De Novo Antimicrobial Peptides with Low Mammalian Cell Toxicity

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De novo antimicrobial peptides with the sequences: $(KLAKKLA)_n$, $(KLAKLAK)_n$ (where n = 1, 2, 3), $(KALKALK)_3$, $(KLGKKLG)_n$, and $(KAAKKAA)_n$ (where n = 2, 3), were prepared as the C-terminus amides. These peptides were designed to be perfectly amphipathic in helical conformations. Peptide antibacterial activity was tested against *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. Peptide cytotoxicity was tested against human erythrocytes and 3T3 mouse fibroblasts. The 3T3 cell testing was a much more sensitive test of cytotoxicity. The peptides were much less lytic toward human erythrocytes than 3T3 cells. Peptide secondary structure in aqueous solution, sodium dodecylsulfate micelles, and phospholipid vesicles was estimated using circular dichroism spectroscopy. The leucine/alanine-containing 21-mers were bacteriostatic at 3−8 μM and cytotoxic to 3T3 cells at about 10 μM concentrations. The leucine/alanine- or leucine/glycine-containing 14-mers and the leucine/glycine 21-mer were bacteriostatic at 6−22 μM but had much lower cytotoxicity toward 3T3 cells and higher selectivities than the natural antimicrobial peptides magainin 2 amide and cecropin B amide. The 7-mer peptides are devoid of biological activity and of secondary structure in membrane mimetic environments. The 14-mer peptides and the glycine-containing 21-mer show modest levels of helicity in model membranes. The leucine/alanine-containing 21-mer peptides have substantial helicity in model membranes. The propensity to α-helical conformation of the peptides in amphipathic media is proportional to their 3T3 cell cytotoxicity.

Introduction

The amphipathic α-helix is a common structural motif of many proteins and biologically active peptides.[1] Amphipathic helical domains are found in membrane recognition sites, such as specific ion channel proteins, signal peptides, and antimicrobial and venom peptides.[2] The interaction of amphipathic peptides with membranes depends at least in part on the relative sizes of the hydrophobic and hydrophilic faces and the charge of the hydrophilic face. Antimicrobial peptides generally have an equivalent number of polar and nonpolar residues within the amphipathic domains and enough basic residues to give the peptide an overall positive charge at neutral pH.[3] Peptide venoms are also positively charged but generally have more extensive hydrophobic surfaces with narrower polar faces. Melittin is a 26-residue peptide found in the venom of the honey bee, *Apis mellifera*.[4] It is cytotoxic and has broad spectrum antimicrobial activity at micromolar concentrations.[5] There are a number of other natural amphipathic peptides that are much less cytotoxic than melittin but have comparable broad spectrum antimicrobial activity. Magainins and cecropins exhibit bacteriostatic and bactericidal activity at concentrations that are not cytotoxic toward normal mammalian cells.[6,7] These peptides are unstructured in dilute aqueous solution but become helical in amphipathic media such as micelles, synthetic bilayers, and cell membranes.[8]

It has been found that sequence homology is not a prerequisite for biological activity. Scores of natural antimicrobial peptides of widely varying sequence have been isolated with amphipathic helical domains as a consistent structural feature.[3] Numerous analogs of native peptides with amino acid substitutions expected to enhance amphipathicity and helicity have shown increased biological activity. Most analogs with increased antimicrobial activity also have increased cytotoxicity.[9] A notable exception are the melittin−cecropin hybrids which are more bacteriostatic than cecropins and less hemolytic than melittin.[10] One of the earliest designed peptides was a melittin analog with a simplified N-terminus and a native C-terminal segment which had hemolytic activity comparable to that of melittin.[11] A designed amphipathic α-helical peptide composed of 2:1 Leu and Lys residues has a narrow polar face and over 10-fold higher hemolytic activity than melittin.[12] D-Melittin, D-magainin, and D-cecropin derivatives were found to have biological activities that were essentially the same as those of the native peptides.[13] De novo peptides that use the amphipathic helix as the only starting point have been synthesized and have bacteriostatic and cytotoxic activity similar to the native peptides.[14−16]

Antimicrobial peptides selectively inhibit and kill bacteria while maintaining low mammalian cell cytotoxicity. The selectivity has been attributed to membrane differences between bacteria and mammalian cells. The exterior membranes of bacteria are negatively charged, whereas mammalian cell exterior membranes are generally neutral.[17] Antimicrobial peptides are positively charged and therefore might preferentially bind to bacteria over mammalian cells.[18] The cholesterol in mammalian cell membranes has also been suggested as the basis of the selectivity of antimicrobial peptides.[19] Membrane disruption by the antimicrobial peptides could be inhibited by the cholesterol in mam-

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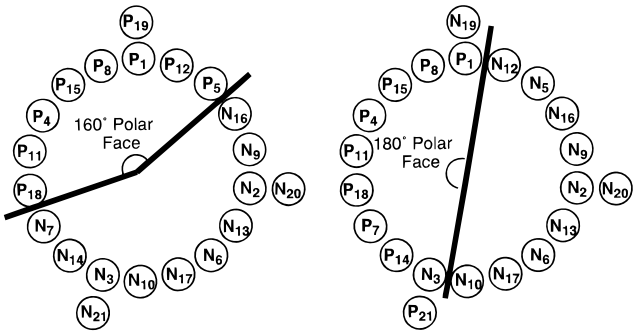

Figure 1. Wheel diagram of peptides with the general sequences $[(PNN)P(PNN)]_n$ and $[(PNN)(PNN)P]_n$.

malian cells. Finally the lower membrane potential across mammalian cells or some combination of the above factors could be responsible for the observed selectivity of the antimicrobial peptides.[20]

Whatever the selectivity basis of antimicrobial peptides, the structural differences between cytotoxic and antimicrobial peptides are subtle. This paper describes our efforts to design, synthesize, and characterize small model peptides that retain or enhance the selectivity of natural antimicrobial peptides. We have used the repetitive heptad approach pioneered by Hodges[21] and DeGrado[22] which places i and $i + 7$ residues of the heptads in close proximity when viewed along the helical axis. The heptad building block scheme has been simplified so that repetitive heptads are composed of repetitive trimers plus an additional residue. Peptides with the general sequences $[(PNN)(PNN)P]_n$ and $[(PNN)P(PNN)]_n$ (P = polar residue, N = nonpolar residue, and $n = 1, 2, 3$) were prepared as the C-terminus amides (Figure 1).

This design allows global substitution of several amino acids by other amino acids of similar polarity with minimal change in the amphipathy of the peptide. We have examined length effects on biological activity and secondary structure by comparing heptad, 14-mer, and 21-mer peptides. The peptide bacteriostatic and bactericidal activities were tested against Gram-negative and Gram-positive bacteria, and cytotoxicity was tested against a mammalian cell line and human erythrocytes. The CD spectra of these peptides were measured in buffer, sodium dodecylsulfate (SDS) micelles, and phospholipid vesicles. The secondary structure of the peptides in model membranes was correlated with their biological activity.

Results

Several multimers of heptads $[(PNN)(PNN)P]_n$ and $[(PNN)P(PNN)]_n$ with 7, 14, and 21 residues with P equal to lysine and N equal to leucine, alanine, or glycine were synthesized. The sequences are shown Table 1. Alanine was replaced by glycine in two peptides, $(KLAKKLA)_{2,3}$ vs $(KLGKKLG)_{2,3}$, to determine if peptide amphipathy would overwhelm the helix destabilization of several glycine residues.[23−25] In another variation, the leucine was replaced with alanine in two peptides, $(KLAKKLA)_{2,3}$ vs $(KAAKKAA)_{2,3}$, to ascertain whether the reduced hydrophobicity of the alanine peptide would reduce its biological activity.

Bioactivity of Designed Peptides. The MICs for these peptides tested against *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* are summarized in Table 1. *E. coli* and *P. aeruginosa* are Gram-negative bacteria, and *S. aureus* is a Gram-positive bacterium. The MIC values for cecropin B amide, magainin 2 amide, and melittin are reported for comparison. The 7-mer peptides are inactive. The leucine/alanine-containing 14-mers inhibit all three bacteria at approximately 6 μM. $(KLGKKLG)_2$ is about 3 times less active than the alanine analog $(KLAKKLA)_2$. $(KAAKKAA)_2$ shows only partial inhibition of *S. aureus* at the highest concentration tested. The leucine-containing 21-mers are active in the 3−4 μM range against the bacteria tested with the exception of $(KALKALK)_3$ which is less active against *S. aureus*. $(KAAKKAA)_3$ is less active than the leucine-containing peptides. Cecropin B amide is the most inhibitory of the peptides tested against *E. coli*. Melittin has MIC values in the range of the most active 21-mers. Magainin 2 amide has activity similar to the moderately active 14-mer $(KLGKKLG)_2$.

Figure 2 shows the minimum bactericidal concentrations for these peptides against *E. coli* (top panel) and *S. aureus* (bottom panel). MBC values should in principle have equal or greater concentrations than the corresponding MIC values, since dead bacteria are also inhibited. The values for the MBC of *E. coli* are within the range expected from the MIC assays for $(KLGKKLG)_2$, $(KLAKLAK)_2$, $(KLGKKLG)_3$, $(KLAKLAK)_3$, and magainin 2 amide but lower than the corresponding MIC values for $(KLAKKLA)_2$, $(KLAKKLA)_3$, $(KLAKLAK)_3$, melittin, and cecropin B amide. For the latter peptides the *E. coli* MIC values are between 1−10 μM, whereas the MBC values are 1 μM. This difference is

Table 1. Biological Activity of Peptides[a]

peptide	*E. coli*	*P. aeruginosa*	*S. aureus*	3T3	HE[b]
KLAKKLA	>100	>100	>100	not lethal	not lytic
$(KLAKKLA)_2$	6	3	6	>272	>750
$(KLAKKLA)_3$	4	4	4	11	>250
KLAKLAK	>95	>95	>95	not lethal	not lytic
$(KLAKLAK)_2$	6	6	6	>517	>750
$(KLAKLAK)_3$	4	4	4	9	>520
$(KALKALK)_3$	4	4	8	11	
$(KLGKKLG)_2$	22		22	not lethal	>780
$(KLGKKLG)_3$	4	4	4	>393	>570
$(KAAKKAA)_2$	47		>256	>525	>670
$(KAAKKAA)_3$	8		8	>440	>1630
melittin	3	6	3	1	1
magainin 2 amide	10	5	19	60	>460
cecropin B amide	1		12	102	>300

[a] Bacterial lysis is measured as MICs (μM), and 3T3 mouse fibroblast lysis is measured as sublethal concentrations (μM), as described in the Experimental Section. [b] Human erythrocytes.

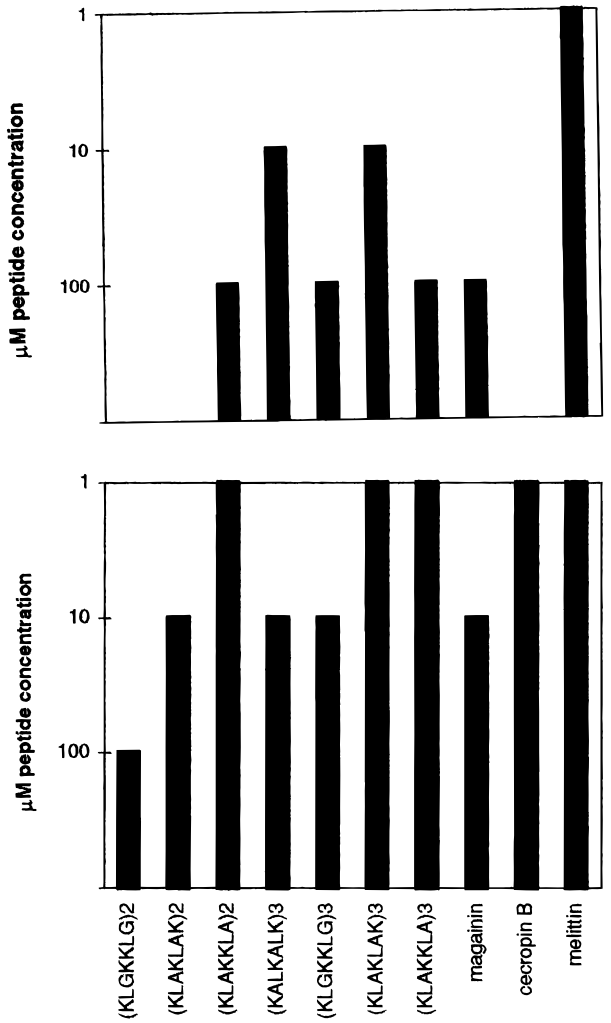

Figure 2. Minimum bactericidal concentrations of peptides against *S. aureus* (top panel) and *E. coli* (bottom panel).

within one dilution between the two assays. The *S. aureus* MBC assays give concentrations that are equal to or greater than the corresponding MIC values except for melittin. All of the 21-mers were effective at 10−100 μM against *S. aureus*. None of the 14-mers were effective at killing 99.9% of the *S. aureus* population at the highest concentration tested (100 μM) except $(KLAKKLA)_2$. Melittin performed better in the *S. aureus* MBC assay than in the MIC assay, but the differences between the two assays are within one dilution.

The activity of these peptides toward lysis of mouse embryo fibroblast 3T3 cells and human erythrocytes is shown in Table 1. The 3T3 cells are much more easily lysed than human erythrocytes. A well-defined sublethal concentration was greater than the highest concentration tested for most peptides with human erythrocytes. The 3T3 cell testing shows a length dependence on bioactivity: 7-Mers are inactive, 14-mers have low cytotoxicity, and 21-mers have greatly increased cytotoxicity except for $(KLGKKLG)_3$ and $(KAAKKAA)_3$.

Helicity of Peptides in Model Membranes. The CD spectra of all the designed peptides in 2.5 mM sodium phosphate buffer, pH 7.4, showed random coil structures at micromolar concentrations. Peptide concentrations as high as 12 mM had no effect on the secondary structure of the 7- and 14-mers. On the other hand, the molar ellipticity at 222 nm of $(KLAKKLA)_3$, $(KLAKLAK)_3$, and $(KLAKKLA)_3$ was concentration dependent, with an apparent increase in helicity with increasing peptide concentration in the range of 8−12 mM (not shown).

Table 2. α-Helicity of Peptides in SDS Micelles[a]

peptide	$[\theta]_{222}$[b]	α-helix[c] (%)
KLAKKLA	−4848	0
$(KLAKKLA)_2$	−10 647	24
$(KLAKKLA)_3$	−29 049	79
KLAKLAK	−1126	0
$(KLAKLAK)_2$	−15 334	37
$(KLAKLAK)_3$	−29 195	79
$(KALKALK)_3$	−25 053	67
$(KLGKKLG)_2$	−911	0
$(KLGKKLG)_3$	−13 856	33
melittin	−33 600	93
magainin 2 amide	−18 300	46

[a] 10 mM sodium phosphate buffer, pH 7.4, except $(KALKALK)_3$; 2.5 mM sodium phosphate buffer, pH 7.4, was used for $(KALKALK)_3$ which was insoluble at higher phosphate concentration. [b] CD spectra were recorded from 250 to 190 nm in 25 mM SDS. [c] The percent α-helix = $-100([\theta]_{222} + 3000)/33\,000$.[33]

Table 2 gives the helical content of all the designed peptides in 25 mM SDS. These results clearly demonstrate that the molar helicity of the peptides in SDS is correlated to the chain length of the peptide. As the number of heptad repeats increases, the helical content of the peptide increases. The alanine-containing peptides of the same length show a similar helical content, whereas the analogous glycine-containing peptides have significantly lower helical content. The helical content of $(KLGKKLG)_3$ and the alanine-containing 14-mers are in the 24−37% range. Magainin 2 is moderately helical; the leucine/alanine-containing 21-mers and mellitin are more helical.

Several CD spectra from a titration of $(KLAKKLA)_n$ (n = 1, 2, 3) in SDS are shown in Figure 3. SDS titration of (KLAKKLA) (panel A) fails to induce any secondary structure. In the absence of SDS, the CD spectra of dilute aqueous solutions of the 14- and 21-mers indicate that $(KLAKKLA)_2$ (panel B) is in a random coil conformation, and $(KLAKKLA)_3$ (panel C) lacks well-defined structure but is not completely random. This is revealed by the weak negative band around 200 nm. In solutions of SDS below the cmc, the CD spectra of $(KLAKKLA)_2$ and $(KLAKKLA)_3$ show a type II β-turn secondary structure.[26] At SDS concentrations of 8−12 mM, the onset of α-helicity occurs, and helicity levels off at the maximum observed for each peptide at about 20 mM SDS (not shown). The CD spectra from the SDS titration of the remaining 14- and 21-mers follow the same trend, except for $(KLGKKLG)_2$ which fails to show helical structure and $(KAAKKAA)_{2,3}$ which precipitates at intermediate SDS concentrations. None of the peptides in the series show an isodichroic point during the SDS titration.

The CD spectra from the titration of a 30 μM aqueous solution of $(KLAKKLA)_3$ (panel A) and $(KLGKKLG)_3$ (panel B) in DLPC/DLPG vesicles are shown in Figure 4. In the absence of vesicles, the peptides display a mostly random coil CD spectrum. Upon vesicle addition, the peptides become α-helical. Table 3 shows a comparison of the helical content of $(KLAKKLA)_3$ and $(KLGKKLG)_3$ in DLPC and DLPC/DLPG vesicles. The peptides show maximum helicity in neutral and negatively charged vesicle preparations at a peptide/lipid ratio of at least 1:100. The amount of helix formed by

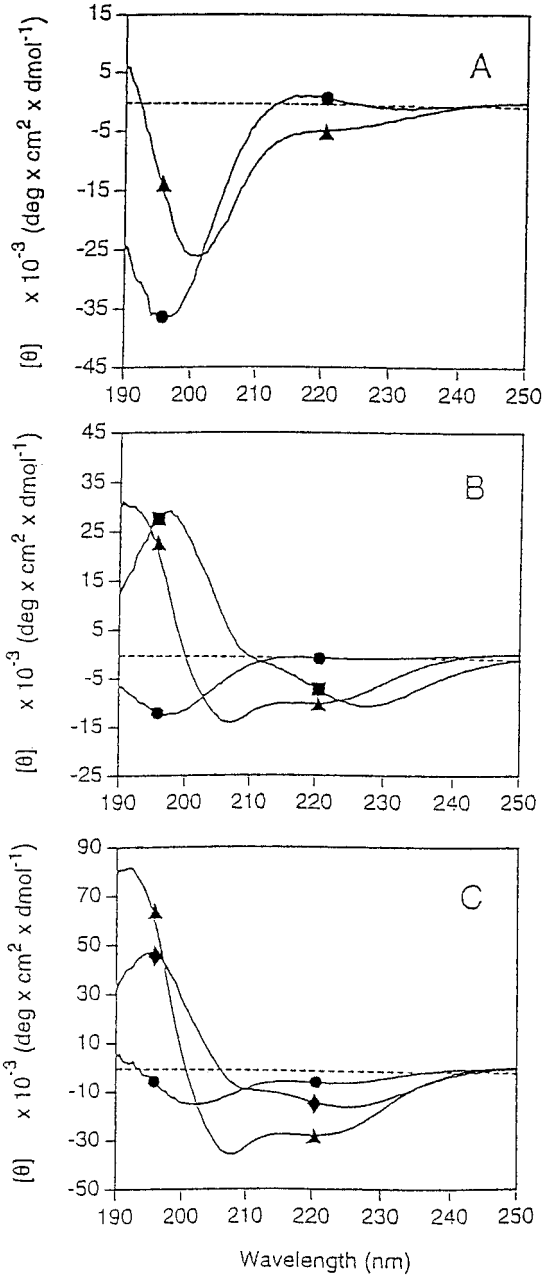

Figure 3. SDS titration of $(KLAKKLA)_n$ (n = 1, 2, 3) CD spectra in 10 mM sodium phosphate buffer, pH 7.4: (A) 240 μM KLAKKLA, (B) 120 μM $(KLAKKLA)_2$, and (C) 32 μM $(KLAKKLA)_3$. The concentration of peptide is constant with varying SDS concentration: ●, no SDS; ■, 1.25 mM SDS; ▲, 2.5 mM SDS; △, 25 mM SDS micelle concentration.

$(KLAKKLA)_3$ in either vesicle preparation is much greater than that for $(KLGKKLG)_3$. Both peptides have higher α-helicity in negatively charged vesicles than in neutral vesicles (Table 3).

Discussion

We have designed a series of highly amphipathic α-helical peptides of the general sequences $(PNNPP-NN)_n$ and $(PNNPNNP)_n$ with P equal to lysine, N equal to leucine and alanine or glycine or just alanine, and n equal to 1−3 or 2 and 3. Leucine and alanine were specifically chosen because of their high helical propensity.[23] Most antimicrobial peptides have several basic residues. Lysine was chosen as the polar residue

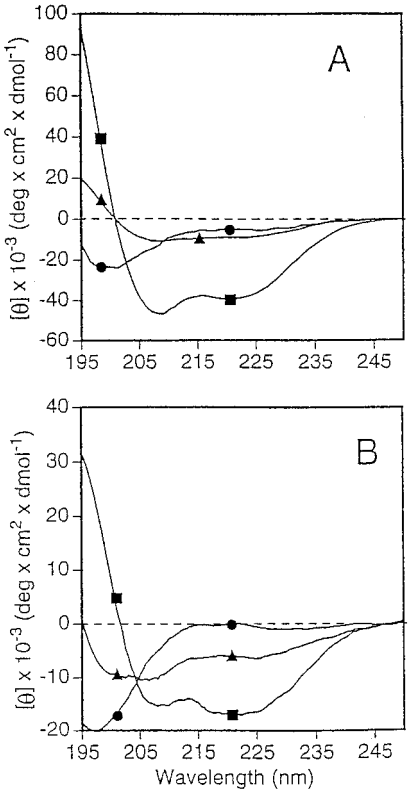

Figure 4. Circular dichroism spectra of (A) $(KLAKKLA)_3$ and (B) $(KLGKKLG)_3$ plus DLPC/DLPG (4:1) liposomes. The ratios are lipid to peptide concentrations: ●, no lipid; ▲, 30:1; ■, 100:1, with constant peptide concentration of 30 μM.

Table 3. Percent α-Helicity[a] of Peptides in DLPC and DLPC/DLPG (4:1) Liposomes[b]

		DLPC		DLPC/DLPG (4:1)	
peptide	free peptide	1:30	1:100	1:30	1:100
$(KLAKKLA)_3$	0	25	60	55	85
$(KLGKKLG)_3$	0	3	12	21	45
melittin	0.5	43	58	48	50
magainin 2 amide	0	13	40	35	38

[a] Percent α-helix = $-100[([\theta]_{222} + 3000)/33\,000]$.[33] [b] 2.5 mM sodium phosphate, 10 mM NaCl, pH 7.4.

because of high helical propensity and ease of peptide synthesis compared to arginine.[16] These peptides were designed to determine the effect of length, sequence, and structure on biological activity. All of the leucine- and alanine-containing peptides of the same length have similar MIC values. The difference in polar face angle predicted from the wheel diagram, 160° for $(KLAKKLA)_n$ and 180° for $(KLAKLAK)_n$, has no discernible effect on the biological activity of these peptides. The 7-mers were inactive. The leucine-containing 21-mers were about 2 times as potent as the 14-mers. The $(KAL-KALK)_3$ 21-mer sequence was slightly less active. The MIC values for melittin, magainin 2 amide, and cecropin B amide provide a basis for comparison with assays performed in other laboratories. Most researchers reporting on the design of new natural or de novo antimicrobial peptides have reported MIC values. It is generally believed that these peptides inhibit bacterial growth by destroying most of the cell population. If this is the case, then MIC assays should agree with MBC assays which measure bactericidal activity. Our results

with *E. coli* show only small or insignificant differences between the two assay procedures. But with assays run against *S. aureus*, MIC values can be several dilutions lower than the corresponding MBC values, suggesting that *S. aureus* is inhibited but not killed. It is clear from this study, using both assays, that bacteriostatic concentrations can be significantly lower than bactericidal concentrations at least for *S. aureus*.

The leucine/alanine-containing peptides with the same number of heptad repeats, $(KLAKKLA)_n$, $(KLAKLAK)_n$ (n = 1, 2, 3), and $(KALKALK)_3$, have nearly equivalent cytotoxicity against 3T3 cells. The 21-mers are only about 10 times less toxic than melittin. The 14-mer peptides and $(KLGKKLG)_3$ have low cytotoxicity against 3T3 cells. The leucine/alanine-containing 14-mers and $(KLGKKLG)_3$ are potent antibacterial agents. These peptides are bacteriostatic at 100−200 times lower concentrations than the sublethal concentration against 3T3 cells. Moreover, they are 2−5 times less cytotoxic than the natural peptides magainin 2 amide and cecropin B amide. The greatly reduced activity of these peptides against human erythrocytes shows that peptide lytic activity can vary greatly among mammalian cells.

The natural antimicrobial peptides magainin 2 amide and cecropin B amide have no defined structure in dilute aqueous media but become helical upon membrane association. Studies of idealized amphipathic peptides have demonstrated that peptide−peptide hydrophobic interactions can induce peptide secondary structure and contribute to the stability of α-helices in aqueous solution.[21,22] All of the designed peptides reported herein were unstructured in aqueous buffer at micromolar concentrations. The leucine/alanine-containing 21-mer peptides showed apparent self-association-induced helicity but at concentrations approximately 100 times higher than the bacteriostatic concentrations. Therefore, potential peptide aggregation in solution is probably not playing a role at the concentrations used in the assays. As expected, we failed to detect any concentration dependent helicity in aqueous buffer for the intrinsically less helical peptide $(KLGKKLG)_3$. The greater conformational flexibility of glycine relative to alanine is the likely cause for this difference.

The CD spectra of peptides in micelles and of representative peptides in phospholipid vesicles suggest a structure/function relationship for these biologically active peptides. SDS possesses an aliphatic tail and a negatively charged head group. SDS forms micelles that partially mimic the amphipathic environment of membranes. The negatively charged head group provides the initial electrostatic interactions needed and enhances the binding affinity of peptides to the nonpolar interior.[26] The percent helicity of the peptides in 25 mM SDS micelles, well above the cmc, correlates with their cytotoxicity to mammalian cells. The SDS titration experiments show that peptide helicity peaks and levels off at less than the 25 mM SDS used for peptide comparisons. Melittin has very high helicity (93%) and cytotoxicity, the leucine- and alanine-containing 21-mers have helicity of 66−79% and high cytotoxicity, magainin 2 amide has modest helicity and cytotoxicity, and $(KLGKKLG)_3$ and the leucine- and alanine-containing 14-mers have lower helicity values and lower cytotoxicity than magainin 2 amide. The 7-mer peptides lack any helicity in membrane mimetic environments and are devoid of biological activity. Figure 5 shows a plot of helicity versus log of the 3T3 cell sublethal concentration that has a correlation coefficient of 0.92. Agawa et al. reported a length dependence on helicity in phospholipid bilayer that correlated with channel formation and that weakly correlated with antimicrobial activity.[27]

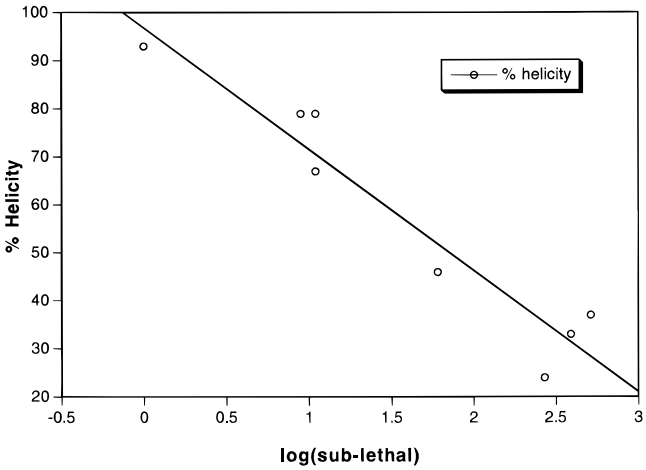

Figure 5. Correlation of log of the 3T3 cell sublethal concentration and percent helicity in 25 mM SDS micelles for peptides showing any cytotoxicity.

The SDS titrations indicate transitions in peptide secondary structure from mostly random coil in aqueous buffer to a type II β-turn in submicellar SDS concentrations and to an α-helix at 8−12 mM SDS. Low concentrations (2−4 mM) of SDS induce β-structure.[26] At SDS concentrations less than 3.5 mM, the 14- and 21-mer peptides show varying levels of β-structure. The induction of a β-structure in submicellar concentrations of SDS is possibly due to hydrophobic interactions between monomeric SDS and the peptides. At SDS concentrations above 8−12 mM, near the SDS cmc, the peptides tend to fold into an α-helical conformation (data not shown). At a ratio of about 1:1 peptide to micelle (micelle aggregation number of 62 SDS monomers), the peptides reach maximum helical content and no change is observed upon further addition of SDS.

The secondary structure of $(KLGKKLG)_3$ and $(KLAKKLA)_3$ in phospholipid vesicles was also determined using CD. The exterior phospholipid surface of bacteria is generally negatively charged, whereas most mammalian cells are neutral.[17] Vesicles were prepared from a mixture of DLPC/DLPG in a 4:1 molar ratio (20% negative charge on the surface) to mimic bacterial exterior cell membranes. Pure DLPC vesicles (neutral) were used as a model membrane for mammalian cells. The peptides show maximum helicity in neutral and negatively charged vesicle preparations at a peptide/lipid ratio of at least 1:100. The amount of helix formed by $(KLGKKLG)_3$ in either vesicle preparation is lower than that for $(KLAKKLA)_3$. Both 21-mer peptides show greater α-helicity in negatively charged vesicles than in neutral vesicles.

In correlating cytotoxicity with α-helicity, we have ignored the binding properties of the peptides. Differences in helical content are likely due to the relative binding affinity of the peptides with the SDS micelles. These considerations are being addressed for some of these peptides in phospholipid model membrane envi-

ronments.[28] These results show that bound peptide is essentially 100% helical and that binding affinity differences account for the reduced helicity of $(KLGKKLG)_3$ compared to $(KLAKKLA)_3$.

The alanine-containing 14-mer peptides have similar bacteriostatic activity to magainin 2 amide and cecropin B amide with substantially lower cytotoxicity. The analogous $(KLGKKLG)_{2,3}$ peptides have slightly less antibacterial activity but are much less cytotoxic than magainin 2 amide or cecropin B amide. Magainin 2 amide, cecropin B amide, and several other selective natural lytic peptides contain one or more helix-breaking residues and are always less perfectly amphipathic than the idealized peptides described herein. The imperfect amphipathy and resulting lower binding affinity of the natural antimicrobial peptides were probably selected to minimize indiscriminate host organism cell lysis.

Experimental Section

Peptide Synthesis. Peptides were synthesized by the solid-phase method using a MilliGen 9050 pep synthesizer. The peptides were cleaved and deprotected with a trifluoroacetic acid-based reagent (88% trifluoroacetic acid (TFA), 5% water, 5% phenol, 2% triisopropylsilane) for 2−4 h, dried, taken up in cold 20% acetic acid, extracted with diethyl ether, and lyophilized. Peptides were purified by reverse-phase preparative HPLC on a Waters 15-μm Deltapak C_4 column, 200 × 25 mm, using a mobile phase of acetonitrile (0.05%, v/v, TFA) and water (0.05%, v/v, TFA) running a gradient of 10−50% of the organic phase over 1 h. Purity was checked on an analytical Vydac 5-μm C_{18} column running a similar mobile phase gradient and monitoring at 220 nm. The molecular weight of purified peptides was verified by plasma desorption mass spectrometry on a BioIon 20 instrument. Peptides were stored as lyophilized powders at −20 °C. Melittin was purchased from Sigma Chemical Co. and purified as above.

Amino Acid Analysis. Peptide concentrations are based on quantitative amino acid analysis. Peptide samples containing a norleucine standard were hydrolyzed in 6 N HCl, 0.1% phenol, for 24 h at 110 °C followed by sodium cation exchange chromatography on a Pickering 3 × 250 mm column at 65 °C and postcolumn derivatization with ninhydrin at 130 °C. Lyophilized peptide samples contain solvent and trifluoroacetate as counterion. The peptide contents of lyophilized powders are between 50% and 80% peptide by weight depending on the charge of the peptide and the efficiency of the solvent removal.

Minimum Inhibitory Concentration Assays. The MIC of peptides were determined against *E. coli* American type culture collection (ATCC) 25922, *P. aeruginosa* ATCC 27853, and *S. aureus* ATCC 25723. Peptide 1:2 serial dilutions were prepared from 512 μg/mL stock solutions to give a range of 256−2 μg/mL in the culture media. Bacterial cultures were grown to midlog phase in nutrient broth and standardized to a 0.5 McFarland turbidity tube before dilution. To each sterile well containing 5×10^4 cells in 50 μL was added an equal volume of peptide solution. The MIC is the lowest concentration that inhibits cell growth as evidenced by absence of turbidity after 4 h.[29] The median assay values are reported for three to seven separate tests. Assay results vary by no more than one dilution from the median value.

Minimum Bactericidal Concentration Assays. MBCs of *E. coli* and *S. aureus* were tested in a concentration range of 100−0.1 μM. Bacteria were grown to midlog phase in nutrient broth, washed in 10 mM phosphate buffer, pH 7, and standardized to a McFarland tube so that the number of cells approximated 10^5 in each test tube. Tubes containing the buffer, cells, and peptide were incubated at 37 °C for 1 h. Serial 1:10 dilutions were made, and aliquots were spread on duplicate tryptic soy agar plates. Surviving cells were counted after 18 h of incubation. A set of control plates containing the same number of cells but no peptide was also counted to determine the actual number of cells present and the survival rate.

Mammalian Cell Assays. The efficacy of peptides to induce cell death of 3T3 mouse fibroblasts was determined by 2-fold serial dilution assay; 50 μL of a stock peptide solution was diluted with an equal volume of MEM, and 1:2 serial dilutions in MEM were prepared. Each dilution was applied to a 1-day old monolayer of 3T3 cells (approximately 1×10^4 cells/well) maintained in a 96-well plate with fresh MEM (50 μL/well). Peptide-treated and control (no peptide) cells were incubated at 37 °C for 30 min. The supernatant was removed, and the cells were gently treated with 0.2% trypan blue stain and viewed in an inverted light microscope. Inclusion of trypan blue dye within a cell is indicative of cell death. A sublethal dose is defined as the highest dilution in which only 1−10 adherent cells are not stained. In Table 1, not lethal means that cell population survival was unchanged from controls when greater than 400 μM peptide was used, and the values in Table 1 listed as greater than mean that at the highest concentration tested, there was some cell lysis but it was less than 99% lysis based on controls defined as sublethal.

Hemolysis Assay. The peptide was dissolved in a minimum amount of serum-free MEM and 2-fold diluted to determine sublethal concentrations as defined above. The diluted peptide solutions (50 μL) were added to a 96-well plate containing 50 μL of 1×10^5 human erythrocytes/well and incubated at 37 °C for 30 min. Several peptides were slightly hemolytic at the highest concentration tested, indicated by a greater than sign in Table 1.

Circular Dichroism Measurements. CD measurements were made using an Aviv 60DS spectropolarimeter. The instrumental outputs were calibrated with (+)-10-camphorsulfonic acid.[30] Measurements were made over a 250−190 nm range in quartz cells of 0.1−0.001 cm path length. All CD spectra were recorded at room temperature and obtained with a 1-nm bandwidth, scan speed of 10 nm/min, and time constant of 5 s. Two scans were obtained to improve the signal to noise ratio. A base line was recorded and subtracted after each spectrum. Ellipticity is reported as the mean residue ellipticity $[\theta]$ in deg cm^2 dmol^{-1}; $[\theta] = [\theta]_{obs}(MRW/10lc)$, where $[\theta]_{obs}$ is the ellipticity measured in millidegrees, MRW is the mean residue molecular weight of the peptide (molecular weight divided by the number of peptide bonds), c is the concentration of the sample in mg/mL, and l is the optical path length of the cell in cm. Since all the peptides were synthesized using a peptide amide linker, the C-terminus acts as an extra residue.

Vesicle Preparation. Phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL). Neutral vesicles were prepared from dilaurylphosphatidylcholine (DLPC) and negatively charged vesicles from a mixture of DLPC and dilaurylphosphatidylglycerol (DLPG) in a molar ratio of 4:1. Unilamellar vesicles of 50−60-nm diameter were prepared from a lipid dispersion in 2.5 mM sodium phosphate, pH 7.4, via extrusion 19 times through a polycarbonate filter (0.05-μm pore size) using a Lipofast extruder device (Avestin, Ottawa, Canada).[31] Vesicle size was determined using dynamic light scattering as previously described.[32]

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Supporting Information Available: Analytical HPLC traces of purified peptides and MS data (36 pages). Ordering information is given on any current masthead page.

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