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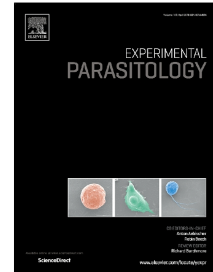

Anti-*Trypanosoma cruzi* action of a new benzofuran derivative based on amiodarone structure

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Anti-*Trypanosoma cruzi* action of a new benzofuran derivative based on amiodarone structure

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Highlights

- A new benzofuran derived compound inhibits *T. cruzi* epimastigotes growth.
- The compound has an antiproliferative effect on *T. cruzi* amastigotes in host cells.
- The drug releases Ca^{2+} from intracellular compartments from this parasite.
- The compound dissipates the mitochondrial electrochemical membrane potential from *T. cruzi*
- The drug induces the alkalinization of the acidocalcisomes from this parasite

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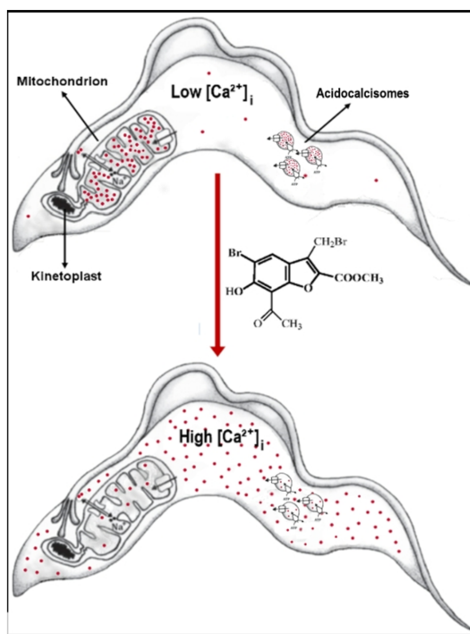
Graphical Abstract



Abstract

Chagas disease is a neglected tropical affection caused by the protozoan parasite *Trypanosoma cruzi*. There is no current effective treatment since the only two available drugs have a limited efficacy and produce side effects. Thus, investigation efforts have been directed to the identification of new drug leads. In this context, Ca^{2+} regulating mechanisms have been postulated as targets for antiparasitic compounds, since they present paramount differences when compared to host cells. Amiodarone is an antiarrhythmic with demonstrated trypanocidal activity acting through the disruption of the parasite intracellular Ca^{2+} homeostasis. We now report the effect of a benzofuran derivative based on the structure of amiodarone on *T. cruzi*. This derivative was able to inhibit the growth of epimastigotes in culture and of amastigotes inside infected cells, the clinically relevant phase. We also show that this compound, similarly to amiodarone, disrupts Ca^{2+} homeostasis in *T. cruzi* epimastigotes, via two organelles involved in the intracellular Ca^{2+} regulation and the bioenergetics of the parasite. We

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demonstrate that the benzofuran derivative was able to totally collapse the membrane potential of the unique giant mitochondrion of the parasite and simultaneously produced the alkalinization of the acidocalcisomes. Both effects are evidenced by a large increase in the intracellular Ca^{2+} concentration of *T. cruzi*.

1. **Introduction**

Chagas' disease, caused by the protozoan parasite *Trypanosoma cruzi*, is the most important parasitic disease in the western hemisphere (Bern, 2015). It affects millions of people around the world, prevailing in endemic areas of 21 Latin-American countries in which economic and medical support are limited (Rueda et al., 2014). Despite the efforts to eradicate vector transmission carried out by blood sucking triatomine bugs in most endemic areas, and the implementation of programs to prevent blood transfusion and congenital transmission respectively (Sosa-Estani and Segura, 2015), the disease remains a large problem owed to occurrence of oral infection outbreaks due to consumption of triatomine-feces-contaminated food (Shikanai-Yasuda and Carvalho, 2012) and migration of affected people to non-endemic countries representing a risk factor (Schmunis, 2007). According to OMS, estimates indicate 56.000 new cases and 12.000 deaths per year. The only treatment available is based on chemotherapy using the nitroaromatic compounds nifurtimox and benznidazole, which are inefficient especially in the chronic patients (Soeiro and de Castro, 2009), and present adverse side effects whereby its administration is limited (Prata, 2001). In fact a clinical study of benzidazol (BENEFIT) showed that although this compound decreases the parasitemia in humans, it does not improve the cardiomyopathy associated with the disease (Morillo et al., 2015). For these reasons, in the last decades many efforts in infectious diseases research are

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directed to the development of new drug leads and the determination of targets in *T. cruzi* for the treatment of the American trypanosomiasis (Duschak, 2011).

Since the 70's many compounds carrying benzofuran in their structure, as well as multiple derivatives of this chemical group, have shown an effect against many trypansomatid species (Bakunov et al., 2009, 2008; Bakunova et al., 2007; Dann et al., 1973, 1972). Another very important compound initially designed and prescribed as an antiarrhythmic which possesses a benzofuran motif in its structure is amiodarone. Analogues derived from amiodarone had previously shown an inhibitory effect on *T. cruzi* (Kinnamon et al., 1998). The direct effect of amiodarone as an antitrypanosomatid drug was reported for the first time on *T. cruzi*, on which amiodarone has a potent synergism with posaconazol (Benaim et al., 2006). Later it was shown to be efficient also against *Leishmania Mexicana* (Serrano-Martín et al., 2009a) acting synergistically with miltefosine (Serrano-Martín et al., 2009b). Dronedarone, synthesized as an amiodarone analogue with an improved safety profile, since it lacks iodine in its structure being the responsible for undesired effects of amiodarone, was also proved to be an efficient agent against *T. cruzi* (Benaim et al., 2012) and *L. mexicana* (Benaim et al., 2014). Furthermore in these reports, it was shown that both compounds, amiodarone and dronedarone, had several mechanisms of action in these parasites. First of all, both benzofuran derivatives inhibited the ergosterol biosynthesis. This inhibition is lethal for trypanosomatids, because they possess this sterol as an essential component of their membranes, instead of cholesterol which is present in humans. On the other hand, both compounds disrupted Ca^{2+} homeostasis in trypanosomatids driving to a large increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Interestingly, the increase in the $[Ca^{2+}]_i$ was due at least by two organelles involved in the regulation of the cytoplasmic concentration of this cation

(Benaim and Garcia, 2011). One of them, the giant unique mitochondrion, in which the electrochemical potential was dissipated, leading to the release of accumulated Ca^{2+}, since the electrochemical membrane potential is the driving force for the large Ca^{2+} buildup observed in this organelle (Benaim et al., 1990). Finally, both benzofuran derivatives showed a potent effect on the acidocalcisomes, essential organelles involved in the bioenergetic of these parasites, since they accumulate large amounts of calcium, polyphosphates and pyrophosphate, the last being an energy source alternative to ATP in trypanosomatids, besides being involved in the parasite Ca^{2+} homeostasis and osmoregulation (Docampo et al., 1995).

We have reported previously the synthesis of a series of benzofuran derivatives inspired by the structure of amiodarone, from which two derivatives showed antifungal activity on *Cryptococcus neoformans*. One of such compounds (denominated **4** in this previous report) was able to completely arrest *C. neoformans* growth at a concentration of 20 μM, while it affected human leukemia K-562 cells only at concentrations higher than 30 μM. Interestingly, this drug was able to enhance the Ca^{2+} mobilization produced by amiodarone in yeast (Hejchman et al., 2012).

In this work we report a new effect for the last mentioned benzofuran derivative (AMIODER), demonstrating that this compound has an inhibitory effect on *T. cruzi* and that this effect is partly related to the disruption of intracellular Ca^{2+} homeostasis in the parasite.

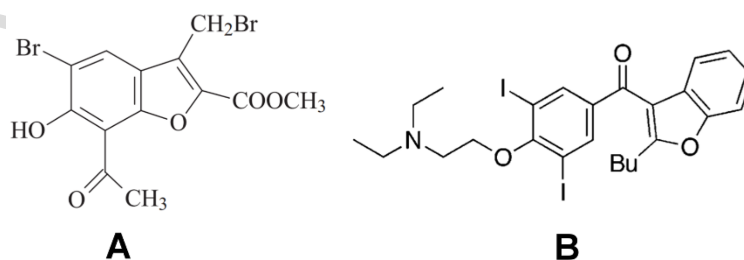

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Fig. 1. Chemical structure of **(A)** the benzofuran derivative (AMIODER), based on the structure of **(B)** amiodarone, used in this work (Hejchman et al., 2012).

2. **Materials and Methods**

2.1. Chemicals. The compound AMIODER (methyl 7-acetyl-5-bromo-6-hydroxy-3-bromomethyl-2-benzofurancarboxylate) used in this work was provided by Dr. Elżbieta Hejchman from the Medical University of Warsaw.

2.2. Mammalian host cell susceptibility to AMIODER. Vero cells (kidney epithelial cells from African green monkey) were employed as model mammalian host cells and were maintained in DMEM (Dulbecco's Modified Eagle Medium), supplemented with 10 % fetal bovine serum at 37 °C, 95 % air-5 % CO_2. For cell viability determinations, Vero cells were cultured in a 96 well plate and exposed to different concentrations of AMIODER for 96 hours. Afterwards cells were washed with phosphate-buffered saline and MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) at 0.5 mg/mL was added followed by a period of incubation (4 hours). The resulting formazan produced by viable cells mitochondrial dehydrogenase activity was determined spectrophotometrically and the IC_{50} was determined using GraphPrism 5.0.

2.3. *Trypanosoma cruzi* epimastigotes culture and susceptibility to AMIODER. *T. cruzi* epimastigotes (CL Brener strain) were grown in LIT (liver infusion tryptose) medium, pH 7.4, supplemented with 10% fetal bovine serum at 29 °C and constant agitation as reported previously (Benaim et al., 2006). For drug susceptibility determinations the parasites were cultured in medium with different drug concentrations. Epimastigotes were placed in glass

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tubes at an initial title of $6x10^6$ parasites/mL and treated with increasing concentrations of AMIODER (0-50 μM), including a control with drug vehicle [dimethyl sulfoxide (DMSO)]. The number of parasites in culture was determined daily by direct counting in a Neubauer chamber. At least 3 independent experiments were performed for each condition and the 50 % inhibitory concentration was determined by using Prisma Graph Pad 5.0.

2.4. Determination of the effect of AMIODER on the cells infected with *T. cruzi* amastigotes.

Amastigotes were cultured in Vero cells. For amastigote susceptibility assays, as reported previously (Benaim et al., 2012), Vero cells were cultured on plastic coverslips placed inside 24 well plates, infected for 5 hours with tissue culture-derived trypomastigotes at a rate of 10 parasites per host cell and then washed 3 times with phosphate-buffered saline (PBS) to remove non-adherent parasites. Medium with different drug concentrations (ranging from 0 to 10 μM) was added and cells were incubated for 72 h. Coverslips with cells were washed with PBS and then stained with Giemsa. The percentage of infected cells was determined directly using a light microscopy. Statistical analysis was carried out and the 50 % inhibitory concentration was estimated using Prisma Graph Pad 5.0.

2.5. Determination of the intracellular Ca^{2+} concentration in *T. cruzi* epimastigotes.

T. cruzi epimastigotes were loaded with the Ca^{2+} radiometric indicator Fura 2 as reported previously (Benaim et al., 2014). The fluorophore Fura 2 is excited by two different wavelengths, 340 nm when it is Ca^{2+}-bound, and 380 nm when it is free of Ca^{2+}, and emission is recorded at a unique wavelength of 510 nm. For this reason, a spectrofluorimeter with the capacity to measure fluorescence ratios at two different wavelengths should be used. Briefly, 1×10^7 parasites were collected by centrifugation at 600 x g for 2 minutes and washed twice in a

"loading buffer" (137 mM NaCl, 4 mM KCl, 1.5 mM KH_2PO_4, 8.5 mM Na_2HPO_4, 11 mM glucose, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 20 mM HEPES-NaOH [pH 7.4]). The pellet was resuspended in 1 µM of Fura 2-AM (the permeable acetoxymethylester derivative of FURA 2). Probenecid (2.4 mM) and pluronic acid (0.05%) were added to the loading buffer. The parasites were incubated at 29 °C in the dark with continuous agitation for 2 hours. Fura 2-AM-loaded parasites were washed twice in the same buffer, in either the presence or absence of Ca^{2+}. Additionally, the chelating agent ethylene glycol tetraacetic acid (EGTA) was added at 500 µM when measurements were made in the absence of extracellular Ca^{2+}. Fluorescence measurements were carried out on a stirred cuvette at 29 °C, using a Perkin-Elmer spectrofluorimeter LS-55 with a double wavelength excitation beam (340 nm and 380 nm) and recording the emission at 510 nm.

2.6. Mitochondrial membrane potential determinations.

The effect of the drug on the mitochondrial membrane potential of *T. cruzi* epimastigotes was evaluated using the fluorescent dye rhodamine 123 as reported previously (Benaim et al., 2006), taking advantage of the internationalization of this fluorophore, according to the mitochondrial electrochemical membrane potential (Δφ). Briefly, 8×10^6 parasites were collected by centrifugation at 600 x g for 2.5 min and washed in phosphate-buffered saline (PBS) plus 1% glucose. The pellet was resuspended in the same buffer in the presence of rhodamine 123 (20 µM) and incubated for 45 min at 29 °C in the dark with continuous stirring. Subsequently, parasites were washed twice and resuspended in the same buffer, and then transferred to a stirred cuvette. Measurements (excitation wavelength [Λ_{ext}], 488 nm; emission wavelength [Λ_{em}], 530 nm) were made in a Hitachi 7000 spectrofluorimeter at 29 °C. The protonophore FCCP (2 µM) was used as a positive control.

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2.7. Determination of acidocalcisomes alkalinization.

The effect of the benzofuran compound on acidocalcisomes was evaluated using acridine orange, which accumulates in acidic compartments (Benaim et al., 2014). Epimastigotes (8×10^6 cells/mL) were collected, washed and incubated in a “loading buffer” (the same used in mitochondrial membrane potential measurements) with acridine orange at 2 µM for 5 min at 29 °C in the dark and with constant stirring. Measurements were performed with λ_{ext} at 488 nm and λ_{em} at 530 nm at 29°C in a Hitachi 7000 spectrofluorimeter under magnetic stirring. Nigericin, a monovalent ionophore acting as a K^+/H^+ exchanger, was used at 2 µM as a positive control.

3. Results and discussion

First we determined the antiproliferative effect of AMIODER on *T. cruzi* epimastigotes grown in axenic medium. As can be seen in Fig. 1, AMIODER showed a dose-dependent inhibitory effect on parasites growth. The calculated IC_{50} was 8,5 µM. A total growth arrest at 30 µM of the benzofuran derivative can also be observed.

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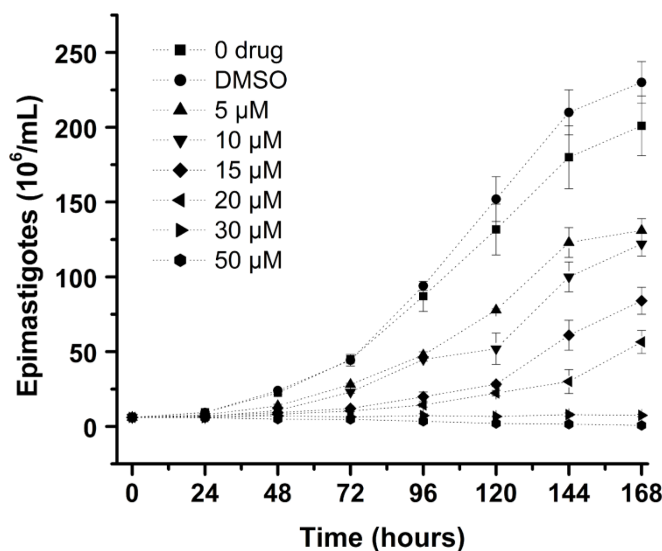

Fig. 2. Susceptibility of *Trypanosoma cruzi* epimastigotes to AMIODER. Cultures of *T. cruzi* epimastigotes were grown in the presence of different concentrations of AMIODER. Experiments were carried out in triplicate and each bar represents the standard deviation of the experimental points.

We then evaluated the effect of AMIODER on cells infected with *T. cruzi* amastigotes which is the clinically relevant stage of the parasite. For this purpose, Vero cells, a classical and very efficient cell line model frequently used to study *T. cruzi* amastigotes infectivity (Benaim et al., 2006), were incubated with *T. cruzi* trypomastigotes, treated with different concentrations of AMIODER for 96 hours, and then the number of cells infected with intracellular amastigotes was determined. As shown in Fig. 3, AMIODER had a clear dose-dependent effect against intracellular amastigotes, diminishing the total percentage of infected cells. The calculated IC_{50} was 1.0 µM. We then determine the effect of the drug on Vero cells, by using the MTT assay, obtaining an IC_{50} value of 39.5 µM and thus, a relatively low cytotoxic effect on these cells (Fig. 4). The calculated Selectivity Index (SI) for this drug determined by

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the ratio of the IC_{50} of Vero cells over the IC_{50} obtained for amastigotes was 39.5, indicating a nearly 40 times more selective cytotoxicity on the clinically relevant amastigote form, when compared with the host cell. Taking in consideration the definition described by Katsuno et al, 2015, in which a SI higher of 10 is considered a significant value, it could be considered that this compound possesses a relatively high selectivity index (Katsuno et al., 2015).

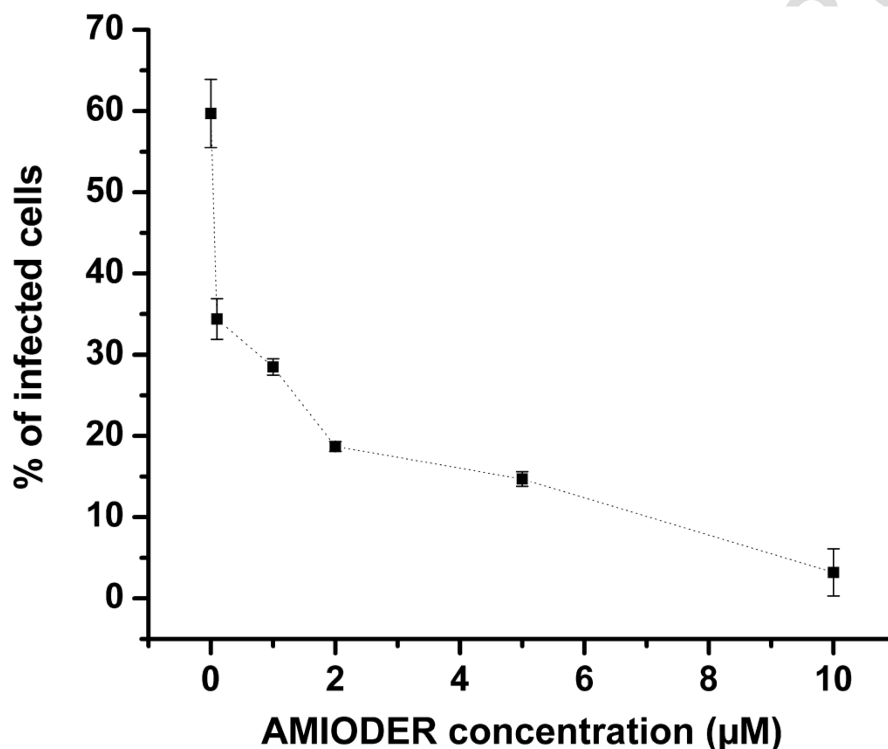

Fig. 3. Effect of AMIODER against intracellular amastigotes of *T. cruzi*. Vero cells were infected with *T. cruzi* trypomastigotes and then treated with different concentrations of the benzofuran derivative. The percentage of infected cells (squares) and the IC_{50} on amastigotes were determined 96 h post-treatment. Experiments were carried out in triplicate and each bar represents the standard deviation of the experimental points.

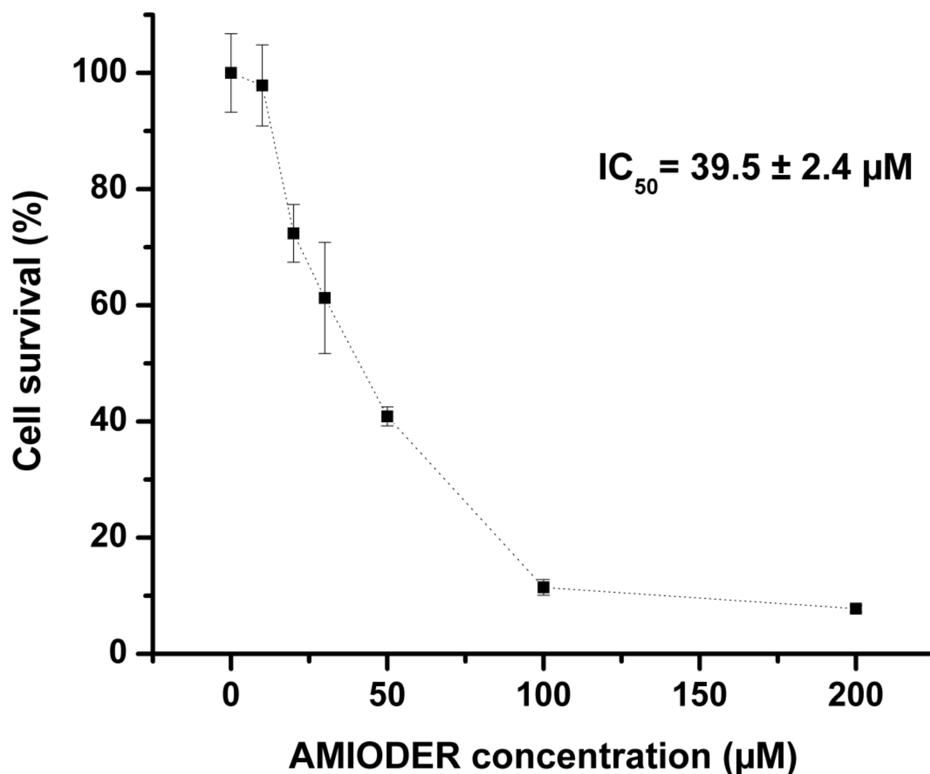

Fig. 4. Effect of AMIODER on Vero cells viability. Cells were exposed to different concentrations of AMIODER and cytotoxicity was measured by the MTT assay. Data points (Cell viability percentage per concentration) and bars represent the arithmetic mean ± SD (N=3). The IC_{50} after 96 hours of treatment is shown.

A comparative analysis on the biological activity of other benzofuran derivatives against *T. cruzi*, reveals that it has been reported that extracts from the Brazilian plant *Piper regnelli var. pallescens* have an antiprotozoal effect. Four of such isolated compounds, namely, eupomatenoid-3, eupomatenoid-5, epupomatenoid-6 and conocarpan showed antiproliferative activity on epimastigotes of *T. cruzi* with IC_{50} values of 90, 23.8, 28.4 and 30 μM, respectively. Interestingly, eupomatenoid-5 showed higher anti-*T. cruzi* activity than the reference drug benznidazol, causing a 100% inhibition of parasite growth at a concentration of 34 μM,

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compared to benznidazole which only produced 78 % of inhibition at 38 μM. Furthermore, eupomatenoid-5 showed a relatively safe chemotherapeutic profile not producing hemolytic effect and a IC_{50} of 849 μM on Vero cells (Luize et al., 2006b). Similar reports, emphasize the study of eupomatenoid-5 on *T. cruzi*, showing that this compound also inhibits the proliferation of the clinically relevant form, amastigotes inside $LLCMK_2$ cells, with an IC_{50} of 17 μM. Additionally, it was observed through electron microscopy, that at the IC_{50}, this compound was able to induce morphological alterations in both epimastigotes and intracellular amastigotes without affecting host cells, causing massive alterations of the cell membrane, the formation of autophagic vacuoles and mitochondrial swelling, as well as an effect on the kinetoplast integrity (Luize et al., 2006a). The same group makes a further evaluation of eupomatenoid-5 on *T. cruzi*, reporting that this compound also affects the trypomastigote stage with an IC_{50} of 40.5 μM and showing that the effects produced on these form are similar to the alterations observed on epimastigotes and amastigotes. A deeper study on the possible mechanism used by this bezofuran derivative showed that, after 24 h of treatment, it induces oxidative stress in both epimastigotes and trypomastigotes, increasing lipid peroxidation, as well as an increase in the production of $NADPH^+$ and on the level of consumption of H_2O_2 by increasing the activity of glucose-6-phosphate (G6PD) and 6-phosphogluconate dehydrogenases (6PGD), enzymes belonging to the pentose phosphate pathway, key in the defense against oxidative stress. When parasites were treated for 96 h with eupomatenoid-5, the activity of both enzymes and the concentration of $NADPH^+$ diminished, meaning that the cell damage caused by the treatment, rendered the parasites unable to be defended against oxidative stress (Pelizzaro-Rocha et al., 2011). Another group of active compounds derived from benzofuran have been studied by Ameta et al, 2012, reporting the synthesis of a series of 2- (substituted

benzylidene)-5,7-dibromo-6-hydroxy-1-benzofuran-3 (2H)-ones and the evaluation of these compounds on *T. cruzi* amastigotes inside Vero cells. The most effective compounds were 2b and 2k with an IC_{50} of 19.4 and 10.7 μM, respectively. Additionally, the IC_{50} on $LLCMK_2$ for 2b and 2k were 58 and 78 μM, respectively, meaning that these derivatives have a relatively good selectivity index, and thus, constitute prospective drug leads for further studies (Ameta et al., 2012). As mentioned before, amiodarone, a benzofuranic compound known for its antiarrhythmic properties, has shown to possess a marked effect against *T. cruzi* epimastigotes and amastigotes *in vitro* with IC_{50} values of 9 and 2.7 μM, respectively. Interestingly, amiodarone had a synergistic activity with the well known antifungal drug posaconazole and showed efficiency *in vivo* on a murine model of Chagas disease, increasing mice survival when both drugs were used in combination (Benaim et al., 2006). Similar results were obtained by the use of dronedarone, showing IC_{50} values of 4.6 and 0.75 μM against epimastigotes and amastigotes respectively (Benaim et al., 2012).

Next we intended to elucidate the possible mechanism of action of AMIODER on *T. cruzi* parasites. Taking in consideration that amiodarone exerts its action on these parasite through the disruption of Ca^{2+} homeostasis we tested the effect of AMIODER on the $[Ca^{2+}]_i$. For this purpose, epimastigotes were loaded with the fluorimetric Ca^{2+} indicator Fura 2 and then exposed to AMIODER, while performing real time fluorescence measurements in a double wavelength fluorimeter, measuring the emission at 510, while exciting at the ratio of 340 and 380 nm. Fig. 5 shows the effect of AMIODER on the $[Ca^{2+}]_i$ in *T. cruzi* epimastigotes. It can be observed a large increase in the $[Ca^{2+}]_i$ upon addition of the drug (10 μM). In order to determine if the large increase in the Ca^{2+} concentration was due to the entrance of Ca^{2+} from the outside *milieu* or instead, from its release from intracellular compartments, we performed

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the same experiment but in the absence of extracellular Ca^{2+} (EGTA). Interestingly, this cytoplasmic Ca^{2+} concentration increase was very similar both in presence (Fig. 5A) and absence of extracellular Ca^{2+} (Fig. 5B), indicating that the cation is released from intracellular compartments, possibly the unique giant mitochondrion and the acidocalcisomes.

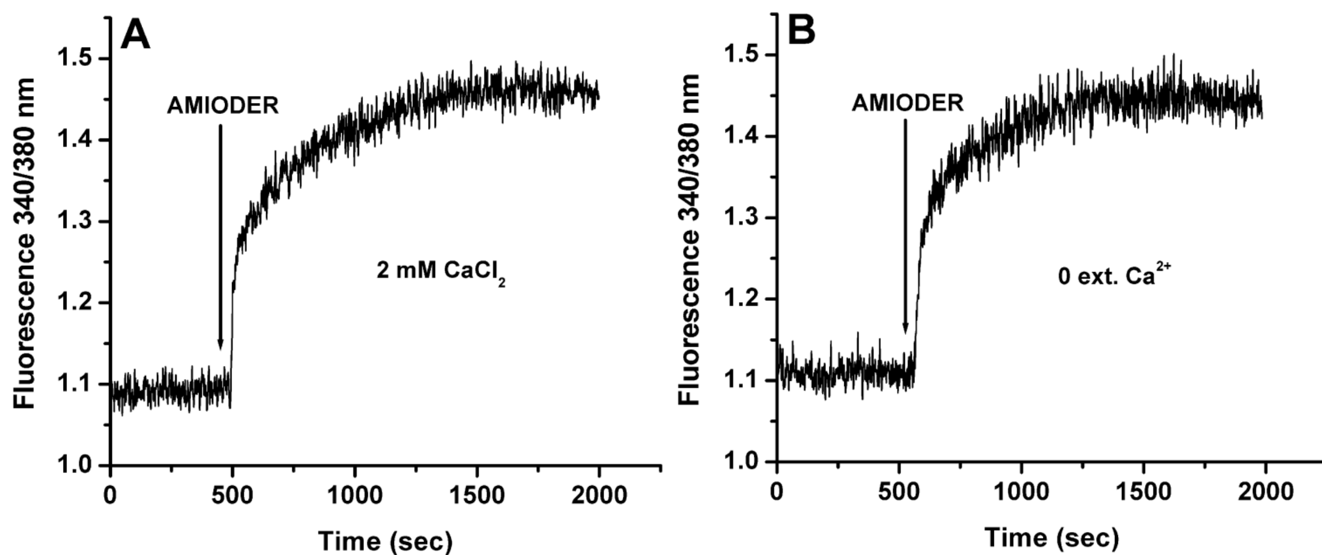

Fig 5. Effects of AMIODER on intracellular Ca^{2+} concentration of *T. cruzi* epimastigotes. **(A)** Effect of AMIODER at 10 μM (arrow) on the intracellular Ca^{2+} concentration of *T. cruzi* epimastigotes in the presence of 2 mM of extracellular Ca^{2+}. **(B)** Effect of AMIODER (10 μM) on the intracellular Ca^{2+} concentration of *T. cruzi* epimastigotes in the absence of extracellular Ca^{2+}.

Next we intended to elucidate the possible role of these two intracellular organelles in the observed $[Ca^{2+}]_i$ increase generated by AMIODER, taking in consideration that both compartments act as intracellular Ca^{2+} reservoirs and also based on previous reports showing the disrupting effect on both organelles by other benzofuran derivatives, like amiodarone and dronedarone on *T. cruzi* (Benaim et al., 2012, 2006) and *L. mexicana* (Benaim et al., 2014;

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Serrano-Martín et al., 2009a). First, we evaluated the effect of AMIODER on the mitochondrial membrane potential of *T. cruzi* epimastigotes by loading the parasites with the fluorescent dye rhodamine 123 which is known to accumulate in the energized mitochondria according to the electrochemical membrane potential ($\Delta\Psi_m$). Concerning the *rationale* of these experiments, it is well known that trypanosomatids are able to accumulate large amounts of Ca^{2+} into their giant unique mitochondrion by mean of a CMU that uses the H^+ electrochemical potential as a driving force for Ca^{2+} accumulation. This CMU is very well conserved among species, being present from humans to all trypanosomatids so far studied (Benaim et al., 1990; Docampo and Vercesi, 1989). As a consequence of this Ca^{2+} transport mechanism the cation accumulates in large quantities inside the organelle and it is released as a product of its deenergization. Fig. 6A, shows a large increase in the fluorescence upon addition of AMIODER, which is interpreted as the dissipation of $\Delta\Psi_m$ and subsequent release of the mitochondrion-accumulated rhodamine 123. After addition of the benzofuran derivative, FCCP, a potent known protonophore and oxidative phosphorylation uncoupler, was added as a positive control and a further increase in fluorescence was observed due to the complete disruption of the $\Delta\Psi_m$. In Fig. 6B, it can be observed the results of the same experiment, but inverting the order of addition of the effectors. As expected, the addition of FCCP produced a large and fast increase in the fluorescence of rhodamine 123. Nevertheless, the subsequent addition of AMIODER was still able to induce a further effect, though small. This could be explained if, under the experimental conditions used in these experiments, FCCP did not have full access to the mitochondrion and therefore could not produce the total dissipation of the $\Delta\Psi_m$.

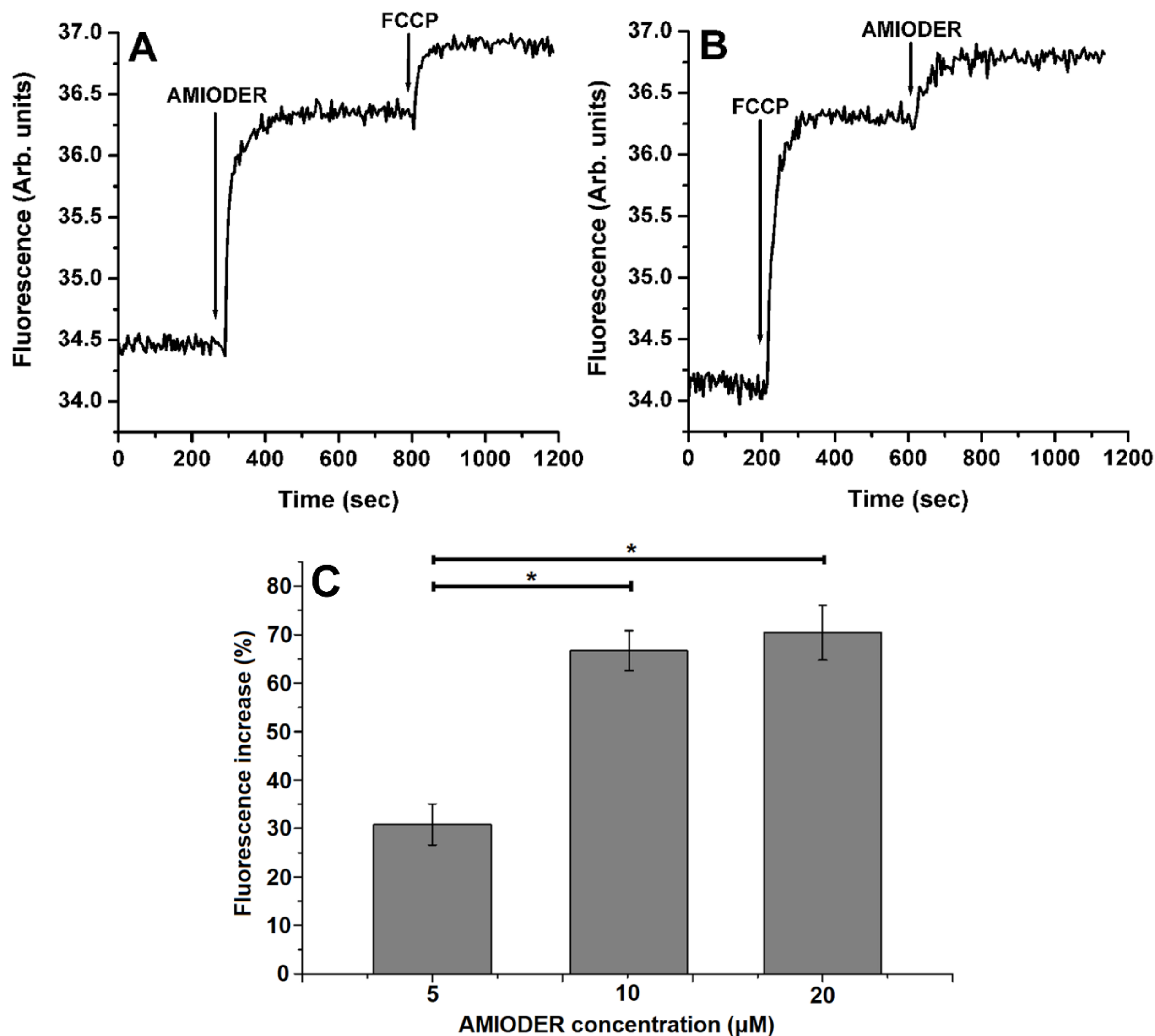

Fig 6. Effect of AMIODER on the mitochondrial electrochemical potential ($\Delta\Psi_m$) of *T. cruzi* epimastigotes. **(A)** AMIODER (10 µM) was added (arrow) to the parasites previously loaded with rhodamine 123, followed by the addition of FCCP (2 µM). **(B)** FCCP (2 µM) was added (arrow) followed by AMIODER (10 µM). **(C)** Percentage of rhodamine 123 fluorescence increase with respect to the basal level after addition of different concentrations of AMIODER to *T. cruzi* epimastigotes. Bars represent the mean ± SD of three independent experiments.

Several studies carried out on *T. cruzi* have shown other benzofuranic compounds targeting related mechanisms to the ones studied in this work. Like AMIODER, amiodarone was able to dissipate the mitochondrial membrane potential and alkalinize the acidocalcisomes with a concomitant increase in the cytoplasmic Ca^{2+} concentration in the parasites, without affecting host cells as seen by fluorescence microscopy. Interestingly, amiodarone was found to target ergosterol biosynthesis in *T. cruzi* epimastigotes, acting as an oxidosqualene cyclase inhibitor (Benaim et al., 2006). Similar mechanisms of action were observed by the use of the amiodarone analog, dronedarone, on *T. cruzi*. Thus, when epimastigotes were treated with a concentration equal to IC_{50}, dronedarone was able to dissipate the mitochondrial membrane potential and alkalinize the acidocalcisomes (Benaim et al., 2012). More extensive studies on the mechanisms involved in the trypanocidal action of eupomatenoid-5 have shown a marked involvement of the mitochondria in triggering a series of cell death events in *T. cruzi* after exposure to this compound. It was shown that eupomatenoid-5 induces an oxidative stress in the three main forms of the parasite, dissipating the mitochondrial membrane potential, increasing the concentration of reactive oxygen and nitrogen species through the damage of electron transport chain in mitochondria, due to a decreased trypanothione reductase activity. This oxidative injure leads to an apoptotic, autophagic and necrotic cell death in *T. cruzi* (Lazarin-Bidóia et al., 2013).

After the study of the mode of action of these benzofuran derivatives on *T. cruzi*, it can be strongly suggested that Ca^{2+} homeostasis could be considered as a common target for these parasites, mainly through the disruption of organelles acting as Ca^{2+} reservoirs. One of such, the mitochondrion, is directly associated with Ca^{2+} regulation, and a brake in its equilibrium, either through an uncoupling in redox metabolism or the dissipation of the

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mitochondrial membrane potential, produces an alteration in the intracellular Ca^{2+} homeostasis, triggering a signaling cascade which would drive to parasite death (Benaim and Garcia, 2011).

Acidocalcisomes are acidic organelles present in trypanosomatids postulated to be involved in osmoregulation and on the bioenergetics of these parasites (Docampo and Moreno, 2011). This organelle accumulates large quantities of polyphosphate and PPi. This later is considered as an energy source alternative to ATP in trypanosomatids. Acidocalcisomes are also very important as a Ca^{2+}-accumulating compartments, possessing a variety of transport mechanisms, including a vacuolar type H^+-ATPase, a Ca^{2+}/H^+ countertransporting ATPase for Ca^{2+} uptake, a Ca^{2+}/H^+ uniporter and a Na^+/H^+ antiporter involved in Ca^{2+} release (Docampo et al., 1995; Vercesi et al., 1994; Vercesi and Docampo, 1996). We next determine whether the benzofuran derivative had an effect on the acidocalcisomes of *T. cruzi* epimastigotes, taking in consideration that other benzofuran derivatives as amiodarone and dronedarone directly affect these important organelles (Benaim et al., 2014, 2012; Benaim and Paniz Mondolfi, 2012). With this purpose, parasites were loaded with the fluorescent dye acridine orange, which is known to accumulate in acidic compartments as acidocalcisomes (Benaim et al., 2012). Upon addition of the drug, while measuring changes in fluorescence, it can be observed (Fig. 7A) a large increase in the fluorescence of acridine orange after addition of AMIODER (10 μM), consequence of the release of the accumulated dye after the alkalinization of the organelle. The subsequent addition of nigericine, a monovalent cation exchanger known to induce the acidocalcisomes alkalinization after the release of H^+ in exchange for cytosolic K^+ (Vercesi and Docampo, 1996), generates a further, but small, acridine orange release. Addition of nigericine after 20

μM AMIODER instead of 10 μM did not produce any further increase in fluorescence, indicating that at this concentration these organelles were already fully alkalinized. When the order of addition of both effectors was inverted (Fig. 7B), nigericine produced a rapid response, as expected. However, this was followed by a further increase in fluorescence, generated by the addition of AMIODER. This additional acridine orange release produced by the drug after nigericine suggests the existence of other acidic compartments on which the benzofuran derivative could also exert an effect (lisosomes, acidic vacuoles, etc). In Fig. 7C it can be observed a dose-dependent increase in acridine orange fluorescence increase with respect to the basal level after addition of different concentration of AMIODER to *T. cruzi* epimastigotes, showing that 20 μM produced the maximal effect. Since the alkalinization of acidocalcisomes is associated with the release of Ca^{2+} from these reservoirs (Benaim and Garcia, 2011; Vercesi and Docampo, 1996), the data from this results explain the observed effect of AMIODER on the $[Ca^{2+}]_i$ in *T. cruzi* observed in the absence of extracellular Ca^{2+} (Fig. 5B). This effect should be additive to that resulted from the release of Ca^{2+} from the mitochondrion (Fig 6) by the action of the drug on the $\Delta\Psi_{m.}$

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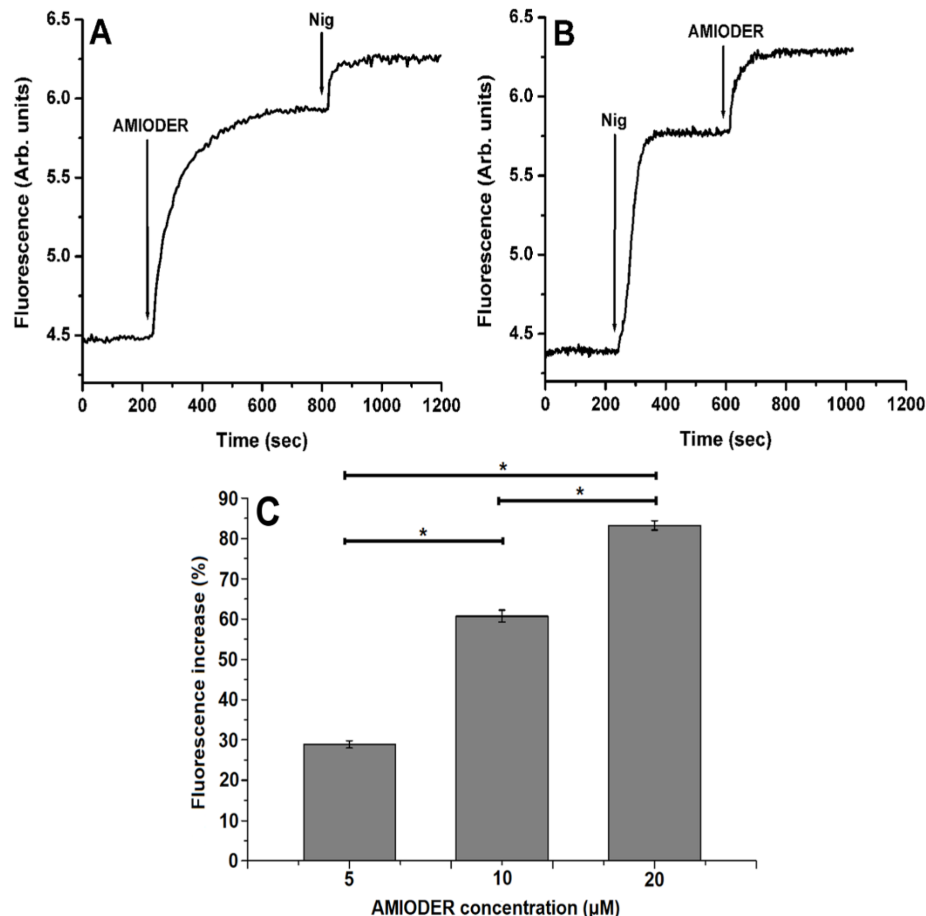

Fig 7. Effects of AMIODER on the level of acidocalcisomes alkalinization in *T. cruzi* epimastigotes. (A) AMIODER at 10 μM was added (arrow) to the stirring cuvette carrying parasites loaded with acridine orange, followed by addition of nigericine (2 μM). **(B)** Addition of nigericine (2 μM) was followed by AMIODER (10 μM). **(C)** Percentage of acridine orange fluorescence increase with respect to the basal level after addition of different concentration of AMIODER to *T. cruzi* epimastigotes. Bars represent the mean ± SD of three independent experiments.

Taking together, in this work we demonstrate that AMIODER has inhibitory effect on the growth of *T. cruzi* epimastigotes. More importantly, this drug also has an effect against amastigotes inside host cells. the clinically relevant phase of these parasites, with an IC_{50}

even lower than that previously reported for this compound against *C. neoformans* (Hejchman et al., 2012), with a relatively low effect on mammal host cells. Interestingly, this benzofuran derivative disrupts the Ca^{2+} homeostasis in *T. cruzi* by affecting to essential organelles, the mitochondrion and the acidocalcisomes, both directly involved in the bioenergetic of this parasite. This results are in contrast to that reported for *S. cereviciae* on which this compound had no effect alone on Ca^{2+} homeostasis on yeast, but potentiates the activity of amiodarone in this respect (Hejchman et al., 2012). The trypanocidal activity of this compound, and its previously reported antifungal effect, place it as a new potential drug lead for further research on protozoan parasites and fungi, for the design of new compounds with enhanced biological activity.

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