Received: 1 February 2017 | Revised: 5 June 2017 | Accepted: 10 June 2017

DOI: 10.1111/cbdd.13058

RESEARCH ARTICLE

WILEY

Role of positively charged residues on the polar and non-polar faces of amphipathic α-helical antimicrobial peptides on specificity and selectivity for Gram-negative pathogens

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We have designed de novo and synthesized eight 26-residue all D-conformation amphipathic α-helical cationic antimicrobial peptides (AMPs), four with "specificity determinants" which provide specificity for prokaryotic cells over eukaryotic cells and four AMPs without specificity determinants. The eight AMPs contain six positively charged Lys residues on the polar face in four different arrangements to understand the role of these residues have on antimicrobial activity against 14 *Acinetobacter baumannii* strains, seven of which were resistant to polymyxin B and colistin; six diverse *Pseudomonas aeruginosa* strains and 17 *Staphylococcus aureus* strains, nine of which were methicillin-sensitive, and eight of which were methicillin-resistant. The four AMPs without specificity determinants are extremely hemolytic. In contrast, the four AMPs with specificity determinants had dramatic improvements in therapeutic indices showing the importance of specificity determinants in removing eukaryotic cell toxicity. The specificity determinants combined with the location of positively charged residues on the polar face provide Gram-negative pathogen selectivity between *A. baumannii* and *S. aureus*. Specificity determinants maintain excellent antimicrobial activity in the presence of human sera, whereas the AMPs without specificity determinants were inactive. This study clearly shows the potential of amphipathic α-helical AMPs with specificity determinants as therapeutics to replace existing antibiotics.

KEYWORDS

Acinetobacter baumannii, amphipathic α-helical peptides, antimicrobial peptides (AMPs), Gram-negative selectivity, *Pseudomonas aeruginosa*, specificity determinants, temperature-profiling by RP-HPLC

1 | INTRODUCTION

The explosion of bacteria resistance to traditional antibiotics and a rapid increase of multi-drug resistance have created an urgency to develop new classes of antimicrobial agents. There are now "Superbugs" that are resistant to most or all of the available antibiotics.[1] The Infectious Diseases Society of America has reported that two-thirds of all healthcare-associated infections are caused by six multi-drug resistant organisms referred to as "ESKAPE" pathogens consisting of two Gram-positive organisms *Enterococcus faecium* and *Staphylococcus aureus* and four Gram-negative organisms consisting of *Klebsiella pneumonia, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* species.[2] A recent study in Mexico[3] demonstrated dramatic increases in the incidence of antibiotic-resistant species. Of 550 clinical isolates of *A. baumannii* and 250 clinical isolates of *P. aeruginosa*, 74% of *A. baumannii* and 34% of *P. aeruginosa* were multi-drug resistant.

Polymyxin B and Polymyxin E (Colistin) are cationic peptides consisting of a cyclic heptapeptide with a tripeptide side

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chain acylated by a fatty acid chain at the amino terminus. These antibiotics were heavily used in the 1960s, but in the 1970s, their clinical use was limited due to serious issues of nephrotoxicity and neurotoxicity[4,5] The revival of these two peptides began in the mid-1990s, due to the lack of novel antibiotics against prevalent multi-drug resistant Gram-negative bacteria. Thus, these compounds have become antibiotics of last resort. Resistance to the current polymyxins could become a major global health challenge because this means that virtually no antibiotics will be available for treatment of serious Gram-negative infections caused by polymyxin-resistant "superbugs"[4,5] Thus, new antibiotics must be effective against Polymyxin B and Colistin resistant organisms.

Antimicrobial peptides (AMPs) represent a ubiquitous response in nature to microbial infections and are produced by bacteria, fungi, plants, insects, amphibians, crustaceans, fish, and mammals, including humans, either constitutively or in response to the presence of a microbe[6] AMPs are rapidly bactericidal, and generally have broad-spectrum activity. There are generally two types of AMPs, those which have specific targets and the all D-versions of the AMPs are inactive, and those where the AMPs are equally active in the all-L or all-D versions. The antimicrobial mechanism of action of many cationic AMPs does not involve a stereoselective interaction with a chiral enzyme or lipid or protein since enantiomeric forms of AMPs with all-D-amino acids have shown equal activities compared to their all-L-enantiomers.[7–15] It is difficult for bacteria to develop resistance to AMPs that do not have specific targets as their mode of action generally involves non-specific interactions with the cytoplasmic membrane of bacteria. The advantage of all D-peptides is that they are resistant to proteolytic enzyme degradation which enhances their potential as therapeutic agents. The disadvantage of native AMPs is their lack of specificity between prokaryotic and eukaryotic cells, that is, they generally are too toxic to be used for systemic treatment of bacterial infections. Toxicity, the ability to lyse mammalian cells, is most frequently expressed as hemolytic activity against human red blood cells.

Obviously, the hemolytic activity, defined as HC_{50} (i.e., the concentration of peptide (μM) that results in 50% hemolysis after 18 hr at 37°C), should be as large as possible. The ratio of hemolytic activity/antibiotic activity defines the therapeutic index for a given AMP and is a measure of specificity of the AMP for bacteria membranes (MIC is the minimum concentration of peptide required to inhibit growth of bacteria after 24 hr at 37°C). The higher the therapeutic index, the more specific is the AMP for prokaryotic cells. To overcome toxicity problem issues, we developed the design concept of "specificity determinant(s)" which refers to substituting positively charged residue(s) in the center of the non-polar face of amphipathic α-helical[15–18]or cyclic β-sheet[19,20] AMPs to create selectivity between eukaryotic and prokaryotic membranes. The objective of substituting "specificity determinant(s)" is to maintain or enhance antimicrobial activity while decreasing or eliminating hemolytic activity or cell toxicity to mammalian cells.[15–18]

In this study, we wanted to significantly reduce the net charge of our de novo designed AMP, D16,[17] by maintaining the identical non-polar face of D16 and decreasing the net positive charge on the polar face from +9 in D16 to +6 in the new analogs and, at the same time, define the importance of the arrangement of these six positively charged residues on the polar face of the amphipathic α-helix on antimicrobial activity, hemolytic activity, and pathogen selectivity. We designed four versions of the new analogs where the arrangement of the positively charged residues on the polar face was varied while maintaining the six positively charged residues on the polar face in each analog. We also prepared the identical four peptides without the two specificity determinants by substituting the specificity determinants by Ala residues. The AMPs were tested against 14 different clinical isolates of *A. baumannii,* seven diverse clinical isolates from different sources and seven isolates resistant to colistin and polymyxin B (antibiotics of last resort to treat Gram-negative infections), six diverse strains of *P. aeruginosa* and 17 different Gram-positive strains of *Staphylococcus aureus*, both methicillin-sensitive (MSSA) and methicillin-resistant (MRSA) strains to determine a Gram-negative pathogen selectivity factor.

2 | METHODS AND MATERIALS

2.1 | Solid-phase peptide synthesis

Standard solid-phase peptide synthesis methodology using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry and Rink Amide-4-methylbenzhydrylamine hydrochloride resin (peptides D33-D36) or Rink Amide-ChemMatrix® resin (Biotage, Charlotte, NC) (peptides D37-D40) using a Focus-XC peptide synthesizer (Aapptec, Louisville, KY). The coupling procedure used (Benzotriazol-1-yloxy)tris(dimethylamino) phosphonium hexafluorophosphate (Bop)/ hydroxybenzotriazole (HOBt) in dimethylformamide (DMF) with N, N-diisopropylethylamine (DIPEA) in N-methyl-2-pyrrolidinone (NMP) with the first coupling at room temperature for one hour and the second coupling at 50°C for another hour. The deprotection procedure (removal of Fmoc protecting group) was carried out by treatment of the resin with 0.1 M HOBt in DMF with 20% piperidine. After completion of the synthesis, the peptide resin was dried under vacuum and the peptide was cleaved from the resin with a mixture of 90% trifluoroacetic acid (TFA), 5% water, and 5% triisopropylsilane (TIS) for 1–2 hr. The resin was removed by filtration, and peptide was precipitated with ice-cooled ethyl ether on ice for 1–2 hr. The pellet was spun down and

redissolved in acetonitrile/water (1:1, with 0.2% TFA) and the solution lyophilized to obtain the crude peptide.

2.2 | Analytical and preparative purification by reversed-phase chromatography

Analytical RP-HPLC: Column, Luna C18 (2), 250 × 4.6 mm I.D., 5 μm particle size, 100 Å pore size from Phenomenex. Run conditions: linear AB gradient (1% acetonitrile/min, starting from 2% acetonitrile) at a flow-rate of 1 ml/min, where eluent A is 0.2% aq. TFA and eluent B is 0.18% TFA in acetonitrile; temperature, 30°C. *Preparative RP-HPLC:* Peptides were dissolved in 0.2% aq. TFA containing 2% acetonitrile to a final concentration of 10 mg/ml. Following filtration through a 0.45-μm Millipore filter and subsequently through a 0.22-μm filter, the peptide solutions were loaded onto the column via multiple 20-ml injections into a 20-ml injection loop at a flow-rate of 5 ml/min. Column, Luna C18 (2), 250 × 30 mm I.D., 10 μm particle size, 100 Å pore size from Phenomenex. Run conditions: 2% acetonitrile/min gradient up to an acetonitrile concentration 15% below that required to elute the peptide during analytical RP-HPLC, then shallow gradient elution (0.1% acetonitrile/min) at a flow-rate of 10 ml/min (same eluents as shown above for analytical RP-HPLC); temperature, room temperature.

2.3 | Temperature profiling of peptides on reversed-phase HPLC

Purified peptides were analyzed on an Agilent 1200 series liquid chromatograph for temperature profiling using a Zorbax 300 SB-C8 column (150 mm × 2.1 mm I.D.; 5 μm particle size, 300 Å pore size) from Agilent Technologies. Conditions: linear AB gradient (0.5% acetonitrile/min) and a flow rate of 0.30 ml/min, where eluent A was 0.20% aqueous TFA, pH 2 and eluent B was 0.18% TFA in acetonitrile. Temperature profiling was carried out on two mixtures of peptides; mixture 1 consisted of peptides RC, D33, D35, D37, and D39 and mixture 2 consisted of RC, D34, D36, D38, and D40. Both mixtures were run at each temperature in 4°C increments from 5 to 77°C (19 different temperatures). Twenty minutes was allowed between runs for temperature equilibration. RC denotes a random coil peptide of 18 residues.

2.4 | Characterization of helical structure

The mean residue molar ellipticities of peptides were determined by circular dichroism (CD) spectroscopy, using a Jasco J-815 spectropolarimeter (Jasco Inc. Easton, MD, USA) at 5°C under KP buffer (50 mM NaH_2PO_4/Na_2HPO_4/100 mM KCl, pH 7.0) as well as in the presence of an α-helix inducing solvent, 2,2,2-trifluoroethanol, TFE, (50 mM NaH_2PO_4/Na_2HPO_4/100 mM KC1, pH 7.0 buffer/50% TFE). A 10-fold dilution of an approximately 500 μM 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.

2.5 | Determination of peptide amphipathicity

Amphipathicity of peptides at pH 7 and pH 2 was determined by the calculation of hydrophobic moment,[21] using the software package EMBOSS 6.5.7 and the Hmoment application, modified to include hydrophobicity scales determined in our laboratory.[22,23] The hydrophobicity scales used in this study are listed as follows: At pH 7, 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 (polar face), Lys, -18.48 (center of non-polar face). These hydrophobicity coefficients were determined from RP-HPLC at pH 7 (10 mM PO_4 buffer containing 50 mM NaCl) of a model random coil peptide with a single substitution of all 20 naturally occurring amino acids.[22] At pH 2, these coefficients were determined in 20 mM trifluoroacetic acid (TFA), Trp, 32.4; Phe, 29.1; Leu, 23.3; Ile, 21.4; Met, 15.7; Tyr, 14.7; Val, 13.4; Pro, 9.0; Cys, 7.6; Ala, 2.8; Glu, 2.8; Thr, 2.3; Asp, 1.6; Gln, 0.6; Ser, 0.0; Asn, -0.6; Gly, 0.0; Arg. 0.6; His, 0.0; Lys, 2.8 (polar face), Lys, -18.48 (center of non-polar face). These HPLC-derived scales reflect 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[23]). The hydrophobicity/hydrophilicity coefficients for Lys residues in the center of the non-polar face at pH 2.0 and pH 7.0 were assigned values of -18.48 determined by reversed-phase chromatography of the identical peptides where Ala was substituted by Lys on the non-polar face at position 13 and 16. Position X was placed in the sequence where these values are to be used in the Hmoment calculations when Lys is in the center of the non-polar face.

2.6 | Gram-negative bacteria strains used in this study

All the *A. baumannii* strains used in this study were (i) obtained from the collection of Dr. Anthony A. Campagnari at the University of Buffalo and originally isolated from different patients and organs/tissues (strain 649, blood; strain 689, groin; strain 759, gluteus; strain 884, axilla; strain 985, pleural fluid); (ii) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) (strain ATCC 17978, fatal meningitis; and strain ATCC 19606, urine); (iii) obtained from MERCK (M89941, M89949, M89951, M89952, M89953, M89955, and M89963). These

seven *A. baumannii* strains were resistant to Polymyxin B and Colistin.

2.7 | Gram-positive bacterial strains used in this study

All the *S. aureus* strains used in this study were (i) nine methicillin-sensitive *S. aureus* strains; M22315, M22274(Spine), M22300 (Finger), M22287(Hip), M22312(Finger), M22075(Axilla), M21913(Finger), BL7429(Blood), and M22097 (Neck) (ii) eight methicillin/Oxacillin-resistant *S. aureus* strains; M22424 (arm), M22111 (ear), M22360 (labia), M22354, M21756 (nose), M22130, M22224 (leg), M21742 (nose).

2.8 | Measurement of antimicrobial activity (MIC)

The minimal inhibitory concentration (MIC) is defined as the lowest peptide concentration that inhibited bacterial growth. MICs were measured by a standard microtiter dilution method in Mueller Hinton (MH) medium. Briefly, cells were grown overnight at 37°C in MH broth and were diluted in the same medium. Serial dilutions of the peptides were added to the microtiter plates in a volume of 50 μl, followed by the addition of 50 μl of bacteria to give a final inoculum of 5×10^5 colony-forming units (CFU)/ml. The plates were incubated at 37°C for 24 hr, and the MICs were determined.

2.9 | Measurement of hemolytic activity

Peptide samples (concentrations determined by amino acid analysis) were added to 1% human erythrocytes in phosphate-buffered saline (100 mM NaCl, 80 mM Na_2HPO_4, 20 mM NaH_2PO_4, pH 7.4), and the reaction mixtures were incubated at 37°C for 18 hr 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 ($800 \times g$). 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. As 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 is generally determined as the peptide concentration that causes 50% hemolysis of erythrocytes after 18 hr (HC_{50}). HC_{50} was determined from a plot of percent lysis *versus* peptide concentration (μM). With the peptides used in this study 50% hemolysis could not be reached, thus we used HC_{30} values, the peptide concentration that causes 30% hemolysis. Hemolysis data are determined at 12 different concentrations up to 1,000 μg/ml for 18 hr at 37°C. The average of three determinations is used with an average variance of less than 4%.

2.10 | Calculation of therapeutic index

The therapeutic index is a widely accepted parameter to represent the specificity of antimicrobial peptides for prokaryotic versus eukaryotic cells. It is calculated by the ratio of hemolytic activity and antimicrobial activity (MIC); thus, larger values of therapeutic index indicate greater specificity for prokaryotic cells. With the peptides used in this study, we used the HC_{30}/MIC ratio value to calculate the therapeutic index.

3 | RESULTS

3.1 | Peptide design, specificity determinants, and amphipathicity

As noted above, enantiomeric forms of AMPs with all-D-amino acids have shown equal activities to their all-L-enantiomers.[7–15] The advantage of all-D-peptides is that they are resistant to proteolytic enzyme degradation, which enhances their potential as therapeutic agents. In this study, we de novo designed, synthesized, purified, and characterized eight all-D amphipathic α-helical antimicrobial peptides, four without "specificity determinants" denoted D37, D38, D39, and D40 and four with "specificity determinants" denoted D33, D34, D35, and D36 (sequences shown in Table 1). Figure 1 shows the amino acid sequences in helical wheel and helical net representations. We have displayed two versions of the helical nets where the polar residues are displayed along the center of the helical net (top) and where the non-polar residues are displayed along the center of the helical net (bottom). Peptides D37, D38, D39, and D40 are all very amphipathic α-helical peptides with amphipathicity values ranging from 5.275 to 5.555 at pH 7 (Table 2). These four peptides have a net positive charge of +6 and vary from one another by the arrangement of the six positively charged Lys residues on the polar face (Figure 1). The hydrophobic/non-polar faces of these four peptides are identical (Figure 1). Peptides D33 to D36 contain two Lys residues ("specificity determinants") in the center of the non-polar face (colored pink) replacing Ala residues in D37 to D40. These two Lys residues dramatically change the amphipathicity of these peptides, as expected, due to locating two very hydrophilic and positively charged residues on the non-polar face. The amphipathicity of peptides D33, D34, D35, and D36 varies from 3.921 to 4.185 at pH 7 as shown in Table 2 and is dramatically different from the peptides without specificity determinants. These four peptides have a net positive charge of +8. Each of the following peptide pairs, D33 and D37,

TABLE 1 Peptides used in this study

Peptide name[a]	Length	Sequence[b] (1 … 13 16 … 26)	Mass
With specificity determinants			
D33	26	Ac-alksllktlskakaaklktllkalsk-amide	2,808.55
D34	26	Ac-alksllktlsaakkkklatllkalsk-amide	2,808.55
D35	26	Ac-alksllatlskakkkklktllaalsk-amide	2,808.55
D36	26	Ac-alasllktlskakkkklktllkalsa-amide	2,808.55
Without specificity determinants			
D37	26	Ac-alksllktlskaaaaalktllkalsk-amide	2,694.36
D38	26	Ac-alksllktlsaaakkalatllkalsk-amide	2,694.36
D39	26	Ac-alksllatlskaakkalktllaalsk-amide	2,694.36
D40	26	Ac-alasllktlskaakkalktllkalsa-amide	2,694.36
RC[c]	18	Ac-ELEKGGLEGEKGGKELEK-amide	1,971.18

[a]The D-denotes that all amino acid residues in each peptide are in the D-conformation.
[b]Peptide sequences are shown using the one-letter code for all amino acid residues and small letters to denote D-configuration; Ac denotes N^{α}-acetyl and amide denotes C^{α}-amide. Positions 13 and 16 are Lys residues for peptides D33, D34, D35, and D36 and are in the center of the non-polar face (see Figure 1) and are Ala residues for peptides D37, D38, D39, and D40.
[c]Peptide RC is used as a random-coil control for temperature profiling in reversed-phase chromatography to determine peptide self-association (Table 2).

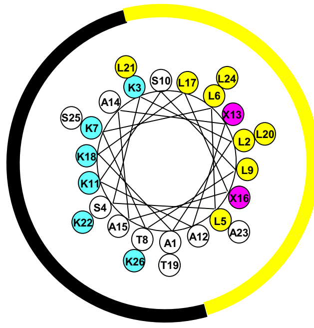
D34 and D38, D35 and D39, and D36 and D40 have identical polar faces, and the positions of the two Lys residues ("specificity determinants") are identical in D33, D34, D35, and D36 (positions 13 and 16 in the center of the non-polar face). The non-polar faces of these four peptides are identical. The polar faces are different and depend on the location of the six Lys residues. These representations in Figure 1 allow easy comparison of different analogs, and these sequence differences will be used to explain their biological and biophysical properties described below.

3.2 | Peptide hydrophobicity

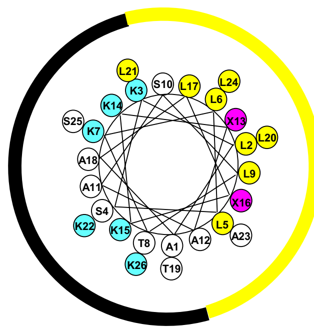
Retention behavior in RP-HPLC is an excellent method to represent overall peptide hydrophobicity. Retention times of peptides are highly sensitive to the conformational status of the peptides upon interaction with the hydrophobic environment of the column matrix.[17,24] The non-polar faces of amphipathic α-helical and amphipathic cyclic β-sheet peptides represent a preferred binding domain for interaction with the hydrophobic matrix of the reversed-phase column.[25] In this study, the observed peptide hydrophobicity was determined by RP-HPLC retention time as described in the methods section and are relative hydrophobicities because they are dependent on the TFA concentration and organic solvent in the mobile phase, gradient rate, temperature, flow rate, and the column used. The four parent antimicrobial peptides lacking specificity determinants, D37, D38, D39, and D40 have only hydrophobic residues on the non-polar face of the helix (8 Leu residues, colored yellow in two clusters (L2, L4, L6, and L9 in the N-terminal cluster and L17, L20, L21, and L24 in the C-terminal cluster and 5 Ala residues, Figure 1). Even though this hydrophobic surface on the non-polar face is the preferred binding domain, the overall hydrophobicity is also affected by the composition of residues on the polar face and their positions. The amino acid composition on the polar face is identical on all four peptides, and the difference between peptides is the location of the six positively charged Lys residues (Figure 1). Thus, overall hydrophobicity varied from 112.5 to 117.5 min (Table 2) showing that the subtle changes in location of positively charged residues on the polar face affect overall hydrophobicity.

The four AMPs with specificity determinants, D33, D34, D35, and D36, have two lysine residues in the center of the non-polar face (Lys 13 and Lys 16). The four AMPs without specificity determinants D37, D38, D39, and D40 have Ala residues at positions 13 and 16 instead of Lys residues. These two Lys residues increase the net positive charge on the peptides from +6 to +8 and dramatically affect the overall hydrophobicity (Table 2). Peptides D33 and D37, D34 and D38, D35 and D39, and D36 and D40 have identical polar faces. The differences in overall hydrophobicity between D33 and D37 are 35.3 min, between D34 and D38 are 35.3 min, between D35 and D39 are 38.3 min, and between D36 and D40 are 38.5 min. These peptide pairs have identical polar faces and differ by either two Lys residues or two Ala residues at positions 13 and 16 in the center of the non-polar face. Thus, the effect of the two specificity determinants on the non-polar face is extremely large (average of 36.85 min for the four peptide pairs compared to the positional effects of Lys residues on the polar face which give a range of 5.0 min for D37 to D40 and a range of 1.8 min for D33 to D36 (Table 2). This

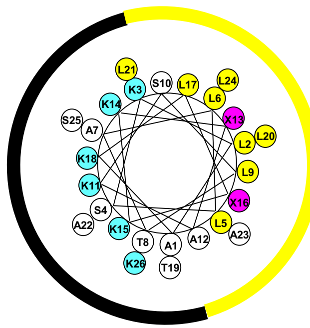
D33(X = K)/D37(X = A)



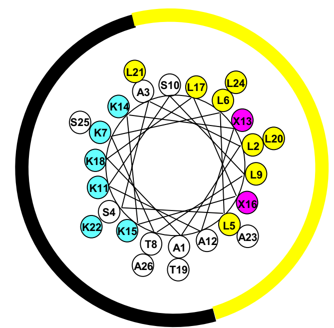
D34 (X = K)/D38(X = A)

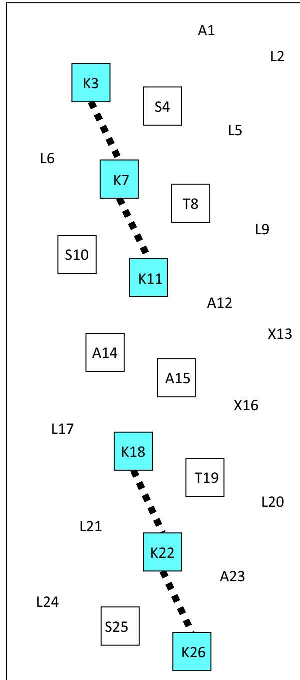


D35 (X = K)/D39(X = A)

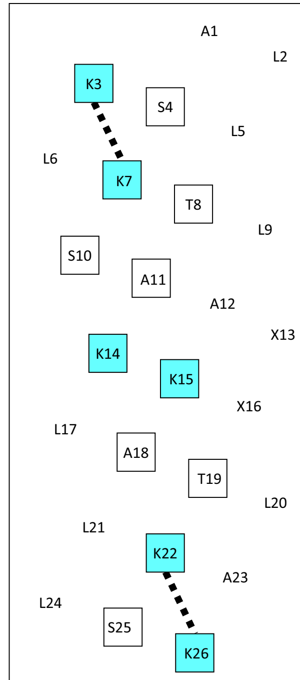


D36 (X = K)/D40(X = A)





Net charge: +8



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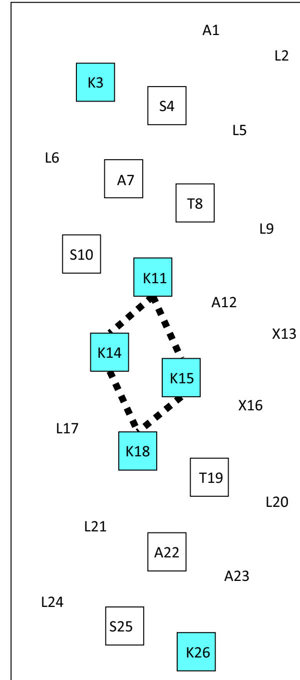

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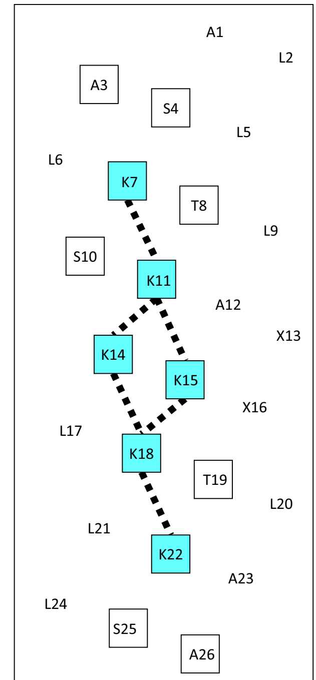

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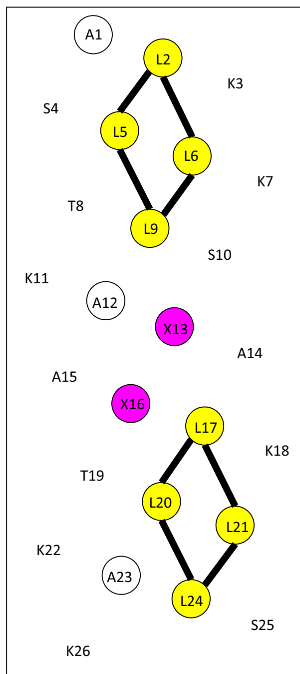

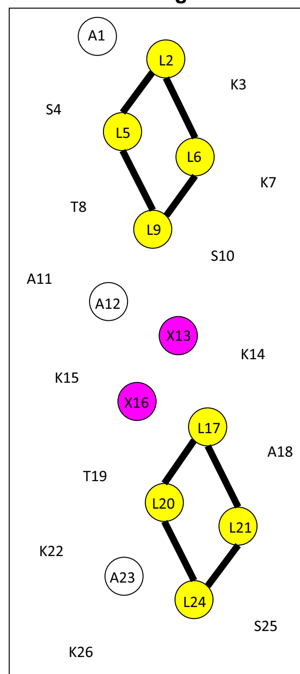

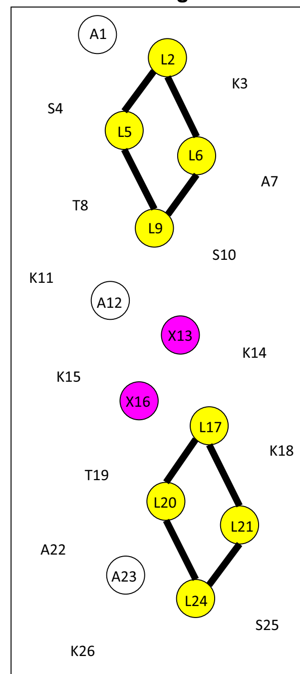

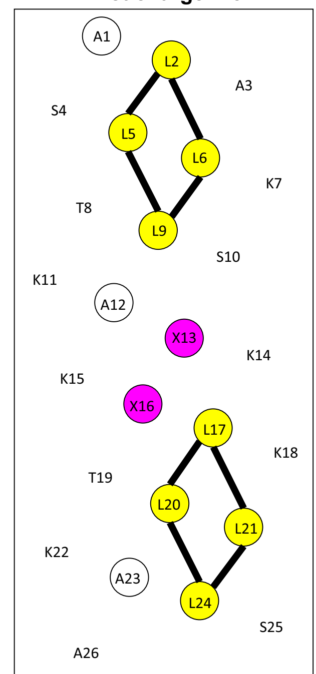

FIGURE 1 Helical wheel (upper panels) and helical net (lower panels) representations of our helical AMPs. In the helical wheels, the non-polar face is indicated as a yellow arc (Leu residues are colored yellow and position 13 and 16 are colored pink). The polar face is indicated as a black arc (Lys residues are colored blue). In the helical nets, the residues on the polar face are boxed (Lys residues are colored blue) and the residues on the non-polar face are circled (Leu residues are colored yellow). The location of the six positively charged Lys residues on the polar face is different between peptides D33, D34, D35, and D36 or between D37, D38, D39, and D40. The polar faces are identical for peptide D33 and D37, D34 and D38, D35 and D39, D36 and D40. Positions 13 and 16 on the non-polar faces are Lys residues (specificity determinants) in peptides D33, D34, D34, and D36 while positions 13 and 16 are Ala residues in peptides D37, D38, D39 and D40. The potential i to i + 3 or i to i + 4 electrostatic repulsions between positively charged residues are shown as black dotted lines. The i to i + 3 or i to i + 4 hydrophobic interactions between large hydrophobes are shown as solid black lines [Colour figure can be viewed at wileyonlinelibrary.com]

emphasizes that changes on the non-polar face are dramatically larger than changes on the polar face in affecting overall hydrophobicity as would be expected as the non-polar face is the preferred binding domain in RP-HPLC. The objective of this study was twofold: first, to determine the role of the six Lys residues and their positions on the polar face; and second, to determine the role of the two lysine residues on the non-polar face on the biophysical and biological properties of these AMPs.

3.3 | Peptide secondary structure

Table 2 shows the circular dichroism spectroscopy results for the eight peptide analogs used in this study in conditions of pH 7 (50 mM PO_4, 100 mM KCl) and in the presence of 50% trifluoroethanol (TFE), a mimic of the hydrophobicity and the α-helix inducing ability of the membrane (pH 7 buffer containing 50% TFE). The objective of substituting two Lys residues in the center of the non-polar face was to disrupt the continuous hydrophobic surface on the non-polar face which stabilizes the α-helical structure. Compare Figure 1 with Figure 2 where the two Lys residues disrupt the continuous hydrophobic surface along the helix on the non-polar face. The average molar ellipticity at 222 nm of peptides D37 to D40 in pH 7 conditions decreased from 20,513 to 7,600 for peptides D33 to D36 with the two Lys residues in the center of the non-polar face (Table 2). This shows that there is a dramatic decrease in helical content when the two specificity determinants are added from an average $[\theta]_{222}$ of 20,513 to 7,600 in pH 7 conditions or the average % helix changes from 54.8% to 20.3%. In the presence of the helical inducing solvent TFE, helical structure is induced in both series of peptides. Peptides D37 to D40 the average $[\theta]_{222}$ increases from 20,513 (average % helix 54.8) in pH 7 conditions to 33,588 (average % helix 89.7). Similarly, peptides D33 to D36 the average $[\theta]_{222}$ increases from 7,600 (average % helix 20.3) to 27,338 (average % helix 73.0) in 50% TFE and an increase in α-helical content of 53%. The specificity determinants dramatically decrease α-helicity in pH 7 conditions, but this helicity can be induced by increasing the hydrophobicity of the environment (50% TFE), a mimic of the hydrophobicity and helix inducing properties of the cell membrane. The inducible α-helix in the presence of 50% TFE increased from an average $\Delta[\theta]_{222}$ of 13,075 for peptides D37 to D40 to an average $\Delta[\theta]_{222}$ of 17,088 for peptides D33 to D36 which contain specificity determinants. Thus, the inducible α-helical structure is larger for peptides D33 to D36.

3.4 | Peptide self-association

Peptide self-association, the ability to oligomerize/dimerize in aqueous solution, is a very important parameter to optimize antimicrobial activity while removing toxicity. We assume that the monomeric random-coil antimicrobial peptides in aqueous solution are best suited to pass through a polysaccharide capsule, the outer membrane lipopolysaccharide and the cell wall peptidoglycan layer of microorganisms prior to penetration into the cytoplasmic membrane, induction of α-helical structure and disruption of membrane structure to kill target cells. On the other hand, if the self-association ability of an AMP in aqueous medium is too strong, stable folded oligomers/dimers through interaction of their non-polar faces are formed which decreases the ability of the AMP to dissociate to monomer and the dimer/oligomer to effectively pass through the capsule and cell wall to reach the cytoplasmic membrane. In this study, the ability of the AMPs to self-associate was determined by a technique developed in our laboratory, referred to as RP-HPLC temperature profiling at pH 2 over the temperature range of 5–80°C.

It is important to understand how the RP-HPLC temperature profiling method works. At low temperature, AMPs are capable of self-associating in aqueous solution via their non-polar faces. As shown in Figure 2, equilibrium is established between monomer and dimer and the concentration of monomer and dimer at any given temperature depends on the strength of the hydrophobic interactions between the two monomers to form the α-helical folded dimer. In RP-HPLC, the hydrophobicity of the matrix disrupts or dissociates the dimer and only the monomeric form of the peptide is bound to the hydrophobic matrix by its preferred binding domain (non-polar face). The monomeric form of the peptide can partition between the hydrophobic surface of the alkyl ligands on the reversed-phase matrix and the mobile phase. At low temperature, the monomer can dimerize in the mobile phase and the retention time is decreased due to the large population of dimers in solution. At higher temperatures, the

TABLE 2 Biophysical data

Peptide name	Net charge	Hydrophobicity t_R [a] (min)	KP Buffer $[\theta]_{222}$ [b]	KP Buffer %Helix[c]	50% TFE $[\theta]_{222}$ [b]	50% TFE %Helix[c]	$\Delta[\theta]_{222}$ TFE-benign	T_p [d] (°C)	P_A [e]	Amphipathicity[f] pH7	Amphipathicity[f] pH2
With specificity determinants											
D33	+8	77.4	5,900	15.8	22,000	58.7	16,100	33	5.3	4.061	3.327
D34	+8	77.2	8,800	23.5	26,500	70.8	17,100	33	5.7	3.921	3.327
D35	+8	78.3	10,000	26.7	34,750	92.8	14,750	33	5.5	3.970	3.327
D36	+8	79.0	5,700	15.2	26,100	69.7	20,400	33	5.6	4.185	3.327
Average		78.0	7,600	20.3	27,338	73.0	17,088	33	5.5	4.034	3.327
Without specificity determinants											
D37	+6	112.7	13,200	35.2	25,000	66.7	11,800	53	18.5	5.424	4.525
D38	+6	112.5	22,800	60.9	37,450	100.0	14,650	45	19.5	5.275	4.525
D39	+6	116.6	23,700	63.2	36,150	96.5	12,450	49	18.5	5.326	4.525
D40	+6	117.5	22,350	59.7	35,750	95.5	13,400	49	18.7	5.555	4.525
Average		114.8	20,513	54.8	33,588	89.7	13,075	49	18.8	5.395	4.525

[a] t_R denotes retention time in RP-HPLC at pH 2 at a temperature of 25°C and is a measure of overall peptide hydrophobicity.

[b] The mean residue molar ellipticities $[\theta]_{222}$ (deg cm^2/dmol) at a wavelength 222 nm were measured at 5°C in KP buffer (100 mM KCl, 50 mM Na_2HPO_4/NaH_2PO_4, pH 7.0) or in KP buffer containing 50% trifluoroethanol (TFE) by circular dichroism spectroscopy.

[c] The helical content (as a percentage) of a peptide relative to the molar ellipticity value of peptides D38 in the presence of 50% TFE.

[d] T_p temperature at which maximum retention time is observed over the temperature range 5–77°C during temperature profiling in RP-HPLC.

[e] P_A denotes the self-association parameter (dimerization/oligomerization) of each peptide during RP-HPLC temperature profiling, which is the maximal retention time difference of ($t_R^t - t_R^5$ for peptide analogs) – ($t_R^t - t_R^5$ for control peptide RC) within the temperature range; $t_R^t - t_R^5$ is the retention time difference of a peptide at a specific temperature (t_R^t) compared with that at 5°C (t_R^5). The sequence of the random coil peptide (RC) is shown in Table 1.

[f] Amphipathicity was determined by calculation of the hydrophobic moment[21] using hydrophobicity coefficients determined by RP-HPLC[22,23]; see section 2 for details.

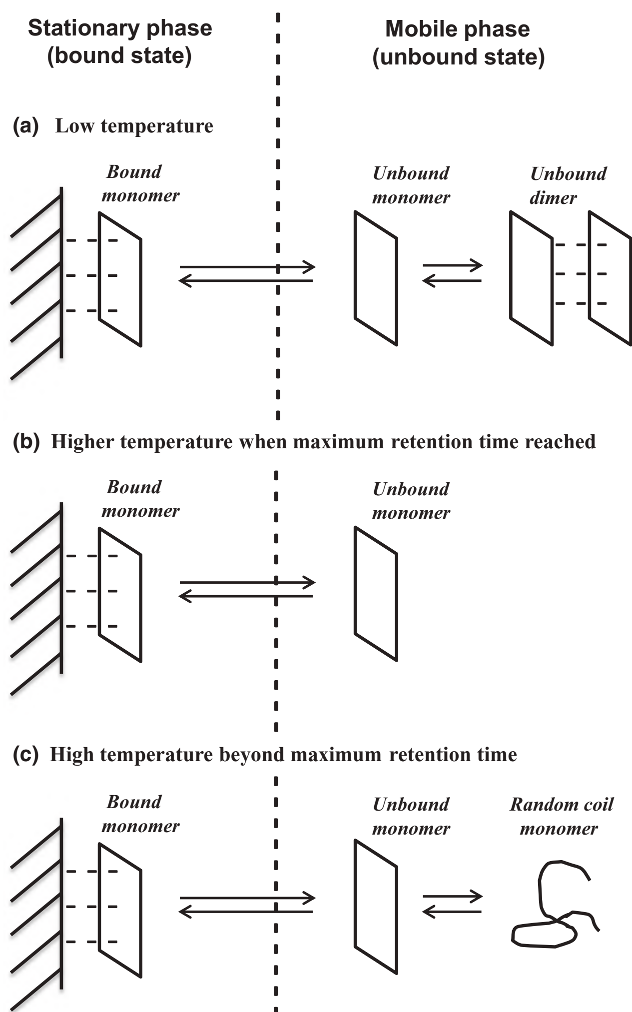

FIGURE 2 Proposed mechanism of temperature profiling by RP-HPLC of amphipathic α-helical antimicrobial peptides. Panel a, at low temperatures, peptides capable of self-association in aqueous solution by their non-polar faces establish an equilibrium during RP-HPLC between the bound helical monomer to the hydrophobic stationary phase, the helical monomer in the mobile phase and the helical dimer in the mobile phase during gradient elution. Panel b, at higher temperatures, the population of dimers in the mobile phase during partitioning decreases, increasing the concentration of the monomeric α-helical peptide which increases peptide retention time. Panel c, at temperatures beyond the point of maximum retention time the unbound helical peptide in the mobile phase, is in equilibrium with the random-coil conformation of the peptide and retention time decreases with further increasing temperature

population of dimers in the mobile phase during partitioning decreases, which increases the concentration of monomeric peptide in solution and thereby increases retention time. At some higher temperature, no dimer exits in the mobile phase and the peptide has its maximum retention time. At temperatures beyond the point of maximum retention time, the unbound helical peptide in the mobile phase is in equilibrium with the random-coil conformation of the peptide and retention time decreases with further increasing temperature. With the random coil control peptide that does not dimerize, the peptide binds to the stationary phase and partitions in the mobile phase as a monomer with undefined structure throughout the temperature range (5–80°C) (Figure 2).

This novel method to measure self-association of small cyclic β-sheet AMPs was first reported by Lee and co-workers in 2003[26] and is a key method in the design and optimization of amphipathic α-helical AMPs.[15,18,27–30] Figure 3 shows the retention behavior of four AMPs without specificity determinants (D37, D38, D39, and D40) and four AMPs with specificity determinants (D33, D34, D35, and D36) over the temperature range of 5–77°C in 4°C increments from 5°C. The eight AMPs are compared to a random-coil control peptide denoted RC. RC is a monomeric random-coil peptide in both aqueous and hydrophobic media and 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. This linear decrease in retention time with increasing temperature represents the general effects of temperature due to greater solute diffusivity and enhanced mass transfer between the stationary and mobile phase. The difference in retention time between the RC control peptide and the amphipathic α-helical antimicrobial peptides is a measure of peptide association. The association parameter, $P_{A,}$ is large for AMPs D37, D38, D39, and D40, ranging from 18.5 to 19.5 min. (Table 2) and is shown by the double headed arrows (Figure 3). The association parameter, $P_{A,}$ is dramatically smaller for the AMPs D33, D34, D35, and D36, which have two Lys residues in the center of the non-polar face (Lys 13 and Lys 16) and range from 5.3 to 5.7 min. (Table 2). Thus, the specificity determinants lower self-association, which is a desired property of effective AMPs. That is, effective AMPs have to have low self-association in aqueous medium in order to more easily pass through the capsule and cell wall to reach the cytoplasmic membrane where the AMPs must be able to be induced into α-helical structure by the hydrophobicity of the membrane. We have shown that when AMPs strongly associate by having a hydrophobic face that is too hydrophobic the AMPs are inactive.[27]

3.5 | Antibacterial activity

Table 3 shows a summary of the antibacterial activities against 14 different strains of *A. baumannii,* seven clinical isolates from different sources and seven different strains resistant to polymyxin B and colistin (antibiotics of last resort). The average of the four MIC_{GM}-values (GM, geometric mean) for peptides D37 to D40, which did not have specificity determinants, was 2.2 μM. In contrast, the average of the four MIC_{GM} values for peptides D33 to D36 with the two specificity determinants was 0.3 μM. Similarly, the average

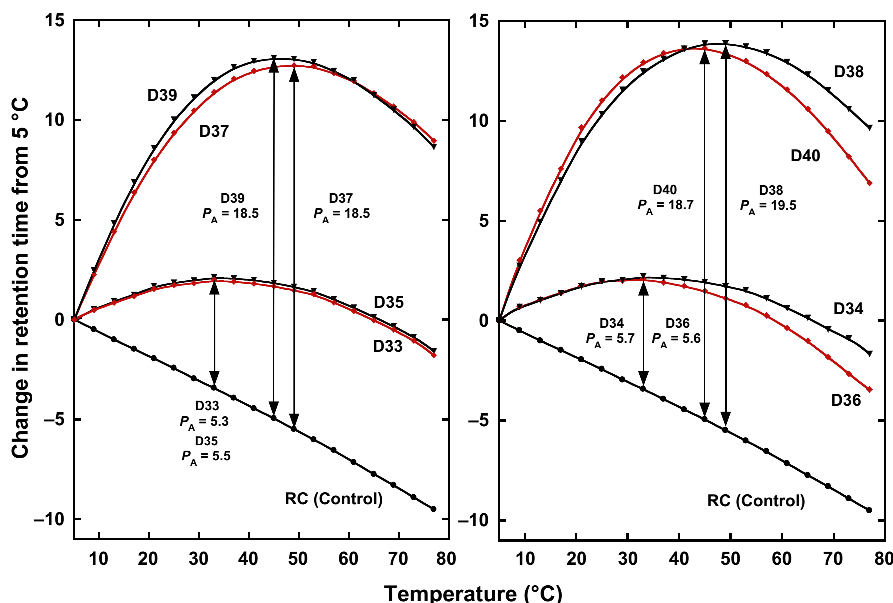

FIGURE 3 Self-association of α-helical antimicrobial peptides determined by temperature profiling in reversed-phase HPLC (RP-HPLC). Retention behavior from RP- HPLC of eight de novo designed amphipathic α-helical antimicrobial peptides (AMPs) after normalization to their retention times at 5°C over the temperature range 5–77°C. Four AMPs have 8 positively charged Lys residues (D33, D34, D35, D36), six positively charged Lys residues on the polar face and two Lys residues on the non-polar face, denoted "specificity determinants." The AMPs without "specificity determinants" have six positively charged residues on the polar face denoted D37, D38, D39, and D40. See Figure 1 for the helical wheel and helical net representations of these 8 AMPs. RC is a random coil control peptide used for RP-HPLC temperature profiling. The peptide self-association parameter, $\mathbf{P_A}$, represents the maximum change in peptide retention time relative to the random coil control peptide, (RC) and is denoted by the double headed arrow. Note the higher the $\mathbf{P_A}$ value, the greater the self-association [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 3 Summary of antimicrobial activity against *A. baumannii*, *P. aeruginosa,* and *S. aureus*

	MIC_{GM}(μM)[a]				
Peptide name	***A. baumannii*** **(7 clinical isolates)**	***A. baumannii*** **(7 resistant strains)**	***P. aeruginosa*** **(6 clinical isolates)**	***S. aureus*** **(8 MRSA)**	***S. aureus*** **(9 MSSA)**
With specificity determinants					
D33	0.1	0.3	1.8	10.8	19.0
D34	0.3	0.4	1.6	19.2	35.1
D35	0.4	0.4	0.9	14.0	28.0
D36	0.4	0.4	1.2	4.4	8.8
Average	0.3	0.38	1.4	12.1	22.7
Without specificity determinants					
D37	3.5	3.2	9.2	–	–
D38	1.2	1.2	36.8	12.3	10.7
D39	1.5	1.5	5.8	5.2	5.8
D40	2.6	3.5	5.2	8.4	8.5
Average	2.2	2.4	14.3	8.6	8.3

[a]MIC_{GM} is the geometric mean of the MIC values (MIC is minimal inhibitory concentration (μM) that inhibited growth of different strains in Mueller-Hinton (MH) medium at 37°C after 24 hr. MIC is given based on three sets of determinations) from seven different clinical isolates of *A. baumannii*, seven different strains of *A. baumannii* resistant to Polymyxin B and Colistin, antibiotics of last resort, six different clinical isolates of *P. aeruginosa*, eight different methicillin-resistant *S. aureus* (MRSA) strains, and nine different methicillin-sensitive *S. aureus* (MSSA) strains. The detailed MIC values of individual strains are shown in Tables 4–6. We did not have peptide D37 available for screening with *S. aureus*.

of four MIC_{GM} values for peptides D37 to D40 (no specificity determinants) for the *A. baumannii* resistant strains to polymyxin B and colistin was 2.4 µM. In contrast, the average of the MIC_{GM} values for peptides D33 to D36 with the two specificity determinants was 0.38 µM. These results show an approximate sevenfold increase in antimicrobial activity of the peptides with specificity determinants against these 14 different *A. baumannii* isolates compared to peptides lacking the specificity determinants. In comparing peptides that have identical polar faces, with and without specificity determinants, the fold increase in antimicrobial activity for the seven clinical isolates of *A. baumannii* and seven resistant strains of *A. baumannii* is as follows: D37/D33 (35-fold and 10-fold); D38/D34 (4-fold and 3-fold); D39/D35 (3.8-fold and 3.8-fold); D40/D36 (6.5-fold and 8.8-fold). These results clearly show the enhancement of antimicrobial activity by incorporating the two specificity determinants into these AMPs. Clearly, AMP D33 shows the greatest improvement in antimicrobial activity with specificity determinants of 35-fold for the seven clinical isolates from diverse *A. baumannii* clinical isolates and 10-fold for the 7 polymyxin B and colistin resistant strains. In the case of *P. aeruginosa* strains (Table 3), the antimicrobial activity (MIC_{GM} values) varied from 5.2 µM for D40 to 36.8 µM for D38. In comparing peptides that have identical polar faces with and without specificity determinants, the increase in antimicrobial activity (MIC_{GM}) was as follows: D37/D33 (5.1-fold), D38/D34 (23-fold), D39/D35 (6.4-fold), and D40/D36 (4.3-fold). Clearly AMP D34 shows the greatest improvement (23-fold) in antimicrobial activity with specificity determinants against *P. aeruginosa* (Table 3). Specificity determinants can make significant improvements in antimicrobial activity with the best peptides showing improvement in the MIC_{GM} values of 35-fold against *A. baumannii* strains and 23-fold against *P. aeruginosa* strains. We also screened the AMPs with and without specificity determinants against 17 *S. aureus* strains, nine methicillin-sensitive strains and eight methicillin-resistant strains (Table 3). The results show that specificity determinants have no advantage in improving antimicrobial activity against Gram-positive organisms represented by *S. aureus*. In fact, the MIC_{GM} values with specificity determinants are clearly higher than without specificity determinants. For example, if we compare peptides with identical polar faces, D38 and D34, there is a loss of antimicrobial activity from a MIC_{GM} value of 10.7 to 35.1 µM; D39 and D35 there is a loss of activity from 5.8 to 28 µM among the MSSA strains. Similarly, for the MRSA strains, with D38 and D34, there is a loss of activity from a MIC_{GM} value of 12.3 to 19.2 µM; for D39 and D35, there is a loss of activity from 5.2 to 14 µM. In general, there is a loss of antimicrobial activity when adding specificity determinants for activity against Gram-positive pathogens which is an advantage when designing Gram-negative selective AMPs. In addition, the activity against Gram-positive organisms represented by *S. aureus* strains is poor with and without specificity determinants (Table 3). The detailed results of all individual strains for *A. baumannii*, *P. aeruginosa,* and *S. aureus* are shown in Tables 4, 5, and 6, respectively.

3.6 | Hemolytic activity and therapeutic indices

The biological activities of the eight peptide analogs, with and without specificity determinants, are shown in Table 7. The four peptides without specificity determinants are extremely hemolytic with HC_{30} values (the peptide concentration required for 30% hemolysis) of 2.6 to 2.8 µM which is comparable with the antimicrobial activity of 1.2 to 3.5 µM. Thus, the therapeutic indices vary from 0.8 to 2.3 (Table 7). The specificity determinants enhance antimicrobial activity by threefold to 10.7-fold depending on the AMP (the average increase in antimicrobial activity for the four peptides D33 to D36 is 6.6-fold) (Table 7). The specificity determinants result in dramatic decreases in hemolytic activities from an average of 2.75 µM for AMPs lacking specificity determinants to 30 to 126 µM depending on the AMP. This corresponds to increases in the therapeutic indices from 39-fold (D35/D39) to 371-fold (D33/D37) (an average improvement of 185-fold for the four peptides with specificity determinants (Table 7). It is obvious that the improvements in the therapeutic indices depend on the location of the positively charged residues on the polar face, which varies between AMPs D33 to D36. The specificity determinants (two positively charged Lys residues at positions K13 and K16) are in identical positions in AMPs D33 to D36 (Figure 1). The positions of the six positively charged residues on the polar face are identical in each peptide pair D33 to D37, D34 to D38, D35 to D39, and D36 to D40, and the only difference is with and without specificity determinants. Depending on the location of the six positively charged residues on the polar face, there are large differences in therapeutic indices from 39-fold for pair D35 to D39, 137-fold for pair D34 to D38, 191-fold for pair D36 to D40, and 371-fold for peptide pair D33 to D37 (Table 7). We designed the location of the six positively charged residues on the polar face to systematically move closer to the center of the polar face as we move from AMP D33 to D36 (Figure 1). The improvement in the therapeutic index changes from a 371-fold improvement for peptide pair D33/D37 to 137-fold improvement for peptide pair D34/D38 to only a 39-fold improvement for peptide pair D35/D39. However, the results of peptide pair D36/D40 which has all six positively charged residues grouped closest to the center of the polar face does not support the trend of decreasing improvement in the therapeutic index as the charged residues are moved closer to the center of the polar face. Instead of an improvement less than 39-fold, we observe a 191-fold

TABLE 4 Antimicrobial activity of peptide analogs against (a) *A. baumannii* clinical isolates and (b) *A. baumannii* strains resistant to Polymyxin B and Colistin

(a)

Strain/source	ATCC 17978 Fatal Meningitis	ATCC 19606 Urine	649 Blood	689 Groin	759 Gluteus	884 Axilla	985 Pleural fluid	MIC_{GM} (μM)[b]
Peptide	**MIC (μM)[a]**							
With specificity determinants								
D33	0.1	0.2	0.1	0.1	0.2	0.1	0.2	0.1
D34	0.2	0.4	0.2	0.2	0.4	0.4	0.4	0.3
D35	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
D36	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
							Average	0.3
Without specificity determinants								
D37	2.9	5.8	2.9	2.9	5.8	2.9	2.9	3.5
D38	1.4	1.4	0.7	0.7	2.9	0.7	1.4	1.2
D39	2.9	2.9	1.4	1.4	1.4	0.7	0.7	1.5
D40	5.8	2.9	2.9	2.9	2.9	1.4	1.4	2.6
							Average	2.2

(b)

Strain	M89941	M89949	M89951	M89952	M89953	M89955	M89963	MIC_{GM} (μM)[b]
Peptide	**MIC (μM)[a]**							
With specificity determinants								
D33	0.2	0.2	0.2	0.7	0.4	0.4	0.2	0.3
D34	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
D35	0.4	0.4	0.4	0.7	0.4	0.4	0.4	0.4
D36	0.4	0.4	0.4	0.7	0.4	0.4	0.4	0.4
							Average	0.38
Without specificity determinants								
D37	2.9	2.9	2.9	2.9	2.9	2.9	5.8	3.2
D38	1.4	1.4	1.4	1.4	0.7	0.4	2.9	1.2
D39	2.9	1.4	0.7	2.9	1.4	0.7	1.4	1.5
D40	5.8	5.8	2.9	2.9	2.9	2.9	2.9	3.5
							Average	2.35
Colistin	>28	>28	>28	>28	>28	>28	>28	>28
PolymyxinB	>25	>25	>25	>25	>25	>25	>25	>25

[a]MIC is minimal inhibitory concentration (μM) that inhibited growth of different strains in Mueller-Hinton (MH) medium at 37°C after 24 hr. MIC is given based on three sets of determinations.

[b]MIC_{GM} is the geometric mean of the MIC values from seven different clinical isolates of *Acinetobacter baumannii* and seven different strains of *A. baumannii* resistant to Polymyxin B and Colistin, antibiotics of last resort.

improvement in the therapeutic index of peptide pair D36/D40. Thus, though there is no systematic trend, the results shown in Table 7 do show that the dramatic differences in the improvement of therapeutic index observed are dependent on the location of the positively charged residues on the polar face.

3.7 | Gram-negative pathogen selectivity

We have shown that the substitution of one or two specificity determinant(s) in broad spectrum native AMPs, Piscidin 1 and Dermaseptin S4 resulted in new AMPs that encode selectivity for Gram-negative pathogens and remove both

TABLE 5 Antimicrobial activity of peptide analogs against clinical isolates of *Pseudomonas aeruginosa*

Peptide	MIC(μM)[a]						
Strain	PAO1	PAK	PA14	CP204	M2	WR5	MIC_{GM} (μM)[b]
With specificity determinants							
D33	1.4	1.4	0.7	2.8	2.8	2.8	1.8
D34	1.4	1.4	0.7	2.8	2.8	1.4	1.6
D35	0.7	1.4	0.4	1.4	1.4	0.7	0.9
D36	1.4	1.4	0.7	1.4	1.4	1.4	1.2
Without specificity determinants							
D37	11.6	5.8	5.8	11.6	11.6	11.6	9.2
D38	23.2	11.6	2.9	>185.6	92.8	92.8	36.8
D39	5.8	2.9	2.9	23.2	5.8	5.8	5.8
D40	5.8	2.9	2.9	11.6	5.8	5.8	5.2

[a]MIC is minimal inhibitory concentration (μM) that inhibited growth of different strains in Mueller-Hinton (MH) medium at 37°C after 24 hr. MIC is given based on three sets of determinations.
[b]MIC_{GM} is the geometric mean of the MIC values from six different clinical isolates of *P. aeruginosa*.

Gram-positive activity and hemolytic activity from broad-spectrum AMPs.[18–20,27–29,31] The Gram-negative selectivity factor for D-Piscidin 1 (I9K) (one-specificity determinant) resulted in a selectivity factor (MIC_{GM}, *S. aureus*/MIC_{GM} *A. baumannii*) of 60 compared to D-Piscidin 1 with a selectivity factor of 1.1 (a 55-fold improvement in selectivity) and D-Dermaseptin S4 (L7K, A14K) (two-specificity determinants) resulted in a selectivity factor >319 compared 3.2 for D-Dermaseptin S4 (a > 99-fold improvement in *A. baumannii* compared to *S. aureus*).[31] These results suggested that amphipathic α-helical AMPs can be designed with selectivity for Gram-negative pathogens. As shown in Table 8, the antimicrobial activity against *A. baumannii* and *P. aeruginosa* is far superior to *S. aureus*. The Gram-negative selectivity factor ranges for the four peptides D33-D36 from 16 to 88 for *A. baumannii* and 5 to 22 for *P. aeruginosa*. AMP D34 has the largest discrimination between *A. baumannii* and *S. aureus* with a selectivity factor of 88 while AMP D35 has the largest discrimination between *P. aeruginosa* and *S. aureus* with a selectivity factor of 22 (Table 8). These results support the concept that Gram-negative selectivity can be controlled by the number and location of the positively charged residues on the polar face of the amphipathic α-helix, as well as by their relative position to the specificity determinants in the center of the non-polar face.

3.8 | Antimicrobial activity of AMPs in the presence of human sera

A critical component to the systemic use of AMPs to treat bacterial infections is the extent of AMP binding to serum proteins. In addition, as only the unbound AMP is available to interact with the therapeutic target, the extent of serum binding can have significant effects on efficacy. To address this issue, we determined the MIC values of our peptide candidates in the presence of Mueller Hinton (MH) medium and MH medium supplemented with human sera (25% v/v). This assay estimates the *in vivo* bioavailability of the AMPs. The appropriate non-specific affinity of a drug for serum proteins can significantly improve *in vivo* half-life and decrease clearance. An increase in MIC in serum is attributed to inhibition of antimicrobial activity due to serum protein binding. As shown in Table 9, the four AMPs without specificity determinants (D37, D38, D39, and D40) have no activity against *A. baumannii* in the presence of 25% human sera. In contrast, the four AMPs with specificity determinants (D33, D34, D35, and D36) have excellent activity against *A. baumannii*. There is only a 1.8 to sevenfold loss of antimicrobial activity depending on the AMP (Table 9) which is due to weak and non-specific binding to human serum proteins, a desired behavior. High affinity binding such as that observed for AMPs (D37 to D40) eliminates these AMPs for further study. These results show the importance of specificity determinants in maintaining weak and non-specific binding to serum proteins and preventing any significant loss of antimicrobial activity. It is also interesting that the specificity determinants enhance antimicrobial activity.

4 | DISCUSSION

The goal of this study was to expand our understanding of the role of positively charged residues in amphipathic α-helical antimicrobial peptides: first, the role of specificity determinants (Lys 13 and Lys 16) in the center of the non-polar face; second, the role of the six positively charged Lys residues and their location on the polar face; and third, the orientation of the Lys residues on the polar face relative to the Lys residues on the non-polar face. We examined the biophysical properties in terms of hydrophobicity, helicity, inducible helical structure,

TABLE 6 Antimicrobial activity of peptide analogs against methicillin-resistant and methicillin-sensitive *Staphylococcus aureus* (a: MRSA, b: MSSA) strains and (c) resistance profile of MRSA and MSSA strains

(a)

Strain	M22424	M22111	M22360	M22354	M21756	M22130	M22224	M21742	
Source	Arm	Ear	Labia	–	Nose	–	Leg	Nose	
Peptide	MIC (μM)[a]								MIC_{GM} (μM)[b]
With specificity determinants									
D33	13.9	13.9	1.4	22.3	11.1	11.1	22.3	11.1	10.8
D34	34.8	17.4	1.4	44.5	22.3	22.3	44.5	22.3	19.2
D35	13.9	27.8	0.7	44.5	22.3	22.3	22.3	11.1	14.0
D36	4.3	4.3	1.4	5.6	5.6	5.6	5.6	5.6	4.4
Without specificity determinants									
D38	14.5	14.5	2.9	23.2	23.2	11.6	11.6	11.6	12.3
D39	7.2	7.2	2.9	5.8	2.9	5.8	5.8	5.8	5.2
D40	9.1	9.1	5.8	11.6	11.6	5.8	11.6	5.8	8.4

(b)

Strain	M22315	M22274	M22300	M22287	M22312	M22075	M21913	BL7429	M22097	
Source	–	Spine	Finger	Hip	Finger	Axilla	Finger	Blood	Neck	
Peptide	MIC (μM)[a]									MIC_{GM} (μM)[b]
With specificity determinants										
D33	27.8	27.8	27.8	5.6	11.1	22.3	11.1	22.3	44.5	19.0
D34	34.8	34.8	34.8	11.1	22.3	44.5	44.5	44.5	89.0	35.1
D35	55.6	13.9	55.6	11.1	22.3	22.3	44.5	22.3	44.5	28.0
D36	4.3	17.4	17.4	5.6	5.6	11.1	5.6	11.1	11.1	8.8
Without specificity determinants										
D38	14.5	14.5	29.0	5.8	5.8	5.8	5.8	11.6	23.2	10.7
D39	7.2	7.2	7.2	5.8	5.8	5.8	5.8	2.9	5.8	5.8
D40	9.1	9.1	18.1	5.8	5.8	5.8	5.8	5.8	23.2	8.5

(Continues)

TABLE 6 (Continued)

(c)

Name	MRSA								MSSA								
	M22424	M22111	M22360	M22354	M21756	M22130	M22224	M21742	M22315	M22274	M22300	M22287	M22312	M22075	M21913	BL7429	M22097
Source	**Arm**	**Ear**	**Labia**	**–**	**Nose**	**–**	**Leg**	**Nose**	**–**	**Spine**	**Finger**	**Hip**	**Finger**	**Axilla**	**Finger**	**Blood**	**Neck**
Antibiotic susceptibility																	
Oxacillin	R[c]	R	R	R	R	R	R	R	S	S	S	S	S	S	S	S	S
Clindamycin	S[c]	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Erythromycin	R	R	R	R	S	R	R	R	S	S	S	–	S	S	S	S	S
Trimethoprim/sulfamethoxazole	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

[a]MIC is minimal inhibitory concentration (μM) that inhibited growth of different strains in Mueller-Hinton (MH) medium at 37°C after 24 hr. MIC is given based on three sets of determinations.
[b]MIC_{GM} is the geometric mean of the MIC values from eight different MRSA strains and nine different strains of MSSA.
[c]R denotes resistant, S denotes sensitive.

helix stability, and amphipathicity. In terms of biological activities, we determined the antimicrobial activity against Gram-negative pathogens, *A. baumannii* and *P. aeruginosa*, Gram-positive pathogens represented by *S. aureus* and the antimicrobial activity against *A. baumannii* in the presence of human sera. With the antimicrobial activities and hemolytic activity, we calculated the therapeutic indices and Gram-negative pathogen selectivity factors for the eight AMPs used in this study. The AMPs were designed in pairs, D33 and D37, D34 and D38, D35 and D39, and D36 and D40 where each pair has identical polar faces and non-polar faces with the only difference being the change of Ala 13 and Ala 16 in D37–D40 to Lys 13 and Lys 16 in D33–D36 (Figure 1). These two specificity determinants dramatically decreased overall hydrophobicity, helicity, helix stability, peptide association, and amphipathicity (Table 2), all desired properties to maintain the AMP in its monomeric unstructured form to allow easier access through the capsule and cell wall of microorganisms to reach the cytoplasmic membrane, the target of amphipathic AMPs. The biophysical studies (Table 2) showed that helicity was inducible in a hydrophobic environment of 50% TFE, a mimic of the hydrophobicity of the membrane. It is our hypothesis that once the peptide is in the membrane, it refolds into its α-helical structure and lies parallel to the membrane surface in prokaryotic cells (carpet model[32]) where its hydrophobic surface interacts with the hydrophobic lipid bilayer in the interface region and the polar and positively charged residues can interact with the head groups of the phospholipids.

The two groups of peptides, with specificity determinants (D33-D36) and without specificity determinants (D37-D40), were active against the 14 different strains of *A. baumannii* (Table 3) and six different strains of *P. aeruginosa* (Table 3) with a significant increase in antimicrobial activity with AMPs containing specificity determinants. As shown in Table 7, the four peptides without specificity determinants were extremely hemolytic with activities similar to their antimicrobial activity and thus extremely poor therapeutic indices (average therapeutic index of 1.45). In contrast, the four AMPs with specificity determinants have excellent therapeutic indices (average therapeutic index of 210) showing the importance of specificity determinants in removing eukaryotic cell toxicity while improving antimicrobial activity by an average of 6.6-fold. In addition, the location of the six positively charged residues on the polar face relative to the location of the two specificity determinants on the non-polar face has a significant impact on the therapeutic index varying from a low of 39-fold improvement to a high of 371-fold improvement in the therapeutic index (Table 7). The specificity determinants also affect Gram-negative pathogen selectivity. The selectivity factor between *A. baumannii* and *S. aureus* was 88 for AMP (D34) while AMP (D35) had the largest discrimination between *P. aeruginosa* and *S. aureus* with a selectivity factor of 22 (Table 8). Lastly, the specificity

TABLE 7 Biological activity of peptide analogs with and without specificity determinants against *A. baumannii* strains resistant to polymyxin B and colistin

Peptide name	Antimicrobial activity MIC_{GM} (μM)[a]	Hemolytic activity HC_{30} (μM)[b]	Therapeutic index HC_{30}/MIC_{GM}[c]
With specificity determinants			
D33	0.3	89	297
D34	0.4	126	315
D35	0.4	30	75
D36	0.4	61	153
Average	0.38	76.5	210
Without specificity determinants			
D37	3.2	2.6	0.8
D38	1.2	2.8	2.3
D39	1.5	2.8	1.9
D40	3.5	2.8	0.8
Average	2.35	2.75	1.45
Fold improvement for peptides with specificity determinants			
Antimicrobial activity		Therapeutic index	
D37/D33	3.2/0.3 = 10.7	D33/D37	297/0.8 = 371
D38/D34	1.2/0.4 = 3.0	D34/D38	315/2.3 = 137
D39/D35	1.5/0.4 = 3.8	D35/D39	75/1.9 = 39
D40/D36	3.5/0.4 = 8.8	D36/D40	153/0.8 = 191
Average	6.6	Average	185

[a]Antimicrobial activity (MIC) is the minimal inhibitory concentration of peptide that inhibits growth after 24 hr at 37°C. MIC_{GM} is the geometric mean of the MIC values from seven different *Acinetobacter baumannii* strains resistant to polymyxin B and colistin (Tables 3 and 4).
[b]HC_{30} is the concentration of peptide that results in 30% hemolysis after 18 hr at 37°C.
[c]Therapeutic index is the ratio of the HC_{30} value (μM) over the geometric mean MIC value (μM). Large values indicate greater antimicrobial specificity compared to human red blood cells.

TABLE 8 Summary of antimicrobial activity and Gram-negative pathogen selectivity

Peptide Name	Antimicrobial activity			Gram-negative selectivity factor[b]	
	A. baumannii MIC_{GM}(μM)[a]	*P. aeruginosa* MIC_{GM}(μM)[a]	*S. aureus* MIC_{GM}(μM)[a]	MIC_{GM} (*S. aureus*) / MIC_{GM} (*A. baumannii*)	MIC_{GM} (*S. aureus*) / MIC_{GM} (*P. aeruginosa*)
D33	0.2	1.8	14.6	73.0	8.1
D34	0.3	1.6	26.4	88.0	16.5
D35	0.4	0.9	20.2	50.5	22.4
D36	0.4	1.2	6.3	15.8	5.3

[a]MIC_{GM} is the geometric mean of the MIC values from 14 strains of *A. baumannii,* seven of which are resistant to polymyxin B and colistin (antibiotics of last resort to treat Gram-negative infections); six diverse clinical isolates of *P. aeruginosa* and 17 strains of *S. aureus*, eight of which are MRSA strains and nine of which are MSSA strains.
[b]The ratio of MIC_{GM} (*S. aureus*) versus MIC_{GM} (*A. baumannii*) or MIC_{GM} (*S. aureus*) versus MIC_{GM} (*P. aeruginosa*) indicates selectivity for Gram-negative versus Gram-positive bacteria where the larger the value, the greater the selectivity for *A. baumannii* or *P. aeruginosa* compared to *S. aureus.*

determinants have a dramatic effect on the antimicrobial activity in the presence of human sera. The peptides without specificity determinants (D37-D40) were inactive in the presence of 25% human serum, whereas AMPs with specificity determinants maintain excellent antimicrobial activity (Table 9). In summary, the key roles of specificity determinants when inserted into the non-polar face of amphipathic α-helical AMPs, whether de novo designed or native AMPs, is as follows: (i) dramatically reduce toxicity to normal cells as measured by hemolytic activity to human red blood cells. It is our hypothesis that reduced toxicity is a result of preventing transmembrane penetration in the bilayer to form channels or pores (barrel-stave model[33,34]); (ii) maintain or enhance antimicrobial activity which depends on the number and location of the positively charged residues on the polar face relative to the location of the specificity determinants on

TABLE 9 Antimicrobial activity against *A. baumannii* strain 649 in the presence and absence of 25% human sera

Peptide Name	MIC (μM)	
	No serum	25% human serum
With specificity determinants		
D33	0.1	0.7
D34	0.2	1.4
D35	0.4	1.4
D36	0.4	0.7
Without specificity determinants		
D37	2.9	>92.8
D38	0.7	>92.8
D39	1.4	>92.8
D40	2.9	>92.8

the non-polar face; (iii) encode selectivity for Gram-negative pathogens by significantly decreasing activity against Gram-positive bacteria; (iv) specificity determinants maintain excellent antimicrobial activity in the presence of human serum by preventing high affinity binding to serum proteins through the non-polar face of the amphipathic α-helix.

One of the important characteristics that affects the biological properties of amphipathic α-helical AMPs is the number of positively charged residues on the polar face and the net charge.[30] In this study, we wanted to maintain the identical non-polar face of a previously de novo designed AMP, D16[17] and design four different versions of the polar face where we maintained a + 6 charge (Figure 1). This would allow us to determine how the location of the positively charged residues on the polar face affected antimicrobial activity, hemolytic activity, and pathogen selectivity. These results clearly show that location of the positively charged residues on the polar face has very little effect on antimicrobial activity against *A. baumannii* and *P. aeruginosa* (Table 3).

D16 had a geometric mean MIC of 0.4 μM,[17] whereas our four AMPs on the same *A. baumannii* strains ranged from 0.1 to 0.4 μM for D33 to D36 (Table 3). Similarly D33 to D36 the geometric mean MIC values ranged from 0.3 to 0.4 μM against 7 *A. baumannii* strains resistant to Polymyxin B and Colistin (Table 3), whereas D16 had a value of 0.7 μM. Thus, the dramatic increase in charge from +8 for the AMPs in this study to +11 for D16 had no significant effect on antimicrobial activity against *A. baumannii*. Similarly, antimicrobial activity against the identical strains of *P. aeruginosa* D33 to D36 ranged from 0.9 to 1.8 μM (Table 3), whereas D16 had a geometric mean value of 1.5 μM.[17] These results clearly show that changing the net charge from +8 (this study) to +11 for D16 has no significant effect on antimicrobial activity. However, this study has demonstrated that changing the location of charged residues on the polar face has significant effects on hemolytic activity which changes by a factor of 4 from 30 μM for D35 to 126 μM for D34 (Table 7). Changing the hemolytic activity also affects the therapeutic index which changes from a low of 75 for D35 to a high of 315 for D34 which remains a factor of 4 since the antimicrobial activity was identical for D34 and D35 (Table 7). Also significant effects were observed on the Gram-negative selectivity factor between *A. baumannii* and *S. aureus* which ranged from 15.8 for D36 to 88 for D34 and for *P. aeruginosa* and *S. aureus* ranged from 5.3 for D36 to 22.4 for D35 (Table 8).

ACKNOWLEDGMENTS

We thank the Biophysics Core Facility at the University of Colorado, School of Medicine, for the use of the Circular Dichroism instrumentation and for Shaun Bevers, Facility Manager of the Biophysics Core, for performing quantitative amino acid analyses of our AMPs. We thank David Farrell for modifying the Hmoment application from the EMBOSS 6.5.7 package with our hydrophobicity/hydrophilicity coefficients at pH 2 and pH 7. We thank MERCK (Christopher Tan and Tomi Sawyer) for providing the *A. baumannii,* polymyxin B, and colistin resistant strains. We acknowledge the John Stewart Endowed Chair in Peptide Chemistry to Robert S. Hodges for providing the financial support for this project.

AUTHOR CONTRIBUTIONS

Robert S. Hodges planned and organized the study including peptide design and was responsible for the manuscript writing. Ziqing Jiang was involved in the synthesis and purification of the peptides, antimicrobial and hemolytic activity assays, secondary structure determination using circular dichroism spectroscopy and manuscript review. Michael Vasil provided the vast majority of the bacteria strains used in this study, microbiology expertise, and manuscript review. Colin T. Mant was responsible for the analytical reversed-phase chromatography including our temperature profiling studies over the temperature range of 5°–77° and manuscript review. Grant sponsors: John Stewart Endowed Chair in Peptide Chemistry to R.S. Hodges.

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How to cite this article: Jiang Z, Mant CT, Vasil M, Hodges RS. Role of positively charged residues on the polar and non-polar faces of amphipathic α-helical antimicrobial peptides on specificity and selectivity for Gram-negative pathogens. *Chem Biol Drug Des*. 2018;91:75–92. https://doi.org/10.1111/cbdd.13058