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Hydroxamic acid derivatives: a promising scaffold for rational compound optimization in Chagas disease

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RESEARCH ARTICLE

Hydroxamic acid derivatives: a promising scaffold for rational compound optimization in Chagas disease

Dayanne da Rocha de Menezes^{1,2}, Claudia Magalhães Calvet³, Giseli Capaci Rodrigues⁴, Mirian Claudia de Souza Pereira³, Igor Rodrigues Almeida⁵, Alcino Palermo de Aguiar⁶, Claudiu T. Supuran⁷, and Alane Beatriz Vermelho¹

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Abstract

This work describes the antitrypanocidal activity of two hydroxamic acid derivatives containing *o*-ethoxy (HAD1) and *p*-ethoxy (HAD2) as substituent in the aromatic ring linked to the isoxazoline ring. HAD1 and HAD2 induced a significant reduction in the number of intracellular parasites and consequently showed activity on the multiplication of the parasite. Treatment of cardiomyocytes and macrophages with the compounds revealed no significant loss in cell viability. Ultrastructural alterations after treatment of cardiomyocytes or macrophages infected by *Trypanosoma cruzi* with the IC₅₀ value of HAD1 revealed alterations to amastigotes, showing initial damage seen as swelling of the kinetoplast. This gave a good indication of the ability of the drug to permeate through the host cell membrane as well as its selectivity to the parasite target. Both compounds HAD1 and 2 were able to reduce the cysteine peptidases and decrease the activity of metallopeptidases

Keywords

Chagas disease, hydroxamic acid derivatives, *Trypanosoma cruzi*

History

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Introduction

Chagas disease, a zoonosis caused by the protozoan *Trypanosoma cruzi*, is currently a global public health challenge. The disease has two distinct clinical phases: acute, mostly asymptomatic with patent parasitemia, and, chronic, which can be asymptomatic (indeterminate stage) or symptomatic (determinate stage), showing severe cardiomyopathy, megacolon, or megaesophagus¹. This disease affects 7–8 million people mostly in Latin American² and it is currently the leading cause of heart failure in South American countries³. However, there is a new epidemiological scenario showing a growing number of infected individuals in non-endemic countries⁴ that is drawing attention to this silent and neglected disease. There are a large number of immigrants with Chagas disease in the United States and European countries^{5,6} and also there is the emerging infection by the congenital and oral

transmission in endemic areas⁷ all of which alert the need to implement strategies to control the transmission and burden of this infectious disease⁸.

The current chemotherapies for clinical practice are benznidazole (BZ) and nifurtimox. The medications are predominantly active during the acute phase of the disease. Resistance to benznidazole (BZ) has been observed with some parasite strains⁹. Additionally, adverse side effects of these drugs, such as digestive and dermatological intolerance, have been implicated in the treatment compliance¹⁰. Also it is important to highlight that the *T. cruzi* polyparasitism and co-infection with other pathogens may interfere with the failure of the present therapeutic interventions¹¹. The failure of promising drugs undergoing clinical trials¹² and also the unsuccessful stem cell therapy¹³ reinforce the need for new strategies in the treatment and management of Chagas' disease¹⁴. These strategies should include an analysis of the advantage of combination therapy¹⁵ and eventual benefits from vaccine development¹⁶, as well as the identification of new drug targets and biomarkers to monitor the treatment response and cure prediction. Thus, the lack of effective drugs for the treatment of this worldwide, emerging disease encourages initiatives on the rational drug development of new trypanocidal drugs.

Given the key role of peptidases in biological systems, these enzymes have been considered a potential therapeutic target against infectious agents^{17,18}. Different classes of peptidases

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participate in important biological events in the *T. cruzi* life cycle, including processes of invasion, differentiation, proliferation as well as mechanisms of escape from host immune responses¹⁹. Thus, peptidase inhibitors are a potential to design trypanocidal drugs. For example, K777, a peptidase inhibitor of the major cysteine proteinase of *T. cruzi* known as cruzain or cruzipain, became a potent candidate to advance into clinical trials in view of its effective activity against different strains of *T. cruzi*²⁰, including its efficiency in protecting against cardiac damage in a canine experimental model²¹ and in response to multi-drug therapy²². Aspartic peptidase from *T. cruzi* has also been proposed as a drug target since treatment of epimastigotes with pepstatin A, an aspartic peptidase inhibitor, inhibited parasite proliferation, induced morphological changes with detachment of flagellum, and increased expression of gp63²³. Moreover, matrix metallopeptidase-9 homologues detected in *T. cruzi* may be a potential therapeutic target, since they could be able to induce a reduction in the expression of matrix metallopeptidase-9 (MMP-9) during interaction with host cells^{24,25}.

A series of novel 4,5-dihydroisoxazoles incorporating hydroxamate moieties were synthesized and evaluated against Chagas disease. These hydroxamic acid derivatives (HAD) proved to be very effective against *T. cruzi*. The assays carried out revealed molecules with low cytotoxicity and efficient action against *T. cruzi* carbonic anhydrase (TcCA) and metallopeptidase²⁶. Carbonic anhydrases (CA, EC 4.2.1.1) are metalloenzymes which catalyze the conversion of CO₂ to the bicarbonate ion and protons. CAs are involved in numerous physiological and pathological processes including microbial diseases caused by fungi and bacteria²⁷. The identification of a gene encoding α -carbonic anhydrase in *T. cruzi* (TcCa) raised interest in the application of this enzyme as a potent therapeutic target in Chagas disease^{26,28}.

Thus, this study aimed to investigate the detailed trypanocidal action of the two derivatives of this new series, since they showed promising results against TcCA²⁶, and their trypanocidal activity has not been investigated until now. Our results showed that these hydroxamic acid derivatives (HAD1 and 2) demonstrated a good activity against intracellular amastigotes, the clinical form found in the chronic phase. An inhibitory effect against parasite peptidases was also observed. Altogether, our data suggest that the hydroxamates scaffold can be considered as interesting candidates to move forward towards the development of more effective, selective and efficient Chagas disease drugs.

Materials and methods

Chemicals

Medium constituents were obtained from Oxoid Ltd. (Hampshire, England). Reagents used in electrophoresis were purchased from Amersham Life Science (Little Chalfont, England). Gelatin and casein were purchased from Merck (Darmstadt, Germany). The metallopeptidase (1,10-PHE – 1,10-phenanthroline), the cysteine peptidase (*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane [E-64]) inhibitors and all reagents used in the synthesis of the 4,5-dihydroisoxazole derivatives were obtained from Sigma-Aldrich (St. Louis, MO). All other reagents were of analytical grade.

Synthesis of 3-(2-ethoxyphenyl)-*N*-hydroxy-4,5-dihydroisoxazole-5-carboxamide (HAD1) and 3-(4-ethoxyphenyl)-*N*-hydroxy-4,5-dihydroisoxazole-5-carboxamide (HAD2)

The hydroxamic acid derivatives were synthesized as described by Rodrigues et al.²⁶ Briefly, the 2-ethoxy and 4-ethoxy-phenylaldehyde were synthesized from 20 mmol of the respective

aldehydes and 60 mmol of NH₂OH.HCl contained in 20 mL of solvent. The reaction medium was maintained for 2 h at a temperature of 27 °C under constant agitation. After which, the respective aldoximes were reacted with trichloroisocyanuric acid (10 mmol) and triethylamine (20 mmol) for 30 min under stirring at room temperature to produce the corresponding hydroximoyl chlorides. Then, dipolarophile (ethyl acrylate) was added to the solutions and, after 24 h, the 4,5-dihydroisoxazol derivatives were generated by 1,3-cycloaddition reactions. Finally, a solution containing NH₂OH (20 mmol) was added to the 4,5-dihydroisoxazol derivatives formed and kept at reflux for 24 h. The respective hydroxamic acid derivatives generated were recrystallized from hot water and filtered. The yields were 40% and 50%, respectively.

Parasites

Trypomastigote forms of *T. cruzi*, Y strain, were isolated from the blood of infected mice at the peak of parasitemia (7 d after infection). To increase the yield of *T. cruzi* trypomastigotes per mouse and reduce the number of animals used in the procedure, mice were immunosuppressed with intraperitoneal (ip) administration of cyclophosphamide 500 mg/kg on the 4th day after infection. To isolate the parasites, blood was collected by cardiac puncture with 3.8% sodium citrate as an anticoagulant and centrifuged at 1200 rpm for 26 min to separate them from the blood cells. The supernatant containing the parasites was then harvested and centrifuged at 4500 rpm for 14 min to pellet the trypomastigotes. The parasites were resuspended in culture medium DMEM-F12 and the concentration of parasites was assessed using a hemocytometer. The amastigote forms were obtained and analyzed through infection of mammalian cultures with trypomastigotes.

Primary culture

Murine peritoneal macrophages were obtained from Swiss Webster mice (18–20 g). Cold Dulbecco's modified Eagle medium (DMEM; Sigma, St. Louis, MO) was injected into the peritoneal cavity and then removed by fluid aspiration with a syringe. Peritoneal exudate cells were centrifuged at 400 × *g* for 10 min and the pellet resuspended in culture medium supplemented with 10% fetal bovine serum (FBS) followed by determination of cell number/mL using a Neubauer chamber. The cells were plated at a density of 1 × 10⁵ and 4 × 10⁵ cells per well in black, clear bottom 96-well (Greiner Bio-One, #655090, Frickenhausen, Germany) and 24-well tissue culture plates, respectively, and maintained at 37 °C with 5% CO₂ atmosphere.

Primary culture of cardiac muscle cells was obtained as previously described²⁹. Briefly, the heart was removed from 18-d old mouse embryos and fragmented ventricles were dissociated with trypsin and collagenase. Isolated cells were resuspended in DMEM-F12 (Sigma, St. Louis, MO) supplemented with 7% FBS, 2% chicken embryo extract, 2.5 mM of CaCl₂, 1 mM L-glutamine and antibiotics. The cells were plated at a density of 5 × 10⁴ and 1.2 × 10⁵ cells per well in 96-well and 24-well tissue culture plates, respectively, and maintained at 37 °C at 5% CO₂ atmosphere. All procedures with animals were performed according to the Oswaldo Cruz Foundation committee of ethics for the use of experimental animals (LW-37/13).

Cell cytotoxicity assay

Primary cardiomyocyte and macrophage cultures were used to evaluate the cytotoxic effect of the compounds. After 24 h of culture, the cells were treated with the synthetic derivatives of 4,5-dihydroisoxazoles incorporating hydroxamate moieties (HAD1 and HAD2) synthesized as described²⁶. A wide range of

concentrations, ranging from 0.22 to 500 $\mu\text{g/mL}$, were tested to evaluate toxicity to mammalian cells. The cell viability analysis (CC_{50} value = the cytotoxic concentration corresponding to 50% reduction of cell viability) was performed after 3 d of treatment with HAD1 and HAD2 compounds using resazurin 0.005% (4 h at 37 °C) as the metabolic function indicator, followed by fluorescence intensity measurements at 530 nm and 630 nm in a Molecular Devices Flexstation 3 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA). Negative controls were performed with the vehicle medium with or without DMSO ($\leq 1\%$) and untreated cell cultures under similar conditions.

Trypanocidal assay

The effect of the HAD1 and HAD2 hydroxamic derivatives on *T. cruzi* was evaluated using bloodstream trypomastigotes of the Y strain. Parasites were isolated from infected Swiss Webster mice at the peak of parasitemia (7 d post-infection). Trypomastigotes (3×10^6 parasites/well) were incubated for 24 h at 37 °C in the presence or absence of serial dilutions of HAD1 or HAD2 compounds (0.04–90 μM). After treatment, the resazurin 0.005% was added to the parasites for 4 h at 37 °C and the fluorescence emission measurements were registered at 530 nm and 630 nm in a Molecular Devices Flexstation 3 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA). The inhibitory concentration (IC_{50}), the drug concentration that reduces 50% of trypomastigotes viability, was determined by fitting the dose–response curve to inhibitory activity compared with the untreated control. A similar curve for treatment with benznidazole (BZ) was also carried out in a parallel assay. Untreated trypomastigotes and cell-free media were used as negative controls.

The efficacy of the compounds was also evaluated, in terms of eliminating the intracellular parasites, by incubating 24 h-infected mammalian cells primary cultures (macrophages and cardiomyocytes) for 72 h at 37 °C with a serial dilution (3.3–90 μM) of HAD1 and HAD2 compounds, followed by fixation with Bouin solution fixative. The percentage of infection was determined by quantification of a minimum of 200 cells in randomly selected microscopic fields using a Zeiss Axioplan microscope (Zeiss, Rochester, NY). The experimental assays were performed at least three times in duplicate.

Transmission electron microscopy (TEM)

Primary cultures of cardiomyocyte and macrophage were infected with Y strain of bloodstream trypomastigotes in a 10:1 host–parasite ratio. After 24 h of infection, free trypomastigotes were removed from the culture supernatant and, after washing with Ringer's solution (154 mM NaCl, 56 mM KCl, 17 mM Na_2HPO_4 , pH 7.0), the cells were treated for 24 h or 48 h with the IC_{50} concentration (30 μM) of the HAD1 compound. Subsequently, the cultures were fixed for 1 h at 4 °C with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, and followed by post-fixation for 1 h at 4 °C with 1% OsO_4 in the same buffer. The samples were dehydrated in serial-graded acetone batches impregnated and embedded in Epon 812. Ultra-thin sections, contrasted with uranyl acetate and lead citrate, were observed in a JEOL transmission electron microscope (JEM/1011).

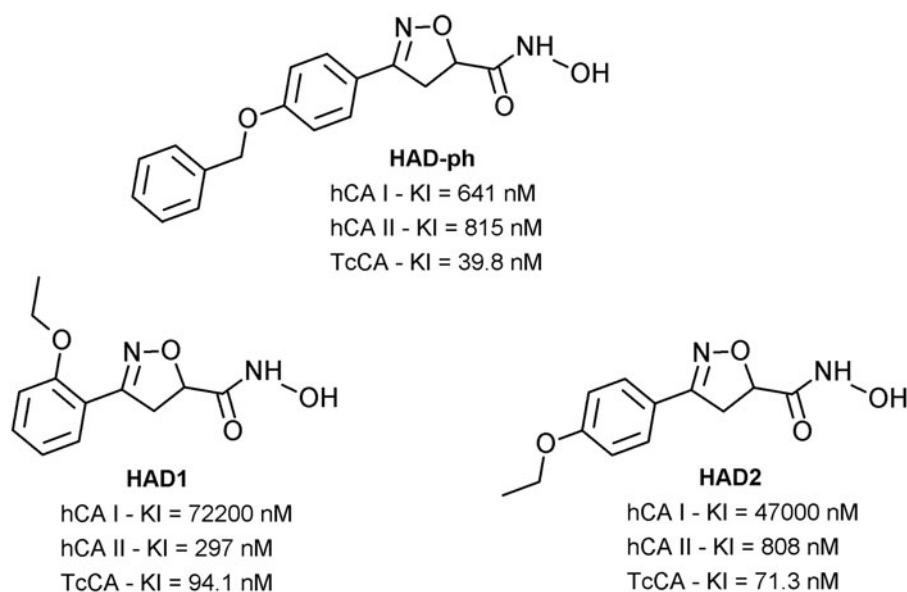
Analysis of peptidase activity

The inhibitory effect on the cellular proteolytic activity was evaluated on 10% SDS–PAGE with 0.1% gelatin incorporated as substrate³⁰. *Trypanosoma cruzi* epimastigote cultures were harvested by centrifugation at $1500 \times g$ for 15 min at 4 °C, and 1×10^7 cells were then lysed at 0 °C by the addition of 100 μL of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer. The gels were loaded with 25 μL of parasite extract per slot (equivalent to 1×10^7 cells) and after electrophoresis at a constant voltage of 170 V for 2 h at 4 °C; then they were soaked for 1 h in 2.5% Triton X-100 under constant agitation. The gel was incubated for 48 h at 37 °C in 50 mM sodium phosphate buffer, pH 5.5, with 100 μM of the HAD1 and HAD2. In order to determine the class of peptidases, 10 μM E-64 (a cysteine-peptidase inhibitor), 10 mM 1,10-phenanthroline (a non-specific metallopeptidase inhibitor) and 2 mM DTT (a cysteine-peptidase activator) were also tested²⁶.

Statistical analysis

Student's *t*-test was used to determine the significance of differences between the means in the amastigote proliferation assays. A *p* value ≤ 0.05 was considered significant.

Figure 1. Hydroxamic acid derivatives (HAD) with potential action against Chagas disease. Carbonic anhydrases human isoforms hCA I and II and *T. cruzi* enzyme TcCA²⁶.



Result and discussion

Chemistry

Previous studies have demonstrated promising antitrypanocidal activities of hydroxamic acid derivatives – HAD²⁶, acting as effective *T. cruzi* carbonic anhydrase (TcCA) inhibitors and ineffective as human CA inhibitors. Also, a lead compound containing a *p*-benzyloxy as a substituent on the aromatic ring (HAD-ph, Figure 1) besides its inhibitory activity against TcCA also showed promising features as an antitrypanosomal agent²⁶.

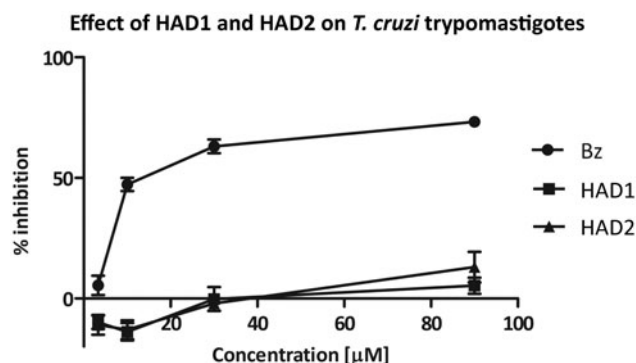


Figure 2. Effect of hydroxamic acid derivatives (HAD1-2) on bloodstream trypomastigotes (Y strain) compared with BZ activity. Trypomastigotes were treated at 37 °C for 24 h with different concentrations of the compounds. HAD1 and HAD2 compounds were inactive against the classical invasive form of *T. cruzi*. In contrast, the reference drug, BZ, showed an IC₅₀ value of 12.2 μM.

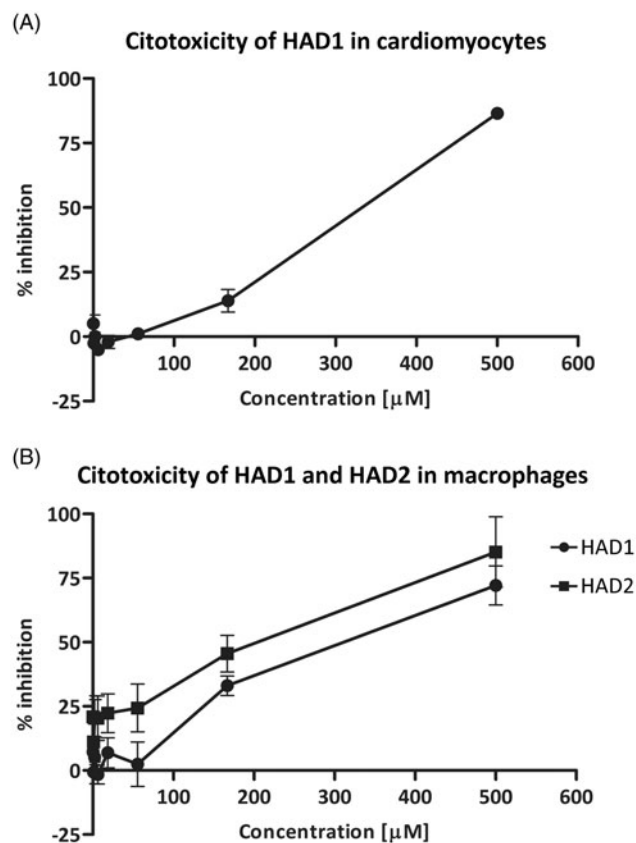


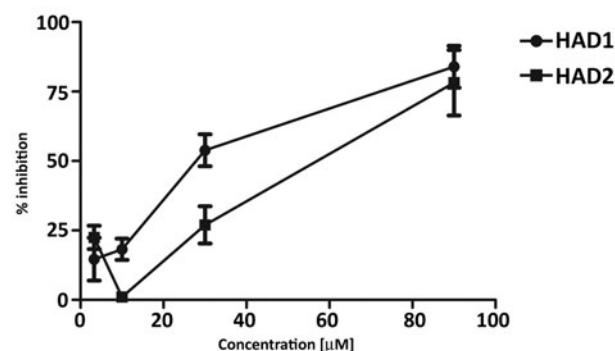
Figure 3. (A) Dose-dependent cardiomyocyte viability curve detected by resazurin in response to HAD1 treatment, resulting in CC₅₀ value = 329.8 μM. (B) HAD1 and 2 cytotoxicity were also assessed in macrophages in the same fashion, resulting in similar viability curves and a CC₅₀ value of 349 μM and 221 μM, respectively.

This led us to analyze the efficacy of the two analogs containing only *o*-ethoxy (HAD1) and *p*-ethoxy (HAD2) moieties as substituents in the aromatic ring (Figure 1) against trypomastigotes and amastigotes.

Effect of the hydroxamic acid derivatives (HAD1-2) against *T. cruzi*

We first analyzed the effect of the synthetic drugs (HAD1 and 2) on the viability of the bloodstream trypomastigotes (Y strain). Our findings demonstrated that even the highest concentration (90 μM) of both compounds (HAD1 and 2) was not effective against the trypomastigote forms of *T. cruzi*, while the IC₅₀ values for benznidazole (BZ), reference drug, was 12.24 μM ± 3.42 (Figure 2). In fact, the synthetic hydroxamic derivatives tested here (HAD1 and 2) were not as effective as their analog (HAD-ph) investigated earlier by Rodrigues et al.²⁶ This drug

(A) Inhibition of amastigote proliferation in cardiomyocytes



(B) Number of amastigotes in cardiomyocytes

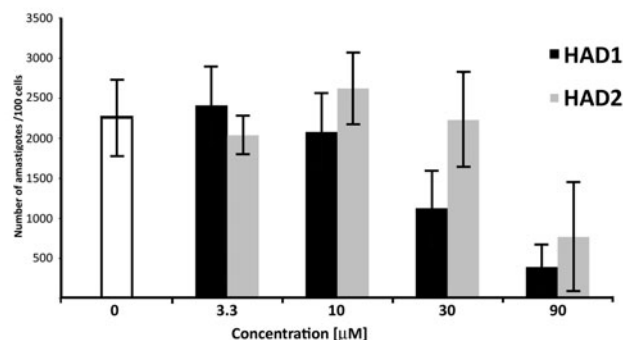


Figure 4. Analyses of the effect of HAD1 and 2 against intracellular amastigotes in cardiomyocyte culture. (A) Treatment with both synthetic compounds, HAD1 and 2, resulted in a dose-dependent inhibition of amastigote proliferation in cardiomyocytes. The lower IC₅₀ value was achieved by HAD1 (30 μM). A significant decrease in the total intracellular amastigotes was observed after HAD1 (≥30 μM) and HAD2 (90 μM) treatment (B). *Statistically significant, $p \leq 0.05$.

Table 1. Antitrypanocidal effect of hydroxamic acid derivatives (HAD).

Compound	Evolutionary forms of <i>Trypanosoma cruzi</i>	
	Trypomastigotes IC ₅₀ (μM)	Amastigotes IC ₅₀ (μM)†
HAD1	NE*	30 ± 10.7
HAD2	NE*	66 ± 19.3

*Not effective in the highest concentration (90 μM) evaluated.

†IC₅₀ values in *T. cruzi*-infected cardiomyocytes.

demonstrated a potent activity (greater than BZ) against all evolutive forms of *T. cruzi*, reaching an IC_{50} value from 7 to $<1 \mu\text{M}$ for different strains of the parasite. The absence of a third aromatic ring in the HAD1-2 structures decreased their inhibitory activity against TcCA and trypanocidal activity trypomastigote forms. Additionally, it was possible to observe that the *ortho* or *para* ethoxy positions of the substituent on the aromatic ring linked to the heterocyclic ring did not influence the trypanocidal activity. This fact highlighted the importance of the *p*-benzyloxy as the substituent on the aromatic ring linked to the heterocyclic

ring (HAD-ph) for trypanocidal activity, either by a steric effect or by increasing the liposolubility of the molecule²⁶.

Screening of cytotoxicity in mammalian cells

Prior to analyze the effect of the synthetic compounds on intracellular amastigotes, we established the non-toxicity concentrations of the compounds to mammalian cells. The cellular toxicity assay was estimated on cardiomyocyte and macrophage cultures using resazurin as a viability indicator. Low toxicity was

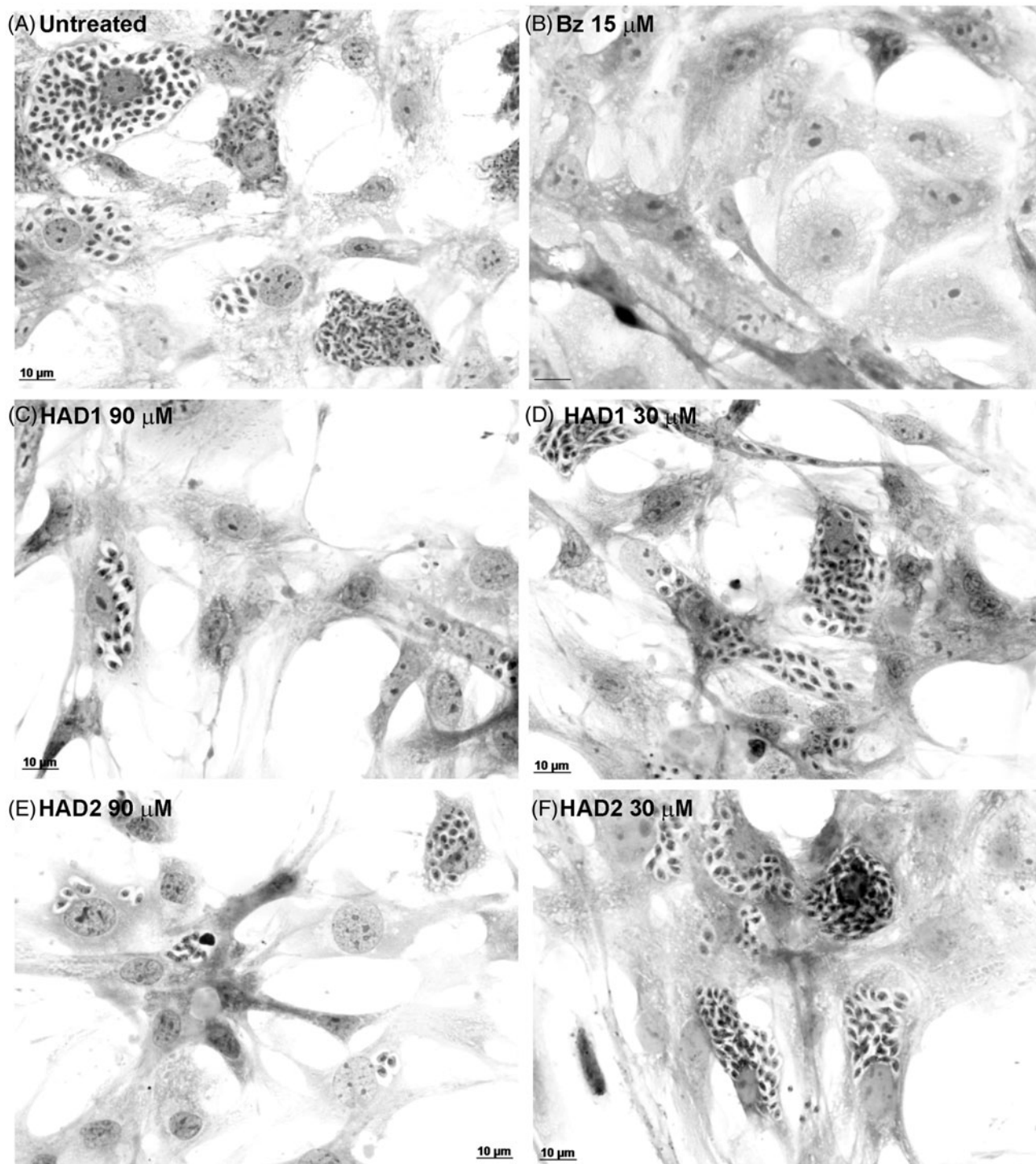


Figure 5. Light microscopy of cardiomyocytes treated with HAD1 and 2 compounds. (A) Untreated cells displaying high number of intracellular amastigotes; (B) benznidazole treatment at $15 \mu\text{M}$ eliminated the parasites. HAD1 treatment resulted in an expressive reduction of amastigote proliferation (C, $90 \mu\text{M}$; D, $30 \mu\text{M}$). Addition of HAD2 in *T. cruzi*-infected cardiomyocyte cultures inhibited amastigote proliferation at high concentrations ($90 \mu\text{M}$, E), but lower concentrations did not produce the same effect ($30 \mu\text{M}$, F). Bar = $10 \mu\text{M}$.

seen for both compounds tested. Treatment of cardiomyocytes with the HAD1 compound revealed no significant loss in viability up to 166 μM , resulting in a CC_{50} value = 329.8 μM (Figure 3A). Also against macrophages, HAD1 and 2 did not demonstrate any cytotoxicity effect, reaching a CC_{50} value of 349 μM and 221 μM , respectively (Figure 3). The lack of toxicity in both cell types was clearly demonstrated by light microscopy, where even the highest concentration of HAD1 (90 μM) did not alter the morphology and viability of the macrophages (data not shown). The absence of *p*-benzyloxy as the substituent on the aromatic ring decreased the antitrypanosomal activity as well as the cellular cytotoxicity, which could be influenced by lower liposolubility of the HAD1 and 2 molecules than the HAD-ph prototype.

Efficacy of compounds against intracellular amastigotes

Considering the previous observations that TcCA inhibitors affect parasite growth²⁸ and that both compounds were effective against epimastigotes²⁶, we hypothesized that these derivatives could have a potential activity on the multiplication of the parasite. Therefore, we investigated the effectiveness of the drugs in clearing the infection. The activity against intracellular amastigotes was assessed by treating *T. cruzi*-infected cardiomyocyte cultures with the HAD1 and 2 compounds. Both drugs induced a significant reduction in the number of intracellular parasites in concentrations of 30 μM and 90 μM , but HAD1 was two-fold more effective than HAD2 at 30 μM (Figure 4). The IC_{50} values, a concentration that reduces 50% of intracellular parasites, in *T. cruzi*-infected cardiomyocytes, were $30 \pm 10.7 \mu\text{M}$ and $66 \pm 19.3 \mu\text{M}$ for HAD1 and 2, respectively. This indicated that although these molecules have a lower liposolubility than the lead compound they are able to act against the intracellular forms of the parasite (amastigotes). Furthermore, it was observed that the intracellular activity was influenced by the ethoxy substituent position in the aromatic ring from the two hydroxamic acids, an important observation for future strategy of rational drug design. Table 1 summarizes the results of the antitrypanocidal effect of hydroxamic acid derivatives

Furthermore, the treatment of *T. cruzi*-infected cardiomyocyte cultures with 90 μM of HAD1 reduced the number of intracellular amastigotes 7-fold when compared with untreated cultures (Figure 4). This phenomenon was clearly observed by light microscopy (Figure 5), which demonstrates a low level of infection in the highest concentration (90 μM) of the HAD1 and 2 compounds compared with control. It is important to highlight that treatment of the *T. cruzi*-infected cardiomyocyte cultures with BZ at 15 μM eliminated all the intracellular parasites.

The fact that HAD1 displayed the highest selectivity index ($\text{SI} \geq 11$) and the lowest IC_{50} prompted us to analyze its activity against *T. cruzi*-infected macrophages, also an important cell involved in the infection by this parasite. Although the IC_{50} value was higher in macrophages (75 μM), the treatment with 90 μM of HAD1 also induced a 5.5-fold reduction of intracellular parasites (Figures 6 and 7). At the highest concentration (90 μM), the macrophages demonstrated normal morphology and spreading, showing the low toxicity of the drug (Figure 7). Although the drugs have shown activity against *T. cruzi*, they were not as efficient in the elimination of parasites as benznidazole, which revealed a reduction of 99% of intracellular amastigotes with 15 μM .

Curiously, HAD1 and 2 were less effective than the HAD-ph from the series of hydroxamic acid derivatives investigated here. The substitution of the *p*-benzyloxy (HAD-ph) with *o*-ethoxy and *p*-ethoxy in HAD1 and HAD2 reduced the efficacy of these compounds to eliminate the intracellular amastigotes in mammalian cells by three-fold and seven-fold, respectively. The strategy

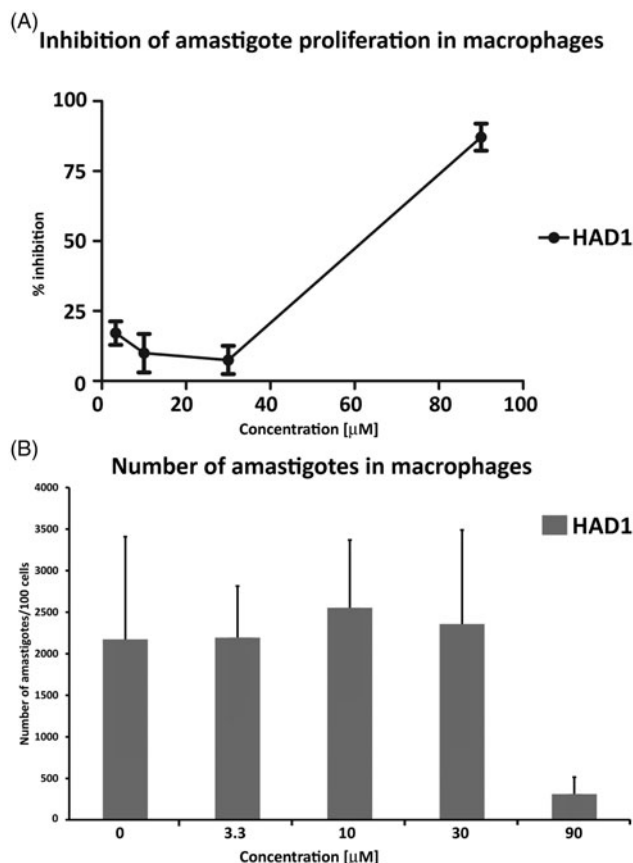


Figure 6. HAD1 compound effect on *T. cruzi* infection in macrophages. (A) Treatment of infected macrophages with HAD1 resulted in a dose-dependent inhibition of amastigote proliferation, achieving an IC_{50} value of 75 μM . (B) A significant decrease in the total number of intracellular amastigotes was detected with 90 μM of HAD1. *Statistically significant, $p \leq 0.05$.

of rational design of drugs has been encouraged in the search for new active drugs for the treatment of neglected diseases³¹. Lead optimization of compounds involving structure–activity relationships (SAR) may improve compound potency and physicochemical properties. However, the synthesis of more active compounds is a challenge and slight structural changes can reduce the compound activity. Therefore, our findings can guide the design of new molecules since the presence of *p*-benzyloxy instead of ethoxy impacts the activity against *T. cruzi*.

Ultrastructural analysis of the effect of HAD1 inhibitor against intracellular parasites

To analyze possible ultrastructural alterations induced by the synthetic compounds, transmission electron microscopy of *T. cruzi*-infected cardiomyocytes and macrophages after HAD1 treatment was performed (Figure 8). Treatment of cardiomyocytes or macrophages infected by *T. cruzi* with the IC_{50} value of HAD1 revealed initial damage in the amastigotes, visualized by swelling of the kinetoplast. One important fact is that both cells, macrophages and cardiomyocytes, did not undergo structural change with this treatment, demonstrating no cytotoxic effect of the compounds for mammalian cells. Thus, our ultrastructural analysis associated with the cytotoxicity findings provided a good indication of the ability of the drug to permeate through the host cell membrane and its selectivity to the parasite target. The ultrastructural analysis of the effect of nonazole CYP51 inhibitors against intracellular amastigotes showed blebbing of cellular and nuclear membranes and autophagic vesicles, in parallel with the

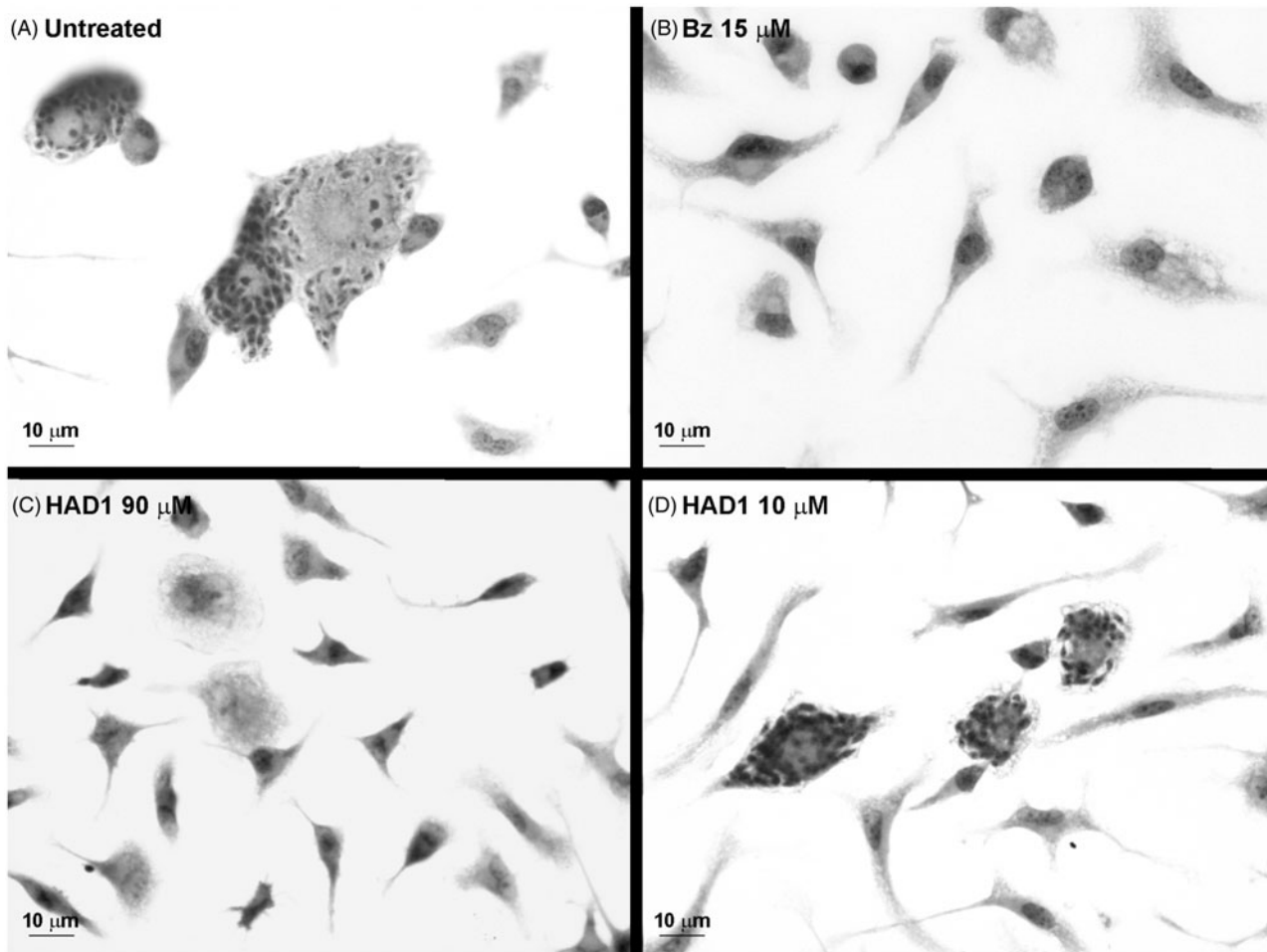


Figure 7. Light microscopy images of *T. cruzi*-infected macrophage cultures treated with HAD1. The cultures were treated 24 h after the establishment of *T. cruzi* infection. (A) General aspect of macrophages cultures after 72 h of infection by *T. cruzi*. (B) Clearance of intracellular parasites after 72 h treatment with 15 μM of benznidazole. Note the effectiveness of HAD1 compound against amastigotes after 72 h of treatment with 90 μM (C) and 10 μM (D).

accumulation of intermediate metabolites from the ergosterol biosynthesis pathway³². Most morphological studies of drug treatment emphasize the direct effect of compounds on the isolated parasites, mainly *T. cruzi* epimastigotes. Ultrastructural changes were also evidenced in *T. cruzi* treated with serine and cysteine protease inhibitors^{33,34}. A Kunitz-type serine protease inhibitor from Caribbean Sea anemone *Stichodactyla helianthus* induced the formation of concentric membrane structures and endoplasmic reticulum surrounding vesicles in the cytoplasm of epimastigotes, suggesting the autophagic cell death as its mechanism of action³³. Cysteine protease inhibitors (morpholine-carbonyl-phenylalanine-homophenylalanine-vinyl sulphone phenyl and morpholineureaphenylalanine-homophenylalanine-fluoromethyl ketone) induced severe alterations in the Golgi complex with chagasin accumulation in enlarged cisternae, suggesting a disturbance in the transport within the ER-Golgi system³⁴. Multiple intracellular compartment damage has been reported with other active drugs against *T. cruzi*³⁵⁻³⁷. Mitochondrial swelling and the presence of concentric structures were frequently noticed in parasites treated with lysophospholipid analogues³⁸ and naphthoquinone derivatives³⁹. Remarkable changes were also induced in the kinetoplast-mitochondrial system, microtubule, and reservosomes organization after treatment of *T. cruzi* with DNA-intercalating drugs and sterol biosynthesis inhibitors, leading to kinetoplast collapse and cell death^{36,37,40}.

Effect of HAD1 and 2 on the peptidase activity of *T. cruzi*

Cell-associated peptidases from *T. cruzi* displayed two distinct proteolytic classes: cysteine-(60 kDa) and metallopeptidase (70 and 80 kDa) activities inhibited, respectively, by E-64 and 1,10-phenanthroline, as shown in Figure 9. Both compounds HAD1 and 2 were able to reduce the cysteine peptidases and decrease the activity of metallopeptidases. Cruzain/cruzipain is a cysteine peptidase required for the survival of *T. cruzi*. This enzyme, present in all life stages of the trypanosome, has been the focus of several studies and patents and is implicated in parasite cellular entry and digestion of immunoglobulin, immune system evasion, and other cellular mechanisms^{19,41}.

In conclusion, this work demonstrated that hydroxamates are a promising class of compound with anti-*T. cruzi* activity. Our data showed that hydroxamates HAD1 and 2 were effective against the replicative forms of the parasite, but not against the infective forms. This could be an indication that its mechanism of action involves the inhibition of parasite peptidases and carbonic anhydrase, essential enzymes for the parasite life cycle. HAD1 and 2 partially inhibited the cysteine and metallopeptidases of *T. cruzi* under the conditions evaluated. Furthermore, previous studies have already demonstrated the selectivity of these compounds against carbonic anhydrase of *Trypanosoma cruzi* (TcAC). In our assays, we observed that the position of the ethoxy substituent on the aromatic ring linked to the heterocyclic one strongly influenced the biological activity. HAD1, which has an

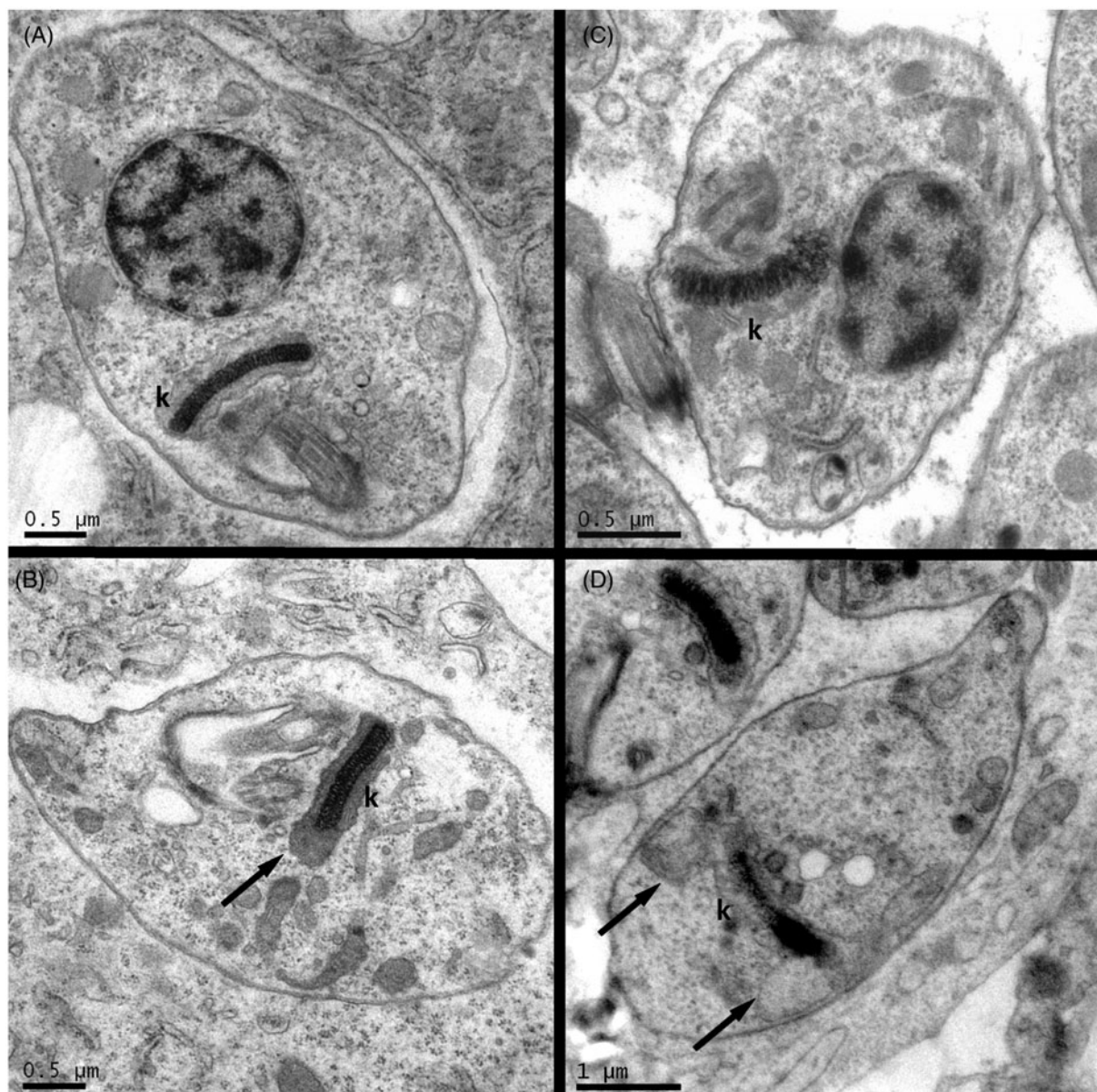


Figure 8. Transmission electron microscopy of mammalian cells infected by *T. cruzi* and treated with HAD1 compound. Untreated controls showing normal general amastigote structure and regular kinetoplasts (k) from cardiomyocyte (A) and macrophage (B) infected cultures. Forty-eight hours treatment of HAD1 at IC₅₀ concentration (30 μM) induced swelling of kinetoplasts (black arrows) both in cardiomyocyte (B) and in macrophage (D)-infected cultures.

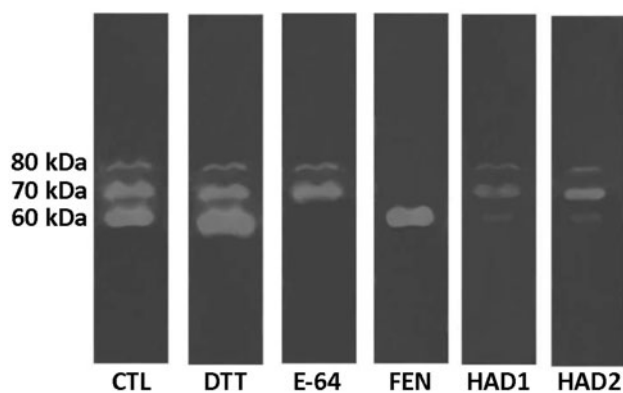


Figure 9. Zymography of *T. cruzi* cellular peptidases after treatment with the HAD1 and HAD2 inhibitors. CTL, control – without treatment; E-64, cysteine peptidase inhibitor; DTT, cysteine peptidase activator; 1,10-PHE, metallopeptidase inhibitor.

ortho-ethoxy, was 2-fold more effective than HAD2, which has a *para*-ethoxy moiety, against the intracellular amastigotes. The absence of *p*-benzyloxy moiety, present in the lead molecule HAD-ph, decreased the lipophilicity of these compounds, but the HAD1 and 2 were still able to be effective against the replicative forms of the parasite. This fact demonstrates that this moiety is important for trypanocidal activity, but not essential. Moreover, new structural changes in the scaffold seem to be important for investigating the trypanocidal activity and the discovery of a more potent agent for the treatment of Chagas disease.

Conclusions

This work demonstrated a promising class of hydroxamates with anti-*T. cruzi* activity. The hydroxamates were effective against replicative forms of the parasite, but not against infective, trypomastigote forms. Ultrastructural analysis of amastigotes, the replicative, forms showed swelling of the kinetoplast after treatment with HAD1. Both compounds reduce the cysteine and

metallopeptidase activities. No cytotoxic effect of the compounds for mammalian cells was observed. HAD1 and HAD2 could be an important scaffold to future rational drug design for anti-chagasic chemotherapy.

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Declaration of interest

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