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Salt Resistance and Synergistic Effect with Vancomycin of α-Helical Antimicrobial Peptide P18

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P18 (KWKLFKKIPKFLHLAKKF-NH_2) is an α-helical antimicrobial peptide designed from a cecropin A–magainin 2 hybrid. In this study, P18 was found to show strong antimicrobial activity against several antibiotic-resistant bacterial and fungal strains. Both the salt resistance on antimicrobial activity and the synergistic effect with clinically used antibiotic agents are critical factors in developing effective peptide antibiotic drugs. For this reason, we investigated the salt resistance of P18 to antagonism by NaCl, $CaCl_2$, and $MgCl_2$ on antimicrobial activity and the synergistic effect of P18 with vancomycin against vancomycin-resistant *Enterococcus faecium* (VREF). Compared to magainin 2, P18 showed strong resistance on antimicrobial activity against bacterial strains and *C. albicans* under high NaCl concentrations of 100–200 mM. In addition, P18 displayed much greater salt resistance on antibacterial activity against Gram-negative bacteria at the physiological or elevated concentrations of $CaCl_2$ and $MgCl_2$ than magainin 2. Furthermore, the combination study revealed that P18 has a relatively effective synergistic effect with vancomycin against VREF. Thus, these results support that P18 may prove to be a salt-resistant antibiotic peptide potentially useful in the treatment of cystic fibrosis patients as well as a valuable adjuvant for antimicrobial chemotherapy. © 2002 Elsevier Science

***Key Words:* α-helical antimicrobial peptide P18; salt resistance; synergistic effect; vancomycin-resistant *Enterococcus faecalis*.**

Many cationic α-helical antimicrobial peptides have been discovered in animal kingdom ranging from insects to humans (1–6). These peptides were known to represent components of the system of the host defense called "innate immunity" (1–6). These peptides are usually cationic and possess broad-spectrum activities against bacteria and fungi. They are known to have an amphipathic structure with clusters of hydrophobic and positively charged region (3, 7–9). This structural property appears to be closely related to their antimicrobial activity.

Among these cationic α-helical antimicrobial peptides, melittin, dermaseptins and SMAP-29 was known to have high toxicity against mammalian cells such as human erythrocytes as well as potent antimicrobial activity against bacteria and fungi (10–13). This undesirable toxicity against mammalian cells might compromise their therapeutic use and should be reduced or eliminated. Thus, a considerable attention has been focused on designing analogue peptides having more potent antimicrobial activity than that of natural peptides without damaging against mammalian cells. In the previous study, we designed a cationic α-helical antimicrobial peptide P18 (KWKLFKKIPKFLHLAKKF-NH_2) having potent antibacterial activity without hemolytic activity (14). Although the therapeutic index is too low for it to be considered an antitumor agent, this peptide displayed potent tumoricidal activity against several human transformed tumor cells with less toxicity against normal NIH-3T3 cells (14).

The ability to NaCl resistance is important for cationic α-helical antimicrobial peptides to function under physiological conditions. In cystic fibrosis (CF) patients, the airways are colonized and chronically infected with a variety of bacteria, including *P. aeruginosa* and *S. aureus* (15). It has been reported that an increase of the NaCl concentration in the bronchopulmonary fluids of a cystic fibrosis (CF) patients decreases the activity of epithelial cell-derived antimicrobial peptide human β-defensin-1 (hBD-1) (16). Moreover, the divalent cations such as $CaCl_2$ and $MgCl_2$ have been reported to inhibit the antimicrobial

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activity of mammalian antimicrobial peptide against Gram-negative bacteria (17, 18). Thus, the resistance to NaCl, $CaCl_2$, and $MgCl_2$ of the peptides on antimicrobial activity is a critical factor in developing an effective antibiotic drug for therapeutic applications.

Recent reports have shown that a significant synergistic effect was observed in several clinically isolated bacterial strains when some α-helical antimicrobial peptides were combined with several clinically used antibiotics (19–22). Therefore, the synergistic effect with clinically used antimicrobial agents against antibiotic-resistant bacterial strains makes the cationic antimicrobial peptides potentially valuable as an adjuvant for antimicrobial chemotherapy.

In this study, we investigated the salt resistance to antagonism by NaCl, $CaCl_2$, and $MgCl_2$ of an α-helical antimicrobial peptide P18 on antimicrobial activity against Gram-negative bacteria, Gram-negative bacteria and *C. albicans*. Furthermore, we investigated its synergistic effect with clinically used the antimicrobial agent vancomycin against vancomycin-resistant *Enterococcus faecium* (VREF).

MATERIALS AND METHODS

Microorganisms. *Escherichia coli* KCTC 1682, *Salmonella typhimurium* KCTC 1926, *Pseudomonas aeruginosa* KCTC 1637, *Bacillus subtilis* KCTC 3068, *Staphylococcus epidermidis* KCTC 1917, *Staphylococcus aureus* KCTC 1916, and *Candida albicans* KCTC 7965 were purchased from the Korean Collection for Type Cultures (KCTC), Korea Research Institute of Bioscience & Biotechnology (KRIBB) (Taejon, Korea). The clinical isolates of vancomycin-resistant *Enterococcus faecium* (VREF), vancomycin-resistant *Enterococcus faecalis* (VREF), *Stenotrophomonas maltophilia*, methicillin-resistant *Staphylococcus aureus* (MRSA) and azole-resistant *Candida albicans* were supplied from the Research Institute of Bacterial Resistance Yonsei University College of Medicine (Seoul, Korea).

Peptide synthesis. P18 and magainin 2 were synthesized the solid phase method using Fmoc-chemistry (23). For each coupling step, the Fmoc-protected amino acid and coupling reagents were added in an 8- to 10 fold molar excess with respect to resin substitution. Coupling (60–90 min) were carried out with 1-hydroxybenzotriazole (HOBT) and *N*,*N*-dicyclohexylcarbodiimide (DCC) in the presence of *N*-methyl-2-pyrrolidone (NMP). Amino acid side chains were protected as follows: 2,2,5,7-pentamethylchroman-6-sulfonyl (Arg), trityl (Asn, Gln, and His) and *t*-butyl (Tyr). Cleavage from resin and deprotection of the synthesized peptide were carried out with a solution of 90% trifluoroacetic acid, 3% water, 1% triisopropylsilane and 2% each of 1, 2-ethanedithiol, thioanisole, and phenol. After repeated precipitation with ether, the crude peptide was purified by RP-HPLC on a Shim-pack C_{18} column (19 × 300 mm), using an appropriate 0–60% acetonitrile gradient in 0.1% trifluoroacetic acid. Molecular weight of the synthetic peptides was confirmed by MALDI-TOF mass spectrometry (data not shown).

Antimicrobial activity. Antimicrobial activity of the peptides against seven selected organisms, including Gram-positive and Gram-negative bacteria and *C. albicans*, was determined by the broth microdilution assay. Briefly, single colonies of bacteria and fungi were inoculated into the culture medium (LB broth for bacteria and YM broth for *C. albicans*) and cultured overnight at 37°C (or 30°C for *C. albicans*). An aliquot of this culture was transferred to 10 ml of fresh culture medium and incubated for an additional 3–5 h at 37°C (for bacteria) or 30°C (for *C. albicans*) to obtain mid-logarithmic phase organisms. A 2-fold dilution series of peptides 1% peptone were prepared, serial dilutions (100 μl) were added to 100 μl of 2×10^6 CFU/ml in 96-well microtiter plates (Falcon), and then were incubated at 37°C (or 30°C for *C. albicans*) for 16 h. The test was performed under the fixed concentrations of NaCl, $CaCl_2$ or $MgCl_2$. The inhibition of growth was determined by measuring absorbance at 620 nm with a Microplate ELISA Reader (Molecular Devices, Sunnyvale, CA). The lowest concentration of peptide that completely inhibited growth of the organisms was defined as the minimal inhibitory concentration (MIC). The MICs were the average of triplicate measurements in three independent assays.

TABLE 1

Antimicrobial Activity of P18 and Magainin 2 (MA-2) against Bacterial and Yeast Strains

Bacterial and yeast strains (MIC: μM)	Peptides	
	P18	MA-2
E. coli (KCTC 1682)	4	32
S. typhimurium (KCTC 1925)	2	16
P. aeruginosa (KCTC 1637)	2	32
B. subtilis (KCTC 3068)	2	2
S. aureus (KCTC 1916)	1	16
S. epidermidis (KCTC 1917)	1	16
C. albicans (KCTC 7965)	4	16
E. faecalis (VREF, clinical isolate)	4	32
E. faecium (VREF, clinical isolate)	1	16
S. aureus (MRSA, clinical isolate)	2	64<
S. maltophilia (clinical isolate)	4	32
C. albicans (azole-resistant, clinical isolate)	1	8
C. albicans (azole-resistant, clinical isolate)	2	32
C. albicans (azole-resistant, clinical isolate)	2	8
C. albicans (azole-resistant, clinical isolate)	2	8

Combination assay. The synergistic effect of between vancomycin and each peptide against vancomycin-resistant *E. faecalis* (VREF) was investigated by the combination assay. Twofold serial dilutions of vancomycin were tested in the presence of a constant amount of peptide, equal to one-quarter and one-eighth of the MIC value of the peptide.

RESULTS AND DISCUSSION

Antimicrobial Activity

The antimicrobial activity of P18 and magainin 2 against Gram-negative bacteria, Gram-positive bacteria, *C. albicans* and antibiotic-resistant bacterial and yeast strains, including MRSA, VREF and azole-resistant *C. albicans*, was measured. When compared with magainin 2 used as a control peptide with amphipathic α-helical structure, P18 showed more potent antibacterial activity with MIC values of 1–4 μM range of concentration against tested all bacterial and yeast strains (Table 1).

Salt Resistance to NaCl, $CaCl_2$, and $MgCl_2$

The MICs of P18 for three Gram-positive bacteria, three Gram-negative bacteria and *C. albicans* were determined in the absence, or the presence of NaCl

TABLE 2

Effect of Increasing Concentration of NaCl on MICs of P18 and Magainin 2 (MA-2) against Gram-Negative Bacteria

	MIC (μM)[b]					
	E. coli (KCTC 1682)		*S. typhimurium* (KCTC 1925)		*P. aeruginosa* (KCTC 1637)	
Concn[a] (mM)	P18	MA-2	P18	MA-2	P18	MA-2
0	4	32	2	16	2	32
100	4	64	2	64	2	64
150	8	128	2	64	4	128<
200	8	128	2	128	4	128<

[a] Assays were performed in 1% peptone supplemented with NaCl to the final indicated concentrations.
[b] Expressed as the most common MIC of ≥3 trials.

(Tables 2 and 3). Although twofold increase in the MICs of P18 toward *E. coli* and *P. aeruginosa* in the presence of 150 or 200 mM NaCl, there was no significant increase in the MICs of P18 against *S. typhimurium* and three Gram-positive bacteria under high NaCl conditions. However, magainin 2 showed a drastic increase in the MIC values against both Gram-positive and Gram-negative bacteria under high NaCl conditions. When compared to bacterial strains, the MICs of P18 and magainin 2 toward *C. albicans* resulted in a remarkable increase under high NaCl conditions. P18 retained good anti-*Candida* activity with the MIC value of 4 μM under the NaCl concentration of 100 mM, whereas magainin 2 was found to have 4-fold decreased anti-*Candida* activity.

The NaCl concentration of 120 mM has been reported to be present in the environment of the epithelial cells of CF patients (16). The major pathogen of CF patients was known to be *P. aeruginosa* and *S. aureus*. Under high NaCl condition of 150–200 mM, P18 retained full antimicrobial activity against *P. aeruginosa* and *S. aureus* (Tables 2 and 3). Thus, P18 may prove to be useful for treatment of the chronic lung infections that afflict CF patients.

TABLE 3

Effect of Increasing Concentration of NaCl on MICs of P18 and Magainin 2 (MA-2) against Gram-Positive Bacteria and *C. alicans*

	MIC (μM)[b]							
	B. subtilis (KCTC 3068)		*S. aureus* (KCTC 1916)		*S. epidermidis* (KCTC 1917)		*C. albicans* (KCTC 7965)	
NaCl concn[a] (mM)	P18	MA-2	P18	MA-2	P18	MA-2	P18	MA-2
0	2	2	1	16	1	16	4	16
100	2	16	1	64	1	64	4	64
150	2	32	1	64	1	64	16	128
200	2	64	1	128	1	128	32	128<

[a] Assays were performed in 1% peptone supplemented with NaCl to the final indicated concentrations.
[b] Expressed as the most common MIC of ≥3 trials.

TABLE 4

Effect of Increasing Concentration of $CaCl_2$ and $MgCl_2$ on MICs of P18 and Magainin 2 (MA-2) against Gram-Negative Bacteria

		MIC (μM)[b]					
		E. coli (KCTC 1682)		*S. typhimurium* (KCTC 1925)		*P. aeruginosa* (KCTC 1637)	
Salt	Concn[a] (mM)	P18	MA-2	P18	MA-2	P18	MA-2
None		4	32	2	16	2	32
$CaCl_2$	1	4	128<	4	128<	8	128<
	3	8	128<	8	128<	16	128<
	5	32	128<	32	128<	32	128<
$MgCl_2$	1	8	128	4	128	4	128<
	3	16	128<	4	128<	16	128<
	5	16	128<	8	128<	32	128<

[a] Assays were performed in 1% peptone supplemented with $CaCl_2$ or $MgCl_2$ to the final indicated concentrations.
[b] Expressed as the most common MIC of ≥3 trials.

Recent reports have shown that cationic antimicrobial peptides cross the outer membrane of Gram-negative bacteria by the self-promoted uptake pathway (24). The initial step of this process should be a high affinity binding of the peptide to surface lipopolysaccharide of Gram-negative bacteria, causing displacement of divalent cations that stabilize adjacent lipopolysaccharide molecules (25, 26). The displacement of divalent cations is hypothesized to destabilize the outer membrane and to lead to self-promoted uptake of the peptides across outer membrane and to subsequent channel formation in the cytoplasmic membrane, resulting in cell death. The actions of cationic antimicrobial peptides are inhibited at this initial step of interacting with bacterial membranes by high concentrations of divalent cations, such as calcium and magnesium. For this reason, the ability to resist is important for cationic antimicrobial peptides to function under high salt conditions of calcium or magnesium.

Therefore, we also examined the antibacterial activities of P18 and magainin 2 against three Gram-negative bacteria in the presence of divalent cations of $CaCl_2$ or $MgCl_2$. As shown in Table 4, P18 was more resistant to $CaCl_2$ and $MgCl_2$ on antimicrobial activity compared with magainin 2. The reported concentrations of calcium and magnesium in human body fluids are the order of 1 mM (27). At the concentration of 1 mM $CaCl_2$ and 1 mM $MgCl_2$, P18 showed good antimicrobial activity with the MIC range of 4–8 μM against

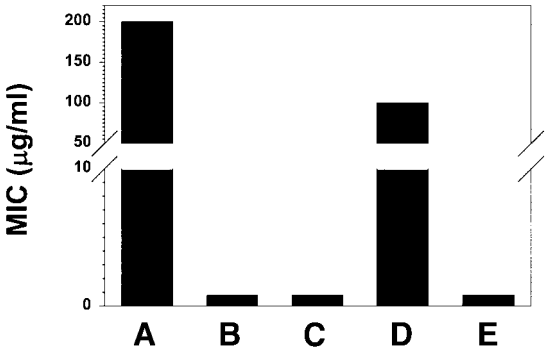

FIG. 1. Synergistic effect of the peptides with vancomycin against vancomycin-resistant *Enterococcus faecalis* (VREF). MIC of vancomycin, alone (A) and in combination with 0.5 μM P18 (0.125× MIC) (B), 1 μM P18 (0.25× MIC) (C), 4 μM magainin 2 (0.125× MIC) (D), and 8 μM magainin 2 (0.25× MIC) (E).

three Gram-negative bacteria (Table 4). Thus, P18 can be described as calcium- and magnesium-resistant at a physiological environment.

Synergistic Effect with Vancomycin against VERF

The recent emergence of antibiotic resistance of Gram-negative bacteria and Gram-positive bacteria has become a serious problem in human medicine throughout the world (18, 28–32). The main reason for this bacterial resistance is thought to be the microorganism's low permeability to the outer membrane of Gram-negative bacteria or peptidoglycan layer of Gram-positive bacteria of antibiotic agents. One way to overcome the problems of the emergence of antibiotic-resistant bacterial strains is to use new antimicrobial compound and/or combination therapy. The combination therapy is generally used to increase the *in vivo* activity, to prevent the emergence of drug resistance and to broaden the antimicrobial spectrum.

In recent years, the emergency of vancomycin-resistant enterococci (VRE) has become a serious problem in immunocompromised patients, such as cancer patients and transplant recipients (30–32). Therefore, we also examined the synergistic effect of P18 and magainin 2 with vancomycin against vancomycin-resistant *Enterococcus faecium* (VREF). One-quarter and one-eighth of the MIC concentration of each peptide against VREF was tested for combination assay. Both P18 and magainin 2 showed to have potent synergistic effect with vacomycin against VREF in the peptide concentration corresponding to one-quarter of the MIC values of each peptide (Fig. 1). However, P18 exhibited more effective synergistic effect in the concentration corresponding to one-eighth of the MIC values of each peptide compared with magainin 2 (Fig. 1). These results suggested that a cationic α-helical antimicrobial peptide P18 is potentially valuable as an adjuvant for antimicrobial chemotherapy in the treatment of VRE infection.

CONCLUSION

P18 showed strong antimicrobial activity against several antibiotic-resistant bacteria and fungi, including MRSA, VREF and azole-resistant *C. albicans*. P18 retained good antimicrobial activity against both bacterial and fungal cells under the physiological or elevated concentrations of salts such as NaCl, $CaCl_2$ and $MgCl_2$. The combination study revealed that P18 has potent synergistic effect with vancomycin against VREF compared with magainin 2. Therefore, these results support that P18 may prove to be a salt-resistant antibiotic peptide potentially useful in the treatment of CF patients as well as a valuable adjuvant for antimicrobial chemotherapy.

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