 2, in which the percentage of bacteria killed is plotted against the base-10 logarithm of the peptide concentration. Actual data points are shown with symbols as identified in the legend, and each curve represents the best fit using a sigmoidal function. LD₅₀ values are read from the fitted curve at 50% mortality. Overall, SC-4 functions best at killing both rough- and smooth-strain Gram-negative bacteria. LD₅₀ values range from 3 nM against *E. coli* J5 to 250 nM against J96. Against strains of *S. aureus*, SC-4 (80–400 nM) and SC-5 (180–350 nM) are comparable in activity with β pep-19 and β pep-25. BG-22, a peptide 28-mer having the amino acid sequence from the β -sheet domain of B/PI protein (residues 82–108) [8] proposed as being that site which promotes bactericidal activity, is less bactericidal than β pep-19 or β pep-25 and considerably less active than SC-4. In general, SC-4 demonstrates the best broad-spectrum bactericidal activity.

Leakage kinetics

Bactericidal peptides are thought to function generally by integrating into the bacterial membrane and creating channels such that bacteria become ‘leaky’ and die. To assess if this mechanism of action occurs with SC peptides, as well as with β pep peptides, membrane-leakage kinetics were investigated against two *E. coli* strains (H5 and J5) and one *S. aureus* strain (MN8). In each case, bacteria became ‘leaky’ within a few minutes after the addition of peptide. For any one of these strains, $t_{\frac{1}{2}}$ values are estimated to be in the range of 10 min.

Haemolytic activity

When considering the potential use of these peptides as antibiotic agents in mammals, it is important to know their potential to lyse eukaryotic cells. For this information, red blood cells were used as a model for all eukaryotic cells. Red blood cell lysis (haemolysis) was checked at peptide concentrations of 100 μM , 10 μM and 1 μM . At 1 μM , SC-1, SC-5 and BG-22 demonstrated 15–30% haemolysis, which increased to about 70% at 100 μM .

Figure 1 Peptide sequences

Peptide sequences are shown for β pep-19 and β pep-25, along with those of eight dodecapeptides which 'walk through' the sequence of β pep-25. The dodecapeptides are referred to as SC peptides. The -NH₂ at the right of each sequence indicates amidation of the C-terminal carboxylate group.

Table 1 Bactericidal and endotoxin-neutralizing activities of β pep-19, β pep-25, BG-22 (see the text) and the SC series of peptides

Bacteria J5, J96, H5 and IA2 are strains of *E. coli*; bacteria MN8 and MNHO are strains of *S. aureus*. *P.a.* is *P. aeruginosa*. S.D. values are about $\pm 30\%$ of the values given. N.A. means not active at concentrations below 10 μ M in the bactericidal assay and below 100 μ M in the endotoxin-neutralization assay.

		Bactericidal activity [LD ₅₀ (μM)]							Endotoxin-neutralizing activity [IC ₅₀ (μM)]
Gram-stain status ...		Gram-negative					Gram-positive		
Peptide	Strain ...	Rough	Smooth	J96	H5	IA2			
Bacterium ...	J5	<i>P.a.</i>							
βpep-19		0.01	0.03	0.1	0.71	0.06	0.25	0.16	2
βpep-25		0.02	0.06	0.13	0.20	0.30	0.16	0.1	1.6
BG-22		0.09	0.05	0.52	0.71	0.32	0.5	0.63	5
SC-1		0.24	0.86	0.12	0.7	0.48	0.65	0.48	3.5
SC-2		0.13	0.05	1.3	0.63	0.8	0.8	0.28	N.A.
SC-3		0.01	0.01	0.63	1.3	0.8	1.2	1	25
SC-4		0.003	0.01	0.25	0.08	0.08	0.35	0.4	1.9
SC-5		0.02	0.13	0.2	0.63	0.33	0.35	0.18	1.6
SC-6		0.4	0.09	1.1	1.3	0.4	0.48	0.35	3.5
SC-7		N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
SC-8		N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.

All other peptides showed less than 5–10 % effect at 1 μ M. Even at 100 μ M, β pep-25 induced only about 5 % haemolysis, while SC-3, SC-4, and SC-8 gave about a 10 % effect, and SC-2, SC-6 and SC-7 gave about a 15 % effect. For most SC peptides, 100 μ M is considerably greater than its bactericidal LD₅₀ value. For example, for SC-4, LD₅₀ values against *P. aeruginosa* and MN-8 are 50000 times and 500 times respectively less than this concentration.

LPS neutralization

Lysis of Gram-negative bacteria produces the endotoxin LPS which triggers the pathological disorder known as sepsis. In this respect, peptides which are not only bactericidal, but also

effectively neutralize LPS, are of considerable importance in combating endotoxaemia and sepsis. These peptides were therefore tested in the LAL assay for their ability to bind and to neutralize LPS. IC₅₀ values were determined from the dose–response curves shown in Figure 3 and are listed in μ M in the right-most column in Table 1. β pep-19, β pep-25 and SC-5 are essentially equipotent (IC₅₀ values of about 2 μ M), with SC-4 being not far behind. SC-1 and SC-6 are slightly less effective having IC₅₀ values of 3.5 μ M. SC-3 is about 10-fold less potent (IC₅₀ = 25 μ M) than either SC-4 or SC-5. SC-2, SC-7 and SC-8 show no LPS binding activity at or below 100 μ M. This was not so surprising for SC-7 and SC-8, which are essentially bactericidally inactive, but SC-2 did demonstrate reasonable bactericidal activity.

CD

CD spectroscopy, used to assess conformational populations of SC peptides, indicates the presence of helical structure. This is demonstrated in Figure 4, which shows selected CD traces for SC-5 and SC-7 acquired at various concentrations of TFE. In all cases, CD traces show a band of negative ellipticity at 222 nm which is characteristic of helix conformation [39–41]. At lower concentrations of TFE, mostly random-coil populations are present, as evidenced by a negative-ellipticity minimum at about 200 nm (rather than at 207/208 nm for helix) and a low $[\theta]_{222}/[\theta]_{200}$ ratio. As the concentration of TFE is increased, helix population increases, since the negative ellipticity band at 200 nm shifts closer to 207 nm and the 222 nm band becomes more negative. At about 20–30 % TFE, both these observable parameters have nearly reached a plateau, as indicated in the inset to Figure 4, in which $[\theta]_{222}$ is plotted against [TFE] in % (v/v). These CD spectral characteristics, however, would result from the presence of either α -helix or 3_{10} -helix or from a mixture of both. The right-handed 3_{10} -helix displays a negative CD band at 207 nm (λ_{min}) and a shoulder centred near 222 nm, with the ratio $[\theta]_{222}/[\theta]_{207}$ being about 0.4 [42,43]. For an α -helix, the 207 and 222 nm bands are also present; however, the ratio $[\theta]_{222}/[\theta]_{207}$ is closer to unity. When λ_{min} is 207 nm and the ratio $[\theta]_{222}/[\theta]_{207}$ is between 0.4 and 1.0, interconverting populations of 3_{10} -helix and α -helix are likely present.

Figure 2 Bactericidal dose-response curves for peptides

A selection of dose-response curves are shown. The percentage of bacteria killed was plotted against the base-10 logarithm of the peptide concentration. Data are provided for three strains of bacteria: (A) rough-strain *E. coli* J5 [SC-2 (◆), SC-4 (■), β pep-19 (▲) and β pep-25 (●)]; (B) smooth-strain *E. coli* IA2 [SC-1 (▼), SC-3 (◆), SC-4 (●), SC-5 (■) and β pep-19 (▲)]; and (C) a strain of Gram-positive *S. aureus*, MNHO [SC-3 (▲), SC-4 (■) and β pep-25 (●)]. Dose-response curves were acquired as described in the Materials and methods section.

For SC peptides, Table 2 lists the wavelength of the far-UV minimum, $\lambda_{\min}$, and the ratio $[\theta]_{222}/[\theta]_{207}$ at 70 % TFE. In cases where the $[\theta]_{222}/[\theta]_{207}$ ratio is closer to 0.4 than to 1.0 and $\lambda_{\min}$ is at 207 nm, the conformation of that SC peptide should have more 3_{10} -helix than α -helix character [42,43]. For the most active peptide, SC-4, $\lambda_{\min}$ is at 207 nm and $[\theta]_{222}/[\theta]_{207}$ is 0.49, indicating the presence of mostly 3_{10} -helix. Even though peptides SC-1,

Figure 3 Dose-response curves for endotoxin neutralization

Dose-response curves, as described in the Materials and methods section, are shown as percentage LPS neutralization plotted against the base-10 logarithm of the peptide concentration. Data are shown for SC-1 (●), SC-3 (▲), SC-4 (▼), SC-5 (◆), SC-6 (□) and for β pep-25 (■).

SC-2, SC-3, SC-5 and SC-6 have $\lambda_{\min}$ values of 207 nm, larger $[\theta]_{222}/[\theta]_{207}$ ratios of 0.6–0.7 indicate less 3_{10} -helix character. Since SC-7 and SC-8 show $\lambda_{\min}$ values of 203 and 204 nm respectively, significant populations of random coil should be present in their conformational ensembles.

NMR conformational analysis

Figure 5 shows the α H–NH and NH–NH regions from NOESY data for SC-4 in the absence (Figure 5A) and presence (Figure 5B) of 30 % (v/v) TFE. In either case, the series of NH–NH NOEs running from F3 to I12 alone indicates the presence of at least nascent helix conformation [44]. Moreover, even in the absence of TFE, four α H–NH $i,i+2$ NOEs are easily distinguishable, supporting the presence of multiple turn or 3_{10} -helix [45,46]. In the presence of TFE, these NOEs remain and other longer-range NOEs appear. For example, two α H–NH $i,i+3$ (8/5, 10/7), three NH–NH $i,i+2$ (8/6, 9/7, 10/8) and two NH–NH $i,i+3$ (10/7, 11/8) NOEs have been labelled in Figure 5 (where i is the residue number; an α H–NH $i,i+3$ indicates an NOE between a particular α H and the NH three residues away in the C-terminal direction.). For pure 3_{10} -helix, only $i,i+2$ and $i,i+3$ NOEs should be observed, whereas for pure α -helix, only $i,i+3$ and $i,i+4$ NOEs should be present [45]. The presence of α H–NH $i,i+2$ NOEs characteristic of 3_{10} -helix is consistent with the CD results discussed above. α -Helical conformation, however, must also contribute to the conformational ensemble as indicated by CD data. Since all SC peptides, to various extents, show similar NOE and CD trends, these peptides exist in aqueous solution in an equilibrium primarily among 3_{10} -helix, α -helix and random coil.

Since some SC peptides are relatively hydrophobic and could tend to self-associate, thereby affecting the presence and/or magnitude of NOEs, PFG-NMR diffusion measurements (results not shown) were made. For all SC peptides, diffusion coefficients derived from these data remain unchanged over the peptide concentration range of 0.1–15 mM, indicating the absence of aggregation.

Figure 4 CD spectra for SC peptides

Far-UV CD spectra for two SC peptides, SC-5 and SC-7, are shown as mean residue ellipticity versus wavelength (nm). Peptide concentration was 40 μ M in 20 mM potassium phosphate, pH 6.3. The temperature was set at 20 $^{\circ}$ C. Other experimental conditions are given in the Materials and methods section. CD traces shown were acquired as a function of TFE concentration from 0 to 70% (v/v). The inset shows the change in $[\theta]_{222}$ versus the concentration (% v/v) of TFE.

Table 2 CD data summary for SC peptides in the presence of 70% TFE

SC-401–SC-409 are SC-4 variants.

Peptide	$\lambda_{\min}$ (nm)	$[\theta]_{222}/[\theta]_{207}$ ratio
SC-1	208	0.83
SC-2	207	0.65
SC-3	207	0.59
SC-4	207	0.49
SC-401	207	0.63
SC-402	204	0.52
SC-403	208	0.79
SC-404	207	0.54
SC-405	207	0.71
SC-406	208	0.67
SC-407	207	0.58
SC-408	207	0.53
SC-409	208	0.65
SC-5	207	0.64
SC-6	207	0.73
SC-7	202	0.5
SC-8	204	0.48

For peptide SC-4, which forms the most stable 3_{10} -helical structure and is generally the most bactericidal, conformational modelling was performed using NOE data acquired for the

peptide in the presence of TFE/water (3:7, v/v). It should be emphasized that most of the same NOEs could be observed in the absence of TFE; however, 30% TFE did apparently stabilize the helix conformation and increase the overall tumbling correlation time, thereby generally increasing the magnitude of NOEs. A total of 140 NOE distance constraints were derived from analysis of NOESY spectra. These include 68 intra-residue, 24 sequential, 20 medium-range ($|i-j| < 5$), and 27 long-range ($|i-j| \geq 5$) constraints. In addition, a total of eight hydrogen bonds could be identified by inspection of initial SC-4 structures and from long-lived backbone NHs, giving rise to 16 hydrogen-bond distance constraints. The total number of experimentally derived constraints was therefore 156, giving an average of 13 constraints per residue.

Initially, 100 structures for SC-4 were calculated, as described in the Materials and methods section. The best-fit superpositions of backbone C^{α} atoms for the final 14 structures are shown in Figure 6 (left). These structures showed no NOE violations greater 0.5 $\text{\AA}$. Structural statistics are summarized in Table 3. The somewhat less structurally defined N-terminus is apparent. The structures satisfy experimental constraints quite well. For residues 3–12, atomic root-mean-square differences with respect to the mean co-ordinate positions are 0.71 ± 0.08 $\text{\AA}$ for backbone (N, C^{α} , C) atoms and 1.4 ± 0.4 $\text{\AA}$ for all heavy atoms. In addition, ϕ and ψ angular order parameters are all > 0.8 . Together, the above data indicate that these structures used to represent the solution conformation of SC-4 are well converged. The helical

Figure 5 NOESY spectra for SC-4

600 MHz ^1H NMR spectra are shown for SC-4 in the absence (**A**) or presence (**B**) of TFE/water (3:7, v/v). The peptide concentration was 6.3 mM in 10 mM potassium phosphate, pH 5.5, and 25 °C. Spectra were accumulated with 8 k data points over 6000 Hz sweep width and were processed with 1 Hz line-broadening. Only spectral regions downfield from the H_2O resonance are shown, and some key NOEs are identified.

Figure 6 NOE-derived structures of SC-4

For dodecapeptide SC-4, 14 final NOE-derived structures have been superimposed on the left of the Figure and structural statistics are given in Table 3. The average structure is shown on the right, and four positively charged residues, K1, K4, R5 and K8, which lie on one surface of the amphipathic helix, are shown in the space-filling mode.

Table 3 Structural statistics for the calculated structures of SC-4 from NMR data

Parameter	Value
Root-mean-square deviations from experimental distance restraints (Å)*	
140 NOEs	0.04 ± 0.0042
16 H-bonds	No deviation
Deviations from idealized geometry	
Bonds (Å)	0.038
Angles (°)	0.65 ± 0.03
Impropers (°)	0.25 ± 0.03
Energies (kcal · mol ⁻¹)	
ENOE†	9 ± 1
ECDIH	1.3 ± 0.6
ENCS	11.6 ± 1
EBOND	3.7 ± 0.4
EANGLE	30 ± 5
EIMPROPER	16 ± 2
ETOTAL	72 ± 7

* None of the 14 final structures exhibited distance restraint violations greater than 0.5 Å or dihedral angle violations greater than 5°. Root-mean-square-deviation values represent the means ± S.D. for the 14 structures.

† The final values of the NOE (ENOE), torsion angle (ECDIH) and NCS (ENCS) potentials have been calculated with force constants of 50 kcal · mol⁻¹ · Å⁻², 200 kcal · mol⁻¹ · rad⁻² and 300 kcal · mol⁻¹ · Å⁻² respectively (note: 1 kcal = 4.184 kJ).

structure of SC-4 is, not surprisingly, between that of an α -helix and a 3_{10} -helix, with the average number of residues per turn being 3.3. From the N-terminus to W9, the helix is amphipathic with K1, K4, R5 and K8 aligned on one face, as illustrated in Figure 6, with these residues being highlighted.

Effects of modifying the SC-4 sequence

Since SC-3 and SC-4 were the most bactericidal and least haemolytic, and SC-4 was one of the best at neutralizing

endotoxin, additional dodecapeptide 'walk throughs' were made to complete the single-residue shifts between SC-3 and SC-4 (the -NH₂ at the right of each sequence indicates amidation of the C-terminal carboxylate group):

SC-401 : QMKLFKRHLKWK-NH₂

SC-402 : MKLFKRHLKWKI-NH₂

In addition, a longer variant which extends SC-4 by one residue on each side of the sequence was investigated:

SC-403 : MKLFKRHLKWKIIV-NH₂

Since the presence of positively charged residues is known to be crucial to bactericidal and LPS-neutralizing activities, a series of lysine/arginine-substituted SC-4 variants was investigated:

SC-404 : XLFKRHLKWKII-NH₂

SC-405 : KLFKRHLKWKII-NH₂

SC-406 : KLFKRHLXWKII-NH₂

SC-407 : KLFKRHLKWXII-NH₂

SC-408 : KLFKKHLKWKII-NH₂

SC-409 : KLFKXHLKWKII-NH₂

SC-404-SC-407 change each of the four lysine residues to norleucine residues (lysine without the side-chain amine group). Variants SC-408 and SC-409 change the arginine, R5, to lysine and norleucine respectively.

CD data indicate that these variants also fold helically, fluctuating between 3_{10} - and α -helical structure like parent peptides SC-3 and SC-4 (results not shown). Table 2 lists $\lambda_{\min}$ and $[\theta]_{222}/[\theta]_{207}$ ratio values at 70 % TFE. Whereas SC-401 and SC-403 both demonstrate $\lambda_{\min}$ values of about 207 nm, their $[\theta]_{222}/[\theta]_{207}$ ratios are larger than those of either SC-3 or SC-4, indicating that these two SC-4 variants have more α -helix and less 3_{10} -helix character than parent peptide SC-4. SC-402, however, has a greater random-coil population in its conformational ensemble, since its $\lambda_{\min}$ value is 204 nm. The lysine/arginine variants, SC-404-SC-409, behave conformationally like parent SC-4, with considerable 3_{10} -helix character. NMR data, acquired in the presence of 30 % TFE (results not shown), support the presence of significant populations of helical conformation as observed for SC-3 and SC-4.

Bactericidal activities from these peptides were acquired against three of the strains used above, namely *E. coli* J5, *P. aeruginosa* and *S. aureus* MNHO. Results are given in Table 4. Against J5, SC-401 and SC-402 are more active than SC-3 and slightly less active than SC-4. Shifting the dodecapeptide unit from SC-3 towards SC-4, therefore, increases activity. On the other hand, addition of hydrophobic residues to both terminal positions in SC-4, i.e., SC-403, increases activity slightly. Using *P. aeruginosa*, bactericidal dose-response curves indicate that either dodecapeptide, SC-401 or SC-402, diminishes activity. This is somewhat surprising, since both SC-3 and SC-4 have the same activity against *P. aeruginosa* (see Table 1). This may have something to do with the way in which these peptides interact with the bacterial cell wall/membrane. Against *S. aureus* strain MNHO, shifting the dodecapeptide window from SC-3 towards SC-4 by only one residue, i.e., SC-401, essentially abolishes activity. Another one-residue shift (SC-402) brings back some activity, and lengthening both termini of SC-4 has no effect.

With the lysine variants, substitution of K1 or K4 for norleucine reduces the bactericidal activity against *E. coli* J5 only slightly, whereas substitution of K8 and K10 for norleucine shows a somewhat more significant decrease in activity. This is especially true for SC-407 (K10X). Nonetheless, bactericidal

Table 4 Bactericidal and endotoxin-neutralizing activities of SC-3 and SC-4 parent peptides and SC-4 peptide variants SC401–SC-409

S.D. values are about $\pm 30\%$ of the values given. N.A. means not active at concentrations below $10\ \mu\text{M}$. J5 and *P.a.* are described in Table 1.

		Bactericidal activity [LD ₅₀ (μM)]			Endotoxin-neutralizing activity [IC ₅₀ (μM)]
Peptide	Gram-stain status ... Strain ... Bacterium ...	Gram-negative		Gram-positive MNHO	
		Rough J5	Smooth <i>P.a.</i>		
Parent peptides					
SC-3		0.01	0.01	1	25
SC-4		0.003	0.01	0.4	2
SC-401–SC-403 variants					
SC-401		0.004	0.06	N.A.	126
SC-402		0.005	0.07	6	25
SC-403		0.002	0.05	0.4	6
Lysine/arginine variants:					
SC-404 (K1X)		0.004	0.22	1	7
SC-405 (K4X)		0.006	0.17	0.4	7
SC-406 (K8X)		0.005	0.05	2	3
SC-407 (K10X)		0.007	0.05	1	3
SC-408 (R5K)		0.003	0.07	2	1000
SC-409 (R5X)		0.001	0.22	0.3	3

activities remain respectable, indicating that removal of a single positive charge alone does not abolish activity. Substitution of R5 for lysine (K), however, shows no change in activity, while substitution of R5 for norleucine causes a significant activity increase. The NMR solution structure of SC-4 shows that K1, K4, R5 and K8 lie on one face of an amphipathic helix with K10 more to the side (Figure 5). This conformation of the peptide sandwiches K4 and R5 between K1 and K8. Removal of the charged group at position 5 may help to reduce charge–charge repulsion and to stabilize, via hydrophobic interactions, the remainder of the lysine core.

Once again, any changes to the SC-4 amino acid sequence reduce bactericidal effects against *P. aeruginosa* (Table 4). In the lysine/arginine series, substitution of K1, K4 and R5 for norleucine has the most detrimental effects. Against *S. aureus* strain MNHO, substitution of any one of these charged residues is more tolerated (Table 4).

These data also indicate that, while the mechanism of action of these peptides against various bacterial strains may be similar, there are discrete and significant differences related to the spatial orientation of charged and hydrophobic groups of the peptides. Moreover, even though CD and NMR data indicate helical conformation for these peptides, interaction with the bacterial cell wall/membrane may certainly modify this conformation, making derivation of precise structure–activity relationships impossible.

The ability of SC-4 variants to neutralize LPS was also investigated. Dose–response curves are shown in Figure 3, and IC_{50} values are listed in Table 4. As shown above, SC-401 and SC-402 are dodecapeptides which shift one residue each from SC-3 to SC-4 respectively. SC-4 has an IC_{50} of $2\ \mu\text{M}$, whereas SC-3 has an IC_{50} more than 10-fold greater ($25\ \mu\text{M}$). With a single residue shift from SC-3 towards SC-4, the activity of SC-401 (IC_{50} value of $126\ \mu\text{M}$) decreases 5-fold relative to that of SC-3. Shifting one more residue to yield SC-402 brings the LPS-neutralizing activity back to the level observed for SC-3 (IC_{50} of $25\ \mu\text{M}$). Comparison of these sequences indicates that it is

apparently crucial to have at least one hydrophobic residue at the C-terminal position. SC-3 has Trp and SC-402 has Ile. Least active SC-401 ends in a Lys residue. The most active peptide, SC-4, has the hydrophobic dipeptide Ile-Ile at its C-terminus. The exact functional significance is not clear, since equipotent SC-5 has only one hydrophobic residue, Leu, at its C-terminus, whereas SC-1 has Phe, SC-2 has His, SC-6 has Gly, and inactive SC-7 also has a Leu.

In the lysine-to-norleucine variants, substitution of the first two Lys residues (K1X and K4X) reduces the activity only about 3-fold. Substitution of the last two Lys residues (K8X and K10X) has essentially no effect on the ability of the peptide to neutralize LPS. In this respect, no one of these lysine residues is crucial to this activity. A surprising finding, however, was observed with SC-408 and SC-409. SC-408 is the R5K variant. Here, the activity decreases dramatically about 500-fold. Alone, this would suggest that the Arg residue is crucial to activity and charge conservation is insufficient to maintain activity. This conclusion, however, is challenged by results on SC-409, which has that Arg replaced with norleucine, completely removes the positive charge, but maintains hydrophobic character from the side-chain methylene groups. In SC-409, nearly full activity relative to that of SC-4 has been preserved. Therefore a small positively charged amine group in that position is detrimental to activity, but the charge itself plays no apparent role in activity.

DISCUSSION

Against bacteria investigated in this study, SC-4, with LD_{50} values falling in the single-digit-nanomolar-to-100-nanomolar range, is, overall, the most effective antibacterial of any of these peptides. Apart from SC-7 and SC-8, which are inactive at concentrations below $10\ \mu\text{M}$, the other SC peptides, particularly SC-3 and SC-5, are reasonably bactericidal as well. In fact, against *S. aureus* strains MN8 and MNHO, activities for peptides SC-1–SC-6 are within about a factor of 2 of each other, but against *E. coli* strain IA2, SC-4 stands out again, being about 4–10-fold more active than any other SC peptide. The observation that bactericidal peptides like SC-4 are more effective against Gram-negative strains as opposed to Gram-positive strains, is generally observed with other known bactericidal peptides. Differences in bactericidal activities against various strains are most likely due to variability in the components of the bacterial membranes and how these peptides interact with those membranes. For instance, the outer membrane surface of Gram-negative bacteria includes LPSs, whereas in Gram-positive bacteria there is no outer membrane and the cell wall includes the acidic polysaccharides, the teichoic acids. SC-4 must capture the appropriate compositional and structural features necessary for interactions with these mostly negatively charged cell surfaces in order to exhibit such broad-spectrum bactericidal activity.

Relative to bacterial membranes, normal mammalian cell membranes, which are composed predominantly of zwitterionic phosphatidylcholine and sphingomyelin phospholipids, have more positive-charge character. Given the significant decrease in the net negative charge of eukaryotic-cell membrane surfaces, bactericidal peptides generally interact less with mammalian cells. Nonetheless, bactericidal peptides can lyse mammalian cells at concentrations near their bactericidal LD_{50} concentrations [9]. In this respect, one must check that these concentration thresholds are not near one another in order to have the most effective and least cytotoxic, therapeutic bactericidal agent. Red blood cells are usually chosen as models for eukaryotic cells, and haemolysis is often used as an index of a peptide's cytolytic activity against mammalian cells. For most SC peptides,

significant haemolysis occurs only at concentrations above 100 μM , whereas LD_{50} values are all in the submicromolar to nanomolar ranges. This low cytotoxicity against mammalian cells is an added advantage to using SC peptides as antibiotics.

Antibacterial peptides have been classified in a number of ways. One classification is based on chemical-structural criteria and is broken down into two broad groups: linear peptides and cyclic peptides. Within the linear group, two subgroups can be distinguished: (1) linear peptides tending to adopt helical structure, and (2) linear peptides of unusual composition, rich in amino acids such as Pro, Arg or even Trp. Given the observation that SC peptides are helical, they are classified in the first subgroup. A list of other linear antibacterial peptides with helical conformation have been tabulated by Andreu and Rivas (see Table I in [47]) and include andropin, BLP-1, bombinin, bombolitin, the cecropins, ceratotoxin, clavanin, CRAMP (cathelin-related antimicrobial peptide), dermaseptin, enbocin, FALL-39, lycotoxin I, magainin, melittin, misgurin, PGLa, pleurocidin, seminalplasmin and styelin. Conformations of peptides within this group are most often discussed as being α -helical, which is a specific type of helix having 13 atoms or 3.6 residues per helical turn. However, it has been observed that many short linear peptides form a mixture of α -helix and 3_{10} -helix in aqueous solution [42,43]. By reviewing structural data (mostly CD measurements) on other known helix-forming antibacterial peptides listed above, it is apparent that some of these peptides also have 3_{10} -helix character as well. In this respect, the term ' α -helical' as used currently in the literature may actually refer to both types of helix, i.e., the 3_{10} -helix should not be excluded.

Both CD and NMR conformational analyses of SC peptides, especially of SC-4, show them to exist in aqueous solution as a mixture of α -helix, 3_{10} -helix and random coil. The degree of specific helix formation depends on the particular sequence and the presence or absence of TFE. With SC peptides, TFE does not appear to induce conformations that, to some extent, are not already present in water. NOE patterns, for example, are essentially the same in water and in TFE. However, in TFE, NOEs are generally larger, indicating that TFE acts to stabilize existing conformations. Moreover, TFE also provides an environment of lower dielectric which, in some ways, may mimic the low dielectric within a membrane. In terms of peptide folding, the presence of either type of helix is interesting, because SC peptides were derived from β pep-25 which forms β -sheet structure in aqueous solution [2]. It appears, therefore, that when removed from the context of the entire β pep 33-mer, SC peptides are free to form helical structure. It has also been observed that bactericidal activity in β pep peptides is negatively correlated with β -sheet stability [1], i.e., more stable β -sheet leads to decreased bactericidal activity. Within the bacterial membrane, this may make conformational rearrangements for some β pep peptides easier, thereby promoting greater bactericidal activity.

On the basis of NOE data, structural modelling shows SC-4 to be conformed as an amphipathic helix. For some time, amphipathic helical structure has been noted as being important for bactericidal activity. Even though the mechanism of action of bactericidal peptides is, for the most part, unclear, it has been shown that peptide-lipid, rather than receptor-mediated recognition processes, plays the major role in their function [48]. At least one antibacterial peptide, KLKLLLLLKLK-NH₂, effective only at a much higher concentration (100 μM) than SC-4, has been shown to function by forming channels in bacterial membranes, thereby increasing membrane permeability and interfering with function, ultimately resulting in cell death [49]. SC peptides probably function similarly, since leakage kinetics occur on the same time scale with a half-kill time of about 10 min. Two

mechanisms of action have been proposed to explain membrane permeation by amphipathic, helical peptides: (1) transmembrane pore formation via a 'barrel-stave' mechanism [50] or (2) membrane destruction/solubilization via a 'carpet-like' mechanism [51,52]. In the generally favoured 'carpet-like' mechanism, amphipathic helical peptides interact with the bacterial membrane surface such that their hydrophobic surfaces face the membrane and their hydrophilic surfaces face the solvent. When a threshold concentration of peptide is reached, the membrane is disrupted and a transient pore is formed. From the present results it is impossible to conclude definitively that SC peptides interact with cell membranes only when in a helical conformation. Nevertheless, when these peptides are conformed as amphipathic helices, their mechanism of action can be more readily explained.

The membrane surface of both Gram-positive and Gram-negative bacteria has a net negative charge, making it a favourable environment for interaction with polycationic peptides like SC-4. In fact, Gray et al. [8] showed that much of the bactericidal activity of the protein B/PI can be accounted for by the positively charged tripeptide sequence KWK. SC peptides, as well as the antibacterial peptides the cecropins [18], also contain the KWK tripeptide motif. Relative to SC-4, however, their bactericidal activities against *P. aeruginosa* are much less, with lethal concentrations, for example, of 6 μM for cecropin P1 [12] compared with 10 nM for SC-4. It is important to emphasize that a high net positive charge alone does not guarantee optimal bactericidal activity. For example, SC-4 and SC-5 both have a net charge of +6, even though SC-4 is the more potent of the two against Gram-negative bacteria. Furthermore, whereas SC-6 and SC-1 have net charges of only +3, SC-6 demonstrates about the same general bactericidal activity as SC-5, and SC-1 is at least as active as SC-4 against *E. coli* strain J96. The presence of hydrophobic residues also modulates bactericidal activity; yet hydrophobicity alone can also not account for these activity differences, since hydrophobic residue content in each SC peptide is about 50–60%. Another mostly positively charged and hydrophobic bactericidal peptide, derived from C-terminal amphipathic α -helix of platelet factor-4 [53], for example, has four lysine residues arrayed in tandem on one face of its helix; yet its activity is about 100 times less than that of SC-4.

Clearly, both composition and sequence contribute to function. Amino acid sequence in turn determines conformation, which, ultimately, is the key to understanding bactericidal effects from SC peptides and from other bactericidal peptides in general. In the case of SC-4, K1, K4, R5 and K8 are aligned on the same face of the helical molecule (see Figure 6). Since CD and NMR data indicate that the other SC peptides also have a propensity to form helical structure in aqueous solution and may also promote their antibacterial activity when in a helical conformation, Figure 7 shows helical-wheel projections for SC-1–SC-8. Note that the most active SC peptides, SC-3 and SC-4, have four positively charged residues aligned in tandem on one face of the helix. Moreover, in both cases, a lysine residue lies at the top of a diamond-shaped array with an R-K pair in the middle and another K at the bottom of this array. For SC-4, the diamond-shaped array is K1, R5-K4, K8, and for SC-3, the array is K4, R8-K7, K11. In SC-4, this array starts at the first turn of the helix from the N-terminus, whereas in SC-3, it starts from the second turn of the helix. This may be the reason why SC-4 is, overall, more active than SC-3. In the next most active peptide, SC-5, residues K1-R2 and K5 on the helix surface form a triangle shape which is part of a diamond shape. In less active peptides SC-1, SC-2 and SC-6, positively charged residues are positioned throughout the helix, and, in least active or non-active peptides SC-7 and SC-8, lysine residues are few and are interspersed with

Figure 7 Helical-wheel projections for SC peptides

SC peptide sequences are shown in helical-wheel projections as discussed in the text.

negatively charged residues. Too much should not be made of this diamond-shaped array in terms of antibacterial potency of SC peptides, since removal of any one those positively charged residues from SC-4 does not reduce its activity to any great extent. Nevertheless, at least part of this charge distribution may be required for optimal activity.

Disintegration of the Gram-negative bacterial cell outer membrane liberates the endotoxin LPS, which, in mammalian white blood cells, triggers the over release of various cytokines such as tumour necrosis factor- α and interleukin-1. This in turn can lead to the pathological disorder of sepsis and possibly eventual death. SC-4 is one such bactericidal peptide which has the added advantage of being able to neutralize LPS. While LPS-binding activity (IC_{50}) of SC-4 (2 μ M) is not as high as that of the 55 kDa protein B/PI (20 nM) [17], SC-4 is, after all, only a dodecapeptide. Mechanistically, the neutralization of LPS is thought to be mediated primarily by interactions between the protein/peptide and the lipid A portion of any given LPS. The nature of LPS varies from bacterial strain to strain; however, all LPS molecules contain a conserved core unit, lipid A, which is composed of a diphosphorylated diglycosaminoglycan with a number of attached phosphorylated oligosaccharides and several acyl chains. LPS neutralization has been proposed to occur via protein/peptide binding to lipid A both via cationic amino acid interactions with the anionic phosphates and via hydrophobic interactions with the acyl chains. In the SC series, positive charge does play a role in LPS neutralization. SC-4 and SC-5, which have the highest net positive charge of +6, also neutralize LPS most effectively. However, as with bactericidal activity, a high net positive charge alone cannot account for LPS neutralization. Consider, for example, SC-2 and SC-3, which both have a net charge of +5; yet SC-3 is considerably

more active than SC-2. Moreover, in the Arg/Lys-substituted norleucine series (Table 4), removal of any one positively charged residue decreases LPS-binding activity only slightly, probably indicating that the positive charge field itself, rather than any one individual charged residue, mediates activity. On the other hand, hydrophobic residues at the C-terminus appear to be more crucial to LPS binding. Removal of the two isoleucine residues from the C-terminus of SC-4 (peptide SC-401) decreases activity 60-fold, whereas removal of only one of these isoleucine residues (peptide SC-402) decreases activity by only 12-fold. One curious finding is that, while removal of R5 (SC-408) does not affect LPS binding, replacing R5 with lysine reduces LPS binding by some 500-fold. More such variants need to be investigated to draw definitive conclusions and structure-function relationships. This work is currently in progress.

Conclusions

Identification of a dodecapeptide with potent bactericidal activity against both Gram-positive and Gram-negative bacteria may lead to the development of a highly effective, therapeutic antibiotic and bacterial-endotoxin-neutralizing agent. Production and purification of bactericidal proteins or peptides with longer sequences present problems which in part can be avoided by the identification of a small peptide like SC-4. As new strains of antibiotic-resistant bacteria continually emerge, such novel bactericidal agents will be particularly useful.

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