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ORIGINAL ARTICLE

Activity of tick antimicrobial peptide from *Ixodes persulcatus* (persulcatusin) against cell membranes of drug-resistant *Staphylococcus aureus*

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Persulcatusin (IP), which is an antimicrobial peptide found in *Ixodes persulcatus* midgut, is active against Gram-positive bacteria such as *Staphylococcus aureus*. Multidrug-resistant bacteria in particular methicillin-resistant *S. aureus* (MRSA), vancomycin-intermediate *S. aureus* (VISA) and vancomycin-resistant *S. aureus* (VRSA) are a worldwide clinical concern. In the present study, to explore the potential of IP as a new agent against multidrug-resistant *S. aureus* infections, we evaluated the antimicrobial activity of IP against multidrug-resistant *S. aureus* strains by MIC_{90}, morphological observation with scanning electron microscope (SEM), and the calcein leakage assay of membrane integrity. Among the six antimicrobial peptides used in this study, IP exhibited the lowest MIC_{90} values for both vancomycin-susceptible and -resistant *S. aureus* strains. The IP MIC_{90} against a VISA strain was equivalent to vancomycin, while the MIC_{90} against VRSA was relatively low. SEM observations indicated that bacterial cells exposed to IP were crumpled and showed prominent structural changes. Moreover, IP influenced the cell membranes of both MRSA and VRSA in a mere 5 min, leading to leakage of the preloaded calcein. Although a VISA strain was resistant to the action of IP on cell membrane, the MIC_{90} of IP was lower than that of Nisin, suggesting that IP had another bactericidal mechanism in addition to cell membrane attack. Our results indicate that the synthetic tick antimicrobial peptide, IP exhibits strong antibacterial activity against multidrug-resistant *S. aureus* strains, including VRSA, via both cell membrane attack and another unknown mechanism. IP represents a promising candidate for a new anti-VRSA therapy.
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INTRODUCTION

Since the late 1980s, clinically important bacterial resistance has progressed rapidly.[1] An organism that is representative of this phenomenon is the methicillin-resistant *Staphylococcus aureus* (MRSA), which has been frequently isolated as a drug-resistant bacterium in nosocomial infections. Vancomycin, a glycopeptide antibiotic, has been used for many years to treat MRSA infection, but the alarming emergence of vancomycin-intermediate *S. aureus* (VISA) and vancomycin-resistant *S. aureus* (VRSA) reported in 1997 and 2002, respectively.[2–4] The appearance of antibiotic-resistant bacteria has resulted in bacterial diseases re-emerging as a threat after the many decades since the introduction of the first antibiotic penicillin.[1]

The development of new antibacterial agents is urgently required, and antimicrobial peptides are viewed as ideal candidate agents.[5] Antimicrobial peptides are integral components of the innate immune system of all living organisms, including mammals, plants and insects.[6] Antimicrobial peptides result in strong natural defense in arthropod, particularly antimicrobial peptides of silk moth[7,8] and beetle,[9] which belong to the defensin family. Persulcatusin (IP), a tick antimicrobial peptide found in the midgut of *Ixodes persulcatus*, exhibits antimicrobial activity against Gram-positive bacteria such as *S. aureus*.[10–12] Furthermore, we have reported previously that *S. aureus* strains could not be isolated from *I. persulcatus* during feeding.[13] This is attributable to the antimicrobial activity of IP, which is highly expressed during blood feeding.[10,11] We hypothesized that IP could exhibit antimicrobial activity against VISA and VRSA, as well as methicillin-susceptible *S. aureus* and MRSA.

In this study, we evaluated the antimicrobial activity of IP against multidrug-resistant *S. aureus* strains using MIC, scanning electron microscopy, and a membrane integrity assay. This study represents an exploration of the potential of IP as a new agent against multidrug-resistant *S. aureus* infections.

MATERIALS AND METHODS

Peptide synthesis

Tick and mammalian peptides were synthesized by the solid-phase method, as previously described.[12] The peptides were purified by reverse-phase HPLC (Model LC-8A; Shimadzu Corporation, Kyoto, Japan) on a YMC-A 302 column. The final products were confirmed by ESI MS and were supplied as trifluoroacetates. The trifluoroacetate forms of the peptides were conserved by suspending in Hanks' Balanced Salt Solution (GIBCO, Grand Island, NY, USA)

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at pH 7.4 and stored at −20 °C. Antimicrobial peptides used in this study were Persulcatusin (IP; GFGCPFNQGACHRHCRSIGRRGGYCAGLFKQTCTCYSR), tick antimicrobial peptide from *I. ricinus* (IR; GGYYCPFFQDKCHRHCR SFGRKAGYCGGFLKKTCICV), tick antimicrobial peptide from *Haemaphysalis longicornis* (HAE; GCPLNQGACHNHCRSIGRRGGYCAGIIKQTCTCYRK), tick antimicrobial peptide from *Ornithodoros moubata* (OMBAC; GFGCPFNQ YECHAHCSGVPGYKGGYCKGLFKQTCNCY), bovine myeloid antimicrobial peptide (BMAP28; GGLRSLGKKILRAWKKYGPIIVPIIRI) and lantibiotic peptide (Nisin). Their sequences have been previously reported.[14–17]

Bacterial strains

Four clinical isolate strains of *S. aureus*, MS-1 (methicillin-susceptible *S. aureus*), MR-1 (MRSA), Mu50 (VISA) and VRS1 (VRSA) were used in the present study.[2,3,14] Strains were stored in Trypto Soya (TS; Nissui, Tokyo, Japan) broth with 20% glycerol at −80 °C until use and maintained on TS agar.

MIC_{90} test

MIC_{90} tests were performed by broth microdilution method. The agents tested included six antimicrobial peptides (IP, IR, HAE, OMBAC, BMAP28 and Nisin) and vancomycin. The bacterial cells were grown in TS broth for 18–19 h at 37 °C. The OD at 660 nm (OD660) of pre-cultured bacteria was measured using an Ubest-35 (JASCO Corporation, Tokyo, Japan). OD660 was adjusted to 0.5 by adding TS broth. The bacteria were diluted to a final concentration of $1–5\times10^5$ CFU ml^{-1} with TS broth, after which 50 μl of a bacterial suspension and 50 μl of peptide solution were mixed together in a 96-well plate. Final inoculum organisms per well were $0.5–2.5\times10^5$ CFU ml^{-1}. The peptide solution was prepared by twofold dilution in TS broth, while the IP solution was prepared to final peptide concentrations of 32, 16, 8, 4, 2, 1, 0.5 and 0.25 μg ml^{-1}. Each mixture of bacteria and peptide solution was incubated at 37 °C. The OD660 of the cell suspension was measured after 24–36 h incubation by using a SynergyTM HT (BioTek, Winooski, VT, USA). A control was prepared by mixing 50 μl of bacterial suspension, 40 μl of TS broth and 10 μl of Hanks' Balanced Salt Solution. The MIC_{90} of the peptides was defined as the lowest concentration of the peptide that reduced growth by >90%.

Scanning electron microscope (SEM) observation

Mid-logarithmic phase MR-1 and VRS1 cells were resuspended at a concentration of 1×10^8 CFU ml^{-1} in Dulbecco's phosphate-buffered saline (DPBS; GIBCO) and incubated with vancomycin or IP for 30 min at 37 °C. Nisin and peptide diluent (DPBS) served as positive and negative controls, respectively. The mixture was then harvested by centrifugation (1000 *g*, 10 min), and bacteria pellets were fixed with 2% glutaraldehyde solution for 2 h at 4 °C. The bacteria were dehydrated in a graded series of alcohols. After that, the samples were then coated with palladium alloy and were observed with a Hitachi SU8000 scanning electron microscope (Hitachi High-Technologies Corporation, Tokyo, Japan).

Table 1 MICs against MSSA, MRSA, VISA and VRSA strains of *Staphylococcus aureus*

	MIC (μg ml^{-1})						
Bacterial strains	*IP*	*IR*	*HAE*	*OMBAC*	*BMAP28*	*Nisin*	*vancomycin*
MS-1 (MSSA)	1	2	>32	8	8	8	1
MR-1 (MRSA)	2	16	>32	8	16	8	1
Mu50 (VISA)	8	>32	>32	>32	>32	>32	8
VRS1 (VRSA)	2	32	>32	8	32	16	>32

Abbreviations: BMAP28, bovine myeloid antimicrobial peptide; HAE, tick antimicrobial peptide from *Haemaphysalis longicornis*; IR, tick antimicrobial peptide from Ixodes ricinus; IP, persulcatusin; MSSA, methicillin-susceptible *S. aureus*; MRSA, methicillin-resistant *S. aureus*; OMBAC, tick antimicrobial peptide from *Ornithodoros moubata*; VISA, vancomycin-intermediate *S. aureus*; VRSA, vancomycin-resistant *S. aureus*.

Calcein leakage assay

Peptide-induced membrane permeabilization of *S. aureus* was quantified by monitoring the leakage of the preloaded fluorescent dye, calcein. Each bacterial strain was grown in TS broth to the logarithmic phase at 37 °C. Cells were then harvested by centrifugation, washed twice with DPBS, and then adjusted spectrophotometrically to an OD600 of 1.0 ($\approx10^9$ CFU ml^{-1}) in PBS containing 10% (vol/vol) TS broth. Cells were then incubated with 3 μM calcein AM for 1.5 h at 37 °C. Calcein-loaded cells were harvested by centrifugation (3000 *g*, 10 min), suspended in DPBS, and diluted to achieve a final inoculum of 10^7 CFU ml^{-1}. A Co^{2+} solution was added to the bacterial suspensions at 2 μM concentration to quench the extracellular calcein fluorescence. Aliquots (100 μl) of the bacterial suspensions were then added to a sterile black-wall 96-well plate. IP was then added at its MIC_{90} value. Bacteria treated with sterile DPBS and Nisin at its MIC_{90} served as negative and positive controls, respectively. Bacteria treated with vancomycin at its MIC_{90} served as a no calcein leakage sample for a short period. Calcein leakage was measured for 30 min using a fluorescence plate reader, Synergy HT (BioTek). The calcein leakage rate (%) was calculated as the absolute percent peptide-induced calcein leakage relative to untreated calcein-loaded cells. Experiments were performed in triplicate.

RESULTS

Antimicrobial activity of IP for four strains having drug resistance different from one another

The antibacterial activity of IP was compared with the other antimicrobial peptides and a glycopeptide antibiotic, vancomycin (Table 1). MIC_{90} end points of IP against all strains were clearly defined. Excluding HAE, all antimicrobial peptides exhibited high antimicrobial activity against strains MS-1 and MR-1. The IP MIC_{90} against strain Mu50 was 8 μg ml^{-1} and was equivalent to vancomycin, while the MIC_{90} of the other antimicrobial peptides were >32 μg ml^{-1}. Thus, IP exhibited the most potent anti-*S. aureus* activity among the antimicrobial peptides evaluated in the present study.

Ultrastructural alternation of MRSA and VRSA cells by IP exposure

To elucidate the effects of IP exposure on bacterial cells, morphological changes in MR^{-1} and VRS1 cells treated with IP and vancomycin were observed by scanning electron microscope. Bacterial cells were exposed to IP and vancomycin at various concentrations for 30 min and subsequently observed. Control bacterial cells, which were exposed only to DPBS, displayed a smooth surface without cellular debris (Figures 1a and e), while the positive control bacteria cells exposed to Nisin exhibited considerable structural changes (Figures 1b and f). Bacterial cells exposed to vancomycin were used as a negative control and showed no morphological changes (Figures 1c and g). In contrast, bacterial cells exposed to IP became crumpled and showed structural changes similar to those observed in the presence of Nisin (Figures 1d and h).

IP-induced membrane permeabilization in MSSA, MRSA, VISA and VRSA cells

To compare the effect on methicillin-susceptible *S. aureus*, MRSA, VISA and VRSA cells between IP and vancomycin, we monitored membrane integrity of *S. aureus* using the calcein leakage assay. Damage to membrane integrity was indicated by calcein leakage from bacterial cells, leading to a reduction of fluorescence intensity. IP-induced membrane permeabilization in strains MS-1, MR-1, Mu50 and VRS1 were quantified by monitoring preloaded calcein leakage over a 30 min period. As shown in Figure 2, vancomycin had no effect on cell membrane integrity over the 30 min period. IP perturbed MS-1, MR-1 and VRS1 cell membranes leading to calcein

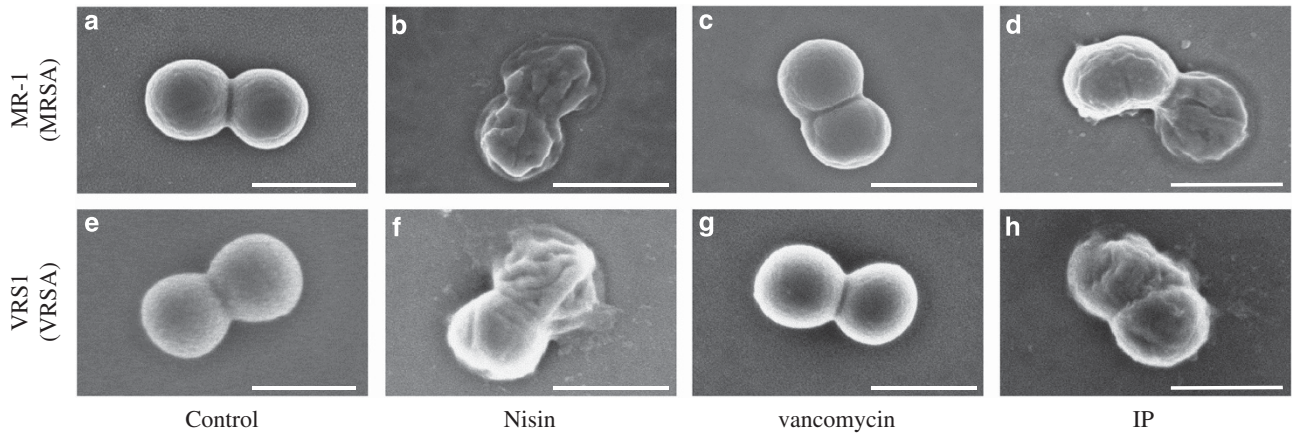

Figure 1 Scanning election microscope images of MRSA (MR^{-1}) and VRSA (VRS1) treated with Nisin, vancomycin, and IP for 30 min. The concentrations of Nisin, IP and vancomycin were 2.5×MIC (20 μg ml^{-1}), 5×MIC (10 μg ml^{-1}) and 5×MIC (5 μg ml^{-1}) in strain MR^{-1}, and 4×MIC (64 μg ml^{-1}), 4×MIC (8 μg ml^{-1}) and 32 μg ml^{-1} in strain VRS1, respectively. Scale bars, 1 μm.

leakage within 5 min, although the effect was not as strong as Nisin. The drug effects of IP were concentration-dependent in strains MS-1, MR-1 and VRS1. On the other hand, in contrast to the anti-membrane activity seen with Nisin, IP did not cause membrane-damage to strain Mu50.

DISCUSSION

MRSA has still been a major clinical concern all over the world; the number of death from MRSA infection has been more than that from HIV/AIDS in the United States.[18] Although there have been few reports on the incidents of VRSA worldwide, VISA strains, exhibiting reduced susceptibility to vancomycin, have been increasingly concerned in clinical practice. The production of new antimicrobials alternative to vancomycin for combatting drug-resistant Gram-positive bacteria has recently become an essential task. To our knowledge, this is the first report to show the antibacterial activity of the synthetic tick antimicrobial peptide persulcatusin against vancomycin-non-susceptible *S. aureus*, VISA and VRSA.

In the present study, the MIC_{90} of IP ranged from 1 to 8 μg ml^{-1} in methicillin-susceptible *S. aureus*, MRSA, VISA and VRSA strains, and were as well or better than those of the synthetic antimicrobial peptide RRIKA (2–4 μg ml^{-1}) and the peptidomimetics LTX-109 (2–4 μg ml^{-1}), previously reported as a new class of drugs with activity against VISA and VRSA strains.[19,20] Also, IP exhibited the most potent anti-*S. aureus* activity among the tick antimicrobial peptides evaluated in the present study. Although VISA strain Mu50 appeared to be resistant to the action of IP on cell membrane, the MIC_{90} of IP was lower than that of Nisin. These results strongly suggested that IP inhibited the bacterial growth mainly via another bactericidal mechanism in addition to cell membrane attack, which was well known as the main effect of antibacterial activity in conventional antimicrobial peptides. We have reported that the structural integrity of IP is maintained by three S–S bonds, unlike antimicrobial peptides from the cathelicidin family, and IP was both cationic and amphipathic.[12] The remarkable and particular antimicrobial activity of IP against staphylococcal strains might result from its unique structure and character.

Antimicrobial peptides have been expected to have fast-acting bactericidal activity, the antibacterial mechanism has been attributed to their pore-forming effect on bacterial membranes.[21] In the present study, rapid onset of pharmacological effect of IP was observed in calcein leakage assay, consistent with the results of the electron-microscopic observation. This obvious difference of bactericidal mechanism from vancomycin suggests that IP is free of cross-resistance to conventional antimicrobial agents.

Mechanisms involved in the resistance to certain antimicrobial peptides have been reported, such as changing the charge on surface molecules and proteolytic cleavage by the release of extracellular proteases.[22–24] Conversely, there are reports that passage under sub-MIC concentrations produced lesser increases in experimental MIC for antimicrobial peptides than conventional antimicrobial agents, and no cross-resistance has been observed among different antimicrobial peptides.[25,26] Thus, it is expected that bacteria will find it difficult to acquire resistance to antimicrobial peptides.[22,27] Antimicrobial peptides interact with bacteria by electrostatic forces between their positive amino acid residues and negative charges present on the bacterial cell surface, thereby causing damage to the cell membrane.[28] Because of the obligatory interaction between antimicrobial peptides and bacterial cytoplasmic membranes, and the consequent necessity to reconfigure the membrane, it is difficult for bacteria to develop resistance to antimicrobial peptides.[29] It is likely that IP has properties suited for the epoch of multidrug-resistant bacteria.

In summary, persulcatusin, IP exhibited strong antibacterial activity *in vitro* against multidrug-resistant *S. aureus* strains including VRSA, via both cell membrane attack and another unknown mechanism. The activity was as good or better than those of vancomycin and other published antimicrobial peptides.[14–17,19,20] Although, host cytotoxicity and instability *in vivo* are major limitations of antimicrobial peptides in their application as antimicrobial drugs, we have reported recently that IP lacked toxicity to mammalian and human cells such as fibroblasts, colon epithelial cells and erythrocytes, and built a stable structure unlike other antimicrobial peptides.[12] Thus, IP combines the most important three advantages for clinical setting, which are potent bactericidal activity, low toxicity and structural stability. This antimicrobial peptide represents a promising candidate as a new agent to combat infections with MRSA, VISA and VRSA.

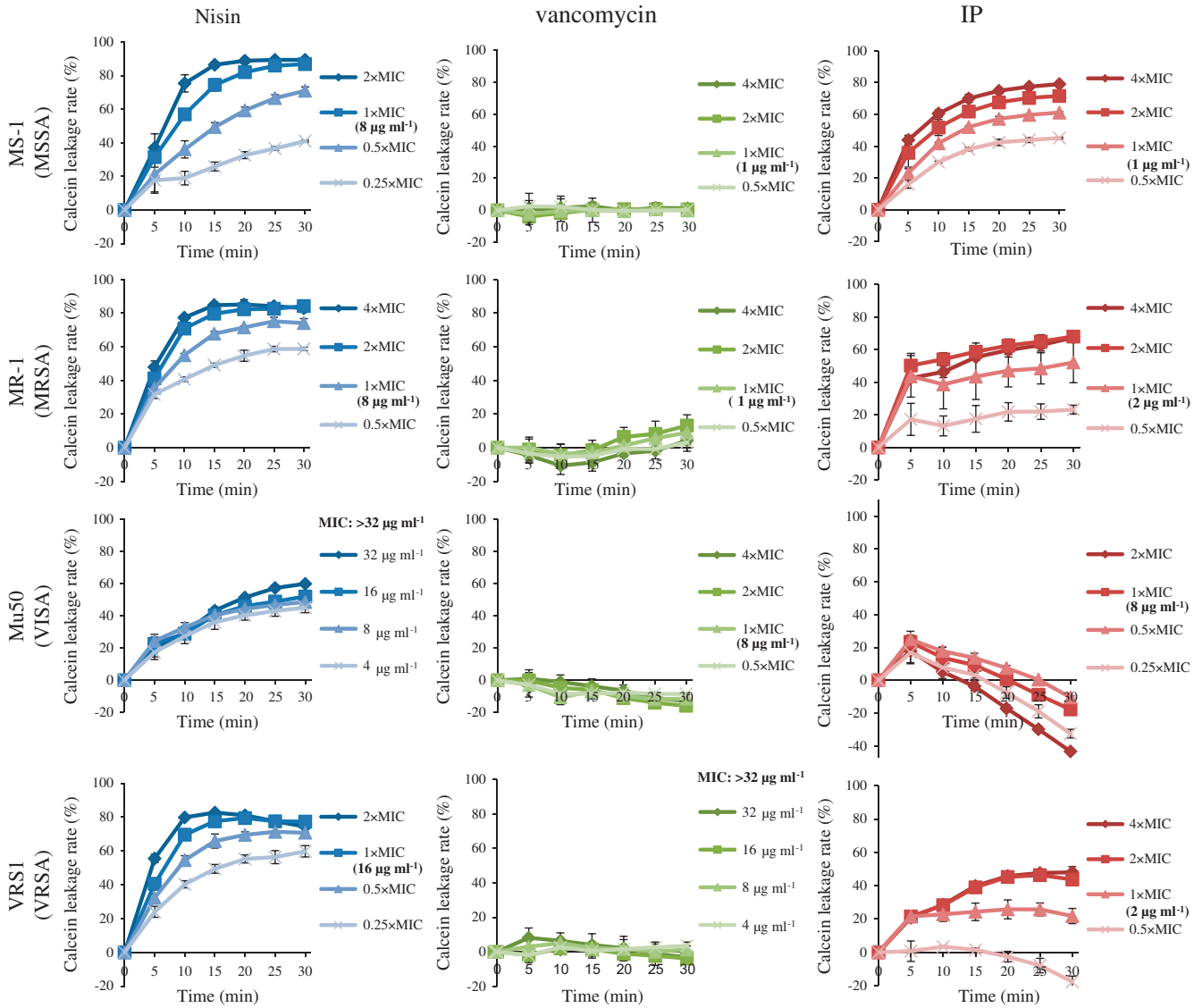

Figure 2 Comparison of IP-induced membrane permeabilization in MSSA (MS-1), MRSA (MR-1), VISA (Mu50) and VRSA (VRS1) strains by calcein leakage assay. Bacteria treated with Nisin and DPBS served as positive and negative controls, respectively. Drug concentrations of Nisin in strain Mu50 and vancomycin in strain VRS1 were fixed from 4 to 32 μg ml^{-1} because of high-level resistance. The means and s.d. of triplicate determinations were presented. MSSA, methicillin-susceptible *S. aureus*.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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