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Stigmurin and TsAP-2 from *Tityus stigmurus* scorpion venom: Assessment of structure and therapeutic potential in experimental sepsis

Alessandra Daniele-Silva [a], Richele J.A. Machado [a, b], Norberto K.V. Monteiro [c], Andréia B. Estrela [a], Elizabeth C.G. Santos [a], Eneas Carvalho [d], Raimundo F. Araújo Júnior [e], Raniere F. Melo-Silveira [b], Hugo Alexandre O. Rocha [b], Arnóbio A. Silva-Júnior [a], Matheus F. Fernandes-Pedrosa [a, b, *]

[a] *Laboratório de Tecnologia e Biotecnologia Farmacêutica, Departamento de Farmácia, Universidade Federal do Rio Grande do Norte, Natal, Rio Grande do Norte, Brazil*
[b] *Programa de Pós-Graduação em Bioquímica, Universidade Federal do Rio Grande do Norte, Natal, Rio Grande do Norte, Brazil*
[c] *Instituto de Química, Universidade Federal do Rio Grande do Norte, Natal, Rio Grande do Norte, Brazil*
[d] *Centro de Biotecnologia, Instituto Butantan, São Paulo, Brazil*
[e] *Departamento de Morfologia, Universidade Federal do Rio Grande do Norte, Natal, Rio Grande do Norte, Brazil*

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ABSTRACT

Microbial resistance to conventional antibiotics is a public health problem worldwide, motivating the search for new therapeutic alternatives in varied natural sources. Cationic peptides without disulfide bridges from scorpions have been targeted in this context, mainly due to their multifunctional action and the limited ability of microorganisms to develop resistance against them. The present study was focused on Stigmurin and TsAP-2, cationic peptides found in the transcriptome of the venom gland from the scorpion *Tityus stigmurus*. The aims were: to assess the secondary structure of TsAP-2 and the structural stability of both peptides by circular dichroism; to evaluate their antiproliferative effect, and antimicrobial activities *in vitro*, *ex vivo* and *in vivo*; and to investigate their therapeutic potential in a murine model of polymicrobial sepsis. Stigmurin and TsAP-2 secondary structures responded similarly to environment polarity changes, and were stable to temperature and pH variation. Both peptides showed antiproliferative effect on tumor cells. TsAP-2 showed lower cytotoxicity to normal cells, and had a mitogenic activity on murine macrophages. Stigmurin demonstrated bactericidal and bacteriostatic activity, depending on the microorganism, whereas TsAP-2 had bactericidal action upon different bacterial strains analyzed. Both peptides were able to reduce leukocyte migration, TNF-α levels and microorganism load in the peritoneal cavity after induction of experimental sepsis, decreasing inflammation in the lung and cecum of septic animals. TsAP-2 also reduced the release of nitric oxide in the peritoneal cavity. Taken together, these data suggest that Stigmurin and TsAP-2 are structurally stable molecules and are efficient in the control of the infectious focus in polymicrobial sepsis, with potential use as a prototype for the rational design of novel therapeutic agents.

1. Introduction

Sepsis is a complex syndrome characterized by an unbalanced immune response triggered by an infectious stimulus, causing high mortality and high costs of hospitalization (Brun-Buisson, 2006; Levy et al., 2003; Lewis et al., 2012). Appropriate antimicrobial therapy has been associated with increased survival in septic patients (Garnacho-Montero et al., 2006; Harbarth et al., 2003). The control of the infection focus is an important therapeutic measure used for treating sepsis. Some studies, however, have shown that treatment with conventional antibiotics may release bacterial components that contribute to the induction of inflammatory

* Corresponding author. Universidade Federal do Rio Grande do Norte, Av. Gal. Cordeiro de Farias, s/n, CEP 59012-570, Natal, RN, Brazil.
E-mail address: mpedrosa@ufrnet.br (M.F. Fernandes-Pedrosa).

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mediators, worsening symptoms of septic patients (Dellinger et al., 2013; Eng et al., 1993; Holzheimer, 2001).

The rapid development of antibiotic resistance by pathogens, combined to a reduced rate of new drugs development, has become a serious public health problem worldwide (Spellberg et al., 2004; Tenover, 2006; Williams and Bax, 2009). Systemic infections caused by multiresistant microorganisms are a grave therapeutic challenge, leading to incessant search for new antimicrobial agents in different natural sources (Ammerlaan et al., 2009; Cosgrove et al., 2003; Gao et al., 2005; Harrison et al., 2014; Tam et al., 2015).

Animal venoms consist of a complex source of active components with high therapeutic potential (Lewis and Garcia, 2003). Non-lethal doses of venom from the scorpion *Tityus serrulatus* or the snake *Vipera aspis* have demonstrated the ability to reduce mortality in experimental sepsis, promoting the control of inflammatory responses (Frolkis et al., 2010; Maciel et al., 2014). The increase in survival of septic animals after pretreatment with *T. serrulatus* venom has been associated to the presence of antimicrobial peptides with antimicrobial activity, as well as to indirect effects on leukocytes, stimulating the production of antimicrobials (Maciel et al., 2014).

Cationic peptides without disulfide bridges have been identified in different species of scorpions, showing multiple activities, including: insecticide, antiviral, antimicrobial, hemolytic, antiproliferative, bradykinin-potentiating, and immunomodulatory (Almaaytah and Albalas, 2014; Du et al., 2014; Erdes et al., 2014; Hancock, 2001; Miyashita et al., 2010; Yan et al., 2011; Zhao et al., 2009, 2012b). Microorganisms have shown reduced ability to develop resistance mechanisms to these non-disulfide-bridged peptides (NDBPs), making them a group of promising molecules for biotechnological applications (Almaaytah and Albalas, 2014; Marr et al., 2006).

Stigmurin is a cationic NDBP found in the venom gland transcriptome of the scorpion *T. stigmurus*. It had its secondary structure investigated, as well as antimicrobial and antiproliferative properties reported *in vitro,* with low hemolytic activity (Melo et al., 2015). The clone TSTI0002C (GenBank: JK483710.1), obtained in the same transcriptomic study as Stigmurin, encodes for an aminoacid sequence identical to TsAP-2 peptide present in the scorpion *T. serrulatus*, which has antimicrobial and antiproliferative effect at non-hemolytic concentrations (Almeida et al., 2012; Guo et al., 2013).

Considering the therapeutic potential of these peptides, the present study was performed to evaluate the secondary structure of TsAP-2, to verify the structural stability of Stigmurin and TsAP-2, and to investigate their antiproliferative and antimicrobial activity *in vitro, ex vivo,* and *in vivo* using a murine model of polymicrobial sepsis.

2. Materials and methods

2.1. Ethical statement

Swiss mice 6–8 weeks old (30–35 g, males and females) were obtained from the Animal Facility at the Health Science Center of the Federal University of Rio Grande do Norte (UFRN). The experimental animals were kept under controlled temperature (23 ± 2 °C) and humidity (50–55%), with commercial diet and water *ad libitum*, considering the animal's circadian cycle. The experimental protocol of the study was approved by the Ethics Committee on Animal Experiments of UFRN under protocol #047/2015, in accordance to the guidelines of the National Council for Animal Experiments Control (CONCEA), and the International Guiding Principles for Biomedical Research Involving Animals, by the Council of International Organizations of Medical Sciences (CIOMS).

2.2. Peptides synthesis

C-terminal amidated Stigmurin (FFSLIPSLVGGLISAFK-NH_2) and TsAP-2 (FLGMIPGLIGGLISAFK-NH_2) were commercially synthesized by Invitrogen Life Technologies (Waltham, Massachusetts, USA) and GenOne Biotechnologies (Rio de Janeiro, Brazil), respectively, and stored at −20 °C until use. The peptides masses were assessed by mass spectrometry with electrospray ionization and their purity confirmed by high performance liquid chromatography (>90% purity).

2.3. Analysis of secondary structure and structural stability by circular dichroism

TsAP-2 (57.7 μM) was evaluated by circular dichroism (CD) on a JASCO J-810 spectropolarimeter (Tokyo, Japan) at 25 °C in 1 mm quartz cuvette. The spectra were recorded five times at wavelengths of 182 nm–260 nm, at 50 nm min^{-1}, using a Peltier system for temperature control. The analyses were performed either in ultrapure water, or in one of the following solutions: 20 mM sodium dodecyl sulfate (SDS), 0.1% (v/v) sodium phosphate buffer (PBS) (NaCl 137 mM, KCl 3 mM, KH_2PO_4 1.5 mM and Na_2HPO_4 10 mM, pH 7.4), or 2,2,2-trifluoroethanol (TFE) in water at 30%, 50% or 70% (v/v). The spectra are presented in molar ellipticity (Juban et al., 1997) as a function of the wavelengths. The secondary structure percentual composition was obtained by deconvolution of the spectra, using the server Dichroweb (Whitmore and Wallace, 2008), applying the algorithms Selcon3 (Sreerama et al., 1999) CONTIN/LL (van Stokkum et al., 1990) and CDSSTR (Compton and Johnson, 1986).

The influence of temperature on the secondary structure of Stigmurin (183.82 μM) and TsAP-2 (57.7 μM) in 20 mM SDS was analyzed by CD at 222 nm in a 1 mm quartz cuvette. Samples were heated from 2 to 98 °C, and then cooled back to the initial temperature. Data collection was performed every 0.1 °C. To evaluate the influence of pH on the secondary structure of Stigmurin (183.82 μM) and TsAP-2 (57.7 μM) in the presence of 20 mM SDS, a buffer system consisting of 0.1 M citric acid ($C_6H_8O_7$)/0.2 M sodium phosphate (Na_2HPO_4) (pH 3.0–7.4), or 0.2 M boric acid (H_3BO_3)/0.05 M sodium borate ($Na_2B_4O_7 \cdot 10H_2O$) (pH 8.0–9.0) was used (Russell et al., 2012). The secondary structure percentual composition was obtained by deconvolution of CD spectra as described above.

2.4. Cell proliferation assay

Human hepatocellular carcinoma cell line (HepG2; ATCC HB-8065), epithelial cells from canine kidney (MDCK) (ATCC CCL-34) and murine macrophages (RAW 264.7; ATCC TIB-71) were kindly supplied by the Laboratory of Biotechnology of Natural Polymers at UFRN. Cells were grown until confluent in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum in 96-well plates, then incubated for 24 h with increasing concentrations of Stigmurin or TsAP-2 (2, 4, 8, 10, 20 and 40 μM). A solution of 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Saint Louis, MO, USA) at a concentration of 2 mg mL^{-1} was added to the plates and incubated for 4 h (Mosmann, 1983). The formazan crystals were made soluble in 96% ethanol and the absorbance measured at 570 nm in a microplate reader (Epoch-Biotek®, USA). Cells incubated in the absence of peptide were used as positive controls. All experiments were performed in triplicate and the percentage of viable cells calculated based on the positive control (100%) (Melo-Silveira et al., 2014).