In vitro anti-Trypanosoma cruzi activity of dronedarone, a novel
amiodarone derivative with an improved safety profile

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Abstract

Amiodarone, a commonly used antiarrhythmic, is also a potent and selective anti-Trypanosoma cruzi agent. Dronedarone is an amiodarone derivative in which the 2,5-diiodophenyl moiety of the parental drug has been replaced with an unsubstituted phenyl group aiming to eliminate the thyroid toxicity frequently observed with amiodarone treatment. Dronedarone has been approved by the Foods and Drug Administration (FDA) and its use as a safe antiarrhythmic has been extensively documented.

We show here that dronedarone also has potent anti-T. cruzi activity, against both extracellular epimastigotes and intracellular amastigotes, the clinically relevant form of the parasite. The IC₅₀ values against both proliferative stages are lower than those previously reported for amiodarone. The mechanism of action of dronedarone resembles that of amiodarone, as it induces a large increase in the intracellular Ca²⁺ concentration of the parasite that results from the release of this ion from intracellular storage sites, including a direct effect of the drug on the mitochondrial electrochemical potential, and through alkalinization of the acidocalcisomes. Our results suggest a possible future repurposed use of dronedarone for the treatment of Chagas’ disease.

Introduction

Trypanosoma cruzi is the causative agent of Chagas’ disease, a chronically debilitating infection that to date has no approved efficient treatment in its chronic stages. However, it is now well established that amiodarone, a commonly used antiarrhythmic drug, has also potent and specific activity against the proliferative stages of T. cruzi (4) as well as Leishmania mexicana.
(23-24) and *L. amazonensis* (17). Recently, the added effect of amiodarone on recovering myocardial contractility in *T. cruzi*-infected cardiac myocytes through a direct action over F-actin fibrils and gap junction proteins such as connexin43 has been reported (1). Notably, amiodarone efficacy has also been demonstrated in at least, one clinical case of human Chagas’ disease (19), and in a case of cutaneous leishmaniasis (18). Amiodarone also supports host survival during malaria episodes (6), opening a whole new perspective in an alternative use for this drug. It has been also found that combination of amiodarone with posaconazole, another potential option for treating Chagas’ disease already in final clinical trials, and other analogs have potent synergistic effects (4). Posaconazole has recently been found to represent a successful treatment, in humans with *T. cruzi* infection (21), and also in a case of cutaneous leishmaniasis (20).

Despite its extensive use in humans, the presence of a 2,5-diodo-phenyl moiety in the structure of amiodarone, which has been associated with significant undesirable side effects related to anti-thyroid activity, makes its prolonged use in Chagas’ disease a difficult therapeutic task for the clinician. Dronedarone, a derivative of amiodarone has been recently synthesized with several significant structural modifications, including replacement of the 2,5-diido-phenyl with an unsubstituted phenyl group and the incorporation of a methyl sulfonyl group, aimed at reducing they thyroid toxicity and lipophilicity of the parental drug (Fig. 1). This new drug has already been FDA-approved for use as an antiarrhythmic in humans, and has begun to replace amiodarone as the drug of choice due to its improved safety profile and apparent absence of
associated thyroid or pulmonary toxicity, therefore resulting in fewer treatment
discontinuations and reduced mortality (22).

In this report we provide evidence that dronedarone has also activity
against extracellular epimastigotes of *T. cruzi*, the proliferative stage equivalent
to that present in the parasite’s insect vector, and the clinically relevant
intracellular amastigote form. Dronedarone and amiodarone act through the
same mechanisms of action, by altering the intracellular Ca\(^{2+}\) homeostasis of
the parasite as a consequence of a direct effect of the drug on the mitochondrial
electrochemical potential, and also by alkalinization of the acidocalcisomes.

**Materials and Methods Chemicals.**

Dronedarone was extracted from commercial tablets (Multaq®), based on the
known fact that this compound is fully soluble in methanol. Dronedarone powder
was obtained by grinding the tablets in a mortar that then was dissolved in
methanol by vigorous mixing. The insoluble material was discarded by
centrifugation. Amiodarone ([(2-butyl-3-benzofuranyl)-[4-[2-(diethylamino)-ethoxi]
3,5-diiodophenyl] methanone hydrochloride), EGTA, digitonin,
fluorocarbonylcyanide *P*-([trifluoromethoxy] phenylhydrazone (FCCP),
bafilomycin A, nigericin were from Sigma (St. Louis, MO). Fura 2-acetoxymethyl
ester (FURA 2-AM), rhodamine 123, rhod 2-AM, and acridine orange were from
Molecular Probes (Eugene, OR).

**Culture of epimastigotes of *Trypanosoma cruzi* and determination of
susceptibility to dronedarone.**
T. cruzi epimastigotes (CL Brener strain) were grown in LIT medium supplemented with 10% fetal-bovine serum at 29 °C, with strong agitation (100 rpm) as reported previously (4), in the absence or presence of either dronedarone or amiodarone at different concentrations. Live parasites were counted daily using a Neubauer chamber. The initial parasite concentration was $10^6$ parasites/mL. Either the drug or vehicle (DMSO) was added after 24 h in each condition. At least 3 independent experiments were performed for each drug and dose, and the EC$_{50}$ value was determined using Prisma GraphPad 5.0.

**Amastigote growth inhibition assay.**

Amastigotes were cultured in Vero cells maintained in Dulbecco’s Minimal Essential Medium, supplemented with 1% fetal-bovine serum and incubated at 37 °C in humidified 95% air/5% CO$_2$ (4). For the assays, Vero cells were placed in a 6-well plate and infected with trypomastigotes (ratio 1:10) for 12 h. After infection, cells were washed three times to remove not interiorized trypomastigotes, and culture medium was added either with or without dronedarone at different concentrations. The infected cells were incubated for 96 h in the conditions previously described (4). At 96 h, the cells were fixed with methanol and stained with Giemsa to determine the percentage of infected cells.

**Determination of the Intracellular Ca$^{2+}$ concentration.**

To evaluate the effect of dronedarone on intracellular Ca$^{2+}$ concentration, we loaded T. cruzi epimastigotes with the fluorescent ratiometric indicator Fura 2. Briefly, 2 x $10^8$ parasites were collected by centrifugation at 600g for 2 min.
and washed twice in PBS buffer plus 1% glucose. Since epimastigotes possess low levels of esterases, which difficult the development of Fura 2 in the cytoplasm, we found very convenient to load the parasites overnight, with Fura 2-AM (6 µM) with Probenecid (12 µM) and Pluronic acid (12 µM) in PBS buffer plus 1% glucose at 29 °C, in dark with continuous agitation. This procedure enabled sufficient loading of the parasites with the Ca\(^{2+}\) fluorophore, to allow precise measurement of the intracellular Ca\(^{2+}\) changes after addition of the either dronedarone or amiodarone. The Fura 2-loaded parasites were washed by centrifugation twice and resuspended in Tyrode buffer in the presence or absence of Ca\(^{2+}\). For Fura 2 measurements these parasites were placed in a cuvette with continuous stirring, and thermostated at 29 °C in a Perkin Elmer 510 Spectrofluorimeter. The conditions of measurement were Ex 340 nm/380 nm and Em 510 nm. Intracellular Ca\(^{2+}\) concentration has been calculated as described by Grynkiewicz et al. (15), by applying the following equation: 

\[ K_d \times \left( \frac{R - R_{\text{min}}}{R_{\text{max}} - R} \right) \times \left[ \frac{F_{\text{min}}(380)}{F_{\text{max}}(380)} \right], \]

where \(K_d\) is the dissociation constant of Fura 2 (244 nM); \(R\) is the ratio of the fluorescence emission obtained after excitation at 340 nm/after excitation at 380 nm; \(R_{\text{max}}\) and \(F_{\text{max}}\) are the ratio of excitation fluorescence at 340 nm/excitation fluorescence at 380 nm and the fluorescence of Fura 2 at 380 nm, respectively, under saturated Ca\(^{2+}\) concentrations; and \(R_{\text{min}}\) and \(F_{\text{min}}\) are the ratio of excitation fluorescence at 340 nm/excitation fluorescence at 380 nm and the fluorescence of Fura 2 at 380 nm, respectively, in the absence of Ca\(^{2+}\). Maximum and minimum values were obtained after the addition of 30 µM digitonin, which allows the flow of Ca\(^{2+}\) to the interior of the cell. Then, 8 mM EGTA was added to chelate all the remaining Ca\(^{2+}\) (9).
Mitochondrial membrane potential.

To evaluate the effect of dronedarone on the mitochondrial membrane potential of *T. cruzi* epimastigotes we used the fluorescent dye Rhodamine 123, as reported previously (23). The parasites were collected by centrifugation at 600 x g for 2 min. and washed in PBS buffer plus 1% glucose. Then the cells were resuspended in the same buffer and loaded with 10 µM of Rhodamine 123 for 40 min. at 29 °C in mild agitation. The loaded parasites were washed twice and resuspended in the same buffer plus 2 µM Probenecid and 2 µM Pluronic acid. Measurements (λ<sub>ext</sub> 488 nm, λ<sub>em</sub> 530 nm) were made in a HITACHI 7000 spectrofluorimeter under stirring at 29 °C. FCCP (1 µM) was added as positive control.

Acidocalcisomes alcalinization.

The effect of dronedarone on the alkalinization of acidocalcisomes was measured using the fluorescence properties of acridine orange as previously described (23). Epimastigotes (10<sup>9</sup> cells/mL) were collected and washed, and then loaded with 2 µM acridine orange in PBS buffer, for 5 min. at 29 °C with constant stirring. Measurements were performed at λ<sub>ext</sub> 488 nm and λ<sub>em</sub> 530 nm at 29 °C in a spectrofluorimeter HITACHI 7000 under continuous stirring. Nigericin (1 µM) was used as positive control.

Results
We first determined the antiproliferative effects of dronedarone and amiodarone on *T. cruzi* epimastigotes grown in axenic LIT medium at 29 ºC. As can be seen in Fig. 2, there is a dose-dependent parasiticidal effect of dronedarone. Interestingly, the IC50 for dronedarone (4.6 µM) was somehow lower than for amiodarone (7.9 µM). Since the clinically relevant stage of *T. cruzi* life cycle is the amastigote form growing inside mammalian host-cells, we determined the effect of dronedarone on the proliferation of these parasites inside VERO cells cultivated at 37 ºC. As shown in Fig. 3, there was a clear dose-dependent response to dronedarone, leading to total disappearance of infected cells at 2 µM of the drug. The IC50 value was 0.75 µM, which again was lower than that obtained previously for amiodarone, 2.7 µM (4).

It has previously been shown that amiodarone is able to induce a large increase in the intracellular Ca2+ concentration in *T. cruzi* epimastigotes (4). Furthermore, it was found that such effect results from the release of Ca2+ from intracellular compartments, since the phenomenon was observed in the presence or absence of extracellular calcium (4). As shown in Fig. 4, dronedarone also induced an increase in the intracellular Ca2+ concentration in these parasites, in the presence (Fig. 4A) or absence (Fig. 4B) of extracellular Ca2+. Interestingly, a biphasic effect on the intracellular Ca2+ is clearly discernible upon addition of dronedarone in both conditions, probably accounting for the induction of the release of Ca2+ from two different intracellular compartments, namely the mitochondrion and the acidocalcisomes, as previously found for the action of amiodarone in *Leishmania mexicana* (23). Thus, amiodarone is now known to cause the death of different trypanosomatids primarily through disruption of intracellular Ca2+ homeostasis, although it also blocks the de novo synthesis of...
sterols at the level of oxidosqualene synthase (4). Specifically, in previous studies it was found that amiodarone acts directly on the parasites’ large mitochondrion, inducing the rapid collapse of the electrochemical proton gradient, and leading to the release of Ca$^{2+}$ to the cytoplasm (4). The electrochemical potential is the driving force for the accumulation of Ca$^{2+}$ in the mitochondrion (2-3,5,27), through a calcium electrophoretic uniporter which its molecular structure has been recently identified (10), and has been found to be very conserved throughout evolution (10,14). For this reason, we next studied the effect of dronedarone on the mitochondrial membrane potential using rhodamine 123, a mitochondrial-specific cationic dye which allows the visualization of the state of the electrochemical potential of this organelle, since this dye is distributed between the internal mitochondrial and cytoplasm depending on the magnitude of the electrochemical potential (4,23). As can be seen in Fig. 5, dronedarone (10 µM) induced a rapid release of rhodamine 123. Addition of FCCP, an uncoupler protonophore (23), did not produce any further effect, thus indicating that dronedarone induced the total collapse of the mitochondrial electrochemical potential. Conversely, when FCCP is added before dronedarone (Fig. 5b), the uncoupler was not able to fully abolish the electrochemical potential, since the further addition of dronedarone induced further release of rhodamine 123. Another interesting result obtained from this experiment was that the rate of the release of rhodamine 123 upon addition of amiodarone (10 µM) was somehow slower than after addition of dronedarone. This was also observed in the experiment where FCCP was added before these drugs (Fig. 5a and 5b).
We also performed experiments on non-infected Vero cells to investigate whether dronedarone affects mammalian cells as well. While dronedarone was not able to exert any effect on the intracellular Ca\(^{2+}\) levels of Vero cells, even at the maximal concentration used in this work (2 µM), a small but reproducible effect was observed on the electrochemical mitochondrial potential with 2 µM dronedarone, as measured by the release of rhodamine 123 (results not shown).

Acidocalcisomes are acidic organelles, also involved in Ca\(^{2+}\) homeostasis in Trypanosomatids, having a larger overall capacity of accumulation of this ion than the mitochondrion (6,9,10). Amiodarone targets the acidocalcisomes in *Leishmania mexicana* (23), inducing their fast alkalinization. Thus, we tested if dronedarone and amiodarone could also affect the acidocalcisomes from *T. cruzi*. The release of acridine orange in the acidocalcisomes was used as a probe for alkalinization, as reported by Docampo *et al.* (11). As depicted in Fig. 6a (grey trace) the addition of 10 µM amiodarone generated a rapid alkalinization of the parasite acidocalcisomes. Additionally, upon adding nigericin, an electroneutral K\(^+\)/H\(^+\) exchanger known to alkalinize the acidocalcisomes of these parasites (11), a further alkalinization response was observed. The same general overall response is observed when dronedarone (10 µM) is added to the preparation (black trace). These results strongly suggested that acidocalcisomes also contribute to the increase in the intracellular Ca\(^{2+}\) concentration induced by both drugs in *T. cruzi*. As was the case for the action of dronedarone on the mitochondrial electrochemical potential, the alkalinization induced by dronedarone occurs more rapidly than that induced by amiodarone.
Additionally, we performed experiments in which nigericin were added before 
dronedarone or amiodarone. (Fig. 6b). In both cases the addition of the drugs 
after the ionophore induced a further release of acridine orange. Since nigericin 
completely disrupts acidocalcisome function, this further effect of the drugs 
probably indicates that another separate compartment is also involved in their 
action.

We also studied the effect of nigericin by itself on the intracellular Ca\textsuperscript{2+} 
concentration (Fig. 4c), showing that this ionophore induced a large Ca\textsuperscript{2+} 
increase. Addition of dronedarone after nigericin induced a further Ca\textsuperscript{2+} 
augment. These experiments support the notion that acidocalcisomes are the 
main intracellular Ca\textsuperscript{2+} compartment in *Trypanosoma cruzi*, but the 
mitochondrion also appears to contribute.

To study whether the effect of dronedarone on epimastigotes was trypanolytic 
or trypanostatic, we measured epimastigote viability through a much shorter 
time course (1-5 hr), demonstrating that during this period there is not any 
discernible effect on cell viability. The trypanocidal action appears only after 24 
hr, observing that 10 µM dronedarone was able to reduce the parasite 
population in about 25% after this period. Thus, dronedarone does not appear 
to induce a rapid trypanosome death (not shown). These experiments 
demonstrate that rapid Ca\textsuperscript{2+} elevation by itself is not able to induce a rapid 
parasite death. However, this was not surprising since it has been previously 
demonstrated that the rapid lethal effect of amphotericin B in *Leishmania 
braziliensis* was not due to a Ca\textsuperscript{2+} entry, since the addition of A-23187, a Ca\textsuperscript{2+} 
ionophore, albeit inducing also a fast intracellular Ca\textsuperscript{2+} increase, failed to induce
the rapid parasiticidal effect observed with amphotericin B (8). In conclusion, the
drug seems to be trypanostatic, at least on epimastigotes over short periods of
time. On the other hand, dronedarone must be trypanolytic on amastigotes
inside Vero cells, since the parasites faded away from the infected cells, after
treatment with the drug.

Discussion

Amiodarone, a commonly used anti-arrhythmic, has recently emerged as
a potential drug candidate for the treatment of Chagas’ disease, either as
monotherapy or in combination with other drugs, such as the azole-based
antifungals posaconazole (4) and itraconazole (19). Nevertheless, and mainly
due to the presence of a 2,5-dihydroxyphenyl moiety in its structure, amiodarone
exhibits a spectrum of undesirable side-effects, causing mainly thyroid,
gastrointestinal and pulmonary toxicity, as well as less toxic side effects such
as cutaneous pigmentation. Dronedarone, an amiodarone-derivative developed
to overcome these limitations, has a similar pharmacological profile, potent anti-
arrhythmic activity, and multichannel blocking properties including Na⁺ channels,
K⁺ currents, L- and T-type Ca²⁺ channels. However, dronedarone has fewer
unwanted side effects and a decreased lipophilicity that improves its
pharmacokinetic properties by lowering its elimination half-time. In this work, we
studied the effects of dronedarone on *T. cruzi*, in order to assess whether this
modified drug retained the trypanocidal properties of amiodarone. Our results
indicate that dronedarone has a dose-dependent effect over the growth of
epimastigotes and on intracellular amastigotes, which are the clinically relevant
parasitic stage. Additionally, we observed that the *IC₅₀* values of dronedarone
against both forms were lower in comparison to that of amiodarone. This difference is higher for the amastigote form (0.75 µM vs. 2.7 µM). It was also found that dronedarone acted more rapidly than amiodarone against its intracellular targets, as depicted in the experiments performed on the mitochondria membrane potential and also on the alkalinization induced by these drugs on the acidocalcisomes.

Similarly to amiodarone, dronedarone induced an increase of intracellular Ca\(^{2+}\) concentration. The scarcity of intracellular esterases, which are essential for the breakdown of the acetoxyimethylesters present in the precursor form of FURA 2, in the epimastigote form of \(T. cruzi\), has been a limitation in previous studies. We successfully addressed this problem by incubating the parasites overnight with the precursor. It is noteworthy that in these experiments it was found that incubation of epimastigotes with amiodarone led to a biphasic response of the intracellular Ca\(^{2+}\) levels, suggesting that this drug induces release of the divalent cation from two different intracellular compartments, the mitochondrion and the acidocalcisomes.

We have previously demonstrated that amiodarone is able to induce alkalinization of acidocalcisomes of promastigotes from \(L. mexicana\) (23). In the present study we found that this effect is also observed in \(T. cruzi\). The interior of these organelles is highly acidic due to the presence of a vacuolar H\(^{+}\)-ATPase which pumps protons from the cytoplasm, inducing in turn the uptake of large amounts of Ca\(^{2+}\) by the combination of numerous ionic transporters, including a Ca\(^{2+}\)-ATPase and several channels and exchangers (12-13). These organelles are essential in Trypanosomatids since they contribute to the internal pH regulation and osmoregulation, and also constitute
the main reservoir of pyrophosphate (and polyphosphates), a known alternative
source of energy, besides ATP, in these parasites (12-13). We found that
dronedarone targets the acidocalcisomes of *T. cruzi*, inducing a rapid
alkalinization, which is enhanced by nigericin, an ionophore that interchanges
K⁺ by H⁺, equilibrating the pH between the interior of the organelle and the
cytoplasm (12).

The other intracellular compartment affected by dronedarone is the
unique mitochondrion present in these parasites. As mentioned, the release of
rhodamine 123 was faster when this drug was used in comparison with
amiodarone. Another interesting effect observed during this work is that the
uncoupler FCCP was not able to fully collapse the electrochemical gradient, but
if amiodarone was added after FCCP full uncoupling action was attained.
However, since we used whole intact parasites (not treated with the sterol-
dependent detergent digitonin) it is possible that the plasma membrane barrier
could limit the access of FCCP to the interior of the cell, thus curtailing its
action. Alternatively, these results are consistent with an effect of dronedarone
on a separate compartment, where FCCP would not have any effect.

The pharmacokinetics of dronedarone is driven by its lipophilicity, with the drug
achieving plasma concentrations that range from 0.01 to 5 µg/mL and
myocardial tissue concentrations ranging from 0.02-500 µg/mL (goats and
dogs) (7,26). Nevertheless, cardiac tissue concentrations have not been
assessed in humans to date. In this work it is shown that the effective range of
dronedarone concentration in amastigote-infected Vero cells varies from 0.75
µM (IC₅₀) to 2 µM (minimal inhibitory concentration). That is approximately 0.4
to 1.1 µg/mL, about two orders of magnitude lower than the maximal level
attained in cardiac tissue under experimental conditions. This reflects by all likelihood that under the reported anti-arrhythmic conventional therapeutic doses, the concentration of dronedarone reached in infected cells would be large enough to exert an adequate parasiticidal effect at the intracellular level.

Based on the present results dronedarone appears to be a promising option for the symptomatic and specific treatment of chagasic cardiomyopathy and other Trypanosomatid infections (such as leishmaniasis), due to its intrinsic antiparasitic activity and better overall safety profile when compared to amiodarone. To verify this hypothesis, work in animal models of Chagas’ disease is currently in preparation.

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References


Induce Parasitological Cure of Mice Infected with *Leishmania mexicana*. 


Figures Legend

Figure 1: Chemical structure of Dronedarone and Amiodarone.

Figure 2: Susceptibility of *Trypanosoma cruzi* epimastigotes to dronedarone. Cultures of *T. cruzi* epimastigotes were exposed to different concentrations of dronedarone and amiodarone, as indicated. Each point represents the mean ± sd of at least triplicate experiments.
Figure 3: Effect of Dronedarone against intracellular amastigotes of *T. cruzi*. Vero cells infected with *T. cruzi* amastigotes were exposed to different concentrations of dronedarone. The percentage of infected Vero cells (filled squares) and the effect on not infected Vero cells (filled circles) were determined at 72 hours after the addition of the drug. At least triplicates were carried out for each experimental condition.

Figure 4: Effect of dronedarone on the intracellular Ca$^{2+}$ concentration of *T. cruzi* epimastigotes. Epimastigotes of *T. cruzi* were loaded overnight, with Fura 2-AM (6 µM) with Probenecid (12µM) and Plouronic acid (12µM) in PBS buffer plus 1% glucose at 29°C, in dark and continuous agitation, and intracellular Ca$^{2+}$ was calculated as described under "Materials and Methods". (A) Effect of 10 µM dronedarone (arrow) on the parasite cytoplasmic Ca$^{2+}$ concentration in the presence of 2 mM Ca$^{2+}$. (B) Effect of 10 µM dronedarone (arrow) on epimastigotes loaded with Fura 2 in absence of external Ca$^{2+}$ (EGTA). (C) Effect of 2 µM nigericin (first arrow) and 10 µM dronedarone (second arrow) on the parasite cytoplasmic Ca$^{2+}$ concentration in the presence of 2 mM Ca$^{2+}$.

Figure 5: Action of amiodarone on the mitochondrial electrochemical potential of *T. cruzi* epimastigotes.. Parasites were incubated in the presence of rhodamine 123 (10µg/mL), for 30 min, at room temperature, as indicated under Materials and Methods. (A) Effect of dronedarone (10 µM), followed by the addition of FCCP (1 µM) on the mitochondrial electrochemical potential (upper black line). Effect of amiodarone (10 µM) followed by the addition of
Effect of FCCP (1 µM), followed by the addition of dronedarone (10 µM) on the mitochondrial electrochemical potential. (upper black line). Effect of FCCP (1 µM) followed by the addition of amiodarone (10 µM) on the mitochondrial electrochemical potential (lower gray line). Arrows indicate the different additions.

Figure 6: Effect of dronedarone on acidocalcisomes from *T. cruzi* epimastigotes. Parasites were loaded with acridine orange (2 µM) as described under Materials and Methods. Excitation wavelength was 488 nm and emission was 530 nm. (A) Upper black trace: Effect of dronedarone (10 µM), followed by the addition of nigericin (Nig, 2 µM) on the acidic level of acidocalcisomes. Lower grey trace: Effect of amiodarone (10 µM), and then nigericin (Nig, 2 µM) on the acidic level of acidocalcisomes. (B) black trace: Effect of nigericin (Nig, 2 µM), followed by the addition of dronedarone (10 µM) on the acidic level of acidocalcisomes. Grey trace: Effect of nigericin (Nig, 2 µM), and then amiodarone (10 µM) on the acidic level of acidocalcisomes.
Fig. 2. Benaim et al. AAC-2012