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Membrane aggregation and perturbation induced by antimicrobial peptide of *S*-thanatin

Guoqiu Wu [a,1,*], Hongbin Wu [b,1], Linxian Li [b], Xiaobo Fan [b], Jiaxuan Ding [b], Xiaofang Li [b], Tao Xi [b], Zilong Shen [b,**]

[a] *Center of Clinical Laboratory Medicine of Zhongda Hospital, Southeast University, Nanjing, PR China*
[b] *Biotechnology Center, Department of Life Science and Biotechnology, China Pharmaceutical University, Nanjing 210009, PR China*

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ABSTRACT

Thanatin, a 21-residue peptide, is an inducible insect peptide. In our previous study, we have identified a novel thanatin analog of *S*-thanatin, which exhibited a broad antimicrobial activity against bacteria and fungi with low hemolytic activity. This study was aimed to delineate the antimicrobial mechanism of *S*-thanatin and identify its interaction with bacterial membranes. In this study, membrane phospholipid was found to be the target for *S*-thanatin. In the presence of vesicles, *S*-thanatin interestingly led to the aggregation of anionic vesicles and sonicated bacteria. Adding *S*-thanatin to *Escherichia coli* suspension would result in the collapse of membrane and kill bacteria. The sensitivity assay of protoplast elucidated the importance of outer membrane (OM) for *S*-thanatin's antimicrobial activity. Compared with other antimicrobial peptide, *S*-thanatin produced chaotic membrane morphology and cell debris in electron microscopic appearance. These results supported our hypothesis that *S*-thanatin bound to negatively charged LPS and anionic lipid, impeded membrane respiration, exhausted the intracellular potential, and released periplasmic material, which led to cell death.

1. Introduction

There is a significantly increasing interest in identifying new classes of antibiotics against drug-resistant bacteria [1]. Antimicrobial peptides (AMPs) have received great interests, which seem to be promising drug candidates for drug-resistant bacteria. AMPs are amphiphilic and positively charged molecules that have been isolated from plants, insects, amphibians and mammals [2]. These peptides exhibit a broad antimicrobial activity against microorganisms and play an important role in the innate immune defense system [3].

Thanatin (GSKKPVPIIYCNRRTGKCQRM), a cationic antimicrobial peptide isolated from the hemipteran insect *Podisus maculiventris*, shows broad antimicrobial activity against bacteria and fungi with low cytotoxicity. The secondary structure of thanatin adopts a well-defined anti-parallel β-sheet structure from 8th amino acid to the C-terminus, which is critical to its antimicrobial activity [4]. *S*-Thanatin, substituting threonine at position 15(Thr15) with serine, shows higher activity against the Gram-positive bacteria, lower toxicity and better tolerance with cations and pH conditions [5].

In the past few years, many studies have been undertaken to understand the mechanisms of AMPs. These studies have shown that cationic antimicrobial peptides including Defensin [6], Cecropin [7], Melittin [8] and Protegrin [9], kill bacteria by forming pores or ion channels in the cytoplasmic membrane, and subsequently lead to the disintegration of cell structure and the spillage of essential metabolites. Nevertheless, it is postulated that these peptides probably possess the same antimicrobial mechanism and particularly act on the bacterial membrane. Several models demonstrating the acting mode of antimicrobial peptides have been proposed [10]. Whether permeabilization occurs by channel formation or lipid destabilization may further depend on membrane site, composition and potential [11].

In this study, we tried to systematically investigate the interaction between *S*-thanatin and bacterial membranes, and to identify the possibility that the cytoplasmic membrane was the target for *S*-thanatin. From this point, we investigated the interactions between the peptide and large unilamellar phospholipids vesicles (LUVs) with various lipid compositions and surface charge densities. The results revealed that the surface charge and compositions of lipid membranes played a major role on the antimicrobial activity of *S*-thanatin. Interestingly, *S*-thanatin could cause acidic vesicles aggregation. Spectroscopic method, flow cytometric analysis and

* Correspondence to: Guoqiu Wu, Center of Clinical Laboratory Medicine of Zhongda Hospital, Southeast University, Nanjing 210009, PR China.
** Correspondence to: Zilong Shen, Biotechnology Center, Department of Life Science and Biotechnology, China Pharmaceutical University, Tongjiaxian 24#, Nanjing 210009, PR China.
E-mail addresses: guoqiuwu@163.com (G. Wu), Zilongshen@sina.com (Z. Shen).
[1] These authors are contributed equally to this work.

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transmission electron microscopy (TEM) were conducted in this study. Besides, the antimicrobial activities against *Escherichia coli* and *Bacillus subtilis* strains and cell-wall deficient L-form bacteria were also determined.

2. Materials and methods

2.1. Materials

S-Thanatin was synthesized using the solid-phase synthesis by Shanghai Sangon Co., Ltd. Brain heart infusion (BHI) medium was from Yocon (Beijing, China). 1-Palmitoyl-2-oleoyl-phosphatidylcholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1′-*sn*-glycerol) (POPG) (sodium salt) and Cardiolipin (CL) (Heart, Bovine) (sodium salt) were purchased from Avanti Polar Lipids (Albaster, AL, USA). Calcein was from Molecular Probes (Eugene, OR, USA) and used without further crystallization. Bis-(1,3-dibutylbarbituric acid) trimethine oxonol [$DiBAC_4(3)$] and propidium iodide (PI) were all purchased from Sigma (St. Louis, MO, USA). Bacitracin and Triton X-100 were purchased from Sangon (Shanghai, China). All other reagents were in high purity reagent grade and were used as received.

2.2. Bacterial strains

Escherichia coli ATCC 25922 and *B. subtilis* ATCC 21332 were used throughout this study; they were obtained from the Institute of Microbiology, China Pharmaceutical University.

2.3. Antimicrobial activity against wild type and L-form bacteria

The minimal inhibitory concentration (MIC) of bacterial growth was determined using a microtiter plate assay as previously described [12]. The peptides were tested against Gram-positive *B. subtilis* (ATCC 21332) and Gram-negative *E. coli* (ATCC 25922) cultivated in BHI. The inoculum was prepared from mid-log-phase cultures (OD_{600} = 0.4 ± 0.1). The final number of cells per well was 5×10^6/ml. The final concentrations of peptides, tested in duplicate, ranged between 2 and 200 μM. L-form *E. coli* and *B. subtilis* were prepared, cultivated in BHI containing 30 mg/ml saccharose and 10 mg/ml yeast extract. The spheroplasts concentration in the wells was approximately 10^8 cells/ml and the final peptide concentration ranged from 8 to 100 μM. After incubating the plates for 18 h at 37 °C, the MICs were determined in 96-well plates and defined as the lowest concentration of peptide for which no change in optical density could be observed. Bacitracin was used as positive control.

2.4. Preparation of liposome and leakage assay

Large unilamellar vesicles (approximately 100 nm in diameter) were prepared by the freeze/thaw and extrusion method as described before [13]. Lipid films were resuspended in 10 mM tris buffer (pH 7.4) containing 70 mM calcein and 0.1 mM EDTA. Calcein which was not entrapped was separated from the vesicles on a Sephadex G-25 column, and was eluted with 10 mM tris buffer (pH 7.4) containing 140 mM NaCl and 0.1 mM EDTA. The prepared vesicles were stable at room temperature for a few days. In calcein leakage assay, fluorescence excited at 490 nm and emitted at 517 nm, was measured on F96 Fluorescence Spectrophotometer (JY Horiba, Longjumeau Cedex, France). The percent of dye-release was evaluated by the equation, leakage (%) = $(F - F_0)/(F_{max} - F_0) \times 100$, where F_0 was the fluorescence intensity of vesicles without peptide or Triton X-100, whereas F and F_{max} were the fluorescence intensities achieved by peptide and 1% (v/v) Triton X-100, respectively.

2.5. Effects of S-thanatin on membrane integrity and potential by flow cytometric determination

The effect of *S*-thanatin on membrane potential and integrity of *E. coli* ATCC 25922 was monitored using flow cytometry by the method of Mason et al. [14]. Bacterial membrane potential was determined by the lipophilic anionic membrane potential-sensitive dye $DiBAC_4(3)$, and membrane integrity was assessed with fluorescent dye PI. A stock solution (100 μg/ml) of $DiBAC_4(3)$ was prepared in ethanol. PI was dissolved in deionized water to a concentration of 100 μg/ml. *E. coli* incubated overnight in 1% proteose peptone was diluted 1:100 with fresh medium and incubated at 37 °C to log-phase growth. Bacteria were washed in fresh medium, then were diluted to a density of 10^7 cells/ml. Bacteria were incubated in final concentration of 100 μg/ml *S*-thanatin in LB, with aliquots taken for analysis at 0, 15, 30, 60, 90 and 120 min. Bacteria were centrifuged at 12,000 rpm for 60 s. $DiBAC_4(3)$ or PI was added to aliquots of cell suspensions at each time point to give a final concentration of 10 μg/ml, and the mixtures were incubated at room temperature for 10 min before flow cytometric analysis. As a positive control for dye performance, a culture of *E. coli* ATCC 25922 in the early logarithmic phase was fixed with 70% ethanol.

The flow cytometer was from BD FACSCanto (Becton Drive, NJ, USA). Fluorescence measurements were conducted through the standard fluorescein isothiocyanate (FITC) filter block with the following characteristics: excitation, 470–490 nm; emission, 520–560 nm (beam splitter at 510 nm). The sheath flow pressure and sample flow rate were set to 0.7 kPa cm^{-2} and 2 μl min^{-1}, respectively.

2.6. Electron microscopy studies of E. coli treated with S-thanatin

Transmission electron microscope (TEM) was employed to confirm morphological changes, which was performed as Daniel S. Chapple described [15]. Log-phase *E. coli* ATCC 25922 (approximately 10^8 bacteria/ml) were incubated for 1 h in LB with *S*-thanatin (500 μg/ml), centrifuged at 4000*g* for 5 min, prefixed in 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.3), postfixed in 0.5% osmium tetroxide in Millonigs constant-osmolarity phosphate buffer (pH 7.4), and dehydrated in graded ethanol solutions. Pellets were embedded in medium Taab Resin and polymerized for 24 h at 60 °C. Sections (50 nm) were stained with aqueous uranyl acetate and lead citrate, and examined with a JEM-1010 transmission electron microscope (JEOL, Tokyo, Japan).

3. Results and discussion

3.1. Antimicrobial activity against wild type and L-form bacteria

MICs against typical Gram-positive and Gram-negative bacteria were summarized in Table 1. Antimicrobial activity of bacitracin against *B. subtilis* was more sensitive than *E. coli*. The cell-wall deficient L-form bacteria were resistant to bacitracin. *S*-Thanatin had more antimicrobial activity against Gram-negative bacteria than Gram-positive bacteria (not only *E. coli* and *B. subtilis*). The different activities against Gram-negative and Gram-positive bacteria

Table 1
Antimicrobial activities of *S*-thanatin against wild type and L-form bacteria.

Peptides	Anti-bacterial activity: MIC (μM)			
	E. coli		*B. subtilis*	
	Wild type	L-form	Wild type	L-form
S-thanatin	6.5	26	104	26
Bacitracin	1000	>2000	125	>2000

must be due to the various mechanisms of *S*-thanatin and bacitracin.

The MIC value of *S*-thanatin against L-form strain reached 4-fold increase compared with wild type for *E. coli*. It seemed that *S*-thanatin interacted with the OM of *E. coli*, but not cytoplasmic membranes. Besides, *S*-thanatin has shown a LPS-binding and -neutralizing activity in our previous study. In contrast, the susceptibility of *B. subtilis* protoplast increased. This showed the importance of cell-wall as a physical barrier for *B. subtilis*. Although there was various composition in cytoplasmic membrane between Gram-negative and Gram-positive bacteria, no distinct differences of MICs against L-form strains were found in Table 1. The outer envelope of Gram-negative bacteria played an opposite role compared with Gram-positive bacteria. The composition of cytoplasmic membrane seemed to be less important for *S*-thanatin's activity. Cationic *S*-thanatin must be attracted to bacterial surfaces, and one possible mechanism was electrostatic bond between *S*-thanatin and bacterial surface. The results underlined the important role of anionic lipids and LPS for antimicrobial activity of *S*-thanatin.

3.2. Peptide-induced calcein release from vesicles

Liposome model was employed to study the interaction between peptide and membrane. Fig. 1 showed the effect of *S*-thanatin on LUVs with different component. Serially diluting peptides (0–45 μM peptide concentrations) achieved peptide-to-lipid molar ratio (P/L) from 0.0125 to 1 in these assays. Inducing the leakage of calcein from the liposomes was not observed in POPC/POPG vesicles. However, *S*-thanatin was more effective in POPE/POPG LUVs than POPC/POPG LUVs. Adding CL to vesicles would cause more leakage, and it showed that CL was important as Hristova reported [16]. Additionally, bacitracin did not induce the leakage of calcein from all the liposomes (data not shown). These findings indicated that *S*-thanatin binding to membrane phospholipid was very important for the antimicrobial activity.

Interestingly, adding *S*-thanatin to anionic vesicles, such as POPE/POPG/CL and POPG/CL, caused visual vesicles aggregation and precipitation. The aggregation was completed within 5 min and the turbidity was dependent on the concentration of peptide. Compared with anionic vesicles, there was no obvious vesicles aggregation in neutral vesicles, such as POPC/POPG, POPE/POPG vesicles. These data suggested that *S*-thanatin induced the aggre-

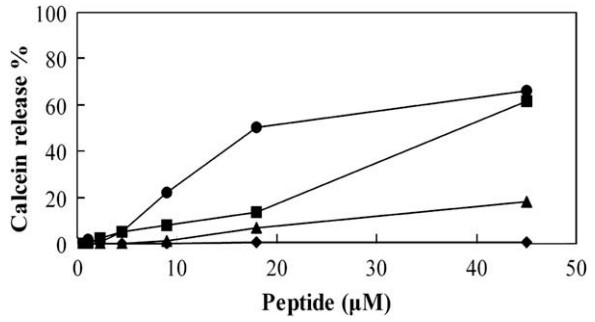

Fig. 1. Leakage percent from the vesicles consisting of (-●-) POPG/CL (1:1), (-■-) POPE/POPG/CL (4:1:1), (-▲-) POPE/POPG (2:1), and (-♦-) POPC/POPG (2:1). Peptide was added into a solution of 45 μM LUVs at 25 °C. The maximum level of fluorescence (100%) was determined by complete lysis of LUVs with Triton X-100. The zero level corresponded to LUVs fluorescence in the absence of peptide. The data represented mean values of three determinations.

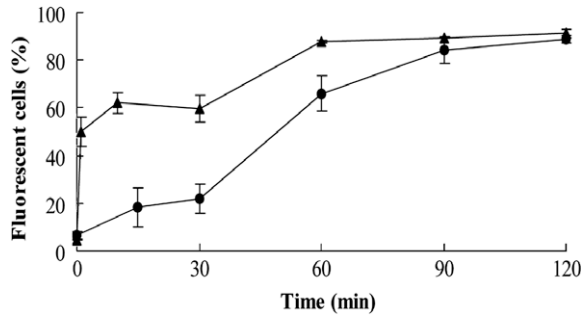

Fig. 3. Time course of *S*-thanatin (100 μg/ml) against *E. coli* ATCC 25922 at 37 °C. Mean percentages of fluorescent cells were shown in the presence of $DiBAC_4(3)$ (-●-) and PI (-▲-). The data represented mean values of three independent experiments. Bars represented the standard errors of the means.

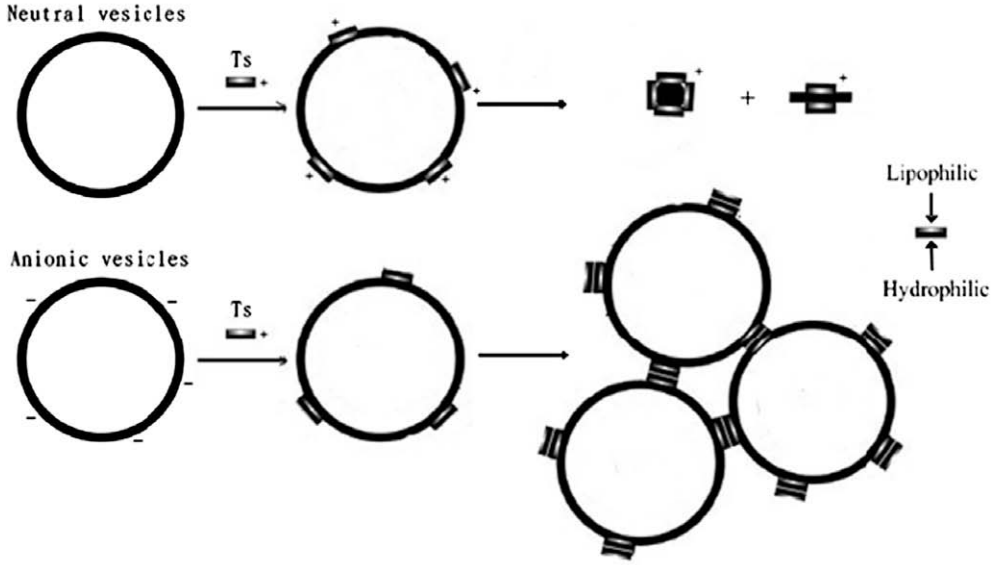

Fig. 2. The model to explain how *S*-thanatin (Ts) interacted with neutral and anionic vesicles. The peptide possesses two faces: the hydrophilic face is presented in black and the lipophilic face in light gray.

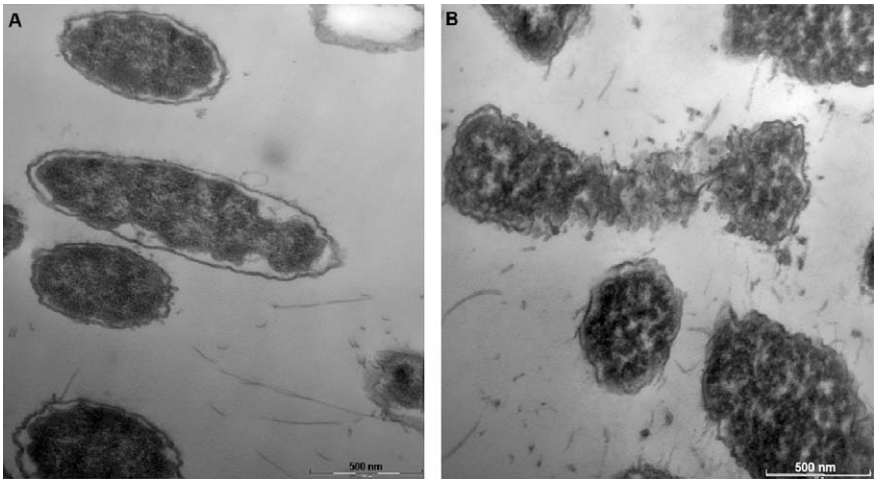

Fig. 4. Electron micrographs of *E. coli* ATCC 25922 cells (10^8 CFU/ml) incubated for 1 h in (A) LB broth alone, (B) LB broth with 500 μg/ml *S*-thanatin, as described in Methods.

gation of vesicles with high surface charge density. *S*-Thanatin could possibly induce aggregation by dehydrating the phospholipid head-groups [17] or causing charge neutralization, thereby decreased the electrostatic repulsions [18]. Fig. 2 showed the model, which explained how *S*-thanatin interacted with vesicles.

3.3. Effect of S-thanatin on E. coli membrane integrity and membrane potential

Adding *S*-thanatin (100 μg/ml) to *E. coli* suspension caused a gradual increase in fluorescent bacteria and membrane depolarization. Time-response assays (Fig. 3) showed that 50% fluorescent cells was observed after approximately 1 min in PI and after approximately 45 min in $DiBAC_4(3)$. It seemed that PI swiftly entered into cells after adding *S*-thanatin, while the cell membrane was still polarized. There was a large increase in fluorescent cells from 30 to 60 min. These findings suggested that *S*-thanatin first disrupted the cell membrane permeability, and then depolarized the cell membrane. Cells lost the ability to maintain membrane potential and metabolites. However, it seemed that collapse of membrane potential occurred before collapse of membrane integrity in the HLP 2 study [15]. Our results demonstrated that *S*-thanatin had a different mechanism from HLP 2, kaliocin-1 [19] and histatin 5 [20].

3.4. Electron microscopy images of E. coli treated with S-thanatin

To further characterize the antimicrobial activity of *S*-thanatin, we used TEM to examine *E. coli* ATCC 25922 pretreated with *S*-thanatin. Log-phase *E. coli* was exposed to *S*-thanatin for 1 h (Fig. 4B), and showed remarkable changes under electron microscopic (Fig. 4A). Additionally, *S*-thanatin induced chaotic membrane morphology and cell debris. *S*-Thanatin showed different mechanism from other antimicrobial peptides. Lactoferricin [21] and defensins [22] could cause electron-dense membrane blisters. HLP 2 could detach the OM at specific sites, either at the peripheral ends of the cells or at the point of division [15].

4. Conclusions

Our results indicated that *S*-thanatin mainly disrupted lipid membranes of bacteria. The inverse effects of *S*-thanatin on wild type bacteria and protoplast showed OM with LPS played an important role for killing bacterial [23]. *S*-Thanatin interacted with membrane and caused similar aggregation as CPPs and RL16 did [24,25], but did not form channels into cytoplasmic membranes as melittin did [26]. *S*-Thanatin had the similar effect with HP(2–20) [27], which disrupted the cytoplasmic membrane either by specific disrupting ion channels or by nonspecific pore formation. These results demonstrated that the *S*-thanatin perturbed membrane lipid bilayers, caused certain cellular material leakage, and removed the electrical potential of the membrane.

Different models describing the process of membrane destruction by these peptides have been developed ranging from the "barrel-stave" model of alamethicin [28], where transmembrane bundles of amphipathic helices should be formed, to the "carpet" and "toroidal-hole" models of cecropin P1 and melittin, where peptides first cover the membrane like a carpet, and form toroidal-like lesions in a way that the peptides are always associated to the lipid head-groups [29]. All in all, our data supported that *S*-thanatin adopted a "carpet" model during the antibacterial process. The antimicrobial action could be divided into four main stages. In the initial stage, *S*-thanatin was attracted to LPS and phospholipid due to the electrostatic and hydrophobic interaction, and then attached to the hydrophilic surface of lipid membrane like "carpet" (limiting step). In the second stage, membrane destabilization and distortion started. The perturbation of membrane caused damage to energization and respiration of cytoplasmic membrane. In the third stage, *S*-thanatin inserts into the cytoplasmic membrane leading to membrane permeabilization and depolarization. In the fourth stage, after disintegration of membranes, bacteria became tattered and cytoplasm material leaks out. Finally, bacteria lose viability.

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