Amiodarone inhibits *Trypanosoma cruzi* infection and promotes cardiac cell recovery with gap junction and cytoskeleton reassembly *in vitro*

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Running title: Amiodarone effects on *Trypanosoma cruzi* infected cardiomyocytes
Abstract

We present the results of the first detailed study of the antiproliferative and ultrastructural effect of amiodarone on Trypanosoma cruzi, the causative agent of Chagas’ disease. Moreover, we report the effects of this compound on the recovery of F-actin fibrils, connexin43 and contractility in T. cruzi infected cardiac myocytes. Amiodarone is the most prescribed class III antiarrhythmic agent and is frequently used for the symptomatic treatment of Chagas’ disease patients with cardiac compromise. In addition, recent studies identified its antifungal and antiprotozoal activities, which take place through Ca^{2+} homeostasis disruption and ergosterol biosynthesis blockage. We tested different concentrations of amiodarone (2.5-10 µM) on infected primary cultures of heart muscle cells and observed a dose- and time-dependent effect on growth of the clinically relevant intracellular amastigote form of T. cruzi. Ultrastructural analyses revealed that amiodarone had a profound effect on intracellular amastigotes, including mitochondrial swelling and disorganization of reservosomes and the kinetoplast and blockade of amastigote-trypomastigote differentiation. Amiodarone showed no toxic effects on host cells, which recovered their F-actin fibrilar organization, connexin43 distribution and spontaneous contractility concomitant with the drug-induced eradication of the intracellular parasites. Amiodarone is, therefore, a promising compound for the development of new drugs against T. cruzi.
Introduction

Chagas disease is the largest parasitic disease burden and a major cause of heart disease and heart-related deaths in Latin America, where it affects approximately 16-18 million people (WHO, 2002; Rassi et al. 2009). The disease is caused by the protozoan parasite *Trypanosoma cruzi* (*T. cruzi*), which possesses a life cycle involving a mammalian host and an insect vector (Tanowitz et al., 1992).

Chemotherapy against *T. cruzi* is limited to two compounds, namely benznidazole (a 2-nitro-imidazole) and nifurtimox (a 5-nitrofuran), which are mostly active in acute and early chronic phase patients but of limited efficacy in the prevalent chronic stage (Coura & de Castro, 2002; Urbina, 2010). Moreover, *T. cruzi* exhibits considerable biological variability, indicating possible variations in virulence, pathogenicity, oxidative stress and drug resistance (Sanchez et al., 1990; Mielniczki-Pereira et al., 2007; Veloso et al., 2001), which may pose an important challenge in the search of safer and more effective chemotherapeutic agents for the specific treatment of Chagas disease.

Studies in the last two decades have permitted the identification of several new drug targets in this parasite. Among the most promising are (i) the essential cathepsin L-like protease cruzipain; (ii) the unique Kinetoplastida enzymes trypanothione reductase and trypanothione synthase; (iii) the inhibitors of de novo sterol biosynthesis pathways, such as imidazole and triazoles derivatives (Buckner et al., 2003; Urbina & DoCampo, 2003; Urbina 2009). There is also strong evidence that bisphosphonates can accumulate in the parasite’s acidocalcisomes and interfere with the activity of enzymes involved in isoprenoid biosynthesis, such as farnesyl
diphosphate synthase (Docampo and Moreno, 2008). Using T. cruzi infected cardiomyocytes we previously demonstrated that the bisphosphonate risedronate has a potent and selective \textit{in vitro} effect against this parasite, resulting in recovery of the cardiac cells after the treatment (Garzoni et al., 2004a). This compound also exhibited marked \textit{in vivo} antiparasitic activity, in a murine model of acute Chagas disease (Garzoni et al., 2004b). Another promising approach is the recent discovery of the anti-T. cruzi activity of the antiarrhythmic drug amiodarone, which is frequently prescribed for the symptomatic treatment of Chagas' disease patients (Benaim et al., 2006). In the heart the effects of this compound include inhibition of Na$^+$ channels, L-type Ca$^{2+}$ channels, K$^+$ channels and Na$^+$/Ca$^{2+}$ exchanger, leading to its characteristic antiarrhythmic action. It was found that the \textit{in vitro} and \textit{in vivo} activity against T. cruzi was mediated by disruption of the parasite's Ca$^{2+}$ homeostasis and a blockade of ergosterol biosynthesis at the level of oxidosqualene cyclase (Benaim et al., 2006).

Although the antiparasitic activity of amiodarone has been demonstrated previously, there is lack of data regarding the effect of this compound on the ultrastructure of T. cruzi and its host cells, and the recovery of these cells after the antiparasitic treatment. Primary cultures of murine cardiac myocytes have been the method of choice to demonstrate alterations in the host cell induced by this parasite. In these studies, many aspects of this relationship were clarified such as alterations in intracellular calcium dynamics (Barr et al., 1996; Garzoni et al., 2003), changes in cell cytoskeleton (Pereira et al., 1993; Taniwaki et al., 2006) and cell-cell junction (Adesse et al., 2008; de Melo et al., 2008). Gap junction channels are critical in maintaining cardiac homeostasis by allowing the free flow of ions and metabolites between cardiac
myocytes, which contributes to the synchronized contraction of and signal exchange throughout the tissue. Gap junctions are composed of the connexin family of transmembrane proteins that assemble as end-to-end alignments of hexameric connexon subunits, thereby forming intercellular conduits for molecules up to 1KDa. Connexin43 (Cx43) is the most abundant gap junction protein in ventricular myocytes, being localized at intercalated disks in normal myocardium (Duffy et al., 2006).

In the present study, we demonstrate the effects of amiodarone on the proliferation and ultrastructure of intracellular amastigote forms of *T. cruzi* growing in cardiomyocytes and the recovery of the host cells. We evaluated the recovery of spontaneous contractility of cardiac myocytes and the distribution of F-actin and Cx43 after treatment.
Materials and Methods

Parasites

The Y (MHOM/BR/1950/Y) strain of T. cruzi was used in this work. Trypomastigote forms of the T. cruzi were obtained from the supernatant of infected heart muscle cells grown in Dulbecco’s Modified Eagle Medium (DMEM, Sigma Aldrich, St. Louis, MO, USA) supplemented with 5% fetal bovine serum (FBS, CultiLab, São Paulo, SP, Brazil), 1mM CaCl$_2$, 1mM L-glutamine, 2% chick embryo extract, 1000 U/ml penicillin and 50 µg/ml streptomycin. After 96 h of infection the parasites were collected, centrifuged and resuspended in DMEM.

Cardiac cell cultures

Hearts of 18-day old Swiss Webster mouse embryos were submitted to mechanical and enzymatic dissociation as previously described (Meireles et al., 1986). Briefly, cells were harvested using 0.05% trypsin and 0.01% collagenase in phosphate-buffered saline (PBS) at 37°C. Ventricular heart muscle cells (HMCs) were plated on 0.02% gelatin-coated plastic flasks, on glass coverslips in 24-well plates or in Petri dishes. Cells were maintained at 37°C in a 5% CO$_2$ atmosphere in DMEM for 72 hours before the experiments. Use and handling of the animals was approved by the Ethics Committee for the Use of Laboratory Animals, FIOCRUZ (CEUA), protocol number P70/09.2.

Infection of cultures and treatments
Heart muscle cells were plated in 24-well plates at a density of 1.5x10^5 cells/well in glass coverslips and infected with culture-derived trypomastigotes (20:1 parasites:host cells), in a final volume of 300 µl DMEM. After 2 h, the cultures were washed with PBS to remove non-adherent parasites, and maintained in DMEM. Treatment with 2.5-10 µM amiodarone was performed by two protocols: (I) immediately after the interaction step; (II) 48 h after infection. The total volume in each well was 500µL. At specific times, coverslips (in triplicate) were collected, fixed with Bouin’s fixative solution (Electron Microscopy Sciences, Hatfield, PA, USA) and stained in Giemsa solution (Merck, Darmstadt, Germany). The percentage of infection was quantified by counting randomly at least 300 cells. In addition, supernatants were collected and released parasites counted in a haemocytometer.

**Ultrastructural studies**

Cells were plated in 35mm plastic Petri dishes and, at chosen times, were fixed (60 min/4°C) with 2.5% glutaraldehyde (Sigma Aldrich), 2.5 mM CaCl₂ and 0.1 M Na-cacodylate buffer (pH 7.2), followed by post-fixation for 1 h in cacodylate buffer solution containing 1% OsO₄, 0.8% potassium ferricyanide and 2.5 mM CaCl₂. Samples were dehydrated in acetone then embedded in Polybed 812 resin (Electron Microscopy Sciences). Thin sections (Leica Ultracuts, UCT, Vienna, Austria) were stained with uranyl acetate and lead citrate, and were examined by transmission electron microscopy using an Zeiss EM10C microscope.

**Immunofluorescence:**
Cells were washed with PBS and fixed with 4% paraformaldehyde for 5 min at 20°C at desired time points. After washes in PBS, cells were permeabilized with 0.5% Triton x-100 and non-specific staining was blocked with 4% BSA. Primary anti-Cx43 antibody (Sigma-Aldrich, St. Louis, MO, USA) was incubated overnight at 4°C, after which cells were washed and incubated with secondary polyclonal goat anti-rabbit Alexa Fluor 488 antibody (Invitrogen, Eugene, OR, USA) for 1 hour at 37°C. F-actin filaments were stained with Alexa Fluor 594 phalloidin (Invitrogen) for 30min at 37°C and DNA was stained with DAPI. Images were acquired with a Olympus Laser Scanning Confocal Microscope.

Statistical analysis
Mean value comparisons were performed by using Anova. P-values below 0.05 were considered significant. IC$_{50}$ values were calculated from dose-response curves by using a non-linear regression analysis with the GraFit software package.

Materials
Amiodarone was purchased from Sigma-Aldrich. Stock solutions were prepared in phosphate buffered saline (PBS) (pH adjusted to 7.4) and sterilized by using a 0.2 μM filter (Millipore, Billerica, MA, USA). Trypsin was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and collagenase was from Worthington Laboratories (Lakewood, NJ, USA). All other reagents were of analytical grade.
Results

Effect of amiodarone on intracellular T. cruzi amastigotes and host cells

Using two different treatment protocols, amiodarone caused a strong inhibition of the infection of HMCs (Figures 1-3). When the drug was added immediately after infection (Protocol I, Figure 1), a dose- and time-dependent reduction in the percentage of infected cells was observed. The inhibition was statistically significant after 24 h of treatment with 10 µM (p<0.05), 48 h with 5 µM (p<0.01) and 96 h with 2.5 µM (p<0.01) (Figure 1A). IC_{50} values with protocol I were 5.85±1.4 μM at 96 h and 3.14±1.2 μM at 120 h. Amiodarone had also an inhibitory effect on the number of intracellular amastigotes (Figure 1B) as revealed by light microscopy (Figure 3), significant for 10 µM at 24 h of treatment (p<0.05). Addition of amiodarone to cultures 48 h after infection (Protocol II) also led to a highly significant reduction in the percentage of infected cells (Figure 2). After 48 h of treatment (96 h of infection), 5 µM of amiodarone already reduced significantly (p<0.05) the parasitism of cultures, which was also observed at 96 h treatment with 2.5 µM (Figure 2A). In this protocol, IC_{50} was 4.47±0.3 μM and 2.24±0.24 μM at 96 and 120 h, respectively. This inhibitory effect was also observed for the number of intracellular amastigotes in the HMCs (Figure 2B). Moreover, amiodarone inhibited the release of trypomastigotes from infected cells in this model, after completion of the intracellular cycle of the parasite. In control (untreated) infected HMC cultures, the first two peaks of trypomastigote release to the supernatant occurred at 96 h and 192 h post-infection (Fig. 2C). Treatment with 5 and 10 µM of amiodarone, starting 48 h post-infection, drastically
inhibited such release after 48 h of treatment (96 h post infection), while at 96 and 144 h, no trypomastigotes were detected in the supernatant (Fig. 2C). Amiodarone, at the concentrations used in the experiments (up to 10 µM) had no apparent cytotoxic effects on the myocytes, as shown in Figures 1C and 2D.

Light microscopy observations showed that treatment with amiodarone induced drastic morphological alterations on intracellular amastigotes (Figure 3B, 3C and 3E, insets). We confirmed this observation through transmission electron microscopy of infected HMCs and released parasites after 144 h of treatment of infected HMCs with 5 µM of amiodarone. Infected untreated HMCs displayed abundant intracellular amastigotes in the cell’s cytoplasm, with the expected kinetoplast’s morphology (bar shaped). A drastic loss of cytoplasmatic content and the formation of membrane inclusions inside the amastigotes were visualized in treated cultures; these cells also exhibited mitochondrial swelling, disorganization of reservosomes and the kinetoplast, probably associated to the disruption of Ca\(^{2+}\) homeostasis (Benaim et al., 2006), as well as blockade of amastigote-trypomastigote differentiation. Untreated amastigotes spontaneously released from their host cells also displayed their characteristic morphology (Figure 4C). There was a marked damage in amastigotes released to the medium form amiodarone-treated (5 µM) cultures, such as kinetoplast alteration and in the Golgi apparatus (Figure 4D).

**Cell physiology recovery after treatment with amiodarone**

In order to assess the recovery of host cell ultrastructure and physiology after the treatment of infected cultures with amiodarone, we evaluated gap junction protein
Cx43 (detected by immunofluorescence) and actin filaments (stained with phalloidin). *T. cruzi* infection is known to disrupt gap junctional communication through Cx43 protein reduction (Adesse et al., 2008). Figure 5 shows that infected untreated HMCs (Figure 5D-F) totally lost Cx43 immunoreactivity at 192 h pi when compared to control uninfected cells (Figure 5A-C), which displayed well formed gap junction plaques (Figure 5C) and abundant myofibrils, as revealed by phalloidin staining. After treatment with 5 µM amiodarone for 144h infection was nearly abolished, as revealed by DAPI staining. In these cultures, Cx43 levels were comparable to age-matched uninfected cultures (Figure 5I) and phalloidin staining revealed the presence of both polygonal and filamentous structures, indicative of cytoskeleton reassembly (Figure 5H).

We also assessed the impact of *T. cruzi* infection on cardiac myocyte spontaneous electrocontractility, and the results are shown in Figure 5J. Parasites' proliferation was associated with a progressive reduction in the cardiomyocytes' contraction, which followed the disorganization of the contractile apparatus, as well as the disappearance of the gap junctions of these cells. After 48, 96 and 144 h of infection (Protocol I), untreated controls displayed 28, 31 and 45% reduction in spontaneous contractility (p<0.05), respectively, whereas treatment with amiodarone (5µM) restored the number of spontaneous beatings to levels indistinguishable to those of uninfected cultures at 96 (p<0.05) and 144 (p<0.01) hours of treatment. We observed no significant effect on contractility when the same dose of amiodarone was added to uninfected cultures (not shown).
Discussion

Human Chagas disease results from the infection by *T. cruzi* and tissue damage arises from both direct parasite action and the inflammatory process that ensues (Chagas, 1909). Cytolysis and fibrosis are key components associated with Chagas disease’s pathological manifestations, along with a sustained and diffuse inflammation of the affected organs (Tanowitz et al., 1992). The growing perception that chronic phase manifestations are associated with the parasite’s persistence in the mammalian tissues (Tarleton et al., 1997) and that the two available drugs (benznidazole and nifurtimox) have important limitations, particularly in the chronic phase (Cançado, 2002), has stimulated the search for new trypanocidal compounds. Amiodarone is frequently used as an anti-arrhythmic in chronic phase Chagas disease patients with cardiac compromise (Rassi et al., 2000; Mason, 1987) and it has also antifungal (Courchesne 2002; Courchesne et al., 2009) and antiprotozoal activities (Benaim et al., 2006; Serrano-Martin et al., 2009), which were recently described. It was shown that the drug disrupts the parasite’s Ca^{2+} homeostasis and also blocks ergosterol biosynthesis, resulting in excellent parasiticidal activity with low cytotoxicity (Benaim et al., 2006). The results obtained in this work clearly confirm that amiodarone has a potent and selective activity against *T. cruzi*, with no significant effects on their preferred host cells, cardiomyocytes, and at doses that do not induce the well-known antiarrhythmic action of the drug on these cells. This selective action was confirmed by ultrastructural analysis of amiodarone-treated cultures (Figure 4), which revealed massive alterations of the parasites, allowing at the same time the full
structural and functional recovery of the cardiomyocytes (see below). The ultrastructural effects of amiodarone on intracellular amastigotes were similar to those previously described for other sterol biosynthesis inhibitors, such as risedronate (Garzoni et al., 2004a), ketoconazole and terbinafine (Lazardi et al., 1990; Vivas et al., 1996).

We also investigated whether amiodarone treatment would induce host-cell recovery, concomitant with the elimination of the intracellular parasites’ burden. We have previously shown that infection by *T. cruzi* induces a cytoskeletal disruption in HMCs due to myofibrillar breakdown (Pereira et al., 1993) and that treatment with risedronate and posaconazole allowed reassembly of cytoskeleton elements (Garzoni et al., 2004a; Silva et al., 2006). The present results show that amiodarone allowed the reorganization of the actin-containing myofibrils to their normal state, with the occurrence of characteristic polygonal arrangement. Lin and co-workers (1989) have described this polygonal configuration in dissociated cardiac myocytes as an indicator of myofibrillar reassembly. Moreover, it is known that the infection of cultured cardiac myocytes with *T. cruzi* decrease gap junction communication and Cx43 expression (de Carvalho et al., 1992; Adesse et al., 2008). These observations were extended to acutely infected mice, which displayed reduced expression of cardiac Cx43 expression (Adesse et al., 2008). Our results demonstrate that Cx43 distribution among cardiac myocytes was fully restored after treatment of infected HMC with amiodarone and treated cultures displayed gap junction plaques comparable to uninfected controls. Cx43 has a turnover of approximately 1.5 hours *in vitro* (Beardslee et al., 1998) and in *T. cruzi*-infected myocytes Cx43 mRNA levels were
largely unaffected (Adesse, D., unpublished data). This may provide evidence that the infection affects Cx43 translation and/or its subsequent trafficking to the plasma membrane, possibly due to microtubular damage (Pereira et al., 1993). After amiodarone-induced disappearance of the intracellular parasites, the host cells were capable of synthesizing new Cx43 molecules and make their successful delivery to the plasma membrane. More importantly, HMCs restored their spontaneous contractility after treatment with 5µM of amiodarone to levels comparable to control cultures. This observation is of interest since it permits to speculate that during in vivo infection, *T. cruzi* disturbs synchronous contractility, which can be reverted with the use of amiodarone alone or in combination with another inhibitor of the parasite's sterol biosynthesis such as posaconazole, which results in synergistic effects (Benaim et al. 2006).

To summarize, our results showed that amiodarone has a selective anti-proliferative effect on *T. cruzi*, in an *in vitro* model of infection of cardiac cells. Treatment induced ultrastructural damage to intracellular amastigotes but promoted full structural and functional recovery of the host cells. This compound should be considered, beyond its known anti-arrhythmic activity, as an important antiparasitic agent and a lead for the development of new specific treatments of this neglected disease.

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Legends for figures:

**Figure 1**: Time and concentration dependence of the effects of amiodarone on the infection of HMC by *T. cruzi* (Protocol I). Amiodarone (2.5-10 µM) was added to the cultures after 2 h of infection. We observed significant effects of the drug on the percentage of infected cells (A) and number of intracellular parasites (B) followed as a function of time. The concentrations of drug used for these studies were not toxic for the cardiac myocytes as shown in C. Asterisks indicate statistical differences in relation to control cultures. The graphs show the mean and standard deviation of triplicates of one representative experiment of four independent experiments.

**Figure 2**: Time and concentration dependence of the effects of amiodarone on the infection of HMC by *T. cruzi* (Protocol II). Amiodarone (5-10 µM) was added to the cultures after 48h of infection and the (A) percentage of infected cells, (B) number of intracellular parasites per host cell and (C) released trypomastigotes in the supernatant were followed as a function of time. We did not observe significant cytotoxic effects of amiodarone in the concentrations used throughout the experiments (D). Asterisks indicate statistical differences in relation to control cultures. The graphs show the mean and standard deviation of triplicates of one representative experiment of four independent experiments.

**Figure 3**: Effect of amiodarone on intracellular cycle of *T. cruzi*. Primary cardiac myocytes were obtained and infected with the Y strain of *T. cruzi*. After 2 hours of infection, cultures were treated with 5 or 10 µM of amiodarone. At 96hpi, untreated cultures (A) displayed cells with trypomastigote forms of the parasite already evading host cells. Cultures treated with 5 µM (B) and 10µM (C) of Amiodarone displayed intracellular parasites with severe morphological alterations (arrowheads). Interestingly the concentration of 10 µM induced a drastic reduction of parasitism (C). After 144 hours of infection (corresponding to 142 hours of treatment), we observed that in non-treated cultures (D) 70-80% of
cardiomyocytes were infected. Treatment with 5 µM (E) reduced drastically the number of parasites, whereas the use of 10 µM of amiodarone (F) almost eliminated the parasitism of the cultures. Insets show in higher magnification details of intracellular parasites. P=Parasites, N=Nucleus. Bars=50µm

**Figure 4:** Effects of amiodarone on the ultrastructure of intracellular *T. cruzi* (Y strain) amastigotes (A). Untreated HMC after 192h of infection with *T. cruzi*, displaying severe intracellular damage and intact amastigote forms (P) presenting bar shaped kinetoplast (K), nucleus (N) and flagellum (F). (B) Infected cultures treated with 5µM Amiodarone for 144h, showed parasites with membrane blebs (arrows) and loss of intracellular material (stars) and kinetoplast alterations (k). Released parasites from untreated cultures (C) displayed acidocalcisomes (AC), bar shaped kinetoplast (K), reservosome (R) and flagellum (F) as expected. Parasites obtained from cultures treated with 5µM of amiodarone (144hr, D) showed important alterations in kinetoplast, and Golgi apparatus (arrow).

**Figure 5:** Amiodarone induced recovery of host cell homeostasis. After eradicating the infection, we observed recovery of morphological and functional aspects of cardiac myocytes cultures, as assessed by immunofluorescence and measuring spontaneous electrocontractility of the cells. Left panel shows that uninfected cultured myocytes displayed abundant connexin43 immunoreactivity (C) as well as striated patterns of F-actin staining (B). Highly infected cultures, at 144 hours of infection, presented destruction of F-actin cytoskeleton (E) and loss of Cx43 plaques (F). Treatment with 5 µM of amiodarone for 142 hours decreased parasitism and induced recovery of host cell, as evidenced by F-actin and Cx43 recovery, which included striations (H) and presence of gap junction plaques (I). The spontaneous electrocontractility of the myocytes culture was evaluated by counting the number of contractions per 10 seconds (J). We observed that *T. cruzi* infected cultures had a progressive decrease in
electrocontractility, whereas treatment with 5 and 10µM of amiodarone restored cultures to normal levels. *:P<0.05; **:P<0.01 (ANOVA).
Figure 1

A. Parasitism

B. Intracellular parasites

C. Cytotoxicity
Figure 2

A. Parasitism
B. Intracellular parasites
C. Released parasite
D. Cytotoxicity
Figure 3

T. cruzi Infected (96hpi)  T. cruzi Infected (144hpi)

Untreated

A

B

C

Amdodarone 5 μM

D

E

F

Amdodarone 10 μM

Figure 3
Figure 4

*T. cruzi* infected (192hpi)

- Untreated
- Amiodarone 5µM (144 hours)
### Table 1: Spontaneous Electrocontractility

<table>
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<th>Time of Treatment</th>
<th>Control 192hr (Spontaneous Electrocontractility)</th>
<th>T. cruzi infected 192hr (Spontaneous Electrocontractility)</th>
<th>Amiodarone 5μM (144hr) (Spontaneous Electrocontractility)</th>
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**Figure 5**

Spontaneous Electrocontractility

- **Cardiac Myocytes (CM)**
- **CM + T. cruzi**
- **CM + T. cruzi + Amio [5μM]**

* denotes statistical significance compared to control.