Peptides 45 (2013) 73–77

Contents lists available at SciVerse ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

Application of S-thanatin, an antimicrobial peptide derived from thanatin, in mouse model of *Klebsiella pneumoniae* infection

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ARTICLE INFO

Article history:
Received 4 March 2013
Received in revised form 19 April 2013
Accepted 19 April 2013
Available online 1 May 2013

Keywords:
S-thanatin
Antimicrobial peptide
Antimicrobial activity
Survival rates
Endotoxin level

ABSTRACT

Thanatin was first discovered from the hemipteran insect *Podisus maculiventris* and showed a promising antimicrobial activity. Multidrug-resistant (MDR) clinical isolates of *Klebsiella pneumoniae* have developed resistance to current therapies. As an attempt to resolve this problem, the efficacy of thanatin and its analogues against clinical isolates of *K. pneumoniae* was studied *in vitro* and *in vivo*. S-thanatin showed an improved antimicrobial activity with the tested MIC values was 2–8-fold lower than those of other thanatin analogs. Antimicrobial assay indicated a high activity of S-thanatin against *K. pneumoniae in vitro* with MIC between 4 and 8 μg/ml. Its *in vivo* activity was evaluated using a *K. pneumoniae*-infected mice model. Adult male ICR mice were randomly grouped and given an intraperitoneal (i.p.) administration of 2×10^{10} colony-forming units of *K. pneumoniae* (CI 120204205). Afterwards, mouse groups were subjected to i.p. administration of saline or S-thanatin (5, 10, or 15 mg/kg). After an inspection of 72 h, the mice were finally sacrificed for analysis of *in vivo* bacterial growth and plasma endotoxin level. The results showed that S-thanatin administration apparently improved the survival rate and reduced the bacterial CFU from intra-abdominal fluid in mice. The plasma endotoxin level was improved as well. All above implied that S-thanatin, as an alternative, may provide a novel strategy for treating *K. pneumoniae* infection and other infections due to multidrug-resistant bacteria.

1. Introduction

The increasing antibiotic resistance from clinic evokes an urgent demand for new antibacterial classes that are not affected by resistance mechanisms. In response to the worldwide severe challenges of drug resistance, the development of new antibacterial compounds has plummeted in the post decades [2,11,16]. Carbapenem antibiotics such as ertapenem and imipenem have been broadly utilized in hospitals [13], however, carbapenem-resistant clinical strains such as *Klebsiella pneumoniae* started to emerge in the last few years. Fortunately, a variety of naturally occurring peptides termed antimicrobial peptides (AMPs) seem to be capable to solve this problem.

AMPs are generally defined as having 12–50 amino acids with 2–9 positively charged lysine or arginine residues and up to 50% hydrophobic amino acids, showing a broad spectra of activity to bacteria, viruses, fungi, and parasites [7,8,10]. They have been isolated from a variety of organisms including plants, invertebrates, prokaryotes and mammals [1,12]. AMPs have emerged as ideal candidates to be utilized as a substitute for antibiotic therapy [15].

Thanatin (GSKKPVPIIYCNRRTGKCQRM), a cationic AMP containing anti-parallel β-sheet constrained by disulfide bonds, was isolated from the hemipteran insect *Podisus maculiventris*. It was found to be the first insect antimicrobial peptide and showed a broad antimicrobial activity against Gram-negative bacteria, Gram-positive bacteria, and fungi [6].

S-thanatin, a novel thanatin analog, is substituted threonine at position 15 of thanatin with serine [19]. Our previous studies showed that S-thanatin has a broad-spectrum antimicrobial activity, especially on Gram-negative bacteria [21]. Moreover, we have reported its antimicrobial activity against a multidrug-resistant (MDR) clinical isolate and confirmed that S-thanatin exert its antibacterial effect through binding to negatively charged LPS and anionic lipid, impeding membrane respiration, exhausting the intracellular potential, and releasing periplasmic material [23]. In this work, we firstly investigated the activity of various thanatin analog on *K. pneumoniae* reference strain and clinical isolates

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http://dx.doi.org/10.1016/j.peptides.2013.04.012

resistant to conventional antibiotics. Furthermore, the antibacterial activities of the peptides on mouse models of sepsis caused by *K. pneumoniae* resistant to ertapenem and imipenem were also assessed *in vivo*.

2. Materials and methods

2.1. Preparation of S-thanatin and thanatin analogs

Thanatin analogs (Table 1.) were synthesized by the solid-phase methodology with 9-fluorenyl-methoxy-carbonyl as protecting group. The crude compounds were purified by reverse-phase high-performance liquid chromatography (RP-HPLC) using an appropriate 0–60% acetonitrile gradient in 0.05% trifluoroacetic acid. Molecular weights were determined by electrospray mass spectrometry using an API instrument (Perkin Elmer SCIEX) as a quality control of the synthesis [18]. The peptides were taken up in oxidation buffer (1 mg/1 ml) [100 mM ammonium acetate (pH 8.5)] to refold for 3 days at room temperature under stirring and purified by RP-HPLC. The prepared peptides were kept in −80 °C after freeze-dried.

2.2. Microorganisms

A total of 18 clinical isolated *K. pneumoniae* strains used in this study were collected between February 2012 to July 2012 in the Center of Medical Laboratory of Zhongda Hospital (Southeast University, China). AST-GN13 cards and GN/CE strips with the VITEK2 system (bio Mérieux, Marcy l' Etoile, France) were used to confirm identities and susceptibilities of clinical isolates. *K. pneumoniae* ATCC 700603 and *Escherichia coli* ATCC 25922 from ATCC were used as reference.

2.3. Animals

Adult male ICR mice (27–30 g) were obtained from the Animal Center, Science Academy of China (Shanghai, China). All animals were housed in individual cages and acclimatized for 1 week in Animal Environmental Control Unit (temperature, 23 ± 3 °C; relative humidity, 50 ± 10%; light–dark cycle, 12 h). All animals were reared in the dark room during the experimental period.

2.4. Minimum inhibitory concentration assay

To test antimicrobial activity of thanatin analogs, the minimum inhibitory concentration (MIC) values were determined by a microdilution assay in sterilized polypropylene 96-wells microtiter plates (Corning Crop) according to the broth microdilution guideline of Clinical and Laboratory Standards Institute (CLSI) [21]. Briefly, a 10-μl aliquot of the purified peptide were added to 90 μl of a logarithmic phase culture of the tested bacteria which were diluted to 10^5–10^6 colony forming units (CFU) per ml. Control wells were inoculated with 10 μl of phosphate-buffered saline (PBS). Final concentration of thanatin analogs in the test well was 0.5 μg/ml, 1 μg/ml, 2 μg/ml, 4 μg/ml, 8 μg/ml, 16 μg/ml, 32 μg/ml, 64 μg/ml, and 128 μg/ml, respectively. Microbial growth was measured as an increase of optical density at 630 nm by a microplate reader (MRX, Dynex) after incubation at 37 °C for 16–24 h. MICs are expressed as the lowest concentration at which antimicrobial peptides cause 100% of growth inhibition [5]. Experiment of each concentration was performed in triplicates.

Table 1
The primary sequences of thanatin analogs.

Peptide	Primary sequence[a]
Thanatin	GSKKPVPIIYCNRRTGKCQRM
Ts	GSKKPVPIIYCNRRSGKCQRM
Tq	GSKKPVPIIYCQRRTGKCQRM
Ta	GSKKPVPIIYCNRRTAKCQRM
Tqs	GSKKPVPIIYCQRRSGKCQRM
Tqa	GSKKPVPIIYCQRRTAKCQRM
Tsa	GSKKPVPIIYCNRRSAKCQRM

[a] Italic indicates the changed amino acid.

2.5. K. pneumoniae challenge and treatments

Septic mouse model was created by an intraperitoneal (i.p.) inoculation of MDR clinical isolate *K. pneumoniae* (CI 120204205) containing 2×10^{10} colony-forming units (CFU). Under the experiment condition, five groups with each randomly containing 10 animals were used in this experiment: (1) Group 1 was control group inoculated with *K. pneumoniae* but without any cure. (2) Group 2 was blank control group injected intravenously a total volume of 150 μl sterile saline. (3) Groups 3–5 were cure groups with administration of 5, 10, or 15 mg/kg of S-thanatin separately. Survival rates were monitored every 6 h for 3 days. Blood samples for culture were collected by aseptic cardiac puncture. To perform quantitative evaluation of bacteria in the intra-abdominal fluid, animals received an i.p. injection of 2 ml sterile saline. Peritoneal fluid samples were serially diluted and spread onto blood agar plates. The limit of detection was ≤1 log CFU/ml. Plates were incubated at 37 °C for 48 h. All experimental protocols complied with the Laboratory Animal Care and Use Guidelines of The Affiliated Zhongda Hospital of Southeast University.

2.6. Endotoxin level assay in experimental animal models

For determination of endotoxin levels in plasma in the animal model, 0.2 ml blood samples were collected by aseptic cardiac puncture, and then transferred to tubes containing ethylene diamine tetra-acetic acid (EDTA) tripotassium salt. Endotoxin concentrations were measured by a commercially available lipopolysaccharide (LPS) assay kit (Hou-regent, Inc.). Plasma samples were serially diluted two-fold with sterile pyrogen-free water and heat-treated for 5 min in a water-bath at 75 °C to destroy inhibitors that can interfere with activation. Endotoxin standards were tested in each run and the concentrations of endotoxin in the text samples (EU/ml) were calculated according to the standard curve.

2.7. Statistical analysis

MICs are presented as average values from three independent measurements. Survival rates and qualitative results for blood cultures between groups were compared using Fisher's exact test (significance level fixed at 0.05). Quantitative evaluation of bacteria in the intra-abdominal fluid cultures is presented as mean ± standard deviation of the mean. Statistical comparisons between groups were performed by analysis of variance (significance level was fixed at 0.05). The difference between data for each group was considered significant at a level of <0.05.

3. Results

3.1. Antimicrobial activities of thanatin analogs

The primary sequences of thanatin analogs were shown in Table 1 MICs of thanatin analogs against *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were summarized in Table 2. S-thanatin showed a superior antimicrobial activity to other analogs. The