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Synergistic Killing Effect of Synthetic Peptide P20 and Cefotaxime on Methicillin-Resistant Nosocomial Isolates of *Staphylococcus aureus*

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Abstract The salt resistance of antibacterial activity and synergistic effect with clinically used antibiotic agents are critical factors in developing effective peptide antibiotic drugs. For this reason, we investigated the resistance of antibacterial activity to antagonism induced by NaCl and $MgCl_2$ and the synergistic effect of P20 with cefotaxime. P20 is a 20-residue synthetic peptide derived from a cecropin A (CA)-melittin (ME) hybrid peptide. In this study, P20 was found to have potent antibacterial activity against clinically isolated methicillin-resistant *Staphylococcus aureus* (MRSA) strains without hemolytic activity against human erythrocytes. The combination study revealed that P20 in combination with cefotaxime showed synergistic antibacterial activity in an energy-dependent manner. We also confirmed the synergism between P20 and cefotaxime by fluorescence-activated flow cytometric analysis by staining bacterial cells with propidium iodide (PI) and bis-(1,3-dibutylbarbituric acid) trimethine oxonol (BOX). This study suggests that P20 may be useful as a therapeutic antibiotic peptide with synergistic effect in combination with conventional antibiotic agents.

Key words: Hybrid peptide, antibacterial activity, synergistic effect, cefotaxime, salts resistance, methicillin-resistant *Staphylococcus aureus* (MRSA)

During the last decades, resistance to most of the clinically available antimicrobial agents has emerged among several pathogens. Today, methicillin-resistant *Staphylococcus aureus* (MRSA) is an important nosocomial problem for the failure of antimicrobial treatments and an increasing problem in community-acquired infections. In the hospital environment, MRSA resistance to a variety of classic agents other than β-lactam antibiotics is responsible for many life-threatening infections [1].

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The threat of resistance demands an increasing effort to search for alternative chemotherapeutic compounds with new action mechanisms. Active anti-staphylococcal agents might become available as a result of research on novel targets for antimicrobials for which no inhibitors are presently used, inhibiting cross-resistance to already existing antibiotics [2]. Cationic antimicrobial peptides are a new generation of antibiotics with unique mode of action mechanism.

Among the reported antibiotics, there are small bioactive peptides secreted by various organisms. It is generally recognized that antimicrobial peptides play an important role in the innate host defense systems against infectious pathogens of living organisms including plants [3], insects [4, 5], amphibians [6, 7], and mammals [8]. The site for the lethal action of these peptides is negatively charged plasma membrane. After the electrostatic binding with the plasma membrane and its hydrocarbon core, the association of several antimicrobial peptides would build pores that would serve as an ion-conducting and anion-selective channel. The membrane pores disrupt the ionic homeostasis of the living organisms and lead to cell death [9]. Furthermore, recent reports showed that these antimicrobial peptides, after insertion into the plasma membrane, may trigger the activity of bacterial murein hydrolases, resulting in damage of the peptidoglycan and lysis of bacteria [10, 11]. Finally, a synergistic effect with several antibiotics, probably due to the membrane-permeabilizing activity of these peptides, has been suggested because the peptides allow maximal entry of these antibiotic substrates inside the bacterial cell [12]. Unfortunately, some peptides demonstrated undesirable lytic properties against eukaryotic cells [13]. Recently, efforts have been made to design hybrids and its analogues containing partial sequences of two peptides with different properties. As the result of these efforts, several hybrids, such as Cecropin A (CA)-Melittin (ME) [14, 15], Cecropin (CA)-Magainin (MA) [16-18], Helicobacter Pylori (HP)-Magainin (MA) [19], and Temporin A (TA)-Cecropin A (CA) [20], have been designed and synthesized to increase

antimicrobial activity with no hemolytic properties. These synthetic peptides are more potent and effective than native antimicrobial peptides, because of changes of hydrophobicity, charge, and structure due to substitution of amino acids. In particular, a positively charged region is important for interaction with bacterial membrane in the first step of the actions of cationic antimicrobial peptides. In a previous study, we designed and synthesized several peptides analogous to the Cecropin A (1-8)-Melittin (1-12) [CA-ME] hybrid, and tested the antibacterial activities and hemolytic activities of these peptides. The results showed that P20-amide, which was derived by substituting Thr for Lys at positions 18 and 19 of the CA-ME hybrid peptide, had the most potent antibacterial activity and the lowest hemolytic activity among these peptides. Therefore, the aim of this study was to evaluate the *in vitro* activity of P20 against MRSA hospital isolates as well as to investigate its *in vitro* interaction with the clinically used anti-staphylococcal agent, cefotaxime.

MATERIALS AND METHODS

Peptide Synthesis

The peptide P20 and melittin used as positive control was synthesized by the solid phase method using Fmoc-chemistry [21]. Rink Amide p-methyl benzhydrylamine (MBHA) resin (0.55-mM/g; Bova Biochem, U.S.A.) was used as support to obtain a C-terminal amidate peptide. The coupling of Fmoc-amino acids was performed by dicyclohexylcarbodiimide (DCC) and N-hydroxybenzotriazole (HoBt). Amino acid side chains were protected as follows: tert-butyl (Asp), trityl (Gln), tert-butyloxycarbonyl (Lys). 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. The crude peptide was then repeatedly washed with diethylether and dried in vacuum. The synthesized peptide was purified by RP-HPLC consisted of Shimadzu 10ADVP and Shimadzu 8A (Shimadzu Corp., Japan) and an Ultrasphere C_{18} column (4.6×250 mm; Beckman, U.S.A.). The column was eluted with Eluent A (0.1% TFA in water) and Eluent B (50% acetonitrile with 0.1% TFA) at a flow rate of 1 ml/min for analytical separations. For separation of the peptides, a linear gradient from 5%–100% B in 30 min was applied. Purity of the peptide was checked by analytical reversed-phase HPLC on an Ultrasphere C_{18} column (Beckman, U.S.A.). The molecular weight of the synthetic peptides was determined by using an AXIMA-CFR MALDI-TOF MS (Shimadzu Corp., Japan) [22].

Bacterial Strains and Antibacterial Activity Assay

Methicillin-resistant *Staphylococcus aureus* (MRSA) strains were isolated from clinical human pathogens at Kyungpook National University Hospital. Single colony of MRSA was placed into the LB (Yeast extract: Tryptone: Sodium chloride, 5 g: 10 g: 10 g per liter) medium and cultured overnight at 37°C. 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 to obtain organisms at mid-logarithmic phase. The bacterial cells were seeded in the wells of a 96-microtiter plate in LB media at a density of 1×10^6 cells (100 ml per well). Twenty ml of the serially-diluted peptide solution was added to each well, and the cell suspension was incubated for 8 h at 37°C. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of sample that gave no visible growth on the plate by the microdilution method [23, 24]. The inhibition of growth was determined by measuring absorbance at 620 nm using a microtiter ELISA reader (Molecular Devices Emax, CA, U.S.A.). The MICs were the average of measurements by triple independent assays. The synergistic effects of peptide P20 together with cefotaxime against MRSA were investigated by the combination assay [25]. The synergistic antibacterial activity was tested at half the MIC of the peptide P20 and one-eighths the MIC of cefotaxime. The synergistic activity assay was performed in the absence and presence of 0.002% NaN_3 as a metabolic inhibitor, and in the presence of salts (20 mM and 100 mM NaCl and $MgCl_2$).

Flow Cytometric Analysis

For analysis of the membrane integrity after peptide treatment, flow cytometric analysis was performed with MRSA bacterial cells. MRSA cells were first harvested at the log phase and mixed with 50 μM P20 and 25 μM cefotaxime. The cells were incubated for another 30 min at 37°C under constant shaking (140 rpm) in the absence or presence of 0.002% NaN_3. After incubation, the cells were harvested by centrifugation and washed three times with ice-cold PBS. Permeabilization of the cell membrane was detected by incubation of the peptide-treated cells in propidium iodide (PI, 5 μg/ml final concentration) and bis-(1,3-dibutylbarbituric acid) trimethine oxonol (BOX, 10 μg/ml final concentration) [26] for 30 min at 4°C, followed by removal of unbound dye through excessive washing with PBS. The fluorescence of PI was monitored in the FL2-H channel, and BOX was monitored in the FL1-H channel. Flow cytometric analysis was performed by the FACScalibur flow cytometer (Becton Dickinson, San Jose, CA, U.S.A.) [27].

Hemolytic Activity

The hemolytic activity of P20 was evaluated by determining hemoglobin release of 4% fresh human erythrocyte cells suspensions at 414 nm. Human red blood cells (hRBCs) were washed three times with PBS (35 mM phosphate buffer/150 mM NaCl, pH 7.0). One-hundred ml of hRBCs diluted to 8% (v/v) with PBS were plated into 96-well

Table 1. Amino acid sequences of peptides used in this study.

Peptides	Sequences	Remarks
CA(1-8)-ME(1-12)	KWKLFKKIGIGAVLKVLTTG-NH_2	
P20	KWKLFKKIGIGAVLKVL**KK**G-NH_2	CA(1-8)-ME(1-12): $T^{18,19}$→K
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2	Native melittin

Peptides were synthesized by the solid phase method using Fmoc (9-fluorenyl-methoxycarbonyl)-chemistry. The amino acids of the peptide were substituted by analysis of a-helical wheel diagram.

plates, and 100 ml of the peptide solution (from 50 mM to 1.56 mM) were added to each well. The plates were incubated for 1 h at 37°C, and then centrifuged for 10 min at 150 g. One-hundred μl of the supernatant was transferred to the 96-well plates. The percent of hemolysis was measured by absorbance at 414 nm with a microtiter ELISA reader (Molecular Devices Emax). Zero and 100 percent of hemolysis were determined in PBS and 0.1% Triton-X 100, respectively. The percent of hemolysis 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 [28].

Results and Discussion

A peptide corresponding to P20-amide and melittin used as positive control was chemically synthesized, and purified to homogeneity and subjected to MALDI-mass spectroscopic analysis (Table 1). Melittin, a toxin from honeybee venom, has been reported to possess potent antimicrobial activity; however, it possesses potent cytotoxicity against eukaryotic cells [29].

Susceptibility of MRSA strains to P20 was examined by MIC test, and the result showed that the P20 has potent antibacterial activity against MRSA strains (Table 2). On the other hand, MRSA strains that were clinically isolated and used as antibiotic-resistant bacterial strains in this study survived in the presence of over 20 μg/ml cefotaxime concentration. The MIC values of cefotaxime demonstrated that the MRSA strains were antibiotic-resistant against cefotaxime. We next assayed the synergistic effects of P20 and cefotaxime against the MRSA strains. The result showed that the synergistic antibacterial activity of P20 in combination with cefotaxime was about 4-fold higher than that of only P20, when about half of its MIC value was used (Fig. 1). In order to visualize this synergistic effect, MRSA cell suspensions were spread on LB agar plate (Fig. 2) [30], and the results demonstrated that the combination of P20 and cefotaxime had strong synergistic antibacterial activity against MRSA, indicating potentially valuable synergistic effect as an adjuvant for antimicrobial chemotherapy in treatment of pathogens and antibiotic-resistant bacteria.

We also tested the effect of NaN_3 as an ATP-depleting agent [31] on the antibiotic activity of P20 and its synergistic effect with cefotaxime, and found that NaN_3 repressed the antibiotic activities of both P20 and its synergistic effect (Fig. 3): Antibacterial activities of P20 alone and its combination with cefotaxime decreased in the presence of 0.002% NaN_3. Therefore, the combination of P20 and cefotaxime exhibits the synergism in an energy-dependent manner, requiring cellular energy consumption [32]. Although the antibacterial activity of P20 was inhibited by NaN_3, P20 in combination with cefotaxime showed about 4-fold more potent antibacterial activity than P20 alone, and this was similar to that obtained with synergistic effect in the absence of NaN_3. The result indicates that the interaction of P20 with MRSA is energy dependent, requiring a cellular metabolite function. Thus, the result suggests that the antibacterial activity of P20 is more effective in combination with cefotaxime as a conventional antibiotic agent, regardless of the physiological conditions of bacterial cells.

In addition, we investigated the effect of cations on the synergistic effect of P20 in combination with cefotaxime in the absence or presence of 20 mM and 100 mM NaCl

Table 2. Antibacterial activity of P20, cefotaxime, and melittin against methicillin-resistant *Staphylococcus aureus* (MRSA) strains.

	MIC (μg/ml)							
	MRSA* strains							
	1	2	3	4	5	6	7	8
P20	10	10	5	1.25	10	5	10	10
Cefotaxime	20>	20>	20>	20>	20>	20>	20>	20>
Melittin	1.25	0.625	1.25	0.31	1.25	1.25	0.625	0.625

*MRSA; Methicillin-resistant *Staphylococcus aureus*.
The bacterial cells were grown to the mid-growth phase on LB (Yeast extract: Tryptone: Sodium chloride, 5 g: 10 g: 10 g per liter) medium. Microbial growth was determined by the increase in optical density at 620 nm after 8 h of incubation at 37°C.

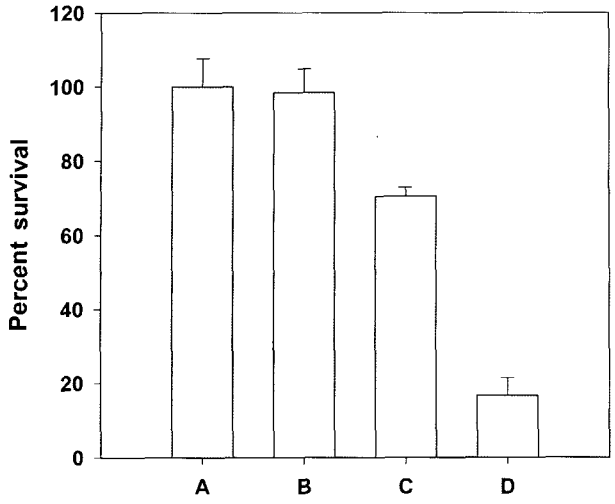

Fig. 1. The synergistic effect of the peptide P20 with cefotaxime against MRSA.
Control (A); 2.5 μg of cefotaxime (0.125×MIC) (B); 5.0 μg of P20 (0.5× MIC) (C); 2.5 μg of cefotaxime and in combination with 5.0 μg of P20 (D).

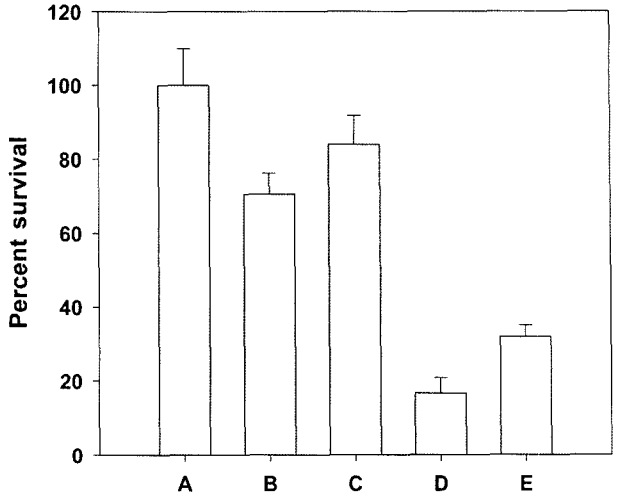

Fig. 3. The effect of sodium azide (NaN_3) on the synergistic effect of P20 with cefotaxime against MRSA.
Exponential phase MRSA cells were treated with 2.5 μg of cefotaxime and 5 μg of P20. The cells were incubated at 37°C for 6 h in the presence of 0.002% NaN_3. Control (A); P20 only (B), P20 and NaN_3 (C); cefotaxime and P20 (D); cefotaxime, P20, and NaN_3 (E).

and $MgCl_2$, respectively (Figs. 4 and 5). Generally, cations have been reported to inhibit the antimicrobial activity of cationic α-helical antimicrobial peptides under physiological conditions; therefore, the resistance of the peptides to cations such as NaCl and $MgCl_2$ is a critical factor for their use as therapeutic agents. It has been reported that the antibiotic action of melittin in independent of salt, temperature, or energy [33]. The antibacterial activities of P20 in

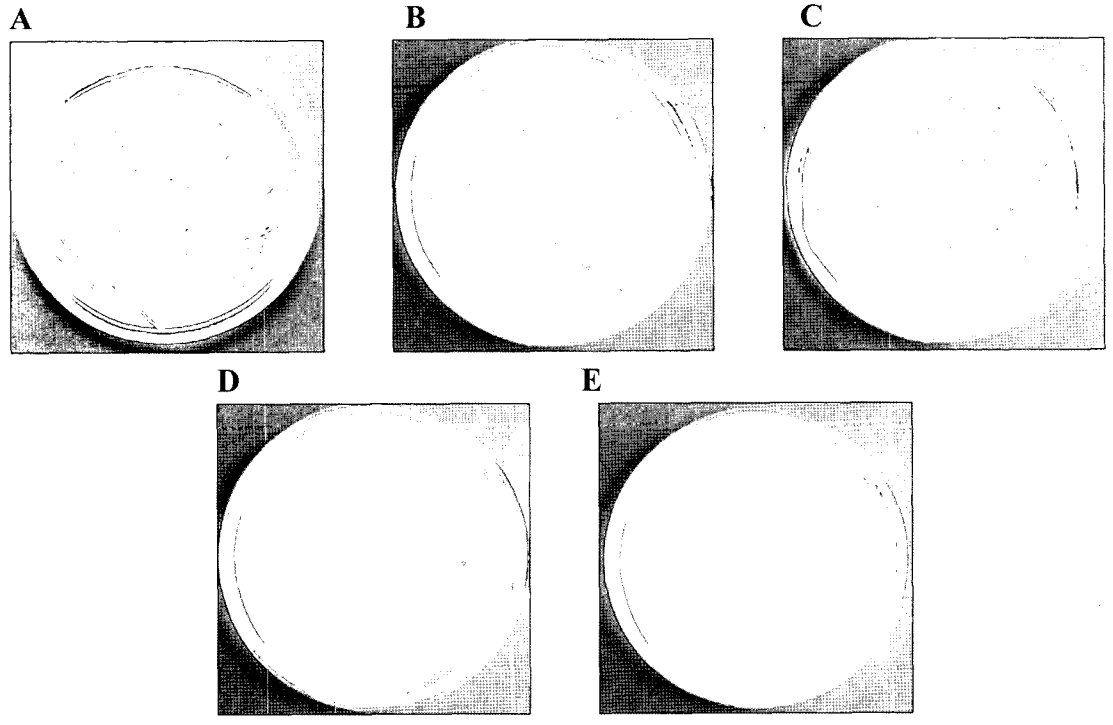

Fig. 2. The antibacterial effects of cefotaxime, P20, and combination of P20 and cefotaxime on MRSA colony formation.
MRSA cells were suspended to density of approx 1×10^6/ml in LB. Each mixture was incubated with 2.5 μg cefotaxime, 5.0 μg P20, 2.5 μg of cefotaxime in combination with 5.0 μg of P20 and 1.25 μg of melittin at 37°C for 2 h. The reaction mixture was spread on a LB agar plate after incubation at 37°C for 8 h. Control (A); 2.5 μg of cefotaxime (B); 5.0 μg of P20 (C); 2.5 μg of cefotaxime and 5.0 μg of P20 (D); 1.25 μg of melittin (E).

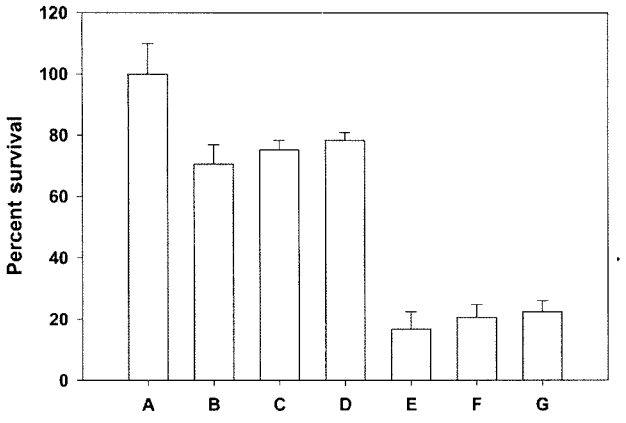

Fig. 4. The effect of NaCl on the synergistic effect of P20 with cefotaxime against MRSA.
Exponential phase MRSA cells were treated with 2.5 μg of cefotaxime and 5 μg of P20. The cells were incubated at 37°C for 6 h in the presence of NaCl (20, 100 mM). The cells incubated in the absence of NaCl were used as positive control. Control (A); (B)–(D) P20-treated cell; (C) and (D) NaCl added 20 mM and 100 mM, respectively; (E)–(G) cefotaxime and P20-treated cell; (F) and (G) NaCl added at 20 mM and 100 mM, respectively.

combination with cefotaxime under monovalent and divalent cations also remained potent, thus indicating that the combination of two agents retained potent antibacterial activity in the presence of either NaCl or $MgCl_2$. Synergistic interactions have already been demonstrated between lysozyme and lactoferrin [34] and between cathelicidin and lysozyme or lactoferrin [35]. In the present study, we found that P20 and cefotaxime acted synergistically in the killing of MRSA strain in energy-dependent and salts-independent manners. Thus, these results indicate that P20 is a potent antibacterial agent when combined with conventional antibiotic agents.

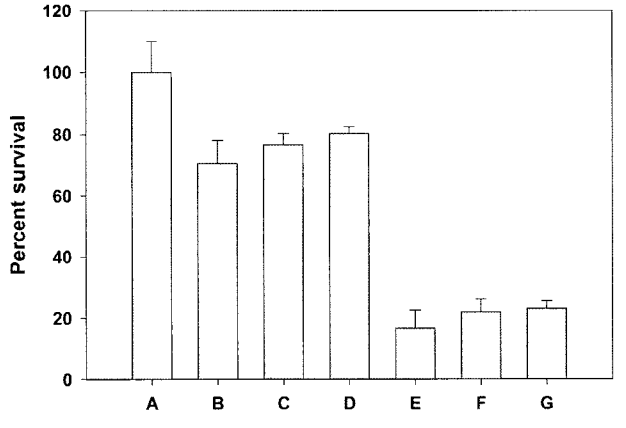

Fig. 5. The effect of $MgCl_2$ on the synergistic effect of P20 with cefotaxime against MRSA.
Exponential phase MRSA cells were treated with 2.5 μg of cefotaxime and 5 μg of P20. The cells were incubated at 37°C for 6 h in the presence of $MgCl_2$ (20, 100 mM). The cells incubated in the absence of $MgCl_2$ were used as positive control. Control (A); (B)–(D) P20-treated cell; (C) and (D) $MgCl_2$ added at 20 mM and 100 mM, respectively; (E)–(G) cefotaxime and P20-treated cell; (F) and (G) $MgCl_2$ added at 20 mM and 100 mM, respectively.

To confirm the synergism between P20 and cefotaxime, we performed flow cytometric analysis by staining bacterial cells with PI and BOX dye. Propidium iodide (PI) dye is taken into the cells and intercalated to DNA, and bis-(1,3-dibutylbarbituric acid) trimethine oxonol (BOX) dye is a membrane-staining dye bound to depolarized membrane [26]. Flow cytometric analysis showed antibacterial effects of P20, cefotaxime, and P20 in combination with cefotaxime on MRSA. In flow cytometric analysis by PI staining (Fig. 6), MRSA was treated with cefotaxime, which inhibits synthesis of the bacterial cell wall [36], and NaN_3. Unlike cefotaxime (Fig. 6A), the cellular uptake of PI molecule in the case of P20 was decreased under NaN_3 (Fig. 6B). It is thought that action of P20 on depolarized bacterial plasma membrane by NaN_3 was inhibited. In the case treated with P20 in combination with cefotaxime, the cellular uptake of PI molecule was increased more than with P20 or cefotaxime only (Fig. 6C), and its effect was also inhibited by NaN_3. We also examined the synergistic effect of P20 with cefotaxime on bacterial membrane by staining with BOX dye. As seen in Fig. 7A, the shift of the peak due to decreased activity of cefotaxime by NaN_3, was found. It seems that the inhibition of cell wall synthesis by cefotaxime exposes the membrane for binding by BOX dye, and much more BOX dye binds to exposed membrane in the absence of NaN_3. Unlike the case of cefotaxime, only P20-treated bacterial cells in the presence of NaN_3 had more BOX dye molecules than in the absence of NaN_3 (Fig. 7B). It is thought that the binding site of BOX dye was decreased by forming pores caused by the peptide P20. This result also suggests that the pore-forming activity of P20 is inhibited by NaN_3, similar to the case of PI staining. However, the shift of the peak was not remarkable in the combination of P20 with cefotaxime. It is highly likely that the effects of P20 and cefotaxime together were canceled out for BOX staining. The result also demonstrated that P20 in combination with cefotaxime is slightly inhibited by NaN_3, but its antibacterial activity is as potent as before. To assess the cytotoxicity of the synthetic peptide P20 against mammalian cells, the hemolysis of human erythrocyte cells was tested at various concentrations of P20 and cefotaxime. Generally, most antimicrobial peptides have some limits, because of cytotoxicity on mammalian cells, such as hemolytic activity on human erythrocytes. Melittin used as a positive control in this study has been known for its hemolytic activity against mammalian cells, such as human erythrocyte cells [37]. As depicted in Table 3, melittin showed potent hemolytic activity at almost the entire concentrations tested, but P20 and cefotaxime showed no hemolytic effect.

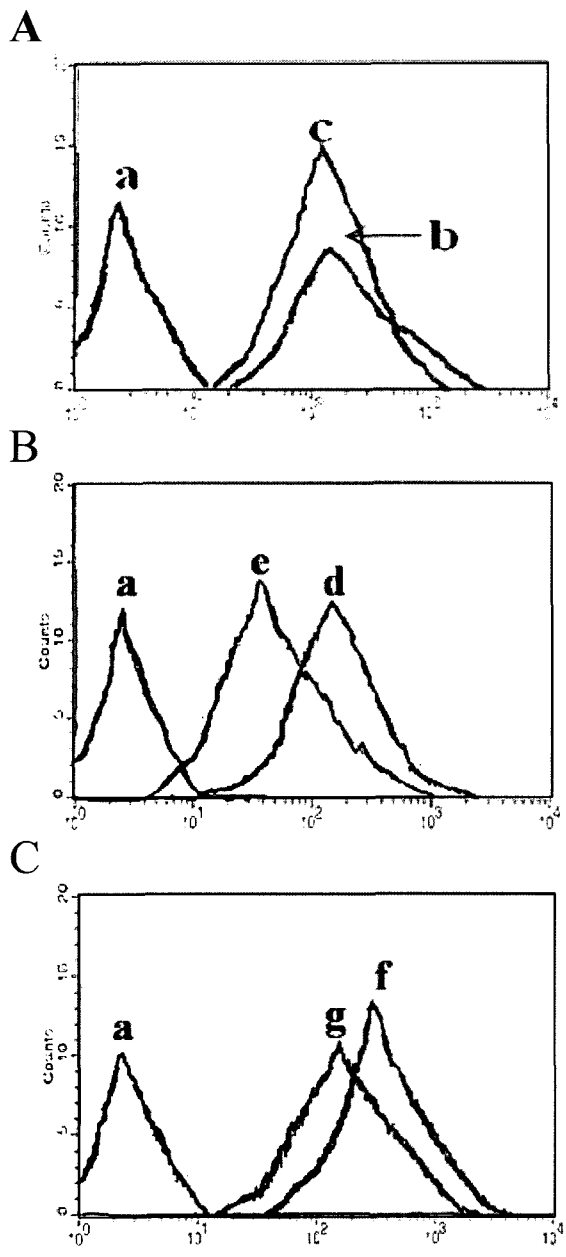

Fig. 6. The synergistic effect of combination of P20 and cefotaxime by flow cytometric analysis with PI-stained MRSA cells.

MRSAs were mixed with P20 at a concentration of 25 μM and cefotaxime at a concentration of 12.5 μM and incubated in the absence (b, d, f) or in the presence (c, e, g) of sodium azide at 37°C for 2 h. The cell membrane was detected by incubation of P20 and cefotaxime treated cells in propidium iodide (PI 5 μg/ml final concentration) at 4°C for 1 h. Control staining without any peptide treatment (a); cefotaxime (b), (c); P20 (d), (e); and P20 in combination with cefotaxime (f), (g).

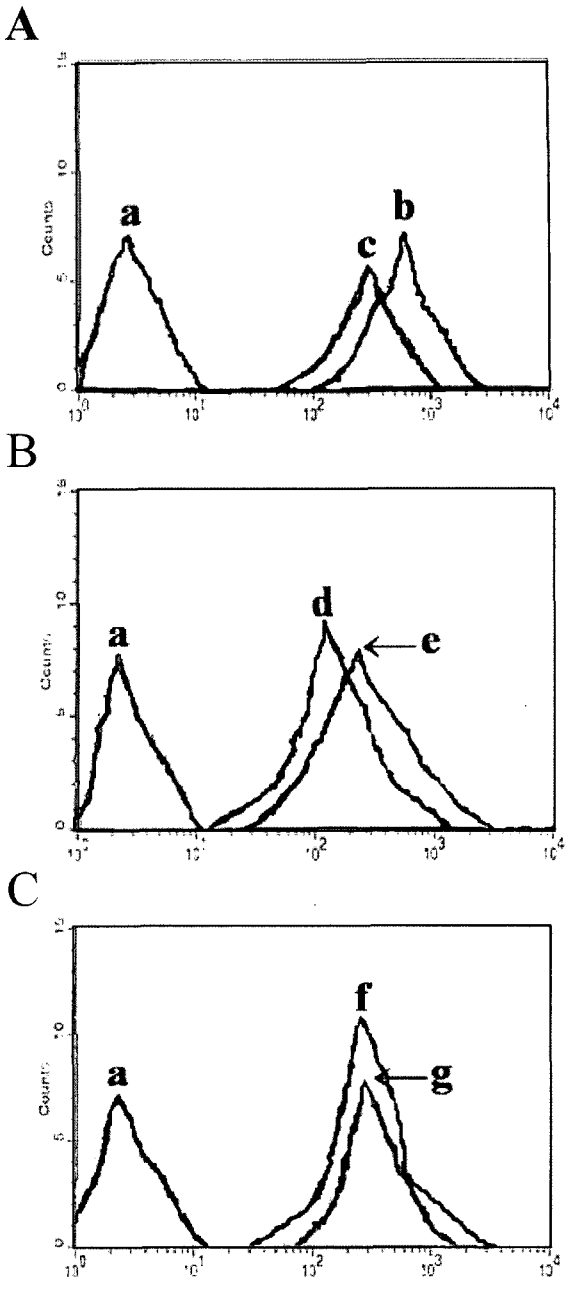

Fig. 7. The synergistic effect of combination of P20 and cefotaxime by flow cytometric analysis with BOX-stained MRSA cells.

MRSAs were mixed with P20 at a concentration of 25 μM and cefotaxime at a concentration of 12.5 μM and incubated in the absence (b, d, f) or in the presence (c, e, g) of sodium azide at 37°C for 2 h. The cell membrane was detected by incubation of P20 and cefotaxime treated cells in BOX (BOX 10 μg/ml final concentration) at 4°C for 1 h. Control staining without any peptide treatment (a); cefotaxime (b), (c); P20 (d), (e); and P20 in combination with cefotaxime (f), (g).

In the present study, we found that the synthetic α-helical peptide P20 has potent antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) strains isolated from clinical human pathogens, without hemolytic effect on human erythrocytes, and that the combination of P20 and cefotaxime showed synergistic effect on antibacterial activity against MRSA that are resistant to cefotaxime. In addition, P20 in combination with cefotaxime showed potent antibacterial activity without significant

Table 3. Hemolytic activity of P20 and cefotaxime against human erythrocytes.

	% Hemolysis (μM)					
	50	25	12.5	6.25	3.12	1.56
P20	0	0	0	0	0	0
Cefotaxime	0	0	0	0	0	0
Melittin	100	100	100	100	92	79

The hemolytic activity of the peptides was evaluated by determining the hemoglobin release of 4% fresh human erythrocytes suspension at 414 nm. The percentage of hemolysis 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.

inhibitions by physiological environments such as NaN_3 and salts condition. Thus, these results suggest that P20, which can possibly be combined with cefotaxime, is a potential therapeutic agent on infection of cefotaxime-resistant bacterial pathogens. Moreover, we expect that P20 is a candidate for a potent antibacterial agent against antibiotic-resistant bacteria, and P20 in combination with cefotaxime will be a potential prescription for bacterial infections.

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