Infestin 1R, an intestinal subtilisin inhibitor from *Triatoma infestans* able to impair mammalian cell invasion by *Trypanosoma cruzi*

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ABSTRACT

Infestins are Kazal-type serine protease inhibitors described in the midgut of *Triatoma infestans*, Chagas disease vector. Of all infestins, only infestin 1R (INF1R) does not control host blood coagulation, due to its inhibitory specificity for chymotrypsin-like proteases. We further investigated the effect of INF1R on cell infection by *Trypanosoma cruzi*. The importance of INF1R reactive site to inhibit *T. cruzi* cell invasion was confirmed using 1RSFTI, a synthetic cyclic peptide containing the inhibitor reactive site region hybridized to the Sunflower Trypsin Inhibitor-1 (SFTI-1). Our results suggest that INF1R efficiently inhibited parasite cell invasion. For the first time, a serine protease inhibitor, derived from *T. infestans*, was shown to impair cell invasion by *T. cruzi*, representing possible new target in parasite cell invasion.

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1. Introduction

Proteases and their inhibitors are fundamental in several biological processes, like reproduction, development, cell cycle, immune system, inflammation, angiogenesis, apoptosis, tissue remodeling, and blood homeostasis (Lalmanach, 2008; Rawlings et al., 2004, 2008), and have been shown to play key roles on parasite–host interactions (Armstrong and Quigley, 2001). In insects, proteases and respective inhibitors are involved in immune response regulation, such as in *Drosophila* (Levashina et al., 1999) and *Manduca sexta* (Kanost et al., 2004), in which immune response is mediated by proteases related to the Toll pathway and prophenoloxidase activation, respectively. Protease inhibitors from insects such as *Galleria mellonella* inhibited parasite proteases with high affinities (Nirmala et al., 2001), and their expression increased after challenges with fungi, bacteria, and several types of antigens derived from microorganisms, known as pathogen-associated molecular patterns (PAMPs) (Frobius et al., 2000; Wedde et al., 1998). Ursic-Bedoya and Lowenberger reported the identification of immune responsive molecules overexpressed after immune challenge in the Chagas disease vector *Rhodnius prolixus* (Ursic-Bedoya and Lowenberger, 2007). In *Anopheles gambiae*, a malaria vector, four serpins were described to be strongly expressed after *Plasmodium* ookinetes invasion of females' midgut (Danielli et al., 2003). Serpin 6 was deleterious for parasite life differentiation (Abraham et al., 2005; Pinto et al., 2008). It has been demonstrated by microarray that a putative Kazal-type inhibitor from the African sleeping sickness vector *Glossina morsitans morsitans* midgut had increased expression after exposure to *Trypanosoma brucei* and bacteria (Lehane et al., 2003). Recently, several Kazal-type protease inhibitors were described as antimicrobial agents in invertebrates.

Kazal-type protease inhibitors are represented by their first described member, pancreatic secretory trypsin inhibitor (PSTI, renamed SPINK5), which compose family I1 in the MEROPS database (Kazal et al., 1948; Rawlings et al., 2008). These protease inhibitors can be found in several species as single domains or in tandem structures expressed as single and large protein precursors processed post-translationally. Basically, each Kazal-type domain consists of a 6–7 kDa structured protein in two alpha-helices, three stranded anti-parallel beta sheets and three disulfide bridges.

In marine invertebrates, protease inhibitors belonging to Kazal-type family also were demonstrated to play a role in immune response against aquatic pathogens, like bacteriostatic molecules (Augustin et al., 2009; Donpudsa et al., 2009). In different cases, subtilisin inhibitors from Kazal-type family are related to immune system response of invertebrates (Rimphanitchayakit and Tassanakajon, 2010).

Triatoma infestans (Hemiptera: Reduviidae) is one of the most important vectors of Chagas disease, a parasitic disease that affects about 10 million people in Latin America (Remme et al., 2006). Chagas disease has not been considered with the deserved attention, and despite all knowledge about *Trypanosoma cruzi* biology, no optimal therapy has been developed as yet. There is neither vaccine nor treatment for chronic chagasic patients (Dias, 2009; Jannin and Villa, 2007). In spite of the high prevalence, the main strategies against Chagas disease transmission rely exclusively on vector and blood transfusion control measures.

We have previously characterized infestin in *T. infestans* midgut as Kazal-type protease inhibitors (Campos et al., 2002). Infestins are derived from a large precursor mRNA encoding for seven Kazal-type domains, most of which are capable of blocking host blood coagulation, except infestin1R, a single Kazal domain, which is a subtilisin and neutrophil elastase inhibitor (Lovato et al., 2006). Infestins are indeed required for triatomine meal acquisition and storage, probably by preventing blood coagulation, as demonstrated using RNAi in *Triatoma brasiliensis* (Araujo et al., 2007).

As proteases play key roles in *T. cruzi*, the agent of Chagas disease, we investigated the role of *T. infestans* protease inhibitor, particularly the infestin 1R domain (INF1R), on the parasite. Proteases are crucial for growth, differentiation and cell invasion by the parasite. Of special importance is cruzipain, a cysteine protease expressed in all *T. cruzi* forms (Martinez et al., 1991; Tomas et al., 1997). Its activity is controlled by its own inhibitor, named chagasin, which is included in a new family of protease inhibitors due to its uncommon structure (Monteiro et al., 2001). In trypomastigotes, cruzipain is associated with cell invasion by inducing kininogen processing and bradykinin release (Scharfstein et al., 2000). Other *T. cruzi* proteases which belong to MEROPS family S9 were described. Oligopeptidase B is involved in cell invasion by releasing a soluble Ca^{2+} signaling factor of unknown structure (Burleigh and Andrews, 1995; Burleigh et al., 1997). Selective inhibitors also blocked mammalian cell invasion by other *T. cruzi* S9 protease, prolyl oligopeptidase Tc80, which is able to hydrolyze extracellular matrix proteins as fibronectin, collagens type I and IV (Grellier et al., 2001; Bastos et al., 2005). Another *T. cruzi* protease was purified from extracellular culture media, and was proven to be inhibited by TPCK and TCLK. This suggested that it is a serine protease that differs from other previously described oligopeptidases (da Silva-Lopez et al., 2008).

In the present study, we investigated the INF1R expression profile after blood ingestion by *T. infestans* and its interaction with *T. cruzi*.

2. Materials and methods

2.1. Insects

Adult *T. infestans* were reared under controlled temperature (28 ± 2.0 °C), 12/12 light/dark and fed weekly on anesthetized mice (ketamine 150 mg/kg and xylazine 7 mg/kg).

2.2. Recombinant protein

INF1R was expressed in *Escherichia coli* and the periplasmic fraction was used to purify the protein following procedures described in (Lovato et al., 2006).

2.3. cDNA synthesis and real-time quantitative PCR

Starved insects were fed on human blood in an artificial feeder through a Parafilm M® membrane. Then, 10^7/ml parasites killed by freezing and thawing (four cycles at temperature varying from −20 to 40 °C, in PBS) were added to human blood for infestin expression analysis. After blood meal, anterior midguts were removed for cDNA synthesis and qPCR experiments. Total RNA from anterior midgut of engorged insects was preserved in RNAlater RNA Stabilization Reagent (QIAGEN) and then extracted using RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. After treatment with DNAse I RNase-free (Fermentas), cDNA was synthesized from approximately 1.0 μg of RNA with ImProm-II™ Reverse Transcription System (Promega) using random primers following the manufacturer's instructions. Relative expression levels of infestin and 18S rRNA (Genbank Accession Nos. DQ309461 and Y18750) were quantified using SYBR® Green PCR Master Mix (Applied Biosystems) in a 7500 Fast Real-Time PCR System (Applied Biosystems). Primer sequences were: PicINF1R 5′-GCGGCCGCTCACTCTACTT-CTTGTTC-3′ and PicINF1F 5′-GGTTGAATTCGAGAAAAAGGATC-CACC-3′ for infestin 1R, and 5′-GGCGGGGGCATTCGTATTG-3′, and 5′-ATCGCTGGCTGGCATCGTTTAT-3′ for 18S rRNA, according to (Faudry et al., 2004). Quantitative PCR (qPCR) conditions were: denaturation at 95 °C for 30 s, annealing at 60 °C for 1 min, and extension at 72 °C for 30 s. qPCR was conducted in 1 μl of tenfold diluted INF1R cDNA and 18S rRNA and primers to a final concentration of 0.5 μM. For each qPCR experiment, three replicates per sample were performed followed by dissociation curve, endogenous 18S RNA control analysis and statistical analysis using the 7500 Fast Real-Time PCR System program (Applied Biosystems).

2.4. Cell cultivation and invasion assay

T. cruzi trypomastigotes Y strains were obtained from the supernatant of infected LLC-MK2 monolayer cells cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, at 37 °C in a humidified 5% CO_2 atmosphere. Trypomastigotes were collected from the extracellular medium 5–7 days after infection and recovered after centrifugation at 2000g for 10 min. LLC-MK2 cells (10^5 cells/ml) for invasion assays were plated in 24-well plates for 24 h. The assays were performed with freshly released parasites in a host/parasite cell ratio of 1/10 (approximately 5×10^6/ml) and parasites were pre-incubated for 15 min with protease inhibitors at different concentrations, before incubation with host cells. Then, parasites were directly added to LLC-MK2 cells for 1 h. After, LLC-MK2 cells were washed three times with DMEM without FBS and fixed with 4% formaldehyde in PBS for 24 h at 4 °C. After fixation, cells were washed four times with PBS and incubated with PBS-2.0% BSA for 1 h and incubated with rabbit polyclonal antibodies against *T. cruzi* (Rubin-de-Celis et al., 2006) diluted 1:100 to detect non-internalized parasites, because cells were not permeabilized. Bound antibodies were detected by incubation 1:400 with FITC-conjugated anti-rabbit IgG (Santa Cruz Biotechnology) in PBS-0.5% BSA containing 100 μM of DAPI for 1 h. Invasion assays were quantified by counting the number of intracellular parasites in a total of 300 cells per slide. Statistical analyses were carried out by using variance analysis with significance level of $p < 0.05$.

3. 1RSFTI peptide synthesis

The cyclic peptide 1RSFTI was synthesized manually by Solid Phase Peptide Synthesis with Fmoc strategy (Fields and Noble, 1990). Cyclization reaction was monitored by liquid chromatography coupled to electrospray ionization mass spectrometer

(LC/ESI-MS) and Ellman's test (Ellman, 1959). After 72 h, the reaction medium was acidified with acetic acid to pH range 4.5–5.0 and lyophilized. Lactam cyclization was performed with the peptide still attached to the resin (Miranda et al., 1994). The crude lyophilized peptides were purified to homogeneity by preparative reversed phase HPLC in a C_{18} column (Vydac). Selected fractions containing the purified peptide were pooled and lyophilized. The purified peptide was characterized by amino acids analysis (Beckman), capillary zone electrophoresis (Waters) and LC/ESI-MS (Waters).

4. Results and discussion

Full length infestin mRNA expression was analyzed at different times after blood feeding (6 h, 12 h, 24 h, 36 h, 7 days and 14 days). qPCRs were performed using specific oligonucleotides which amplify the INF1R domain, the first Kazal-type domain of the infestin precursor (Fig. 1A). The qPCRs result showed a decreasing infestin precursor expression after feeding reached up to one third of original pre-feeding levels, which occurred 24 h after blood meal. Seven to 14 days later, infestin precursor expression returned to levels observed for unfed insects (Fig. 1B). This recovery time is compatible with the *T. infestans* feeding cycle (Lent and Wygodzinsky, 1979), and suggests a relationship between infestin expression and blood feeding. It is possible that higher infestin levels are required before blood feeding in order to block host blood coagulation cascade, or control other blood hazardous proteases, like inflammatory related proteases from tissue and immune cells. These results are in accordance with RNAi experiments with intestinal Kazal-type precursor in *T. brasiliensis* called brasiliensin, which showed a decrease in blood intake (Araujo et al., 2007).

In order to verify a possible interference of *T. cruzi* proteins in infestin precursor expression after blood meal, heat-killed trypomastigotes, which are the protozoan form ingested by the insect vector, were added to blood. As shown in Fig. 1C, specifically after 12 h, infestin expression in insects fed on blood containing killed
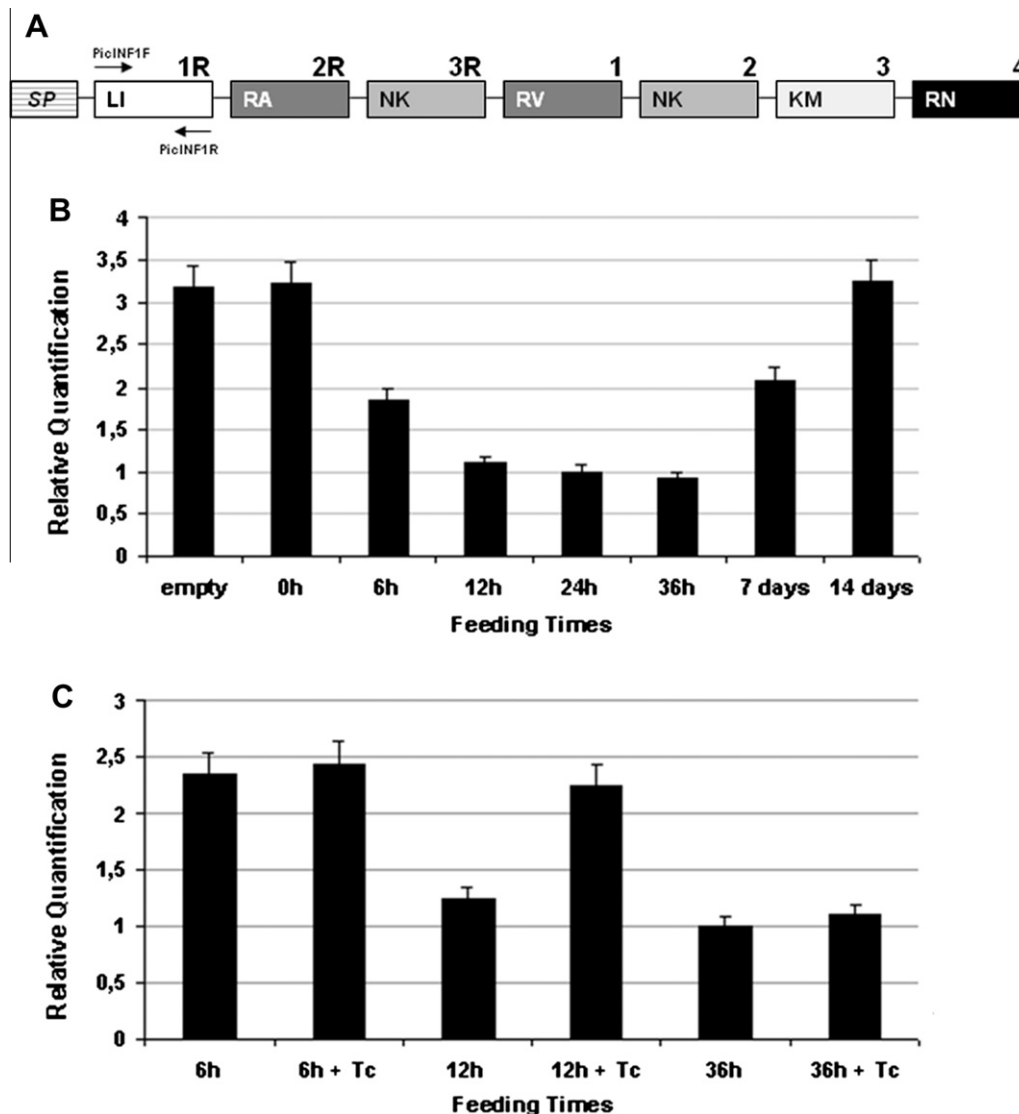

Fig. 1. Relative quantification of infestin mRNA in *T. infestans* midgut after blood meal in artificial feeder. (A) Diagram of full length infestin cDNA representing predicted signal peptide (SP) in dashed box and respective Kazal-type domains with reactive site P1–P1′ amino acid residues (Schechter and Berger, 1967) in gray scale boxes. The arrows represent the primers PicINF1F and PicINF1R used in qPCR. (B) Full length infestin mRNA expression after feeding for 14 days. The value of infestin mRNA was arbitrarily set to 1.00 for the insects 24 h after feeding and analyses were performed relative to 18S RNA levels previously described (Faudry et al., 2004). Differences are statistically significant (*t*-test, $p < 0.05$). (C) Infestin expression after blood meal containing killed *T. cruzi* trypomastigotes forms (Tc). The value of infestin mRNA was arbitrarily set to 1.00 for the insects 36 h after feeding. Differences are statistically significant just for 12 h versus 12 h + Tc (*t*-test, $p < 0.05$). Similar results were obtained in three independent experiments.

T. cruzi decreased less as compared to the drop in infestin expression measured in insects that ingested pure blood (control), remaining two times as high. No significant differences were observed among the insects fed on blood containing killed parasites and respective controls, after 6 and 36 h. These results indicated that the presence of parasite molecules could alter infestin expression level after blood feeding, and point to a possible signalization in midgut, where parasite molecules could probably act as PAMPs, known to induce host responses against pathogens. In *R. prolixus*, another kissing bug species that also is a Chagas disease vector, different immune challenges elicited NO system response in midgut, suggesting that kissing bugs can also recognize and respond against pathogens, including *T. cruzi* (Whitten et al., 2007). Ursic-Bedoya and Lowenberger also identified immune related molecules in *R. prolixus* after immune challenge (Ursic-Bedoya and Lowenberger, 2007), but the authors used injection of microorganisms into hemocoel as experimental strategy, not oral infection, which leads to quite different gene expression of immune-related genes.

In the natural infection, *T. infestans* is infected by bloodstream trypomastigote form of *T. cruzi*, the infective and non-replicated life cycle form, which is released from infected cells from mammalian host tissues. Despite some differences, bloodstream trypomastigotes are similar to trypomastigotes obtained from infected mammalian culture cells. In order to evaluate possible interaction between INF1R and the parasite, the recombinant inhibitor was used in cell invasion assays. *T. cruzi* does not invade cells in *T. infestans* midgut during its life cycle and neither does it cross intestinal epithelium, like other trypanosomes, such as *Trypanosoma rangeli*. We performed cell invasion assays as a model to detect and analyze the effect of protease inhibitors during this event, specially the effect of subtilisin inhibitors. INF1R was added to LLC-MK2 epithelial cell culture, it impaired cell invasion by *T. cruzi* in a dose-dependent manner. The IC_{50} of INF1R was between 0.1 and 1.0 µM, and INFR1 10 µM caused a drastic drop in intracellular parasites inside host cells (Fig. 2). No morphological and motility alterations were observed in parasites after incubation with INF1R and during invasion assays, by visualization under light microscopy (data not shown). When these findings are considered together, it is possible to suggest that INF1R inhibited cell invasion by *T. cruzi*. Indeed, mainly cysteine proteases, but also other proteases, like oligopeptidases, have been found to participate in *T. cruzi*

cell invasion (Burleigh et al., 1997; Grellier et al., 2001). Several cysteine protease inhibitors were discovered to be able to interfere in *T. cruzi* biology, especially concerning cell invasion, replication and differentiation (Aparicio et al., 2004; Monteiro et al., 2001; Tomas et al., 1997). Based on this knowledge, a cysteine protease inhibitor named K777 was developed and demonstrated to be a promising clinical candidate (McKerrow et al., 2009). E-64, a classical cysteine protease inhibitor, was demonstrated to inhibit *T. cruzi* cell invasion due to inhibition of cysteine proteases cruzipains (Aparicio et al., 2004).

Other results have shown that several *T. cruzi* trypomastigotes forms incubated with 10 µM INF1R-rhodamine presented surface labeling (data not shown). Similar result was observed using anti-INF1R polyclonal antibodies by indirect immunofluorescence (data not shown). These results of INF1R impairing cell invasion and binding to trypomastigotes suggest that INF1R may interact with the surface of trypomastigotes, probably with a serine protease.

To elucidate if the reactive site of INF1R is responsible for inhibition of cell invasion, and consequently investigate the mechanism that prevents invasion, a cyclic peptide was synthesized containing the INF1R reactive site in the structure of the smallest serine protease inhibitor Sunflower Trypsin Inhibitor-1 (SFTI-1), a Bowman-Birk inhibitor (BBI) formed by 14 amino acid residues
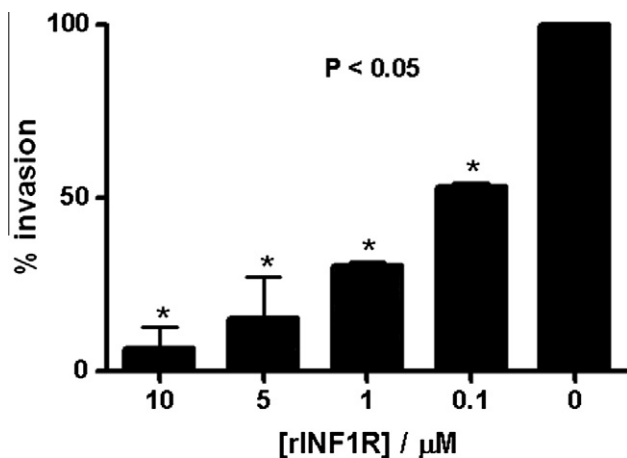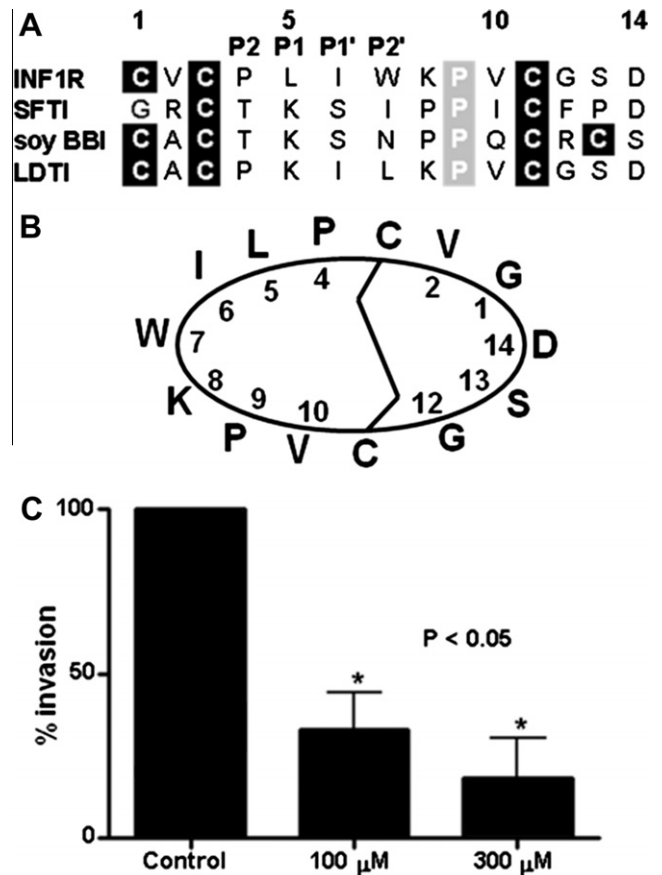
Fig. 3. (A) Amino acid sequence alignment of reactive site of different protease inhibitors. INF1R, infestin 1R, SFTI, Sunflower Trypsin Inhibitor, soy BBI, Bowman-Birk soy bean trypsin inhibitor, LDTI, Leech-derived tryptase inhibitor. The amino acid residues at P2, P1, P1′ e P2′ positions of reactive site are the important residues to interact with serine proteases. (B) SFTI-like peptide designed based on amino acid alignment of infestin 1R and SFTI-1 according to (Korsinczky et al., 2001). (C) Effect of 1RSFTI on epithelial cells invasion by *T. cruzi* trypomastigotes strain Y. Three independent experiments were performed in triplicates. The results of cell invasion (%) were analyzed using *t*-test and a significance level of $p < 0.05$ for control (∗).

Fig. 2. Effect of INF1R on epithelial cell invasion by *T. cruzi* trypomastigotes strain Y. The assays were performed with freshly released parasites in a host/parasite cell ratio of 1/10 (approximately 5×10^6/ml) and parasites were pre-incubated for 15 min with INF1R at different concentrations before incubation with LLC-MK2 cells. Then, parasites were directly added to LLC-MK2 cells for 1 h of infection. Three independent experiments were performed in triplicates. The results of cell invasion (%) were analyzed using *t*-test and a significance level of $p < 0.05$ for control (∗).
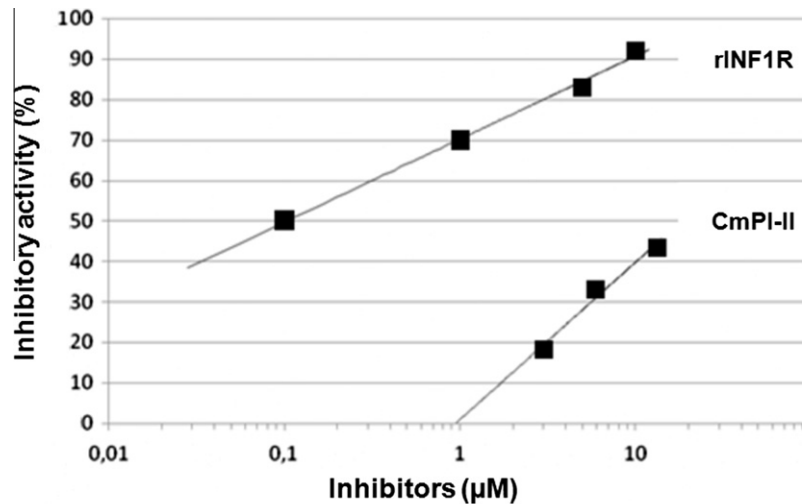
Fig. 4. Inhibition curves of *T. cruzi* cell invasion for two subtilisin inhibitors, INF1R and CmPI–II. Graphic shows inhibitory activity toward *T. cruzi* cell invasion versus log of protease inhibitor concentrations. The assays were performed with freshly released parasites in a host/parasite cell ratio of 1/10 (approximately 5×10^6/ml) and parasites were pre-incubated for 15 min with INF1R in different concentrations (0.1–10 μM INF1R or 3.0–13 μM CmPI2) before incubation with LLC-MK2 cells. Then, parasites were directly added to LLC-MK2 cells for 1 h at 37 °C. These data represent two independent experiments in triplicates.

naturally occurring in cyclic form. The new cyclic peptide, called 1RSFTI, was designed taking into account the similarity between the amino acid sequence of reactive sites of Kazal-type and BBI inhibitor families previously described (Korsinczky et al., 2001) (Fig. 3A). We have replaced the amino acid Cys for Gly in order to promote the cyclization of the recombinant peptide named 1RSFTI (Fig. 3B). 1RSFTI 100 μM used in cell invasion by *T. cruzi* assay impairs more than 50% invasion (Fig. 3C). Differences in the inhibitory activity between INF1R and 1RSFTI occur probably due to interaction with other regions in the INF1R structure. Despite that, 1RSFTI could be a target molecule to design new molecules able to block *T. cruzi* cell invasion and elucidate the involved mechanism.

In sum, the influence of INF1R on cell invasion by *T. cruzi* may be imputed to the inhibition of parasite proteases. Another serine protease inhibitor, TiPI1, which belongs to pacifastin-like inhibitors family (I19, according to the MEROPS database) and is a strong elastase inhibitor (Ki = 0.4 nM) similar to INF1R but without subtilisin inhibitory activity (de Marco et al., 2010), was used in the cell invasion assay. *T. cruzi* cell invasion was inhibited in culture media containing up to TiPI1 8 μM final concentration, (data not shown). Another serine protease inhibitor used in invasion assays was CmPI-II, a trypsin (Ki = 1.1 nM), elastase (Ki = 2.6 nM), and subtilisin A (Ki = 30.8 nM) inhibitor (Gonzalez et al., 1997). CmPI-II was able to impair *T. cruzi* cell invasion and presented an IC$_{50}$ around 10 μM (Fig. 4). When compared to INF1R, CmPI2 presented lower inhibitory activity, probably due to the tenfold difference in the subtilisin inhibitory constant (Ki).

More experiments should be conducted to identify the INF1R target molecule in the *T. cruzi*, to demonstrate and clarify possible new roles of INF1R and related molecules in this Chagas disease vector.

Acknowledgments

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