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Effects of a marine serine protease inhibitor on viability and morphology of *Trypanosoma cruzi*, the agent of Chagas disease

Natália Pereira de Almeida Nogueira[a], José Andrés Morgado-Díaz[b], Rubem Figueiredo Sadok Menna-Barreto[c], Marcia Cristina Paes[a], Raquel Elisa da Silva-López[d,*]

[a] *Laboratório de Interação de Tripanosomatídeos e Vetores, Departamento de Bioquímica, Instituto de Biologia Roberto Alcântara Gomes, Universidade do Estado do Rio de Janeiro, UERJ, Rio de Janeiro, RJ, Brazil*
[b] *Laboratório de Biologia Estrutural, Divisão de Biologia Celular, Pesquisa Básica, Instituto Nacional de Câncer, Rio de Janeiro, RJ, Brazil*
[c] *Laboratório de Biologia Celular, Instituto Oswaldo Cruz, FIOCRUZ, Rio de Janeiro, RJ, Brazil*
[d] *Laboratório de Proteases e Inibidores de Proteases de Origem Natural, Departamento de Química de Produtos Naturais, Farmanguinhos, FIOCRUZ, Av. Brazil 4365, 21045-900 Rio de Janeiro, RJ, Brazil*

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ABSTRACT

It has been reported that serine peptidase activities of *Trypanosoma cruzi* play crucial roles in parasite dissemination and host cell invasion and therefore their inhibition could affect the progress of Chagas disease. The present study investigates the interference of the *Stichodactyla helianthus* Kunitz-type serine protease inhibitor (ShPI-I), a 55-amino acid peptide, in *T. cruzi* serine peptidase activities, parasite viability, and parasite morphology. The effect of this peptide was also studied in *Leishmania amazonensis* promastigotes and it was proved to be a powerful inhibitor of serine proteases activities and the parasite viability. The ultrastructural alterations caused by ShPI-I included vesiculation of the flagellar pocket membrane and the appearance of a cytoplasmic vesicle that resembles an autophagic vacuole. ShPI-I, which showed itself to be an important *T. cruzi* serine peptidase inhibitor, reduced the parasite viability, in a dose and time dependent manner. The maximum effect of peptide on *T. cruzi* viability was observed when ShPI-I at 1×10^{-5} M was incubated for 24 and 48 h which killed completely both metacyclic trypomastigote and epimastigote forms. At 1×10^{-6} M ShPI-I, in the same periods of time, reduced parasite viability about 91–95% respectively. Ultrastructural analysis demonstrated the formation of concentric membranar structures especially in the cytosol, involving organelles and small vesicles. Profiles of endoplasmic reticulum were also detected, surrounding cytosolic vesicles that resembled autophagic vacuoles. These results suggest that serine peptidases are important in *T. cruzi* physiology since the inhibition of their activity killed parasites *in vitro* as well as inducing important morphological alterations. Protease inhibitors thus appear to have a potential role as anti-trypanosomatidal agents.

1. Introduction

Chagas disease, or American trypanosomiasis, is a poverty-related neglected parasitic disease that triggers a chronic inflammatory condition. It is caused by the intracellular obligatory flagellated protozoan parasite *Trypanosoma cruzi*. The disease is widely distributed in Latin America, where it causes significant morbidity and mortality and also occurs sporadically in non endemic countries such as United States and Spain (Schmunis, 2007; Machado et al., 2012). It is estimated that more than 25 million people live in risk areas and about 10 million people are currently infected with *T. cruzi*, resulting in over 15,000 deaths. Approximately 50,000 new cases are diagnosed every year and the disease is the major cause of cardiopathy in endemic areas (Rassi et al., 2012). The disease comprises two stages, an acute phase which occurs about two months after initial infection and is characterized by a high number of parasites in the blood and a chronic phase developed by about 30–40% of the infected patients when parasite affects the cardiovascular, gastrointestinal, and nervous systems of the human host (Py, 2011). The life cycle of *T. cruzi* involves obligatory passages through vertebrate mammals and

Abbreviations: L-BAPNA, N_{α}-Benzoyl-L-arginine 4-nitroanilide hydrochloride; BPTI, bovine pancreatic trypsin inhibitor; BHI, brain heart infusion; EM, electron microscopy; FBS, fetal bovine serum; IC_{50}, half maximal inhibitory concentration; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PBS, phosphate buffer saline; ShPI-I, Kunitz-type serine protease inhibitor from Sea anemone *Stichodactyla helianthus*; TAU, triatomine artificial urine; Tc80, *T. cruzi* prolyl oligopeptidase with 80-kDa.

* Corresponding author. Tel.: +55 21 3977 2475; fax: +55 21 3977 2422.
E-mail addresses: rlopez@far.fiocruz.br, raquelelisa@globo.com (R.E. da Silva-López).

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invertebrate hematophagous triatomine bug hosts. The metacyclic trypomastigote ingested by the vector differentiate into proliferative epimastigote which, on reaching the posterior intestine, evolve into metacyclic trypomastigote. These forms invade vertebrate host cells and differentiate into non-flagellated amastigotes, which transform once again to metacyclic trypomastigotes that are responsible for the dissemination of the infection (Romano et al., 2012). However, the parasite can be transmitted through blood transfusion, organ transplant, congenital, and oral transmission due to contaminated food (Coura, 2007). In addition, the parasite can be transmitted by blood transfusion, infected mothers during pregnancy, organ transplantation and oral infection by consumption of food contaminated with triatomines or their feces (Coura, 2007).

The control of Chagas disease requires an integrated approach that addresses its underlying bio-eco-socio-economic causes (Morel et al., 2005). Despite programs, that reduced significantly the vectorial and transfusional transmission, the disease remains an important public health concern because of its clinical variability, the absence of a vaccine against *T. cruzi* forms, the low efficacy of chemotherapeutic agents, parasite resistance, and infected donor transmission. Importantly, nifurtimox and benznidazole, the two drugs used for the current treatment of this illness, have significant drawbacks, as they are at best moderately effective in the chronic stages of the infection and cause severe side effects (Coura, 2009; Guedes et al., 2011). Hence, elucidation of the molecular mechanisms involved in the establishment of *T. cruzi* infection is critical both to understanding the pathogenesis of Chagas disease and to developing strategies to produce efficient non-toxic drugs. There are a large number of trypanosomatid enzymes and/or biochemical pathways that have been identified as targets for drug development, and peptidases are considered to be promising candidates (Silva-López, 2012). The study of these enzymes in trypanosomatids has attracted considerable attention over the last decade, since some of peptidases play important roles in host/parasite interactions (Barrett et al., 1999) and are important virulence factors (Duschak et al., 2006; Kosec et al., 2006; Silva-López et al., 2007; Motta et al., 2012; Alvarez et al., 2012). Peptidases, also known as proteases, are hydrolytic enzymes that cleave peptide bounds in proteins and peptides, releasing peptides with variable sizes and free amino acids. Unlike most enzymes, proteases lack specificity toward a substrate, they are very specific for a peptide containing the scissile peptide bond and the amino acids involved in the neighborhood of the peptide bonds (Silva-López, 2012). *T. cruzi* has been shown to contain several biochemically characterized proteolytic activities, such as cysteine, serine, threonine, aspartic and metalloproteases (McKerrow et al., 2006; Alvarez et al., 2012) and other peptidases have been predicted from the data of the genome project (El-Sayed et al., 2005). Cruzipain, the major acidic cysteine protease of *T. cruzi* is the best characterized proteases and its inhibitors have been proposed as potential therapies for the infection by this protozoa (Sajid et al., 2011), however these compounds also inhibited cysteine proteases of hosts and caused many adverse side effects (Beaulieu et al., 2010). On the other hand, the activity of *T. cruzi* serine peptidases was also proved to be essential for parasite survival and proliferation for infection maintenance (Grellier et al., 2001; Motta et al., 2012).

Serine peptidases are among the most extensively studied enzymes, found in all living organisms and participate in a vast number of other biological phenomena (Song et al., 2013). They are grossly subdivided into two major groups: (1) exopeptidases (oligopeptidases), which hydrolyze peptide bonds in the proximity of the amino or carboxyl termini of the substrate and (2) endopeptidases (proteases), which hydrolyze internal peptide bonds (Rawlings and Barret, 1994; Rawlings et al., 2012). The first report about serine peptidase activity in *T. cruzi* evidenced an alkaline 120-kDa oligopeptidase B purified and characterized from a cytosolic fraction of epimastigote forms (Santana et al., 1992). This oligopeptidase B play crucial roles for the generation of Ca^{2+} signaling in the host mammalian cell that results in the recruitment and fusion of lysosomes at the parasite binding site and cell invasion (Burleigh and Andrews, 1995; Burleigh et al., 1997; Yoshida and Cortez, 2008; Motta et al., 2012). Inhibition of peptidase activity prevents the entry of *T. cruzi* into host cells and the silencing of this enzyme gene, importantly inhibited the infective potential of metacyclic trypomastigotes (Caler et al., 1998; Burleigh and Woolsey, 2002; Yoshida and Cortez, 2008; Motta et al., 2012). In the same cytosolic fraction of *T. cruzi* was also detected a prolyl oligopeptidase with 80-kDa, named Tc80, which demonstrated significant collagenase activity against human collagen types I and IV at neutral pH, and fibronectin (Santana et al., 1997). This enzyme is located nearby to the flagellar pocket – the only part of the cell surface that supports exocytosis and endocytic traffic in trypanosomes (Landfear and Ignatushchenko, 2001) – and is secreted to the extracellular environment and thus may be important for degrading the host extracellular matrix, parasite dissemination and host cell invasion (Grellier et al., 2001). The other *T. cruzi* serine peptidase was a lysosomal high-mannose glycoprotein carboxypeptidase with a molecular mass of about 54 kDa and optimal pH at 4.5. Unlike other enzymes this carboxypeptidase seems to be associated to membrane and catalyze in acidic pH range (Parussini et al., 2003). Finally, a secreted and glycosylated 75-kDa serine oligopeptidase was obtained from culture supernatant of epimastigote forms and differently from Tc80 it was not able to hydrolyze collagen types or other protein substrates (Silva-López et al., 2008). Besides their location in flagellar pocket this enzyme was also found in reservosomes (Silva-López et al., 2008) – a lipid/protein storage organelle and the main site of proteolysis in *T. cruzi* (Sant'Anna et al., 2009). This enzyme possibly participates in protein processing, endocytic pathway or in protein hydrolysis of host tissues since they are secreted to extracellular environments (Silva-López et al., 2008).

In this regard, inhibitors of serine protease activity of *T. cruzi* could interfere in parasite survival and consequently in the Chagas disease progression. In the present work we evaluate the importance of serine proteases in *T. cruzi* survival using a serine protease inhibitor, considering two criteria: (1) cell viability and (2) parasite morphological alterations. The ShPI-I which is a 55-amino-acid peptide Kunitz-type serine protease inhibitor of 6110.6 Da from the Caribbean Sea anemone *Stichodactyla helianthus* was employed. This peptide exhibits a broad specificity of inhibition of serine, cysteine and aspartic proteases from human, bovine and plants (Delfin et al., 1996). Regardless of the fact that ShPI-I inhibits other peptidases, it was demonstrated that it has a great specificity for serine proteases, exemplified by the lowest *Ki* values obtained for serine, such as bovine trypsin ($Ki = 1.1 \times 10^{-10}$ M and very strong inhibition) in comparison to other types, such as papain and bromelain (*Ki* = not determined and weak inhibition) plant cysteine and aspartic proteases respectively (Delfin et al., 1996). Furthermore, the higher specificity of ShPI-I observed for serine proteases might also be explained by the fact that this inhibitor demonstrated a nearly identical molecular architecture to the bovine pancreatic trypsin inhibitor (BPTI), an important mammalian serine protease inhibitor (García-Fernández et al., 2012). ShPI-I is usually purified by affinity chromatography using trypsin, a known pancreatic serine protease immobilized in Sepharose matrix (Antuch et al., 1993). This peptide was an important inhibitor of serine protease activities of *Leishmania amazonensis* promastigotes and this inhibition reduced significantly the viability of parasites *in vitro* and induced the parasite death by the formation of autophagic vacuole (Silva-López et al., 2007). It is important to point out that protease inhibitors are valuable tools for the investigation of the biochemical properties and biological functions of proteolytic enzymes, as well as representing leads to selective and possibly potent chemotherapeutic agents for