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Antibacterial synergism of novel antibiotic peptides with chloramphenicol

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Abstract

HP (2–20) is an antimicrobial sequence derived from the N-terminus of *Helicobacter pylori* ribosomal protein L1. We previously tested whether several analogues of HP (2–20), with amino acid substitutions that increased or decreased net hydrophobicity, could be useful as therapeutic agents. In the present study, we show that substituting Gln and Asp for Trp at positions 17 and 19, respectively, of HP (2–20) (peptide A3) had potent antibacterial activity in minimal inhibition concentration and minimal bactericidal concentration without having hemolytic activity. In contrast, when we decreased hydrophobicity by substituting Leu or Phe for Ser at positions 12 and 19, respectively, of HP (2–20) (Anal 4, Anal 5), there was no significant effect on antibacterial activity. We found that A3 acted synergistically with chloramphenicol against bacterial cells. Fluorescence activated flow cytometry showed that A3-treated cells had higher fluorescence intensity than untreated cells, similar to that of melittin-treated cells. Furthermore, A3 caused significant morphological alterations of *Staphylococcus aureus* and *Pseudomonas aeruginosa*, as shown by scanning electron microscopy. Our results suggest that peptide A3 may be useful for the design of novel antibiotic peptides that possess high bacterial cell selectively and synergistic effects with conventional antibiotic agents but lack hemolytic activity.

Keywords: Hydrophobicity; A3; Antibiotic effect; Scanning electron microscopy

Antibiotics have become indispensable in the modern health care system, assisting and complementing the natural immune system. The appearance of antibiotic-resistant strains, however, has led to a continuous search for more potent and efficient antibiotic agents. During the past decade, interest in peptide antibiotics has increased greatly. These peptides, which are widely distributed in nature and have been identified from many living species, take part in innate defense against microbes [1–4]. Among the more potent of these compounds are small bioactive peptides such as cecropin A, magainin 2, melittin, and plant defensin [5].

It is generally recognized that antimicrobial peptides are important in the innate host defense mechanisms of most living organisms, including plants [5], insects [6], amphibians [7], and mammals [8]. These antimicrobial peptides possess potent activity against bacteria, fungi, and even certain enveloped viruses [9]. Although their primary sequences are highly heterogeneous, these peptides are generally cationic and amphipathic. One of the main targets for these peptides is the lipid bilayer of the cytoplasmic membrane [10]. Permeabilization of the membrane leads to dissipation of the transmembrane potential, allowing the cell contents to be released, finally resulting in cell death [11]. Cell lysis by these peptides requires two steps: initial binding to the cell surface, followed by membrane permeabilization [11].

Recently, it has been suggested that the N-terminal region of *Helicobacter pylori* ribosomal protein L1 (RPL1) possesses antimicrobial activity, but that *H. pylori* itself is resistant to this 19-amino acid sequence, known as HP (2–20), which has been shown to be a Cecropin-like N-terminal peptide [12,13]. In our previous

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study, we sought to obtain peptides with improved antibiotic activity but little or no cytotoxicity, and we therefore designed novel analogue peptides with amino acid substitutions based on the sequence and α-helical wheel diagram of HP (2–20). In the present study, we tested the antibacterial effect of these synthetic peptides against Gram-positive and Gram-negative bacteria, as well as their synergism with the antibiotic agent, chloramphenicol. Finally, the antibacterial effect of these peptides on *Staphylococcus aureus* was examined by fluorescence activated flow cytometric analysis and scanning electron microscopy (SEM).

Materials and methods

Peptide synthesis. Peptides (Table 1) were synthesized by the solid phase method using 9-fluorenyl-methoxycarbonyl (Fmoc) chemistry [14]. Rink Amide 4-methyl benzhydrylamine (MBHA) resin (0.55 mmol/g) was used as the support to obtain a C-terminal amidated peptide. The coupling of Fmoc-amino acids was performed using *N*-hydroxybenzotriazole (HOBt) and dicyclohexylcarbodiimide (DCC). Asp side chains were protected with tert-butyl, Gln side changes with trityl, and Lys side chains with tert-butyloxycarbonyl groups. Deprotection and cleavage from the resin were carried out using a mixture of trifluoroacetic acid, phenol, water, thioanisole, 1,2-ethandithiol, and triisopropylsilane (88:2.5:2.5:2.5:2.5:2.0, v/v) for 2 h at room temperature. Each crude peptide was repeatedly washed with diethyl ether, dried in vacuum, and purified using a reversed-phase preparative HPLC on a Waters 15-μm Deltapak C_{18} column (19 × 30 cm). Peptide purity was determined by analytical reversed-phase HPLC on an Ultrasphere C_{18} column (Beckman, USA), 4.6 × 25 cm. The purified peptides were hydrolyzed with 6 N-HCl at 110 °C for 22 h, dried in a vacuum, dissolved in 0.02 N HCl, and assayed on an amino acid analyzer (Hitachi Model, 8500 A, Japan). Peptide concentrations were determined by amino acid analysis. The molecular weights of the synthetic peptides were determined using a matrix-assisted laser desorption ionization MALDI-mass spectrometer (data not shown) [15].

Antibiotic activity. Microdilution assays to establish minimal inhibition concentration (MIC) and minimal bactericidal concentration (MBC) values of synthetic peptides were performed. Cells were grown to mid-phase in 10 g/L bactotryptone, 5 g/L yeast extract, and 10 g/L NaCl, pH 7.0. The peptides were filtered through a 0.22 μm filter and stepwise-diluted in a medium of 1% bactopeptone. Each organism to be tested was suspended at 2×10^6 colony formation units (CFU)/ml in growth medium, and 100 μl was mixed with 100 μl of each 2-fold serial solution of peptide in a microtiter plate well; there were three replicates for each test sample. The plates were incubated for 18 h at 37 °C. The MIC was defined as the lowest concentration of peptide that gave no visible growth on the plate [16]. MBC was evaluated from the same test by viable counting assay and defined as the lowest concentration of peptide that killed 99.9% of the test inoculum [17].

RBC preparation and hemolytic activity. Human red blood cells were centrifuged and washed three times with phosphate-buffered saline solution, which is 35 mM phosphate buffer with 0.15 M NaCl at pH 7.0. The hemolytic activities of the peptides were evaluated by determining the hemoglobin release of 8% suspensions of fresh human erythrocytes at 414 nm. One hundred microliter aliquots of the 8% suspended red blood cells were transferred to the 96-well plates. Hemolysis was measured by absorbance at 414 nm with an ELISA plate reader (Molecular Devices Emax, Sunnyvale, CA, USA). Zero percentage hemolysis and 100% hemolysis were determined in PBS and 0.1% Triton X-100, respectively. The hemolysis percentage was calculated using the following equation: % hemolysis = [($Abs_{414\,nm}$ in the peptide solution − $Abs_{414\,nm}$ in PBS)/($Abs_{414\,nm}$ in 0.1% Triton X-100 − $Abs_{414\,nm}$ in PBS)] × 100.

Hemolytic effect of the peptide under RBC morphology. RBCs were incubated at 37 °C for 1 h with 60% MIC of HP-A3 and melittin. Negative controls were run without peptides. The RBCs were fixed with equal volumes of 4% glutaraldehyde and 1% paraformaldehyde in 0.05 M cacodylate buffer (pH 7.2). After lyophilization and gold coating, the samples were examined on a HITACHI S-2400 (Tokyo, Japan).

Effects of two-agent combinations on cell growth. The effects of two-agent combinations were analyzed as described previously [18]. Serial dilutions of two antimicrobial agents were mixed in checkerboard fashion in a microtiter plate so that each row contained a fixed amount of one agent and increasing amounts of the second agent. The concentration ranges used were based upon the MICs of each antimicrobial agent and bacteria. As controls, the MIC of each of the agents acting alone was determined in every tray. The trays were incubated overnight at 37 °C and inspected visually for bacterial growth, which was confirmed by reading optical density at 570 nm (Reader 510; Organon Teknika, Boxtel, The Netherlands).

FACScan analysis. For analysis of membrane integrity after peptide treatment, 2×10^6 cells harvested at log phase were mixed with HP (2–20) (14.5 μg/ml) and incubated for 30 min at 28 °C with constant shaking (140 rpm). The cells were harvested by centrifugation, washed three times with PBS, and incubated with propidium iodide (PI, 50 μg/ml final concentration) at 4 °C for 30 min, followed by removal of the unbound dye through washing with an excess of PBS. Flow cytometry was performed using a FACScan (Becton–Dickinson, San Jose, CA).

Scanning electron microscopy. Bacteria cells were suspended at 10^8 CFU/ml in Na-phosphate buffer, pH 7.4, supplemented with 100 mM NaCl (buffer A), and incubated at 37 °C with HP (2–20) and ciprofloxacin. Controls were run in the absence of peptide and ciprofloxacin solvent. After 30 min the cells were fixed with an equal volume of 5% glutaraldehyde in 0.2 M Na-cacodylate buffer, pH 7.4, 2 h at 4 °C, filtered on Isopore filters (0.2 μm pore size, Millipore, Bedford, MA, USA), and extensively washed with 0.1 M Na-cacodylate buffer, pH 7.4. The filters were treated with 1% osmium tetroxide, washed with 5% sucrose in cacodylate buffer, and dehydrated with a graded

Table 1
Amino acid sequences of the synthetic antimicrobial peptide, HP (2–20) derived from the N-terminus of *H. pylori* ribosomal protein L1 and its analogues

Peptides	Amino acid sequences	Remarks
HP (2–20)	AKKVFKRLEKLFSKIQNDK-NH_2	Native
Anal 1	AKKVFKRLEKLFSKIQNWK-NH_2	($D^{19} \rightarrow W^{19}$)
Anal 2	AKKVFKRLEKLFSKIWNDK-NH_2	($Q^{17} \rightarrow W^{17}$)
Anal 3	AKKVFKRLEKLFSKIWNWK-NH_2	(Q^{17} $D^{19} \rightarrow W^{17}$ W^{19})
Anal 4	AKKVFKRLEKSFSKIQNDK-NH_2	($L^{12} \rightarrow S^{12}$)
Anal 5	AKKVSKRLEKLFSKIQNDK-NH_2	($F^{6} \rightarrow S^{6}$)

ethanol series. After lyophilization and gold coating, the samples were examined on a HITACHI S-2400 instrument (HITACHI, Japan).

Results

Design and synthesis of the peptides

The amino acid sequences used in this study are summarized in Table 1. The synthetic peptides were purified by the reverse-phase HPLC and the correct molecular weights of the synthetic peptides were confirmed by MALDI mass spectrometry (Table 2). In order to investigate a possible correlation between the overall peptide hydrophobicity and biological activities on bacterial and human erythrocyte membranes, the hydrophobic characteristics of the peptides were investigated by comparing the retention times on RP-HPLC (Table 2). As expected, Anal 1, 2, 3 eluted later and Anal 4, 5 eluted earlier than HP (2–20) (see Figs. 1 and 2).

Susceptibility of various bacterial strains and human erythrocyte to peptides

We then tested the in vitro activity of HP (2–20) and its analogues against various Gram-negative and Gram-positive bacterial strains by determining the minimum inhibitory (MIC) and bactericidal (MBC) concentrations of each by the microdilution method [16] (Table 3). Substitution of each Trp residue at positions 17 and 19 of HP (2–20) (peptides A1, A2, and A3) enhanced the antibacterial activity compared with HP (2–20). In particular, substitution of Gln and Asp for Trp at positions 17 and 19, respectively (peptide A3), caused a dramatic increase in antibacterial activity. The MIC values of A3 ranged between 0.39 and 3.13 μM, whereas the MBCs were 1–4 times higher than the MICs, consistent with a bactericidal mechanism of action for the peptides (Table 4).

To assess the cytotoxicity of these peptides against mammalian cells, we tested the ability of each to lyse human erythrocytes. Each of the synthesized peptides showed no hemolytic activity, whereas melittin exhibited a strong hemolytic activity (data not shown). In addition, SEM showed that HP (2–20) and A3 did not alter the surface of normal RBCs, whereas erythrocytes treated with a half MIC of chloramphenicol and melittin exhibited rough surfaces and pores (Fig. 4).

Table 2
Molecular masses of the synthetic peptides, as determined by MALDI-MS

Peptides	Calculated value	Observed value	Retention time (min)
HP (2–20)	2320	2319.38	17.82
Anal 1	2390	2390.43	19.54
Anal 2	2377	2377.40	20.95
Anal 3	2448	2448.45	22.38
Anal 4	2295	2293.33	10.68
Anal 5	2261	2259.34	15.41

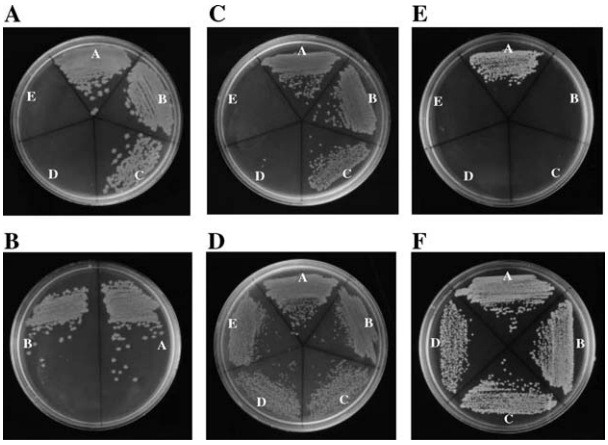

Fig. 1. Minimal bactericidal concentration (MBC) of A3 and chloramphenicol against Gram-positive bacteria. (A) A, 0.39 μM; B, 0.78 μM; C, 1.56 μM; D, 3.13 μM; and E, 6.25 μM in A3 against *Bacillus subtilis*; (B) A, 12.5 μM; B, 25 μM in chloramphenicol against *B. subtilis*; (C) A, 0.39 μM; B, 0.78 μM; C, 1.56 μM; D, 3.13 μM; and E, 6.25 μM in A3 against *Staphylococcus epidermidis*; (D) A, 0.78 μM; B, 1.56 μM; C, 3.13 μM; D, 6.25 μM; and E, 12.5 μM in chloramphenicol against *S. epidermidis*; (E) A, 1.56 μM; B, 3.13 μM; C, 6.25 μM; D, 12.5 μM; and E, 25 μM in A3 against *S. aureus*; and (F) A, 3.13 μM; B, 6.25 μM; C, 12.5 μM; and D, 25 μM in chloramphenicol against *S. aureus*.

Effects of two-agent combinations on cell growth

When we tested combinations of synthetic peptides and antibiotics with different modes of action, we found synergy between A3 and chloramphenicol, a blocker of ribosomal peptidyl transferase, against *S. aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli* (Fig. 3). This was especially evident against *S. aureus*, in that the MIC of chloramphenicol plus A3 was much higher than that of chloramphenicol alone.

Of the peptides tested, peptide A3 showed the most potent antibiotic against bacterial cells, as well as synergistic activity with chloramphenicol, with no hemolytic activity against human erythrocytes. These findings suggest that this peptide may facilitate not only an understanding of the mechanism of action of α-helical antibiotic peptides but also the design of novel antibiotic peptides.

Antibiotic efficiency of FACScan flow cytometry

Although the overall antibiotic mechanism(s) of antibiotic peptides has not been fully elucidated, disruption of the cell structure by pore formation [19] or ion channel generation seems to be the most likely mechanism [20]. To determine whether peptide A3 has an effect on

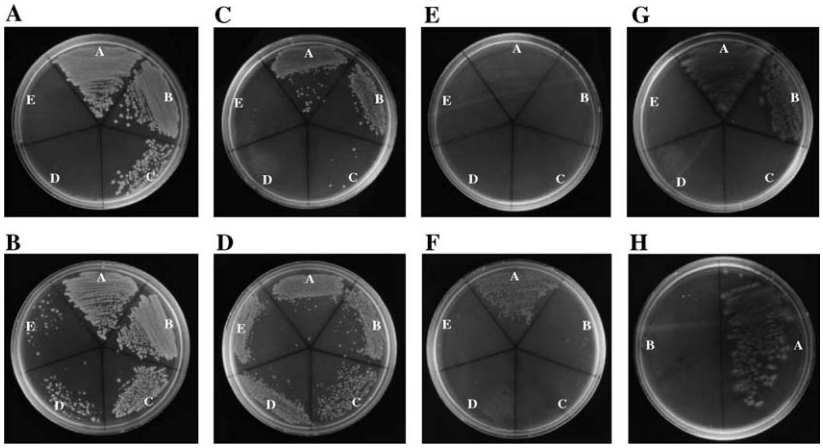

Fig. 2. Minimal bactericidal concentration (MBC) of A3 and chloramphenicol against Gram-negative bacteria cells. (A) A, 0.39 μM; B, 0.78 μM; C, 1.56 μM; D, 3.13 μM; and E, 6.25 μM in A3 against *E. coli*; (B) A, 0.78 μM; B, 1.56 μM; C, 3.13 μM; D, 6.25 μM; and E, 12.5 μM in chloramphenicol against *E. coli*; (C) A, 0.78 μM; B, 1.56 μM; C, 3.13 μM; D, 6.25 μM; and E, 12.5 μM in A3 against *P. vulgaris*; (D) A, 1.56 μM; B, 3.13 μM; C, 6.25 μM; D, 12.5 μM; and E, 25 μM in chloramphenicol against *P. vulgaris*; (E) A, 0.39 μM; B, 0.78 μM; C, 1.56 μM; D, 3.13 μM; and E, 6.25 μM in A3 against *S. typhimurium*; (F) A, 0.39 μM; B, 0.78 μM; C, 1.56 μM; D, 3.13 μM; and E, 6.25 μM in chloramphenicol against *S. typhimurium*; (G) A, 1.56 μM; B, 3.13 μM; C, 6.25 μM; D, 12.5 μM; and E, 25 μM in A3 against *P. aeruginosa*; and (H) A, 12.5 μM; B, 25 μM in chloramphenicol against *P. aeruginosa*.

Table 3
Minimal inhibition concentration (MIC) of HP (2–20) and its analogues

	MIC (μM)						
	Positive bacteria			Negative bacteria			
	B. substilis	*S. epidermides*	*S. aureus*	*E. coli*	*P. vulgalis*	*S. typhimurium*	*P. aeruginosa*
HP (2–20)	1.56	3.13	12.5	6.25	3.13	0.78	12.5
A1	1.56	0.78	3.13	1.56	1.56	0.39	6.25
A2	1.56	1.56	3.13	1.56	1.56	0.78	6.25
A3	0.78	0.78	3.13	0.78	1.56	0.39	3.13
A4	6.25	12.5	>12.5	12.5	6.25	1.56	>12.5
A5	3.13	6.25	>12.5	6.25	3.13	3.13	>12.5
Chloramphenicol	25	1.56	6.25	1.56	3.13	0.78	12.5–25

Table 4
Minimal bactericidal concentration (MBC) of A3 and chloramphenicol

	MBC (μM)						
	Gram-positive bacteria			Gram-negative bacteria			
	B. substilis	*S. epidermides*	*S. aureus*	*E. coli*	*P. vulgalis*	*S. typhimurium*	*P. aeruginosa*
A3	3.13	3.13	3.13	3.13	3.13	0.39	6.25
Chloramphenicol	>25	>12.5	>25	>12.5	>25	0.78	25

the cell membrane or on cell physiology, we tested its effect on bacterial cells incubated with the DNA intercalating dye propidium iodide (PI). In this study, PI staining of the peptide treated cells was expected to give further information about cytotoxic mechanism of A3. We hypothesized that, if A3 disrupted the bacterial cell membrane, it would be permeabilized and therefore would allow the free diffusion of small dyes such as PI into cytoplasm. Detection of internal PI was analyzed on single cells by FACScan flow cytometry. Whereas normal cells and cells treated with chloramphenicol showed no PI fluorescence, cells treated with 3.13 μM HP (2–20) or A3 showed a shift of the dots to the right of 23.19% and 84.32%, respectively (Fig. 5). The fluorescence intensity of these cells serves as a marker for the relative amount of internalized PI in case of a permeabilized cell membrane like that of disintegrating cells.

Effect of A3 and chloramphenicol on cell morphology

When we tested the effects of A3 and/or chloramphenicol on bacterial cell morphology by SEM, we

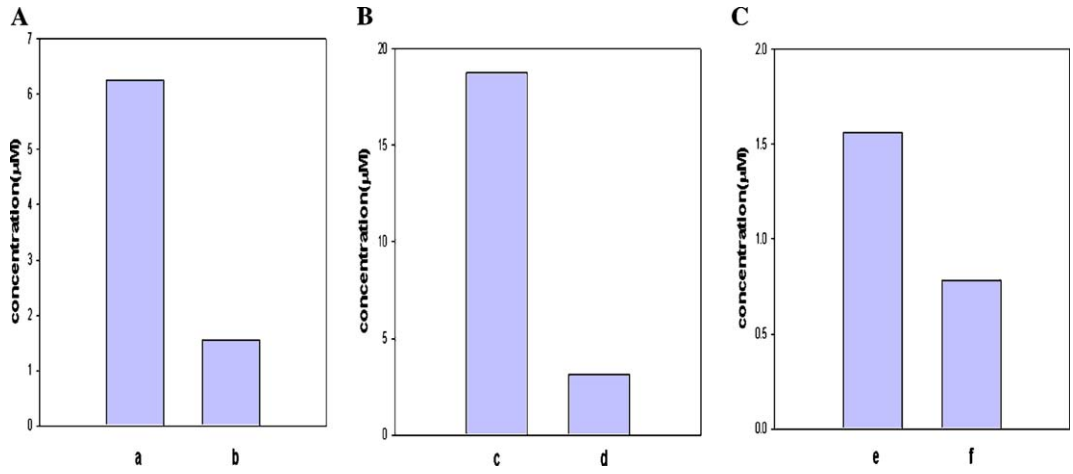

Fig. 3. Synergistic effect of A3 and chloramphenicol against *S. aureus* (A), *P. aeruginosa* (B), and *E. coli* (C). Serial dilutions of chloramphenicol were tested in the presence of a constant amount of A3, equal to one-eighth its MIC. (a) MIC of chloramphenicol against *S. aureus*, (b) MIC of chloramphenicol plus 0.39 μM A3 against *S. aureus*, (c) MIC of chloramphenicol against *P. aeruginosa*, (d) MIC of chloramphenicol plus 0.39 μM A3 against *P. aeruginosa*, (e) MIC of chloramphenicol against *E. coli*, and (f) MIC of chloramphenicol plus 0.39 μM A3 against *E. coli*.

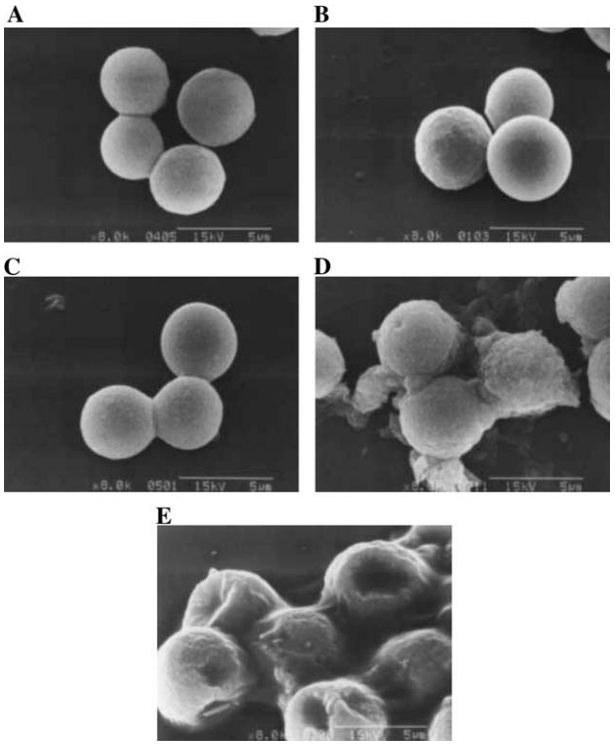

Fig. 4. Hemolytic effect of peptide A3, chloramphenicol, and melittin on human red blood cells. (A) Negative control; (B) HP (2–20); (C) peptide A3; (D) chloramphenicol; and (E) melittin.

found that, while untreated cells had a normal smooth surface (Fig. 6A), cells treated with A3 or chloramphenicol showed surface pores (Figs. 6B and C) and cells treated with both agents showed that they acted synergistically, disrupting the cell membrane (Fig. 6D). These results provide morphological evidence of the potent permeabilizing and synergistic activity of A3.

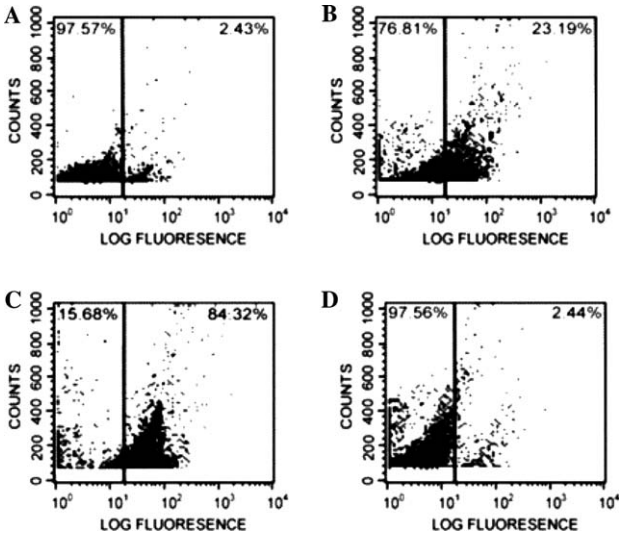

Fig. 5. Flow cytometric analysis. Exponential phase *S. aureus* cells were treated with 3.13 μM of peptide A3, and cellular fluorescence was analyzed by FACScalibur flow cytometry. The increments of the log fluorescence signal represent PI uptake by peptides. (A) no peptide, (B) HP (2–20), (C) peptide A3, and (D) chloramphenicol.

Discussion

Amphipathic α-helical model peptides with ratios of hydrophilic to hydrophobic amino acid residues of 9:9 have been shown to possess potent antimicrobial activity with no hemolytic effects, whereas peptides with increased numbers of hydrophobic residues (ratios of 11:7 and 13:5) showed potent hemolytic as well as antimicrobial activity [21]. These results suggest that a balance between hydrophilic and hydrophobic residues is crucial in designing novel α-helical peptides with antimicrobial activity and no cytotoxicity. In our previous

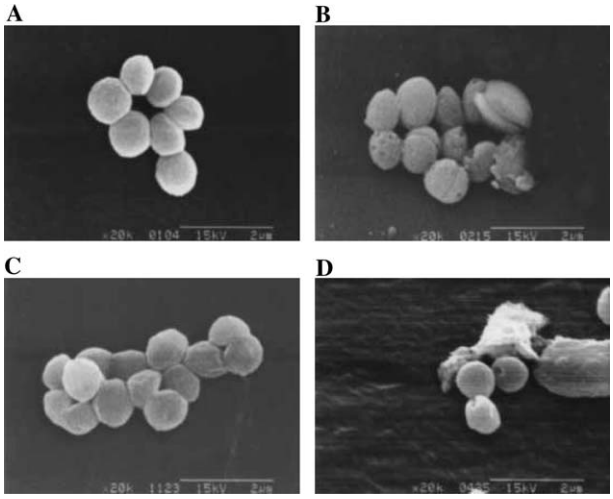

Fig. 6. Scanning electron micrographs of (A) untreated *S. aureus* and *S. aureus* treated with (B) peptide A3, (C) chloramphenicol, or (D) peptide A3 plus chloramphenicol.

study, we sought to elucidate the relationship between hydrophobic and hydrophilic regions of antibiotic peptides, and we therefore designed and synthesized analogues of HP (2–20) that had more potent antibiotic activity but no cytotoxicity. In this study, we tested the importance of peptide hydrophobicity on antibacterial activity, and we found a synergistic interaction between the conventional antibiotic agent chloramphenicol and the HP (2–20) analogue peptide A3. In this peptide, the hydrophilic residues Gln and Asp were substituted for the hydrophobic amino acid Trp at positions 17 and 19 of HP (2–20). Our finding, that A3 possesses potent antibiotic activity, both alone and together with chloramphenicol, but no hemolytic activity, suggests that this peptide may facilitate not only an understanding of the mechanism of action of α-helical antibiotic peptides on the bacterial plasma membrane but may also contribute to the design of novel antibiotic peptides with enhanced antibacterial activity without cytotoxicity.

We found that peptide A3 inhibited the in vitro growth of both Gram-positive and Gram-negative bacterial isolates. The small differences we observed between MIC and MBC values indicate that the effects of A3 are bactericidal, while our PI-based permeabilization assay and SEM images of A3-treated bacteria suggest that bacterial membranes are an important target of this molecule.

The combination of chloramphenicol and A3 peptide acted synergistically against *S. aureus*, *P. aeruginosa*, and *E. coli*. That is, when combined with peptide A3, the in vitro antibacterial effect of chloramphenicol against *S. aureus* was increased up to 8-fold, while the in vitro antibacterial effect of chloramphenicol against *P. aeruginosa* and *E. coli* was increased 2- to 4-fold. Macrolides are large hydrophobic antibiotic molecules usually ineffective against Gram-negative bacteria owing to the outer membrane barrier or efflux of the antibiotics [22,23]. Several antimicrobial peptides are known to interact with the outer membranes of Gram-negative bacteria, making this outer protective shield more permeable [24]. It is possible that our peptides may cause an increase in this permeability by interacting with the LPS, allowing chloramphenicol easier access to its cytoplasmic target. Another possible mode of action may be that the peptides block macrolide efflux pumps. A study of the synergy between a macrolide and antimicrobial peptides concluded that this is a complex mechanism that probably involves the peptide-induced entrance of large lipophilic, amphiphilic molecules into the cell [25]. Synergy between rifampicin and temporin H, a 10-mer peptide isolated from frog secretions, was reported to be caused by the ability of temporin H to facilitate the entrance of rifampicin into the bacterial cell [26]. Chloramphenicol acts on the ribosomal 50S subunit in bacteria, inhibiting translation by blocking either the peptidyl transferase reaction or the translocation step [27]. While not entirely excluding the hypothesis that our peptides increase chloramphenicol uptake by increasing the permeability of the LPS, it is possible that antibacterial peptides and erythromycin may inhibit sequential steps in the protein biosynthesis. A hypothesis that includes increased uptake and accessibility to the target, combined with drugs acting on a common pathway, can explain the observed synergy of peptide A3 and chloramphenicol.

To provide further information on its mechanism of action, we assayed uptake of PI by bacterial cells. Our results suggest that the *S. aureus* cell membrane was damaged by peptide A3, allowing the free diffusion of PI into the cytoplasm. This fluorescence signal was comparable to that observed with melittin, suggesting that both A3 and melittin may act in a similar way (data not shown).

SEM offers a unique method by which to observe cells, allowing examination of cell surfaces at high resolution [28]. When we used SEM to assay the morphological changes induced by peptide treatment, we found that, while chloramphenicol-treated cells were similar to untreated cells, cells treated with A3 showed cell surface pores, and cells treated with both two agents showed synergistic enhancement of cell membrane pores and disruption, providing further evidence that the bacterial membrane is an important target of this peptide.

In conclusion, we have shown synergy between chloramphenicol and antibacterial peptide against bacterial cells. This synergy may result from enhanced uptake owing to increased permeability of the cytoplasmic membrane and/or LPS layer, or the sequential inhibition of the same biosynthetic pathway (i.e., protein synthesis).

Acknowledgments

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