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Cathelicidin-derived Trp/Pro-rich antimicrobial peptides with lysine peptoid residue (Nlys): therapeutic index and plausible mode of action

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Abstract: Recently, we designed a novel cell-selective antimicrobial peptide (TPk) with intracellular mode of action from Pro → Nlys (Lys peptoid residue) substitution in a noncell-selective cathelicidin-derived Trp/Pro-rich antimicrobial peptide, tritrpticin-amide (TP; VRRFPWWWPFLRR-NH_2) (*Biochemistry* 2006; 45: 13007–13017). In this study, to elucidate the effect of Pro → Nlys substitution on therapeutic index and mode of action of other noncell-selective cathelicidin-derived Trp/Pro-rich antimicrobial peptides and develop novel short antimicrobial peptides with high cell selectivity/therapeutic index, we synthesized Nlys-substituted antimicrobial peptides, TPk, STPk and INk, in which all proline residues of TP, symmetric TP-analogue (STP; KKFPWWWPFKK-NH_2) and indolicidin (IN; ILPWKWPWWPWRR-NH_2) were replaced by Nlys, respectively. Compared to parent Pro-containing peptides (TP, STP and IN), Nlys substituted peptides (TPk, STPk and Ink) had 4- to 26-fold higher cell selectivity/therapeutic index. Parent Pro-containing peptides induced a significant depolarization of the cytoplasmic membrane of intact *Staphylococcus aureus* at their MIC, whereas Nlys-substituted antimicrobial peptides did not cause visible membrane depolarization at their MIC. These results suggest that the antibacterial action of Nlys-substituted peptides is probably not due to the disruption of bacterial cytoplasmic membranes but the inhibition of intracellular components. Taken together, our results showed that Pro → Nlys substitution in other noncell-selective Trp/Pro-rich antimicrobial peptides such as STP and IN as well as TP can improve the cell selectivity/therapeutic index and change the mode of antibacterial action from membrane-disrupting to intracellular targeting. In conclusion, our findings suggested that Pro → Nlys substitution in noncell-selective Trp/Pro-rich antimicrobial peptides is a promising method to develop cell-selective antimicrobial peptides with intracellular target mechanism.
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Keywords: cathelicidin-derived Trp/Pro-rich antimicrobial peptide; tritrpticin; indolicidin; cell selectivity; therapeutic index; lysine peptoid residue (Nlys)

INTRODUCTION

AMPs are important components of innate host defense in species from microorganisms to arthropods, vertebrates and mammals including humans [1,2]. They are positively charged, amphipathic small molecules, and have the capacity to kill a broad range of microorganisms, including gram-positive and gram-negative bacteria, protozoa, and fungi [3–5]. The widespread clinical use of traditional antibiotics has led to the growing emergence of a great number of antibiotic-resistant strains nowadays [6,7]. Therefore, much interest has been attached from AMPs as a new class of antibiotics to combat such challenge due to the low potential of AMPs for development of resistance [5].

One major class of AMPs is called cathelicidin, which are evolutionarily conserved antimicrobial peptides present in all mammals. They are synthesized in precursor form with a highly conserved *N*-terminal signal sequence (cathelin domain) and an extremely variable *C*-terminal domain, which is cleaved to release a peptide with antimicrobial activity [8–10]. Despite the wide variety of cathelicidin family peptides in origination, they can be classified into three groups on the basis of their diverse amino acid compositions and secondary structures. First group is amphipathic α-helical peptides, e.g. CAP18 [11], CRAMP [12], and SMAP29 [13]. A second group, like protegrin [14], is β-sheet peptides that are a few in cathelicidin family. The third group is Pro/Arg-rich or Trp/Pro-rich peptides, e.g. PR-39 [15], Bac7 [16], OaBac5 [17], tritrpticin [18] and indolicidin [19].

In the present study, we focused on two Trp/Pro-rich antimicrobial peptides, tritrpticin (VRRFPWWWPFLRR) and indolicidin (IILPWKWPWWPWRR-NH_2), which

Abbreviations: Abbreviations: AMPs, antimicrobial peptides; $DiSC_3$-5, 3,3′-dipropylthiadicarbocyanine iodide; EYPC, egg yolk L-α-phosphatidylcholine; EYPE, egg yolk L-α-phosphatidylethanolamine; EYPG, egg yolk L-α-phosphatidyl-DL-glycerol; Fmoc, fluoren-9-yl-methoxycarbonyl; hRBCs, human red blood cells; MALDI-TOF MS, matrix-assisted laser-desorption ionization-time-of-flight mass spectrometry; MHC, minimal hemolysis concentration; MIC, minimal inhibitory concentration; Nlys (lysine peptoid residue), [*N*-(4-aminobutyl)glycine]; RP-HPLC, reversed-phase high performance liquid chromatography; SUVs, small unilamellar vesicles.

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belong to the cathelicidin family derived from the porcine myeloid mRNA [18] and the cytoplasmic granules of bovine neutrophils [19], respectively. These two peptides are relatively short peptides consisting of only 13 residues, and contain high fraction of tryptophan and proline. Although both peptides have broad spectrum of antimicrobial activities against bacteria and fungi, their relatively high toxicity toward eukaryotic cells prevents their usage as antibiotics [20,21]. Therefore, much effort has been done in the past decade to decrease the cytotoxicity or increase the cell selectivity of both tritrpticin and indolicidin [20,22–25]. Furthermore, we recently reported that the substitution of two proline residues of tritrpticin-amide (TP) with lysine peptoid residue (Nlys) is an effective method to decrease the cytotoxicity toward eukaryotic cells of TP while retaining strong antimicrobial activity [26].

Here, to elucidate the effect of Pro → Nlys substitution on therapeutic index and mode of action of two Trp/Pro-rich antimicrobial peptides and develop novel antimicrobial peptides with high therapeutic index, we introduced Nlys to replace all Pro residues of symmetric TP-analogue (STP) (KKFPWWWPFKK-NH_2) and indolicidin (IN). Biological activity assays and fluorescence experiments showed that Pro → Nlys substitution can also affect the cytotoxicity and antibacterial action mode of these two Trp/Pro-rich peptides, STP and IN as well as TP.

MATERIALS AND METHODS

Materials

Rink amide 4-methylbenzhydrylamine resin, Fmoc-amino acids and other reagents for the peptide synthesis were purchased from Calibochem-Novabiochem (La Jolla, CA). EYPC, EYPE, EYPG, cholesterol, acrylamide, EDTA, HEPES, and gramicidin D were supplied from Sigma Chemical Co. (St Louis, MO). $DiSC_3$-5 was obtained from Molecular Probes (Eugene, OR). All other reagents were of analytical grade. The buffers were prepared in double glass-distilled water.

Peptide Synthesis

We designed three Nlys-substituted antimicrobial peptides, TPk, STPk and INk, in which all proline residues of TP, STP and IN were replaced by Nlys, respectively (Table 1). All the peptides used in this study were prepared by the standard Fmoc-based solid-phase method on rink amide MBHA resin (0.54 mmol/g). Fmoc-Nlys-OH was synthesized by the previous method [27]. Dicyclohexylcarbodiimide (DCC) and *N*-hydroxybenzotriazole (HOBt) were used as coupling reagent, and ten-fold excess Fmoc-amino acids were added during every coupling cycle. After cleavage and deprotection with a mixture of trifluoroacetic acid/water/thioanisole/ethanedithiol/triisopropylsilane (81.5:5:5:5:2.5:1, v/v) for 2 h at room temperature, the crude peptides were repeatedly extracted with diethyl ether and purified by reverse phase HPLC on a preparative Vydac C_{18} column (15 μm, 20 × 250 mm) using an appropriate 0–80% water/acetonitrile gradient in the presence of 0.05% trifluoroacetic acid. The final purity of the peptides (>98%) was assessed by reverse phase HPLC on an analytical Vydac C_{18} column (5 μm, 4.6 × 250 mm) and their identities were confirmed by MALDI-TOF MS (Shimadzu, Japan) (Table 1).

Antimicrobial Activity Assay

The antibacterial activities of the peptides against two gram-positive bacterial strains, two gram-negative bacterial strains and four antibiotic-resistant clinical isolates were examined in sterile 96-well plates using the broth microdilution method as our previous reports [13,20,22,23,26]. Aliquots (100 μl) of a bacterial suspension at 2×10^6 colony-forming units (CFU)/ml in 1% peptone were added to 100 μl of peptide solution (serial two-fold dilutions in 1% peptone). After incubation for 18–20 h at 37 °C, the inhibition of bacterial growth was determined by measuring the absorbance at 620 nm with a microplate autoreader EL 800 (Bio–Tek Instruments). The MIC is defined as the minimal peptide concentration that inhibits bacteria growth. Two types of gram-positive bacteria (*Staphylococcus epidermidis* [KCTC 1917] and *Staphylococcus aureus* [KCTC

Table 1 Amino acid sequences, calculated and observed molecular masses and charges of peptides

Peptide	Amino acid sequence[a]	Molecular mass (Da)		Net charge
		Calculated	Observed[b]	
TP	VRRFPWWWPFLRR-NH_2	1901.2	1903.2	+5
TPk	VRRP**k**WWW**k**FLRR-NH_2	1963.3	1965.2	+7
STP	KKFPWWWPFKK-NH_2	1576.9	1577.3	+5
STPk	KKF**k**WWW**k**FKK-NH_2	1639.1	1638.8	+7
IN	ILPWKWPWWPWRR-NH_2	1906.3	1906.1	+4
Ink	IL**k**WKW**k**WW**k**WRR-NH_2	1999.5	1999.2	+7

[a] Bold **k** are Nlys [NH_2–$(CH_2)_4$–NH–CH_2–COOH].
[b] Molecular mass was determined by MALDI-TOF-MS.

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1621]) and two types of gram-negative bacteria (*Escherichia coli* [KCTC 1682] and *Pseudomonas aeruginosa* [KCTC 1637]) were procured from the Korean collection for type cultures (KCTC) at the Korea Research Institute of Bioscience and Biotechnology. Methicillin-resistant *S. aureus* (MRSA) (CCARM 3001 and CCARM 3543) and multidrug-resistant *P. aeruginosa* (MDRPA) (CCARM 2095 and CCARM 2109) were obtained from the culture collection of antibiotic-resistant microbes (CCARM) at Seoul Women's University in Korea.

Hemolytic Activity Assay

Fresh hRBCs were washed 3 times with PBS (35 mM phosphate buffer, 0.15 M NaCl, pH 7.4) by centrifugation for 7 min at 1000 g and resuspended in PBS. The peptide solutions (serial two-fold dilutions in PBS) were added to 100 μl suspension of hRBCs (4% (v/v) in final) in PBS to the final volume of 200 μl and were incubated for 1 h at 37 °C. The samples were then centrifuged at 1000 g for 5 min, and the release of hemoglobin was monitored by measuring the absorbance of the supernatant at 405 nm by Microplate ELISA Reader. The MHC is defined as the minimal peptide concentration that produces hemolysis. For negative and positive controls, hRBCs in PBS (A_{blank}) and in 0.1% Triton X-100 (A_{triton}) were used, respectively. The percentage of hemolysis was calculated according to the equation:

$$\% \text{ hemolysis} = 100 \times [(A_{sample} - A_{blank})/(A_{triton} - A_{blank})]$$

Preparation of Small Unilamellar Vesicles (SUVs)

SUVs were prepared by a standard procedure with required amounts of either EYPE/EYPG (7 : 3, w/w) or EYPC/cholesterol (10 : 1, w/w) for tryptophan fluorescence. Dry lipids were dissolved in chloroform in small glass vessel. Solvents were removed by rotary evaporation to form a thin film on the wall of a glass vessel and then lyophilized overnight. Dried thin films were resuspended in tris-HCl buffer by vortex mixing. The lipid dispersions were then sonicated in ice water for 10–20 min with a titanium-tip ultrasonicator until the solution became transparent.

Tryptophan Fluorescence Blue Shift

The fluorescence emission spectrum of tryptophan of peptides was monitored in aqueous tris-HCl buffer, and in the presence of vesicles composed of either EYPE/EYPG (7 : 3 w/w) SUVs or EYPC/cholesterol (10 : 1 w/w) SUVs. In these fluorometric studies, SUVs were used to minimize differential light scattering effects [28,29]. The tryptophan fluorescence measurements were taken with a model RF-5301PC spectrophotometer (Shimadzu, Japan). Each peptide was added to 3 ml of tris-HCl buffer (10 mM tris, 0.1 mM EDTA, 150 mM NaCl, pH 7.4) containing 0.3 mM liposomes, and the peptide/liposome mixture (a molar ratio of 1 : 200) was allowed to interact at 20 °C for 10 min. The fluorescence was excited at 280 nm, and the emission was scanned from 300 to 400 nm.

Membrane Depolarization

The membrane depolarization activity of peptides was determined using intact *S. aureus* cells and the membrane potential-sensitive fluorescent dye, $DiSC_3$-5 based on the methods of Friedrich *et al.* [30]. Briefly, *S. aureus* was grown at 37 °C with agitation to mid-log phase ($OD_{600} = 0.4$) were harvested by centrifuge. The cells were washed twice with washing buffer (20 mM glucose, 5 mM HEPES, pH 7.4) and resuspended to an OD_{600} of 0.05 in a similar buffer containing 0.1 M KCl. Following this, the cells were incubated with 20 nM $DiSC_3$-5 until a stable reduction of fluorescence was achieved, indicating the incorporation of the dye into the bacterial membrane. Membrane depolarization was then monitored by observing the change in the intensity of fluorescence emission of the membrane potential-sensitive dye $DiSC_3$-5 ($\lambda_{ex} = 622$ nm, $\lambda_{em} = 670$ nm) after the addition of peptides. Full dissipation of the membrane potential was obtained by adding gramicidin D (final concentration, 0.2 nM). The membrane potential-dissipating activity of the peptides is expressed as follows:

$$\% \text{ Membrane depolarization} = 100 \times [(F_p - F_0)/(F_g - F_0)]$$

where F_0 is the stable fluorescence value after addition of the $DiSC_3$-5 dye, F_p is the fluorescence value 5 min after addition of the peptides, and F_g is the fluorescence signal after the addition of gramicidin D.

RESULTS AND DISCUSSION

Antimicrobial and Hemolytic Activity

In the antimicrobial activity assay, we introduced the geometric mean (GM) of the MIC values from all bacterial strains including antibiotic-resistant clinical isolates to provide an overall evaluation of antimicrobial activity of the peptides against bacteria. Compared to parent Pro-containing peptides, Nlys-substituted peptides had the same or approximate two-fold enhanced antimicrobial activity. Furthermore, TPk, STPk, and INk had broad spectrum of antimicrobial activities against bacteria like TP, STP, and IN (Table 2).

To exhibit the hemolytic activity of the peptides, we here introduced the MHC, which is defined as the minimal peptide concentration that produces hemolysis. The MHC value of TP, STP and IN was 50, 100, and 25 μM, respectively. However, none of Nlys-substituted peptides caused hemolysis at concentrations as high as 200 μM (Table 2).

Cell Selectivity/Therapeutic Index

To assess the cell selectivity of our peptides, therapeutic index, which is a widely accepted parameter to represent the specificity of antimicrobial reagents through calculating the ratio of MHC/MIC, was here introduced. A larger therapeutic index indicates greater cell selectivity. In contrast to parent Pro-containing peptides, Nlys-substituted peptides had 4- to 26-fold higher cell selectivity/therapeutic index (Table 2).

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Table 2 Antimicrobial and hemolytic activities of peptides

Cell types	TP	TPk	STP	STPk	IN	INk
Bacterial cells (MIC: μM)[a]						
E. coli (KCTC 1682)	8	2	4	1	8	4
P. aeruginosa (KCTC 1637)	8	4	2	2	16	2
S. epidermidis (KCTC 1917)	1	1	0.5	1	1	1
S. aureus (KCTC 1621)	2	1	1	1	1	1
MRSA1[b] (CCARM 3001)	2	1	1	2	1	2
MRSA2 (CCARM 3543)	2	0.5	0.5	0.5	1	1
MDRPA1[c] (CCARM 2095)	16	4	4	4	16	4
MDRPA2 (CCARM 2109)	32	8	8	8	16	8
GM (μM)[d]	4.8	1.8	1.7	1.7	3.7	2.2
MHC (μM)[e]	50	>200	100	>200	25	>200
Therapeutic index[f]	10.4	222.2	58.8	235.3	6.8	181.8

[a] Each MIC is the mean determined from three independent experiments performed in triplicate with a standard deviation of 23.0%.
[b] Methicillin-resistant *S. aureus*.
[c] Multidrug-resistant *P. aeruginosa*.
[d] The geometric mean of MIC values from all bacterial strains in this table.
[e] MHC is defined as the minimal peptide concentration that produces hemolysis. When no detectable hemolysis is observed at 200 μM, we use a value of 400 μM to calculate the therapeutic index.
[f] The ratio of the MHC (μM) over the geometric mean MIC (μM).

Blue Shift of Trp Residues

The presence of tryptophan residues at each peptide allowed us to monitor the binding specificity of the peptides to artificial liposomes by the fluorescence emission of the tryptophan. In negatively charged phospholipids [EYPE/EYPG (7 : 3, w/w) SUVs] which mimic bacterial membranes, the fluorescence emission maxima of all peptides exhibited a significant blue shift compared to peptides in tris-buffer, indicating all peptides anchor into the hydrocarbon region of bilayer. In zwitterionic phospholipids membranes [EYPC/cholesterol (10 : 1, w/w) SUVs] which mimic the outer surface of mammalian membranes, parent Pro-containing peptides, TP, STP, and IN also exhibited a blue shift in the fluorescence emission maxima similar to that observed in the bacterial membrane-mimic liposomes. In contrast, Nlys-substituted peptides exhibited no or less blue shift in zwitterionic phospholipids membranes (Table 3 and Figure 1). These results suggest that parent Pro-containing peptides have good binding affinity to both bacterial and mammalian membrane-mimics artificial liposomes, but Nlys-substituted peptides preferentially bind to negatively charged artificial liposomes. Furthermore, the increase in intensity of the fluorescence emission maxima of Nlys-substituted peptides was greater in EYPE/EYPG vesicles than in EYPC/cholesterol vesicles, suggesting Nlys-substituted peptides have a strong interaction with bacterial membranes rather than mammalian membranes (Figure 1). This lipid specificity of Nlys-substituted peptides, TPk, STPk, and INk, for artificial liposomes corresponds well with their cell selectivity/therapeutic index (Table 2).

Table 3 Tryptophan emission maxima of 1.5 μM peptides in tris-buffer (pH 7.4) or in the presence of 0.3 mM EYPE/EYPG (7 : 3, w/w) SUVs and 0.3 mM EYPC/cholesterol (10 : 1, w/w) SUVs

Peptides	Tris-buffer (nm)	EYPE/EYPG (7 : 3, w/w) (nm)	EYPC/cholesterol (10 : 1, w/w) (nm)
TP	347	338 (−9)[a]	340 (−7)
TPk	349	341 (−8)	349 (0)
STP	347	336 (−11)	342 (−5)
STPk	349	341 (−8)	349 (0)
IN	350	340 (−10)	342 (−8)
INk	350	341 (−9)	348 (−2)

[a] Blue shift of Trp emission maxima compared to tris-buffer.

Depolarization of the Membrane Potential in Intact Bacteria

Bacterial membrane potential depolarization assay is a widely adopted method to examine whether antimicrobial peptides possess the ability of disrupting bacterial membranes. If a peptide induces membrane permeation, it will, in the meanwhile, cause a dissipation of the transmembrane potential monitored by an increase in fluorescence due to the release of the membrane potential-sensitive fluorescent dye $DiSC_3$-5 from the membrane. Figure 2 showed that three parent Pro-containing peptides can depolarize cytoplasmic membrane of intact *S. aureus* at their MIC,

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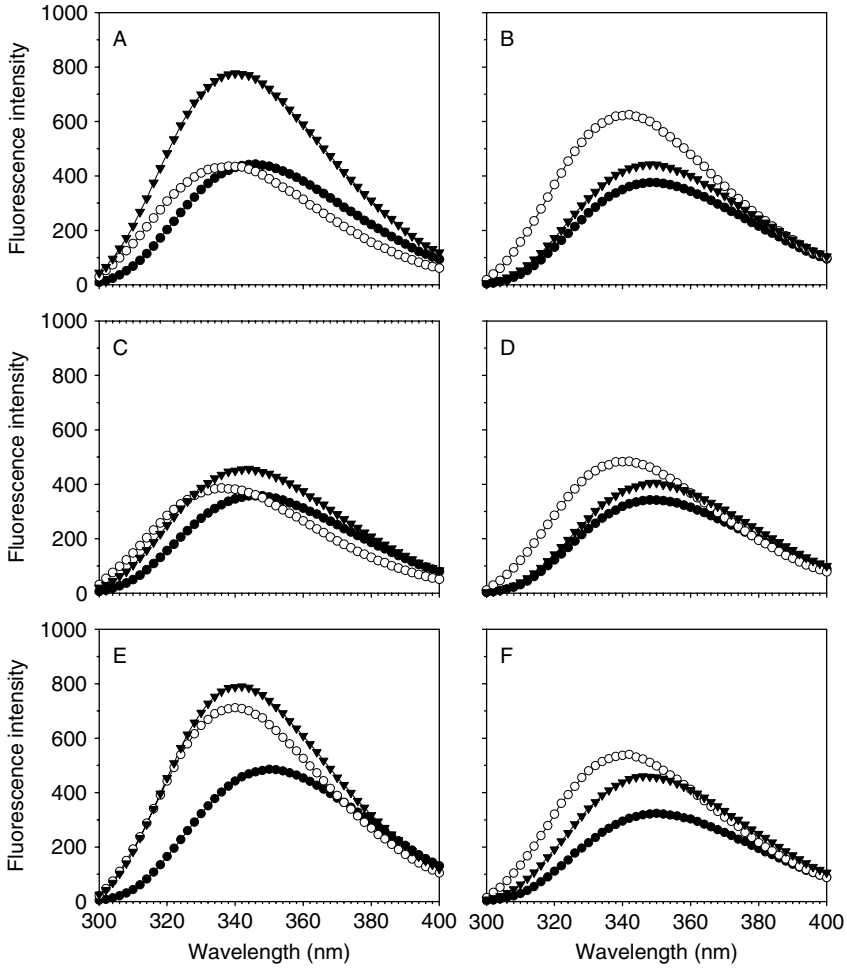

Figure 1 Trp fluorescence emission spectra of 1.5 μM peptides in tris-buffer (pH 7.4) (●), or in the presence of 0.3 mM EYPE/EYPG (7 : 3, w/w) SUVs (○) and 0.3 mM EYPC/cholesterol (10 : 1, w/w) SUVs (▼). TP (A), TPk (B), STP (C), STPk (D), IN (E), and INk (F).

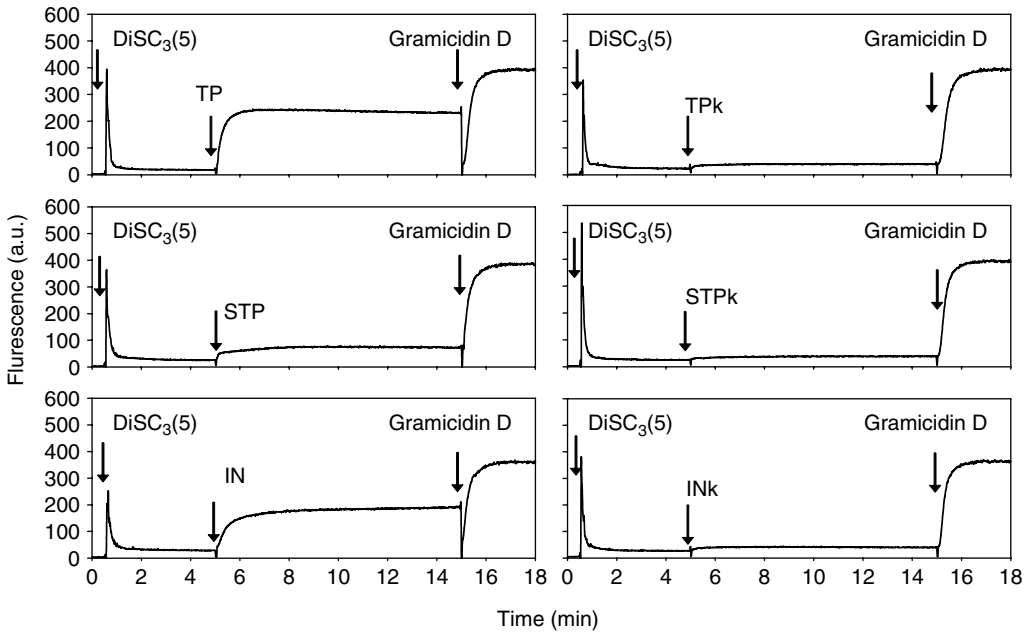

Figure 2 Effect of the peptides on the membrane potential of intact *S. aureus* cells (OD_{600} = 0.05). Peptides concentrations are their MIC as follows: 2 μM TP, 1 μM TPk, 1 μM STP, 1 μM STPk, 1 μM IN and 1 μM INk. Experiments were repeated independently three times to ensure reproducibility.

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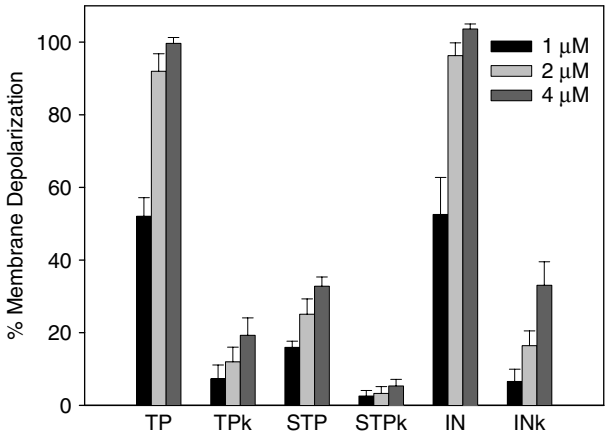

Figure 3 Dose-dependent dissipation of the transmembrane potential of *S. aureus* cells ($OD_{600} = 0.05$) by the peptides. The membrane potential-dissipating activity of the peptides is expressed as follows: % membrane depolarization $= 100 \times [(F_p - F_0)/(F_g - F_0)]$, where F_0 is the stable fluorescence value after addition of the $DiSC_3$-5 dye, F_p is the fluorescence value 5 min after addition of the peptides, and F_g is the fluorescence signal after the addition of gramicidin D. Values are the means of three independent experiments ± SD values (error bars).

whereas Nlys-substituted peptides did not cause visible membrane potential depolarization at their MIC. The dose-dependent dissipation of the transmembrane potential of *S. aureus* by peptides (Figure 3) showed that two membrane-disruption peptides, TP [20] and IN [21] can completely depolarize the cytoplasmic membrane of intact *S. aureus* at 4 μM, respectively. Also, STP, which was thought to kill bacteria via both membrane depolarization and secondary intracellular targeting [31], caused about 40% membrane depolarization at 4 μM. In contrast, TPk, STPk, and INk cause a much weaker dose-dependent depolarization of the transmembrane potential than their related parent Pro-containing peptides (Figure 3). These results suggest that the antibacterial action of STPk and INk is probably not due to the disruption of bacterial membrane but the inhibition of intracellular components like TPk.

CONCLUSIONS

Collectively, our results showed that Pro → Nlys substitution in other Trp/Pro-rich antimicrobial peptides such as indolicidin and symmetrical tritrpticin analogue as well as tritrpticin can improve the cell selectivity/therapeutic index and change the mode of antibacterial action from membrane-disrupting to intracellular targeting. Finally, our findings suggested that Pro → Nlys substitution in noncell-selective Trp/Pro-rich antimicrobial peptides is a promising strategy to develop cell-selective antimicrobial peptides with the intracellular targeting mechanism.

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