MDL28170, a Calpain Inhibitor, Affects *Trypanosoma cruzi* Metacyclogenesis, Ultrastructure and Attachment to *Rhodnius prolixus* Midgut

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Abstract

**Background:** *Trypanosoma cruzi* is the etiological agent of Chagas’ disease. During the parasite life cycle, many molecules are involved in the differentiation process and infectivity. Peptidases are relevant for crucial steps of *T. cruzi* life cycle; as such, it is conceivable that they may participate in the metacyclogenesis and interaction with the invertebrate host.

**Methodology/Principal Findings:** In this paper, we have investigated the effect of the calpain inhibitor MDL28170 on the attachment of *T. cruzi* epimastigotes to the luminal midgut surface of *Rhodnius prolixus*, as well as on the metacyclogenesis process and ultrastructure. MDL28170 treatment was capable of significantly reducing the number of bound epimastigotes to the luminal surface of the midgut insect. Once the cross-reactivity of the anti-Dm-calpain was assessed, it was possible to block calpain molecules by the antibody, leading to a significant reduction in the capacity of adhesion to the insect guts by *T. cruzi*. However, the antibodies were unable to interfere in metacyclogenesis, which was impaired by the calpain inhibitor presenting a significant reduction in the number of metacyclic trypomastigotes. The calpain inhibitor also promoted a direct effect against bloodstream trypomastigotes. Ultrastructural analysis of epimastigotes treated with the calpain inhibitor revealed disorganization in the reservosomes, Golgi and plasma membrane disruption.

**Conclusions/Significance:** The presence of calpain and calpain-like molecules in a wide range of organisms suggests that these proteins could be necessary for basic cellular functions. Herein, we demonstrated the effects of MDL28170 in crucial steps of the *T. cruzi* life cycle, such as attachment to the insect midgut and metacyclogenesis, as well as in parasite viability and morphology. Together with our previous findings, these results help to shed some light on the functions of *T. cruzi* calpains. Considering the potential roles of these molecules on the interaction with both invertebrate and vertebrate hosts, it is interesting to improve knowledge on these molecules in *T. cruzi*.

Introduction

Chagas’ disease is a neglected tropical disease, which remains a major health problem in Latin America. Over eight million people are infected with this disease and an estimated of 14,000 people die as a consequence of the infection every year [1]. The etiological agent *Trypanosoma cruzi* undergoes profound morphological changes during its development in a complex life cycle involving mammalian and invertebrate hosts. The protozoa life cycle comprises three major morphological stages: epimastigotes, trypomastigotes, and amastigotes [2]. During the infection of the invertebrate host, a hemipteran insect of the Reduviidae order, non-infectious epimastigotes adhere to the insect host midgut, begin to proliferate and differentiate (metacyclogenesis process) into metacyclic trypomastigotes, which are non-proliferative forms that are able to infect a mammalian host [3]. The adhesion to the luminal midgut surface of the insect appears to be necessary for the metacyclogenesis, but there is a general lack of information about which molecules are implicated in this process [3,4]. In this context, peptidases, a class of hydrolytic enzymes responsible for breaking peptide bonds, has attracted the attention of our research group because of their role in several crucial steps of the life cycle of the trypanosomatid parasites [5]. Among *T. cruzi* different peptidases that we considered, the calpains have been presenting interesting findings and seem to be a remarkable target for the development of an alternative target to treat Chagas’ disease and leishmaniasis [6,7,8].
Calpains constitute a large family of calcium-regulated cytosolic cysteine peptidases that have been characterized mainly in humans and whose role still remains poorly understood [9]. Some evidence indicates that these enzymes may participate in a variety of cellular processes, including the rearrangement of cytoskeletal proteins, different signal transduction pathways and apoptosis. In this context, a variety of calpain inhibitors are under development and the potential clinical utility of these compounds have been shown mainly in the treatment of neurodegenerative disorders [10,11,12,13]. In this sense, a classical study employing whole genome analyses showed the presence of a large and diverse family of calpains in Trypanosoma brucei, Leishmania major and T. cruzi [14]. Some years before, the same group had already characterized a trypsinosomatid calpain-like protein in procyclic forms of T. brucei [15]. In addition, our group described the presence of calpain-related proteins in T. cruzi epimastigote forms and Leishmania amazonensis promastigote forms and the effects of the calpain inhibitor III (MDL28170) on growth, viability and infectivity. These studies showed the presence of a large and diverse family of trypanosomatid calpain-like protein in procyclic forms of T. brucei [16].

In this regard, we have conducted a study to investigate the effect of the calpain homologues in the life cycle of T. cruzi. Here, we have analyzed the effect of anti-calpain antibodies on the interaction of epimastigote forms to the midgut surface of the insect and on the metacyclogensis.

Methods

Ethics Statement

The experiments were carried out in accordance with the guidelines established by the FIOCRUZ Committee of Ethics for the Use of Animals (CEUA L-028/09).

Chemicals

The calpain inhibitor III, MDL28170 (carbobenzoxy-valylphe-nylalanil; Z-Val-Phe-CHO), was purchased from Calbiochem (San Diego, CA, USA). Stock solutions of the drug (5 mM) were prepared in dimethylsulfoxide (DMSO). All other reagents were analytical grade or superior.

Parasite culture

Epimastigote forms of T. cruzi were grown in 3.7% brain heart infusion medium (BHI), containing hemin and folic acid and supplemented with 10% heat-inactivated fetal bovine serum, at 28°C for 4 days to reach late-log phase growth. For the following experiments, epimastigotes were collected, washed three times in 0.15 M NaCl, 0.01 M phosphate-buffer pH 7.2 (PBS) and immediately used. The Y strain of T. cruzi was used in all experiments except for the metacyclogenesis assay, in which the Dm28c strain is the best characterized model for in vitro differentiation [18].

Insects

Rhodnius prolixus were reared and maintained as previously described [19]. Briefly, fifth-instar larvae were starved for 30 days after the last eclosion and then allowed to feed on rabbit blood through a membrane feeder. Ten days after the feeding, insects were dissected; the posterior midguts were then removed, longitudinally sectioned and washed three times in PBS to expose their luminal surfaces. After the washing, the tissue fragments were processed as described below. The insects were obtained from the insectary of the Laboratório Nacional e Internacional de Referência em Taxonomia de Triatomíneos, Instituto Oswaldo Cruz, FIOCRUZ.

Identification of calpain homologues by flow cytometry and fluorescence microscopy

Epimastigotes (1 x 10⁷ cells) from the Y strain used for these experiments were fixed at 4°C in 0.4% paraformaldehyde in PBS (pH 7.2) for 30 min, followed by extensive washing in the same buffer. The fixed cells maintained their morphological integrity, as verified by optical microscopic observation. After this step, the cells were incubated for 1 h at room temperature with a 1:100 dilution of the anti-calpain antibodies. Cells were then incubated for an additional hour with a 1:200 dilution of fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG [20]. The cells were then washed 3 times in PBS and observed in a Zeiss epifluorescence microscope (Axioplan 2). Alternatively, the parasite associated fluorescence was excited at 488 nm and quantified on a flow cytometer (FACS Calibur, BD Bioscience, USA) equipped with a 15 mW argon laser emitting at 488 nm. Non-treated cells and those treated with the secondary antibody alone were run in parallel as controls. Each experimental population was then mapped by using a two-parameter histogram of forward-angle light scatter versus side scatter. The mapped population (n = 10,000) was then analyzed for log green fluorescence by using a single parameter histogram. The calpain antibodies tested were: a rabbit antiserum raised against Drosophila melanogaster calpain (anti-Dm-calpain) [21], anti-C21, anti-C23 or anti-C24 raised against the whole molecule, the cysteine active site and the histidine active site, respectively, of human brain m-calpain [22], anti-CAP5.5, raised against the cytoskeleton-associated protein from Trypanosoma brucei [13], and anti-CDP11b and anti-Ho-CalpM raised against Homarus americanus calpains [23,24].

In vitro inhibition of T. cruzi-Rhodnius interaction

Live epimastigote forms (Y strain) were resuspended in 200 µl of fresh BHI to a density of 1 x 10⁷ cells and treated with the calpain inhibitor for 1 h with sub-inhibitory concentrations (6.25–50 µM) and washed in PBS. Under this experimental condition, the parasite maintains their viability, as previously described [7]. Dilutions of DMSO corresponding to those used to prepare the drug solution were assessed in parallel for control. Alternatively, the parasites were incubated for 1 h with anti-calpain antibodies, or rabbit pre-immune sera. After that, binding of protozoa to insect gut was performed by a method similar to that described previously [25]. Briefly, the parasites were incubated for 20 min at 28°C with R. prolixus dissected posterior midguts that were sliced open longitudinally. Subsequently, the explanted midguts were spread onto glass slides to count the number of attached parasites per epithelial cells. The experiment was performed three times independently, for each experimental group 4 insect midguts were used and 100 epithelial cells were counted randomly.

In vitro inhibition of T. cruzi metacyclogensis

For in vitro differentiation, epimastigotes (clone Dm28c) in the stationary phase of growth were incubated for 2 h at 28°C in triatominergic artificial urine (TAU) medium [190 mM NaCl, 17 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, 8 mM phosphate buffer pH 6.0] at a density of 5 x 10⁶ cells/ml. The parasites at a dilution of 1:100 were further incubated for 96 h in TAU3AAG medium.
(TAU supplemented with 10 mM L-proline, 50 mM L-sodium glutamate, 2 mM L-sodium aspartate, and 10 mM D-glucose) in culture flasks [10]. For inhibition assays, epimastigotes were incubated in the presence, or absence (control), of increasing concentrations of MDL28170 (6.25–30 μM) in TAU3AAG medium or anti-calpain antibodies. Culture supernatants were collected after 24, 48, 72, and 96 h of incubation in TAU3AAG medium and the number of epimastigotes and metacyclic trypomastigotes was determined by cell counting in a Neubauer chamber. Under this experimental condition, the parasite maintains their viability, as previously described [7]. These morphological stages can be easily differentiated on morphological grounds by light microscopy, since epimastigotes are broader and have a quite rigid body, while trypomastigotes are slimmer and present a vigorous wavy movement of the whole body. Three independent experiments were performed in triplicate and a DMSO dilution corresponding to highest drug concentration was assessed in parallel.

Effects of MDL28170 on T. cruzi trypomastigotes viability

Bloodstream trypomastigotes forms, obtained from infected albino Swiss mice at the peak of parasitemia by differential centrifugation, were resuspended in Dulbecco’s modified Eagle medium supplemented with 10% fetal calf serum (DMEM). This suspension was incubated at 37°C for 24 h in the presence of increasing concentrations of MDL28170 (6.25–50 μM). Viability was assessed by mobility of the parasite flagellum and lack of staining after challenge with trypan blue. Dilutions of DMSO corresponding to those used to prepare the drug solutions were assessed in parallel. Therafter, the number of viable motile trypomastigotes was quantified by counting the flagellates in a Neubauer chamber.

Transmission electron microscopy

Briefly, epimastigotes from the Y strain (5×10⁶ cells/ml) were treated with the 50% inhibitory concentration (IC₅₀) of MDL28170, as previously determined [7] for 72 h in BHI medium at 28°C. Afterwards, the parasites were fixed with 2.5% glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.2) at room temperature for 40 min at 25°C and post-fixed with solution of 1% OsO₄, 0.8% potassium ferricyanide and 2.5 mM CaCl₂ in the same buffer for 20 min at 25°C [26]. The cells were dehydrated in an ascending acetone series and embedded in PolyBed 812 resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in Jeol JEM1011 transmission electron microscope. Alternatively, untreated epimastigotes were submitted to pre-embedding protocol, whereas the parasites were fixed, permeabilized (Triton X-100 0.1%), and incubated with polyclonal rabbit anti-Dm-calpain (dilution 1:10), followed by labeling with the secondary anti-rabbit-gold (10 nm) antibody (dilution 1:10) before the routine protocol described above.

Statistical analysis

All experiments were repeated at least three times, and media or representative images of these experiments are shown. The data were analyzed statistically using Student’s t test using EPI-INFO 6.04 (Database and Statistics Program for Public Health) computer software. P values of 0.05 or less were considered statistically significant.

Results

The presence of calpain-like molecules in epimastigote forms of T. cruzi Y strain was achieved by flow cytometry assay and fluorescence microscopy analyzes using a panel of antibodies raised against different calpains. The antibodies anti-Dm-Calpain, anti-CDP1b, anti-Ho-Calpm and anti-CAP5.5 were capable of strongly binding to T. cruzi cells, while anti-C21, anti-C23 and anti-C24 did not recognize epitopes on the parasite cell surface (Fig. 1A). However, no cross-reactive of the antibodies with trypomastigote forms were observed. Fluorescence microscopy (Fig. 1B) and FACS (Fig. 1A) with non-permeabilized parasites are
suggestive of a surface distribution of calpain-related molecules in epimastigote forms of \textit{T. cruzi}. It has been previously shown by our research group that the anti-\textit{Dm}-Calpain recognizes a polypeptide band migrating at approximately 80 kDa in cellular extracts from the Dm28c strain \cite{7}. Here, we showed that this antibody also recognized a protein with a similar molecular mass (80 kDa) in cellular extracts of \textit{T. cruzi} Y strain (Fig. 1A, inset). For control, lysates of \textit{Drosophila melanogaster} were run in parallel, revealing a reactive band in the same molecular range (Fig. 1A, inset). As previously reported, the fragment of the \textit{D. melanogaster} protein CAAX5297.1 that was employed to generate the antibody used in the present report \cite{21} was compared in a BlastP analysis with \textit{T. cruzi} proteins found in GenBank data base. The first 15 hits (homologues with e-value ranging from 2e-8 to 0.003) all corresponded to calcium-dependent cysteine peptidases and had their theoretical molecular mass determined, and 4 out of these 15 homologues presented a molecular mass around 80 kDa: XP_816697.1 (78.3 kDa), XP_803757.1 (80.8 kDa), XP_820102.1 (82.4 kDa) and XP_816696.1 (82.6 kDa), which supports the recognition of \textit{T. cruzi} 80 kDa calpain by the anti-\textit{Dm}-calpain antibody \cite{7}.

In order to assess a potential function for calpain-like molecules in \textit{T. cruzi}, we have performed binding assays with parasites previously treated with the anti-\textit{Dm}-calpain antibody, at concentrations that did not promote cell agglutination. The blockage of calpain molecules by the antibody led to a significant reduction in the capacity of adhesion to the insect guts by \textit{T. cruzi} in a dose-dependent manner, the inhibition ranged from 30\% to 60\% as antibody concentration rose from 1:250 to 1:50 (Fig. 2A), similar results were obtained with the other antibodies (data not shown). On the other hand, parasites treated with the pre-immune serum at the highest concentration adhered to the guts at a rate similar to that of the control (Fig. 2A).

Some studies dedicated to clarify the functional mechanisms of \textit{T. cruzi} proteins resorted to specific inhibitors as a methodological alternative \cite{6,7,8,27,28}. In this sense, we performed binding assays with parasites previously treated with MDL28170, a potent calpain inhibitor. The compound reduced significantly the number of parasites adhered to the insect luminal midgut surface in all drug concentrations tested (6.25–50 \textmu M). At the highest doses 25 and 50 \textmu M, the adhesion rate was approximately 0.6 epimastigotes per midgut cell while the control was about 1.4 (Fig. 2B), which represents a reduction of 65\%. DMSO at a dose equivalent to the highest concentration used to dissolve the drug did not promote any significant effect on the parasite adhesion (Fig. 2B). The effect of anti-calpain antibodies on parasite adhesion to \textit{R. prolixus} gut together with FACs and fluorescence analyses support a surface localization of the calpains. Therefore, we performed pre-embedding assays with fixed and permeabilized parasites incubated with anti-\textit{Dm}-Calpain at 4\degree C, afterwards parasites were routinely fixed and the ultrathin sections analyzed by transmission electron microscopy. The results showed labeling mainly at \textit{T. cruzi} cytoplasm (Fig. 3), but scarce labeling in few fields was also detected at the parasite membrane (Figure S1). The parasites incubated with rabbit pre-immune serum (control) showed no labeling (Fig. 3A).

These results led us to investigate whether MDL28170 might have any effect on the metacyclogenesis process. For this purpose, we performed experiments in which late-log phase epimastigotes form the Dm28c clone were submitted to the differentiation assay, and treated or not for 4 days with MDL28170 at concentrations ranging from 6.25 to 50 \textmu M. The Dm28c was selected instead of \textit{Y} strain because this strain did not yield a satisfactory rate of differentiation to metacyclic trypomastigotes. Under this experi-

![Figure 2. Effect of MDL28170 and anti-calpain antibody in the interaction process between \textit{T. cruzi} and explanted guts of \textit{Rhodnius prolixus}. (A) Epimastigotes (1.0x10^7 cells) were treated for 1 h at 28\degree C with the anti-\textit{Dm}-calpain (1:50, 1:100 and 1:250) or the pre-immune serum (1:50). (B) Epimastigotes (1.0x10^7 cells) were treated for 30 min at 28\degree C with increasing concentrations of MDL28170 (6.25 to 50 \textmu M) in 200 \textmu l of BHI. The data from DMSO represents the concentration present in the highest dose of the drug. The viability of the parasites was not affected by the treatments used in this set of experiments. Then, parasites were washed, and incubated for 20 min at 28\degree C with \textit{R. prolixus} dissected posterior midguts that were sliced open longitudinally. Subsequently, the explanted midguts were spread onto glass slides to count the number of attached parasites per epithelial cells. For each experimental group 4 insect midguts were used, and 100 epithelial cells were counted randomly. The results are shown as the mean ± standard error of the mean of three independent experiments. Symbols denote systems treated with MDL28170 or anti-calpain antibody that had a adhesion rate significantly different from the control (P<0.05 or P<0.001; Student’s t test). doi:10.1371/journal.pone.0018371.g002]
that the drug present a time and dose-dependent inhibition profile, since in cultures without MDL28170 the number of metacyclic trypomastigotes increased over time, whereas MDL28170 treatment inhibited the differentiation process almost to 50% in the highest drug concentration. DMSO at a dose equivalent to the highest concentration used to dissolve the drug did not interfere in the metacyclogenesis (Fig. 4). Then, we decided to investigate whether anti-calpain antibodies would prevent metacyclogenesis, all antibodies tested even at the highest concentration (1:50) did not promote a significant effect on parasite differentiation (data not shown).

Finally, we decided to evaluate the direct effect of the MDL28170 on bloodstream trypomastigotes viability in vitro after 24 h of treatment at 37°C. The inhibitor at 25 μM powerfully reduced parasite viability at about 63% in comparison to control (Fig. 5). Bloodstream trypomastigotes cultured in the presence of DMSO at a dose used to dissolve the highest drug concentration presented no significant effect (Fig. 5).

Given that the calpain inhibitor can reduce the growth of epimastigote forms with a trypanostatic effect [7], interfere in the parasite adhesion to the insect midgut and in the differentiation process of epimastigotes into metacyclic trypomastigotes, we decided to investigate the ultrastructural effects of MDL28170 against the epimastigote forms, by transmission electron microscopy. For this purpose, the morphology of non-treated cells

![Figure 3. Ultrastructural immunolabelling of calpains in T. cruzi epimastigotes.](image1)

Figure 3. Ultrastructural immunolabelling of calpains in T. cruzi epimastigotes. (A) Secondary antibody control presenting no gold particles. (B,C) Parasites showing anti-calpain immunolabelling in the cytosol (arrows). In detail, it was observed in 10 nm-gold particles. Bars = 200 nm. Inset bars = 100 nm. doi:10.1371/journal.pone.0018371.g003

![Figure 4. Effect of MDL28170 on T. cruzi metacyclogenesis in vitro.](image2)

Figure 4. Effect of MDL28170 on T. cruzi metacyclogenesis in vitro. Epimastigotes from the stationary phase of growth (5.0 × 10⁸ cells) were incubated in TAU3AAG medium for 96 h to induce cellular differentiation to metacyclic trypomastigotes. Parasites were treated with increasing concentrations of MDL28170 (6.25 to 50 μM). The data from DMSO represents the concentration present in the highest dose of the drug. At each time point, culture supernatants were collected and the number of epimastigotes and metacyclic trypomastigotes was determined by cell counting in a Neubauer chamber. The results correspond to the mean of three independent experiments performed in triplicate. Symbols denote systems treated with MDL28170 that had a percentage of metacyclic trypomastigotes significantly different from the control (*P<0.05 or **P<0.001, Student’s t test). doi:10.1371/journal.pone.0018371.g004
Figure 5. In vitro efficacy of MDL28170 on T. cruzi bloodstream trypomastigotes viability. Bloodstream trypomastigote forms obtained from Swiss mice (5.0 x 10⁶ cells) were treated with increasing concentrations of MDL28170 (6.25 to 50 μM) for 24 h. The data from DMSO represents the concentration present in the highest dose of the drug. Thereafter, viable parasites were counted by trypan blue exclusion and mobility. The results are expressed in viability percentage in relation to control. The results correspond to the mean of three independent experiments performed in triplicate. Symbols denote significantly different from the control (P < 0.05 or P < 0.001; Student’s t test).

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(Fig. 5a–b) was compared with the ultrastructure of parasites treated for 24 h with 34 μM of MDL28170, the 50% inhibitory concentration (IC₅₀) of MDL28170, as previously determined [7] (Fig. 6c–g). Results showed an important injury to the reservosomes (Fig. 6c–e), plasma membrane (Fig. 6d,g) and Golgi (Fig. 6f,g). Reservosomes and Golgi were severely affected, showing a washed-out appearance with loss of organelles’ electron-density and complete disruption of their membranes.

Discussion

Over the past few years some studies have described the presence of calpain-related proteins in trypanosomatids. At first, a classical study employing whole genome analyses showed the presence of a large and diverse family of calpains in T. brucei, L. major and T. cruzi [14]. In this sense, our group became involved in the study of these molecules though the report of the expression of calpain-like proteins following reparable mechanical damage of the plasma membrane and membranes [34]. At least two acylated calpain-like proteins in the kinetoplastids L. major and T. brucei are biochemically associated or co-localize with cellular membranes [35,15]. Acylated proteins are often associated with the cytoplasmic face of membranes and lipid rafts, where they are implicated in signal transduction [35,15]. Reversible externalization of intracellular proteins following reparable mechanical damage of the plasma membrane has been recently reported for cells in tissues of multicellular organisms [36]. Calpains have been described in the surface of the tegument of male adult worms, being secreted by the kinetoplastids since it is speculated that most calpain-like proteins do not have proteolytic activity, since the amino acid residues essential for catalytic activity are altered [14]. It has been speculated that calpains devoid of activity are involved in regulatory processes [14,31], for instance, calpain-6 is involved in microtubules stabilization through a non-catalytic mechanism [32]. Up to date, a calpain with proteolytic activity in trypanosomatids was only described in C. deanei, an insect trypanosomatid. However, since its microsequencing was not performed, it is still an open question. Also, since C. deanei genome is not available it is not possible to search for calpain sequences with the conserved catalytic triad [16]. It is interesting to note that with few exceptions, most organisms outside the animal kingdom have only a single calpain gene, while in the Trypanosomatidae family, there is a surprising expansion of genes, which may reflect parasite plasticity to face distinct environments, such as the mammalian host and the insect vector [14,33]. Therefore, although the results employing MDL28170 must be interpreted with caution, the anti-calpain antibodies were capable of significantly reducing the number of bound parasites to the luminal midgut surface of the insect host, which supports the possible involvement of calpains.

A first pre-requisite for calpain-like molecules to act on parasite binding to the insect midgut would be its surface location. Typically, calpains are cytosolic enzymes. Although membrane binding is not well substantiated for classical calpains, predicted transmembrane segments in phytocalpain and some ciliate calpains suggest an evolutionary link between calpain function and membranes [34]. At least two acylated calpain-like proteins in the kinetoplastids L. major and T. brucei are biochemically associated or co-localize with cellular membranes [35,15]. Acylated proteins are often associated with the cytoplasmic face of membranes and lipid rafts, where they are implicated in signal transduction [35,15]. Reversible externalization of intracellular proteins following reparable mechanical damage of the plasma membrane has been recently reported for cells in tissues of multicellular organisms [36]. Calpains have been described in the surface of the tegument of male adult worms, being secreted by cercarian penetration glands [37]. Whether calpains are transiently or constantly present at T. cruzi surface remains to be elucidated. Bioinformatics analysis gives indication of putative acylation, myristoylation and palmitoylation motifs in T. cruzi calpains, suggesting that they may be membrane-associated [38] (Ennes-Vidal, unpublished data). Accordingly, TcCALPx11 (XP_816697.1) partitioned in the insoluble fraction after detergent extraction, suggesting an association with membranes [39]. Additionally, calpains were identified in a proteomic analysis of detergent-solubilized membrane proteins from T. cruzi [38]. FACS analysis revealed a clear predominance of calpains in T. cruzi cytosol, since detergent permeabilization increased the fluorescence intensity [7]. The immunofluorescence analysis with non-permeabilized parasites is suggestive of calpain surface localization, and the effect of anti-Dn-calpain on parasite binding to the
insect midgut also supports a surface location. Curiously, the pre-embedding technique did not reveal significant membrane labeling. Calpains localization in *T. cruzi* is still an open issue, and the main goal of our laboratory is to help to elucidate this question. One possible explanation would be that some isoforms, recognized by the same antibody, are abundantly expressed in the cytosol and recruits more efficiently the antibody. Indeed, some membrane labeling was detected, in scarce fields.

The metacyclogenesis process consists in the differentiation of non-infectious *T. cruzi* epimastigotes into pathogenic metacyclic trypomastigotes. During this process, epimastigotes adhere to the epithelium of the insect midgut before transforming into metacyclic trypomastigotes [2]. The adhesion is thought to be a pre-requisite for differentiation to the infective form, but less is known about how epimastigote adhesion triggers the differentiation process after the nutritional stress [40]. Our findings from *T. cruzi* in vitro metacyclogenesis showed that the calpain inhibitor impaired the differentiation process, which is not surprising since previous studies have shown that cysteine peptidases are required during the metacyclogenesis process [41,42]. Once again, it should be taken into account that MDL28170 might be acting on other *T. cruzi* cysteine peptidases. In addition, this approach was performed with Dm28c instead of the Y strain. Distinct levels of expression of calpains were detected between Y strain and Dm28c: higher levels

**Figure 6. Ultrastructural effects of MDL28170 in *T. cruzi* epimastigotes.** (a,b) Control parasites showing typical elongated morphology with normal kinetoplast (K), mitochondrion (M), nucleus (N) and Golgi (G). (c–g) The treatment of epimastigotes with 34 μM of the inhibitor for 72 h led to an extensive disorganization in the reservosomes (asterisks), plasma membrane alterations (arrows) as well as to the disruption of Golgi cisternae (stars). Bars = 0.5 μm.

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(twice as many) were found for Y strain in comparison to Dm28c
[7]. Therefore the better performance of the Dm28c to
differentiate in vitro does not have a direct correlation with calpain
expression, at least for the isoforms detected by the anti-Dm-
calpain. When live parasites were allowed to differentiate in
the presence of the antibody, no significant effect was observed. Since
we are dealing with live parasites and the incubation time required for
differentiation is too long, the live parasites could be removing
the proteins from the surface, since antibodies can be quickly
interiorized by endocytosis and degraded [42,43], or even,
subjected to constant secretion to the surface such that bound
calpains are sloughed off and replaced. Although our data do not
allow to infer if calpain-like molecules are actually involved in T.
cruzi metacyclogenesis, the transriptome of several T. cruzi
indicated that TcCALPx11 is up-regulated in epimastigotes under
nutritional stress [39], a requirement for differentiation to
metacytic trypomastigotes [4].

Finally, the ultrastructural data demonstrated severe effects of
MDL28170 in the reservosomes and Golgi, which showed a
washed-out appearance with loss of organelles electrondensity and
MDL28170 in the reservosomes and Golgi, which showed a
T. cruzi peptidase of epimastigotes treated with inhibitors from the major cysteine
somes [44,45]. Similar ultrastructural alterations were observed in
the main peptidase involved in protein degradation in reservo-
to metacyclogenesis inhibition. Cruzipain has been implicated as
Therefore, the effects promoted by MDL28170 in crucial
dependence during metacyclogenesis, differentiating parasites
forms. Proteins and lipids accumulated in reservosomes of
epimastigotes forms are used as an energy source during
metacyclogenesis, resulting in the disappearance of these organ-
elles in the vertebrate stages of the parasite [44]. Besides protein
degradation during metacyclogenesis, differentiating parasites
must quickly synthesize new trypomastigote-specific proteins.
Therefore, the effects promoted by MDL28170 in crucial
organelles for protein degradation and synthesis are in accordance to
metacyclogenesis inhibition. Cruzipain has been implicated as
the main peptidase involved in protein degradation in reservo-
somes [44,45]. Similar ultrastructural alterations were observed in
epimastigotes treated with inhibitors from the major cysteine
peptidase of T. cruzi, the cruzipain [46]. The cysteine peptidase
inhibitor was capable of modifying the intracellular localization of
cruzipain and induced its accumulation in peripheral dilations of
Golgi cistern, these abnormalities were followed by distention of
endoplasmatic reticulum and nuclear membranes [46]. It should
be taken into account that MDL28170 could act nonspecifically
on cruzipain. Nevertheless, a recent proteomic analysis showed the
presence of calpain-like molecules in a purified fraction of
reservosomes [45].

It has been almost ten years since the first calpain-like protein
CAPS5 was characterized in T. brucei [15]. The distribution of
calpains in the distinct life stage of T. cruzi is still unknown. Here, we
showed that the anti-calpain antibody reacts with epimastigotes,
but not bloodstream trypomastigotes, while amastigotes were not
assessed. In 2008, Giese and collaborators [39] also reported an
epimastigote-specific calpain, which is one of the calpains
recognized by the antibody used in the present work. Since
calpains are a multigenic family, it cannot be ascertained if all
calpains are indeed epimastigote-specific. Nevertheless, at least the
four proteins recognized by the anti-Dm-calpain are epimastigote-
specific, which could suggest a role in the adaptation of this life
stage to the insect vector environment, as previously suggested [39]. Corroborating this view, we are demonstrating a probable
involvement of calpains in insect midgut attachment, and
metacyclogenesis. However, it should be reinforced that at least
these four proteins have the catalytic triad altered and it is unlikely
that they restrained catalytic properties. Therefore, our main goals
now are a careful analysis of calpain expression in the distinct life
forms, which will help to shed some light on calpain functions.

Supporting Information

**Figure S1** Ultrastructural immunolabelling of calpain
in T. cruzi epimastigotes surface. (A) The parasite presented
cytosolic labeling and scarce gold-particles in the plasma and
flagellar membranes (squares). (B,C) The high magnification of
plasma membrane and the flagellar pocket regions evidenced the
10 nm-gold particles (arrows). A, Bar = 200 nm. B,C, bars =
100 nm. (TIF)

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Author Contributions

Conceived and designed the experiments: VEV RFSMB ALSS MHB CMdL. Performed the experiments: VEV RFSMB. Analyzed the data:
VEV RFSMB ALSS MHB CMdL. Wrote the paper: VEV RFSMB CMdL.

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