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Modifications on amphiphilicity and cationicity of unnatural amino acid containing peptides for the improvement of antimicrobial activity against pathogenic bacteria

Junichi Taira,[a,b‡] Yutaka Kida,[c‡] Hiroshi Yamaguchi,[b] Koichi Kuwano,[c] Yuichiro Higashimoto[a] and Hiroaki Kodama[b*]

The widespread natural sources-derived cationic peptides have been reported to reveal bacterial killing and/or growth-inhibiting properties. Correspondingly, a number of artificial peptides have been designed to understand antibacterial mechanism of the cationic peptides. These peptides are expected to be an alternative antibiotic against drug-resistant pathogenic bacteria because major antimicrobial mechanism of cationic peptides involves bacterial membrane disorder, although those availabilities have not been well evaluated. In this study, cationic peptides containing Aib were prepared to evaluate the availability as an antimicrobial agent, especially against representative pathogenic bacteria. Among them, BRBA20, consisting of five repeated Aib-Arg-Aib-Ala sequences, showed strong antibacterial activity against both Gram-negative and Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus*. Additionally, growth of *Serratia marcescens* and multidrug-resistant *Pseudomonas aeruginosa*, known as proteases-secreting pathogenic bacteria, were also completely inhibited by BRBA20 under 20 μg/ml peptide concentrations. Our results suggested availabilities of Aib-derived amphiphilicity and protease resistance in the design of artificial antimicrobial peptides. Comparing BRBA20 with BKBA20, it was also concluded that Arg residue is the preferred cationic source than Lys for antimicrobial action of amphiphilic helices.

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Keywords: Aib; amphiphilicity; antibiotic-resistant bacteria; antimicrobial peptide; membrane permeation

Introduction

Antibiotics are indispensable to treat various infectious diseases. On the other hand, dissemination and abusing of antibiotics have led to the development of antibiotic-resistant pathogenic bacteria. Cationic antimicrobial peptides are considered one reasonable alternative for conventional antibiotics, hence bacterial target of the peptides is the cytoplasmic membrane [1]. Cationic peptides are generally able to interact electrostatically with negatively charged bacterial phospholipids and then insert into the membranes, forming transient 'pores' [2,3]. In alternative mechanistic views, the antibacterial peptides kill bacteria by the 'carpet' model in which the peptides cooperatively destroy the membrane barrier without channel formation [4,5]. Those natural antimicrobial peptides are typically from 10 to 40 amino acid length and may be roughly categorized according to their secondary structures. However, structural complexity and diversity of the natural peptides make it difficult to design practical antimicrobial peptides. Consequently, *de novo* designed peptides have been recognized as potential tools for systematically understanding the structure–activity relationships of the antimicrobial peptides [6–11], and the chemical strategies allow design of peptides composed of desired amino acids including unnatural one. Nevertheless, antimicrobial effect of the cationic peptides against drug-resistant pathogenic bacteria has not been well evaluated and several issues still remain to utilize such antimicrobial peptides. For example, acquisition of protease resistibility is one of the issues toward the practical utilization. Several reports, including our previous work, mentioned that peptides composed of α,α-dialkyl amino acids avoided protease processing [12,13]. Additionally, it has been known that α,α-dialkyl amino acid residues maintain ridged secondary structure of peptide chain [9,10]. Thus, knowledge on the usage of α,α-dialkyl amino acids expected to be powerful tools in the design of artificial

* *Correspondence to: Hiroaki Kodama, Department of Chemistry, Faculty of Science and Engineering, Saga University, Saga 840-8502, Japan. E-mail: hiroaki@cc.saga-u.ac.jp*

a *Department of Chemistry, Kurume University School of Medicine, Kurume 830-0011, Japan*

b *Department of Chemistry, Faculty of Science and Engineering, Saga University, Saga 840-8502, Japan*

c *Division of Microbiology, Department of Infectious Medicine, Kurume University School of Medicine, Kurume 830-0011, Japan*

‡ *These authors contributed equally to this study.*

antimicrobial peptides. In fact, such amino acids have been identified in peptaibols, a representative of naturally occurring channel-forming antibiotics [14].

In this study, correlation between antimicrobial activities and structures of Aib-containing peptides was systematically explored to find out the optimum prototype of peptide antibiotics. As described in the following section, brief antimicrobial activities of several analogs, measured by classical diffusion assay, were reported previously, although total evaluation and comparison of antimicrobial activity throughout the series have not been done. The repeated sequence of Aib-Xxx-Aib-Ala was used as the framework in this study and the Xxx positions were occupied by cationic residues. Peptide length, numbers and positions of Aib, and cationic residues were varied and antimicrobial activity was evaluated. In addition, peptide structures on lipid bilayer and other biophysical properties, including membrane permeation activity and ion channel-forming property, were also measured on the peptides, which showed strong antimicrobial activity to explore antimicrobial mechanism. The consequence of this study would contribute as a part of knowledge to the development of design of peptide antibiotics.

Materials and Methods

Materials

N-α-Fmoc-protected amino acids and Rink amide resin were obtained from Merck (Tokyo, Japan). DCC, HOBt, and TFA were products of Peptide Institute (Osaka, Japan). Other reagents were obtained from Sigma (Tokyo, Japan) or Wako Pure Chemical Industries (Osaka, Japan) and used as received.

Bacterial Strains

Staphylococcus aureus ATCC6538P, methicillin-resistant *S. aureus* (MRSA), *Escherichia coli* ATCC25922, clinical isolated *Serratia marcescens*, and multidrug-resistant *Pseudomonas aeruginosa* (MDRP) were used.

Peptide Synthesis

Syntheses and confirmation of homogeneity of BKBA20, BKBA12, AKAA20, BKAA20, and AKBA20 were reported previously [9,10]. Same strategy was applied for the syntheses of BKBA28 and BRBA20. Briefly, the peptide elongation was carried out by conventional Fmoc chemistry on Rink amide resin. *N*-termini were acetylated by AcOH pre-activated with DCC-HOBt. Crude linear products were cleaved from the resins by TFA treatments and following ether precipitation. Crude linear peptides were purified by RP-HPLC on a Wakosil 5C4-200 column (ϕ 10.0 $\times$ 250 mm). Purities of peptides were confirmed by analytical RP-HPLC. Structures of peptides were verified by MALDI-TOF MS analyses. Amino acid compositions and peptide concentrations of stock solutions used in various assays were determined by quantitative amino acid analysis as described previously [10].

Antimicrobial Assays

Bacteria were grown at 37 °C in Luria Bertanii (LB) broth containing 50 mM $NaHCO_3$ (pH 7.4) to stationary phase, diluted 1 : 10 into LB broth, and grown to early log phase. Bacteria were harvested by centrifugation (12 000*g*; 5 min), and then re-suspended in Mueller-Hinton broth containing 50 mM $NaHCO_3$ (pH 7.4) [15]. Evaluated peptides were dissolved in phosphate-buffered saline (PBS) (pH 7.4). Fifty microliters of the peptide solutions was added to 50 µl of the bacterial suspension (2×10^5 CFU/ml) in 96-well flat bottom plates (Nunc, Tokyo, Japan). The plates were incubated at 37 °C for 3 h. Then, 10 µl of alamerBlue (Invitrogen, Tokyo, Japan) was added and further incubated for 4 h at 37 °C. Absorbances at 570 and 595 nm were measured by Bio-Rad Model 680 micro-plate reader (Bio-Rad, Hercules, CA, USA). Inhibition of bacterial growth was determined according to the manufacturer's instruction manual.

Membrane Permeability Measurement

Membrane permeabilizations in *E. coli* and *S. aureus* were determined by fluorometric measurement. Bacteria were grown at 37 °C in LB broth containing 50 mM $NaHCO_3$ (pH 7.4) to stationary phase, diluted 1 : 10 into LB broth, and grown to early log phase [15]. Bacteria were harvested by centrifugation (12 000*g*; 5 min) and re-suspended in Mueller-Hinton broth containing 50 mM $NaHCO_3$ (pH 7.4) at a density of 10^8 CFU/ml. Evaluated peptides were dissolved in 10 mM Na-phosphate buffer (pH 7.4). Final peptide concentrations in the experiments with *S. aureus* and *E. coli* were 5 and 10 µg/ml, respectively. Fifty microliters of the peptide solutions and 50 µl of the bacterial suspensions were mixed in 96-well black flat bottom plates (Nunc), and then, 5 µl of CYTOX-Green (Invitrogen) was added to each well. Suspensions were immediately excited at 485 nm and emissions at 535 nm were measured using Twinkle LB 970 (Berthold, Bad Wildbad, Germany).

CD Measurement

Preparation of small unilamellar vesicles (SUVs), composed of dipalmitoylphosphatidylcholine (DPPC)/dipalmitoylphosphatidylglycerol (DPPG) (3 : 1) or DPPC only, was described previously [16]. CD spectra were recorded on a Jasco J-720 spectropolarimeter (Jasco, Tokyo, Japan) at room temperature. Peptide concentration was 10 µM. Cylindrical cell with 0.2 cm path lengths was used for the measurement. CD experiments were carried out in the wavelength range between 190 and 260 nm. Four scans were averaged for each sample and blank was subtracted from all spectra. The results were expressed as the mean residue ellipticity $[\theta]_M$ with units of degrees cm^2/dmol.

Single Channel Measurement

Single channel measurement was performed using tip-dip method as described previously [9]. Briefly, the electrolyte composition was symmetrical for both sides of the diphytanoyl phosphatidylcholine (DPhPC) membrane. The final peptide concentration was 100 nM. Electrolyte solution was 0.5 M KCl solutions buffered with 5 mM *N*-(2-hydroxethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) at pH 7.4. Single channel currents were amplified and measured by Axopatch 1D patch-clamp amplifier (Axon Instruments, Union City, CA, USA) controlled with pClamp 6 (Axon Instruments). Data were filtered at 2 kHz and analyzed with AxoGraph 3.5 (Axon Instruments).

Results

Peptide Design and Synthesis

The peptide structures were shown in Figure 1. BKBA12, BKBA20, and BKBA28 are peptides that possess repeated sequence of Aib-Lys-Aib-Ala and are composed of 12, 20, and 28 amino

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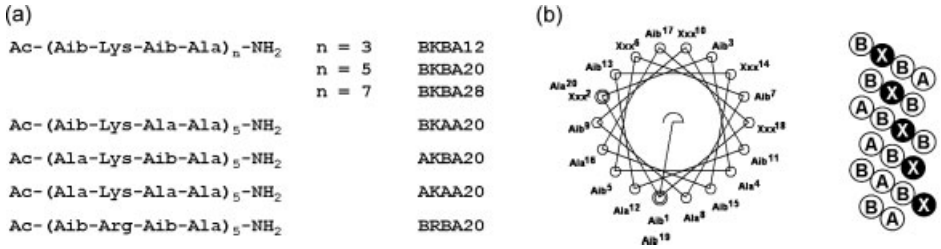

Figure 1. Structures of synthetic peptides. (a) Primary structures and abbreviated names of model peptides evaluated in this study. (b) Diagrams of (left) helical wheel and (right) helical net of five repeated Aib-Xxx-Aib-Ala sequence. When Xxx or X positions in helical wheel and helical net were occupied by cationic residues, the α-helix takes amphiphilic structures, where B stands for Aib.

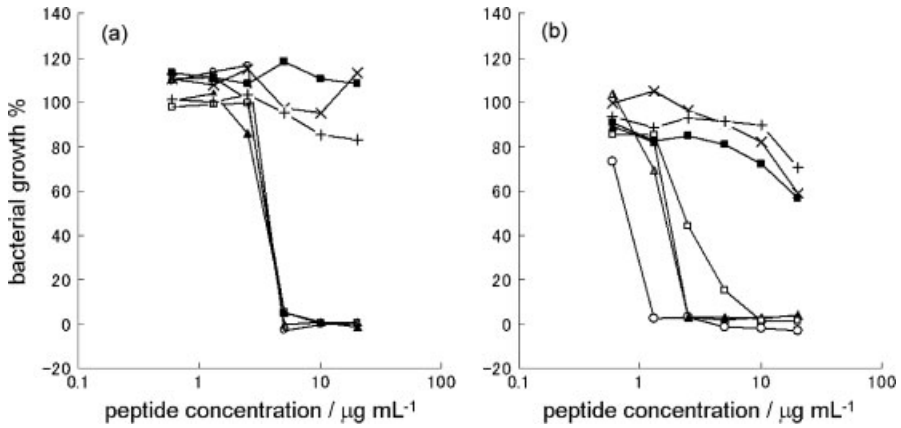

Figure 2. Growth inhibition of *E. coli* and *S. aureus* by synthetic peptides. Symbols represent BKBA12 (×), BKBA20 (△), BKBA28 (▲), BKAA20 (□), AKBA20 (■), AKAA20 (+), and BRBA20 (○). Bacterial growth was estimated by reduction of alamerBlue. (a) Growth inhibition of *E. coli* by synthetic peptides. BKBA20, BKBA28, BKAA20, and BRBA20 completely inhibited bacterial growth at 5 μg/ml peptide concentration. (b) Growth inhibition of *S. aureus* by synthetic peptides. BKBA20 and BKBA28 completely inhibited growth of *S. aureus* at 2.5 μg/ml peptide concentration, whereas BRBA20 inhibited at 1.3 μg/ml.

acid residues, respectively. AKAA20, AKBA20, and BKAA20 were designed to evaluate the effects on number and positions of Aib residues. We previously reported brief antimicrobial activities of BKBA20, AKAA20, AKBA20, and BKAA20 [9,10], but details of the antimicrobial mechanism and antimicrobial activities on various pathogenic bacteria have not been evaluated. To assess whether cationic residues play an important role in antimicrobial activity, Lys residues of BKBA20 were substituted with Arg residues to yield Arg-substituted analog (BRBA20).

Antimicrobial Activities of Synthetic Peptides

Antimicrobial activities of model peptides were evaluated using *S. aureus* and *E. coli* as representative Gram-positive and Gram-negative bacteria. Detection and quantification of antimicrobial activity were determined by reduction of alamerBlue. Relationships of bacterial growth inhibition *versus* peptide dose are illustrated in Figure 2. BKBA20 and BKBA28 inhibited growth of *S. aureus* and *E. coli* at 2.5 and 5 μg/ml peptide concentrations, respectively, whereas BKBA12 did not exhibit the activities. AKAA20 also failed to show antimicrobial activities through all tested concentrations. Notably, BRBA20 showed highest antimicrobial activities in the experiment with *S. aureus*, and it worked even at 1.3 μg/ml peptide concentration. BKAA20 and AKBA20 have same amino acid composition and only the positions of Aib are different. Antimicrobial activity of BKAA20 was comparable to BKBA20, whereas the activities of AKBA20 failed to observe. According to the report of Dorschner *et al*., the presence of physiological carbonate ion species dictates microbial susceptibility to antimicrobial

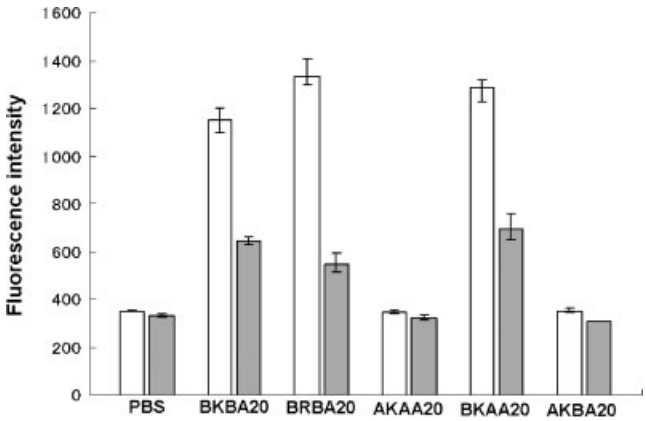

Figure 3. Bacterial membrane permeations caused by synthetic peptides. Membrane permeations were assessed by the uptake of CYTOX-Green in *S. aureus* (white column) and *E. coli* (gray column). Increase in relative fluorescence intensities was observed in bacteria treated with BKBA20, BRBA20, or BKAA20.

peptides [15]. Although carbonate is a ubiquitous molecule in many microenvironments of the body, it increases the sensitivity of antimicrobial peptides by suppressing bacterial gene expressions such as Sigma factor. Actually, no antimicrobial activities of BKBA20 and BKAA20 against *E. coli* were reported in our previous study [9,10]. The previous experiments had been performed by classical diffusion assay without carbonate ions. Discrepancy between

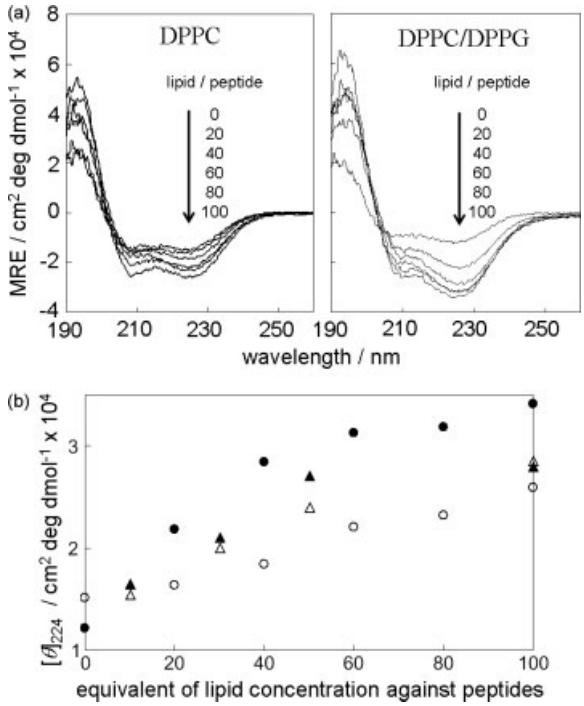

Figure 4. (a) CD spectra of BRBA20 in the presence of 1 mM DPPC (left) or DPPC/DPPG (3 : 1) SUVs (right). The spectrum exhibits typical absorption of α-helix with double minima at 207 and 224 nm. (b) Transition of mean residue ellipticities at 224 nm $[\theta]_{224}$ against addition of SUVs. Closed symbols (●) and (▲) represent the values of BRBA20 and BKBA20, respectively, in the presence of DPPC/DPPG (3 : 1) SUVs. Open symbols (○) and (△) represent the values of BRBA20 and BKBA20, respectively, in the presence of DPPC SUVs. The value of $[\theta]_{224}$ reflects helix contents of peptide. BRBA20 showed higher helical contents than BKBA20 on the anionic membranes. Peptide concentrations were 10 μM. The spectra were corrected in the presence of 0–1 mM SUVs.

the present and previous result would represent the effect of carbonate ions.

Membrane Permeability

Next, we elucidated whether the Aib peptides promote membrane permeability on *S. aureus* and *E. coli*. The membrane permeability was estimated using CYTOX-Green and observed relative fluorescence intensity is shown in Figure 3. BKBA20, BRBA20, and BKAA20, which have antimicrobial activities, exhibited increment of the fluorescence intensities on both bacteria. On the other hand, the fluorescence intensity of AKAA20 and AKBA20, the analogs lacking antimicrobial activities, were as low as PBS. These results indicate that the peptides possessing antimicrobial activities can promote membrane permeability of *S. aureus* and *E. coli*.

CD Study

To evaluate structures of BKBA20 and BRBA20 on phospholipid bilayer, liposome titration CD spectra were measured. Phosphatidylcholine (PC)/phosphatidylglycerol (PG) lipids are often used to mimic the electrostatically charged bacterial membranes, thus, DPPC/DPPG (3 : 1) SUVs were used as bacterial model membrane and zwitterionic DPPC SUVs were used as erythrocytic model membrane. Spectra of BRBA20 exhibited typical patterns of helix structure in the presence of DPPC and DPPC/DPPG SUVs (Figure 4a). Increment of $[\theta]_{224}/[\theta]_{207}$ ratio reflects association of

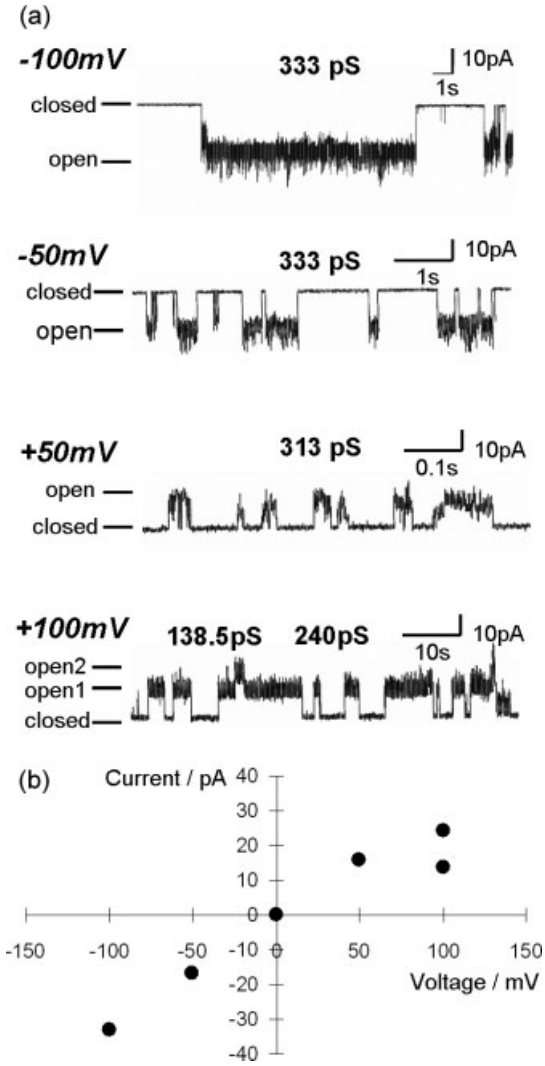

Figure 5. Channel-forming properties of BRBA20. (a) Ion conductance patterns of BRBA20 under various electrical potentials and histogram analyses. The experiment was carried out by tip-dip technique. (b) Current and voltage (I–V) relation of BRBA20 channel.

helices, and specific association of BRBA20 in anionic membranes was suggested [16]. Helical contents of peptides were estimated and compared according to $[\theta]_{224}$ [17]. Relationships of SUV concentration and $[\theta]_{224}$ values of BKBA20 and BRBA20 were plotted in Figure 4b. The values of BKBA20 were comparable in both of DPPC and DPPC/DPPG SUVs. On the other hand, BRBA20 showed larger $[\theta]_{224}$ values in DPPC/DPPG SUVs and the values were also larger than those of BKBA20. Specific helix forming and association propensity of BRBA20 in anionic membrane were suggested.

Channel-forming Property of BRBA20

We previously reported ion channel forming of BKBA20 [9]. BKBA20 showed single-state pattern with 228 pS conductance fluctuation. In this study, channel forming of BRBA20 was evaluated and its property was compared with that of BKBA20. As shown in Figure 5a, conductance of BRBA20 also showed a single-state pattern and a linear I–V relation was observed below ±100 mV (Figure 5b). Observed conductance values were slightly, but larger than that

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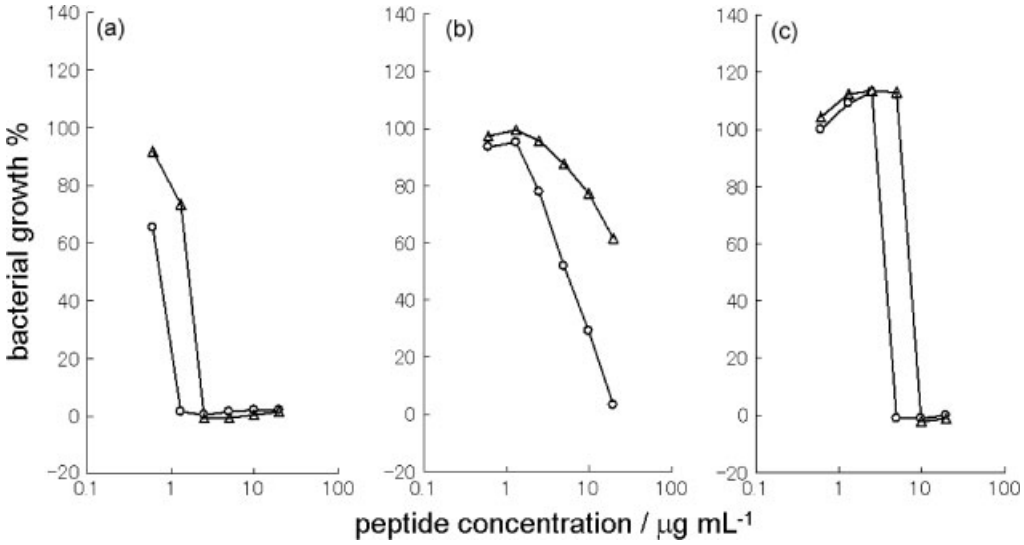

Figure 6. Growth inhibitions of clinically important bacterial strains by BKBA20 and BRBA20. Symbols represent BKBA20 (△) and BRBA20 (○). Bacterial growth was estimated by reduction of alamerBlue. Growth inhibition of (a) MRSA, (b) *S. marcescens*, and (c) MDRP. BRBA20 inhibited all tested bacterial growths at lower concentration than BKBA20.

of BKBA20. These observations suggested that both BRBA20 and BKBA20 have similar channel-forming properties.

Antimicrobial Activities of BKBA20 and BRBA20 Against Clinically Important Bacteria

Antimicrobial activities of BKBA20 and BRBA20 against MRSA, *S. marcescens*, and MDRP, known as clinically important bacteria, were evaluated. As shown in Figure 6, both peptides inhibited bacterial growth, especially, BRBA20 showed higher efficiency in suppressing growth than BKBA20. BRBA20 completely inhibited growth of *S. marcescens* at 20 μg/ml peptide concentration. These results indicate that both peptides are able to inhibit growth of drug-resistant bacteria such as MRSA and MDRP.

Discussion

As shown in Figure 2, BKBA20 and BKBA28 exhibited antimicrobial activities against *E. coli* and *S. aureus*. In contrast, BKBA12 was found to be inactive to detect. It is conceivable that the structure of BKBA20 is a minimum requisite for the effective antimicrobial action among the Lys containing evaluated peptides. As Aib has α,α-dimethylgroups, the residue appears to be a potent inducer of helical structures when incorporated into peptide chain [18]. Accordingly, the Aib-promoted helical conformation would be reinforced peptide amphiphilicity, which is provided by the clustering of hydrophobic and hydrophilic region of molecular surfaces (Figure 1b). The amphiphilicity of peptide has been shown to play an important role in interaction and insertion of peptides to lipid bilayer, followed by bacterial membrane disorder [19,20]. Thus, these reports support our data that the peptide amphiphilicity contributes to antimicrobial activity via bacterial membrane disorder (Figures 2 and 3). We previously reported that BKAA20 and AKBA20 have similar conformational property, hydrophobicity, and amphiphilicity in each other [10]. In this study, BKAA20 showed comparable antimicrobial activity of BKBA20; however, AKBA20 failed to show the activity (Figure 2). In addition, membrane permeability of AKBA20 was also lower than that of BKAA20 (Figure 3). The differences in the biological properties remained for further evaluation in future works.

BRBA20 showed higher antimicrobial activities than BKBA20 through all tested bacteria (Figures 2 and 6). High helix-forming propensity and association nature of BRBA20 on the anionic membrane were suggested from CD study (Figure 4). The strong antimicrobial properties of BRBA20 would be attributed not only to the higher peptide helicity and amphiphilicity but also to Arg-specific interactions with phospholipids of membrane. Preferred membrane insertion and channel formation with larger conductance would reflect the specific interaction of Arg residues with phospholipids [21].

Both BKBA20 and BRBA20 showed antimicrobial activities against *S. marcescens* and *P. aeruginosa* (MDRP) (Figure 6). These bacteria have been shown to secrete various proteases [22,23]. Microbial proteases have also been reported to degrade and inactivate various antimicrobial proteins and peptides [24,25]. Aib-containing natural peptides, such as trichogin, exhibit anti-proteolysis against various proteases [26]. We also previously reported that peptides with high contents of Aib are unaffected by tryptic digestion [13]. Thus, these findings support the idea that Aib-derived protease resistance of peptide plays a critical role in the growth inhibitions of *S. marcescens* and MDRP. Considered together, we propose that BRBA20 has the most appropriate structures as antibiotics, and Arg residue is a preferred cationic source than Lys in antimicrobial action of the peptides.

Peptide cationicity correlates as key features to selective killing of bacteria, because the major difference between eukaryotic and prokaryotic membranes is the much higher concentration of negatively charged lipids on the surface monolayer of bacterial cytoplasmic membrane [19]. Although BKBA20 and BRBA20 have high cationicity, these peptides exhibited 25–55% hemolysis at 20 μg/ml peptide concentration (see supporting information). As the cytoplasmic membranes of red blood cells scarcely contain any anionic phospholipids, interaction between the membranes and cationic peptides is mainly considered to be hydrophobic interaction [19,20]. Agawa *et al*. reported that an amphiphilic helical peptide, 4_6 peptide, contains Arg residues as much as BRBA20 [27]. Although 4_6 peptide showed negligible antimicrobial

activities and strong hemolytic activities, it was improved by the modifications on peptide hydrophobicity and hydrophobic moment [11]. Similar modifications would remain to overcome the hemolysis problem.

In recent years, some of cationic peptides have been reported to reveal immunomodulating properties [28,29]. For example, cathelicidin-related peptide LL-37 has been shown to be involved in chemotaxis of mast cells, neutrophils, and CD4 T cells and can modulate immune responses in dendritic cells and keratinocytes [30–32]. Multiple roles of the cationic peptide are considered to play an important role in innate immune system, and the immunomodulating properties have an ability to generate synergistic effects with antimicrobial properties. Evaluations on the immunomodulating function of the Aib peptides are undergoing in our laboratory. Achievement to obtain both antimicrobial and immunomodulating properties in the artificial peptide would accelerate the step toward practical use of the peptides.

Acknowledgements

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Supporting information

Supporting information may be found in the online version of this article.

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