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Anti-Tuberculosis Activity of α -Helical Antimicrobial Peptides: *De Novo* Designed L- and D-Enantiomers Versus L- and D-LL37

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Abstract

With the emergence of multi-drug resistant (MDR) and extensively drug resistant (XDR) *Mycobacterium tuberculosis* (Mtb), new classes of anti-mycobacterial agents with very different modes of action compared to classical antibiotics, are urgently needed. In this study, a series of 26-residue, amphipathic α -helical antimicrobial peptides consisting of all D-amino acid residues and synthetic human L-LL37 (L-enantiomer) and D-LL37 (D-enantiomer) were investigated against *M. tuberculosis* susceptible strain (H37Rv) and a clinical multi-drug resistant strain (Vertulo). Minimal inhibitory concentrations (MICs) were determined through a peptide killing assay. D5, the most active analog against *M. tuberculosis* had a MIC value of 11.2 μ M (35.2 μ g/ml) against H37Rv strain and 15.6 μ M (49 μ g/ml) against the MDR strain. Peptide D1 had similar activity as D5 against the MDR strain (57 μ g/mL), a 9-fold improvement in hemolytic activity and a 7.4-fold better therapeutic index compared to D5. Surprisingly, LL37 enantiomers showed little to no activity compared to the *de-novo* designed α -helical antimicrobial peptides.

Keywords

Antimicrobial peptides; *Mycobacterium tuberculosis*; hemolysis; all D-enantiomer

INTRODUCTION

Mycobacterium tuberculosis (Mtb) are classified as acid-fast Gram-positive bacteria due to their lack of a typical outer cell membrane [1]. Contagious tuberculosis spreads through the air. According to World Health Organization's report [2]: more than 2 billion people, one-third of the world's population, are infected with *M. tuberculosis*. In 2008, 9.4 million new cases of tuberculosis (TB) were reported including 1.4 million cases among people living with human immunodeficiency virus (HIV). There were 1.8 million people who died from TB in 2008, including 500,000 people with HIV. TB is a leading killer of HIV patients. 5% of all TB cases involve multi-drug resistant (MDR) Mtb, which is a form of Mtb that is difficult and expensive to treat and fails to respond to standard first-line drugs. The

emergence and rapid spread of MDR Mtb strains represents a worldwide health care problem. *M. tuberculosis* within host cells is surrounded by a capsule outside the bacterial wall and membrane which represents a passive barrier impeding the diffusion of molecules towards inner parts of the envelope [3]. The capsule consists of a complex mixture of polysaccharides, proteins, lipids and enzymes including pro-teases and lipidases all of which may participate to the active resistance of the bacterium to the hosts' microbicidal mechanisms. Since, the lipid-rich cell wall structure of mycobacteria makes the cell surface hydrophobic, the permeability to anti-tuberculosis drugs is reduced. It is believed that the resistance to anti-mycobacterial drugs is mainly due to the peculiar properties of the mycobacterial cell envelope [4]. Actively growing bacilli have to transport the breakdown products of host macromolecules and nutrients to ensure their survival [3].

The importance of the protective function of the mycobacterial envelope is demonstrated by the fact 1M sodium hydroxide is sufficient to kill most microorganisms but many treated samples of *M. tuberculosis* remain viable and can be grown in uncontaminated cultures after treatment with sodium hydroxide [3]. Antibiotic resistance, due to the extensive clinical use of classical antibiotics, is a growing health concern worldwide. Thus, there is an urgent need for a new class of antibiotics not only to treat *M. tuberculosis* infection but other microorganisms in general. Cationic antimicrobial peptides (AMPs) have been proposed as a potential new class of antibiotics with the ability to kill target cells rapidly, broad spectrum activity and activity against some of the most serious antibiotic-resistant pathogens isolated in clinics. Also it is thought that the development of resistance to membrane active peptides whose sole target is the cytoplasmic membrane is considerably reduced.

We believe the factors important for α -helical AMPs to have the desired properties of a clinical therapeutic to treat bacterial infections now include the following [5–9]: (1) an amphipathic nature with a non-polar face and a polar/charged face; (2) the presence of high number of positively charged residues on the polar face and an overall net positive charge; (3) an optimum overall hydrophobicity; (4) the importance of lack of structure in aqueous conditions but inducible structure in the presence of the hydrophobic environment of the membrane; (5) the presence of “specificity determinant(s)”, that is, positively charged residue(s) in the center of the non-polar face which serve as determinant(s) of specificity between prokaryotic and eukaryotic cell membranes. These “specificity determinant(s)” reduce or eliminate toxicity (as measured by hemolytic activity against human red blood cells) by decreasing or eliminating transmembrane penetration into eukaryotic membranes but allowing antimicrobial peptide access to the interface region of prokaryotic membranes; (6) the importance of reducing peptide self-association in aqueous environment which allows the monomeric unstructured peptide to more easily pass through the cell wall components to reach the bacterial cytoplasmic membrane; (7) the sole target for the peptide should be the bacterial membrane and the peptide should not be involved in any stereoselective interaction with chiral enzymes or lipids or protein receptors; (8) the peptides should be prepared in the all D-conformation to provide resistance to proteolysis; and (9) the extent of binding to serum proteins must be modulated since only the unbound peptide is available to interact with the bacterial target.

Although the exact mechanism of action of AMP's is not known and can differ depending on the AMP, it is clear that the positively charged residues of the AMP are attracted to the negatively charged surface of the bacterial membrane. The non-polar face of the AMP must be incorporated into the lipid bilayer where peptide accumulation in the membrane results in increased permeability and loss of barrier function, leakage of cytoplasmic components and cell death. Antimicrobial peptides whose sole target is the cytoplasmic membrane must pass through the capsule and bacterial cell wall of Mtb to reach the membrane and must be

resistant to proteases in the capsule. For this reason we have designed our antimicrobial peptides in the all D-conformation.

The 37-residue peptide LL37, the only human member of the cathelicidin family of antimicrobial peptides [10], is considered to play an important role in the innate immune response to *M. tuberculosis* infection. LL37 is expressed by mononuclear phagocytes and neutrophils [11]. *In vitro* studies showed that LL37 mRNA is upregulated in a dose- and time-dependent manner in A549 cells in response to stimulation with mycobacterium cells [11]. This process is vitamin D-mediated in human macrophages [12]. According to Martineau *et al.* [13], the synthetic peptide LL37 induced a dose dependent reduction in CFU (colony forming unit)/mL of *M. tuberculosis* H37Rv strain in iron-depleted broth (10 nM Fe, 7H9 medium). At 100 µg/mL, the CFU/mL was reduced from around 10^8 to 10^7 in 7 days.

Previously, we demonstrated that a hybrid peptide, V681 (Cecropin A (1–8) + Melittin B (1–18) derivative), had excellent antimicrobial activity but also exhibited high toxicity to human red blood cells as measured by hemolytic activity [5]. We showed that a single valine to lysine substitution in the center of the non-polar face (V13K) dramatically reduced toxicity and increased the therapeutic index [5]. This was the first report that a single substitution of a positively charged residue in the center of the non-polar face could act as a “specificity determinant” in an α -helical antimicrobial peptide that is creating specificity for prokaryotic cells. Furthermore, the all-D *de-novo* designed peptide was resistant to proteolytic enzyme degradation [6], which enhances the potential of D-V13K and analogs as clinical therapeutics as well as anti-tuberculosis drugs. Our D-V13K analogs have been tested on many different types of microorganisms, including Gram-negative bacteria, Gram-positive bacteria and Zygomycota and Ascomycota fungi [5–8].

In the current study, we used the D-V13K and analogs to investigate the effect of increasing hydrophobicity of the antimicrobial peptides on their anti-tuberculosis activity toward two *M. tuberculosis* strains, a standard strain, H37Rv and a multi-drug resistant strain, Vertulo. As a comparison, the synthetic D- and L-LL37 were used. We showed that hydrophobicity has significant effects on anti-tuberculosis activity and must be optimized to provide the highest therapeutic index.

MATERIALS AND METHODS

Peptide Synthesis and Purification

Synthesis of the peptides was carried out by standard solid-phase peptide synthesis methodology using t-butyloxy-carbonyl (t-Boc) chemistry and 4-methylbenzhydrylamine resin (substitution level 0.97 mmol/g) followed by cleavage of the peptide from the resin as described previously [5–7]. Peptide purification was performed by reversed-phase high-performance liquid chromatography (RP-HPLC) on a Zorbax 300 SB-C₈ column (250×9.4 mm I.D.; 6.5 µm particle size, 300 Å pore size; Agilent Technologies, Little Falls, DE, USA) with a linear AB gradient (0.1% acetonitrile/min) at a flow rate of 2 mL/min, where eluent A was 0.2% aqueous trifluoroacetic acid (TFA), pH 2, and eluent B was 0.2% TFA in acetonitrile, where the shallow 0.1% acetonitrile/min gradient started 12% below the acetonitrile concentration required to elute the peptide on injection of analytical sample using a gradient of 1% acetonitrile/min [14].

Analytical RP-HPLC and Temperature Profiling of Peptides

The purity of the peptides was verified by analytical RP-HPLC and the peptides were characterized by mass spectrometry (LC/MS) and amino acid analysis. Crude and purified peptides were analyzed on an Agilent 1100 series liquid chromatograph (Little Falls, DE,

USA). Runs were performed on a Zorbax 300 SB-C8 column (150_2.1 mm I.D.; 5 μ m particle size, 300 Å pore size) from Agilent Technologies using a linear AB gradient (1% acetonitrile/min) and a flow rate of 0.25 mL/min, where eluent A was 0.2% aqueous TFA, pH 2, and eluent B was 0.18% TFA in acetonitrile. Temperature profiling analyses were performed on the same column in 3°C increments, from 5°C to 80°C using a linear AB gradient of 0.5% acetonitrile/min, as described previously [5–7, 15].

Characterization of Helical Structure

The mean residue molar ellipticities of peptides were determined by circular dichroism (CD) spectroscopy, using a Jasco J-810 (D1 to D5) or J-815 (LL37) spectropolarimeter (Easton, MD, USA) at 5°C under benign (non-denaturing) conditions (50 mM NaH₂PO₄/Na₂HPO₄/100 mM KCl, pH 7.0), hereafter referred to as benign buffer, as well as in the presence of an α -helix inducing solvent, 2,2,2-trifluoroethanol, TFE, (50 mM NaH₂PO₄/Na₂HPO₄/100 mM KCl, pH 7.0 buffer/50% TFE). A 10-fold dilution of an approximately 500 μ M stock solution of the peptide analogs was loaded into a 0.1 cm quartz cell and its ellipticity scanned from 195 to 250 nm. Peptide concentrations were determined by amino acid analysis.

Determination of Peptide Amphipathicity

Amphipathicity of peptides were determined by the calculation of hydrophobic moment [16], using the software package Jemboss version 1.2.1 [17], modified to include a hydrophobicity scale determined in our laboratory [18, 19]. The hydrophobicity scale used in this study is listed as followed: Trp, 33.0; Phe, 30.1; Leu, 24.6; Ile, 22.8; Met, 17.3; Tyr, 16.0; Val, 15.0; Pro, 10.4; Cys, 9.1; His, 4.7; Ala, 4.1; Thr, 4.1; Arg, 4.1; Gln, 1.6; Ser, 1.2; Asn, 1.0; Gly, 0.0; Glu, -0.4; Asp, -0.8 and Lys, -2.0. These hydrophobicity coefficients were determined from reversed-phase chromatography at pH7 (10 mM PO₄ buffer containing 50 mM NaCl) of a model random coil peptide with a single substitution of all 20 naturally occurring amino acids [18]. We proposed that this HPLC-derived scale reflects the relative difference in hydrophilicity/hydrophobicity of the 20 amino acid side-chains more accurately than previously determined scales (see recent review where this scale was compared to other scales [19]).

Mycobacterium tuberculosis Strains

M. tuberculosis H37Rv, a susceptible strain, was used along with Vertulo a multi-drug resistant strain which is used as a quality control strain at National Jewish Health mycobacteriology reference laboratory. Cultures were grown in 7H9 broth for 7–10 days and then diluted to an optical density of McFarland Standard No. 1. This density of cells is approximately 10⁸ CFU/ml. The bacterial suspension was then preserved in 1 ml aliquots at -70°C until the time of use.

Peptide Recovery from *M. tuberculosis* Culture Filtrate

M. tuberculosis strain H37Rv was grown in RMPI 1640 (Fisher scientific) tissue culture media (10⁶ CFU/mL) for 3 days. The bacteria culture was centrifuged at 1500g for 10min. The supernatant was removed and filtered (0.2 micron). The peptides were added to the culture filtrate for 24 hours or 7 days and peptide recovery was determined by RP-HPLC from 3 different samples at each time period. The analytical RP-HPLC condition is as describe above. The recovery rate is based on the peak integration values compared to a standard curve.

Peptide Killing Assay and Measurement of Anti-tuberculosis Activity (MIC)

A fresh suspension of 10^6 CFU/ml of either H37Rv or Vertulo strain was made from the frozen stock in Middlebrook 7H9 liquid media (Becton Dickinson, Franklin Lakes, New Jersey) into 5 ml polypropylene tubes. To the fresh bacterial suspension the peptides were added at the desired concentration and incubated for 7 days at 37° C and 5% CO₂. Samples were plated on Middlebrook 7H11 (Hardy Diagnostics Santa Maria, CA) whole plates on day 0 and day 7. The plates were incubated for 3 weeks at 37°C before counting to determine CFU/ml. Peptide concentrations of 1, 10 and 100 µg/ml determined by amino acid analysis, were used. On the concentration-response format Figs. (4,5 and 6), the point at which the curve crossed the concentration of the initial inoculum (dashed line) was reported as the minimal inhibitory concentration (MIC). MIC is given as mean value of 4 sets of determinations.

Measurement of Hemolytic Activity (HC₅₀)

Peptide samples (concentrations determined by amino acid analysis) were added to 1% human erythrocytes in phosphate-buffered saline (100 mM NaCl, 80 mM Na₂HPO₄, 20 mM NaH₂PO₄, pH 7.4) and the reaction mixtures were incubated at 37°C for 18 h in microtiter plates. Twofold serial dilutions of the peptide samples were carried out. This determination was made by withdrawing aliquots from the hemolysis assays and removing unlysed erythrocytes by centrifugation (800×g). Hemoglobin release was determined spectrophotometrically at 570 nm. The control for 100% hemolysis was a sample of erythrocytes treated with water. The control for no release of hemoglobin was a sample of 1% erythrocytes without any peptide added. Since erythrocytes were in an isotonic medium, no detectable release (<1% of that released upon complete hemolysis) of hemoglobin was observed from this control during the course of the assay. The hemolytic activity was determined as the peptide concentration that caused 50% hemolysis of erythrocytes after 18 h (HC₅₀). HC₅₀ was determined from a plot of percent lysis versus peptide concentration.

Calculation of Therapeutic Index (HC₅₀/MIC Ratio)

The therapeutic index is a widely accepted parameter to represent the specificity of antimicrobial reagents for prokaryotic versus eukaryotic cells. It is calculated by the ratio of HC₅₀ (hemolytic activity) and MIC (anti-tuberculosis activity); thus, larger values of therapeutic index indicate greater anti-tuberculosis specificity.

RESULTS

Peptide Design

According to our previous results (data not shown), peptides with all-D-amino acid residues were more active against *M. tuberculosis* than peptides with all-L-amino acid residues. The all-D-peptides were resistant to proteolytic enzymes present in the culture filtrate of *M. tuberculosis*. Peptide D-V13K (D1) is a 26-residue amphipathic peptide consisting of all D-amino acid residues, which adopts an α -helical conformation in a hydrophobic environment and contains a hydrophilic and positively-charged lysine residue in the center of the non-polar face (position 13) as a “specificity determinant”, Table 1, Fig. (1) [5–7]. In the current study, we systematically substituted one, two or three alanine residues with the more hydrophobic leucine residues to generate peptides D2, D3 and D4, Table 1, Fig. (1). To increase the antimicrobial activity and avoid the high level of self-association [7], peptide D5 was designed with a substitution of valine with lysine at position 16 (creating a second “specificity determinant”) compared to D4. This modification proved to decrease hydrophobicity, amphipathicity, helicity, self-association and hemolytic activity compared to peptide D4 (Table 2) and increase antibacterial and antifungal activity [8].

The anti-tuberculosis activity of our de-novo designed peptide analogs were compared to human α -helical cathelicidin, LL37, which has been shown to have anti-tuberculosis activity [13]. The all D-LL37 and all L-LL37 were synthesized, purified and characterized by the same methods as D1 analogs (Table 1).

Secondary Structure of Peptides

Fig. (2) shows the CD spectra of the peptide analogs (D1, D4 and D5) in different environments, i.e., under benign conditions (non-denaturing) Fig. (2A) and in buffer with 50% TFE to mimic the hydrophobic environment of the membrane, Fig. (2B). Fig. (2C) shows D- and L-LL37 in both benign and hydrophobic (50% TFE) conditions. All-D helical peptides exhibited a positive spectrum while all-L helical peptides exhibited a negative spectrum as expected [6]. D1 and D5 showed negligible secondary structure in benign buffer, Fig. (2A) and Table 2. D4, the triple-Leu-substituted peptide and LL37, exhibited an α -helical spectrum under benign conditions, Fig. (2A and 2C) compared to the spectra of the other analogs. Regardless of the different secondary structures of the peptides in benign buffer, a highly helical structure was induced by the nonpolar environment of 50% TFE, a mimic of hydrophobicity and the α -helix-inducing ability of the membrane, Fig. (2B) and Table 2. All the peptide analogs showed a typical α -helix spectrum with double maxima at 208 nm and 222 nm. The helicities of the peptides in benign buffer and in 50% TFE relative to that of peptide D4 in 50% TFE were determined (Table 2). As expected, the D- and L-LL37 exhibit spectra that were exact mirror images compared to each other, with ellipticities equivalent but of opposite sign both in benign buffer and in 50% TFE, Fig. (2C).

Peptide Self-Association

Peptide self-association (i.e., the ability to oligomerize/dimerize) in aqueous solution is a very important parameter for antimicrobial activity [5–7]. We assumed that monomeric random-coil antimicrobial peptides are best suited to pass through the capsule and cell wall of microorganisms prior to penetration into the cytoplasmic membrane, induction of α -helical structure and disruption of membrane structure to kill target cells [7]. Thus, if the self-association ability of a peptide in aqueous media is too strong (e.g., forming stable folded dimers/oligomers through interaction of their non-polar faces) this could decrease the ability of the peptide to dissociate to monomer where the dimer/oligomer cannot effectively pass through the capsule and cell wall to reach the membrane [7]. The ability of the peptides in the present study to self-associate was determined by the technique of RP-HPLC temperature profiling at pH 2 [15, 20, 21]. The reason pH 2 is used to determine self-association of cationic AMPs is that highly positively charged peptides are frequently not eluted from reversed-phase columns at pH 7 due to non-specific binding to negatively charged silanols on the column matrix. This is not a problem at pH 2 since the silanols are protonated (i.e., neutral) and non-specific electrostatic interactions are eliminated. At pH 2, the interactions between the peptide and the reversed-phase matrix involve ideal retention behavior, i.e., only hydrophobic interactions between the preferred binding domain (nonpolar face) of the amphipathic molecule and the hydrophobic surface of the column matrix are present [22]. Fig. (3A) shows the retention behavior of the peptides after normalization to their retention times at 5°C. Control peptide C shows a linear decrease in retention time with increasing temperature and is representative of peptides which have no ability to self-associate during RP-HPLC. Control peptide C is a monomeric random coil peptide in both aqueous and hydrophobic media; thus, its linear decrease in peptide retention behavior with increasing temperature within the range of 5°C to 80°C represents only the general effects of temperature due to greater solute diffusivity and enhanced mass transfer between the stationary and mobile phase at higher temperatures [23]. To allow for these general temperature effects, the data for the control peptide was subtracted from each temperature profile as shown in Fig. (3B). Thus, the peptide self-association parameter, P_A ,

represents the maximum change in peptide retention time relative to the random coil peptide C. Note that the higher the P_A value, the greater the self-association.

By replacing a single valine with lysine in the center of the nonpolar face (D1 in the present study), there was a dramatic decrease in self-association [5, 6]. However, by systematically increasing the hydrophobicity of the nonpolar face from peptide D1 to D4, Fig. (1), the self-association ability also increased, Fig. (3) and Table 2. Peptide D1 had an overall hydrophobicity as measured by retention time in RP-HPLC of 76.8 min compared to peptide D4 with a value of 101.6 min (Table 2). The self-association parameter for D1 was 2.78 compared to D4 with a value of 9.63. By replacing a second valine with lysine in the center of the non-polar face (position 16) of D4 generating D5, there was a dramatic decrease in self-association ability, Fig. (3B). Peptide D4 had a self-association parameter of 9.63 compared to D5 with a value of 4.35 (Table 2). That is, the substantial positive effect of a triple Ala_Leu substitution (D4) on self-association was partially overridden by the additional Lys residue in the center of the non-polar face of D5 (V16K). Thus, peptide D5 maintains the three Leu residues and an increase in hydrophobicity in the two hydrophobic patches Fig. (1) while maintaining low self-association compared to peptide D4, Table 2, Fig. (3B). LL37 has a very high ability to self-associate similar to peptide D4, Fig. (3) and Table 2.

Anti-Tuberculosis Activity

The time-kill curve of anti-tuberculosis activity against H37Rv strain of peptide D1 to D5 are shown in Fig. (4) left panel. Peptide concentrations of 1, 10 and 100 $\mu\text{g/ml}$, 10-fold serial concentrations were used. After 7 days incubation with peptides, the CFU/ml of each sample was calculated compared with day 0. The samples treated with D1 or D5 at 100 $\mu\text{g/ml}$ had dramatic reductions in CFU/ml. The data were converted to a concentration-response format, Fig. (4) right panel. The point at which the line crossed the concentration of the initial inoculum (dashed line) was reported as the MIC. The MIC value of peptide D5 is 35.2 $\mu\text{g/ml}$, the most active peptide against *M. tuberculosis* strain H37Rv. This is a two-fold improvement compared with D1 (70.7 $\mu\text{g/mL}$). The less active peptide is D4, with a MIC value of >200 $\mu\text{g/mL}$ (Table 3). Peptide D5 increased the anti-tuberculosis activity by about 5.7-fold compared to D4, which is due to a valine to lysine substitution at position 16. Our lead compound, peptide D1 had a 2.8-fold improvement in anti-tuberculosis activity compared to that of D4.

The time-kill curve of anti-tuberculosis activity against the MDR strain Vertulo and concentration-responses of peptides D1 to D5 are shown in Fig. (5). Peptide D5 was also the best analog against Vertulo strain with a MIC value of 49 $\mu\text{g/mL}$. D1 had the same level of the antituberculosis activity against MDR strain as D5 with a MIC value of 57 $\mu\text{g/mL}$ (Table 3).

D-LL37 inhibited the growth of H37Rv strain significantly more than L-LL37, Fig. (6) Top panel. However, no MIC value could be determined at 100 $\mu\text{g/mL}$, the highest peptide concentration that was tested. For the MDR strain Vertulo, there was only slight inhibition from either D- or L-LL37. The difference in inhibitory activity of D-LL37 against H37Rv strain and MDR Vertulo strain can be explained by the fact that different strains may differ in terms of architecture of their cell envelopes [3]. However, it is interesting that peptide D1 and D5 are equally active against both strains (Table 3).

Hemolytic Activity

The hemolytic activities of the peptide against human erythrocytes were determined as a measure of peptide toxicity toward higher eukaryotic cells. The HC_{50} values, the peptide

concentration that produces 50% hemolysis of human red blood cells after 18 hours in the standard microtiter dilution method, are shown in Table 3. From the strongest hemolytic peptide D4 to the weakest hemolytic peptide D1, there is a 120-fold difference in HC_{50} values (D4 3.5 $\mu\text{g/mL}$ compared to D1, 421.5 $\mu\text{g/mL}$). The most active peptide in anti-tuberculosis activity, D5 showed a 13-fold improvement in hemolytic activity compared to D4. The difference between their sequences is at position 16, with valine in D4 and lysine in D5, respectively, Fig. (1).

Therapeutic Index

The therapeutic indices were shown in Table 3. Large values indicate greater antimicrobial specificity. For *M. tuberculosis* H37Rv strain, the best analog is D1, with a therapeutic index value of 6.0; while the worst peptide is D4, the most hydrophobic analog, with a therapeutic index value of 0.018. There is a 333-fold difference between them. The most active peptide in anti-tuberculosis activity, D5 has a therapeutic index value of 1.3, which is a 72-fold improvement compared to D4. The therapeutic index for D-LL37 against H37Rv strain was 0.63, a 35-fold improvement compared to D4, but almost 10-fold worse than peptide D1. For MDR strain Vertulo, the best analog is D1, with a therapeutic index value of 7.4 and D5 has a therapeutic index value of 1.0. Since no observed MIC values could be determined, the therapeutic index for LL37 enantiomers could not be calculated. Both enantiomers were essentially inactive against MDR vertulo strain, Fig. (6).

Enantiomeric Forms of Peptides

In our previous study [6], we showed that L- and D-enantiomers of peptide V13K had equal activities against other microorganisms, and the all D-enantiomers were resistant to proteolytic enzyme degradation. In the current study, we compared L- (L1) and D-enantiomers (D1) of peptide V13K in their anti-tuberculosis activity, Fig. (7A). D1 dramatically inhibited the growth of H37Rv strain, while L1 had a slight effect. The proteolytic enzymes in the cell envelop of *M. tuberculosis* could cause the degradation of L1 and reduced its activity.

We also used the *M. tuberculosis* culture filtrate to treat the all L- and D-enantiomers of one of our peptides (D5), the all L-form (L5) was obviously degraded by proteases in the *M. tuberculosis* culture filtrate with a recovery of 71% after 1 day and only 26% after 7 days exposure, Fig. (7B). The all D-form (D5) was resistant to degradation by proteases with recovery of 91% after 1 day and 89% after 7 days exposure, Fig. (7B).

DISCUSSION

Hydrophobicity is a key parameter, which affects the activity of antimicrobial peptides (AMPs) [7, 8, 24–26]. In our previous study [7], we showed that increasing hydrophobicity increased hemolytic activity, however, there was an optimum hydrophobicity window where high antimicrobial activity to *Pseudomonas aeruginosa* could be obtained. Increasing or decreasing hydrophobicity outside of this window dramatically decreased antimicrobial activity. The decreased antimicrobial activity at high peptide hydrophobicity may be due to helical structure formation and strong peptide self-association in aqueous conditions that prevents the peptide from readily crossing through the cell envelope in prokaryotic cells to reach the membrane. RP-HPLC retention behavior is a particularly good method to evaluate overall peptide hydrophobicity, and the retention times of peptides are highly sensitive to the conformational status of peptides upon interaction with the hydrophobic surface of the column matrix [5, 27]. The HC_{50} value (hemolytic activity), MIC value against the *M. tuberculosis* H37Rv strain (anti-tuberculosis activity) and the corresponding therapeutic index (antimicrobial specificity) to the increase of hydrophobicity (expressed as RP-HPLC

retention time) was plotted in Figs. (8A, 8B, and 8C), respectively. For peptide D1 to D4, increasing hydrophobicity dramatically increased hemolytic activity, Fig. (8A), up to 120-fold, Table 3, Fig. (8C), and decreased anti-tuberculosis activity (Fig. 8B) by almost 3-fold (Table 3). As a result, increasing hydrophobicity decreased antimicrobial specificity (therapeutic index) up to 333-fold (Table 3). The similar result was observed for *P. aeruginosa*, due to the unwanted self-association introduced by the higher hydrophobicity [7]. By altering one valine with lysine at position 16 of D4 to generate D5, the hydrophobicity decreased from 101.6 min (D4) to 80.4 min (D5) (Table 2), anti-tuberculosis activity increased 5.7-fold while hemolytic activity decreased 13-fold and thus, the therapeutic index increased 72-fold (Table 3). This substitution to create peptide D5 decreased hydrophobicity and self-association [8], however peptide D5 retained the high anti-tuberculosis activity and decreased hemolytic activity compared to peptide D4.

The above observations can be explained by our membrane discrimination mechanism [5–7]. We suggest that if antimicrobial peptides have activity against zwitterionic eukaryotic membranes they do so by the pore-formation mechanism (“barrel-stave” mechanism [28, 29]). That is, the peptides must be able to form a transmembrane pore. However the combination of lower hydrophobicity of the non-polar face and the introduction of a “specificity determinant” (a positively charged residue in the center of the non-polar face) prevents transmembrane penetration in the bilayer of eukaryotic cells. Peptides with higher hydrophobicities will penetrate deeper into hydrophobic core of the red blood cell membrane [30] causing strong hemolysis by forming pores or channels. On the other hand, interaction of antimicrobial peptides with negatively charged prokaryotic cell membranes utilizes the detergent-like mechanism (“carpet” mechanism [31]) and transmembrane insertion is not required for antimicrobial activity. The peptides can lie parallel to the membrane surface with their hydrophobic surface buried in the interface region of the bilayer and the positively charged residues are still able to interact with the negatively charged phospholipid head groups of the bilayer. The peptides are still able to disrupt the lipid bilayer causing cytoplasmic leakage and cell death. Increasing hydrophobicity can still improve antimicrobial activity but only up to an optimum. If the hydrophobicity is too high, an unwanted high level of peptide self-association is introduced and the highly folded α -helical dimer/oligomer cannot easily pass through cell capsule and cell wall to reach the cytoplasmic membrane. Thus, antimicrobial activity can be dramatically decreased. This explanation agrees with the results of the peptides used in this study. Peptide LL37 and D4 were basically inactive against MDR strain Vertulo, Figs. (5 and 6). Both peptides have the highest overall hydrophobicity and self-association parameter (Table 2), which stabilizes the folded α -helical dimer/oligomer in aqueous conditions and thus these peptides may have been unable to penetrate through the capsule and cell wall to reach the membrane. In contrast, peptide D1 has the lowest overall hydrophobicity and self-association parameter and is an unfolded monomeric random coil peptide in aqueous conditions and could more easily penetrate through the capsule and cell wall to reach the membrane. Peptide D5 has the increased hydrophobicity of D4 Leu residues 12, 20 and 23 in place of Ala residues in D1, Fig. (1) but valine 16 was substituted with lysine in D5, which decreased the hydrophobicity and disrupted the consistency of the hydrophobic surface, Fig. (1). This reduces transmembrane penetration in eukaryotic cells since burial of two positively charged residues in the center of the non-polar face is more difficult. The net result was decreased hemolytic activity and self-association and increased antimicrobial activity because of the higher hydrophobicity compared to D1. Though D5 is the most active compound against both strains of *M. tuberculosis* used in this study, the anti-tuberculosis activity was within a factor of 2 between peptide D1 and D5. Peptide D1 had 9-fold improvement in hemolytic activity compared to D5 and the resulting therapeutic index was still 7.4-fold better for D1 compared to D5 against the MDR strain Vertulo (Table 3).

Several of the peptides designed from first principles had improved activity relative to LL37. In our studies we observed that only D-LL37 exhibits growth inhibitory activity against H37Rv, but does not appear to kill *M. tuberculosis* at concentration below 100 µg/mL. Careful examination of data from others reveals that our results with L-LL37 are consistent with previous studies [4, 11, 12]. Therefore, application of the design principles introduced in this study to create novel peptides with potent anti-tuberculosis activity and low hemolytic activity have been successful.

Biography

Dr. Hodges is presently at the University of Colorado, School of Medicine as Professor of Biochemistry and Molecular Genetics, Director of the Program in Structural Biology and Biophysics and holder of the John Stewart Endowed Chair in Peptide Chemistry. In 2002 he won the Vincent Du Vigneaud Award from the American Peptide Society for outstanding achievements in peptide research. His research interests are diverse from the development of broad spectrum antimicrobial peptides, a universal synthetic peptide vaccine against influenza, understanding protein folding and stability, to developing new separation methodology for peptides and proteins.

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Figure 1.

Helical net representation of the sequences of antimicrobial peptides D1, D4 and D5 vs D-LL37 in Table 1. Peptides D1, D4 and D5: The alanine to leucine substitutions (position 12, 20 and 23) are colored green. The “specificity determinant(s)” lysine residues in the center of the non-polar face are denoted pink triangles (lysine residue at position 13 in peptide D1 and D4 and lysine residue at position 16 in peptide D5). The amino acid residues on the nonpolar face are circled and the large hydrophobes are colored yellow (Trp, Phe, Leu, Val and Ile). The $i \rightarrow i+3$ and $i \rightarrow i+4$ hydrophobic interactions between large hydrophobes along the helix are shown as black bars. Peptide D-LL37: The amino acid residues on the nonpolar face are circled and the ones on the polar face are boxed. The residues that are not in the α -helix are denoted with diamonds. The large hydrophobes are colored yellow, the positively charged residues are colored blue and the negatively charged residues are colored red. The $i \rightarrow i+3$ and $i \rightarrow i+4$ hydrophobic interactions along the helix are shown as black bars. The number of hydrophobic interactions on the non-polar face, the number of positively charged residues and the number of negatively charged residues are indicated at the bottom of each helical net. The one-letter code is used for amino acid residues. The sequences are shown in Table 1.

Figure 2.

Circular dichroism (CD) spectra. Panel **A** shows the CD spectra of peptides D1, D4 and D5 in benign buffer (100 mM KCl, 50 mM NaH₂PO₄/Na₂HPO₄ at pH 7.0, 5° and panel **B** shows the spectra in the presence of buffer-trifluoroethanol (TFE) (1:1, v/v). Panel **C** shows D- and L-LL37 in both benign buffer and benign buffer containing 50% TFE. Peptides D2 and D3 (data not shown) are between D1 and D4.

Figure 3.

Peptide self-association ability as monitored by reversed-phased chromatography (RP-HPLC) temperature profiling. In panel **A**, the retention time of peptides are normalized to 5° through the expression $(t_R^t - t_R^5)$, where t_R^t is the retention time at a specific temperature of an antimicrobial peptide or control peptide **C**, and t_R^5 is the retention time at 5 °C. In panel **B**, the retention behavior of the peptides was normalized to that of control peptide **C** through the expression $(t_R^t - t_R^5 \text{ for peptides D1-D5}) - (t_R^t - t_R^5 \text{ for control peptide C})$. The maximum change in retention time from the control peptide **C** defines the peptide association parameter, denoted P_A . The sequences of the peptides and the random coil control peptide are shown in Table 1.

Figure 4.

Anti-tuberculosis activity of peptides D1-D5 against *M. tuberculosis* strain H37Rv. Left panels: Time-kill analysis was used to determine the growth of *M. tuberculosis* in the presence of increasing concentrations of the peptides for 7 days (the open symbols diamonds, squares, triangles and circles: ◇, □, △, ○ represent peptide concentrations of 0, 1, 10 and 100 µg/mL). Right panels: The data were then converted to a concentration-response format. The point at which the line crossed the concentration of the initial inoculum (dashed line) was used as the MIC value.

Figure 5.

Anti-tuberculosis activity of peptides D1-D5 against *M. tuberculosis* multi-drug resistant strain vertulo. Left panels: Time-kill analysis was used to determine the growth of *M. tuberculosis* in the presence of increasing concentrations of the peptides for 7 days (the open symbols diamonds, squares, triangles and circles: $\diamond$, $\square$, $\triangle$, $\circ$ represent peptide concentrations of 0, 1, 10 and 100 μ g/mL). Right panels: The data were then converted to a concentration-response format. The point at which the line crossed the concentration of the initial inoculum (dashed line) was used as the MIC value.

Figure 6. Anti-tuberculosis activity of L-LL37 (open symbols) and D-LL37 (closed symbols) against *M. tuberculosis* strain H37Rv (top panels) and multi-drug resistant strain Vertulo (bottom panels). Left panels: Time-kill analysis was used to determine the growth of *M. tuberculosis* in the presence of increasing concentrations of the peptides for 7 days (symbols open and closed diamonds: ◇, ◆; open and closed squares: □, ■; open and closed triangles: △, ▲ and open and closed circles: ○, ● represent peptide concentrations of 0, 1, 10 and 100 µg/mL where open symbols are L-LL37 and closed symbols are D-LL37). Right panels: The data were then converted to a concentration-response format. The point at which the line crossed the concentration of the initial inoculum (dashed line) was used as the MIC value.

Figure 7.

L- and D-enantiomers of peptides V13K and V13K/V16K/A12L/A20L/A23L. Panel A is the anti-tuberculosis activity of peptide L1 (open diamonds ◇) versus D1 (closed diamonds ◆) against H37Rv strain. Panel B, Peptide recovery from *M.tuberculosis* culture filtrate at 24 hours and 7 days: *M.tuberculosis* strain H37Rv was grown in tissue culture media as described in the methods. The peptides were added to the culture filtrate and peptide recovery was determined by RP-HPLC from 3 different samples. 24 hours and 7 days exposure time are denoted by the black bars and the open bars, respectively. Peptide L5 is the all L-enantiomer of peptide D5 (all D-enantiomer).

Figure 8.

Correlation of peptide hydrophobicity with hemolytic activity (HC₅₀) (Panel A), anti-tuberculosis activity (MIC) (Panel B) and therapeutic index (Panel C). Hydrophobicity is expressed as retention times of peptides in RP-HPLC at room temperature. Lines are drawn through peptides D1 to D4 only, since these peptides systematically increase in hydrophobicity as shown in Table 1. Peptide D5 has the same two hydrophobic patches as D4 except valine 16 in peptide D4 was substituted by lysine 16 in peptide D5.

Peptides Used in this Study

Table 1

Peptide Name	Substitution ^a	Sequence ^b															37
		1		13	16											26	
D1	D-(V13K)	Ac- K- W- K- S- F- L- K- T- F- K- S- A- K - K- T- V- L- H- T- A- L- K- A- I- S- S- amide															
D2	D-(V13K, A20L)	Ac- K- W- K- S- F- L- K- T- F- K- S- A- K - K- T- V- L- H- T- L - L- K- A- I- S- S- amide															
D3	D-(V13K, A12L, A20L)	Ac- K- W- K- S- F- L- K- T- F- K- S- L- K - K- T- V- L- H- T- L - L- K- A- I- S- S- amide															
D4	D-(V13K, A12L, A20L, A23L)	Ac- K- W- K- S- F- L- K- T- F- K- S- L- K - K- T- V- L- H- T- L - L- K- L - I- S- S- amide															
D5	D-(V13K, A12L, A20L, A23L, V16K)	Ac- K- W- K- S- F- L- K- T- F- K- S- L- K - K- T- K - L- H- T- L - L- K- L - I- S- S- amide															
L-LL37		NH ₂ - L- L- G- D- E- F- R- K- S- K- E- K- I- G- K- E- F- K- R- I- V- Q- R- I- K- D- F- L- R- N- L- V- P- R- T- E- S- OH															
D-LL37		NH ₂ - L- L- G- D- E- F- R- K- S- K- E- K- I- G- K- E- F- K- R- I- V- Q- R- I- K- D- F- L- R- N- L- V- P- R- T- E- S- OH															
Control C		Ac- E- L- E- K- G- G- L- E- G- E- K- G- G- K- E- L- E- K- amide															

^aThe L- or D- denotes that all amino acid residues in each peptide are in the L or D conformation

^bPeptide sequences are shown using the one-letter code for amino acid residues; Ac- denotes N α -acetyl and -amide denotes C α -amide. The “specificity determinants”, Lys residues incorporated in the center of the nonpolar face are bolded. The Ala to Leu substitutions are bolded

Table 2

Biophysical Data of D-(V13K) Analogs and D-LL37

Peptide Name	Hydrophobicity		Benign		50% TFE		P _A ^e	Amphipathicity ^f
	t _R ^a (min)	Δt _R (X-D1) ^b (min)	[θ] ₂₂₂ ^c	%Helix ^d	[θ] ₂₂₂ ^c	%Helix ^d		
D1	76.8	0	1,150	3	34,100	81	2.78	4.92
D2	86.7	9.9	2,300	5	37,550	89	4.62	5.71
D3	94.8	18.0	4,850	12	38,450	91	7.67	5.86
D4	101.6	24.8	10,550	25	42,050	100	9.63	6.34
D5	80.4	3.7	3,700	9	35,500	84	4.35	5.78
D-LL37	108.2	31.4	23,100	55	33,200	79	8.31	5.89 ^g

^at_R denotes retention time in RP-HPLC at pH 2 and room temperature, and is a measure of overall peptide hydrophobicity.

^bΔt_R(X-D1) is the difference in retention time between the peptide analogs and peptide D1, as a measure of the change in hydrophobicity

^cThe mean residue molar ellipticities [θ]₂₂₂ (deg cm²/dmol) at wavelength 222 nm were measured at 5 °C in benign conditions (100 mM KCl, 50 mM NaH₂PO₄/Na₂HPO₄, pH 7.0) or in benign buffer containing 50% trifluoroethanol (TFE) by circular dichroism spectroscopy.

^dThe helical content (as a percentage) of a peptide relative to the molar ellipticity value of peptide D4 in the presence of 50% TFE.

^eP_A denotes oligomerization/dimerization parameter of each peptide during RP-HPLC temperature profiling, which is the maximal retention time difference of (t_R-t_R⁵ for peptide analogs)–(t_R-t_R⁵ for control peptide C) within the temperature range; t_R-t_R⁵ is the retention time difference of a peptide at a specific temperature (t_R) compared with that at 5°C (t_R⁵). The sequence of control peptide C is shown in Table 1.

^fAmphipathicity was determined by calculation of hydrophobic moment [15] using hydrophobicity coefficients determined by reversed-phase chromatography [17, 18] see methods for details.

^gFor LL37, only residues 1–32 were used to calculate amphipathicity.

Table 3
Biological Activity of D-(V13K) Analogs and LL37 against *M. tuberculosis*

Peptide Name	Hemolytic Activity		Anti-Tuberculosis Activity to H37Rv Strain		Therapeutic Index to H37Rv Strain		Anti-Tuberculosis Activity to MDR Strain		Therapeutic Index to MDR Strain
	HC ₅₀ ^a μg/ml	Fold ^b	MIC ^c μg/ml	Fold ^b	HC ₅₀ /MICd	Fold ^b	MIC ^c μg/ml		
D1	421.5	120	70.7	2.8	6.0	333	57	7.4	
D2	83	24	83.7	2.4	1.0	56.0	72	1.2	
D3	14	4	109.2	1.8	0.13	7.2	100	0.14	
D4	3.5	1	200	1.0	0.018	1.0	1 ^e	1 ^e	
D5	47	13	35.2	5.7	1.3	72.0	49	1.0	
L-LL37	43.5	12	1 ^e	—	1 ^e	—	1 ^e	1 ^e	
D-LL37	125	36	200	1.0	0.63	35.0	1 ^e	1 ^e	

^a HC₅₀ is the peptide concentration that produces 50% hemolysis of human red blood cells after 18 h in the standard microtiter dilution method.

^b The fold improvement in HC₅₀/MIC and therapeutic index compared to that of D4.

^c MIC is minimal inhibitory concentration that inhibited 99.9% growth of *M. tuberculosis* in killing assay.

^d Therapeutic index is the ratio of the HC₅₀ value over the geometric mean MIC value. Large values indicate greater antimicrobial specificity. To calculate the therapeutic index for which an MIC was not observed at 100 μg/mL, a value of 200 μg/mL was used.

^e I denotes inactive.