Potent Antibacterial Activity of Synthetic Peptides Designed from Salusin-β and HIV-1 Tat(49–57)

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Salusin-β is an endogenous bioactive peptide that was identified in a human full-length enriched cDNA library using bioinformatics analyses. In our previous study, we found that synthetic salusin-β exhibits antibacterial activity against only Gram-positive microorganisms such as *Staphylococcus aureus* NBRC 12732. Salusin-β has an ability to depolarize the cytoplasmic membrane of this bacterium, and this phenomenon may be linked to the antibacterial activity of this peptide. A cell-penetrating peptide (CPP), human immunodeficiency virus (HIV)-1 transactivator of transcription (Tat) (49–57) is a short cationic peptide that can traverse cell membranes. In this report, synthetic peptide conjugates of salusin-β and HIV-1 Tat(49–57) showed potent antibacterial activities against both Gram-positive *Staphylococcus aureus* NBRC 12732 and Gram-negative *Escherichia coli* NBRC 12734. The synthetic peptides also depolarized the cytoplasmic membrane of *Escherichia coli* NBRC 12734 as well as *Staphylococcus aureus* NBRC 12732. These results suggested that HIV-1 Tat(49–57) is a protein transduction domain or CPP that changes the interaction mode between salusin-β and the cell membrane of *Escherichia coli* NBRC 12734. By binding to HIV-1 Tat(49–57), salusin-β showed a broad antibacterial spectrum regardless of whether the target was a Gram-positive or Gram-negative bacterium.

Key words salusin-β, antibiotic activity, conjugate, human immunodeficiency virus-1 transactivator of transcription (49–57), antibacterial action mechanism

Introduction

Shichiri et al. discovered the multifunctional endogenous bioactive peptides, namely salusin-α and salusin-β. Salusin-β has been detected in various human tissues such as the nervous system, cardiovascular system, kidneys, monocytes, and macrophages, as well as body fluids such as plasma and urine.1–3 Several studies have demonstrated that these peptides are associated with conditions such as hypertension, atherosclerotic cardiovascular disease, acute coronary syndrome, and vascular resistance.1–8 In a previous study,5 we found that salusin-β exhibits antibacterial activity against Gram-positive microorganisms, such as *Staphylococcus aureus* NBRC 12732, but had no activity against Gram-negative bacteria. We also reported that salusin-β depolarizes the bacterial cytoplasmic membrane of *S. aureus* NBRC 12732, which may be linked to its antibacterial activity.

Frankel and Pabo6 and Green and Loewenstein7 found that the human immunodeficiency virus (HIV)-1 transactivator of transcription (Tat) protein involved in the transcriptional control of HIV translocates through the cell membrane and into cells. Vives et al.8 showed that 11 amino acids in the RNA-binding domain are important for the intracellular translocation of Tat protein. Furthermore, Weeks et al.9 suggested that Tat attachment to cells is mediated through a 90-kDa cell surface protein that binds to a Tat domain between amino acids 49 and 57. The cell-penetrating peptide (CPP), HIV-1 Tat(49–57) is a short cationic peptide that can traverse cell membranes. Methods for using these membrane-permeable peptides have been used for intracellular delivery of molecules that are difficult to introduce into cells due to their high molecular weight and high hydrophilicity. These transmembrane peptides are called protein transduction domains (PTDs) or CPPs, and it has been reported that various substances such as small molecule drugs, oligonucleic acids and their derivatives, and high molecular weight polymers can be efficiently delivered into cells using these peptides.10–13

In order to develop salusin-β analogs that are active against various bacteria, we characterized the HIV-1 Tat(49–57) as a PTD/CPP. In the present study, we synthesized peptides combining salusin-β and HIV-1 Tat(49–57), and investigated the antibacterial activity and antimicrobial mechanism of these synthetic peptides. The results from this study confirmed that the HIV-1 Tat(49–57) is a suitable PTD/CPP to alter the antibacterial spectrum of salusin-β.

Results and Discussion

Synthesis of Peptides The synthesis of salusin-β, HIV-1 Tat(49–57), HIV-1 Tat(49–57)-salusin-β and salusin-β-HIV-1 Tat(49–57)-Lys-NH2 (Fig. 1) was performed as reported in Experimental. The product was extensively purified by HPLC and gel-filtration prior to the examination of its biological activity. The homogeneity of the purified peptide was confirmed by analytical HPLC and matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry. The purity of the synthetic peptides was >98%.

Antimicrobial Activity of the Synthetic Peptides The minimum inhibitory concentration (MIC), which is the minimum amount of peptide necessary to completely inhibit bacte-
Salusin-β
H-Ala-Ile-Phe-Ile-Phe³-Ile-Arg-Trp-Leu-Leu⁶-Lys-Leu-Gly-His-His¹⁵-Gly-Arg-Ala-Pro-Pro³⁰-OH
HIV-1 Tat (49–57)
H-Arg-Lys-Lys-Arg-Arg³-Gln-Arg-Arg-Arg-OH
[1] HIV-1 Tat (49–57)-Salusin-β
H-Arg-Lys-Lys-Arg-Arg³-Gln-Arg-Arg-Ala¹⁰-Ile-Phe-Ile-Phe-Ile¹³-Arg-Trp-Leu-Leu-Lys⁹.
Leu-Gly-His-His-Gly³⁵-Arg-Ala-Pro-Pro-OH
[2] Salusin-β-HIV-1 Tat (49–57)-Lys-NH₂
H-Ala-Ile-Phe-Ile-Phe³-Ile-Arg-Trp-Leu-Leu⁶-Lys-Leu-Gly-His-His¹⁵-Gly-Arg-Ala-Pro-Pro³⁰.
Arg-Lys-Lys-Arg-Arg³-Gln-Arg-Arg-Lys⁹-NH₂

Fig. 1. Structures of Salusin-β, HIV-1 Tat(49–57), Synthetic Peptides [1] and [2]

Table 1. Antibacterial Activities of Salusin-β, HIV-1 Tat(49–57), Synthetic Peptides [1] and [2]

| Peptides          | MIC*  |
|-------------------|-------|
|                   | A     | B    |
| Salusin-β         | 2     | >128 |
| HIV-1 Tat (49–57) | 640   | 1280 |
| [1] HIV-1 Tat (49–57)-Salusin-β | 0.5   | 1    |
| [2] Salusin-β-HIV-1 Tat (49–57)-Lys-NH₂ | 0.5   | 1    |
| Salusin-β + HIV-1 Tat (49–57) | 4**   | >32**|

A: Staphylococcus aureus NBRC 12732, B: Escherichia coli NBRC 12734.
* MIC values in nmol/mL were determined using a microplate dilution method with 10⁶ organisms per mL medium. Antibacterial activity tests were performed 7–10 times for each peptide. **: These values indicate the concentrations of salusin-β and HIV-1 Tat(49–57), respectively.

On the other hand, peptides [1] and [2], which were designed from salusin-β and HIV-1 Tat(49–57), showed lower antibiotic activities against both bacteria than peptide [1] and [2], especially for E. coli NBRC 12734. The chemical binding of HIV-1 Tat(49–57) to salusin-β made this peptide more potent as antibiotics against both bacteria. These results indicated that HIV-1 Tat(49–57) is a PTD/CPP that enhances the antimicrobial activity of salusin-β against both S. aureus NBRC 12732 and E. coli NBRC 12734, and that the peptide synthesized here may have a broad antibacterial spectrum. Lee et al. reported that binding of magainin to a PTD/CPP induces only a 2- to 4-fold increase in antimicrobial activity against Gram-positive bacteria, but elicits antimicrobial activity against Gram-negative bacteria by a 4- to 16-fold. In our study, we demonstrated for the first time that salusin-β, which has antibacterial activity only against Gram-positive bacteria, also exerts potent antibacterial activity against Gram-negative bacteria by the chemical binding to a PTD/CPP.

Cytoplasmic Membrane Depolarization Inhibiting the biological synthesis of bacterial nucleic acids, proteins, and the cell wall are the mechanisms of action for antibiotics. The principal mode of antibiotic action has been proposed to result from an interaction between antibacterial peptides and the cell membrane of target microorganisms. In a previous study, as shown in a part of Fig. 2(A), we investigate the mode of antibacterial action of salusin-β using S. aureus NBRC 12732, which is the most sensitive toward this peptide. The addition of salusin-β causes the leakage of 3,3′-dipropylthiadicarbocyanine iodide (DiSC₃(5)) dye from this bacterium. A cytoplasmic membrane-depolarizing assay measured using fluorescent dye revealed increases in fluorescence units. This indicated that salusin-β depolarizes the bacterial cytoplasmic membrane of S. aureus NBRC 12732. These results suggested that cytoplasmic membrane depolarization activity of the peptide is linked to its antibacterial activity.

In this study, we investigated the activity of synthetic peptides using S. aureus NBRC 12732 and E. coli NBRC 12734. As shown in Fig. 2(A), addition of 0.5 nmol/mL peptides [1] and [2] to S. aureus NBRC 12732 caused an increase of approximately 60 fluorescence units at 150 s after the addition of the peptides. The addition of 0.9 nmol/mL of salusin-β to E. coli NBRC 12734 caused no leakage of dye from this bacterium (Fig. 2(B)). On the other hand, addition of peptides [1] and [2] to E. coli NBRC 12734 increased the reading of fluorescence units in the assay buffer. The fluorescence intensity of the leaked dye depended on the concentration of the peptides in both bacterial strains. Peptides [1] and [2] depolarized the cytoplasmic membrane of both S. aureus NBRC 12732 and E. coli NBRC 12734.
HIV-1 Tat(49–57) is a PTD/CPP that changes the interaction mode between salusin-β and the cell membrane of *E. coli* NBRC 12734, and that HIV-1 Tat(49–57) may alter the antimicrobial spectrum of antibacterial peptides such as salusin-β.

**Conclusion**
Salusin-β shows high antibacterial activity against Gram-positive bacteria but not against Gram-negative bacteria. Peptides [1] and [2], in which salusin-β and HIV-1 Tat(49–57) were chemically bonded, showed potent antibacterial activity against both Gram-positive and Gram-negative bacteria. Peptides [1] and [2] also had the ability to depolarize the cytoplasmic membrane, and this may be linked to the antibacterial activity of this agent. These results suggested that conjugation of HIV-1 Tat(49–57) to salusin-β causes the peptide to have a broad antimicrobial spectrum.

**Experimental**
**General** 9-Fluorenylmethoxycarbonyl (Fmoc) amino acids and all reagents for peptide synthesis were purchased from Watanabe Chem., Ind., Ltd. (Hiroshima, Japan) and Peptide Institute Inc. (Osaka, Japan), respectively. Unless otherwise noted, all materials were obtained from commercial suppliers and used without further purification. HPLC was performed on an apparatus equipped with two 510 pumps (Waters Corp., MA, U.S.A.), a U6K injector (Waters), Lambda-Max Model 481 LC Spectrophotometer (Waters), 680 Automated Gradient Controller (Waters), and Chromatocorder 21 (System Instruments Co., Ltd., Tokyo, Japan). Gel filtration column chromatography was performed using Toyopearl HW-40-S (Tosoh Corp., Tokyo, Japan). MALDI-TOF mass spectrometry was conducted on a Model Voyager RP BioSpectrometry Workstation (PerSeptive Biosystems, Inc., MA, U.S.A.).

**Preparation of Synthetic Peptides** All peptides used in this study were synthesized using a continuous flow solid phase method with an Fmoc-strategy using an automated peptide synthesizer (Model Pioneer; Life Technologies, CA, U.S.A.). The peptides including salusin-β, HIV-1 Tat(49–57), HIV-1 Tat(49–57)-salusin-β, and salusin-β-HIV-1 Tat(49–57)-Lys-NH₂ prepared by the procedure in accordance with a previous report. The protective groups of the amino acid side chains used here were 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf), trityl (Trt), and tert-butyloxycarbonyl (Boc) for Arg, His, Gln, and Lys, respectively.

**Determination of the Antibacterial Activities of the Synthetic Peptides** *Staphylococcus aureus* NBRC 12732 and *Escherichia coli* NBRC 12734 were grown overnight at 37°C on nutrient agar medium and harvested in sterile saline. The densities of the bacterial suspensions were determined...
at 600 nm, using a standard curve relating absorbance to the number of colony forming units.

MIC values of the synthetic peptides against Gram-positive and Gram-negative bacterial strains were assayed using the microplate dilution method in accordance with the method described previously. IC10. MIC was expressed as the lowest final concentration (nmol/mL) at which no growth was observed. Antibiotic activity tests were performed 7–10 times for each peptide and we obtained the same values for each peptide within the limits of error.

**Cytoplasmic Membrane Depolarization Assay** The interaction between the synthetic peptides and the bacterial cytoplasmic membrane was determined using a membrane potential-sensitive cyanine dye, DiSC3(5) (Sigma-Aldrich Co., LLC, MO, U.S.A.). S. aureus NBRC 12732 and E. coli NBRC 12734 were grown to mid-log phase at 37°C in tryptosoya broth (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). A 100 µL aliquot of the bacterial suspension (OD600 = 0.5) in assay buffer (5 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 20 mmol/L glucose containing 0.1 mol/L KCl, pH 7.4) was added to 889 µL of the same buffer followed by 1 µL of 1 mmol/L DiSC3(5) in DMSO. The bacterial suspension was then incubated at 20°C until a stable reduction in fluorescence from dye uptake and quenching in the bacteria in response to an intact membrane potential. The synthetic peptides were then added at adequate final concentrations. Changes in fluorescence in response to membrane depolarization were recorded with a Hitachi F-2700 Fluorescence Spectrophotometer (Hitachi, Ltd., Tokyo, Japan) at an excitation wavelength of 622 nm and emission wavelength of 670 nm at 20°C.

In the case of E. coli NBRC 12734, all assay procedures were performed at 25°C and bacterial cells collected in mid-log phase were preincubated in the assay buffer containing 15 mmol/L ethylenediaminetetraacetic acid for 15 min. IC11.

**Conflict of Interest** The authors declare no conflict of interest.

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