**Lentivirus-derived antimicrobial peptides: increased potency by sequence engineering and dimerization**

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We have previously described a family of cationic amphipathic peptides derived from lentivirus envelope proteins that have properties similar to those of naturally occurring antimicrobial peptides. Here, we explored the effects of amino acid truncations and substitutions on the antimicrobial potency and selectivity of the prototype peptide, LLP1. Removal of seven residues from the C-terminus of LLP1 had little effect on potency, but abrogated haemolytic activity. Replacement of the two glutamic acid residues of LLP1 with arginine resulted in a peptide with greater bactericidal activity. We discovered that the cysteine-containing peptides spontaneously formed disulphide-linked dimers, which were 16-fold more bactericidal to *Staphylococcus aureus*. Monomeric and dimeric LLP1 possessed similar alpha helical contents, indicating that disulphide formation did not alter the peptide’s secondary structure. The dimerization strategy was applied to magainin 2, enhancing its bactericidal activity eight-fold. By optimizing all three properties of LLP1, a highly potent and selective peptide, named TL-1, was produced. This peptide is significantly more potent than LLP1 against Gram-positive bacteria while maintaining high activity against Gram-negative organisms and low activity against eukaryotic cells. In addition to new antimicrobial peptides, these studies contribute useful information on which further peptide engineering efforts can be based.

**Introduction**

Antimicrobial peptides are a recently discovered arm of the immune system. However, because of their existence in many species ranging from insect to human, it appears that they are among the most basic forms of innate immunity. These host defence peptides are particularly abundant in epithelial and immune cells, and their expression is induced in response to immune challenge.

In recent years, there has been a surge in the occurrence of bacteria that are resistant to conventional antibiotics. However, antibiotic-resistant bacteria are very often susceptible to antimicrobial peptides; this property has made natural bacterial agents attractive candidates for development of new antimicrobial therapies. Although many natural antimicrobial peptides exist, they often do not have all of the properties that would make them acceptable therapeutics, so efforts are under way to engineer peptides that possess more favourable properties.

Antimicrobial peptides can be classified according to well-defined structures. The two largest classes of motif are ‘linear’ alpha helices, exemplified by cecropins and magainins, and beta sheets folded by two or three disulphide bonds, as found in alpha and beta defensins and protegrins. These common structures (particularly the alpha helical peptides) are often created by highly diverse amino acid sequences. One approach to the development of antimicrobial peptides as chemotherapeutic agents involves optimization of the functional properties of natural peptides by sequence engineering of their structural features. A though some structure-function studies have been performed with magainin and...
cecropin analogues,\textsuperscript{17} as well as hybrid peptides,\textsuperscript{18,19} their highly diverse sequences make elucidation of general structure-function relationships arduous. However, primary amino acid homology is less important than secondary structure: many of these peptides possess sequences that, when modelled as alpha helices, display strong amphipathic character. This conserved structural motif highlights those features that may be important for antimicrobial activity.

We have described a group of peptides, derived from lentivirus envelope proteins, that interact with membranes and, in most cases, are antibacterial.\textsuperscript{12,20–22} These peptides, collectively referred to as Lentivirus Lytic Peptides (LLPs) are alpha helical in the presence of membrane-mimetic solvents,\textsuperscript{23} thus displaying a secondary structure common to magainins and cecropins. However, LLPs possess no amino acid sequence homology to these natural antimicrobial peptides. It was originally believed that they were non-selectively cytolytic, on the basis of their ability to kill both bacteria and a human T-cell line at similar peptide concentrations,\textsuperscript{20} as well as their moderate haemolytic activity.\textsuperscript{22,24} More recently, we demonstrated that several of the peptides are selective, killing bacteria at 50- to 100-fold lower concentrations than those required to lyse red blood cells (RBCs).\textsuperscript{12} LLP1, the peptide derived from the C-terminus of HIV-1 gp41 transmembrane protein, has been the focus of several studies of its membrane-interactive\textsuperscript{20–22,24,25} and calmodulin-binding properties.\textsuperscript{22,26–28}

In an effort to understand better the structure-function relationships that exist in the LLP family of amphipathic alpha helical peptides, we took a site-specific engineering approach. Several LLP1 analogues were synthesized and characterized with respect to their antibacterial and haemolytic activities to assess the effects of charged residues and peptide length. During the course of this investigation we discovered an unexpected role for cysteine in the antimicrobial activity of LLP1. These results provide insight into the structural requirements for antimicrobial and haemolytic activity, which may be applied to engineering of antimicrobial peptides for therapeutic use.

**Materials and methods**

**Peptide synthesis**

Peptides were synthesized as C-terminal amides using Fmoc synthesis protocols as described previously.\textsuperscript{26,29} Synthetic peptides were characterized and purified by reverse-phase high-performance liquid chromatography (HPLC), and the identity of each peptide was confirmed by mass spectrometry. Magainin 2 was either purchased from Calbiochem (La Jolla, CA, USA) and used without further purification or synthesized as above. The sequences of the peptides used in this study are listed in Table I.

**Structural analysis and design of peptide sequences**

The amino acid sequences of LLP1 and magainin 2 were analysed for similarity and optimal alignment using the Genetics Computer Group (Madison, WI, USA) package of programs through the Pittsburgh Supercomputing Center. The residue in magainin 2 to be replaced by Cys was chosen such that it would be in a similar environment to that found in LLP1. The program SIMPLIFY was used to categorize the amino acid residues as basic, hydrophobic or other, then the GAP alignment program was applied; it was determined that Cys 837 of LLP1 optimally aligns with Gly 3 of magainin 2. This is illustrated in helical wheel diagrams of LLP1 and magainin 2 (Figure 1), where each aforementioned residue is found at the interface of the hydrophobic and hydrophilic regions of the respective peptide. The engineered peptide, magG3C, in which Gly 3 is replaced by Cys (Table I), was synthesized as described above. Solutions of Cys-containing peptides contained a two-fold molar excess of dithiothreitol (DTT) to prevent disulphide formation. Unless otherwise specified, activities reported are those of the fully reduced peptide. Peptide concentrations were determined by quantitative ninhydrin assay as described previously,\textsuperscript{12} analytical HPLC by integration (A\textsubscript{220}) of the peptide peak, or simply measuring A\textsubscript{220} of a dilute solution (before adding DTT).

<table>
<thead>
<tr>
<th>Peptide name</th>
<th>Amino acid sequence\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>LLP1</td>
<td>RVIEVVQGA C R A I R H I P P R I R Q G L E R I L</td>
</tr>
<tr>
<td>LP21</td>
<td>RVIEVVQGA C R A I R H I P P R I R</td>
</tr>
<tr>
<td>LP17</td>
<td>V V Q G A C R A I R H I P P R I R</td>
</tr>
<tr>
<td>LP14</td>
<td>G A C R A I R H I P P R I R</td>
</tr>
<tr>
<td>Analogue 5</td>
<td>RVIRVVQGA C R A I R H I P P R I R Q G L R R I L</td>
</tr>
<tr>
<td>C837Y</td>
<td>RVIEVVQGA Y R A I R H I P P R I R Q G L E R I L</td>
</tr>
<tr>
<td>C837S</td>
<td>RVIEVVQGA S R A I R H I P P R I R Q G L E R I L</td>
</tr>
<tr>
<td>magG3C</td>
<td>G I C K F L H S A K K F G K A F V G E I M N S</td>
</tr>
<tr>
<td>TL1</td>
<td>RVIRVVQGA C R A I R H I P P R I R</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Sequences are aligned with respect to their Cys residues, indicated in bold.
Engineering lentivirus antimicrobial peptides

Dimerization of Cys-containing peptides by formation of disulphide linkages

Peptides containing a single Cys residue were oxidized under gentle conditions to produce disulphide-linked dimer peptides. This was accomplished using dimethyl sulphoxide (DMSO) in a manner similar to that described by Tam et al.30 Peptide solutions (2 mg/mL) in 10 mM ammonium acetate buffer, pH 6, and 20% DMSO were stirred vigorously for 18–24 h at room temperature. The extent of dimerization was monitored by reverse-phase HPLC and was usually evident from the slight difference in retention time of the dimeric peptide relative to the monomer (data not shown). Dimerized peptide was purified from DMSO and monomer peptide by preparative HPLC; this was followed by lyophilization or, if the reaction was complete, by dialysis of DMSO-containing mixtures in Spectra/Por 7 Membrane dialysis tubing (molecular weight cutoff = 1000; Spectrum Industries, Houston, TX, USA) against 0.1 mM acetic acid. Mass spectrometry as well as its mobility in a tricine acrylamide gel (see below) confirmed dimerization of each peptide.

Tricine gel electrophoresis of peptides

The method of Schagger and von Jagow,31 as described in detail,32 was used. Briefly, 1.5 nmol peptide samples were diluted in non-reducing sample buffer and loaded into a 16.5% acrylamide Tris-tricine Ready Gel (Bio-Rad, Hercules, CA, USA). Electrophoresis was carried out at 100 V for 1.5 h. The gels were fixed, stained in brilliant blue G (Bio-Rad), and destained according to the manufacturer’s instructions.32

Bacterial killing assay

Bacterial killing assays were conducted as described in detail previously12 using clinical patient isolates or laboratory isolates of several Gram-positive and Gram-negative bacteria. Briefly, bacterial suspensions ((0.5–1) \times 10^6 cfu/mL) in 10 mM potassium phosphate buffer, pH 7.2, were incubated with two-fold dilutions of peptides for 1 h at 37°C. Serial dilutions were performed and each was plated on tryptic soy agar (Difco, Detroit, MI, USA); colonies were counted the next day. MBC, the lowest two-fold dilution of peptide sufficient to cause 99.9% or 3 logs killing of bacteria, was determined by comparing colony counts on the controls (1:1000 dilution) with those on the undiluted test plates. The MBC was determined by comparing colony counts in the 1:1000 dilutions of both the control and treated plates, and is the lowest peptide concentration that resulted in fewer than half the number of colonies compared with the controls. The range of MBC values obtained from two to four experiments is given.

Erythrocyte lysis assay

As a measure of selectivity, peptides were screened for haemolytic activity as previously described.12,22 The average (two or three trials) peptide concentration required to lyse 50% of treated RBCs is reported.

Circular dichroism studies

Peptides were examined by circular dichroism (CD) as described previously.23 Briefly, CD spectra were recorded at ambient temperature (20°C) using a JASCO J-500C CD spectropolarimeter (Jasco, Inc., Easton, MD, USA) using a

Figure 1. Helical wheel diagrams of antimicrobial peptides LLP1 and magainin 2. Peptides were modelled as alpha helices; the view is down the centre of the helix. Hydrophobic residues are indicated by shaded circles. Numbering of LLP1 residues corresponds to the sequence of HIV-1 gp160 (HXB2 isolate). The amphipathicity is evident from the segregation of hydrophobic and hydrophilic residues on either side of the helix.
cell path length of 0.1 cm. The concentration of the peptide was 10 μM in 10 mM phosphate buffer. Several samples of peptide in various ratios of trifluoroethanol (TFE) and phosphate buffer were examined. The percentage of alpha helix under each condition was calculated by the method of Scholtz et al.33

Results

Table I lists the sequences of the synthetic peptides used in the studies described below. The peptides are analogues of LLP1, the carboxyl-terminal sequence of HIV-1 transmembrane protein (isolate HXB2R). The analogues may be grouped into two types; one set of analogues, referred to as truncated, includes peptides with 7–14 residues removed from the parent LLP1 sequence (see Table I). The other group comprises analogues possessing one or two amino acid substitutions in Glu or Cys residues.

Effect of peptide length on antimicrobial activity

LLP1 is a potent antimicrobial agent; at concentrations of 16 μM and above it reduces the number of viable bacteria in a culture up to a million-fold.12 However, with 28 amino acid residues, LLP1 is rather long compared with other antimicrobial peptides, and LLP-like peptides of shorter length have been shown to be membrane-active.34–36 To determine whether LLPs of reduced length are potent antibacterials, a series of truncated LLP1 peptides (LP21, LP17 and LP14; Table I) were synthesized and their antimicrobial activities tested. Antibacterial activity (Table II) was diminished as a function of the number of residues removed in analogues possessing 21, 17 or 14 residues compared with the native 28-residue peptide. The MBC values ranged from 8 to 16 μM for the full-length peptide to 64 μM for the shortest peptide. All of the truncated peptides possessed diminished antimicrobial activity, but LP21, with seven residues removed from the C-terminus, was only two-fold less active against Staphylococcus aureus than the full-length LLP1. In addition, haemolytic activity was abrogated by removal of seven residues from the C-terminus of LLP1; each truncated peptide was more selective, lysing <5% of RBC at its respective MBC.

Effect of net positive charge on antimicrobial activity

It has been previously shown that increasing the net positive charge of the peptide, by replacement of acidic residues by basic ones, enhances the antimicrobial activity in the context of LLP1; conversely, reducing the net positive charge drastically reduces activity.21 The replacement of two Glu residues by Arg generated a peptide, named analogue 5 (Table I), that was more active against both Gram-positive and Gram-negative bacteria than was LLP1. This analogue was found to be 8- to 16-fold more active against S. aureus than was LLP1 (Table II). In spite of the increase in bactericidal potency, analogue 5 displayed low haemolytic activity at the MBC.

Structure of Cys-containing peptides

In the course of these analogue studies, batch-to-batch variations in antimicrobial and haemolytic activity among preparations of LLP1 were often observed. In addition, LLP1 analogues in which the single Cys residue was replaced by Tyr or Ser (Table II and data not shown) were not as active as earlier batches of the parent peptide in bactericidal and haemolytic assays.12 These findings suggested that the single Cys residue contributed in some way to the membrane activity of LLP1. Furthermore, peptides that had been previously purified to homogeneity eluted as two very close peaks in reverse-phase HPLC (data not shown). Treatment of LLP1 with a 10-fold excess of DTT for 1 h at 37ºC resulted in a single peak on reverse-phase HPLC, suggesting that the second peak was due to the formation of disulphide-linked dimers. A nalysis by mass spectrometry revealed that ions corresponding to the molecular mass twice that of LLP1 were present in peptide preparations not subject to DTT treatment. The dimerized state of the peptides was demonstrated by non-reducing

Table II. Antimicrobial activity against S. aureus and haemolytic activity of LLP1 and analogue peptides

<table>
<thead>
<tr>
<th>Peptide name</th>
<th>Bactericidal activity MBC (μM)</th>
<th>MBC50 (μM)</th>
<th>% RBC lysis at the MBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>LLP1</td>
<td>8–16</td>
<td>1–2</td>
<td>13</td>
</tr>
<tr>
<td>LP21</td>
<td>16–32</td>
<td>4–8</td>
<td>5</td>
</tr>
<tr>
<td>LP17</td>
<td>&gt;64</td>
<td>32</td>
<td>4</td>
</tr>
<tr>
<td>LP14</td>
<td>&gt;64</td>
<td>32–64</td>
<td>4</td>
</tr>
<tr>
<td>Analogue 5</td>
<td>1</td>
<td>0.25–0.5</td>
<td>7</td>
</tr>
<tr>
<td>CB37Y</td>
<td>16–32</td>
<td>4–8</td>
<td>6</td>
</tr>
</tbody>
</table>

Figure 2. Tricine gel of peptides. The oligomerization states of the test peptides were examined by electrophoresis in a non-reducing tricine gel system (30). Each well contained 1.5 nmol peptide. Lane 1, CB37Y; lane 2, bis-LLP1; lane 3, LLP1; lane 4, bis-analogue 5; lane 5, analogue 5; lane 6, bis-TL1; lane 7, TL1; lane 8, bis-magG3C; lane 9, magG3C; lane 10, magainin 2.
tricine gel electrophoresis (Figure 2). This gel system resolves species with molecular weights in the 2000–5000 Da range better than does conventional glycine-containing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE), and the peptides migrate as a function of both mass and charge because of their high content of positively charged residues. The peptides C837Y and magainin 2, which cannot form disulphide-linked dimers because of their lack of Cys, were run as controls (lanes 1 and 10, respectively). The dimeric peptides migrated at a significantly reduced rate compared with the monomeric peptides. It was therefore concluded that this peptide, over time in solution, became oxidized to a disulphide-linked dimer. In some cases, peptides favoured the dimeric state and several hours of DTT treatment, as well as storage in this reducing agent, was required to maintain the monomeric state. Peptides dimerized by DMSO remained disulphide linked.

Role for disulphide-linked dimerization in antimicrobial activity of LLP1

With pure preparations of monomer (LLP1) and dimer (bis-LLP1) peptides, the effect of oxidation (dimerization) on antimicrobial activity was addressed. Striking differences in the membrane-permeabilizing activities between dimeric and monomeric peptides were observed. Figure 3 shows that bis-LLP1 killed more S. aureus than did monomeric LLP1 at equal peptide concentrations. With an MBC value of 0.5–1 μM, bis-LLP1 was 16-fold more active against S. aureus than its monomeric counterpart (Table III). C837Y, the analogue without disulphide-forming capability, possessed bactericidal activity similar to that of monomeric LLP1 (Table II and Figure 3) thus further illustrating the importance of the Cys residue for antimicrobial potency of bis-LLP1 against this Gram-positive organism. The dimerization strategy was also applied to analogue 5: bis-analogue 5 also had increased antimicrobial activity against S. aureus relative to its monomer (Figure 3). In contrast, dimerization did not improve the peptides’ activity against Pseudomonas aeruginosa; the monomer and dimer peptides killed the Gram-negative test isolate at equal concentrations (Table III). This result was confirmed with different isolates of P. aeruginosa and Serratia marcescens (Table IV, below).

Haemolytic activity of LLP1 was greatly affected by its oligomerization state. It had previously been reported that LLP1 lysed 50% of RBCs at concentrations of 20–40 μM. However, these values were obtained using a mixture of monomer and dimer peptides. Figure 4 depicts the haemolytic activities of monomer and dimer LLP1 and analogue peptides. Lysis of RBCs became notable (>10%) only at high peptide concentrations, where the differences between monomeric and dimeric peptides can be observed. For example, monomeric LLP1 lysed less than 10% of RBCs at concentrations below 20 μM, which is above the MBC. Although bis-LLP1 was more haemolytic than mono-LLP1 at high concentrations (Figure 4), both peptides were only slightly haemolytic at their effective bactericidal concentrations. Likewise, bis-analogue 5 displayed enhanced antimicrobial (Figure 3) and haemolytic activity (Figure 4) compared with monomeric analogue 5. Thus, it appears that, in the context of this panel of peptides, haemolytic activity is more a function of dimerization state than the net positive charge on the peptide.

Table III. A ntimicrobial and haemolytic activities of dimeric peptides

<table>
<thead>
<tr>
<th>Peptide name</th>
<th>MBC (μM)</th>
<th>% RBC lysis at the MBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>LLP1</td>
<td>8–16</td>
<td>1–2</td>
</tr>
<tr>
<td>bis-LLP1</td>
<td>0.5–1</td>
<td>2</td>
</tr>
<tr>
<td>Analogue 5</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>bis-Analogue 5</td>
<td>8–16</td>
<td>2</td>
</tr>
<tr>
<td>magainin 2</td>
<td>32–64</td>
<td>1–2</td>
</tr>
<tr>
<td>magG 3C</td>
<td>32–64</td>
<td>1</td>
</tr>
<tr>
<td>bis-magG 3C</td>
<td>8–16</td>
<td>1–2</td>
</tr>
<tr>
<td>TL1</td>
<td>8–16</td>
<td>1</td>
</tr>
<tr>
<td>bis-TL1</td>
<td>1–2</td>
<td>0.5–2</td>
</tr>
</tbody>
</table>

Influence of dimerization on secondary structure

To ascertain whether the presence of the disulphide bond influenced the secondary structure, the monomeric and dimeric peptides were compared by circular dichroism in phosphate buffer and TFE, which can mimic conditions of
membranes. LLP1, like other lentivirus antimicrobial peptides, was found to have a disordered conformation in aqueous solution and a predominantly alpha helical conformation in the presence of TFE. Monomeric LLP1 was 11.2% helical in 10 mM phosphate buffer and 65.6% helical in TFE-phosphate buffer; the corresponding values for the C837S analogue, 10.5% and 62.5%, were similar. The secondary structure of the dimer was much like that of the monomer: bis-LLP1 was 12.2% helical in phosphate buffer and 61.2% helical in TFE-phosphate buffer. In addition, all of the peptides reached maximum helicity at approximately 30% TFE (data not shown). These values suggest that dimerization does not predispose the peptide to an alpha helical conformation; it is likely that the amphipathic helical structure does not form unless the peptide is associated with a membrane, regardless of whether it is covalently dimerized.

**Use of dimerization as a general motif to enhance activity**

To determine whether the intermolecular disulphide motif could be engineered into another peptide to enhance its potency, an analogue of magainin 2 was engineered to contain a Cys residue in place of Gly 3. This residue is at the interface of the hydrophobic and basic-hydrophilic faces of the peptide when in an alpha helical conformation, as it is found in LLP1 (Figure 1). This engineered analogue, named magG3C, was compared with magainin 2 in both its monomeric and dimeric forms. A was found for LLP1, disulphide-linked dimerization of magG3C conferred 4- to 8-fold greater antibacterial potency against S. aureus compared with monomeric magG3C or the parent magainin 2 (Table III). In addition, each of these peptides had very low haemolytic activity (Table III). The assays performed with P. aeruginosa indicated no change in anti-Gram-negative activity. These results show that this motif, unique to LLP1 among antimicrobial peptides, may be applied to other peptides to enhance their activities against Gram-positive bacteria while maintaining micromolar potency against Gram-negative bacteria.

**Combination of peptide engineering approaches**

LLP1 was engineered to create a new analogue named TL1, which combines the desirable features of high positive charge (increased bactericidal activity) and shortened length (reduced haemolytic activity). Like LP21, this peptide is 21 amino acid residues long; however, it possesses an additional Arg residue (Table I). Comparing the monomeric peptides, TL1 had bactericidal activity against S. aureus equal to LLP1 (Table II); TL1 was 25% more potent on a per-gram basis than LLP1. Dimerization of TL1 led to an eight-fold increase in potency against S. aureus compared with monomeric TL1, and bis-TL1 was nearly as potent as bis-LLP1. Importantly, dimerization did not significantly affect the potency of TL1 against P. aeruginosa.

**Table IV.** Bactericidal activity of bis-TL1 and mono-LLP1 against a panel of seven isolates

<table>
<thead>
<tr>
<th>Bacterial isolate</th>
<th>bis-TL1 (µM)</th>
<th>LLP1 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MBC</td>
<td>MBC&lt;sub&gt;50&lt;/sub&gt;</td>
</tr>
<tr>
<td>Gram-positive:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. aureus</td>
<td>1–2</td>
<td>0.5–2</td>
</tr>
<tr>
<td>methicillin-resistant S. aureus</td>
<td>1</td>
<td>0.25–0.5</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>8–16</td>
<td>0.5–1</td>
</tr>
<tr>
<td>vancomycin-resistant E. faecalis</td>
<td>1</td>
<td>0.25–1</td>
</tr>
<tr>
<td>Gram-negative:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. marcescens (646)</td>
<td>1</td>
<td>0.25–0.5</td>
</tr>
<tr>
<td>S. marcescens (231)</td>
<td>4</td>
<td>0.5</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>0.5–2</td>
<td>0.25–0.5</td>
</tr>
</tbody>
</table>

**Figure 4.** RBC lysis by monomeric and dimeric peptides. RBCs were incubated for 1 h in PBS containing two-fold dilutions of peptides. Per cent lysis is measured as the amount of haemoglobin release compared with a standard curve (RBCs in water). Data plotted are the average (two to four trials) per cent lysis at each peptide concentration. ■, bis-LLP1; □, LLP1; ▽, bis-analogue 5; ▼, analogue 5; ○, bis-TL1; ●, TL1; ×, C837Y.
increase its haemolytic activity: bis-TL1 was much less haemolytic than bis-LLP1 (Figure 4). Consequently, by sequence engineering a highly potent and selective antimicrobial peptide was achieved.

TL1 was further evaluated against a panel of bacteria described previously comprising Gram-positive and -negative isolates with different antimicrobial susceptibilities. The engineered peptide bis-TL1 was compared with the original, monomeric sequence LLP1; TL1 was more potent against these isolates compared with LLP1 (Table IV); the difference in potency between the two peptides varied with the organism being treated. Whereas monomeric LLP1 was more effective against Gram-negative than Gram-positive isolates, bis-TL1 was bactericidal, at low-micromolar concentrations, to both types of bacteria. Thus by engineering LLP1 to create bis-TL1 the anti-Gram-positive activity was improved and high anti-Gram-negative activity was maintained. In addition, Table IV shows that the peptides were effective against antibiotic-resistant pathogens; the methicillin-resistant clinical isolate of S. aureus was equally susceptible to the peptides as the laboratory-cultured, non-resistant isolate, and the vancomycin-resistant isolate of Enterococcus faecalis was even more susceptible to the antimicrobial peptides than was the non-resistant isolate. These data demonstrate the activity of the bis-TL1 engineered peptide against a variety of clinically relevant bacteria.

Discussion

The three major goals or requirements for antimicrobial peptides are potency, selectivity or specificity, and activity under physiological conditions. Potency refers to the activity per amount of reagent: a more potent peptide requires less of it to be effective, reducing unwanted side effects and production costs. Selectivity is achieved by peptides possessing high antimicrobial activity yet low eukaryotic toxicity; this is desirable so that concentrations of peptide that are effective against bacteria yet do not damage host tissue can be used. A third goal is to create peptides that are active under physiological conditions, particularly ionic strength. To date, only a few peptides possessing bactericidal activity in high-salt environments have been reported; a notable example is the family of clavanins from tunicates. A’s most antimicrobial peptides suffer from this limitation; they have not yet been used successfully to treat systemic infections. In this study we have improved both potency and selectivity of the parent peptide, LLP1, by changing specific amino acids, and engineered a unique motif into magainin 2 to enhance its activity.

The potency of LLP1 against S. aureus can be altered by replacement of acidic residues with basic ones, resulting in an increase in the net charge of the peptide. This is demonstrated by the difference in activity of analogue 5 compared with LLP1. A analogue 5 has two acidic-to-basic (Glu-to-A rg) substitutions and possesses 16-fold higher antibacterial activity. This peptide is an example of what can be done to engineer an increase in potency. A’s haemolytic activity did not appreciably increase as a result of the additional charge, this peptide is both more potent and more selective than LLP1. In the case of analogue 5, dimerization did not dramatically improve antibacterial activity (two-fold increase), and it made the peptide slightly more haemolytic (Table III). This suggests that there may be some inherent limit for antibacterial activity by peptides of this general structure. Curiously, the above alterations in peptide composition resulted in little difference in activity against our Gram-negative test isolates (Tables III and IV); similar results have been obtained for other Gram-negative organisms. These results indicate that differences in the membrane structures that distinguish these two classes of bacteria make them differentially susceptible to this family of cationic antimicrobial peptides.

Reducing the length of LLP1, as demonstrated by peptide LP21, reduces haemolytic activity but largely retains bactericidal activity. By combining the two approaches of addition of positive charge and length reduction, we achieved a new peptide, TL1, which is potent bactericidal. That TL1 and bis-TL1 have low haemolytic activities suggests that there may be a critical minimum length required for haemolytic activity of LLP-like peptides; bis-magG3C, with a peptide length of 23 amino acids, was haemolytic at higher concentrations (data not shown). A brogation of haemolytic activity in the shortened LLP1 analogues may also be due to the nature of the seven-residue sequence removed. The presence of Gly in the sequence (Table I) may cause the full-length peptide to be bent in this region, which may facilitate interactions with RBC membranes. Whatever the explanation, the shorter sequence is an attractive feature of an antimicrobial peptide as it is less costly to synthesise.

The most surprising result is the role of the single Cys residue in antibacterial potency. The presence of a single Cys residue allows peptides to form disulphide-linked dimers, which improves the Gram-positive bactericidal activity of both LLP1 and magainin 2. Although oligomerization of peptides to form a ‘channel’ or ‘pore’ has been hypothesized for many and shown for some membrane-active peptides (e.g. melittin), the peptide-peptide associations have always been proposed to be non-covalent. This is the first example of a covalent interaction playing a role in the oligomerization of an antimicrobial peptide. The interaction is not absolutely necessary for activity, as evidenced by the antimicrobial activities of the LLP1 analogue C837Y (Table II) and other lentivirus-derived antimicrobial peptides that do not possess Cys. The CD data suggest that the presence of the Cys does not alter the conformation of the peptide backbone, but instead enhances peptide activity by providing a nucleation site for oligomerization, perhaps shifting the equilibrium to favour oligomers.

E ngineering lentivirus antimicrobial peptides
Cysteine is a common amino acid in some classes of antimicrobial peptides; for example, defensins contain six Cys residues,11 and protegrins have four.16 The Cys residues in these peptides occur in pairs and are involved in intramolecular disulphide bond formation. LLP1 is unique in that its single Cys residue forms an intermolecular bond resulting in a disulphide-linked dimer. It is also different from defensins and protegrins in that the secondary structure of LLP1 (in buffer–TFE mixtures) primarily consists of alpha helix, whereas the others comprise beta sheets and turns.12–14

In conclusion, by sequence engineering of LLP1 we created peptides that have high antimicrobial potency and selectivity. In addition, a unique structural motif, the intermolecular disulphide bond, was identified in LLP1 and shown to contribute to its high bactericidal activity. This motif was successfully engineered into magainin 2 to improve its antimicrobial activity. The combination of the engineering approaches used here is illustrated in peptide bis-TL1, which is potently bactericidal to both Gram-positive and -negative bacteria while maintaining very low haemolytic activity. These strategies may be applied in other ways to create even better antimicrobial agents that are effective in a variety of environments.

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