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ORIGINAL RESEARCH PAPER

# Effect of dimerization of a β-turn antimicrobial peptide, PST13-RK, on antimicrobial activity and mammalian cell toxicity

**Sung-Tae Yang · Jae Il Kim · Song Yub Shin**

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**Abstract** PST13-RK (KKKFPWWWPFKKK-$NH_2$) is an improved derivative of tritrpticin adopting a β-turn structure. In order to investigate the effect of dimerization of PST13-RK on antimicrobial activity and mammalian cell toxicity, we designed and synthesized its Cys- and Lys-linked dimers. The dimerization of PST13-RK resulted in a 2–4 fold decreased antimicrobial activity against Gram-positive and Gram-negative bacteria. However, the dimers showed a large increase in mammalian cell toxicity against mouse NIH-3T3, human MDA-MB-361, and human A549 cells. These results suggested that PST13-RK is active as a monomer to bacterial cells but as an oligomer to mammalian cells. Since the dimeric PST13-RK is much more effective against the cancer cells than the monomer, it might be an attractive candidate for anticancer chemotherapeutic drugs.

S.-T. Yang · J. I. Kim
Department of Life Science, Gwangju Institute of Science and Technology, Gwangju 500-712, Korea

S. Y. Shin (✉)
Department of Cellular & Molecular Medicine, School of Medicine, Chosun University, Gwangju 501-759, Korea
e-mail: syshin@chosun.ac.kr

S. Y. Shin
Department of Bio-Materials, Graduate School and Research Center for Proteineous Materials, Chosun University, Gwangju 501-759, Korea

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## Introduction

The 13-residue cationic peptide tritrpticin (VRRFP WWWPFLRR) is a member of the cathelicidin family, a group of diverse antimicrobial peptides found in neutrophil granules (Lawyer et al. 1996). Tritrpticin has a broad spectrum of antimicrobial activity against Gram-positive and Gram-negative bacteria and fungi (Lawyer et al. 1996). However, the clinical potential of tritrpticin is limited by its relatively strong cytotoxicity against human red blood cells (Yang et al. 2002). It is thought that tritrpticin binds to microbial membranes and causes disruption of the membranes leading to bacterial cell death (Schibli et al. 1999). It adopts a unique amphipathic β-turn structure when bound to SDS micelles with the Trp side chains separated from the Arg residues (Schibli et al. 1999). Three Trp residues of tritrpticin are essential for its antibacterial and hemolytic activities and membrane-disrupting activity (Yang et al. 2002). In the recent study on several tritrpticin derivatives with a β-turn structure, we found that the PST13-RK (KKKFPWWWPFKKK-$NH_2$) exhibits the most powerful antimicrobial activity (Yang et al. 2006).

In this study to investigate the effect of dimerization of PST13-RK on antimicrobial activity and mammalian cell toxicity, two dimeric peptides (di-PST13-RK-C and di-PST13-RK-K) were prepared. We examined the antimicrobial activity against four Gram-positive and four Gram-negative bacterial cells and the mammalian cell toxicity against mouse NIH-3T3, human A-549, and human MDA-MB-361 cells. To examine the structural change by dimerization, circular dichroism (CD) spectra were measured in the presence of membrane-mimicking SDS micelles.

## Materials and method

### Materials

Rink amide MBHA resin and 9-fluorenylmethoxycarbonyl (Fmoc) amino acid derivatives used for peptide synthesis were obtained from Calibiochem-Novabiochem (La Jolla, CA).

### Bacterial and mammalian cells

The bacterial strains were purchased from the Korean Collection for Type Cultures (KCTC), Korea Research Institute of Bioscience & Biotechnology (KRIBB) (Taejon, Korea). Four Gram-negative bacteria included *Escherichia coli* (KCTC 1682), *Salmonella typhimurium* (KCTC 1926), *Pseudomonas aeruginosa* (KCTC 1637) and *Proteus vulgaris* (KCTC 2433). The four Gram-positive bacteria were *Bacillus subtilis* (KCTC 1918), *Streptococcus pyogenes* (KCTC 3096), *Staphylococcus aureus* (KCTC 1621) and *Staphylococcus epidermidis* (KCTC 1917). Human lung carcinoma cancer cells (A-549: ATCC CCL-185), human breast adenocarcinoma cells (MDA-MB-361: ATCC HTB-27) and mouse-3T3 fibroblastic cells (NIH-3T3) were obtained from the Genetic Resources Center, KRIBB.

### Peptide synthesis and purification

The peptides (see Table 1) used in this study were synthesized by Fmoc-based solid-phase method and cleaved from the resin by treatment with trifluoroacetic acid/$H_2O$/thioanisole/phenol/ethanedithiol/triisopropylsilane (81.5:5:5:5: 2.5:1, by vol.) for 2 h at room temperature. The crude peptides were purified using a $C_{18}$ preparative column (Vydac $C_{18}$, 20 × 250 mm, 300 Å) and peptide purity was verified using a $C_{18}$ analytical column (Vydac $C_{18}$, 4.6 × 250 mm, 300 Å). Characterization of the peptides was performed by molecular mass determination using MALDI-TOF MS (matrix-assisted laser-desorption ionization-time-of-flight mass spectrometry) (Shimadzu, Japan) (see Table 1). Peptide concentrations were determined by amino acid analysis (Hitachi Model, 8500 A, Japan).

### Antimicrobial activity (MIC)

The antibacterial activities of peptides against four Gram-positive bacterial strains and four Gram-negative bacterial strains were examined by the microdilution method as described previously (Zhu et al. 2006). Aliquots (100 μl) of a bacterial suspension at $2 \times 10^6$ c.f.u./ml in 1% peptone were added to 100 μl peptide (serial 2-fold dilutions in 1% peptone). After incubation for 18–20 h at 37°C, bacterial growth was determined from the $OD_{620}$ with a Microplate autoreader. The MIC was defined as the minimum peptide concentration that inhibits bacteria growth.

### Mammalian cell toxicity ($IC_{50}$)

Mammalian cells (A-549, MDA-MB-361 and NIH-3T3) were cultured in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 units penicillin G/ml, and 100 μg streptomycin/ml. The cells were maintained under 5% $CO_2$ at 37°C. Cytotoxicity of the peptides against the mammalian cells was determined by MTT assay as previously reported (Scudiero et al. 1988) with minor modifications. The cells were seeded on 96-well microplates at $2 \times 10^4$ cells/well in 150 μl DMEM containing 10% (v/v) FBS. The plates were then incubated for 24 h at 37°C in 5% $CO_2$. Then 20 μl peptide solution (serial 2-fold dilutions in DME) was added to each well and then the plates were incubated for 2 days. Wells containing cells without peptides served as controls. Subsequently, 20 μl MTT (5 mg MTT/ml in phosphate buffered saline) was added and further incubated for 4 h at 37°C. The precipitated MTT formazan was dissolved in 40 μl 20% (w/v) SDS containing 0.01 M HCl

**Table 1** Primary structure of the peptides used in this study

| Peptides | Amino acid sequences | MALDI-TOF-MS | |
|---|---|---|---|
| | | Observed | Calculated |
| Tritrpticin | VRRFPWWWPFLRR | 1903.3 | 1902.3 |
| PST13-RK | KKKFPWWWPFKKK-$NH_2$ | 1833.1 | 1833.3 |
| di-PST13-RK-C | (KKKFPWWWPFKKKC-$NH_2$)$_2$ | 3870.0 | 3870.8 |
| di-PST13-RK-K | (KKKFPWWWPFKKK)$_2$K-$NH_2$ | 3777.2 | 3777.8 |

overnight. The absorbance at 570 nm was measured using a microplate ELISA reader. Percent cell survival was expressed as a percent ratio of $A_{570}$ of cells treated with peptide over cells only.

Circular dichroism (CD) analysis

CD spectra of the peptides were recorded using a Jasco J-720 spectropolarimeter (Japan Spectroscopic Co. Tokyo, Japan). All peptide samples were maintained at 25°C during analysis. Four scans per sample were performed over wavelength range 190–240 nm at 0.1 nm intervals. The spectra were measured in 10 mM sodium phosphate buffer (pH 7.0) or 30 mM SDS at 25°C using a 1 mm pathlength cell. The peptide concentrations were 100 μg/ml. The mean residue ellipticity, [$\theta$], is given in deg cm$^2$ dmol$^{-1}$: [$\theta$] = [$\theta$]obs (MRW/10lc), where [$\theta$]obs is the ellipticity measured in millidegree, 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. The spectra are expressed as molar ellipticity [$\theta$] versus wavelength.

## Results and discussion

The Lys-linked dimer (Di-PST13-RK-K) was synthesized by the coupling of Fmoc-Lys(Fmoc)-OH to Rink amide MBHA resin and the Cys-linked dimer (Di-PST13-RK-C) was prepared by cysteine oxidation of the monomer (KKKFPWWWPFKKKC-$NH_2$) in 10% (v/v) DMSO (peptide concentration: 1 mg/ml) for 48 h at room temperature. The formation of dimers was checked by comparison of analytical RP-HPLC profiles between monomer and dimer and confirmed by MALDI-TOF-MS (Table 1). The minimal inhibitory concentration (MIC) of the peptides is listed in Table 2 and their mammalian cell toxicity is shown in Fig. 1. The $IC_{50}$ (50% inhibitory concentration) is calculated by fitting the dose-dependent cell survival (Table 2). PST13-RK exhibited a 2–8 fold increased

**Table 2** Antimicrobial activity and mammalian cell toxicity of the peptides

| Cells | Tritrpticin | PST13-RK | di-PST13-RK-C | di-PST13-RK-K |
|---|---|---|---|---|
| Gram-negative bacterial strains (MIC: μg/ml) | | | | |
| *E. coli* | 32 | 8 | 16 | 16 |
| *S. typhimurium* | 16 | 2 | 4 | 4 |
| *P. aeruginsa* | 64 | 8 | 16 | 8 |
| *P. vulgaris* | 64 | 8 | 16 | 8 |
| Gram-positive bacterial strains (MIC: μg/ml) | | | | |
| *B. subtilis* | 8 | 2 | 4 | 4 |
| *S. pyogenes* | 64 | 8 | 8 | 8 |
| *S. aureus* | 16 | 4 | 8 | 8 |
| *S. epidermidis* | 8 | 2 | 8 | 8 |
| Mammalian cells ($IC_{50}$: μg/ml) | | | | |
| A549 (human) | >200 | 90 | 17 | 15 |
| MDA-MB-361 (human) | 142 | 80 | 17 | 10 |
| NIH-3T3 (mouse) | 145 | 83 | 20 | 10 |

MIC is defined as the minimal inhibitory concentration that inhibits bacteria growth

$IC_{50}$ indicates the peptide concentration that inhibits 50% cell growth

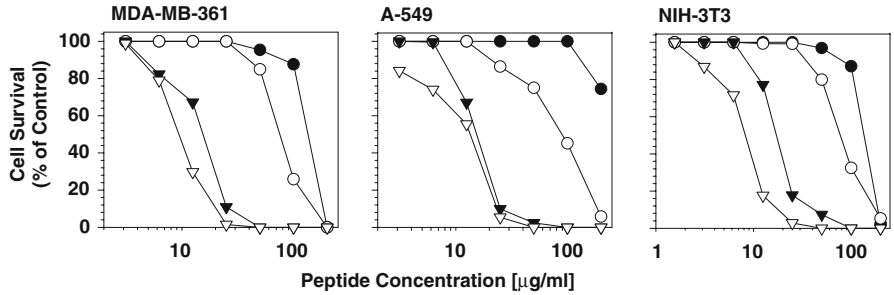


**Fig. 1** Concentration-response curves of the mammalian cell toxicity of the peptides against human A-549 and human MDA-MB-361 and mouse NIH-3T3 cells. Peptides are indicated as follows: tritrpticin (●), PST13-RK (○), di-PST13-RK-C (▼) and di-PST13-RK-K (▽)

antimicrobial activity against eight bacterial stains tested and slightly higher mammalian cell toxicity, compared with the parental tritrpticin. Interestingly, dimerization of PST13-RK (di-PST13-RK-C and di-PST13-RK-K) decreased antimicrobial activity against both Gram-negative and Gram-positive bacteria by 2–4 fold, whereas it induced a significant increase in mammalian cell toxicity against mouse NIH-3T3, human MDA-MB-361, and human A549 cells.

These results suggest that PST13-RK is active as a monomer to bacterial cells but as an oligomer to mammalian cells. On the other hand, the conformational change by the dimerization of PST13-RK was evaluated by measuring the CD spectra of the peptides in the membrane-mimicking environments. As shown in Fig. 2, both dimeric peptides displayed a CD spectral pattern similar to that of PST13-RK with a positive mean residue molar ellipticity at 212 nm in SDS micelles (Fig. 2). This result indicated that dimerization of PST13-RK does not affect on its overall $\beta$-turn structure in the membrane-mimicking environments.

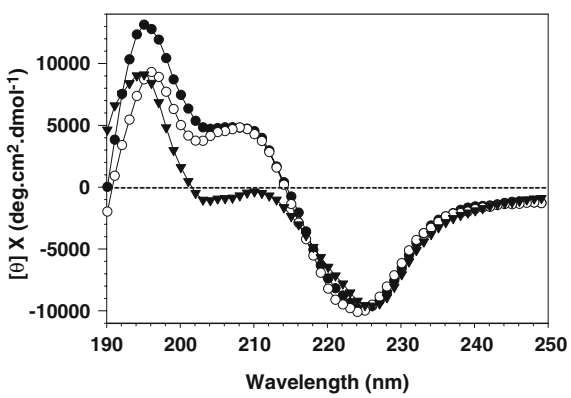


**Fig. 2** CD spectra of the peptides in the presence of 30 mM SDS micelles. Peptides are indicated as follows: PST13-RK (●), di-PST13-RK-C (○) and di-PST13-RK-K (▼)

The length, charge, hydrophobicity, $\alpha$-helicity, dipole moment and amphipathicity of $\alpha$-helical antimicrobial peptides are responsible for their antimicrobial activity and mammalian cell toxicity (Giangaspero et al. 2001). In addition, the oligomerization of $\alpha$-helical antimicrobial peptides in an aqueous environment is important for controlling their cell selectivity between bacterial and mammalian cells (Tossi et al. 2000). A disulfide-linked dimer of $\alpha$-helical magainin 2 had greater antimicrobial and hemolytic activities and formed larger pores in a membrane with a 3 fold-longer lifetime than the corresponding monomers (Dempsey et al. 2003). In addition, a dimer of lentivirus-derived $\alpha$-helical antimicrobial peptide showed a 16 fold increased bactericidal activity, compared with its monomeric form (Tencza et al. 1999). The authors proposed that $\alpha$-helical antimicrobial peptides form pores or ion channels through phospholipid membranes of bacterial cells by oligomerization of several peptide molecules, resulting in the microbial cell death. Unlike $\alpha$-helical antimicrobial peptides, dimers of PST13-RK with a $\beta$-turn structure were less active than monomer for antimicrobial activities. However, they exerted much more potent anticancer activities. Since peptides are becoming more and more important as potential drug candidates, we suggest that the significant anticancer potency of the dimers makes them attractive candidates for chemotherapeutic drugs.

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