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Antimicrobial peptides containing unnatural amino acid exhibit potent bactericidal activity against ESKAPE pathogens

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ABSTRACT

A series of 36 synthetic antimicrobial peptides containing unnatural amino acids were screened to determine their effectiveness to treat *Enterococcus faecium, Staphylococcus aureus, Klebsiella pnemoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter species* (ESKAPE) pathogens, which are known to commonly infect chronic wounds. The primary amino acid sequences of these peptides incorporate either three or six dipeptide units consisting of the unnatural amino acids Tetrahydroisoquinolinecarboxylic acid (Tic) and Octahydroindolecarboxylic acid (Oic). The Tic-Oic dipeptide units are separated by SPACER amino acids with specific physicochemical properties that control how these peptides interact with bacterial cell membranes of different chemical compositions. These peptides exhibited minimum inhibitory concentrations (MIC) against these pathogens in the range from >100 to 6.25 μg/mL. The observed diversity of MIC values for these peptides against the various bacterial strains are consistent with our hypothesis that the complementarity of the physicochemical properties of the peptide and the lipid of the bacteria's cell membrane determines the resulting antibacterial activity of the peptide.

1. Introduction

Over the past two decades the global emergence and spread of antimicrobial resistant strains of commonly encountered pathogens has been observed.[1–5] Many nosocomial clinical isolates, such as *Acinetobacter*, *Staphylococcus* and *Pseudomonas* are resistant to many of the traditional antibiotics.[6–8] This has resulted in a major crisis in modern medicine which has stimulated major research efforts to develop novel antibiotic therapeutics.[9–15] Antimicrobial peptides are a potential source of novel antimicrobial agents currently being extensively investigated.[4,5,16–18]

As a class, natural and synthetic antimicrobial peptides exhibit a very high potential as new therapeutic agents because of their novel mechanism of antimicrobial activity, coupled with the difficulty of bacteria to develop resistance to them.[11,19–21] Antimicrobial peptides (AMP) have evolved in almost every class of living organisms as a host defense mechanism against invading microorganisms including bacteria, fungi, protozoa and parasites.[22,23] AMPs have also been shown to be key components of the innate immune response.[24,25] AMPs are generally small (5–50 amino acid residues) highly positively charged (+3 to +9)[26] amphipathic peptides with well-defined region of hydrophobicity and hydrophilicity.[21,27] As of January 2009, more than 1330[28] natural and synthetic antimicrobial peptides exhibiting a wide range of biological activity have been reported.[29] AMPs are broadly divided into two mechanistic classes, membrane-disruptors and non-membrane-disruptors.[30,31] Five different mechanisms have been proposed at one time or another to explain membrane disruption.[32–35] The absence of a specific target or receptor for membrane-disruptor AMPs is supported by the observation that analogs containing D-amino acid replacements do not exhibit reduced anti-bacterial activity.[36] The selectivity of AMPs for prokaryotic versus eukaryotic cells is believed to be derived from the differences in the chemical compositions of their respective cell membranes.[23] Bacterial cells contain a high percentage of negatively charged phospholipids while mammalian cells contain a much higher concentration of Zwitterionic phospholipids.[12,13,15,35,37–39] Other differences also exist including; membrane composition (sterols, lipopolysaccharide, peptidoglycan etc.),[35] structure, transmembrane potential and polarizability. Hancock and co-workers[30] have extended this hypothesis to propose that the differences in membrane composition between different strains of bacteria are responsible for the diversity in the potency and selectivity exhibited by a particular AMP against different strains of bacteria. The lipid bilayer of Gram-positive bacteria is covered by a porous layer of peptidoglycan, while the membrane structure of Gram-negative bacteria is more complex with two lipid membranes containing lipopolysaccharides and a cytoplasmic membrane.[23,40] There is a developing preponderance of evidence in the literature, supporting the concept that the selectivity and potency of a specific AMP is determined in a large measure by the

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complementarity of the physicochemical properties of the peptide and of the target membrane.[30,41]

The intent of this investigation was to determine the role played by various physicochemical properties on the antibacterial activity of AMPs containing unnatural amino acids against Gram-positive and negative bacteria with high clinical relevance.

2. Methods

2.1. Peptide synthesis

Peptide synthesis was performed either manually using tBOC chemistry or with an automated peptide synthesizer using FMOC chemistry[42,43] as previously reported.[44–46] All peptides were purified by Reverse Phase HPLC using an Agilent 1100 Series Preparative Instrument and a Vydac C18 Reverse Phase Preparative HPLC Column as previously reported.[45,46] All purified peptides were analyzed again by HPLC and Mass-Spec. Mass Spectral analyses were carried out using a Finnigan LTQ ESI-MS instrument running Xcalibur 1.4SR-1 or a Kratos PC Axima CFR Plus instrument (MALDI) running Kompact V2.4.1. ESI-MS showed multiply charged ions and the accurate mass was calculated. MALDI analyses were performed in reflectron mode.[45,46]

2.2. In vitro susceptibility study

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined by a procedure based on the National Committee for Clinical Laboratory Standards broth microdilution method. MIC is defined as the lowest concentration of the test antimicrobial that would inhibit the growth of the target organism as detected spectrophotometrically. MBC is defined as the lowest concentration of the test antimicrobial required to kill the target organism as determined by subculturing of the treated organism to agar media. The susceptibility study was performed as previously described.[47] Briefly, Meuller Hinton (VWR International, Radnor, PA) was used as the assay medium for the test organisms. Freshly grown bacterial cultures at exponential phase were adjusted to an optical density (OD) at 600 nm of 0.05 (equivalent to 2×10^7 CFU/mL) and further diluted to obtain a concentration of 2×10^6 CFU/mL to be used as the inoculum. Aqueous peptide solution (100 μL) at two times of the highest test concentration was added to each well of a sterile 96-well, flat bottomed plate (TPP, Switzerland). The antimicrobial peptide solution was twofold serially diluted with sterile distilled water in the wells, with the final peptide concentrations ranging from 0.78 to 100 μg/mL. After adding 100 μL aliquots of the suspension of the target organisms to the wells, the plates were incubated at 37 °C for 24 h. The MIC was determined by recording the lowest concentration of the peptide that prevented visible turbidity of the target organism, as measured at 600 nm by using an ELISA reader (Biotek synergy HT). Visible turbidity was determined by the OD readings of the tested samples that were significantly greater than that of the medium or background. The MBC was determined by plating 100 μL from each clear well (⩾MIC) onto 5% sheep blood agar plates. After incubation for 24 h, the MBC was determined as the lowest concentration of the test peptide that did not permit visible growth on the surface of the agar.

2.3. In vitro toxicity studies

Normal human dermal fibroblasts (NHDF), obtained from Lonza (Walkersville, MD, USA), were used as the target for in vitro toxicity studies. NHDF were cultured in fibroblast growth media containing FGM-2 bullet kit (Lonza) which contained 2% fetal bovine serum at 37 °C in a CO_2 incubator prior to exposure to various concentrations of either peptide **53** or **79**, which included concentrations that were at least 10-fold (up to 1000 μg/mL) above the effective antimicrobial doses used in the bactericidal assay. The assays were performed as previously described.[47] In these assays, cells that were exposed to medium alone served as controls. Untreated and cells that had been exposed to different concentrations of test peptides were examined for viability, as determined by their ability to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO) and their membrane integrity, as a function of the amount of cytoplasmic lactate dehydrogenase (LDH) released into the medium. Detection of cellular conversion of MTT to water-insoluble colored formazan and determination of total (cytoplasmic and extracellular) and extracellular LDH of treated and untreated cells were done according to the instructions of the manufacturer of a commercially available in vitro toxicity assay kit (Sigma). Reaction products of the LDH assay were measured spectrophotometrically by using a test wavelength of 490 nm and a reference wavelength of 690 nm. For measuring acid/isopropanol-solubilized formazan, a test wavelength of 570 nm and a reference wavelength of 690 nm were used.

3. Discussion of antibacterial activity

One of the major drawbacks of many of the existing AMPs limiting their therapeutic effectiveness is their susceptibility to enzymatic degradation. This creates a barrier for the stability of AMPs for use in hostile host environments such as wounds for controlling infections. The focus of the research conducted in our laboratories is to develop antimicrobial peptides containing unnatural amino acids as effective therapeutic agents.[44] The incorporation of unnatural amino acids into the primary sequence of peptide based drugs is increasing because of their inherent increased metabolic stability as compared to the 20 RNA encoded amino acids.[21,39,48–50] Incorporation of unnatural amino acids into a peptide sequence also provides a 'toolbox' of different physicochemical properties that are not available in peptides incorporating only the 20 RNA encoded amino acids.[49–53] These physicochemical properties include molecular flexibility, the ability to adopt novel helical conformations via unique intramolecular hydrogen bonding schemes, modification of the electronic character of the aromatic ring of Phe residues, distance of a positively charged nitrogen side change atom to the peptide backbone, charge delocalization and hydrophobicity among others. We are employing this 'toolbox' of functionality to facilitate the development of novel peptides with specific physicochemical properties that have the ability to interact selectively with membranes of different chemical compositions.[45,54,55] Thus providing a methodology for the design of bacteria strain specific AMPs.[56,57] The peptide skeleton of the unnatural AMPs designed in our laboratories is based on the placement of multiple L-Tic(Tetrahydroisoquinolinecarboxylic acid)-L-Oic (Octahydroindolecarboxylic acid) dipeptide units into the polypeptide backbone to induce an ordered structure onto the peptide.[44] The Tic-Oic dipeptide units are connected via one or two amino acid residue SPACERs with defined properties of charge and hydrophobicity. These SPACERs also maintain sufficient conformational flexibility to allow these peptides to adopt different conformations on interacting with membranes with different chemical compositions.[44]

It is well documented that the chemical compositions of the membranes of different bacterial strains vary greatly;[58–61] therefore, the resulting physicochemical surface properties presented by the membranes to the external environment will be different as well as specific for each bacterial strain.[4,16,62] The documented difference in the chemical composition of bacterial membranes

explains why the physicochemical properties of charge, hydrophobicity and conformation, among others, are known to play a major role in defining antimicrobial activity and organism selectivity of peptides.[15,40,63,64] Our research hypothesis evolved from the above observations, which in its simplest form states; the 3D-physicochemical surface properties of target cell membrane interacts with the 3D-physicochemical surface properties of the approaching AMP in a very specific way (via bioactive conformation) thus defining the resulting organism selectivity and potency.[62] This hypothesis was confirmed by the observation that the AMPs developed in our laboratories exhibited very different in vitro activity against *Staphylococcus aureus* and *Mycobacterium ranae* bacteria. The chemical compositions of these two bacteria's cell membranes are very different. The results of two 3D-QSARs (quantitative structure–activity relationship) studies determined that there are five major physicochemical descriptors necessary to define the activity of these AMPs in the *S. aureus* QSAR model.[62] Five different physicochemical descriptors were also necessary to define the activity of these AMPs in the *M. ranae* QSAR model.[62] These results support the hypothesis that for any particular AMP, organism selectivity and potency are controlled by the chemical composition of the target cell membrane.[15,40,62–64]

3.1. Analogs containing three Tic-Oic dipeptide units

The basic skeleton of the AMPs based on three Tic-Oic dipeptide units is given in Figure 1.[44] SPACER #1 defines the distance between the two Tic-Oic dipeptide units. This SPACER is involved in defining the flexibility of any induced turn or helical structure, as well as any intramolecular hydrogen bonding schemes (e.g., 10-helix or 12-helix) that would stabilize one secondary structure over another. SPACER #2 defines the distance between the polypeptide backbone atoms and the positively charged side chain nitrogen as well as determining the overall surface charge density of the molecule. SPACER #3 defines the distance between the last Tic residue and the C-terminal Lys residues and provides additional conformational flexibility for binding to the cell membrane surface. The amino acid sequences of the peptide analogs containing three Tic-Oic dipeptide units used in the study are given in Table 1. Rationale used in the design of these analogs is discussed in detail in the literature.[46,56,65,66]

As pointed out by several researchers in order to develop AMPs as potential therapeutic agents it is critical to develop an understanding of the basis for the different interactions that occur between the AMP and Zwitterionic and anionic lipids.[21,67,68] To accomplish this goal extensive circular dichroism (CD) spectroscopy, isothermal titration calorimetry (ITC) and induced calcein fluorescence leakage experiments were conducted to investigate the interactions of these AMPs with various model membrane systems.[46,65,66,69] Large unilamellar vesicles (LUVs) and small lamellar vesicles (SUVs) consisting of 1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine (POPC) were selected as a simple model for the Zwitterionic membranes of mammalian cells and membrane models consisting of (4:1) POPC/**1**-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)] (sodium salt) (POPG) were selected as a simple model for the anionic membranes of bacteria cells.[70] In addition, we used anionic sodium dodecyl sulfate (SDS) and Zwitterionic dodecylphosphocholine (DPC) micelles to isolate and investigate the surface binding interaction of these peptides from the aggregation and pore forming interactions that occur on binding to lipids. The CD spectra of these AMPs in the presence of Zwitterionic DPC micelles and anionic SDS micelles are very different indicating that these compounds adopt different conformations on binding to the surface of anionic and Zwitterionic membrane models.[69] These AMPs also exhibited very different CD spectra in the presence of Zwitterionic

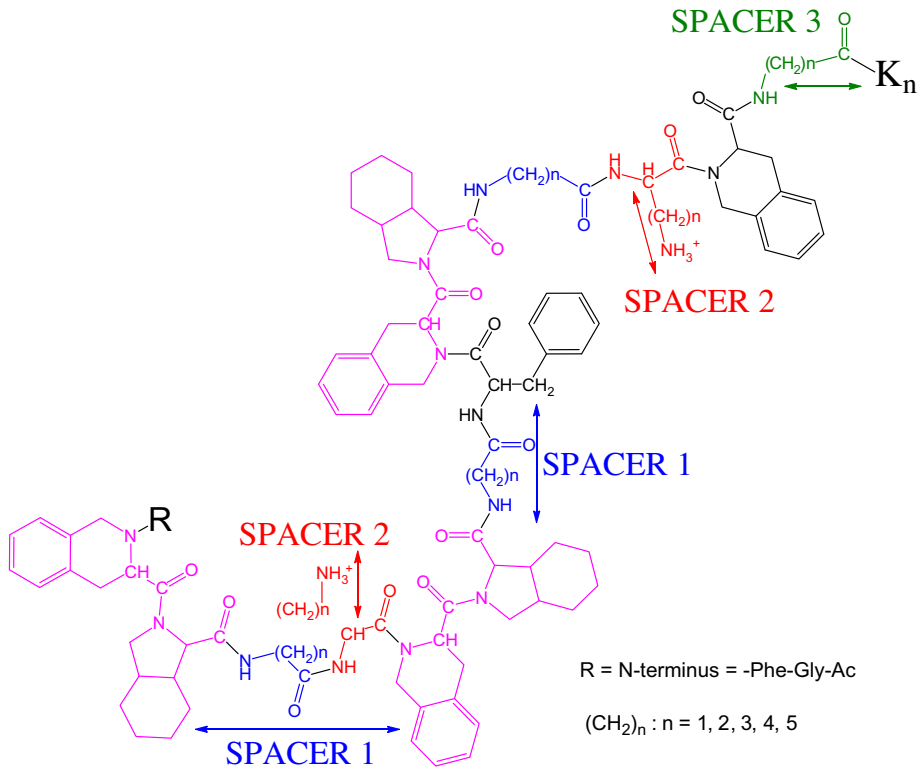

Figure 1. Skeleton of the analogs containing three Tic-Oic dipeptide units. The Tic-Oic dipeptide units are shown in PURPLE, SPACER #1 is shown in BLUE, SPACER #2 is shown in RED and SPACER #3 is shown in GREEN.

Table 1
Amino Acid sequence of peptide analogs containing Tic-Oic dipeptide units

Compd #	Amino acid sequence
Analogs containing three Tic-Oic dipeptide units	
14	Ac-GK-Tic-Oic-GLGKE-Tic-Oic-GLGK-Tic-Oic-ELMGER-$CONH_2$
23	Ac-GF-Tic-Oic-GK-Tic-Oic-GF-Tic-Oic-GK-Tic-KKKK-$CONH_2$
24	Ac-GF-Tic-Oic-GK-Tic-Oic-GF-Tic-Oic-GK-Tic-KKKK-CONH-CH2-$CH2-NH_2$
29	Ac-Gaba-F-Tic-Oic-Gaba-K-Tic-Oic-Gaba-F-Tic-Oic-Gaba-K-Tic-KKKK-$CONH_2$
36	Ac-βAla-F-Tic-Oic-βAla-K-Tic-Oic-βAla-F-Tic-Oic-βAla-K-Tic-KKKK-$CONH_2$
40	Ac-Gaba-F-Tic-Oic-Gaba-K-Tic-Oic-Gaba-F-Tic-Oic-Gaba-K-Tic-KKKKK-$CONH_2$
42	Ac-G-Fpa-Tic-Oic-GK-Tic-Oic-G-Fpa-Tic-Oic-GK-Tic-KKKK-$CONH_2$
43	Ac-GF-Tic-Oic-G-Orn-Tic-Oic-GF-Tic-Oic-G-Orn-Tic-Orn-Orn-Orn-Orn-$CONH_2$
45	Ac-GF-Tic-Oic-G-Dpr-Tic-Oic-GF-Tic-Oic-G-Dpr-Tic-Dpr-DprDpr-Dpr-$CONH_2$
46	Ac-βAla-Fpa-Tic-Oic-βAla-Dpr-Tic-Oic-βAla-Fpa-Tic-Oic−βAla-Dpr-Tic-Dpr-Dpr-Dpr-Dpr-$CONH_2$
47	Ac-G-DF-Tic-Oic-GK-Tic-Oic-G-DF-Tic-Oic-GK-Tic-KKKK-$CONH_2$
48	Ac-GF-Tic-Oic-G-DK-Tic-Oic-GF-Tic-Oic-G-DK-Tic-KKKK-$CONH_2$
49	Ac-G-Nph-Tic-Oic-GK-Tic-Oic-G-Nph-Tic-Oic-GK-Tic-KKKK-$CONH_2$
50	Ac-GF-Tic-Oic-GK-Tic-Oic-GF-Tic-Oic-GK-Tic-βA-KKKK-$CONH_2$
51	Ac-GF-Tic-Oic-GK-Tic-Oic-GF-Tic-Oic-GK-Tic-Gaba-KKKK-$CONH_2$
52	Ac-GF-Tic-Oic-GK-Tic-Oic-GF-Tic-Oic-GK-Tic-Ahx-KKKK-$CONH_2$
53	Ac-GF-Tic-Oic-G-Dab-Tic-Oic-GF-Tic-Oic-G-Dab-Tic-Dab-Dab-Dab-Dab-$CONH_2$
55	Ac-GF-Tic-Oic-GW-Tic-Oic-GF-Tic-Oic-GW-Tic-KKKK-$CONH_2$
56	Ac-GF-Tic-Oic-GR-Tic-Oic-GF-Tic-Oic-GR-Tic-RRRR-$CONH_2$
58	Ac-G-Cph-Tic-Oic-GK-Tic-Oic-G-Cph-Tic-Oic-GK-Tic-KKKK-$CONH_2$
60	Ac-βAla-Fph-Tic-Oic-βAla-K-Tic-Oic-βAla-Fph-Tic-Oic-βAla-K-Tic-Dpr-Dpr-Dpr-Dpr-$CONH_2$
61	Ac-KKKK-GF-Tic-Oic-GK-Tic-Oic-GF-Tic-Oic-GK-Tic-$CONH_2$
62	Ac-GF-Oic-Oic-GK-Oic-Oic-GF-Oic-Oic-GK-Tic-KKKK-$CONH_2$
64	Ac-GF-Tic-Oic-GK-Tic-Oic-GF-Tic-Oic-GK-Tic-KKK-$CONH_2$
Analogs containing six Tic-Oic dipeptide units	
22	H_2N-KL-Tic-Oic-K-Tic-Oic-F-Tic-Oic-K-Tic-Oic-F-Tic-Oic-K-Tic-Oic-KR-$CONH_2$
70	Ac-KL-Tic-Oic-K-Tic-Oic-F-Tic-Oic-K-Tic-Oic-F-Tic-Oic-K-Tic-Oic-KKKK-$CONH_2$
71	H_2N-Orn-L-Tic-Oic-Orn-Tic-Oic-F-Tic-Oic-Orn-Tic-Oic-F-Tic-Oic-Orn-Tic-Oic-Orn-Orn-Orn-Orn-$CONH_2$
72	H_2N-Dpr-L-Tic-Oic-Dpr-Tic-Oic-F-Tic-Oic-Dpr-Tic-Oic-F-Tic-Oic-Dpr-Tic-Oic-Dpr-Dpr-Dpr-Dpr-$CONH_2$
73	H_2N-Dab-L-Tic-Oic-Dab-Tic-Oic-F-Tic-Oic-Dab-Tic-Oic-F-Tic-Oic-Dab-Tic-Oic-Dab-Dab-Dab-Dab-$CONH_2$
74	H_2N-KL-Tic-Oic-GK-Tic-Oic-F-Tic-Oic-GK-Tic-Oic-F-Tic-Oic-GK-Tic-Oic-KKKK-$CONH_2$
75	H_2N-KL-Tic-Oic-K-Tic-Oic-GF-Tic-Oic-K-Tic-Oic-GF-Tic-Oic-K-Tic-Oic-KKKK-$CONH_2$
76	H_2N-KL-Tic-Oic-GK-Tic-Oic-GF-Tic-Oic-GK-Tic-Oic-GF-Tic-Oic-GK-Tic-Oic-KKKK-$CONH_2$
77	H_2N-KL-Tic-Oic-K-Tic-Oic-FG-Tic-Oic-K-Tic-Oic-FG-Tic-Oic-K-Tic-Oic-KKKK-$CONH_2$
78	H_2N-KL-Tic-Oic-KG-Tic-Oic-F-Tic-Oic-KG-Tic-Oic-F-Tic-Oic-KG-Tic-Oic-KKKK-$CONH_2$
79	H_2N-KL-Tic-Oic-KG-Tic-Oic-FG-Tic-Oic-KG-Tic-Oic-FG-Tic-Oic-KG-Tic-Oic-KKKK-$CONH_2$
80	H_2N-KL-Tic-Oic-GK-Tic-Oic-βA-F-Tic-Oic-GK-Tic-Oic-βA-F-Tic-Oic-GK-Tic-Oic-KKKK-$CONH_2$

Residues in blue indicate D amino acids. Residues in red indicate unnatural amino acids

POPC and anionic mixed 4:1 POPC/POPG SUVs and LUVs, indicating the formation of different conformations on interaction with the two membrane types. In order to obtain selectivity for bacterial cells versus mammalian cells these AMPs must interact differently with anionic and Zwitterionic membranes. This observation is also supported by isothermal titration calorimetry (ITC) and calcein leakage data. ITC data clearly indicated that these AMPs interact via different thermodynamic process with POPC and 4:1 POPC/POPG LUVs.[46,65,66,69] This observation again supports the hypothesis that organism selectivity is possible within this series of AMPs. ITC data suggested these AMPs interact primarily with the surface of Zwitterionic LUVs and was further supported by fluorescence experiments where the interactions do not appear to be concentration dependent. In the presence of anionic membranes, the interactions appear more complex and the calorimetric and fluorescence data both imply pore formation is dependent on peptide concentration.[46,65,66,69]

Here we chose to test the bactericidal activity of these peptides (both groups of analogs) against the ESKAPE pathogens (*Enterococcus faecium, S. aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter* species). These pathogens are responsible for a very high percentage of nosocomial infections and are highly resistant to antimicrobial agents. The MIC and MBC values that are associated with each of the test compound against the ESKAPE pathogens as described in the following sections are listed in Table 2. The MIC and MBC values against one of the test strains of *S. aureus*, strain USA300, are not listed in Table 2, but presented only in the text instead. The differences in the in vitro antibacterial activity of these analogs shown in Table 2 are not unexpected. It is consistent with our expectations.

3.1.1. Activity against Gram-positive pathogens

The cell membranes of the two strains of Gram-positive bacterial *S. aureus* and *Enterococcus faecium* are known to consist of 57%[71] and 23%[72] of the anionic lipid POPG and 30%[73] and 44%[72] of the doubly negatively charged lipid caridiolipin respectively. Because of this variation in lipid composition variations in the observed antibacterial activity of these AMPs should also be observed. This was indeed the case.

Modifications of SPACER #1 (compounds **23**, **29** and **36**) had very little effect on the activity against *S. aureus* UAMS-1 (Gram-positive), thus suggesting that molecular flexibility does not play a major role in defining the interactions that occur between these AMPs and the membrane of *S. aureus*. However, for *E. faecium* modifications of SPACER #1 had the following effect on the MIC and MBC values against *E. faecium*. Compounds **23** and **36** exhibited MIC and MBC values of 25 and 50 μg/mL respectively. However increasing the length of SPACER #1 to three carbon atoms (Gaba residue (**29**)) decreased the MIC and MBC values to 12.5 and 25 μg/mL respectively. *E. faecium* was the only bacterial strain (Gram-positive or negative) investigated where SPACER 1 was found to exhibit an effect on the antibacterial activity. The higher concentration of the doubly negatively charged lipid, caridiolipin may be responsible for the observed difference in activity. The

Table 2
In vitro susceptibility of antimicrobial peptides containing unnatural amino acids toward ESKAPE pathogens

	S. aureus UAMS-1		*A. baumannii* WRAIR #13		*K. pneumoniae* BAMC 07-18		*P. aeruginosa* PAO1		*E. aerogenes*		*E. faecium* MMC4	
Comp#	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
14	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
23	>100	>100	6.25	25	100	100	100	100	100	100	25	50
24	50	>100	6.25	6.25	25	50	50	100	50	50	12.5	25
29	>100	>100	12.5	25	100	100	>100	>100	>100	>100	12.5	25
36	>100	>100	12.5	25	>100	>100	>100	>100	>100	>100	25	50
40	>100	>100	not tested	not tested	>100	>100	>100	>100	>100	>100	not tested	not tested
42	100	100	12.5	25	100	100	>100	>100	>100	>100	25	50
43	100	100	6.25	25	50	50	50	50	100	>100	12.5	50
45	>100	100	12.5	12.5	12.5	25	25	25	50	50	50	100
46	>100	>100	12.5	25	12.5	25	25	50	25	25	50	>100
47	100	100	12.5	12.5	50	100	50	50	100	100	12.5	50
48	>100	>100	12.5	25	100	100	>100	>100	>100	>100	12.5	50
49	100	>100	12.5	12.5	50	50	50	100	100	100	25	50
50	>100	>100	12.5	25	>100	>100	>100	>100	>100	>100	12.5	50
51	>100	>100	12.5	50	>100	>100	>100	>100	>100	>100	50	>100
52	100	>100	12.5	25	100	100	100	>100	100	>100	25	>100
53	50	50	6.25	12.5	12.5	12.5	12.5	25	25	25	6.25	50
55	>100	>100	25	100	>100	>100	>100	>100	>100	>100	25	100
56	50	>100	6.25	12.5	50	100	100	>100	50	50	6.25	25
58	25	50	6.25	25	50	100	50	50	>100	>100	6.25	25
60	100	>100	12.5	12.5	50	100	50	>100	>100	>100	not tested	not tested
61	>100	>100	12.5	25	100	100	>100	>100	>100	>100	25	>100
62	>100	>100	12.5	12.5	>100	>100	>100	>100	>100	>100	12.5	100
64	100	>100	12.5	50	100	>100	>100	>100	>100	>100	25	100
22	50	50	100	>100	>100	>100	>100	>100	>100	>100	12.5	25
70	50	50	100	100	>100	>100	>100	>100	>100	>100	12.5	25
71	50	100	12.5	100	100	>100	100	>100	100	>100	12.5	50
72	50	100	12.5	50	100	100	50	100	100	100	12.5	25
73	25	25	25	100	50	100	50	100	50	50	12.5	25
74	50	50	12.5	50	>100	100	>100	>100	>100	>100	6.25	25
75	50	50	25	100	>100	100	50	50	>100	>100	6.25	25
76	50	50	12.5	12.5	50	50	50	50	50	50	6.25	25
77	25	25	12.5	50	50	100	50	50	100	100	6.25	25
78	50	50	12.5	12.5	50	100	50	50	>100	>100	6.25	6.25
79	12.5	50	12.5	25	50	50	50	50	50	50	6.25	25
80	50	50	6.25	25	50	50	25	50	50	100	6.25	25

All MIC and MBC values are in μg/mL.

increased molecular flexibility of the GABA residue and the unique intramolecular hydrogen bonding scheme available to that residue may stabilize the membrane binding process and those increase activity. This hypothesis is yet to be proven. Another possible explanation of the increased activity is that increasing the hydrophobicity (GABA is more hydrophobic than the other two residues) of the backbone increases the binding interaction between these AMPs and the membrane of *E. faecium*.

SPACER #2 (compounds **23**, **43**, **45**, **53** and **56**) seems to play a major role in defining the interactions that occur between these AMPs and the membrane of *S. aureus*. Replacing the four carbon side chain of the Lys residues (**23**) with three carbon side chain of the Orn residues (**43**) decreased the MIC and MBC values to100 μg/mL. Decreasing the number of carbon atoms in the side further to two carbon of the Dab (**53**) residue decreased the MIC and MBC values against *S. aureus* UAMS-1, the osteomyelitis

Table 3
Properties of the positively charged residues used in this investigation

Compd #	Amino acid	# Carbons	Distance[a]	hydrophobicity[b]	hydrophobicity net[c]
45	Dpr	1	2.56	−9.3	−57.0
53	Dab	2	2,98	−9.5	−55.8
43	Orn	3	3.55	−9.0	−54
23	Lys	4	4.76	−9.9	−59.4
56	Arg	3	5.12	−10.0	−60.0

[a] Average distance in angstroms from peptide backbone to the positively charged nitrogen atom as calculated using ChemDraw 3D.
[b] Combined consensus scale hydrophobicity for each charge residue.
[c] Total combined consensus scale hydrophobicity for all of the charged residues in the molecule.

Table 4
In vitro activity, MIC activity (μg/mL), against selected Gram-negative bacterial strains as previously reported by Hicks and co-workers[45]

Bacteria	Compound	**23**	**64**	**61**
Yersinia pestis C092		500	62.5	32.2
Brucella melitensis 16M		500	125	125
Brucella abortus 2308		500	62.5	125
Francisella tularensis		250	0.04	125
Burkholderia mallei		500	0.04	500
Burkholderia pseudomallei		500	0.04	500

clinical isolate, to 50 μg/mL. This strain is sensitive to methicillin (methicillin-sensitive *S. aureus*, MSSA). Compound **53** exhibited greater MIC activity against *S. aureus* USA300 with a MIC value of 25 μg/mL. USA300 is a strain of community-associated methicillin-resistant *S aureus* (CA-MRSA). However, decreasing the carbon chain to one carbon atom (**45**) increased the MIC and MBC values against *S. aureus* UAMS-1 to 100 μg/mL. This data suggest that there is an optimal distance, the two carbon atoms of the Dab residue, from the peptide backbone and the positively charge nitrogen on the side chain that favors membrane binding and distribution leading to cell death. It should be pointed out that changing the number of carbon atoms in the side chain of SPACER #2, also changes the overall hydrophobicity of the residue. These basic residues exhibit negative hydrophobicity and by shortening the side chain the protonated nitrogen is moved closer to the peptide backbone. This results in reducing the solvent accessibility of the protonated nitrogen and thus increasing the hydrophobicity. The combined consensus scale (CCS) (hydrophobicity) developed by Tossi and co-workers[74] for each residue used as SPACER #2 is given in Table 3. The net hydrophobicity resulting from the incorporation of six residues into the AMPs is also given in Table 3. Compound **56** were the Lys residues have been replaced with Arg residues exhibited greater activity against *S. aureus* UAMS-1 and *S. aureus* USA300 with MIC values of 50 and 25 μg/mL respectively. Compound **56**, contains a side chain guanidinium group where the positive charge is delocalized over two nitrogen atoms instead of only one nitrogen atom as is the case with the other four analogs. The increased activity of compounds **53** and **56** are consistent with the fact that the lipid composition of some strains of *S. aureus* contain as much as 38% lysylphosphatidylglycerol which is a cationic lipid that is known to repel antimicrobial peptides.[75–77] The shortening of the length of SPACER #2 (**53**) will result in less side chain flexibility and also bring the side chain positive charge closer to the peptide backbone. This will result in the nitrogen being somewhat delocalized onto the peptide backbone instead being localized on the side chain nitrogen atom. This is because the positive charge on the nitrogen atom is now close to the electron density associated with the carbonyl oxygen of the amide bond. This proximity has the net effect of shielding the lipid surface from the full positive charge of the nitrogen atom. This argument is supported by the observation that the guanidinium group of compound **56** also delocalizes the side chain positive charge and also exhibits increased activity. Any delocalization of the side chain positive charge will reduce the repulsion of the AMPs by the positively charged lysylphosphatidylglycerol.

Similar trends were observed for variation of SPACER #2 and increased activity against *E. faecium* (see Table 2 for details). The optimal side chain length of SPACER #2 for maximal against *E. faecium* is once again the Dab residue. Compound **56** where the Lys residues have been replaced with Arg residues exhibited greatly increased activity against *E. faecium* with MIC and MBC values of 6.25 and 25 μg/mL respectively. The overall increase in activity of these AMPs against *E. faecium* verse *S. aureus* may be a result of the higher concentration of the doubly negatively charged lipid, caridiolipin found in *E. faecium* verse *S. aureus*. This hypothesis has yet to be confirmed,

E. faecium was the only strain of bacteria investigated where variation of SPACER #3 (compounds **50**, (β-Ala) **51**, (Gaba) and **52** (Ahx)) had an effect on the observed MIC values. (Please see Table 2 for details.) It is interesting to point out that decreasing the number of C-terminal Lys residues from 4 to 3 (**64**) and relocating the four Lys residue cluster from the C-terminus to the N-terminus (**61**) had no effect on the observed activity against *E. faecium.* (As seen in Table 4, these modifications have dramatically increased the activity against other strains of bacteria.) Thus confirming the hypothesis it is the distance and flexibility of the Lys cluster relative to the hydrophobic core of the peptide that is important and is not position (C or N-terminus) or total positive charge. The incorporation of positive charge in the hydrophobic core of the peptide is also not important in controlling the interaction of these peptides with the cell membrane of *E. faecium*. In compound **55** the two internal Lys residues were replaced with Trp and the MIC values remained the same, whereas in all the other bacterial strains the MIC values increased. It is also interesting to point out that converting the stereochemistry of the Lys from L to D (**48**) and of Phe from L to D (**47**) decreased the MIC values to 12.5 μg/mL, this also was not observed in the other strains. The unique chemical composition of the lipid membrane of *E. faecium,* particularly the high concentration of caridiolipin may be responsible for the unique activity.

The effect of changing the electron density of the aromatic rings of the Phe residues was investigated by preparing the 4-chloro (**58**), the 4-fluoro (**42**) and the 4-nitro (**49**) analogs. Only the 4-chloro analog exhibit significant MIC and MBC activity against both UAMS-1 and *S. aureus* USA300 with MIC values of 25 μg/mL and MBC values of 50 μg/mL. Only the 4-chloro analog increased activity against *E. faecium* with MIC values of 6.25 μg/mL and MBC values of 25 μg/mL. The increased activity of the 4-chloro analog (**58**) is unexplained at this time.

3.1.2. Activity against Gram-negative pathogens

The lipid compositions of the cell membranes of the Gram-negative bacteria investigated in this study vary. For example the average lipid composition of membranes of *Acinetobacter baumannii,* consist of 55% POPE (palmitoyl-oleoyl-phosphoethanolamine) and 30% POPG and 17% caridiolipin.[78,79] The lipid composition of the membranes of *Klebsiella pneumoniae* contains 5% POPG, 82% POPE and 6% caridiolipin.[5] The lipid composition of the membranes of *Pseudomonas aeruginosa* contains 21% POPG and 60% POPE and 11% caridiolipin.[5]

All of the AMPs tested exhibited very good activity against the Gram-negative *A. baumannii* WRAIR #13 (a clinical isolate obtained from Walter Reed Army Medical Center; this strain exhibited multidrug resistance to ampicillin, gentamicin, kanamycin,

streptomycin, and tetracycline) with MIC values in the range 3.1–12.5 μg/mL. *K. pneumoniae* BAMC 07-18 (a clinical isolate obtained from Brooke Army Medical Center that is resistant to ampicillin, azithromycin, chloramphenicol, gentamicin, and tetracycline) unlike *A. baumannii* exhibited less sensitivity to these AMPs (these AMPs exhibited lower activity against this strain). Modifications of SPACER #1 had little effect on activity, however SPACER #2 (compounds **23**, **43**, **45**, **53** and **56**) seems to play a major role in defining the interactions that occur between these AMPs and the membranes of *K. pneumoniae*. Replacing the four carbon side chain of the Lys residues (**23**) with three carbon side chain of the Orn residues (**43**) decreased the MIC and MBC values to 50 μg/mL. Decreasing the carbon side further to two carbon atoms of the Dab (**53**) and one carbon atom of the Dpr (45) residues decreased the MIC and MBC values against *K. pneumoniae* to 12.5 μg/mL. In the case of *P. aeruginosa* SPACER #2 also played a major role in defining activity of these AMPs. In fact very similar activity was observed against both organisms. Replacing the four carbon side chain of the Lys residues (**23**) with three carbon side chain of the Orn residues (**43**) decreased the MIC and MBC values to 50 μg/mL. Decreasing the carbon side chain further to two carbon atoms of the Dab (**53**) decreased the MIC and MBC values against *P. aeruginosa* to 12.5 μg/mL. Decreasing the side chain length to only one carbon atom of the Dpr (**45**) residues increased the MIC and MBC values against *P. aeruginosa* to 25 μg/mL.

In summary the charge delocalization and the position of the charge density relative to the peptide backbone of SPACER #2, as well as, the electronic character of the aromatic rings of the Phe residues (see Table 2 for details) seem to control the interactions of these peptides with the cell membranes of *K. pneumoniae* and *P. aeruginosa* to a greater degree than molecular flexibility and hydrophobicity of the peptide.

Comparing the lipid compositions of the membranes of *A. baumannii, K. pneumoniae* and *P. aeruginosa* consisting of 55%, 80% and 60% POPE, 30%, 5% and 21% POPG as well as 17%, 6% and 11% caridiolipin respectively, it is difficult to explain the observed antibacterial activity. The ratio of the Zwitterionic lipid POPC to the anionic lipids POPG and caridiolipin for these three bacteria are 1.2:1, 7.3:1 and 1.9:1 respectively. Based on the ratio of POPC to POPG it would be expected that the activity of these AMPs against *A. baumannii,* and *P. aeruginosa* would be very similar and very different against *K. pneumonia,* instead of the observed similarity in activity against *K. pneumoniae* and *P. aeruginosa.* However the total percent of anionic lipids for the three bacterial strains are 47%, 11% and 32%. This explains in part the increased activity of these AMPs against *A. baumannii* compared to the other two strains, however it does not explain the similar activity against *K. pneumoniae* and *P. aeruginosa,* unless there is a minimum threshold for anionic lipid composition above 32% for maximal activity. Additional investigations are required to explain the similarity in activity against *K. pneumoniae* and *P. aeruginosa.*

The Gram-negative bacteria *Enterobacter aerogenes*, a clinical isolate, is relatively insensitive to the analogs with only compound **53** exhibiting a relative good MIC and MBC values of 25 μg/mL. Compounds **45** and **56** exhibit similar MIC and MBC values of 50 μg/mL. All other analogs exhibited MIC values of 100 μg/mL or higher. We are unable to explain the observed activity of these AMPs against *E. aerogenes* as a function of lipid composition at this time.

3.2. Analogs containing six Tic-Oic dipeptide units

AMPs incorporating six Tic-Oic dipeptide units as well as four additional SPACERS, A, B, C and D on either side of the intervening hydrophobic and charged residues are shown in Figure 2.[45,80] These SPACERS define the overall conformational flexibility of the peptide backbone. These SPACERS are defined in Table 5. A fifth SPACER, E, which defines the distance between the polypeptide backbone and the positively charged side chain amine group, is involved in determining the overall surface charge density of the peptide as well as defining the distance between the membrane surface and the polypeptide backbone.[45,80]

3.2.1. Activity against Gram-positive pathogens

Increasing the number of Tic-Oic dipeptide units from three to six increased the activity of these peptides against both *S. aureus* UAMS-1(MSSA) and USA300 (CA-MRSA) with MIC and MBC values ranging from 50 to 12.5 μg/mL. These peptides were slightly more active against *S. aureus* USA300 (MRSA) as compared to *S. aureus* UAMS-1(MSSA). It is not know at this time if the increase in activity is simply due to the increase length of the peptides or to a combination of the changes in the three-dimensional placement of hydrophobic and changed groups within the peptide. Modification of SPACERs A and C had no effect on the observed MIC and MBC values against both strains. Modification of SPACERS B and D did affect the observed MIC values with compounds **77** and **79** (25 and 12.5 μg/mL respectively being more active than compounds **78** and **80,** both had the MIC at 50 μg/mL against strain UAMS-1). Based on this data SPACER D is required for increased activity, while the incorporation of SPACER B decreases somewhat the activity against both strains. From this data it appears that backbone flexibility after the hydrophobic or charged residue and the following Tic residue is more important in the interactions of these peptides with the cell membranes of *S. aureus* USA300 (MRSA) and *S. aureus* UAMS-1(MSSA) than the flexibility of the hydrophobic or charge residue and the preceding Oic residue. For SPACER E the Dab residue (MIC = 25 μg/mL) seems to be the most favorable for the interaction of these peptides with the cell membranes of *S. aureus* UAMS-1. The importance of the Dab residue was also observed in compound **53** of the series containing only three Tic-Oic dipeptide units. No other effect from the variation of SPACER E was observed.

Increasing the number of Tic-Oic dipeptide units from three to six without the incorporation of SPACERs A, B, C, D and E resulted in analogs with very good activity against *E. faecium* exhibiting MIC values in the range of 6.25–12.5 μg/mL and MBC values in the range of 6.25–50 μg/mL against *E. faecium.* Again the increase in the activity of the analogs containing six Tic-Oic dipeptide units against *E. faecium* as compared to *S. aureus* is believed to be a direct result of the previously discussed differences in lipid composition of the respective membranes.

3.2.2. Activity against Gram-negative pathogens

Increasing the number of Tic-Oic dipeptide units from three to six without the incorporation of SPACERs A, B, C and D in compound **70** (100 μg/mL) resulted in a dramatic loss in activity against all of the Gram-negative bacteria tested as compared to compound **23**. However, incorporation of any of the SPACERs A, B, C, or D resulted in an increase in activity against *A. baumannii* with MIC values in the range of 12.5–25 μg/mL. Compound **80** where SPACER B is a β-Ala residue and SPACER D is a Gly exhibited the greatest activity against *A. baumannii* with a MIC value of 6.25 μg/mL. Of the SPACER E analogs compound **73** which contains the Dab residues was the least active against *A. baumannii* exhibiting a MIC value of 25 μg/mL compared to MIC values of 12.5 μg/mL for the other SPACER E analogs (**71**, **72** and **74**). The decreased activity of the Dab containing analog (**73**) compared to the other SPACER E analogs was not observed in the other five strains of bacteria.

For the bacterium *K. pneumoniaee* BAMC 07-18, inserting either SPACERs A (**74**) or C (**75**) did not improve the activity of these AMPs against *K. pneumoniaee*, which is in contradiction to the

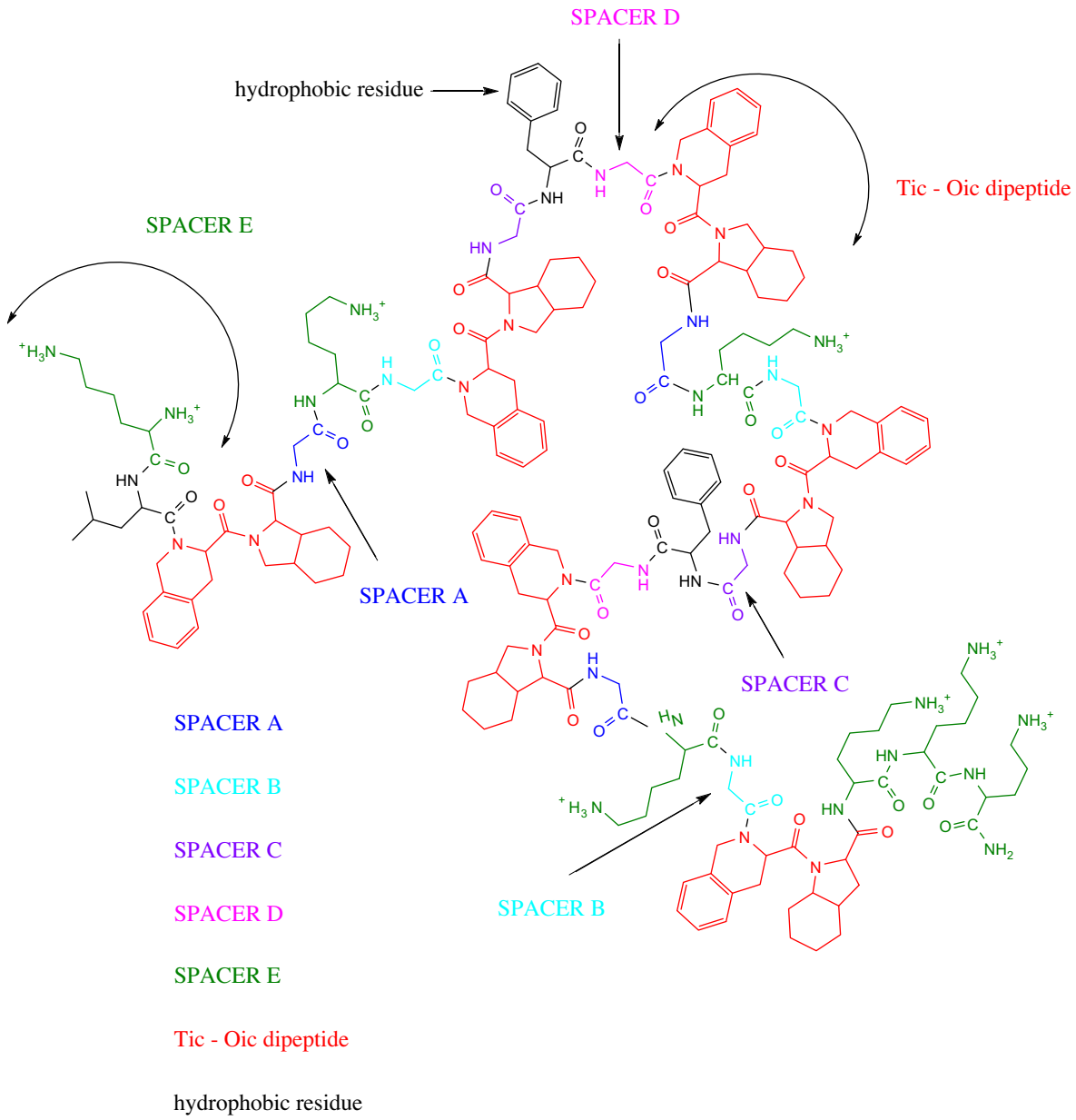

Figure 2. The basic skeleton of the analogs containing six Tic-Oic dipeptide units and the five SPACER residues.

increase in activity observed against *A. baumannii*. However, inserting both SPACERs A and C (**76**) increase the activity against *K. pneumoniae* (50 μg/mL). The insertion of either or both SPACERs B and D (**77**, **78**, **79** and **80**) increased the activity of these AMPs against *K. pneumoniae* (50 μg/mL). From this data it appears that backbone flexibility after the hydrophobic or charged residue and the following Tic residue is more important in the interactions of these peptides with the cell membrane of *K. pneumoniae* than the flexibility of the hydrophobic or charge residue and the preceding Oic residue.

For the bacterium *P. aeruginosa* PAO, again inserting SPACERs A (**74**) did not improve the activity of these AMPs. However inserting SPACER C (**75**) or both SPACERs A and C (**76**) increased the activity against *P. aeruginosa* (50 μg/mL). This indicates that SPACER C plays a critical role in defining the interactions that occur between these AMPs and the cell membranes of *P. aeruginosa*. The insertion of either or both SPACERs B and D (**77**, **78**, **79** and **80**) increased the activity of these peptides against *P. aeruginosa* with MIC and MBC values for all three of these analogs of 50 μg/mL. The MIC value for compound **80** was decreased slightly to 25 μg/mL. From this data it appears that backbone flexibility after the hydrophobic or charged residue and the following Tic residue is more important in the interactions of these peptides with the cell membranes of *P. aeruginosa* than the flexibility of the charge residue and the preceding Oic residue.

Inserting only SPACER A (**74**) or C (**75**) did not improve the activity of these peptides against *E. aerogenes*; however, inserting both SPACERs A and C (**76**) increase the activity against *E. aerogenes* with a MIC value of 50 μg/mL. This indicates that both SPACERs A and C plays a critical role in defining the interactions that occur between these peptides and the cell membrane of *E. aerogenes*. This same trend was observed with *K. pneumoniae*. The insertion of either SPACER B (**77**) or SPACER D (**78**) decreased the activity of these peptides against *E. aerogenes*. This trend was not observed in the other five strains of bacteria investigated. However insertion of both SPACERs B and D increased activity against *E. aerogenes*

Table 5
Definition of the SPACERS found in the six Tic-Oic containing analogs

Compd #	SPACER A[a]	SPACER B[b]	SPACER C[c]	SPACER D[d]	SPACER E[e]
22	Gly	None	None	None	Lys/Arg
70	None	None	None	None	Lys
71	None	None	None	None	Orn
72	None	None	None	None	Dpr
73	None	None	None	None	Dab
74	Gly	None	None	None	Lys
75	None	None	Gly	None	Lys
76	Gly	None	Gly	None	Lys
77	None	None	None	Gly	Lys
78	None	Gly	None	None	Lys
79	None	Gly	None	Gly	Lys
80	None	β-Ala	None	Gly	Lys

[a] Spacer A is the residue preceding each internal Lys residues (N-terminal side of the Lys).
[b] Spacers B is the residue following each internal Lys residues (C-terminal side of the Lys).
[c] Spacer C is the residue preceding each internal Phe residue (N-terminal side of the Phe).
[d] Spacer D is the residue following each internal Phe residues (C-terminal side of the Phe).
[e] Spacer E replaces the charged Lys residues with charged residues with progressively shorter side chains.

(50 μg/mL). From this data it appears that backbone flexibility after both the hydrophobic and charged residue and the following Tic residue or the flexibility of the charged and hydrophobic residue and the preceding Oic residue are more important for the interaction of these peptides with the cell membranes of *E. aerogenes* than the flexibility of just one.

Variations of SPACER E except for compound **73** (Dab residue), did not affect the activity of these AMPs against, *K. pneumonia, P. aeruginosa* and *E. aerogenes*.

The same trend was observed for the activity of the six Tic-Oic dipeptide containing analogs against the Gram-negative strains as was observed for the three Tic-Oic dipeptide containing analogs. These analogs were more active against *A. baumannii* than they were against the other four Gram-negative strains. We believe that the observed differences in activity are a result of the differences in the lipid compositions of the membranes of the four bacterial strains.

4. In vitro toxicity studies

Among the peptides tested, six peptides showed MIC values being 50 μg/mL or lower against each of the ESKAPE pathogens. **53** from the group of analogs containing three Tic-Oic dipeptide units and **79** from the group of analogs containing six Tic-Oic dipeptide units were selected in testing their toxicity against normal human dermal fibroblasts. These peptides at the MIC level (50 μg/mL) neither induced cell death nor compromised the membrane integrity of the treated fibroblasts, as indicated by the ability of the treated fibroblasts to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Fig. 3A) and the presence of low level of extracellular lactate dehydrogenase (LDH) (Fig. 3B), respectively. Fibroblasts contain a significant level of intracellular LDH.[47] At higher concentrations (above the MIC), these peptides exhibited some degree of cellular toxicity.

In previous investigations conducted in our laboratories LUVs and SUVs consisting of POPC were selected as a simple model for the Zwitterionic membranes of mammalian cells and membrane models consisting of (4:1) POPC/POPG were selected as a simple model for the anionic membranes of bacteria cells.[70] Different CD spectra were observed for these AMPs binding to Zwitterionic POPC and anionic mixed 4:1 POPC/POPG LUVs and SUVs, indicating that they adopt very different conformations on interaction with these two types of LUVs and SUVs.[46,65,66,69] In addition, ITC and calcein leakage data indicated that all the peptides studied interact via very different mechanisms with anionic and Zwitterionic LUVs.[46,65,66,69] This data suggests to us that electrostatic interactions are the major physicochemical property controlling binding with anionic lipids and hydrophobic interactions are the major physicochemical property controlling binding to Zwitterionic lipids. Thus reducing the hydrophobicity of the peptide should result in reduced mammalian toxicity.[75,81,82] There are several ways to accomplish this from replacing the Phe residues with Ala residues,[83] or to increasing the number of basic amino acid residues[82] in the peptide backbone.

5. Conclusion

This investigation clearly demonstrated that we were successful in obtaining increased organism potency and selectivity by modification of the physicochemical properties of these peptides. More importantly, this investigation indirectly revealed differences in the physicochemical properties of these cell membranes of these various bacteria strains. In vitro toxicity studies of the two selected peptides (**53** and **79**) that showed a wide spectrum of bactericidal activity did not induce cellular toxicity against human fibroblasts at the bacterial inhibition concentration or lower. Analysis of this data has and will continue to provide critical information that can be used to design new peptides that will exhibit greater organism potency and selectivity. In summary we feel compounds **53** and **79** are very strong candidates for continued development as

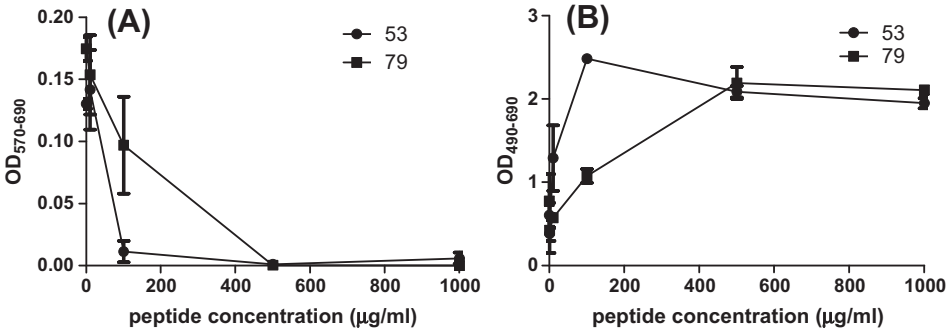

Figure 3. Determination of reduction of (A) MTT and (B) LDH release by the antimicrobial peptide (**53** or **79**) treated or untreated fibroblasts. Points and bars represent the mean and standard deviation (SD) of triplicate determinations. 2×10^5 fibroblasts were used in each determination of LDH release and MTT reduction. Cells were cultured for 72 h at 37 °C in a CO_2 incubator before exposure to different concentrations of **53** or **79**. NHDF were exposed to **53** and **79** for 6 h prior to measurements. The data represent the results of one of the two separate experiments.

topical treatments for wound infections caused by the bacteria strains investigated in this study.

DOD disclaimer

Some of the authors (J.J.A. and K.P.L.) are employees of the U.S. Government. The work presented is part of their official duties. The opinions or assertions contained herein are the private views of these authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

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