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Effects of positively charged arginine residues on membrane pore forming activity of Rev–NIS peptide in bacterial cells

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ABSTRACT

Here, we investigated antibacterial effects of Rev–NIS and suggested the role of positively charged amino acids on membrane pore forming activity of the peptide in bacterial cells, by synthesizing two analogs, Anal R and Anal S. Based on the amphipathic property of Rev–NIS, Anal R and Anal S were designed by substituting E^1 and L^3 to R and L^3 to S, respectively. The circular dichroism (CD) spectroscopy showed that Anal R and Anal S have the same conformation of Rev–NIS, with a significant fraction of helical structure. In succession, the antibacterial susceptibility testing showed that Rev–NIS and its analogs possessed significant activities (Anal R > Rev–NIS > Anal S), without hemolytic effects, against bacterial pathogens including antibiotics-resistant strains. Moreover, the membrane studies, 3,3′-dipropylthiadicarbocyanine iodide ($diSC_35$) staining and FITC-dextran (FD) leakage assay demonstrated that the analogs as well as Rev–NIS acted on the bacterial membranes and potently made pores, with the hydrodynamic radius between 1.4 nm and 2.3 nm. Especially, Anal R made larger pores than other peptides, with the radius between 2.3 nm and 3.3 nm. These results also corresponded to the result of antibacterial susceptibility testing. In summary, this study indicates that the two arginine residues are more influential than the hydrophobicity or the helicity, regarding the molecular activity of the peptide, and finally suggests that Anal R peptide may be applied to novel antibacterial agents.
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1. Introduction

Recently, due to the emergence of organisms possessing resistance to several antibiotics [1,2], a variety of diseases are becoming increasingly difficult to treat, including microorganisms responsible for infections in hospitalized patients, food-borne pathogens, and sexually transmitted pathogens, some of which are known to be resistant to most antimicrobial drugs [3–5]. The clinical impact of the above noted resistance is regarded as immense, characterized by increased cost, morbidity, or mortality [6,7]. Therefore, the increasing resistance against various commonly used antibiotic agents requires immediate actions, continuous studies and the eventual suggestion of novel bactericidal and fungicidal agents [8,9]. Although the search for novel naturally occurring antimicrobial molecules such as antimicrobial peptides (AMPs) has focused on the molecules isolated from plants or animals [10], actually, there is no limitation with respect to the sources for the selection of AMPs. Most AMPs can interact, permeate and physically disrupt the cell plasma membrane, causing damages that are hard to repair and sometimes fatal, in contrast to conventionally used antibiotics that are targeting specific intracellular enzymes or DNA. Conclusively, resistance may occur with lower probability [11].

HIV-1 encodes the regulatory protein Rev constantly shuttling between the nucleus and the cytoplasm in the host cells. This protein is concerned in the control of a signal-mediated active import and export system of HIV-1, and the function of NIS region is especially critical [12–15]. If the NIS does not carry out its role, Rev may migrate back through the nuclear pore against the well-controlled nucleocytoplasmic shuttling to conduct its own role in each compartment. In the whole sequence of NIS, residues 11–20 (Rev–NIS, ELLKAVRLIK) are composed of the conserved hydrophilic motif forming an amphipathic helix. This helical motif was regarded to be critical with respect to the function of NIS and Rev itself [16]. Previously, we found that Rev–NIS exerted antifungal activities by disrupting fungal membranes of *Candida albicans* and this result suggested a therapeutic potential as new antifungal agents [17]. In this study, first, we investigated the antibacterial activity of Rev–NIS. Thereafter, we designed and synthesized two analogs, Anal R and Anal S, to investigate the effect of positively charged amino acids regarding the antibacterial activity of the peptides. Concretely, Anal R and Anal S were designed by substituting the specific amino acids, Glu^1 and Leu^3 to Arg (Anal R) and Leu^3 to Ser (Anal S), respectively.

Abbreviations: CD, circular dichroism; $diSC_35$, 3,3′-dipropylthiadicarbocyanine iodide; FITC, fluorescein isothiocyanate; FD, FITC-dextran; AMP, antimicrobial peptide; NIS, nuclear entry inhibitory signal; TFE, 2,2,2-trifluoroethanol; SDS, sodium dodecyl sulfate; MIC, minimum inhibitory concentration; ARPA, antibiotics-resistant *Pseudomonas aeruginosa*; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; DCM, dichloromethane; DMF, *N,N*-dimethylformamide; Fmoc, fluorenylmethyloxycarbonyl; HOBt, 1-hydroxybenzotriazole; DIC, 1,3-diisopropylcarbodiimide; TFA, trifluoroacetic acid; CLSI, clinical and laboratory standards institute; ITO, indium tin oxide

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2. Materials and methods

2.1. Solid-phase peptide synthesis

Anygen Co. (Gwangju, Korea) carried out the peptide synthesis. Anygen Co. offers the following procedures for peptide synthesis. The assembly of peptides consisted of a 60-min cycle for each residue at ambient temperature as follows: (1) the 2-chlorotrityl (or 4-methylbenzhydrylamine amide) resin was charged to a reactor and then washed with DCM and DMF, respectively, and (2) a coupling step with vigorous shaking using a 0.14 mM solution of Fmoc-L-amino acids and Fmoc-L-amino acids preactivated for approximately 60 min with a 0.1 mM solution of 0.5 M HOBt/DIC in DMF. Finally, the peptide was cleaved from the resin using a TFA cocktail solution at ambient temperature [18,19].

2.2. Peptide characterization

Analytical and preparative reverse-phase HPLC runs were performed with a Shimadzu 20 A or 6 A gradient system. Data was collected using an SPD-20 A detector at 230 nm. Chromatographic separations were achieved with a 1%/min linear gradient of buffer B in A (A = 0.1% TFA in H_2O; B = 0.1% TFA in acetonitrile (CH_3CN)) over 40 min at flow rates of 1 and 8 ml/min using Shimadzu C_{18} analytical (5 μm, 0.46 cm × 25 cm) and preparative C_{18} (10 μm, 2.5 cm × 25 cm) columns, respectively. The HPLC retention time (min) of Rev–NIS, Anal R, and Anal S is 18.992, 13.550, and 15.433, respectively. The mass spectrometry was also performed. The observed mass of Rev–NIS, Anal R, and Anal S is 1183.2, 1251.9, and 1155.8, respectively.

2.3. CD analysis

CD spectra of the peptides were recorded using a spectropolarimeter (Jasco J720; Japan). All samples were maintained at 25 °C during the analysis. Four scans per sample were performed over a wavelength range of 190–250 nm at 0.1 nm intervals. The spectra were measured in 50% (v/v) TFE and 30 mM SDS in 10 mM sodium phosphate buffer, pH 7.2, respectively, at 25 °C using a 1-mm path-length cell. The peptide concentrations were 50 μM. The mean residue ellipticity, [θ], is given in deg·cm^2·dmol^{-1}: $[\theta] = [\theta]_{obs}$ (MRW/10lc), where $[\theta]_{obs}$ is the ellipticity measured in millidegrees, MRW is the mean residue molecular weight of the peptide, c is the concentration of the sample in mg/ml, and l is the optical path-length of the cell in cm [20,21].

2.4. Isolation of bacterial strains and determining of antibacterial susceptibility

All bacterial strains were isolated from a tertiary teaching hospital in Daegu, South Korea. All isolates were processed by the MicroScan WalkAway 96 system for genus and species identification and the determination of the antimicrobial susceptibility testing. MicroScan Gram positive MIC/combo (PC1A) panel, Gram negative MIC/combo (NC44) panel, and Gram negative breakpoint combo (NBC39) panel were used in determining antimicrobial agent susceptibility and identification to the species level of *Enterococcus faecium*, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*, respectively.

Inoculum suspensions for the system were processed on the same day using the same fresh 18- to 24-h subculture plate. The Prompt Inoculation System-D was used to standardize inocula for microdilution antimicrobial susceptibility tests. All procedures were performed according to the manufacturers' recommendations. All antimicrobial susceptibility tests were performed in compliance with current CLSI methods as recommended by the manufacturers' package inserts with the products. Quality control was monitored by using the following organisms: *S. aureus* ATCC 29213, *P. aeruginosa* ATCC 27853, and *E. coli* ATCC 25922.

2.5. Antibacterial susceptibility testing

Bacterial cells (2×10^7/ml) were inoculated into a Mueller-Hinton broth and 0.1 ml/well was dispensed onto 96-well microtiter plates. The bacterial numbers were calculated by measurement of turbidity with a spectrophotometer (DU530; Beckman, Fullerton, CA, U.S.A.). MICs were determined by a serial two-fold dilution of test peptides, following the recommendations of the Clinical and Laboratory Standards Institute (CLSI). After 24 h of incubation at 37 °C, the minimal peptide concentration required to prevent the growth of a given test organism was determined and was defined as the MIC. The growth was assayed with a microtiter ELISA Reader (Molecular Devices Emax, CA, U.S.A.) by monitoring absorption at 620 nm [22].

2.6. Hemolytic activity assay

The hemolytic activity of the peptides was evaluated by measuring the absorbance at 414 nm with an ELISA Reader to determine the release of hemoglobin from a 4% suspension of human erythrocytes [17]. The hemolysis percentage was calculated using the following equation: %Hemolysis = 100[($Abs_{414\ nm}$ in the peptide solution − $Abs_{414\ nm}$ in PBS)/($Abs_{414\ nm}$ in 0.1% Triton X-100 − $Abs_{414\ nm}$ in PBS)].

2.7. Membrane depolarization assay

Measurements of cytoplasmic membrane depolarization were made using a membrane potential-sensitive probe, diSC$_3$5. DiSC$_3$5 was purchased from Sigma Chemical Co. (USA). *E. coli* O-157 cells were grown at 37 °C to mid-log phase, centrifuged (3500 rpm, 7 min) and washed with 5 mM HEPES buffer (pH 7.2) containing 20 mM glucose and resuspended in buffer (5 mM HEPES buffer, 20 mM glucose, 100 mM KCl, pH 7.2) to an OD_{600} of 0.05. Changes in fluorescence due to the collapse of the cytoplasmic membrane potential were continuously monitored at 20 °C using a Shimadzu RF-5301 spectrofluorophotometer at an excitation wavelength of 622 nm and an emission wavelength of 670 nm. When the dye uptake was maximal, as indicated by a stable reduction in fluorescence due to quenching of the accumulated dye in the membrane interior, peptides (at the MIC) were added to the cells. Measurements were repeated two times under each condition to ensure reproducibility [23].

2.8. Preparation of FD-loaded liposome and leakage assay

FITC-labeled dextrans (FD4, FD10, and FD20) were employed to evaluate the extent of membrane damage induced by the peptides. All FDs were purchased from Sigma Chemical Co. (USA). To prepare FD-entrapped liposome, buffer I (1 ml, 50 mM potassium phosphate, pH 7.4, with 0.1 mM EDTA) containing 2 mg/ml of FD, was sonicated for 30 min with 20 mg/ml of lipid [PE/PG (7:3, w/w)] solution in chloroform on ice. Chloroform was removed by using a rotary vacuum evaporator for 2 h at 25 °C, resulting first in the formation of a viscous gel, and then a liposome suspension. Buffer I (2 ml) was added and the suspension was evaporated further for the removal of eventual traces of chloroform. The liposome suspension was sonicated, centrifuged and washed for several cycles at 13,000 rpm for 30 min to remove unentrapped-FD. For the assay, the peptides (at the MIC) were treated in a suspension of FD-loaded liposome. The mixture (3 ml, final volume) was stirred for 10 min in the dark and then centrifuged at 13,000 rpm for 20 min. The supernatant was recovered and its fluorescence intensity was recorded by measuring a fluorescent intensity, at wavelengths (λ_{ex} = 494 nm, λ_{em} = 520 nm), with RF-5301PC spectrofluorophotometer (Shimadzu, Japan). 20 μl of 10%

Triton X-100 was added to vesicles to determine 100% dye leakage [24,25]. The percentage of dye leakage was calculated as follows: % FD leakage = $100 \times (F - F_0)/(F_t - F_0)$, where F represents the fluorescent intensity achieved by the peptides treatment (at the MIC) and F_0 and F_t represent the fluorescent intensities without the peptides treatment and with Triton X-100 treatment, respectively.

3. Results

3.1. Design and secondary structures of the analogs of Rev–NIS

As noted, we designed the analogs of Rev–NIS, Anal R, and Anal S, based on the boundary of the hydrophobic and the charged/polar regions of the peptide (Fig. 1). Specifically, Anal R was designed to increase the net positive charge, considered the significant factor regarding the interaction between the peptide and the bacterial cell plasma membranes, and Anal S was designed to decrease the hydrophobicity, related to both the cytotoxicity and the hydrophobic interaction with the membranes (Table 1). As a result, the net positive charge of Anal R has increased from +2 to +5 and no change has been made to Anal S. Regarding the hydrophobicity, we did calculation by using Eisenberg–Weiss scale and came up with the conclusion that Anal R had the value of −0.502 while Anal S had the value of −0.24 and Rev–NIS had that of −0.161.

First, to investigate the alteration of the secondary structure of the analogs, after the amino acid substitution, CD spectroscopy was performed in membrane-like environments. Previously, Rev–NIS was known to have an α-helical structure [16], and as shown in Fig. 2, the results showed that the analogs, Anal R and Anal S, were characterized by exhibiting double negative ellipticity in both 50% TFE and 30 mM SDS. It meant that the two analogs maintained the Rev–NIS-like helix, regardless of the substitution.

3.2. Antibacterial and hemolytic activities of Rev–NIS and its analogs

In this study, melittin was used as a positive control peptide for comparing the potency of the peptides. Melittin is the main component in the venom of the honey bee *Apis mellifera* and is one of the typical membrane-disruptive AMPs, helping researchers to investigate lipid–peptide interactions. It also possesses potent antibacterial and hemolytic activities [26–28]. As shown in Table 2, although melittin showed the most potent activities, Rev–NIS and its analogs also showed remarkable antibacterial activities, with MIC values in the 2.5–40 μM range, against 6 Gram-positive bacteria and 6 Gram-negative bacteria, including antibiotics-resistant bacterial strains, such as MRSA or ARPA. Furthermore, the cytotoxicity assay was conducted against human erythrocytes. The result showed that Rev–NIS did not cause any hemolysis, such as the experiment performed in the previous study [17], and that the analogs did not

Fig. 1. Helical wheel diagram of the analogs of Rev–NIS. The diagram is drawn at a rotation angle of 108° per peptide bond [16]. The oblique line indicates the boundary between the hydrophobic (left) residues and the charged/polar (right) residues.

Table 1
Physicochemical properties of Rev–NIS and its analogs.

Peptide	Amino acid sequence	Calculated value	Observed value	Net charge	Retention time (min)	Hydrophobicity
Rev–NIS	ELLKAVRLIK	1182.5	1183.2	+2	18.992	−0.161
Anal R	RLRKAVRLIK	1252.6	1251.9	+5	13.550	−0.502
Anal S	ELSKAVRLIK	1156.4	1155.8	+2	15.433	−0.24

induce hemolytic effects at any of the tested concentrations that are similar to that of Rev–NIS (Table 3). The results of the antibacterial susceptibility testing and the hemolysis assay suggest that the analogs, as well as Rev–NIS, can be developed as novel antibacterial agents without remarkable cytotoxicity.

3.3. Rev–NIS and its analog-induced membrane leakage in intact bacterial cells, using diSC$_3$5 assay

In the previous study, Rev–NIS has shown to exert its antifungal activity via membrane-active mechanism [17]. Like Rev–NIS, many AMPs are considered to have a common target site, the cytoplasmic membrane, regarding their antimicrobial activity. Therefore, to investigate the effects of Rev–NIS and its analogs on the bacterial cytoplasmic membrane, the membrane-depolarization experiments using the membrane-sensitive dye diSC$_3$5 was conducted against *E. coli* O-157, as compared to melittin. The addition of all the peptides resulted in an increase of diSC$_3$5 fluorescence intensity, and this increase demonstrated that the peptides induced membrane depolarization in *E. coli* O-157 (Fig. 3). Also, the pattern in the result of the diSC$_3$5 assay mostly corresponded to the result of the antibacterial susceptibility testing (Anal R>Rev–NIS>Anal S).

3.4. Size of the pores formed by Rev–NIS and its analogs in bacterial model membranes, using FITC-dextran leakage

To elucidate the extent of membrane damage induced by Rev–NIS and its analogs, the release of FD of manifold sizes (FD4, FD10, and FD 20) from liposome was assessed. The liposome consisted of PE/PG (7:3, w/w) and this composition was derived from the inner membrane of *E. coli*. The average molecular mass of FD4, FD10, and FD20 is 3.38, 10.1, and 14.8 kDa, respectively. Also, the hydrodynamic radius (Stokes radius) of FD4, FD10, and FD20 is known to be 1.4, 2.3, and 3.3 nm, respectively [29]. As shown in Fig. 4, on average, Rev–NIS, Anal R, and Anal S released 16.828%, 21.147%, and 2.568% of FD4 from the liposome. Interestingly, Anal R further induced the release of FD10, with 7.158%. However, all the peptides, except for melittin, could not induce the leakage of FD20 from liposome. This result indicated that Rev–NIS and Anal S made pores in the bacterial model membranes, with the radius between 1.4 nm and 2.3 nm. The radius of the pores made by Anal R is thought to exceed 2.3 nm, but below 3.3 nm.

4. Discussion

AMPs are one of the most significant components of the natural defenses of many living organisms against invading pathogenic microorganisms. Although the sequence, length, or structure of AMPs is not consistent, their molecular weight is relatively small (<10 kDa) and specifically, they have two representative properties such as cationicity and amphipathicity. With respect to the structure of AMPs, the peptides are classified into five groups as followings: (a) α-helical peptides, (b) cysteine-rich peptides, (c) β-sheet peptides, (d) histatin, arginine, and proline-rich peptides, and (e) peptides composed of uncommon or modified amino acids. These AMPs are known to exert

Fig. 2. CD spectra of the analogs of Rev–NIS. (A) Rev–NIS. (B) Anal R. (C) Anal S.

broad-spectrum activities against a variety of microorganisms, including bacteria, fungi, yeast, protozoa, viruses, and antibiotics-resistant strains. Additionally, AMPs have been isolated from various vertebrates, invertebrates, animals, plants, bacteria and fungi. Moreover, some AMPs are derived from macrophages, neutrophils, epithelial cells, hemocytes, fat body, and the reproductive tract [30].

The Rev protein of HIV-1 is known as a post-transcriptional transactivator for structural and enzymatic viral gene expression. Rev protein also allows both unspliced and incompletely-spliced viral mRNAs to be efficiently transported out of the nucleus of the host cells, and translated on polysomes [31–34]. This series of processes are carried out by the direct interaction between the Rev protein and its structural RNA target, on such HIV-1 mRNAs, the specific region entitled the Rev responsive element (RRE) [35–37]. Furthermore, many studies regarding HIV-1 have suggested that Rev is shuttling between the cytoplasm and the nucleus of the host cells [12–15], mediated by the NLS (nuclear localization signal), the NOS (nucleolar targeting signal) [38] and the NES (nuclear export signal) of Rev itself [39–41]. As mentioned above, NIS region is also crucial for the cycle survival of HIV-1.

Table 2
Antibacterial activity of Rev–NIS and its analogs.

	MIC (μM)			
	Rev–NIS	Anal R	Anal S	Melittin
Gram-positive bacteria				
E. faecium	10	5	10	2.5
MRSA 1	5	2.5	10	1.25
MRSA 2	10	5	20	2.5
MRSA 3	10	5	20	2.5
MRSA 4	10	5	10–20	5
MRSA 5	20	10–20	40	5
Gram-negative bacteria				
ARPA 1	10	5	20	2.5
ARPA 2	5	2.5–5	10	1.25
ARPA 3	10	10	20	5
ARPA 4	10	5	20	5
ARPA 5	5	2.5	10–20	2.5
E. coli O-157	10	5	20	1.25

E. faecium: *Enterococcus faecium*, MRSA: methicillin-resistant *Staphylococcus aureus*, ARPA: antibiotics-resistant *Pseudomonas aeruginosa*, *E. coli* O-157: *Escherichia coli* O-157.

We focused on the AMPs from the species that are not mentioned above and found the Rev–NIS peptide derived from the HIV-1 Rev protein. Rev–NIS is not only cationic but also amphipathic, and according to our previous study, this peptide is known to contain an anticandidal property. Actually, short peptides such as Rev–NIS have been specifically considered as the model for therapeutic agents. Regarding not only the production costs but also the susceptibility to human proteolytic enzymes, short peptides are thought to possess many advantages. Long peptides are very susceptible to enzymes in the gastrointestinal track [42]. Contrarily, short peptides are less susceptible to the enzymes and can be absorbed after oral administration, without difficulty [43]. Those are the reasons that this short peptide consisting of 10 amino acids, Rev–NIS, is considered as a potential antibacterial peptide. Furthermore, in this study, we designed two analogs, to investigate the effects of the substituted amino acids, arginine and serine, on the activity of Rev–NIS.

Prior to the investigation of the antibacterial effects of the peptides, we checked the variation of secondary structures of the analogs. The CD spectroscopy results were interesting. Both Anal R and Anal S displayed the same conformation of Rev–NIS, with a significant fraction of helical structure, and as mentioned above, the amino acid-substitutions did not affect the peptide secondary structure. The degree of peptide helicity is also different according to the environments. Next, Rev–NIS and its analogs are active against Gram-negative and Gram-positive bacteria, mainly the antibiotic resistant strains such as MRSA and ARPA in this study; however, they do not lyse eukaryotic cells such as human erythrocytes. Particularly, Rev–NIS and its analogs possessed a significant activity pattern, Anal R > Rev–NIS > Anal S. However, there was no specific discrepancy between Gram-positive and Gram-negative bacteria, regarding the activity of the peptides. Significantly, in the case of Anal R and Anal S, the net positive charge was thought to be more crucial than the hydrophobicity and the helicity, regarding the antibacterial activity. In

Table 3
Hemolytic activity of Rev–NIS and its analogs.

Peptide	%Hemolysis (μM)						
	100	50	25	12.5	6.25	3.13	1.56
Rev–NIS	0	0	0	0	0	0	0
Anal R	0	0	0	0	0	0	0
Anal S	0	0	0	0	0	0	0
Melittin	100	100	100	100	100	100	98

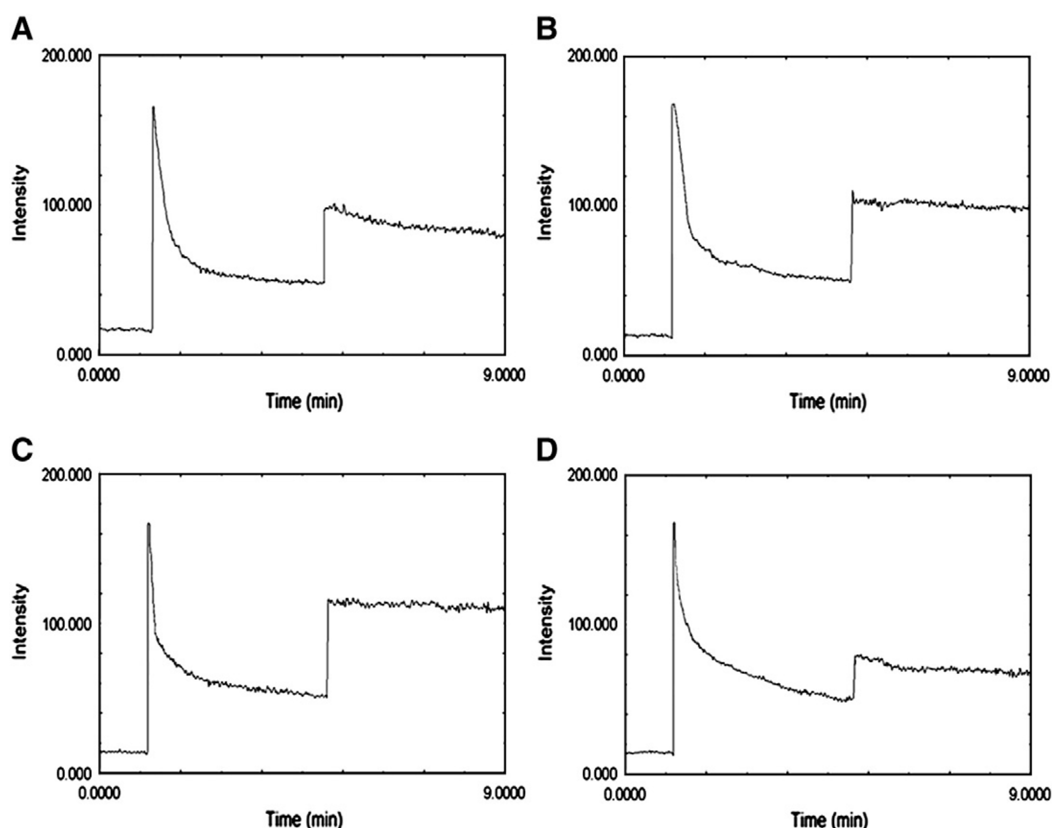

Fig. 3. Effects of melittin, Rev–NIS, and its analogs on the membrane potential of intact *E. coli* O-157 cells (OD_{600} = 0.05). Di$SC_3$5 fluorescence was measured as described in Materials and methods. The peptides were treated at the MIC value. (A) Cells treated with melittin. (B) Cells treated with Rev–NIS. (C) Cells treated with Anal R. (D) Cells treated with Anal S.

summary, the peptides including Rev–NIS are expected to overcome the resistance of bacteria, and they can be one of the most promising potential next generation antibiotic models.

The amphipathic property can induce the effective interactions of the peptides with the cell plasma membranes, consisting of phospholipids. Thus, a representative characteristic, observed for membrane-active peptides, is their disruptive action toward bilayer integrity, like depolarization and pore formation. This action on the membranes can often lead to the collapse of the transmembrane electrochemical gradients and, consequently, provide a description regarding the cell killing activities [44,45]. The pore formation affects cellular respiration [46], deprives organisms of an energy source by disrupting the electrochemical gradient, across free-energy transduction within the membranes [47,48]. Lastly, this process results in cell swelling and osmolysis, due to the flow of ion or water [27,49].

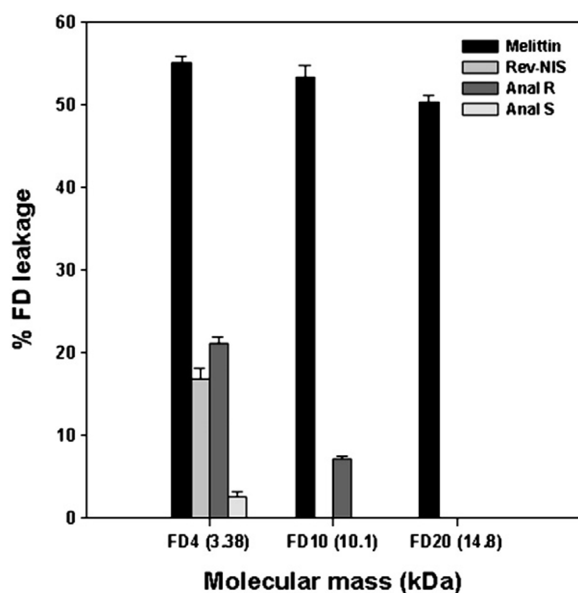

Fig. 4. Percentage of FITC-dextran (FD) leakage induced by melittin, Rev–NIS, and its analogs from PE/PG [7:3 (w/w)] liposome. The peptides were treated at the MIC value. The error bars represent the standard deviation values for three independent experiments, performed in triplicate.

In the previous study, Rev–NIS showed the antifungal activity via the membrane-disruptive action against *C. albicans*. To examine that Rev–NIS also showed antibacterial effects by a similar mode of action, we first performed di$SC_3$5 assay using *E. coli* O-157. This dye is a membrane potential-sensitive fluorescent probe [50]. If it crosses the outer membrane, di$SC_3$5 can be taken up by the cells, according to the magnitude of the electrical potential gradient of the cytoplasmic membrane, and thereafter, it accumulates in the membrane, where it self-quenches its own fluorescence. Providing that the peptides disrupt the cytoplasmic membrane and that this action results in the depolarization of the $\Delta\Psi$, the electrical potential gradient, we can check the release of di$SC_3$5 through the increase of fluorescence [51]. The result indicated that all the peptides remarkably disrupted the cytoplasmic membrane of *E. coli* O-157 and that the pattern of potency was consistent with the result of MIC assay (Anal R>Rev–NIS>Anal S). It further suggests that the substituted positively charged arginine (R) is very significant in the interaction between the peptide and the negatively charged LPS, PE or PG of the *E. coli* O-157. Regarding PE, in *E. coli* cells, the fluorescence signal of Ro (Ro09-0198)-bound PE is uniformly distributed in the cell surface, and this observation indicates that PE is uniformly localized over the whole bacterial cell membrane [52]. Ro is the cyclic peptide probe that can bind specifically to PE [53]. Moreover, it was thought that the decreased hydrophobicity, by substituting serine (S), weakened the hydrophobic interactions between the hydrophobic residues of Rev–NIS and the

hydrophobic tail region of the bacterial membranes, after the interactions of the positively charged amino acids, such as K^4, R^7, and K^{10}, with the negatively charged components. When considering the pattern of hydrophobicity, this fact supported that the net positive charge is one of the most crucial factors.

To confirm the mode of action of the peptides, the FD leakage assay was conducted. This assay, using FDs of both the different molecular weight and the radius, is a method to elucidate the pore-forming mechanism in the membrane perturbation process [54,55]. The radius of Rev–NIS and its analogs-induced pores may be anywhere from 1.4 nm to 2.3 nm. Also, the radius of pores induced by Anal R is considered to be from 2.3 nm to 3.3 nm. This result indicated that the substituting with two arginines increased the net positive charge of the peptide, and thereafter, the increased positive charge makes the interaction between the peptide and the negative charged components more potent. It meant that the high affinity regarding the interaction between the positively charged residues of Anal R induced larger pores than other peptides. In conclusion, these results suggest that the peptides in this study make pores in the bacterial cell membranes, can affect cellular respiration, and cause the membrane to transport water or ions. Finally, the peptides can be thought to cause the bacterial cell death. Considering that the negatively charged phospholipids are uniformly distributed in both the outer and the inner membranes of bacteria, as compared with those of fungi and mammals, specifically, Anal R possessing many arginines is thought to be a potent candidate as a novel antibacterial agent.

Our ultimate goals have been to develop and suggest novel non-toxic antibiotic peptides with noticeable potency and range of susceptible microorganisms to be of practical utility. The pore forming peptides studied herein, Rev–NIS and its analogs, Anal R, and Anal S, were conducive with the conditions mentioned above, and among the peptides, Anal R can be a novel potential antibiotic itself or a template for designing new therapeutic agents.

Acknowledgments

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