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Design of New Antimicrobial Peptides (AMPs) with "Specificity Determinants" that Encode Selectivity for Gram-negative Pathogens and Remove both Gram-positive Activity and Hemolytic Activity from Broad-spectrum AMPs

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Introduction

Antimicrobial peptides (AMPs) are widely distributed in nature, generally have broad-spectrum activity and represent a promising class of new antimicrobial agents. However, it is widely accepted that native AMPs lack specificity and may be too toxic (ability to lyse mammalian cells, normally expressed as hemolytic activity against human red blood cells) to be used for systemic treatment [1,2]. To overcome this problem, we developed the design concept of "specificity determinants" which refers to substituting positively charged residue(s) in the center of the non-polar face of amphipathic cyclic β-sheet [3,4] or amphipathic α-helical AMPs [5] to create selectivity between eukaryotic and prokaryotic membranes; that is, antimicrobial activity is improved or maintained and hemolytic activity or cell toxicity to mammalian cells is decreased or eliminated. We showed that a single valine to lysine substitution in the center of the non-polar face of an AMP dramatically reduced toxicity and increased the therapeutic index [5-7].

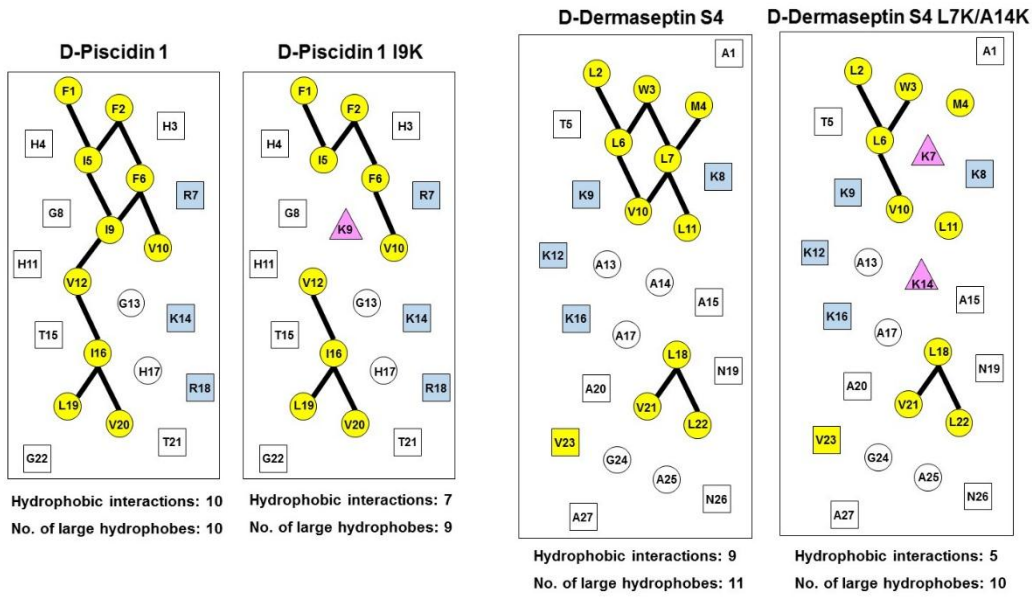
The question arose could we take such a broad spectrum AMP in the all-D conformation and use a rational design approach to enhance further the biological properties if the focus was to develop a better Gram-negative AMP rather than maintain broad-spectrum activity. Our final AMP had a 746-fold improvement (i.e., decrease) in its hemolytic activity, improved antimicrobial activity and improved therapeutic indices by 1305-fold and 895-fold against *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, respectively [7]. We applied this design concept to native AMPs, piscidin 1 (isolated from mast cells of hybrid striped bass - *Morone saxatilis* male × *Morone chrysops* female) and dermaseptin S4 (isolated from the skin of tree-dwelling, South American frogs of the *Phyllomedusa* species), where substitution of one or two lysine residues at different positions in their non-polar faces enhanced or maintained Gram-negative activity, dramatically decreased hemolytic activity and significantly improved the therapeutic indices (55-fold and 730-fold for D-piscidin 1 I9K and D-dermaseptin S4 L7K, A14K against *A. baumannii*, respectively) [8].

In the current study, we used the above 2 native AMPs and tested their activity against 2 different pathogens: 11 and 20 diverse clinical isolates of *A. baumannii*, and *Staphylococcus aureus* (12 Methicillin-sensitive *S. aureus* strains and 8 Methicillin/Oxacillin-resistant *S. aureus* strains), respectively. We showed that substitution of "specificity determinant(s)" in broad spectrum AMPs, encode selectivity for Gram-negative pathogens and simultaneously remove both Gram-positive activity and hemolytic activity of these 2 diverse amphipathic α-helical AMPs which differ dramatically in amino acid composition, net positive charge and amphipathicity, showing generality of our approach.

Results and Discussion

The peptide analogs (D-piscidin 1, D- piscidin 1 I9K, D-dermaseptin S4 and D-dermaseptin S4 L7K, A14K) (Figure 1) were synthesized by standard solid-phase peptide synthesis methodology using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry on Rink amide 4-methylbenzhydrylamine resin using a CEM Liberty microwave peptide synthesizer and using Boc-chemistry on 4-methylbenzhydrylamine (MBHA) solid support. Peptides were cleaved from the resins by TFA cleavage cocktail for Fmoc chemistry or by HF for Boc chemistry. Peptide purification was performed by reversed-phase high-performance liquid chromatography (RP-HPLC) on a Zorbax 300 SB-C_8 column (250 × 9.4 mm I.D.; 6.5 μm particle size, 300 Å pore size; Agilent Technologies, Little Falls, DE, USA). The minimal inhibitory concentration (MIC) was measured by a standard microtiter two-fold serial dilution method

in Mueller Hinton (MH) medium after incubation at 37°C for 24h. The hemolytic activity was determined as the peptide concentration that caused 50% hemolysis (compared to human erythrocytes treated with water or 0.1% Triton-X 100 as 100% lysis) of erythrocytes after 18h (HC_{50}).



Peptide Name	Length	Sequence	MW
D-Piscidin 1	22	NH_2-FFHHIFRGIVHVGKTIHRLVTG-amide	2571
D-Piscidin 1 I9K	22	NH_2-FFHHIFRG**K**VHVGKTIHRLVTG-amide	2586
D-Dermaseptin S4	27	NH_2-ALWMTLLKKVLKAAAKALNAVLVGANA-amide	2778
D-Dermaseptin S4 L7K, A14K	27	NH_2-ALWMTL**K**KKVLKA**K**AKALNAVLVGANA-amide	2851

Fig. 1. Helical net representation of D-piscidin 1 and D-dermaseptin S4 analogs. The one-letter code is used for amino acid residues. D denotes that all residues in the peptides are in the D-conformation. Positively charged residues (Lys and Arg) are colored light blue, large hydrophobic residues (Val, Ile, Leu, Phe, Met and Trp) are colored yellow. The "specificity determinant(s)" are denoted by pink triangles. The residues on the polar face are boxed and the residues on the non-polar face are circled. The i→i+3 and i→i+4 potential hydrophobic interactions along the helix are shown as black bars. D-dermaseptin S4 (27-mer) reported here involved a deletion of Ala18 from the 28-mer sequence reported in the literature [9]. This deletion results in 10 out of 11 large hydrophobes being located on the non-polar face with only Val23 being on the polar-face as shown above.

Antibacterial activities against 11 strains of *A. baumannii* and 20 strains of *S. aureus* are compared in Tables 1 and 2, respectively. Geometric mean of MIC values was calculated to provide an overall view of antibacterial activity of different analogs. It is clear that all four of our peptides were effective in killing Gram-negative pathogen *A. baumannii*. The single specificity determinant analog (D-piscidin 1 I9K) and D-dermaseptin S4 L7K, A14K with two specificity determinants exhibited similar or improved antibacterial activity compared to their parent peptides: D-piscidin 1 and D-dermaseptin S4 (Table 1). However, the new analogs have a dramatic decrease in antibacterial activity against Gram-positive pathogen *S. aureus* (Table 2).

As summarized in Table 3, a single "specificity determinant" had a dramatic effect in lowering the hemolytic activity of D-piscidin 1 from a HC_{50} value of 1.8 μM to 98 μM for D-piscidin 1 I9K, a 54-fold improvement (Table 3). In addition, two "specificity determinants" in D-dermaseptin S4 gave the analog D-dermaseptin S4 L7K, A14K which decreased hemolytic activity from 0.6 μM to a HC_{50} value of 241 μM, a 402-fold improvement in hemolytic activity (Table 3).

Table 1. Peptide antimicrobial activity (MIC[a] (μM)) against Gram-negative A. baumannii clinical isolates.

Strain	Source	Peptide Name			
		D-Piscidin 1	D-Piscidin 1 I9K	D-Dermaseptin S4	D-Dermaseptin S4 L7K,A14K
ATCC 17978	Fatal meningitis	3.0	3.0	2.8	0.7
ATCC 19606	Urine	3.0	1.5	2.8	0.7
649	Blood	3.0	3.0	1.4	0.7
689	Groin	1.5	3.0	1.4	0.7
759	Gluteus	3.0	3.0	1.4	1.4
821	Urine	3.0	3.0	2.8	0.7
884	Axilla	3.0	3.0	2.8	1.4
899	Perineum	3.0	3.0	1.4	1.4
964	Throat	3.0	3.0	1.4	2.7
985	Pleural fluid	1.5	3.0	0.7	0.7
1012	Sputum	6.1	6.0	2.8	2.7
GM[b]		2.8	3.0	1.8	1.1

[a]MIC is minimal inhibitory concentration (μM) that inhibited growth of different strains in Mueller-Hinton (MH) medium at 37°C after 24h; MIC is given based on three sets of determinations; [b]GM is the geometric mean of the MIC values from 11 different isolates of A. baumannii.

Table 2. Peptide antimicrobial activity (MIC[a] (μM)) against Gram-positive S. aureus clinical isolates.

Strain	Source	Peptide Name			
		D-Piscidin 1	D-Piscidin 1 I9K	D-Dermaseptin S4	D-Dermaseptin S4 L7K,A14K
M22315		3.0	773.4	11.3	>350.8
M22274	Spine	3.0	773.4	11.3	>350.8
M22300	Finger	3.0	773.4	5.6	>350.8
M22287	Hip	3.0	773.4	5.6	>350.8
M22312	Finger	6.1	773.4	11.3	>350.8
M21935	Resp.	3.0	386.7	5.6	>350.8
M22111	Ear	1.5	3.0	5.6	87.7
M22075	Axilla	3.0	386.7	2.8	>350.8
M21913	Finger	3.0	193.3	5.6	>350.8
BL7429	Blood	3.0	193.3	2.8	>350.8
M22097	Neck	6.1	193.3	5.6	>350.8
M21991	Resp.	6.1	193.3	5.6	>350.8
M22424	Arm	3.0	773.4	11.3	>350.8
M22111	Ear	3.0	96.7	5.6	>350.8
M22360	Labia	1.5	3.0	5.6	>350.8
M22354		3.0	773.4	5.6	>350.8
M21756	Nose	3.0	386.7	2.8	>350.8
M22130		3.0	12.1	5.6	>350.8
M22224	Leg	3.0	96.7	5.6	>350.8
M21742	Nose	3.0	96.7	5.6	>350.8
GM[b]		3.1	180	5.8	Inactive

[a]MIC is minimal inhibitory concentration (μM) that inhibited growth of different strains in Mueller-Hinton (MH) medium at 37°C after 24h; MIC is given based on three sets of determinations; [b]GM is the geometric mean of the MIC values from 12 different isolates of Methicillin-sensitive S. aureus (from strain M22315 to M21991) or 8 different isolates of Methicillin/Oxacillin-resistant S. aureus (from strain M22424 to M21742).

The corresponding therapeutic indices when comparing hemolytic activity and antimicrobial activity against *A. baumannii* showed a dramatic improvement of 55-fold and 730-fold, respectively (Table 3).

However, the antibacterial activity against the Gram-positive pathogen *S. aureus* was lost. The new peptide analogs turned into Gram-negative pathogen-selective AMPs with a Gram-negative selectivity factor of 60 for D-piscidin 1 I9K and >319 for D-dermaseptin S4 L7K, A14K (Table 3).

Table 3. Summary of biological activity.

Peptide Name	*Hemolytic activity*		*Antimicrobial activity*				*Gram-negative selectivity factor*[f]
			A. baumannii			*S. aureus*	$\frac{MIC_{GM}\ (S.\ aureus)}{MIC_{GM}\ (A.\ baumannii)}$
	HC_{50}[a] *(μM)*	*Fold*[b]	MIC_{GM}[c] *(μM)*	*T. I.*[d]	*Fold*[e]	MIC_{GM}[c] *(μM)*	
D-Piscidin 1	1.8	1.0	2.8	0.6	1.0	3.1	1.1
D-Piscidin 1 I9K	98	**54**	3.0	33	**55**	180	60
D-Dermaseptin S4	0.6	1.0	1.8	0.3	1.0	5.8	3.2
D-Dermaseptin S4 L7K,A14K	241	**402**	1.1	219	**730**	>351[g]	>319

[a]*HC_{50} is the concentration of peptide (μM) that results in 50% hemolysis after 18h at 37°C;* [b]*The fold improvement in HC_{50} compared to that of native D-Piscidin 1, D-Dermaseptin S4 are bolded;* [c]*MIC is the minimum inhibitory concentration (μM) of peptide that inhibits growth of bacteria after 24h at 37°C, MIC_{GM} is the geometric mean of the MIC values from 11 different isolates of A. baumannii or 20 different isolates of S. aureus;* [d]*"T.I." denotes therapeutic index, which is the ratio of the HC_{50} value (μM) over the geometric mean MIC value (μM), where large values indicate greater antimicrobial specificity;* [e]*The fold improvement in therapeutic index compared to that of native D-Piscidin 1, D-Dermaseptin S4 are bolded;* [f]*The ratio of the MIC_{GM} (S. aureus) versus MIC_{GM} (A. baumannii) indicates selectivity for Gram-negative versus Gram-positive bacteria, where the larger the value, the greater the selectivity for A. baumannii;* [g]*Inactive against S. aureus.*

In summary, we have taken two examples of native AMPs, piscidin 1 and dermaseptin S4, to demonstrate the universality of our "specificity determinant" design concept to effect a dramatic reduction in AMP hemolytic activity and antibacterial activity against the Gram-positive pathogen *S. aureus*, while maintaining or improving antibacterial activity against Gram-negative pathogen *A. baumannii* to successfully generate Gram-negative pathogen-selective AMPs as potential drug candidates. To us, the excitement in the field of amphipathic α-helical AMPs lies in our demonstration that a single or double substitution in nature's AMP sequences can have such a dramatic effect on changing their biological profiles for drug development.

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