Effects of the calpain inhibitor MDL28170 on the clinically relevant forms of *Trypanosoma cruzi* in vitro

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**Objectives:** There is a general lack of effective and non-toxic chemotherapeutic agents for treating Chagas’ disease. In the present work, we evaluated the in vitro activity of the calpain inhibitor MDL28170 against *Trypanosoma cruzi* relevant clinical forms.

**Methods:** The effect of MDL28170 on bloodstream trypomastigotes at different concentrations was assessed by counting the parasites in a Neubauer chamber, which allowed the determination of IC₅₀ values. Subsequently, parasite–macrophage interaction was assessed by two approaches: (i) peritoneal mouse macrophages were pre-infected with trypomastigotes for 3 h and then treated daily for 72 h with MDL28170; or (ii) bloodstream trypomastigotes were pre-treated with the calpain inhibitor for 1 h and then subjected to the infection assay.

**Results:** MDL28170 was capable of significantly reducing the viability of bloodstream trypomastigotes, presenting an IC₅₀/24 h value of 20.4 μM. Also, parasites pre-treated with the inhibitor, at subinhibitory drug concentrations, prior to macrophage infection presented a clear dose-dependent inhibition profile, where the inhibition increased from 20% to 50% (in relation to control) as MDL28170 concentration rose from 6.25 to 50 μM. In addition, macrophages experimentally infected with *T. cruzi* that were treated with the calpain inhibitor presented a significant reduction in the percentage of infection even at the lowest concentrations (6.25 μM).

**Conclusions:** These data may contribute to the study of the calpains in *T. cruzi* infection and add new in vitro insights into the possibility of exploiting calpains as promising targets to treat Chagas’ disease.

**Keywords:** Chagas’ disease, chemotherapy, proteases, peptidases, cysteine peptidases

**Introduction**

*Trypanosoma cruzi* is the aetiological agent of Chagas’ disease, which affects over 16 million people, with more than 100 million exposed to the risk of infection.¹ The parasite life cycle involves several stages of differentiation: in the mammalian host, the intracellular amastigotes and the bloodstream trypanomastigotes are the main evolutive forms, whereas the replicative epimastigote is the major form in the insect vector. To effectively combat the disease, a promising trypanocidal agent should impair parasite development in mammalian host cells with minimal side effects. Unfortunately, the currently accepted drugs for this disease, nifurtimox and benznidazole, are still unsatisfying, due to their low efficacy and high toxicity.² In this context, several research groups have become involved in the study and identification of potential targets for the treatment of Chagas’ disease;³ particularly, our research group has invested in the study of trypanosomatid peptidases. The search for specific inhibitors of these enzymes is a reasonable strategy that might lead to the design of powerful chemotherapeutic agents against these pathogens.⁴ One such enzyme is calpain, which is a calcium-regulated cytosolic cysteine peptidase whose role remains poorly understood. Nevertheless, some evidence indicates that it may be involved in crucial cellular functions, such as rearrangement of cytoskeletal proteins and protein cleavage in order to activate various receptors and...
pro-enzymes. A variety of calpain inhibitors are under development and the potential clinical utility of these inhibitors has been shown in the treatment of neuromuscular and neurodegenerative diseases in which calpains have been shown to be up-regulated. By means of distinct biochemical and molecular approaches, our group and other researchers have described the presence of calpain-like proteins in T. cruzi epimastigote forms. In addition, a potent calpain inhibitor, designated MDL28170, impaired parasite growth and promoted morphological alterations of epimastigote forms; however, no data are available in relevant clinical forms of the parasite. Herein, we have evaluated the effect of MDL28170 on the viability and infectivity of T. cruzi trypomastigotes in vitro, as well as on amastigote intracellular development.

Methods

Effects of MDL28170 on T. cruzi trypomastigote viability

Bloodstream trypomastigote forms (Y strain) were obtained from infected albinino Swiss mice at the peak of parasitaemia by differential centrifugation, and resuspended in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. This suspension was incubated at 37°C for 24 h in the presence of increasing concentrations of MDL28170 (6.25–50 μM). Trypomastigote viable cells were counted in a Neubauer chamber, allowing the determination of the 50% inhibitory concentration (IC_{50}/24 h). 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).

Effects of MDL28170 on T. cruzi–macrophage interaction

Mouse peritoneal macrophages (6–8 weeks old) were collected from Swiss mice, and parasite–macrophage interaction was assessed by two approaches, as previously described. Briefly, the drug was added to the parasites either before (pre-treatment) or after (post-treatment) the interaction with macrophage cells, and the percentage of infection was determined as described elsewhere. The toxic effects of MDL28170 on mammalian cells were assessed by a dye-reduction assay employing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). All experiments were repeated at least three times in triplicate. P < 0.05 was considered statistically significant by means of Student’s t-test.

Results

The direct effect of the calpain inhibitor MDL28170 on bloodstream trypomastigote viability revealed an IC_{50} after 24 h of 20.4 μM. DMSO at a dose used to dissolve the highest drug concentration presented no significant effect on parasite viability (data not shown). These results led us to investigate whether the calpain inhibitor might have any effect on the interaction between T. cruzi and macrophages. For this purpose, we performed experiments in which the bloodstream trypomastigote forms of the parasite were treated for 1 h, before the interaction, with MDL28170 at concentrations ranging from 6.25 to 50 μM. Under this experimental condition, the parasites maintained their viability, as judged by their morphology, motility and propidium iodine staining, in which >95% of the trypomastigotes were viable (data not shown). The drug presented a clear dose-dependent inhibition profile, where inhibition increased from 20% to 50% (in relation to control) as MDL28170 concentration rose from 6.25 to 50 μM. DMSO at a dose equivalent to the highest concentration used to dissolve the drug did not promote any significant effect (Figure 1).

Given that the calpain inhibitor can interfere in the early steps of parasite infection, since the compound was added exclusively to T. cruzi trypomastigotes and that the interaction process was stopped after only 3 h, we resolved to investigate the association index of T. cruzi with macrophage cells during in vitro treatment for 24, 48 and 72 h with MDL28170. First of all, the toxicity of MDL28170 was determined on mouse peritoneal macrophages by the MTT assay. A significant deleterious effect was only observed at 50 μM (data not shown). Finally, we tested the effect of MDL28170 on T. cruzi interaction with macrophage cells after parasite invasion. For this purpose, untreated bloodstream trypomastigotes were allowed to interact with macrophages for 3 h, unbound parasites were washed away and the cultures were treated daily with MDL28170 at 6.25, 12.5 and 25 μM and followed for 24, 48 and 72 h. A clear time- and dose-dependent effect of the compound on the infection rate was observed, while the highest drug concentration (25 μM) was capable of reducing almost the entire infection after 72 h (Figure 2) resulting in a moderate 2- to 4-fold selectivity index. DMSO at a dose equivalent to the highest concentration used to dissolve the drug did not promote any significant effect (Figure 2).

Discussion

In the present study we demonstrated the effect of the calpain inhibitor MDL28170 on trypomastigotes and amastigotes, the...
Effect of MDL28170 on *Trypanosoma cruzi*

Figure 2. Susceptibility of intracellular parasites to MDL28170 in macrophages. Murine macrophages were infected with bloodstream trypomastigotes for 3 h at 37°C, unbound parasites were washed away and the cultures were treated daily with MDL28170 (6.25–25 µM), and followed for 24, 48 and 72 h. DMSO corresponds to the dose used to dissolve the highest drug concentration. The drug concentrations followed for 24, 48 and 72 h. DMSO corresponds to the dose used to dissolve the highest drug concentration. The drug concentrations correspond to the mean of at least three independent experiments.

clinically relevant forms of *T. cruzi*. This compound is just one of the available calpain inhibitors screened for several human diseases that are believed to be calpain-associated disorders. Calpain activity in humans is tightly regulated by its natural and highly specific endogenous inhibitor calpastatin. Inappropriate regulation of the calpain–calpastatin proteolytic system is associated with several important human pathological disorders including muscular dystrophy, cancer, Alzheimer’s disease, neurological injury, ischaemia/reperfusion injury, atherosclerosis, diabetes and cataract formation. In this context, calpain inhibitors in advanced clinical trial steps could be an interesting alternative in the treatment of neglected diseases such as leishmaniasis and Chagas’ disease, once the effects of the drugs under development are confirmed.

Several studies have described the presence of calpain-related proteins in trypanosomatids. The first characterized member of the calpain-related genes was CAP5.5, a *Trypanosoma brucei* cytoskeleton protein associated with the cell membrane, which is selected exclusively in procyclic forms. In the last year, the infection percentage was determined by light microscopy, counting 300 cells randomly in each duplicate coverslip. The results correspond to the mean of at least three independent experiments.

The functional roles of calpain-related proteins in *T. cruzi* remain an open question. The expression of a calpain-related protein from *T. cruzi* during nutritional stress preceding metacyclogenesis is 2.5 times higher than that observed in epimastigotes. However, the overexpression of this gene in transfected parasites induced no altered phenotype. Nevertheless, a proteomic analysis of *T. cruzi* resistance to benznidazole revealed that a calpain-like cysteine peptidase is exclusively detected in samples of the resistant phenotype. The effects of calpain inhibitors on trypanosomatids raise the interesting possibility of using these inhibitors to better understand the calpain functions in these parasites. Some studies dedicated to clarifying the functional mechanisms of *T. cruzi* proteins resorted to specific inhibitors as a methodological alternative. In this context, further studies assessing the effects of calpain inhibitors on the ultrastructure of the parasite and on the overall protein expression of treated parasites, through a proteomic approach, for instance, might help to shed some light on calpain function in trypanosomatids. These, together with molecular approaches, will probably unveil calpain functions and add new insights into the possibility of using calpain inhibitors as a promising alternative in the treatment of neglected diseases such as Chagas’ disease. Therefore, our results suggest that *T. cruzi* calpains could be a remarkable target for the development of potent and selective drugs.

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Transparency declarations

None to declare.

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