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Easy Strategy To Increase Salt Resistance of Antimicrobial Peptides▽

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The efficacies of many antimicrobial peptides are greatly reduced under high salt concentrations, limiting their development as pharmaceutical compounds. Here, we describe an easy strategy to increase salt resistance of antimicrobial peptides by replacing tryptophan or histidine residues with the bulky amino acids β-naphthylalanine and β-(4,4′-biphenyl)alanine. The activities of the salt-sensitive peptide P-113 were diminished at high salt concentrations, whereas the activities of its β-naphthylalanine and β-(4,4′-biphenyl)alanine-substituted variant were less affected.

Antimicrobial peptides play important roles in the host innate defense mechanism by interacting and permeabilizing microbial membranes (21). With an increase of antibiotic resistance, the potential for the development of antimicrobial peptides as novel therapeutic agents could overcome the problem of resistance (5).

The development of antimicrobial peptides has been hindered by several problems. One of these problems is salt sensitivity (13). The efficacy of human β-defensin-1 is greatly reduced in the presence of high salt concentrations in bronchopulmonary fluids in cystic fibrosis patients (4). Similar problems were observed in the clinically active histidine-rich peptide P-113, indolicidins, gramicidins, bactenecins, and magainins (12, 14, 17, 20). A number of studies have been reported on the design of salt-resistant antimicrobial peptides. However, most of them were focused on overall structure modifications, such as structure rigidity, helix stability, hydrophobicity, and amphipathicity (2, 3, 6, 12, 13, 15, 16).

Previously, we found that a novel Trp-rich peptide, Ac-KWRRWVRWI-NH_2, designated Pac-525, demonstrated improved activity against both bacteria and fungi and showed reduced hemolytic activity (18). Nal-Pac-525, with all of its tryptophans replaced by the bulky amino acid β-naphthylalanine (7), was shown to have a higher antimicrobial activity than Pac-525 (19). More importantly, unlike Pac-525, the antifungal activity of Nal-Pac-525 against fluconazole-resistant fungal pathogens was not blocked by high-salt incubation conditions (17). However, Nal-Pac-525 has a high hemolytic activity that prohibits its further clinical application (Fig. 1). Thus, the advantages and disadvantages of bulky amino acid substitution to salt-resistant antimicrobial peptides remain to be elucidated. P-113 is a 12-amino-acid histidine-rich peptide derived from the saliva protein histatin 5. The anti-*Candida* activity of P-113 has been documented previously (14). Recently, a clinical trial involving HIV patients showed that P-113 has a good outcome for oral candidiasis therapy, but the activity of P-113 is restricted to a low-salt condition, limiting its application (8).

Based on the studies of Pac-525 and Nal-Pac-525, it is hypothesized that the substitution of tryptophan residues by the bulky amino acid β-naphthylalanine may generate a potent peptide with improved antimicrobial activity and salt resistance. β-Naphthylalanine residues may position themselves deeper into the bacterial and fungal cell membranes, making the peptide more efficient in disrupting the membranes, hence compensating the competition from the cations to the negatively charged microbial cell surface. However, the hemolytic activity of Nal-Pac-525 increases dramatically with the β-naphthylalanine substitutions (Fig. 1). These observations lead to the hypothesis that the replacement of the aromatic residues with bulky aromatic amino acids of salt-sensitive and low-hemolytic antimicrobial peptides may result in salt-resistant peptides with slight increases of their hemolytic activities.

The histatin derivative P-113, AKRHHGYKRKFH-NH_2, was

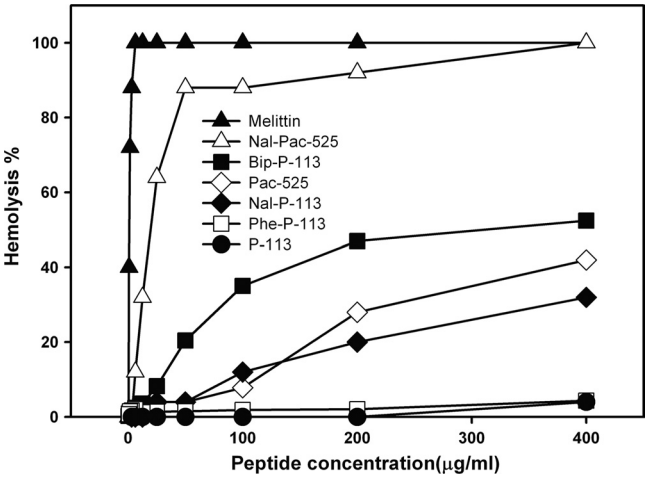

FIG. 1. Hemolytic activities of P-113 (●), Phe-P-113 (□), Nal-P-113 (◆), Bip-P-113 (■), Pac-525 (◇), and Nal-Pac-525 (△). Melittin (▲) was used as a control.

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TABLE 2. MIC values for ampicillin, P113, Phe-P-113, Nal-P-113, and Bip-P-113 in different pH conditions and concentrations of $MgCl_2$

Species	MIC (μg/ml) in LYM broth[a]																											
	Control					$MgCl_2$ concn (mM)												pH condition											
						0.5				1.5				2.5				6.5				5.5				4.5			
	AP	P-113	Phe	Nal	Bip	P-113	Phe	Nal	Bip	P-113	Phe	Nal	Bip	P-113	Phe	Nal	Bip	P-113	Phe	Nal	Bip	P-113	Phe	Nal	Bip	P-113	Phe	Nal	Bip
E. coli ATCC 25922	25	12.5	12.5	3.1	6.25	12.5	25	3.1	6.25	25	50	6.25	6.25	25	>50	12.5	6.25	12.5	50	6.25	3.1	12.5	>50	12.5	12.5	>50	>50	12.5	12.5
S. aureus ATCC 25923	12.5	12.5	6.25	1.56	3.1	50	6.25	3.1	3.1	50	>50	3.1	3.1	50	>50	3.1	3.1	12.5	6.25	3.1	3.1	50	50	12.5	3.1	6.25	50	6.25	3.1
S. aureus ATCC 29213	12.5	12.5	6.25	1.56	3.1	12.5	6.25	1.56	3.1	25	12.5	1.56	3.1	25	>50	1.56	3.1	12.5	>50	1.56	3.1	6.25	>50	12.5	6.25	3.1	50	25	6.25
S. aureus ATCC 19636	6.25	50	25	6.25	6.25	>50	25	12.5	6.25	>50	25	12.5	6.25	>50	>50	25	6.25	>50	25	3.1	6.25	>50	>50	6.25	6.25	>50	>50	6.25	3.1
P. aeruginosa ATCC 27853	X	25	25	1.56	3.1	50	50	3.1	3.1	50	>50	3.1	6.25	50	>50	6.25	12.5	12.5	>50	1.56	6.25	50	>50	3.1	6.25	>50	>50	12.5	6.25
P. aeruginosa ATCC 9027	X	12.5	25	3.1	3.1	>50	50	6.25	3.1	>50	50	6.25	6.25	>50	>50	12.5	6.25	>50	>50	6.25	25	>50	>50	12.5	25	>50	>50	25	6.25

[a] X, ampicillin resistant; AP, ampicillin; Phe, Phe-P-113; Nal, Nal-P-113; Bip, Bip-P-113.

TABLE 1. MIC values for ampicillin, P-113, Phe-P-113, Nal-P-113, and Bip-P-113 in different concentrations of NaCl

Species	MIC (μg/ml)[a]																											
	MH broth					LYM broth																						
						Control					NaCl concn (mM)																	
											50				100				200				300					
	AP	P-113	Phe	Nal	Bip	AP	P-113	Phe	Nal	Bip	P-113	Phe	Nal	Bip	P-113	Phe	Nal	Bip	P-113	Phe	Nal	Bip	P-113	Phe	Nal	Bip		
Escherichia coli ATCC 25922	25	>50	>50	12.5	12.5	25	12.5	12.5	3.1	6.25	50	50	3.1	12.5	50	>50	12.5	12.5	>50	>50	25	12.5	>50	>50	50	12.5		
Staphylococcus aureus ATCC 25923	3.1	>50	>50	6.25	3.1	12.5	12.5	6.25	1.56	3.1	50	12.5	1.56	6.25	50	50	3.1	12.5	>50	>50	12.5	12.5	>50	>50	50	25		
S. aureus ATCC 29213	12.5	>50	>50	6.25	6.25	12.5	12.5	6.25	1.56	3.1	50	25	1.56	6.25	50	>50	3.1	6.25	>50	>50	6.25	6.25	>50	>50	25	12.5		
S. aureus ATCC 19636	25	>50	>50	6.25	12.5	6.25	50	25	6.25	6.25	50	50	12.5	6.25	50	>50	25	6.25	>50	>50	50	12.5	>50	>50	50	12.5		
Pseudomonas aeruginosa ATCC 27853	X	>50	>50	50	50	X	25	25	1.56	3.1	50	50	1.56	3.1	50	50	12.5	6.25	>50	>50	50	6.25	>50	>50	>50	12.5		
P. aeruginosa ATCC 9027	X	>50	>50	50	50	X	12.5	25	3.1	3.1	50	>50	6.25	6.25	50	>50	50	6.25	>50	>50	50	6.25	>50	>50	>50	50		

[a] X, ampicillin resistant; AP, ampicillin; Phe, Phe-P-113; Nal, Nal-P-113; Bip, Bip-P-113.

TABLE 3. Susceptibilities of *Candida* strains to fluconazole, P-113, and Nal-P-113 in modified LYM medium

Candida sp.	Source	Strain	MIC (μg/ml)							
			Fluconazole	Control		50 mM NaCl		100 mM NaCl		150 mM NaCl	
				P-113	Nal	P-113	Nal	P-113	Nal	P-113	Nal
C. krusei	ATCC 6258	YLO6	32	6.25	6.25	6.25	6.25	100	6.25	100	12.5
C. parapsilosis	ATCC 22019	YLO7	8	6.25	6.25	50	12.5	>100	12.5	>100	25
C. glabrata	ATCC 9003	YLO8	16	12.5	6.25	>100	25	>100	25	>100	25
C. albicans	ATCC 90028	YLO12	1	6.25	6.25	25	12.5	100	12.5	>100	12.5
C. tropicalis	ATCC 13803	YLO86	>64	3.1	6.25	3.1	6.25	12.5	6.25	12.5	6.25
C. albicans	HIV patient	YH050001	2	6.25	6.25	25	12.5	100	12.5	>100	12.5
C. albicans	HIV patient	YH050005	1	6.25	6.25	50	25	>100	25	>100	25
C. tropicalis	HIV patient	YH050007	>64	3.1	6.25	3.1	6.25	25	6.25	25	12.5
C. tropicalis	HIV patient	YH050013	>64	3.1	6.25	3.1	6.25	50	6.25	50	12.5
C. albicans	HIV patient	YH050072	>64	6.25	6.25	25	25	>100	25	>100	25
C. krusei	HIV patient	YH050075	64	6.25	6.25	25	12.5	>100	12.5	>100	12.5
C. dubliniensis	HIV patient	YH050092	0.5	6.25	6.25	12.5	12.5	>100	12.5	>100	12.5
C. glabrata	HIV patient	YH050105	16	6.25	6.25	50	12.5	100	25	>100	50
C. tropicalis	HIV patient	YH050114	>64	3.1	6.25	3.1	6.25	100	6.25	100	12.5

used to test this hypothesis. We have designed and synthesized Nal-P-113, with His 4, 5, and 12 replaced by β-naphthylalanines (19). Bacterial and fungal strains from ATCC and clinical isolates (17) used in this study are listed in Tables 1, 2, and 3. The antibacterial activities of P-113 and Nal-P-113 were determined by the standard broth microdilution method of the National Committee for Clinical Laboratory Standards with Mueller-Hinton (MH) broth and LYM broth (14). The anti-*Candida* activities of fluconazole, P-113, and Nal-P-113 were determined in LYM broth medium (14) with different salt concentrations. The MIC value is the lowest concentration of peptide at which there was no change in optical density. All tests were measured in triplicate. The hemolytic activities of the peptides were determined from hemolysis against human red blood cells (hRBC) (18). The MIC values of Nal-P-113 were found to be more potent than those of P-113 (Tables 1 and 2), with only a slight increase of hemolysis of hRBCs (Fig. 1).

Several studies reported that the efficacy of P-113 is greatly reduced in the presence of high salt concentrations (9–11). As can be seen in Tables 1 and 2, P-113 demonstrates activities against various bacterial strains in the LYM broth medium. However, the activity of P-113 was reduced by the addition of 50 mM NaCl or 0.5 mM $MgCl_2$ into the LYM medium and was further diminished by the addition of 200 mM NaCl or 1.5 mM $MgCl_2$. On the other hand, the MIC values of Nal-P-113 were found to be more potent than P-113 in both Mueller-Hinton broth and modified LYM broth medium. Nal-P-113 still retained its antibacterial activities with 300 mM NaCl or 2.5 mM $MgCl_2$ added.

The anti-*Candida* activities of fluconazole, P-113, and Nal-P-113 were determined in LYM culture medium under different salt conditions. There were six resistant strains with high fluconazole MICs (≥32 μg/ml). As expected, both P-113 and Nal-P-113 had similar MICs in the LYM medium, ranging from 3.1 to 12.5 μg/ml (Table 3). The activity of P-113 was inhibited by the addition of 100 mM NaCl in the LYM medium and was strongly blocked by the addition of 150 mM NaCl. Again, Nal-P-113 retained its antifungal activities in the media containing high salt concentrations (Table 3).

To compare if other bulky amino acids can achieve similar results, we have synthesized Phe-P-113 and Bip-P-113 with His 4, 5, and 12 replaced by phenylalanine or the bulky amino acid β-(4,4′-biphenyl)alanine (Bip) (7). As expected, the antimicrobial and hemolytic activities of Phe-P-113 are similar to those of P-113, and its activities are diminished under high-salt conditions (Tables 1 and 2). On the other hand, the MIC values of Bip-P-113 are found to be more potent than those of P-113, and Bip-P-113 still retains its antimicrobial activities under high-salt conditions (Tables 1 and 2). Even though Bip-P-113 has slightly higher hemolytic activity than Nal-P-113 (Fig. 1), it exhibits less than 5% hemolytic activity at its effective MICs.

Certain antimicrobial peptides containing potent and broad-spectrum activities against various microbial pathogens are already in clinical trials (5). However, the antimicrobial activities of some agents were found to diminish under physiological and high-salt conditions (1, 15, 22). In this study, the β-naphthylalanine and β-(4,4′-biphenyl)alanine-substituted peptides, Nal-P-113 and Bip-P-113, have potent activity against microbial pathogens, including methicillin-, ampicillin-, and fluconazole-resistant strains. Moreover, the antimicrobial activity is no longer hindered by high salt concentrations.

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