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Subacute toxicity of antimicrobial peptide S-thanatin in ICR mice

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ABSTRACT

Antibiotics are commonly used for infectious diseases and saved a lot of lives since its discovery, but the emergence of drug-resistant microorganism has brought a tremendous challenge to clinical therapy at present. Antimicrobial peptides, which are of broad antimicrobial spectrum and rare resistance development in pathogens, are expected to replace conventional antibiotics. S-thanatin, a novel antimicrobial peptide with 21 amino acid residues, was proved of significant benefit on therapy of pathogens infection. To evaluate the security of S-thanatin, its subacute toxicity was examined in ICR mice by continually intravenous injection with 125, 50, 20 mg/kg (1/4, 1/10, 1/25 LD_{50}) or saline with equal volume for two weeks. Results demonstrated that neither significant difference of serum chemistry and hematology, nor pathological changes were changed in major organs caused by S-thanatin between groups. In conclusion, S-thanatin appears to be a safe antimicrobial peptide for further preclinical trials.

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1. Introduction

Antibiotic resistance induced by abuse of antibiotics is a growing problem in the treatment of clinical infectious diseases caused by bacteria, fungi, parasites and viruses. In particular, the superbugs, a variety of bacterial resistant to multiple or all antibiotics and lack effective treatment, have emerged over the past decades as a major health problem over the world [2,19], which is especially severe in hospitals and chronic care circumstances for the strong selective pressure to the occurrence of resistance [1,13,14,19]. These superbugs include methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococci* (VRE), and multidrug-resistant *Pseudomonas*, and cause thousands of infections annually [1].

In response to the worldwide severe challenges, only three antibacterial antimicrobials with novel structure entered the market over the last 40 years [1,11,15]. Therefore, it is imperative to develop new antibacterial drugs with potentials to kill these drug-resistant bacterial pathogens. Recently, a variety of natural peptides antimicrobial peptides (AMPs) seem like a promising candidate to solve this problem. By and large, they are small, cationic and amphipathic peptides with variable length, sequence and structure, and present in a wide variety of organisms including prokaryotes, plants, and vertebrates [5,12,18]. Such peptides are typically 12–50 amino acids in length with 2–9 excess basic residues (arginine or lysine) and up to 50% hydrophobic amino acids, having a broad spectra of activity to bacteria, fungi, viruses, and parasites [7]. In recent years, AMPs have received extensive attention as a possible source of novel antimicrobials due to their rapid efficacy to a broad range of microbes, rare resistance development, and very limited immunogenicity [1]. To date, approximately 500 AMPs have been reported, which can be retrieved online at http://www.bbcm.univ.trieste.it/~tossi/antimic.html. For development of therapeutic drugs, some of them have already been evaluated in clinical trials [5].

In 1996, thanatin (GSKKPVPIIYCNRRTGKCQRM), a cationic AMP containing a β-sheet structure from 8th amino acid to the C-terminus constituted by a disulphide bond, was isolated from the hemipteran insect *Podisus maculiventris* [3]. It is identified as the first insect antimicrobial peptide and shows broad antibiotic activity against Gram-negative bacteria, Gram-positive bacteria, and fungi without cytotoxicity [6,10]. S-thanatin, previously obtained by substituting the fifteenth amino acid of threonine with serine in the disulphide loop of thanatin, which plays an important role in its antimicrobial activity [9]. The antibacterial activity of S-thanatin is superior to thanatin against some multidrug-resistant bacteria [20,21]. Moreover, our previous researches indicated that it could improve the survival rates of sepsis mice and had a good synergy with conventional antibiotics [21]. These promising efficacies were thought to benefit from its activities of endotoxin binding and directly bactericidal potency, which was totally different from the conventional antibiotics [21,25]. Although the

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production procedure, antibacterial activities in vitro and in vivo have been comprehensive investigated, the security of S-thanatin has never been studied. For this purpose, the study was designed to investigate the subacute toxicity of S-thanatin in ICR mice.

2. Materials and methods

2.1. Animals and maintenance

All ICR mice (28–31 g) were obtained from the Laboratory Animal Center, Science Academy of China (Shanghai, China), and caged in groups by sex and dose levels at random. All animals were raised in GLP laboratory under specific pathogen-free conditions with temperature of $23 \pm 1\,^{\circ}C$ and relative humidity of $50 \pm 10\%$. Artificial light was provided continuously in a light/dark cycle (12 h/12 h). The mice were provided food and water freely.

2.2. Preparation of S-thanatin

S-thanatin was synthesized by the solid-phase method with a 9-fluorenyl-methoxycarbonyl (Fmoc) protecting group. Peptides were cleaved from the resin with a solution containing 95% trifluoroacetic acid (TFA), 2.5% water and 2.5% triisopropylsilane. After repeating precipitation with diethyl ether, the peptides were purified by reverse-phase high-performance liquid chromatography (RP-HPLC) using an appropriate 0–60% acetonitrile gradient in 0.05% trifluoroacetic acid. Molecular mass was determined by electrospray mass spectrometry using an API instrument (Perkin Elmer SCIEX) as a quality control of the synthesis. The peptide was taken up in oxidation buffer (1 mg/1 ml) [100 mM ammonium acetate (pH 8.5)], allowed to refold for 3 days at room temperature under stirring and purified by RP-HPLC. The purity of prepared S-thanatin was analyzed by HPLC–MS. It was kept in $-80\,^{\circ}C$ after freeze-drying.

2.3. Experimental protocol

To evaluate the possible toxicity of S-thanatin in mice, 80 mice were divided into four groups (10 males and 10 females for each group): high, middle, low dosage groups and the control group on the basis of weight. Before the experiment, the animals were maintained in GLP laboratory to acclimatize for one week. All groups were continually injected with 125, 50, 20 mg/kg (1/4, 1/10, 1/25 LD_{50}) of S-thanatin or saline with equal volume respectively via tail veins once daily (once a day) for two weeks. The mice were raised for one more week as the convalescent stage after the administration was accomplished. The routine behaviors were observed daily after administration till the end of convalescent stage.

All animals were sacrificed in batch at the end of administration and the convalescent stage after starvation for 12 h to conduct routine hematology test, plasma biochemical examination and pathological assay. EDTA-2K anti-coagulated blood samples collected from inner canthus of the mice were used for routine blood testing on a fully automated blood cell analyzer (M150KX-21N, Japan). For biochemical studies, blood was drawn from orbital plexus before sacrificing the animals. Serum samples harvested from the mice were used to determine activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein (TP), albumin (ALB), g-glutamyl transpeptidase (g-GT), glucose (GLU), blood urea nitrogen (BUN), and creatinine(Cr), total cholesterol (TC) and triglyceride (TG) on an auto-biochemic alanalyzer (HITACHI7020, Hitachi, Japan).

Soon after blood sampling, animals were sacrificed through cervical dislocation at the end of administration and the convalescent stage. Then the hearts, livers, spleens, lungs, and kidneys were dissected, fixed in 10% neutral buffered formalin, embedded in paraffin wax, sectioned at 5 μm, and processed for routine histology with hematoxylin and eosin stain.

2.4. Statistical analysis

All data were presented as mean ± SD and analyzed by one-way analysis of variance, then the statistical differences between administration groups and the control group were evaluated by paired two-tailed Student's *t*-test. A probability of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Assay of prepared S-thanatin

To guarantee the prepared S-thanatin was appropriate for conduction of the subacute toxicity study, the purity of S-thanatin was analyzed by HPLC–MS, and HPLC result showed that there was only one peak with purity of 95.27% (>95%), and MS spectrum demonstrated that the molecular weight of synthesized S-thanatin was identical to the theory value.

3.2. Routine behaviors observation

There was no death during the experiment period. The weight of mice increased steadily, with no significant differences between groups. The routine behaviors were normal throughout the experiment, including food consumption, movement and excretion. The results showed that S-thanatin did not influence routine behaviors of the experimental mice.

3.3. Routine hematology test

To evaluate the effect of S-thanatin on hematology for a diagnosis of possible diseases and lesions, all of the routine hematology parameters were measured by a fully automated blood cell analyzer. The platelet count (PLT) value of 50 mg/kg group was higher than other three groups without significance ($P > 0.05$), and no dose-dependent relationship was observed among the groups (Table 1A). Therefore, it could be considered that the dose of S-thanatin had no effect on platelet counts in mice. It was shown in Table 1B that white blood cell (WBC) of 125 mg/kg group was prone to decrease but not significant at the convalescent stage in contrast to the control group, and no other routine hematology parameters of the administration groups were observed of significant difference over the control group (Table 1A and B). Therefore, S-thanatin was supposed to be harmless for the routine hematology of mice.

3.4. Plasma biochemical examination

Several basic blood biochemical parameters were examined at the end of administration period, and at the end of a 3-week drug-free convalescent stage. It was concluded in Table 2, although some biochemical parameters were a little higher or lower than the control group, the differences were not significant and did not show a dose-dependent relationship. The results indicated that S-thanatin was safe to the basic metabolism of ICR mice.

3.5. Pathological assay

Histopathology examination of heart, liver, spleen, lung and kidney indicated no structural abnormalities. H&E staining result showed that the heart and kidney of each group were intact, with normal cell morphology and no obvious abnormalities (Fig. 1A and B). Spleens of all groups had integrate structure, but the multinucleated giant cells increased and aggregated (Fig. 1C and D), which

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