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Antiproliferative effect of a benzofuran derivate based on the structure of amiodarone on *Leishmania donovani* affecting mitochondria, acidocalcisomes and intracellular Ca^{2+} homeostasis

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ABSTRACT

Leishmaniasis is a parasitic disease representing an important problem of public health. Visceral leishmaniasis, resulting from infection with *Leishmania donovani*, causes considerable mortality and morbidity in the poorest region of the word. At present there is no current effective treatment, since the approved, drugs are expensive and are not free of undesirable side effects. Therefore, there is a need for the identification of new drugs. In this context, the parasite Ca^{2+} regulatory mechanisms in which mitochondria and acidocalcisomes are involved have been postulated as important targets for several trypanocidal drugs. Thus, amiodarone and dronedarone, common human antiarrythmics, exert its known action on these parasites through the disruption of the intracellular Ca^{2+} homeostasis. AMIODER is a benzofuran derivate based on the structure of amiodarone that recently demonstrates a significant effect on *Trypanosoma cruzi*. We now report the effect of AMIODER on *Leishmania donovani* demonstrating that it inhibit the growth of promastigotes and also of amastigotes inside macrophages, the clinically relevant stage of the parasite, obtaining IC_{50} values significantly lower than those reported for *T. cruzi*. We also show that this compound disrupted Ca^{2+} homeostasis in L. *donovani*, through its action on two organelles involved in the intracellular Ca^{2+} regulation and on the bioenergetics of the parasite. AMIODER totally collapsed the electrochemical membrane potential of the unique giant mitochondrion and simultaneously induced the alkalinization of acidocalcisomes, driving together to a large increase in the intracellular Ca^{2+} concentration of the parasite as the main mechanism of action of this benzofurane derivative.

1. Introduction

Leishmaniasis is a parasitic disease found through tropical and subtropical regions that generates large morbidity and mortality, affecting mainly the poorest areas of the world [1]. Visceral leishmaniasis, also known as Kala-Azar, is the most severe form of this human infection and is caused by *Leishmania donovani* and *Leishmania infantum* [2]. Around 350 million people are at risk of been infected by this parasite [3] and according to the World Health Organization (WHO, 2017), it is estimated that between 50,000 and 90,000 new cases occur each year. Unfortunately, the prevalence of visceral leishmaniasis has increased over the last years, due to lack of effective treatments [2].

The most used drugs are amphotericin B (liposomal or deoxycholate formulations) [4] and miltefosine, which is the only orally active FDA accepted drug [5]. However, the first drug is very expensive and causes many side effects, while the second has been reported to be teratogenic and also induces development of resistance [5]. Nevertheless, miltefosine is frequently used. Very recently, its mechanism of action has been demonstrated [6], pointing to the disruption of the intracellular Ca^{2+} homeostasis of the parasite, thus supporting the notion that the mechanisms responsible for the Ca^{2+} regulation are possible therapeutic targets against these parasites [7]. Thus, miltefosine collapse the mitochondrial membrane potential ($\Delta\Psi m$) of the unique large mitochondrion (12% of the total volume of the parasite), necessary for the storage of Ca^{2+} in this essential organelle, since $\Delta\Psi m$ is the driving force for the accumulation of Ca^{2+} in these compartment in *Leishmania spp* and other trypanosomatids [8]. In this same context miltefosine affects directly the acidocalcisomes [6], essential organelles involved in the bioenergetic of these parasites, which similar to mitochondria are able to accumulate large amounts of Ca^{2+}. Acidocalcisomes also accumulates a large quantity of polyphosphates and pyrophosphate, the last being an alternative source of energy partially substituting ATP [9].

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Therefore, these two facts constitute an essential difference with humans, so that it has been claimed that these organelles should be considered as possible therapeutic target [9,10].

As mentioned, there is an urgent need for the discovery of new treatments for visceral leishmaniasis. In the last few decades, new alternative therapies have been investigated. Accordingly, since the 1970's many compounds carrying benzofuran and benzofuran derivatives have shown to be effective against L. *donovani* [11,12]. For example, amiodarone, a compound that was designed and is prescribed as an antiarrhytmic drug has been shown to have a potent inhibitory effect on the growth of *Trypanosoma cruzi* [13] and *Leishmania mexicana* [14]. Amiodarone also inhibited the parasite ergosterol biosynthesis, which unlike humans that possess cholesterol, is the sterol present in trypanosomatids. In accordance with the previous statement, it has been shown that amiodarone disrupted the intracellular Ca^{2+} homeostasis in trypanosomatids, affecting the mitochondrion and the acidocalcisomes [7].

A new recently synthesized benzofuran derivative namely AMIODER has been studied in *T. cruzi* [15]. This compound showed an inhibitory effect on the growth of *T. cruzi* epimastigotes and amastigotes, inducing an increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), by disrupting the Ca^{2+} regulation in the mitochondrion and the acidocalcisomes [15]. In this work, we report a potent inhibitory effect of AMIODER on the growth of L. *donovani* promastigotes and amastigotes. Our results showed that AMIODER is even more effective against L. *donovani* when compared to *T. cruzi*, demonstrating that this effect is also related to the disruption of the parasite intracellular Ca^{2+} homeostasis (Fig. 1).

2. Materials and methods

2.1. Chemicals

The compound methyl 7-acetyl-5-bromo-6-hydroxy-3-bromomethyl-2-benzofurancarboxylate (AMIODER) was synthesized according to the method previously reported [16]. Digitonin, FCCP, nigericin, Fura 2-AM, rhodamine 123 and acridine orange were obtained from Sigma Chemical Co (Saint Louis MO. USA).

2.2. Leishmania donovani promastigote cultures

Leishmania donovani promastigotes (DD8 strain) were grown in Liver infusion tryptose (LIT) medium, pH 7.4, supplemented with 10% fetal bovine serum at 29 °C.

2.3. Determination of the effect of AMIODER on L. donovani promastigotes

Promastigotes (5×10^5 parasites/mL) were treated with increasing concentrations of AMIODER, including a control with the drug vehicle, dimethylsulfoxide (DMSO).

The increase in the population of parasites was determined daily by direct counting in a Neubauer chamber. The IC_{50} was determined using GraphPad Prism 5.0.

2.4. Determination of macrophages susceptibly to AMIODER

Macrophages (J774 strain) were maintained in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum at 37 °C, at 5% CO_2. For cell viability determinations, macrophages were cultured in a 96 well plates and exposed to different concentrations of AMIODER for 48 h. Cells were washed with phosphate-buffered saline (PBS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at 1 mg/mL was added, followed by 4 h incubation. The formazan produced by the mitochondrial dehydrogenase activity of viable cells was determined reading the absorbance at 570 nm, using a plate spectrophotometer. The IC_{50} was determined using GraphPad Prism 5.0.

2.5. Quantification of the effect of AMIODER on macrophages infected with L. donovani amastigotes

Amastigote susceptibility assays were performed as reported previously [15], with slight modifications. Macrophages were cultured on plastic coverslips placed inside 24 well plates, infected for 24 h with promastigotes at a proportion of 15 parasites per host cell, and then washed 3 times with PBS to remove non-adherent parasites. Medium with different drug concentrations was added and cells were incubated for 72 h, 37 °C in under 5% CO_2. Coverslips with cells were washed with PBS and then stained with Giemsa. The percentage of infected cells was determined directly using a light microscopy with a 100x objective under immersion. The IC_{50} was determined using GraphPad Prism 5.0.

2.6. Determination of the intracellular Ca^{2+} concentration

The changes in the $[Ca^{2+}]_i$ were followed essentially as previously described [17]. *L. donovani* promastigotes were loaded with the Ca^{2+} radiometric indicator Fura 2, by the use of Fura-2 AM. Ethylene glycol tetra acetic acid (EGTA, 500 μM) was added when measurements were made in the absence of extracellular Ca^{2+}. Digititonin (40 μM) was used to permeabilize the parasites for calibration. Fluorescence measurements were carried out using a Perkin-Elmer spectrofluorimeter LS-55 adapted with a device that allow the excitation by a double wavelength beam (340 nm and 380 nm) and registering the emission at 510 nm [13]. For the determination of the relative value of the fluorescence increase after AMIODER addition in the presence or absence of Ca^{2+}, the values of fluorescence at the plateau with respect to the resting rate was plotted in each case. For the determination of the change of fluorescence *versus* time, to quantify the initial velocity of fluorescence increase, it was considered the fluorescence value in which half the increase in intracellular Ca^{2+} concentration was obtained in the presence or absence of extracellular $CaCl_2$. Each column represents the result of three independent experiments. The bars at the top of columns represent the standard deviation. Bars represent the mean ± SD of four independent experiments.

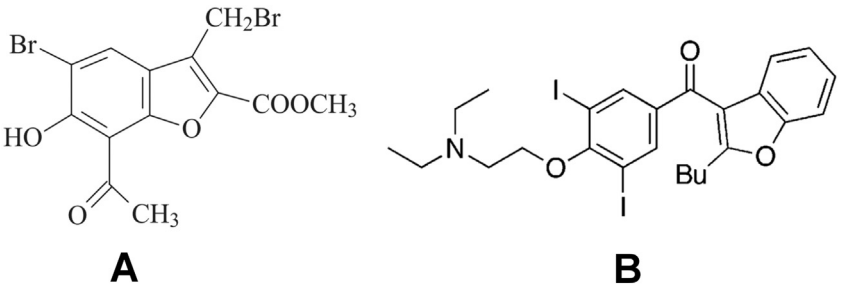

Fig. 1. Chemical structure of the benzofuran derivate AMIODER (A), based on the structure of Amiodarone (B).

2.7. Measure of mitochondrial electrochemical membrane potential ($\Delta\Psi_m$)

The effect of the drug on the mitochondrial membrane potential of L. *donovani* promastigotes was evaluated using the fluorescent dye rhodamine 123 as reported previously [13], taking advantage of the internationalization of this fluorophore, according to the mitochondrial electrochemical membrane potential ($\Delta\Psi_m$). Briefly, 7.5×10^6 parasites were collected by centrifugation at 600 X g for 2.5 min and washed in loading buffer (130 mM KCl, 1 mM $MgCl_2$, 2 mM KH_2PO_4, 20 mM Tris-HCl) plus 1% glucose. The pellet was resuspended in the same buffer in the presence of rhodamine 123 (10 μM) and incubated for 40 min at 29 °C and in the dark under 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. For the determination of the increase in the effect of AMIODER after FCCP addition, the whole effect of both effectors was taking as 100%, and the increase of AMIODER after the increment induced by FCCP was plotted at each drug concentration. Bars represent the mean ± SD of four independent experiments. The asterisk (*) indicates a statistically significant difference with p value < .05 (*) and < 0.01(**).

2.8. Determination of the effect of AMIODER on acidocalcisomes

The effect of the benzofuran compound on acidocalcisomes was evaluated using acridine orange, which accumulates in acidic compartments [17]. Promastigotes (7×10^6 cells/mL) were collected, washed and incubated in the same loading buffer described above, with acridine orange at 2 μM for 5 min, 29 °C, in the dark and under 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. Nigericin, a monovalent ionophore acting as a K^+/H^+ exchanger was used at 2 μM as a positive control.

2.9. Statistic analysis

Statistical analysis was carried out using the student's *t*-test, using the Past Statistical Program.

3. Results and discussion

In this study we determined the effect of AMIODER on L. *donovani*, a human parasite belonging to a genus related to *T. cruzi* but producing a totally different disease. AMIODER affected in a dose-dependent manner the proliferation of promastigotes of L. *donovani* (Fig. 2). The IC_{50} determined after 72 h of treatment of the parasites was 3.62 μM. This effect is greater than that observed for *T. cruzi* epimastigotes which showed an IC_{50} value of 0.8 μM [15].

We also evaluated the inhibitory effect of AMIODER on the growth of amastigotes inside macrophages, which is the clinically relevant phase of the parasite, obtaining an IC_{50} of 0.14 μM (Fig. 3A). When the effect of the drug was evaluated on non-infected macrophages for comparative purposes, an IC_{50} value of 14.43 μM was measured (Fig. 3B). The selectivity index (SI) for this drug, which was determined by the ratio of the IC_{50} for macrophages over the IC_{50} obtained for amastigotes, was 103.1, indicating about 100 times more selective cytotoxicity against the amastigote form of the parasite than against the host cell. Since SI higher than 10 is considered a significant value, it could be considered that this compound possesses a relatively high selectivity index [18]. Compared with *T. cruzi* which SI was 40, AMIODER again appears to be much more potent for *Leishmania donovani*.

The IC_{50} for amastigotes was approximately 30 times lower than for promastigotes, probably due to the fact that promastigotes are covered

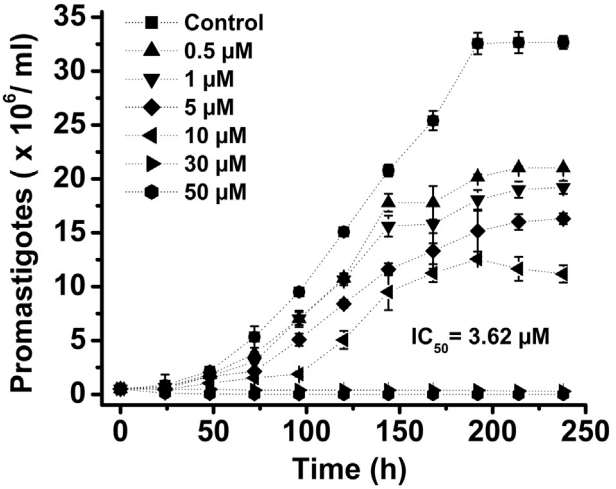

Fig. 2. Susceptibility of L. *donovani* promastigotes to AMIODER. Cultures of L. *donovani* promastigotes were grown in the presence of different concentrations of AMIODER (0–50 μM). Experiments were carried out in triplicate. Each point represents the mean ± SD.

by a glycocalyx of 7 nm to 17 nm thick [19]. On the contrary, the amastigotes practically do not have glycocalyx, which would allow the passage of the compound in a more efficient and fast way [19]. Moreover, there are differences between the two forms on the catabolism of glucose, utilization of fatty acids, purine metabolism, gene expression, gp63 metalloproteases and lipophosphoglycans [20], which may increase the effectiveness of the drug in the amastigote stage. A comparative analysis on the biological activity of other benzofuran derivatives against *Leishmania* sp. reveals that eupomatenoid-5 shows an important *in vitro* activity against L. *amazonensis* [21,22]. Eupomatenoid-5 also induced a time-dependent decrease in reduced thiol levels of treated parasites. The trypanothione system is unique in trypanosomatid parasites and plays an important role in the homeostasis of the parasite redox metabolism. Additionally, eupomatenoid-5 produced the externalization of phosphatidylserine and a reduction of parasite volume [21]. Similar results were obtained in *T. cruzi* [23].

It has been shown that in *T. cruzi*, the mechanism of action of AMIODER is through the disruption of the mitochondrial electrochemical potential and the alkalinization of the acidocalcisomes, driving to a large increase in the $[Ca^{2+}]_i$ [15]. We obtained that AMIODER (10 μM) induced a large increase in $[Ca^{2+}]_i$ of these parasites (Fig. 4A). It worthwhile to remind here that the 340/380 nm ratio is directly proportional to $[Ca^{2+}]_i$ [24]. In order to determine if this increase was due to the influx of the cation from the extracellular *milieu* the same experiment was performed but in the absence of extracellular Ca^{2+}, using EGTA as a chelating agent (Fig. 4B). As can be seen, the results were very similar indicating that Ca^{2+} was released from intracellular compartments, possibly from the unique giant mitochondrion and/or from the acidocalcisomes.

We proposed to elucidate the possible role of these two intracellular organelles in the observed $[Ca^{2+}]_i$ increase generated by AMIODER, taking into consideration that both compartments act as intracellular Ca^{2+} reservoirs and also based on previous reports showing the effect on both organelles by other benzofuran derivatives, like amiodarone and dronedarone on *T. cruzi* [13,15] and L. *mexicana* [14,17]. First, the effect of AMIODER was evaluated on the mitochondrial membrane potential of L. *donovani* promastigotes 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 ($\Delta\Psi_m$). Ca^{2+} accumulates in large quantities inside the organelle and it would be released to the extracellular *milieu* as a product of its deenergization. Fig. 5A shows a large increase in the fluorescence upon addition of AMIODER, which is interpreted as the dissipation of

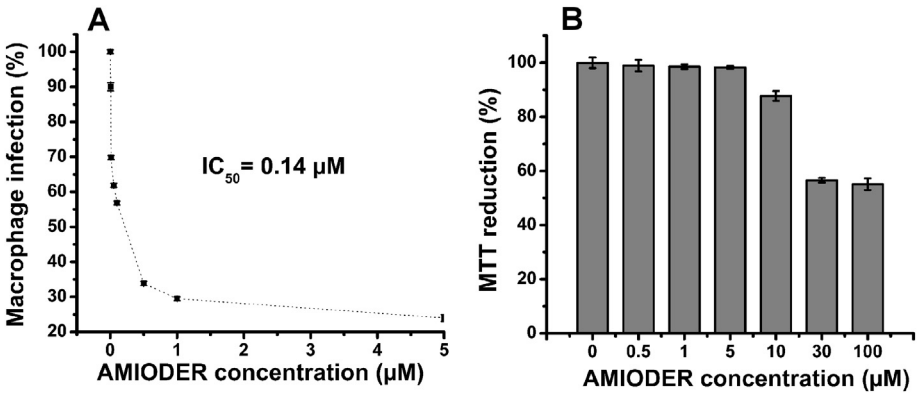

Fig. 3. AMIODER on the viability of macrophages and against intracellular amastigotes of L. *donovani*. (A) Macrophages were infected with L. *donovani* promastigotes and then treated with different concentrations of AMIODER. The percentage of infected cells (squares) and the IC_{50} on amastigotes were determined 96 h post-treatment. (B) Cells were exposed to various concentrations of AMIODER and cytotoxicity was measured by the MTT assay. The calculated IC_{50} was 14.43 ± 1.79 µM. Experiments were carried out in triplicate and values represent the mean ± SD.

$\Delta\Psi_m$ and subsequent release of rhodamine 123 accumulated in the mitochondrion. After adding AMIODER, addition of FCCP, a protonophore known to collapse the $\Delta\Psi_m$ as a positive control, produced only a small increase in the fluorescence (Fig. 5A) indicating that the drug had already collapsed the $\Delta\Psi_m$. A similar experiment but inverting the order of addition of the effectors is illustrated in Fig. 5B. As expected, further addition of AMIODER after FCCP showed only a faint effect. The quantification of the effect of AMIODER is shown in Fig. 5C, in which a dose-dependent behavior is clearly seen.

Consequently AMIODER was able to dissipate the mitochondrial membrane potential from L. *donovani*. Other extensive studies on other benzofuran derivates as eupomatenoid-5 showed a similar mechanism of action on its trypanocidal action, targeting the mitochondria which trigger a series of cell death events in L. *amazonensis* after exposure to this compound [21]. Thus, it was shown that eupomatenoid-5 induces an oxidative stress in the two forms of the parasite, dissipating the mitochondrial membrane potential and increasing the concentration of reactive oxygen and nitrogen species (ROS) in the cytoplasm through the damage of the electron transport chain of the mitochondrion, due to a decrease in trypanothione reductase activity. This oxidative damage leads to an apoptotic, autophagic and necrotic cell death in L. *amazonensis* [21] and in *T. cruzi* [23].

As mentioned before, acidocalcisomes are acidic organelles present in trypanosomatids postulated to be involved in osmoregulation and on the bioenergetics of these parasites [10]. 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^+ counter-transporting ATPase for Ca^{2+} uptake, a Ca^{2+}/H^+ uniporter and a Na+/H+ antiporter involved in Ca^{2+} release [25–27]. We studied whether AMIODER had an effect on the acidocalcisomes of L. *donovani* promastigotes, taking in consideration that other benzofuran derivatives such as amiodarone and dronedarone [14,17,28,29] and AMIODER itself [15] directly affect these important organelles. With this purpose, parasites were loaded with the fluorescent dye acridine orange, which is known to accumulate in acidic compartments as acidocalcisomes [14]. It can be observed (Fig. 6A) a large increase in the fluorescence of acridine orange upon addition of AMIODER (10 µM), consequence of the release of the accumulated dye after the alkalinization of the organelle by the drug. The subsequent addition of nigericin, a monovalent cation exchanger known to induce the acidocalcisomes alkalinization after the release of H^+ in exchange for cytosolic K^+ [27], generates a further, but small, acridine orange release

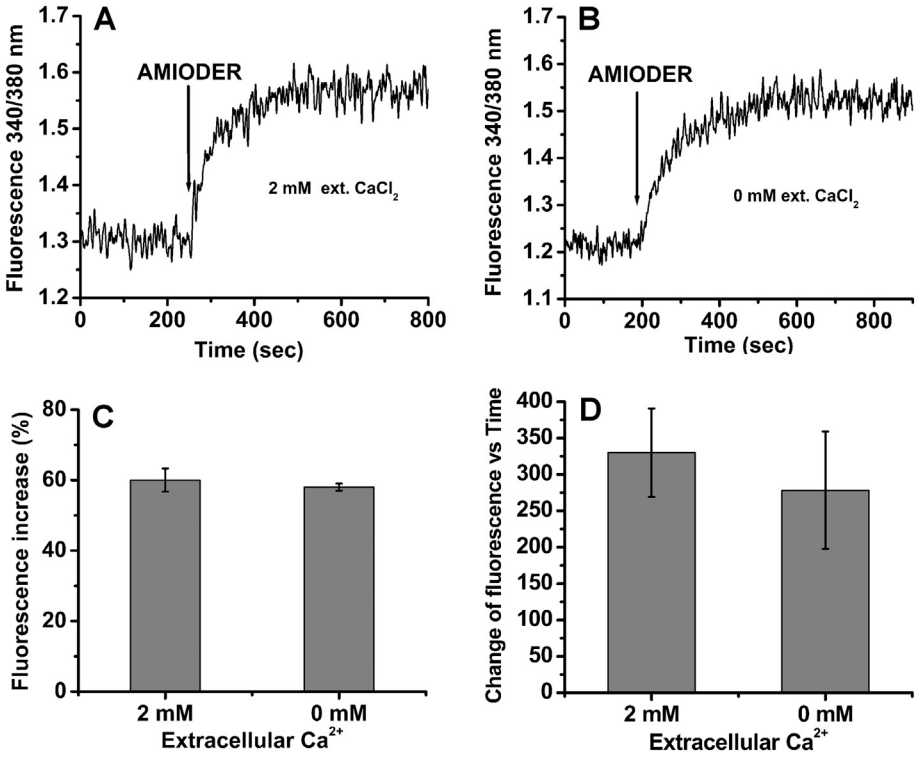

Fig. 4. Effect of AMIODER on intracellular Ca^{2+} concentration of L. *donovani*. (A) Outcome AMIODER at 10 µM (arrow) on the intracellular Ca^{2+} concentration of *L. donovani* promastigotes in the presence of 2 mM of extracellular Ca^{2+}. (B) Effect of AMIODER (10 µM) on the intracellular Ca^{2+} concentration of *L. donovani* promastigotes in absence of extracellular Ca^{2+}. The traces represent typical results from at least three different experiments. (C) Percentage increase of the 340/380 nm fluorescence ratio by adding 10 µM of AMIODER to promastigotes of L. *donovani* after reaching the *plateau*. (D) Change of fluorescence at the time in which half the increase in intracellular Ca^{2+} concentration was obtained. Each column represents the result of three independent experiments in the presence (left) and absence (right) of extracellular Ca^{2+}. The bars at the top of each column represent the standard deviation. The difference between both experiments is not statistically significant.

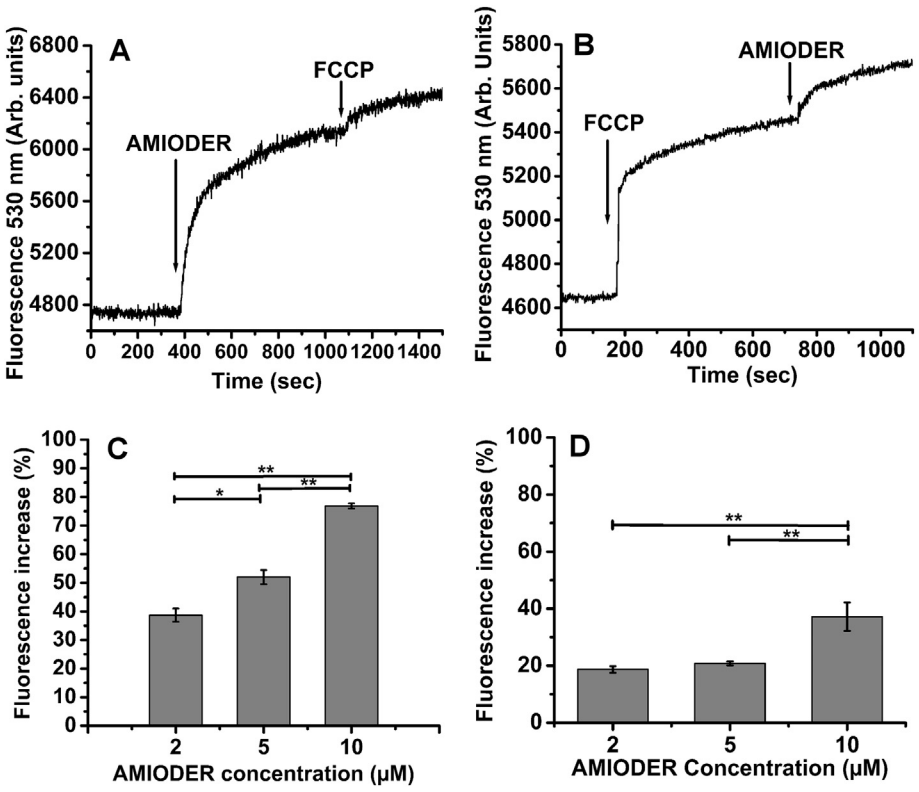

Fig. 5. Effect of AMIODER on the mitochondrial electrochemical potential (ΔΨm) of *Leishmania donovani* promastigotes. (A) AMIODER (10 μM) was added (arrow) to the parasites previously loaded with rhodamine 123, followed by the addition of FCCP (2 μM). (B) FCCP (2 μM) was added (arrow) followed by AMIODER (10 μM). The traces represent typical results from at least three different experiments. (C) Percentage of rhodamine 123 fluorescence increase with respect to the basal level after addition of different concentrations of AMIODER to L. *donovani* promastigotes. (D) Percentage of AMIODER fluorescence increase after addition of FCCP. Bars represent the mean ± SD of four independent experiments. The asterisk (*) indicates a statistically significant difference with p value < .05 (*) and < 0.01(**).

(Fig. 5A). When the order of addition of both effectors was inverted (Fig. 6B), nigericin 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 after nigericin suggests the existence of other acidic compartments such as phagolysosomes and/or acidic vacuoles, which could accumulate small quantities of the fluorophore and cause its release after the addition of the drug. However, acidocalcisomas are the predominant acidic organelles in trypanosomatids [27,30], thus inducing the larger and faster release of acridine orange in these experiments. In Fig. 6C it can be observed a dose-dependent increase in acridine orange fluorescence with respect to the basal level after addition of different concentration

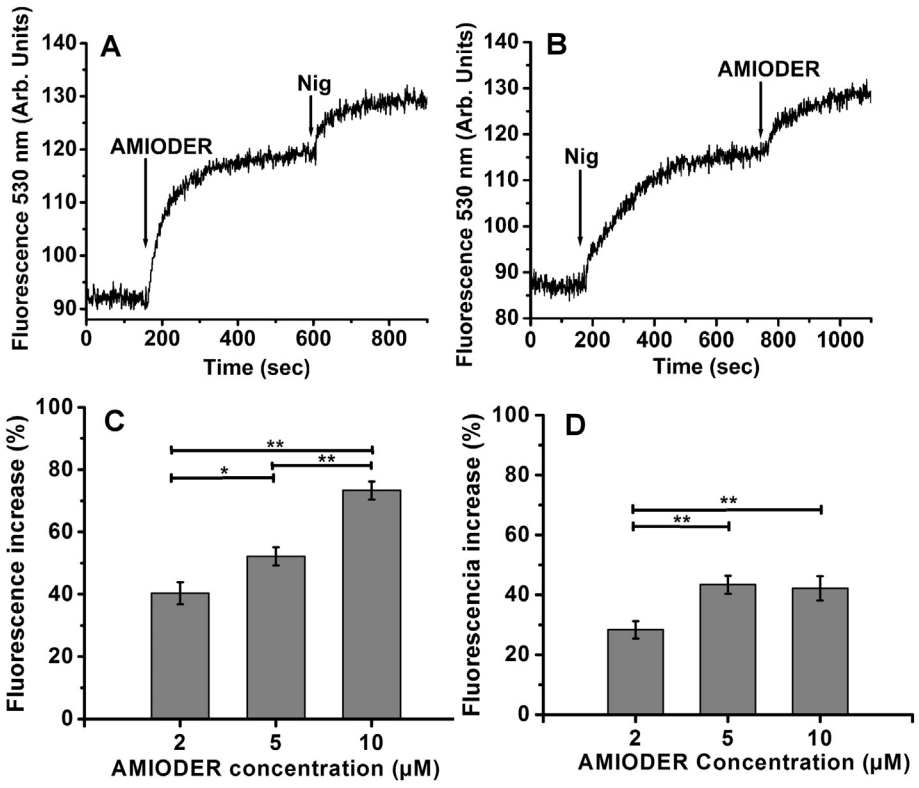

Fig. 6. Effects of AMIODER on the level of acidocalcisomes alkalinization in L. *donovani* promastigotes mastigotes. (A) AMIODER at 10 μM was added (arrow) to the stirring cuvette carrying parasites loaded with acridine orange, followed by addition of nigericin (2 μM). (B) Addition of nigericin (2 μM) was followed by AMIODER (10 μM). The traces represent typical results from at least three different experiments. (C) Percentage of acridine orange fluorescence increase with respect to the basal level after addition of different concentration of AMIODER to L. *donovani* promastigotes. (D) Percentage of AMIODER fluorescence increase after addition of nifericin. Bars represent the mean ± SD of four independent experiments. The asterisk (*) indicates a statistically significant difference with p value < .05 (*) and < 0.01(**).

of AMIODER to L. *donovani* promastigotes. Since the alkalinization of acidocalcisomes is associated to the release of Ca^{2+} from these reservoirs [7,27], this effect of AMIODER supports the notion that its action would contribute to the large increase in the $[Ca^{2+}]_i$ obtained in Fig. 4 upon addition of the benzofuran derivative, jointly with its action on the mitochondria.

Taking all these results together, it could be strongly suggested that the increase in intracellular Ca^{2+} concentration observed upon addition of AMIODER was due to the release of this cation from the mitochondria and the acidocalcisomes. These results are in contrast to that reported for *S. cerevisiae* on which this compound had no effect alone on Ca^{2+} homeostasis on yeast, but potentiates the activity of amiodarone in this respect [16]. The trypanocidal activity of AMIODER, and its previously reported antifungal effect, places 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, and also supports the need to deepen these studies in search of benzofuran derivatives based on the structure of amiodarone, oriented toward the investigation of a possible therapy against visceral leishmaniasis.

Declarations of interest

None

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