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Mode of action of Nifurtimox and *N*-oxide-containing heterocycles against *Trypanosoma cruzi*: Is oxidative stress involved?

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ABSTRACT

Chagas disease is caused by the trypanosomatid parasite *Trypanosoma cruzi* and threatens millions of lives in South America. As other neglected diseases there is almost no research and development effort by the pharmaceutical industry and the treatment relies on two drugs, Nifurtimox and Benznidazole, discovered empirically more than three decades ago. Nifurtimox, a nitrofurane derivative, is believed to exert its biological activity through the bioreduction of the nitro-group to a nitro-anion radical which undergoes redox-cycling with molecular oxygen. This hypothesis is generally accepted, although arguments against it have been presented. In the present work we studied the ability of Nifurtimox and five *N*-oxide-containing heterocycles to induce oxidative stress in *T. cruzi*. *N*-Oxide-containing heterocycles represent a promising group of new trypanosomicidal agents and their mode of action is not completely elucidated. The results here obtained argue against the oxidative stress hypothesis almost for all the studied compounds, including Nifurtimox. A significant reduction in the level of parasitic low-molecular-weight thiols was observed after Nifurtimox treatment; however, it was not linked to the production of reactive oxidant species. Besides, redox-cycling is only observed at high Nifurtimox concentrations (>400 μM), two orders of magnitude higher than the concentration required for anti-proliferative activity (5 μM). Our results indicate that an increase in oxidative stress is not the main mechanism of action of Nifurtimox. Among the studied *N*-oxide-containing heterocycles, benzofuroxan derivatives strongly inhibited parasite dehydrogenase activity and affected mitochondrial membrane potential. The indazole derivative raised intracellular oxidants production, but it was the least effective as anti-*T. cruzi*.

1. Introduction

Chagas disease or American trypanosomiasis is caused by the trypanosomatid parasite *Trypanosoma cruzi* (*T. cruzi*) and threatens millions of lives in South America. As other neglected diseases, it has not received much attention by the pharmaceutical industry mainly due to economic considerations. Actual treatment of Chagas' disease relies on two drugs, Nifurtimox (Nfx, Lampit®, recently discontinued by Bayer) and Benznidazole (Bnz, Rochagan®, Roche, now produced by LAFEPE, Brazil) discovered empirically more than three decades ago [1]. Both clinically used drugs are nitroheterocycle compounds, Nfx is a nitrofurane derivative (Fig. 1) while Bnz is a nitroimidazole one. Whereas several studies have been performed in the mode of action of these drugs, knowledge on this subject is still incomplete [2–4]. Similar to other nitrocompounds, Nfx and Bnz are believed to exert their biological activity through the bioreduction of the nitro-group [3,5]. This process begins with the reduction of the nitro-group to a nitro-anion radical, in a reaction catalyzed by a putative NADPH/NADH-nitroreductase [3]. The fate of this radical differs among these drugs. Nifurtimox radical undergoes redox-cycling with molecular oxygen, which produces superoxide anion ($O_2^{\bullet -}$) and consequently hydrogen peroxide (H_2O_2) through a superoxide dismutase catalyzed reaction (Scheme 1). These species, in the presence of iron, form the strong oxidant hydroxyl radical via Haber–Weiss reaction. Increased production of reactive oxidant species (ROS) would eventually lead to oxidative stress in the parasite that, in addition, was thought to be weak in endogenous

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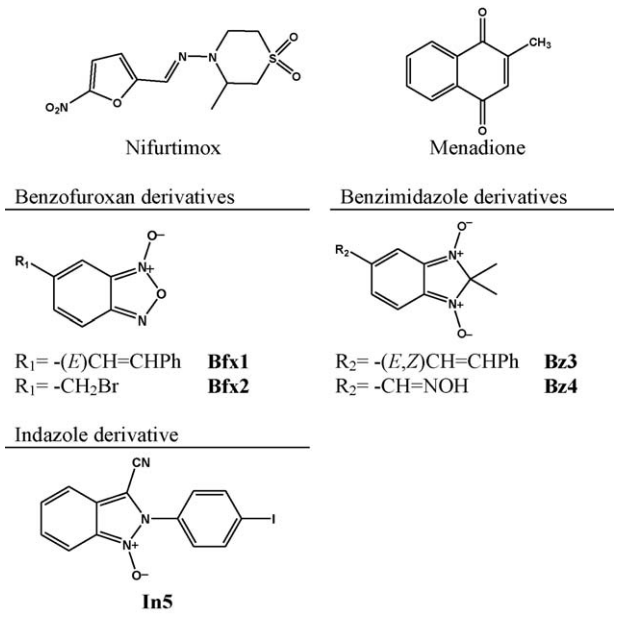
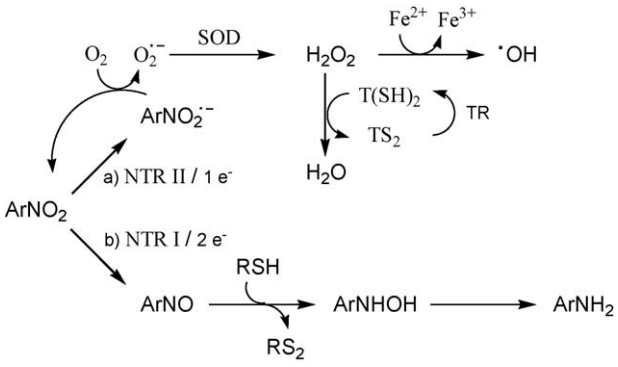


Fig. 1. Structure of the reference drugs, Nifurtimox and Menadione, and the five *N*-oxide-containing heterocycles, Bfx1, Bfx2, Bz3, Bz4 and In5, used in the present study.

anti-oxidant enzymes. The hypothesis that the trypanosomicidal activity of Nfx is dependent on its redox-cycling with O_2 is supported by different lines of evidence: (a) addition of Nfx to *T. cruzi* homogenates led to the formation of the corresponding nitro-anion radical as observed by electron spin resonance (ESR) [6], (b) Nfx increased cyanide-insensitive oxygen uptake [6] and steady-state concentration of $O_2^{\bullet -}$ and H_2O_2 [3,7] and (c) depletion of low-molecular-weight thiols (mainly glutathione and trypanothione) in the parasite was observed after Nfx treatment [8]. Other possibilities, however, cannot be completely excluded. Among them, nitro-reduction in a multiple electron process would lead to the intermediate formation of the deleterious nitroso- and hydroxylamine derivatives [9–12] (Scheme 1).

The action of Nfx through oxidative damage has also been grounded in the differences observed between trypanosomatids and higher eukaryotes regarding anti-oxidant defenses. In contrast to all other eukaryotes, trypanosomatids lack catalase (except *Crithidia fasciculata*) and selenium-containing glutathione perox-



Scheme 1. Bioreduction of nitroaromatic drugs ($ArNO_2$). Adapted from [12]. (a) One-electron reduction by type II nitroreductases (NTR) promoting redox-cycling with superoxide/H_2O_2 formation and drug regeneration. ROS production oxidizes parasite thiols. (b) Two-electron reduction by type I NTR yielding the nitroso-compound (ArNO), a good scavenger of thiols. SOD = superoxide dismutase; $T(SH)_2$ = reduced trypanothione, TS_2 = oxidized trypanothione; TR = trypanothione reductase.

idases, the thiol metabolism being based on trypanothione, a glutathione spermidine conjugate [13–15]. The absence of glutathione-dependent hydrogen peroxide metabolism was regarded for a long time as a target for trypanosomicidal drug design through increased intracellular H_2O_2 steady-state concentrations [16–20]. More recently, however, five peroxidases have been identified in *T. cruzi*: two tryparedoxin peroxidases, one cytosolic (TcCPX) and one mitochondrial (TcMPX), which efficiently detoxify H_2O_2, small-chain organic hydroperoxides [21] and peroxynitrite [22,23]; two glutathione-dependent peroxidases (TcGPX I and TcGPX II also able to work with the tryparedoxin/trypanothione system) that detoxify fatty acid and phospholipid hydroperoxides but not H_2O_2 [24,25], and an ascorbate-dependent hemeperoxidase (TcAPX) located at the endoplasmic reticulum, that efficiently detoxify H_2O_2 [26]. Similar to higher eukaryotes, $O_2^{\bullet -}$ elimination in trypanosomatids is based on the activity of superoxide dismutases (SODs), but trypanosome SODs belong to the Fe class, a group restricted to protozoans, prokaryotes and plants [27]. Overall, the parasite has an effective and complex system to deal with oxidative stress in contrast to previous reports. Besides, there is conflicting data regarding Nfx mechanism. Overexpression of peroxidases in *T. cruzi* is not able to protect the parasite from Nfx damage [21] and *C. fasciculata*, a trypanosomatid that has catalase activity, is as susceptible as *T. cruzi* to Nfx [28]. More recently, *T. cruzi* old yellow enzyme (TcOYE), a NADH oxidoreductase that contains flavin mononucleotide as the prosthetic group, was shown to reduce a variety of trypanosomicidal drugs, including napthoquinones and nitroheterocycles [29]. Interestingly, Nfx reduction by TcOYE occurs via two electrons and no radical intermediate was observed. When this protein was immunoprecipitated from epimastigotes lysates, the majority of the reductase activity was abolished, which implicates TcOYE as a key drug-metabolizing enzyme [29]. Therefore, it would be worthwhile to revise the mode of action of Nfx on this new scenario.

Whereas Nfx (and Bnz) are effective in the treatment of acute Chagas' disease, efficacy in the chronic stage is limited probably due to inadequate pharmacokinetics [30]. Besides, toxic side effects are often encountered, which have been assigned to the nitro-moiety that acts as a nonselective redox-damaging function. In the search for more selective and less toxic anti-chagasic drugs, we have developed heterocyclic derivatives possessing an *N*-oxide moiety as the bioreductive pharmacophore [31], representing different skeletal types: benzofuroxans [31–34], benzimidazole *N*-oxides [35,36], indazole *N*-oxides [37] and quinoxaline dioxides [38]. Among these families, a number of promising candidates for the development of anti-chagasic drugs have been found and have been subjected to further studies (mammal metabolism, toxicity and *in vivo* activity) [35,39]. The structural features determining the ability of these derivatives to inhibit parasite growth are not quite clear, but a bioreduction process is presumed to be involved. The electrochemical and biological (with *T. cruzi* homogenates) reduction have been verified for benzofuroxan and indazole *N*-oxide derivatives using cyclic voltammetry and ESR [31,37,40]. While only the heterocyclic radical was observed for benzofuroxans, the hydroxyl radical was also observed when the indazole *N*-oxides were incubated with *T. cruzi* homogenates. Theoretical studies performed on all the *N*-oxide derivatives pointed to the significance of the electrophilic character of the molecule and the participation of the heterocycle in the reduction process [41]. Altogether the data lead to propose that, similarly to Nfx, these *N*-oxide-containing heterocycles could promote oxidative stress.

Recently, plans to extend the use of Nfx in the treatment of African sleeping sickness (caused by *T. brucei*) are being evaluated [42], which have led to a renewed interest in the study of its mode of action. Besides, the search for new anti-chagasic drugs continues

to help overcome drug resistance and drug toxicity, and probably requires looking for compounds that have different mechanisms of action than the reference drugs. In this work, we studied the ability of Nfx and five *N*-oxide-containing heterocycles (Fig. 1) to induce oxidative stress in *T. cruzi* parasites. The naphthoquinone Menadione (Mena), a well-known inducer of oxidative stress, was included as a positive control. The findings presented herein have implications for both the use of Nfx and the development of new anti-chagasic drugs.

2. Materials and methods

2.1. Chemicals

Unless otherwise stated, all chemicals were from Sigma–Aldrich (USA) or Merck (Germany). The following *N*-oxide-containing heterocycles (Fig. 1) were obtained from our in-house chemical library [43]: 5-*E*-phenylethenylbenzo[1,2-*c*][1,2,5]-oxadiazole *N*-oxide (Bfx1) [32], 5-bromomethylbenzo[1,2-*c*][1,2,5]oxadiazole *N*-oxide (Bfx2) [34], 2,2-dimethyl-5-phenylethenyl-2*H*-benzimidazole 1,3-dioxide (Bz3) (as an isomeric mixture (*E*:*Z*, 2:8)) [35], 2,2-dimethyl-5-(hydroxyimino)methyl-2*H*-benzimidazole-1,3-dioxide (Bz4) [35], 3-cyano-2-(4-iodophenyl)-2*H*-indazole *N*-oxide (In5) [37]. Nfx was purchased from Bayer AG Leberkusen (Germany).

2.2. Parasite culture

T. cruzi epimastigotes (Tulahuen 2 strain) were grown at 28 °C in axenic medium containing brain-heart infusion (33 g L^{-1}), disodium hydrogen phosphate (4 g L^{-1}), 10% fetal calf serum, streptomycin (0.2 g L^{-1}), and penicillin (200 000 U/L^{-1}) (culture medium) [44]. Mid-log phase parasites were harvested (approximately 5 days) by centrifugation and washed twice in 0.14 M NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 15 mM KH_2PO_4, pH 7.4 (medium A). Parasites were counted using a Neubauer chamber and protein concentration was determined by the BCA protein assay kit (Sigma–Aldrich Co.) (1 mg of protein ~2×10^8 cells).

In order to determine the effects of the drugs on growth, mid-log phase parasites, 4×10^6 cells mL^{-1} in culture medium were seeded in 24-well plates, and DMSO-stock solutions of the compounds were added (DMSO in the culture medium never exceeded 1% (v/v)). A control condition using 1% DMSO was included in all experiments. Parasite growth was followed by nephelometry (absorbance at 610 nm) for 5 days and the percentage of growth inhibition at different drug concentrations were determined as previously described [31]. Absorbance readings were directly proportional to cell density. The IC_{50} value corresponds to the drug concentration that produces a 50% reduction in parasite growth as compared to control (no drug added). In a second protocol, parasites (4×10^6 cells mL^{-1}) were exposed to different concentrations of the drugs for 4 h and afterwards, cells were harvested and washed twice in culture medium to eliminate non-incorporated drugs. Treated parasites were then resuspended in sterile drug-free culture medium and growth was followed as described above for 5 days. The IC_{50} determined in this case was termed LC_{50} (Table 1).

2.3. Drug uptake

Mid-log phase *T. cruzi* parasites (4–8 $\times 10^7$ cells mL^{-1}) were seeded in 6 mL of medium A supplemented with 5.5 mM glucose (medium A/G). Drugs were added to the medium at 40 μM and the mixture was incubated at 28 °C. Every 15 min and for a 1 h period, 700 μL aliquots were centrifuged (5000 rpm, 5 min) and the concentration of the drug was determined in the supernatant using the corresponding extinction coefficient (ε): Nfx, ε_{402} = 13200 M^{-1} cm^{-1}; Bfx1, ε_{295} = 8100 M^{-1} cm^{-1}; Bfx2, ε_{352} = 7300 M^{-1} cm^{-1}; Bz3, ε_{465} = 6000 M^{-1} cm^{-1}; Bz4, ε_{502} = 4600 M^{-1} cm^{-1}. Stability of each compound in culture medium A was confirmed (up to 1 h), and only derivative In5 precipitated with time. Initial rates of uptake are expressed in nanomoles of compound h^{-1} mg^{-1} of protein (Table 2).

Table 1
Anti-proliferative activity against *T. cruzi* epimastigotes (Tulahuen 2 strain).[a].

Drug	IC_{50} (μM)	LC_{50} (μM)[b]
Nfx	7.0[c]	25.0
Bfx1	9.5[c]	6.0
Bfx2	7.0[c]	<10.0
Bz3	4.0[c]	15.0
Bz4	12.5[c]	100.0
In5	25.0[c]	>100.0
Mena	25.0	ND

ND = not determined.
[a] For experimental details see Section 2.2.
[b] The values are the means of three different experiments within an error less than 10%.
[c] Taken from Ref. [43] and references therein.

Table 2
Drug uptake by *T. cruzi* epimastigotes.[a].

	Drug uptake (nmol h^{-1} mg^{-1} protein)
Nfx	27
Bfx1	86
Bfx2	82
Bz3	133
Bz4	88

[a] For experimental details see Section 2.3.

2.4. Analysis of intracellular low-molecular-weight thiols

T. cruzi parasites (4×10^7 cells mL^{-1}) in culture medium were incubated with the drugs for 4 h at ~4 times the IC_{50} (g) values (Nfx, Bfx1, Bfx2, and Bz3 at 25.0 μM, In5, and Bz4 at 100.0 μM). Mena (25.0 μM) was used as a positive control. After treatment, parasite low-molecular-weight thiols were determined using Ellman's method as previously described [45]. Briefly, parasites (2.8×10^8 cells mL^{-1}) were permeabilized with 0.5% Triton X-100, followed by centrifugation at 13 000 rpm for 3 min. Supernatants were treated with 0.5 M $HClO_4$ to precipitate proteins, then neutralized before adding Ellman's reagent. Calibration curves were performed with known amounts of glutathione (GSH).

2.5. Potential direct reaction of Bfx1 and Bfx2 with GSH

The possible participation of benzofuroxan derivatives in a direct reaction with thiols was studied using GSH, following the reaction by UV-spectroscopy. Spectral changes were examined using a SHIMADZU-UV 1603 dual wavelength spectrophotometer with two chambers each with 1 cm optical path length allowing spectra to be obtained before and after mixing. Ethanolic stock solutions of Bfx1 or Bfx2 (0.1 mM, 0.2 mM and 1.0 mM) were diluted in phosphate buffer (50 mM, pH 7.4) to give final concentrations of 20.0–200.0 μM (ethanol 2% (v/v)), and these were mixed with freshly prepared GSH (100 mM, 1 mM diethylenetriaminopentaacetate) to give a final GSH concentration of 1.0 mM. Spectral changes in the region 260–600 nm were recorded with time (up to 70 min).

2.6. Oxygen uptake

Oxygen uptake was determined with a water-jacketed Clark-type electrode (YSI Model 5300) using 1.5 mL cell suspension (6–15 × 10^7 parasites) in medium A/G at 28 °C as previously [44]. The electrode was calibrated with air-saturated distilled water at 28 °C (220 μM O_2). The amount of parasites used in the assay was equivalent to 0.2–0.5 mg protein mL^{-1}. Drugs were directly added to the chamber using a gas-tight Hamilton syringe, and the oxygen consumption was registered, in the absence and in the presence of 2 mM KCN.

2.7. Drug-dependent intracellular oxidants production

The non-fluorescent probe dihydrorhodamine 123 (DHR) was used to detect the intracellular production of reactive oxygen and nitrogen species (ROS and RNS) by measuring its oxidation to the fluorescent product rhodamine 123 (RH) [46,47]. Parasites (4 × 10^7 cells mL^{-1}) were loaded with DHR (50 μM) for 20 min at 28 °C and washed with medium A in order to eliminate non-incorporated probe. Cells were resuspended in medium A and drugs were added at 25.0 μM. At different time intervals, RH fluorescence was determined at 520 nm (λ_{exc} = 485 nm) in a Fluostar Galaxi BMG, Germany. When interference due to drug absorption was observed (for In5, Bz3 and Bz4) the cells were washed and resuspended in drug-free medium A right before RH detection.

In order to determine the ability of the drugs to produce redox-cycling increasing the production of H_2O_2, the release of this molecule to the medium was analyzed. Mid-log phase parasites were resuspended in medium A at different cell densities (0.5–3.5 × 10^8 mL^{-1}) and incubated in the absence or presence of 25.0 μM of the drugs for 30 min at 28 °C. Mena at 10.0 μM was used as positive control. At different time points, cells were pelleted and H_2O_2 was determined in the supernatant using *p*-hydroxyphenylacetic acid (*p*HPA, 2 mM) and horseradish peroxidase (HRP, 10 nM) in 100 mM pyrophosphate buffer, pH 9.2. After 5 min, *p*HPA dimer formation was determined by fluorescence at 400 nm (λ_{exc} = 323 nm) in a Aminco-Bowman Series 2 spectrofluorimeter [48].

Protein nitrotyrosine formation was used as a marker of peroxynitrite ($ONOO^-$) production [49]. Cells were lysed with 0.1% Triton X-100 and proteins (100 μg) were analyzed on SDS-PAGE (10%) followed by Western blotting onto nitrocellulose membranes probed with a polyclonal anti-nitrotyrosine antibody prepared in our lab as previously described [45]. Bovine serum albumin (BSA) treated with a slight excess of peroxynitrite was used as a positive control for nitrated protein and the BSA treated with previously decomposed peroxynitrite as a negative control.

2.8. Dehydrogenase activity

Reduction of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by NAD(P)H-dependent dehydrogenases is widely used as a measure of cell proliferation and viability. Herein, the MTT test was used to reflect dehydrogenase activities following parasite exposure to the different compounds [50,51]. Parasites (4 × 10^7 cells mL^{-1}) in medium A/G were seeded in 24-well plates (1 mL $well^{-1}$) and 5 μL of each drug was added in duplicates at ~4 times the IC_{50} (concentrations indicated in Fig. 6). Two wells with untreated parasites were maintained as controls. Cultures were incubated at 28 °C and at different time points, 100 μL aliquots were used for the colorimetric MTT dye-reduction assay in 96-well plates [52]. Briefly, 10 μL of a solution containing 5 mg mL^{-1} MTT in PBS was added to each well and the plates were incubated for an additional 2 h. The reaction was stopped by addition of 100 μL of acidic isopropanol (0.4 mL 10 N HCl in 100 mL isopropanol) and after 30 min, the absorbance was measured at 610 nm in a microplate reader (Bio-tek EL301, USA).

2.9. Mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\Psi_m$) was evaluated using the lipophilic cation dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) [53]. JC-1 presents potential-dependent accumulation in mitochondria, indicated by a shift in fluorescence emission from green (λ_{em} 520 nm) to red (λ_{em} 590 nm). Parasites (3 × 10^8 cells mL^{-1}) were incubated with Bfx1 (25.0 μM) in medium A or just DMSO as control. At time intervals, aliquots (3 × 10^7 cells mL^{-1}) were taken and cells were loaded with JC-1 (2 μM) 20 min before measurement. Fluorescence detection was performed using black 96-well plates in a fluorescence microplate reader at λ_{ex} 485 nm and λ_{em} 520 and 590 nm. A decrease in the red/green fluorescence intensities is indicative of mitochondrial membrane depolarization.

3. Results

3.1. Drug effect on cell viability and proliferation

The effect of the drugs on parasite proliferation was analyzed by determining two parameters, cell growth and cell viability (Table 1). In the cell growth assay, parasites were grown in the presence of the drug, whereas in the viability assay, parasites were exposed to the drug for 4 h and then resuspended in drug-free medium to follow growth. As previously observed, all the drugs were able to inhibit parasite growth, Bz3 being the most potent and In5 the least active compound (IC_{50} = 4.0 and 25.0 μM, respectively). The naphthoquinone derivative Mena, a well-known redox-cycling agent, showed anti-proliferative activity similar to the least active derivative In5. A different activity profile was observed for the cell viability assay. Here, the benzofuroxan derivatives Bfx1 and Bfx2 were the most potent (LC_{50} < 10 μM), whereas the benzimidazole derivative Bz4 was the least active compound (LC_{50} = 100.0 μM). Notably, the indazole In5, had no effect on cell viability at the concentrations studied (LC_{50} > 100 μM).

3.2. Drug uptake

Drug uptake by the parasite, determined as disappearance of the compound from the media, was similar among all *N*-oxide-containing heterocycles assayed and dependant on cell density (Table 2). Nfx uptake by *T. cruzi* was determined at a rate of 27 nmol h^{-1} mg^{-1} protein, in agreement with a previous report [6], and it was 2–3-fold lower than the rate of uptake observed for the *N*-oxide derivatives used in this study.

3.3. Drug-induced low-molecular-weight thiol depletion

Since low-molecular-weight thiols are the first line of defence against oxidative damage, the effect of these drugs on free thiol levels was determined and compared to control (untreated parasites) using Ellman's reagent (Fig. 2). The effect of Nfx on low-molecular-weight thiols in *T. cruzi* has already been studied using different strains and life-cycle stages [8,54]. Using the epimastigote form, Morello and co-workers [54] observed around 30–40% decrease in free thiols after 2-h treatment with Nfx. Herein, using a different *T. cruzi* strain, we also observed a 30% decrease in reduced thiols after a short period of treatment (2 h, data not shown). At longer incubation periods (4 h in this study), the effect was less pronounced, around 20% decreased (Fig. 2). These results could imply that, following an initial drop, thiols are being recovered. In fact, when thiols were analyzed after a

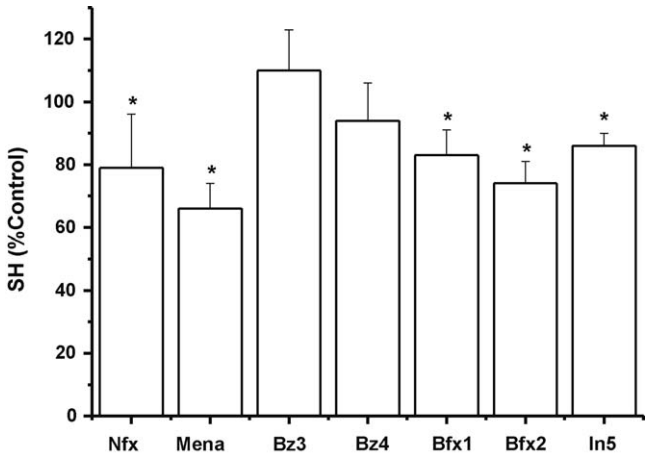


Fig. 2. Effect of Nfx, Mena, and the *N*-oxide derivatives on intracellular low-molecular-weight thiol levels in *T. cruzi* determined by Ellmans' method as described in Section 2. Thiol concentration in the control condition was 26.8 nmol SH mg^{-1} protein. Drug concentrations were 25.0 μM for Nfx, Mena, Bfx1, Bfx2, Bz3, and 100.0 μM for Bz4, In5. The incubation time was 4 h. Results are expressed as the percentage of the control and represent means ± SD for at least three independent experiments. Asterisks indicate statistical difference with control in Mann–Whitney's test ($p < 0.01$).

prolonged drug treatment (5 days) no difference with control was observed (data not shown).

Glutathione depletion has been associated with Mena toxicity in different cell lines [55] but information on its effect on *T. cruzi* cells is scanty. We observed that after treating the parasites with Mena (25.0 μM for 4 h), reduced thiols dropped by 30% (Fig. 2).

Among the *N*-oxide derivatives, Bfx1, Bfx2 and In5 were also observed to decrease thiol levels, approximately 10–20% after 4 h. Since benzofuroxan itself has previously been suggested as a thiol probe [56], we analyzed the possibility that benzofuroxan derivatives (Bfx1 and Bfx2) could be participating in a direct reaction with thiols. Benzofuroxan heterocycle presents three electrophilic centers likely to be attacked by a thiolate anion, leading to oxidation of the thiol to the corresponding disulphide and the transformation of the benzofuroxan to the corresponding *o*-benzoquinone dioxime. Bfx1 and Bfx2 were incubated in the presence of GSH and the reaction was followed spectrophotometrically at 25 °C. Different concentrations of the drugs and GSH and different incubation times were assayed, but, as previously observed for another 5-substituted benzofuroxan [57], no change in Bfx1 nor Bfx2 spectrum was detected. Since Bfx1 and Bfx2 are 5-substituted benzofuroxan, this result could imply that a non-substituted benzofuroxan is necessary for the reaction with a thiolate. Therefore, thiol depletion observed after Bfx1 and Bfx2 treatment cannot be explained by direct chemical reaction of thiols with the drugs.

3.4. Oxidant species production

Having established that some of the drugs (Nfx, Bfx1, Bfx2 and In5) induce loss of low-molecular-weight thiols, any corresponding increase in the levels of ROS/RNS within the cell was monitored using the cell permeant probe DHR (Fig. 3A). In cellular systems, DHR can be oxidized to the fluorescent cationic product RH by different ROS/RNS via a radical mechanism [47].

A time-dependent increase in RH fluorescence was observed upon treatment with Mena, in agreement with its redox-cycling activity and concomitant ROS production (Fig. 3A). Parasites treated with Nfx, however, showed no DHR oxidation even at concentrations as high as 300.0 μM, one-order of magnitude above the concentration required to depress thiols (Fig. 3A and B). Regarding the *N*-oxide-containing heterocycles, different outcomes were observed in accordance to the results obtained with cellular thiols levels. Benzimidazole derivatives Bz3 and Bz4 had no effect on DHR oxidation, whereas, indazole derivative In5 led to an increase in fluorescence (Fig. 3B). Remarkably, Bfx1 and Bfx2 showed a time-dependant loss of basal fluorescence. The possible implication of this is discussed later.

According to Giulivi et al. [7] Nfx is able to increase intracellular H_2O_2 concentration in *T. cruzi* epimastigotes due to its putative redox-cycling activity, stimulating the release of H_2O_2 to the extracellular medium, which is in contradiction with our findings on DHR oxidation. In that work [7] parasites were incubated with the drug, and H_2O_2 concentration was measured in the culture supernatant following the formation of HRP-compound II in a dual wavelength spectrophotometer. Using this technique these authors [7] reported a basal parasite intracellular concentration of H_2O_2 of 1.5 μM, while others argue that no H_2O_2 release is observed in intact *T. cruzi* cells [58]. To address this question, parasites were incubated with the drugs and H_2O_2 was measured in the supernatant using the *p*HPA/HRP system (detection limit = 50 nM) (Fig. 4). Under our assay conditions no release of H_2O_2 was observed in control parasites (no drug added) in accordance with Docampo and Moreno [58], but surprisingly, the same result was obtained for Nfx (25.0 μM) treatment, that

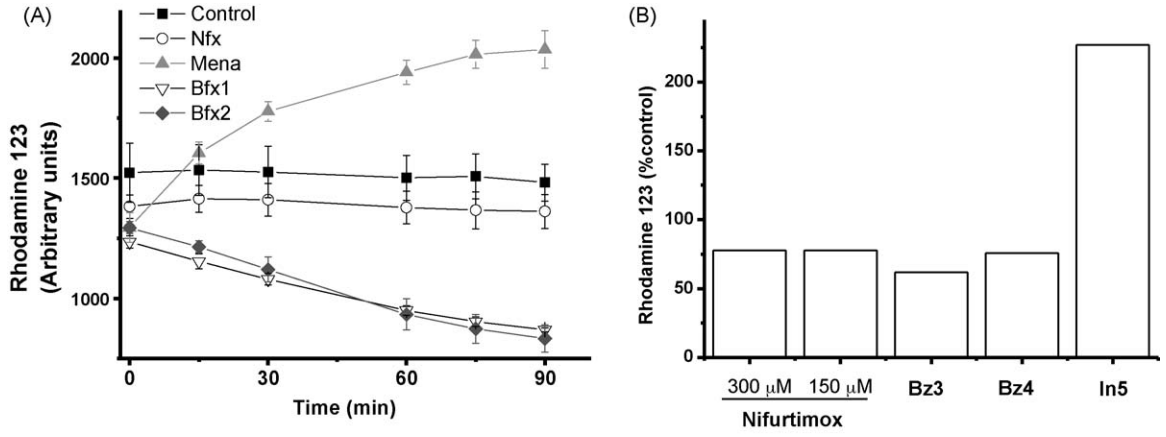


Fig. 3. Production of ROS/RNS on *T. cruzi* parasites after drug treatment (for details see Section 2). (A) Parasites pre-loaded with DHR were incubated with 25.0 μM dose of the drugs and at intervals, RH fluorescence was measured. Results are expressed in arbitrary units as means ± SD of triplicate measurements. (B) Parasites pre-loaded with DHR were incubated with the drugs during 30 min. Cells were then washed and RH fluorescence was measured. Results are expressed as percentage of the control and are average of two experiments with less than 10% SD.

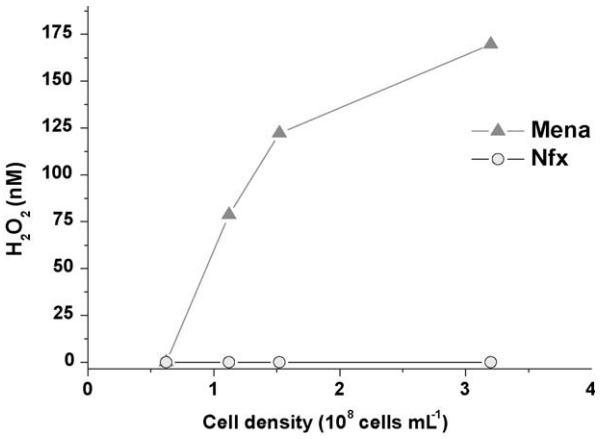


Fig. 4. Production of H_2O_2 by *T. cruzi* treated with Nifurtimox and Menadione. Parasites were incubated with the drugs (10.0 μM Mena, 25.0 μM Nfx) for 30 min and then H_2O_2 concentration was determined in the supernatant using *p*HPA/HRP as described in Section 2.7. Results represent one typical experiment out of three performed.

reinforces our previous observation on absence of DHR oxidation by Nfx. Mena treatment (10.0 μM), on the other hand, promoted H_2O_2 release that was detected in the supernatant as a function of cell density (Fig. 4). Regarding the *N*-oxide-containing heterocycles, none of them were able to produce detectable amounts of H_2O_2 using this sensitive fluorimetric method.

Finally, the possible participation of these drugs in a nitrosative process was analyzed by western blot detection of protein-3-nitrotyrosine formation. Nitrotyrosine is used as a biological marker of RNS formation *in vivo* [49]. Faint bands appeared (molecular weights lower than 30 kDa) but no significant increased intensities were observed for the treated parasites (25–100 μM) *versus* control (data not shown), suggesting the mechanism of action of these drugs do not involve the increased generation of peroxynitrite.

3.5. Drug effect on oxygen uptake and redox-cycling

Mitochondrial oxidative processes play a central role in the maintenance of cellular energy supply, thus, a deleterious effect of drugs on mitochondrial function could have serious consequences on parasite viability. Normal mitochondrial aerobic metabolism yields some ROS production that could increase by drug-mediated mitochondrial alterations. The ability of the drug to act as a redox inhibitor or a redox cycler was analyzed by measuring the oxygen consumption (QO_2) immediately after drug addition to *T. cruzi* cells. While a redox inhibitor would lead to a reduction in QO_2, a

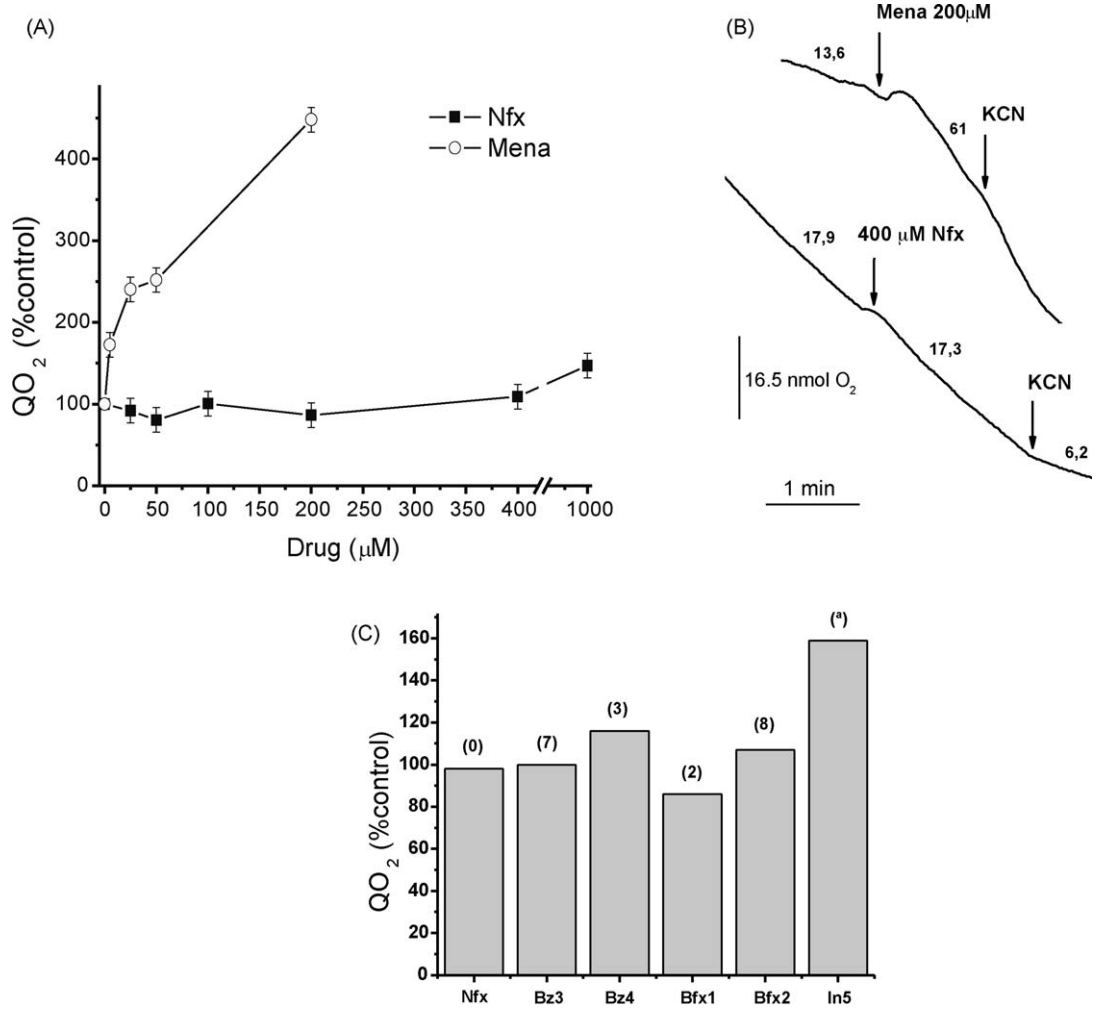


Fig. 5. Drugs effect on oxygen uptake (QO_2) and redox-cycling. (A) Dose dependent effect of Nfx and Mena upon *T. cruzi* QO_2. Results are expressed as percentage of the control (QO_2 = 13.6–17.9 nmol O_2 min^{-1} mg^{-1} protein) and correspond to mean ± SD of three independent experiments. (B) Effect of KCN (2 mM) on QO_2 by Mena (200.0 μM) and Nfx (400.0 μM). Numbers above traces indicate the rate of oxygen uptake in nmol O_2 min^{-1} mg^{-1} of protein. (C) QO_2 after the addition of 200.0 μM drugs. Results are expressed as percentages of the control and correspond to the average of two independent experiments. Numbers in parentheses indicate percentage of QO_2 remanent after the addition of 2 mM KCN. [a]Direct reaction of In5 with KCN is observed.

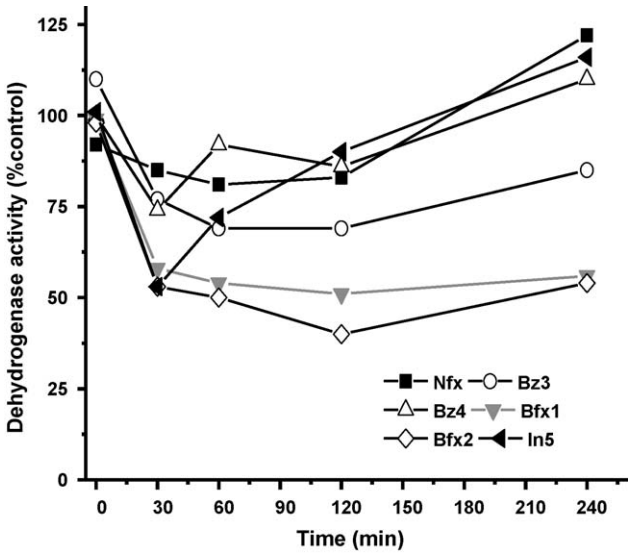


Fig. 6. Effect of *N*-oxides and Nfx on *T. cruzi* dehydrogenase activity. Parasites were incubated with the drugs (Nfx, Bfx1, Bfx2, Bz3 at 25.0 μM and Bz4, In5 at 100.0 μM) and at intervals an aliquot was taken to measure dehydrogenase activity as described in Section 2.8. Results are expressed as percentage of the control and correspond to averages of two independent experiments with less than 10% SD.

redox cycler is expected to increase oxygen uptake, being this consumption insensitive to cyanide, a known inhibitor of c-terminal oxidase. The presence of alternative terminal oxidases in *T. cruzi* [59] led to a small amount of QO_2 insensitive to cyanide inhibition (7% in our assay conditions).

Previous data on the effect of Nfx on intact *T. cruzi* cells showed a concentration-dependent increase in QO_2, but using high Nfx concentration (above 300 μM) [6,60]. There is no previous data on the effect of low Nfx concentrations, in the range required for anti-proliferative activity (around 25 μM, Table 1). Therefore, a study of Nfx effect on QO_2 was conducted using a wide range of concentrations (25.0–1000.0 μM) (Fig. 5). Interestingly, in the range of 25.0–200.0 μM, Nfx rendered similar QO_2 to control and it was inhibited in the presence of cyanide (Fig. 5A and B). At 400.0 μM Nfx, no increase in total oxygen uptake was observed but cyanide-insensitive QO_2 rose to 36%, in agreement with previous data (Fig. 5B). Only at 1 mM Nfx, a net increase in QO_2 was observed and approximately 50% was cyanide-insensitive. It is difficult to link this redox-cycling activity, observed at high Nfx concentrations, with the anti-proliferative activity observed at low Nfx concentration. For comparison, the effect of Mena on QO_2 was studied (Fig. 5A and B). Mena was able to increase QO_2 even at 5.0 μM and the effect was more pronounced than that observed for Nfx at 1 mM (Fig. 5A).

Among *N*-oxide derivatives, indazole derivative In5 showed a marked increase in QO_2 (50%) immediately after addition (Fig. 5C). Although cyanide-insensitive QO_2 could not be measured due to drug reaction with KCN, this result along with data obtained on thiols and DHR assays support the existence of In5 redox-cycling activity.

3.6. Drug effect on dehydrogenase activity

It is generally believed that MTT is reduced to form a formazan product by accepting electrons from cellular reducing equivalents (i.e. NADH, NADPH), indicating that MTT reduction is dependent on cellular redox activity, thus, a reflection of mitochondrial function. However, different cell types differ markedly in their substrate-preferences for MTT reduction. Whereas for some cell types MTT reduction reflects mitochondrial dehydrogenase activity, for other

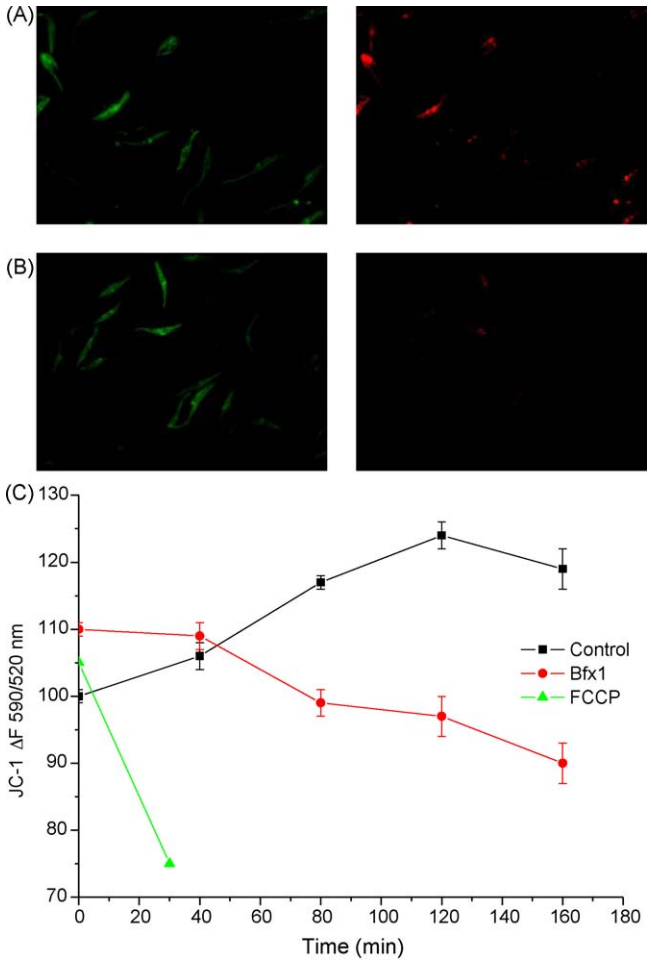


Fig. 7. Effect of Bfx1 on mitochondrial membrane potential. $\Delta\Psi_m$ was evaluated using the cationic dye JC-1 in entire parasites incubated in the presence of DMSO (control) or 25.0 μM Bfx1. Fluorescence microscopy of control parasites (A) and Bfx1-treated parasites (B) after 3 h. Parasites were charged with JC-1 20 min prior fluorescence reading. JC-1 monomer was visualized using 485 nm excitation and 520 nm emission (left panel) and J-aggregate accumulated in mitochondria was visualized using 535 nm excitation and 590 nm emission (right panel). (C) Ratio of fluorescence 590/520 nm (λ_{exc} 485 nm) as a function of time of incubation with the drug. FCCP was added as a positive control of membrane depolarization (experimental details in Section 2.9).

cell types it is mainly ascribed to cytosolic redox activity [61]. In the present work, MTT reduction was used as a measure of overall dehydrogenase activity. Parasites were incubated with the drugs for 4 h and at time intervals formazan formation was determined (Fig. 6).

Nfx showed no effect on dehydrogenase activity at the assayed concentration (25.0 μM). Interestingly, all *N*-oxide-containing heterocycles produced inhibition on formazan formation. Benzimidazole derivatives, Bz3 and Bz4, showed an initial drop on dehydrogenase activity, but recovered activity with time. For indazole derivative In5, a similar behaviour was observed but the initial drop was more pronounced (~50%) and it took longer to recover activity. On the other hand, benzofuroxan derivatives Bfx1 and Bfx2 showed an important decrease on dehydrogenase activity (~50%) that was maintained up to 4 h.

3.7. Effect of benzofuroxan derivative Bfx1 on mitochondrial membrane potential

In the previous experiment using DHR (Fig. 3) we observed a loss of basal RH fluorescence when parasites were treated with both benzofuroxan derivatives, Bfx1 and Bfx2. Since rhodamine

RH, a cationic probe, is accumulated in the mitochondria due to membrane potential ($\Delta\Psi_m$), the signal loss could be indicative of membrane depolarization. In order to assess this, we used a more sensitive and specific probe for mitochondrial potential $\Delta\Psi_m$, the lipophilic cation dye JC-1, which exists as a green fluorescent monomer (λ_{em} 520 nm) at low concentration in the cytosol and forms red fluorescent J-aggregates (λ_{em} 590 nm) at higher concentrations in the mitochondria where it concentrates due to $\Delta\Psi_m$. Microscopic views of epimastigotes stained with the potentiometric probe JC-1 after 3 h incubation with DMSO (control) or Bfx1 (25.0 μM) are shown in Fig. 7. Note the red-stained mitochondria with high $\Delta\Psi_m$ in control parasites (Fig. 7A), whereas almost no red-stained mitochondria appeared in the Bfx1-treated group, evidencing mitochondrial membrane depolarization (Fig. 7B). The ratio of red/green fluorescence increased with time for the untreated parasites, indicating accumulation of the JC-1 probe in the mitochondria, whereas the benzofuroxan-treated parasites did not increase the red emission characteristic of JC-1 aggregates, suggesting depolarization of mitochondrial membrane (Fig. 7C). As a positive control, parasites were treated with the known uncoupler FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone) at 50 μM that after 30 min incubation provoked a 35% decreased in 590/520 nm fluorescence ratio.

4. Discussion

In the late 70s and early 80s *T. cruzi* parasites were regarded as an organism particularly susceptible to intermediates of oxygen reduction ($O_2^{\bullet-}$, H_2O_2, $^{\bullet}OH$) due to the absence of catalase activity and mainly, the lack of knowledge on trypanosomatid anti-oxidant defences [58]. Accordingly, many naphthoquinones derivatives were assayed as anti-*T. cruzi* agents as intracellular generators of ROS. Among these we could mention β-Lapachone and Mena. Both drugs were active *in vitro* and their anti-proliferative activity was correlated to the induction of O_2^- release from quinone treated epimastigotes [19]. Herein, we included Mena as a positive control of oxidative stress generation in *T. cruzi* parasites. At a concentration close to its IC_{50}, Mena decreased parasite thiols levels, induced the release of H_2O_2, increased oxygen consumption (cyanide-insensitive) and led to oxidation of the intracellular probe DHR, in agreement with its redox-cycling activity. Altogether, this information supports the oxidative stress hypothesis for Mena mechanism of trypanosomicidal action. On the other hand, the results obtained with Nfx pointed in a different direction. Whereas a reduction in the level of low-molecular-weight thiols was observed, this could not be linked to the production of ROS/RNS, such as $O_2^{\bullet-}$, H_2O_2 or peroxynitrite. Redox-cycling activity was only observed at high Nfx concentration (>400 μM), two orders of magnitude higher than the Nfx concentration required for anti-proliferative activity. Nitroheterocycles have largely been considered pro-drugs whose activity relies on activation through bioreduction. Oxidative stress has been proposed as the main mode of action of nitrocompounds, such as Nfx, against trypanosomatids. The results herein obtained argue against this hypothesis, however, it is clear the interplay between drug action and drug metabolism. Wilkinson et al. [4] identified a nitroreductase (NTR) responsible for Nfx and Bnz activation in *T. cruzi* and *T. brucei*. This NTR is a bacterial-like, type I NTR and functions via a series of two-electron reductions, using NADH as cofactor. Down-regulation of its activity confers resistance to nitroheterocyclic drugs, demonstrating its participation in Nfx and Bnz activation in trypanosomes, and draws attention to two-electron reduction products, such as the nitroso- and the hydroxylamine derivatives, instead of the nitroanion radical. In addition, nitroso-compounds are known to react with thiols which may relate the observed thiol depletion to drug metabolism instead of thiol consumption by ROS [9,12].

The importance of SOD activity in trypanosomatids for the resistance of the parasite towards superoxide-generated drugs has been documented [62]. Four isoforms of iron-dependent SOD are present in trypanosomatids: SODA, SODC (in mitochondria), SODB1 (cytosolic, glycosomal) and SODB2 (mainly in glycosome). Down-regulation of TbSODA using RNAi provoked higher sensitivity towards paraquat (superoxide generator) but not towards Nfx, in accordance with our observations that low concentrations of Nfx are not redox-cycling producing $O_2^{\bullet-}$ [63]. However, a more recent work showed that Tbsodb1 mutant displayed 3-fold-increased susceptibility to Nfx, underlying the importance of subcellular localization of NTR responsible for drug activation and the SOD isoform [64].

Regarding *N*-oxide-containing heterocycles, all the compounds displayed comparable anti-proliferative activity but the effect in cell viability was more dissimilar (Table 1). Remarkably, benzofuroxan derivatives, Bfx1 and Bfx2, and benzimidazole Bz3 severely compromised cell viability after 4 h of exposure, while benzimidazole Bz4 and indazole In5 required more time or higher concentrations to attain similar cytotoxicity. Since the uptake is similar among heterocyclic families (Table 2), this result implies the existence of different mechanisms of cellular damage. Concerning the postulated mode of action (generation of oxidative stress) the results observed were diverse and dependent on structural family. Indazole derivative In5 was shown to decrease thiol levels and to produce ROS in the parasite (Figs. 2 and 3). However, the participation of the drug in a redox cycle with oxygen may not be the main cause of thiol loss. It has been suggested that the protonated *N*-oxide radical is a source of $^{\bullet}OH$ via homolysis of the N–OH bond [65,66], and the formation of $^{\bullet}OH$ has been observed when In5 was incubated with *T. cruzi* homogenates [37]. Benzimidazole derivatives, Bz3 and Bz4, on the other hand, showed no effect on free thiols or in the production of ROS/RNS, ruling out their action as oxidative stress promoters. Treatment with benzofuroxan derivatives, Bfx1 and Bfx2, produced a reduction of free thiols (Fig. 2) but there was no evidence of increased production of ROS/RNS (Fig. 3A). Similar to Nfx, drug-mediated loss of free thiols could be ascribed to conjugation with drug reduced metabolites. In fact, we recently reported that Bfx1 is metabolised by the parasite to the corresponding *o*-nitroaniline, that could act as thiol trapping after metabolisation of the nitro-moiety to the nitroso-group, like it occurs with the nitrofuran Nfx [67].

All *N*-oxide-containing heterocycles reduced formazan formation but the effect of benzofuroxans was more pronounced and long-lasting. As previously mentioned, a bioreduction step is presumed to be involved in *N*-oxide derivative's activity and oxidoreductases are likely candidates for this process. Therefore, *T. cruzi* drug metabolism may directly or indirectly affect parasite dehydrogenase activity by enzymatic inhibition and/or depletion of NAD(P)H cofactors. Furthermore, in analogy with mammalian mitochondria where a close communication among pyridine nucleotide, matrix and inner membrane thiols could lead to changes in membrane permeability, we observed that benzofuroxan derivatives produce depolarization of *T. cruzi* mitochondrial membrane (Fig. 7).

The enzymes involved in *N*-oxides metabolism in *T. cruzi* are not completely characterized but some recent data point to a cytosolic NADPH-dependent enzyme and *o*-nitroanilines as the main metabolites [67] suggesting different parasite metabolism for *N*-oxides than for nitroheterocycles like Nfx. The present work shows that all *N*-oxides assayed had an immediate effect on dehydrogenase activity (whereas Nfx showed no effect), thus, a possible implication of these enzymes in *N*-oxides metabolism may be inferred.

In summary, while the mode of action of Nfx remains an open subject, our results indicate that oxidative stress via ROS and RNS production is not the answer. Considering that Nfx along with Bnz are the only drugs used clinically to treat Chagas' disease, an effort should be made to identify their cellular targets. A similar conclusion could be drawn for the potential *N*-oxide drugs, which showed different effects among the studied heterocyclic families. A complete characterization of their metabolism and mode of action may lead to the discovery of new drug targets that help overcome problems related to drug toxicity and drug resistance.

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