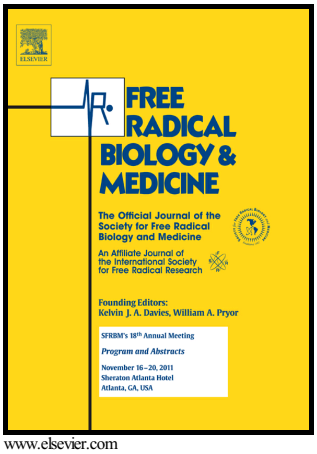

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Mitochondrial disfunction and ROS production are essential for anti-*Trypanosoma cruzi* activity of β-lapachone-derived naphthoimidazoles

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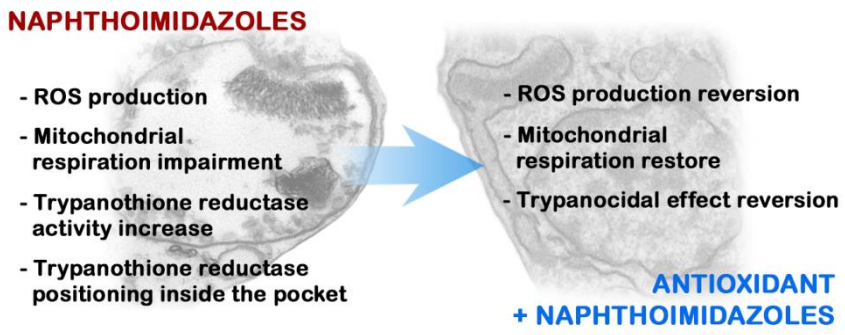
ABSTRACT

Chagas disease is caused by the hemoflagellate protozoa *Trypanosoma cruzi* and is one of the most important neglected tropical diseases, especially in Latin American countries, where there is an association between low-income populations and mortality. The nitroderivatives used in current chemotherapy are far from ideal and present severe limitations, justifying the continuous search for alternative drugs. Since the1990s, our group has been investigating the trypanocidal activity of natural naphthoquinones and their derivatives, and three naphthoimidazoles (N1, N2 and N3) derived from β-lapachone were found to be most effective *in vitro.* Analysis of their mechanism of action via cellular, molecular and proteomic approaches indicates that the parasite mitochondrion contains one of the primary targets of these compounds, trypanothione synthetase (involved in trypanothione production), which is overexpressed after treatment with these compounds. Here, we further evaluated the participation of the mitochondria and reactive oxygen species (ROS) in the anti-*T. cruzi* action of naphthoimidazoles. Preincubation of epimastigotes and trypomastigotes with antioxidants (α-tocopherol and urate) strongly protected the parasites from the trypanocidal effect of naphthoimidazoles, decreasing the ROS levels produced and reverting the mitochondrial swelling phenotype. The addition of pro-oxidants (menadione and H_2O_2) before the treatment induced an increase in parasite lysis. Despite the O_2 uptake and mitochondrial complex activity being strongly reduced by N1, N2 and N3, urate partially restored the mitochondrial metabolism only in N1-treated parasites. In parallel, MitoTEMPO, a mitochondrial-targeted antioxidant, protected the functionality of the mitochondria in N2- and N3-treated parasites. In addition, the trypanothione reductase activity was remarkably increased after treatment with N1 and N3, and molecular docking demonstrated that these two compounds were positioned in pockets of this enzyme. Based on our findings, the direct impairment of the mitochondrial electron transport chain by N2 and N3 led to an oxidative misbalance, which exacerbated ROS generation and led to parasite death. Although other mechanisms cannot be discounted, mainly in N1-treated parasites, further investigations are required.

Graphical Abstract:



Keywords: *Trypanosoma cruzi*, Chagas disease, chemotherapy, naphthoimidazoles, mitochondria, reactive oxygen species, antioxidant defenses.

1. Introduction

Chagas disease is a neglected tropical disease caused by the trypanosomatid parasite *Trypanosoma cruzi* and represents a severe health problem in Latin America, especially due to the mortality and morbidity associated with poverty [1, 2]. In recent decades, Chagas disease has spread globally, resulting in the migration of infected individuals to non-endemic areas, where transmission occurs via contaminated blood bags or even by congenital routes [3, 4]. The common treatment of this disease involves benznidazole or nifurtimox, two nitroderivatives that have been available since the 1960sand that have severe side effects and restricted activity in the chronic phase [5, 6]. As the currently available chemotherapy is far from ideal, intense efforts are underway to find alternative drugs, including natural products [7].

β-Lapachone is a naturally occurring naphthoquinone purified from the heartwood of "Ipês" trees (*Tabebuia* sp.); β-lapachone is bioactive against different pathogens, including *T. cruzi* [8-12]. Medicinal chemistry studies have explored the reactivity of the quinoidal carbonyls of β-lapachone towards nucleophilic reagents, and many synthetic derivatives have been assayed against the infective bloodstream forms of *T. cruzi* [9]. Three naphthoimidazoles, N1, N2 and N3, have shown high trypanocidal activity [9, 13-15] and have been researched in subsequent mechanistic studies. Using electron microscopy, flow cytometry and biochemical techniques, our group previously assessed the mode of action of the three naphthoimidazoles and indicated that the parasite mitochondrion and autophagic pathway are the central and primary targets of these compounds [16-18].

In *T. cruzi*, the single and ramified mitochondrion presents peculiar characteristics, including the presence of a specialized region named the kinetoplast, where the mitochondrial DNA of the parasite is concentrated. In relation to bioenergetics and redox metabolism, the functional plasticity of this organelle was proposed to be important during the parasite cycle depending on substrate availability and the environmental conditions of the host [19, 20]. The absence of classical antioxidant machinery in trypanosomatids, such as the glutathione/glutathione reductase system and catalase, reinforces their rudimentary defenses against reactive oxygen species (ROS) and points to the mitochondrion as a promising target for drug development because the mitochondrial electron transport system represents a critical point of electron leakage and consequent ROS production in *T. cruzi* [20, 21].

More recently, by employing distinct proteomic approaches, we identified a large number of mitochondrial proteins that were differentially expressed in epimastigotes and bloodstream

trypomastigotes treated with naphthoimidazoles [22, 23], reinforcing the pivotal role of this organelle in trypanocidal activity. Surprisingly, proteomics showed an increased level of trypanothione synthetase in treated epimastigotes [22]. Trypanothione synthetase is crucial for trypanothione production, which is one of the main antioxidant pathways in the parasite [24, 25], suggesting that naphthoimidazoles may be able to induce oxidative stress, even though they lack the redox properties that generally lead to ROS production. In the present work, we further investigated the trypanocidal activity, mitochondrial functionality and ultrastructure, and ROS generation after treatment with the three naphthoimidazoles in the presence of different antioxidants. In addition, we evaluated the effects of the naphthoimidazoles on the antioxidant machinery of *T. cruzi* epimastigotes.

2. Materials and methods

2.1. Reagents

α-Tocopherol, β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (NADPH), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), antimycin A from *Streptomyces* sp. (AA), cytochrome c from equine heart, glutaraldehyde grade I, horseradish peroxidase (HRP), menadione, osmium tetroxide (OsO_4), sodium dithionite, succinic acid, tetrabutylammonium hexafluorophosphate ($TBAPF_6$), trypanothione trifluoroacetate salt ($T(SH)_2$) and uric acid were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Dimethylsulfoxide (DMSO), hydrogen peroxide (H_2O_2) and potassium cyanide (KCN) were obtained from Merck (Darmstadt, Hessen, Germany). PolyBed 812 resin was purchased from Polysciences, Inc. (Warrington, Florida, USA). Amplex red was purchased from Molecular Probes (Eugene, Oregon, USA). (2-(2,2,6,6 Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl (MitoTEMPO) was purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). Fetal bovine serum (FBS) and RPMI medium were purchased from Cultilab (Campinas, São Paulo, Brazil). Extradry *N*,*N*-dimethylformamide (DMF; 99.8%) was acquired from Acros Organics. Ultrapure water (18.2MΩ cm) was purified from a Milli-Q system (Merck Millipore, Burlington, Vermont, USA). All other reagents were of analytical grade or better.

2.2. Naphthoimidazole synthesis

4,5-Dihydro-6,6-dimethyl-6H-2-(phenyl)-pyran[b-4,3]naphth[1,2-d]imidazole(N1),4,5-dihydro-6,6-dimethyl-6H-2-(3´-indolyl)-pyran[b-4,3]naphth[1,2-d] imidazole (N2) and 4,5-dihydro-6,6-dimethyl-6H-2-(4´-methylphenyl)-pyran[b-4,3]naphth[1,2-d]imidazole (N3) were obtained from the reaction of acetic acid diluted β-lapachone in the presence of ammonium acetate with benzaldehyde, indolylaldehyde and 4-methylbenzaldehyde, respectively, as previously described [13-15] (Fig. 1). Naphthoimidazole stock solutions were prepared in DMSO.

2.3. Electrochemical analysis

Cyclic voltammetry (CV) experiments were performed with a conventional three-electrode cell in an AutolabPGSTAT-30 potentiostat (Echo Chemie, Utrecht, Netherlands) coupled to a PC microcomputer using GPES 4.9 software. The working electrode was a glassy carbon electrode (GCE) BAS (d = 3 mm), the counter electrode was a Pt wire, and the reference electrode was Ag|AgCl, Cl^- (sat.). All of the electrodes were contained in a one-compartment

electrochemical cell with a volumetric capacity of 10 mL. The GCE was cleaned by polishing with alumina on a polishing felt (BAS polishing kit). The solvent used was extra dryN,N-dimethylformamide (DMF). In CV experiments, the scan rate varied from 10 to 500 $mV.s^{-1}$. Electrochemical reduction/oxidation was performed in aprotic media (DMF + TBAPF60.1 mol.L-1) at room temperature (25 ± 2°C). Each compound (2 x $10^{-3}$$mol.L^{-1}$) was added to the supporting electrolyte, and the solution was deoxygenated with argon before each CV measurement. Different potential ranges were used for cathodic to anodic scanning. The most representative range was from 0 to -3.0 V vs Ag|AgCl, Cl^- (sat.) in the cathodic direction and 0 to +1.2 V in the anodic direction.

2.4. Parasites

T. cruzi (Y strain) was employed in all of the experimental procedures. Proliferative epimastigotes were maintained at 28°C in liver infusion and tryptose medium (LIT) supplemented with 10% heat-inactivated FBS and were harvested during the exponential phase of growth. Trypomastigotes were purified from the bloodstream of infected mice at the peak of parasitemia [19]. This study was performed in accordance with the guidelines of the Colégio Brasileiro de Experimentação Animal (COBEA) and under biosafety conditions. All animal procedures were reviewed and approved by the Fiocruz Committee of Ethics in Animal Research (L-005/2017) according to resolution 196/96 of the National Health Council of Brazilian Ministry of Health.

2.5. Direct effect of preincubation with pro-oxidants or antioxidants

Epimastigotes and bloodstream trypomastigotes (5 x 10^6 parasites/mL) were resuspended in LIT or RPMI medium, respectively. Parasites were preincubated with pro-oxidants (175 µM $H_2O_2$and 1.0 µM menadione in epimastigotes and 360 µM $H_2O_2$and 1.5 µM menadione in trypomastigotes) or antioxidants (20-40 µM α-tocopherol and/or 50-100 µM urate) in 96-well microplates and incubated at 28°C (epimastigotes) or 37°C (trypomastigotes) for 30 min. After this incubation, the parasites were treated with previously determined IC_{50}/24 h concentrations [16, 17] of the three naphthoimidazoles for 24 h and then quantified in a Neubauer chamber.

2.6. ROS release analysis

H_2O_2 release was measured using the previously described Amplex Red method [26]. Epimastigotes (5 x 10^6 parasites/mL) were incubated with 80 µM α-tocopherol, 100 µM urate or 10 nM MitoTEMPO in LIT medium at 28°C for 30 min and treated with the IC_{50}/24 h concentrations of the naphthoimidazoles for 24 h. Then, parasites (5 x 10^8 parasites/mL) were incubated with 5 µM Amplex Red reagent and 200 µg/mL HRP in 100 µL of respiration buffer. Resorufin fluorescence was monitored at excitation and emission wavelengths of 530 nm and 590 nm, respectively, in a SpectraMaxM3 fluorimeter (Molecular Devices, Sunnyvale, California, USA). Menadione (4 µM) was added as a positive control 2 h before reagent addition.

2.7. Ultrastructural analysis

Following a similar experimental design, epimastigotes (5 x 10^6 parasites/mL) were incubated with 100 µM urate and then treated with naphthoimidazoles at IC_{50}/24 h doses. Then,

the parasites were washed with 10 mM phosphate-buffered saline (PBS, pH 7.2) and fixed with 2.5% glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.2) for 30 min at 25°C. After washing in the same buffer, the samples were post fixed with 1% OsO_4, 0.8% potassium ferricyanide and 2.5 mM $CaCl_2$ for 20 min, dehydrated in an ascending acetone series and embedded in PolyBed 812 resin. Ultrathin sections stained with uranyl acetate and lead citrate were analyzed using a JEM1011 transmission electron microscope (Jeol, Tokyo, Japan) located in Plataforma de Microscopia Eletrônica at Instituto Oswaldo Cruz (Fiocruz).

2.8. Oxygen uptake analysis

Epimastigotes(5 x 10^6 parasites/mL) were incubated with 100 µM urate or 10 nM MitoTEMPO followed by treatment with naphthoimidazoles at the IC_{50}/24 h concentrations as described above. Then, parasites were resuspended in 2 mL of respiration buffer (2.5 x 10^7 parasites/mL): 125 mM sucrose, 5 mM succinate, 65 mM KCl, 10 mM Tris-HCl (pH 7.2), 1 mM $MgCl_2$, and 2.5 mM monobasic potassium phosphate at 28°C under continuous stirring and analyzed in a high-resolution oxygraph 2K (Oroboros Instruments, Innsbruck, Tyrol, Austria). The oxygen concentration and flux were recorded using DatLab software as previously described [19]. AA (2 µM) was added to reach residual oxygen consumption (ROX).

2.9. Analysis of the mitochondrial complex activity

As described above, epimastigotes(5 x 10^6 parasites/mL) were incubated with 100 µM urate or 10 nM MitoTEMPO followed by treatment with naphthoimidazoles at the IC_{50}/24 h concentrations. Then, the parasites were washed with PBS and maintained at −20°C. The parasites were thawed, the homogenates were obtained by sonication as previously described [27], and the protein concentration was assessed by using a Pierce™ BCA Protein Assay Kit (Thermo Fisher, Waltham, Massachusetts, EUA). The AA-sensitive succinate:cytochrome c oxidoreductase activity (complexes II–III) was measured by the increase in the absorbance at 550 nm due to the reduction of ferricytochrome c (ε=19 mM−1 cm−1). The reaction mixture consisted of 100 mM potassium phosphate buffer (pH 7.4), 150 µM equine heart cytochrome c, 3 mM succinic acid and 1 mM KCN. KCN-sensitive cytochrome c oxidase (complex IV) activity was measured based on the decrease in absorbance due to the oxidation of ferrocytochrome c at 550 nm (ε=19 mM−1 cm−1). The reaction mixture consisted of 100 mM potassium phosphate buffer (pH 7.4) and 50 µM sodium dithionite reduced equine heart cytochrome c. Alterations in absorbance were monitored at room temperature using a SpectraMaxPlus384 (Molecular Devices) after the addition of 0.5 mg protein/ml, and the controls were generated with the addition of 2 µM AA (complex II–III) and 1 mM KCN (complex IV) [28].

2.10. Trypanothione reductase activity analysis

Trypanothione reductase activity was measured at room temperature in a total reaction volume of 200 µL in a SpectraMaxPlus384 spectrophotometer (Molecular Devices) based on 2TNB production measured at 412 nm [29]. Epimastigotes (5 x 10^6 parasites/mL) were treated for 24 h with the naphthoimidazoles at the IC_{50}/24 h concentrations. Then, the parasites were washed with PBS and maintained at −20°C. The parasites were thawed, the homogenates were obtained by sonication as previously described [27], and the protein concentration was assessed by using a Pierce™ BCA Protein Assay Kit (Thermo Fisher). The assay mixture contained 1 µM

$T(SH)_2$, 150 μM NADPH, 400 mM HEPES(pH 7.5), 25 μM DTNB and 1 mM EDTA. The reactions were initiated by the addition of 100 μL of the assay mixture and 100 μL of epimastigote homogenates (0.25 mg protein/mL).

2.11. Molecular docking analysis

For each model, hydrogen atoms were added using UCSF Chimera, and the protonation states of the ionizable side chains at the appropriate pH were defined using PROPKA [30]. The two-dimensional (2D) files of the N1, N2 and N3 compounds were transformed into a three-dimensional (3D) format by Open Babel [31] and saved as molecule files. All of the molecules in the compounds were prepared with UCSF Chimera. The preparation included the generation of tautomers and ionization states at the same pH as the proteins. As the binding sites of the compounds were not known, blind docking was performed for each protein using the Dockthor server (https://dockthor.lncc.br/v2/). The center of mass of the proteins was defined as the center of the grid that covered the entire protein.

The amino acid sequences of γ-glutamylcysteine synthase, cystathionine-β-synthase, serine acetyltransferase, cysteine synthase, trypanothione synthetase, trypanothione reductase, mitochondrial tryparedoxin peroxidase, cytosolic tryparedoxin peroxidase and superoxide dismutase were retrieved from TrytripDB (http://tritrypdb.org/tritrypdb/) in FASTA format. Initially, we used the BLASTP program (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) and Protein Data Bank (https://www.rcsb.org/) as a search set to select the template structures for comparative modeling for each protein. The template and target sequences were then aligned with the clustal omega program (https://www.ebi.ac.uk/Tools/msa/clustalo/). One hundred models were generated with the standard 'auto model' routine of Modeller version 9.18 [32] for each target sequence. Each model was optimized via the variable target function method (VTFM) until 300 iterations were achieved. The resulting modeled structures were selected according to their discrete optimized protein energy (DOPE) score and evaluated by a Ramachandran plot, ERRAT and Verify3D using the SAVES server (http://servicesn.mbi.ucla.edu/SAVES/). The three-dimensional structures were generated with UCSF Chimera [33].

2.12. Statistical analysis

Statistical analysis was performed using the nonparametric Mann-Whitney test in the IBM SPSS Statistics 22.0 software (IBM Corporation, Armonk, New York, USA), with the threshold for significance set at $p \leq 0.05$. In all experiments, the pairwise comparisons were as follows: control x treated parasites (naphthoimidazoles or antioxidant treatment) and naphthoimidazoles treated parasites x treatment with antioxidant preincubation.

3. Results

3.1. Naphthoimidazoles do not have redox activity, although preincubation with antioxidants protects T. cruzi from their trypanocidal activity and decreases ROS generation

To analyze the participation of oxidative stress in the mechanism of action of the N1, N2 and N3naphthoimidazoles, epimastigotes and trypomastigotes were preincubated in the presence of α-tocopherol or urate and then challenged by the IC_{50}/24 h doses of the compounds that were previously determined [16, 17]. Preincubation with these two antioxidants completely

abolished the trypanocidal effect of the naphthoimidazoles on both parasite stages in a dose-dependent manner (Fig. 2). In parallel, using a similar approach, the addition of H_2O_2 or menadione (pro-oxidants) 30 min before treatment with the three naphthoimidazoles promoted an increase in the anti-*T. cruzi* activity of the compounds (Fig. S1). These results encouraged us to quantity ROS production. The kinetics of reactive species generation was determined (Fig. S2). N1, N2 and N3 promoted a strong increase of 2.9-, 4.6- and 3.3-fold, respectively, in ROS generation compared with that of control epimastigotes. Both α-tocopherol and urate decreased the ROS production induced by the three compounds; however, urate showed better results, completely abolishing ROS generation (Fig. 3).

To exclude the possibility of direct ROS generation from the redox activity of the naphthoimidazoles, we analyzed their electrochemical properties using cyclic voltammetry in aprotic (DMF/$TBAPF_6$) media. Electrochemical analysis indicated the very negative redox potential of N1, N2 and N3, which displayed one main wave, with a monoelectronic irreversible electron transfer, generating the corresponding anion radical (Fig. S3). Reduction of these compounds is not a favorable process; once heteroaromatic systems are generated, N2 is reduced at more negative potential (-2.421 V) (Table 1).

3.2. *Naphthoimidazoles collapse the parasite mitochondrial metabolism, and antioxidants protect the mitochondrial ultrastructural architecture from N1, N2 and N3 activity*

Because the mitochondria of trypanosomatids are the major ROS source and based on preliminary studies on the mode of action of these compounds, which demonstrated that the mitochondrion was their primary target [16, 17], we evaluated whether antioxidant protection would change the previously described mitochondrial swelling phenotype in treated parasites. In the absence of urate, N1, N2 and N3 treatment induced strong injury in the mitochondrion (Fig. 4C, E, G), leading to a remarkable washed-out phenotype in the mitochondrial matrix of N2-treated epimastigotes (Fig. 4E). However, no ultrastructural effect could be detected in the mitochondria of treated parasites that were preincubated with urate (Fig. 4D, F, H).

In the next step, we analyzed whether treatment with naphthoimidazoles affected mitochondrial functionality. Clearly, treatment with N1, N2 and N3 reduced the oxygen uptake (routine stage), reaching 58, 72 and 40%, respectively, of the decrease in mitochondrial functionality in *T. cruzi* epimastigotes. In addition, the possible protective effect of the antioxidant preincubation was also evaluated. Despite the decrease in oxygen consumption in all treated parasites, the protective effect of urate was only observed in N1-treated parasites. The addition of AA strongly reduced O_2 consumption, guaranteeing that this consumption was mitochondrial (ROX stage) (Fig. 5A). Treatment with N1, N2 and N3 also decreased complex II-III activity by 50, 61 and 57%, respectively, and complex IV activity by 72, 53 and 47%. Similar to the results for mitochondrial O_2 uptake, preincubation with urate only protected the parasites from the effect on complex II-III after treatment with N1 (Fig. 5B). On the other hand, pretreatment with urate reverted (at least partially) the inhibition of complex IV activity in parasites treated with the three compounds (Fig. 5C).

3.3. *MitoTEMPO, a specific mitochondrial antioxidant, completely abolishes the damage caused by N3 treatment*

Because urate did not prevent the effect of the naphthoimidazoles on parasite O_2 uptake, a new strategy to define the action of the naphthoimidazoles was employed. Epimastigotes were preincubated with MitoTEMPO, a mitochondria-targeted antioxidant with superoxide and alkyl

radical scavenging properties. First, the capacity of this antioxidant to decrease the ROS production induced by N1, N2 and N3 was analyzed. Similar to urate, preincubation with MitoTEMPO reduced ROS production by 20-40% in N2- and N3-treated parasites; however, this antioxidant did not influence oxidative stress after treatment with N1 (Fig. 6A). However, MitoTEMPO reversed the impairment in O_2 uptake and in complex II-III activity in N2- and N3-treated parasites (2.7- and 1.8-fold and 2.0- and 2.4-fold, respectively) (Fig. 6B and C). A similar effect of this antioxidant was also detected after incubation with N1. MitoTEMPO also protected complex IV activity from the inhibition caused by N1 and N3 (approximately 2.8- and 2.1-fold, respectively) (Fig. 6D).

3.4. Treatment with N1 and N3 leads to an increase in trypanothione reductase activity

Because the oxidative metabolism was triggered by the compounds, we investigated the antioxidant defenses of the parasite. Using a classical biochemical approach, we assessed trypanothione reductase activity in epimastigote homogenates. Our data indicated an important increase in this enzyme activity after treatment with N1 and N3, approximately 2.8- and 1.9-fold, respectively, while treatment with N2 did not affect the enzyme activity (Fig. 7).

3.5. NaphthoimidazolesN1 and N3 are positioned in pockets of trypanothione reductase

The experimental results suggest that compounds N1, N2 and N3act in the oxidative stress pathway. Thus, the enzymes γ-glutamylcysteine synthase, cystathionine-β-synthase, serine acetyl transferase, cysteine synthase, trypanothione synthetase, trypanothione reductase, mitochondrial tryparedoxin peroxidase, cytosolic tryparedoxin peroxidase, superoxide dismutase, nonselenium glutathione peroxidase-like enzymes, glutathionyl spermidine synthetase and ascorbate peroxidase, which are present in the oxidative stress pathways of the parasite, were selected for analysis with the purpose of computationally predicting the direct interaction between N1, N2 and N3 with these proteins. Among these selected proteins, we obtained a three-dimensional structure for four molecules: (i) trypanothione synthetase (the model was obtained by comparative modeling using PDB id: 2VOB as the template, with 59% identity and 98% coverage with the target protein); (ii) trypanothione reductase (PDB id: 1NDA); (iii) mitochondrial tryparedoxin peroxidase (the model was obtained by comparative modeling using PDB id: 4BK3 as the template, with 74% identity and 84% coverage with the target protein); (iv) and cytosolic tryparedoxin peroxidase (PDB id: 4LLR). All of the generated models obtained ERRAT, Verify 3D and Ramachandran plot values greater than 89, 91 and 96%, respectively. The models generated for the other proteins did not obtain a sufficient resolution for use in molecular docking assays due to the low identity and coverage with the selected templates.

Virtual screening of the N1, N2, and N3 compounds on the four selected proteins using a blind docking approach yielded the highest Dockthor score (-8,143) for N1 at a trypanothione reductase protein binding site. For all proteins, compound N2 obtained the worst score (Table 2). The total energy represents the intermolecular energy between the ligand-receptor plus the intramolecular energy of the ligand (interaction energy). Compound N2 obtained the worst total energy for all proteins. Only compounds N1 and N3 were positioned in pockets formed in trypanothione reductase (Fig. 8), showing a possible interaction with residues positioned up to 5 Å away from the ligand.

4. Discussion

The involvement of mitochondrial metabolism in the molecular mode of action of drugs has been extensively described in all eukaryotic cells. In trypanosomatids, the single mitochondrion exhibits peculiar functional and morphological characteristics, such as the presence of noncanonical antioxidant machinery and an alternative oxidase in *Trypanosoma brucei*, making this organelle an attractive target in parasites [20]. Due to their chemical/electrochemical behavior, the biological activity of quinones is usually related to their favorable redox potential. As strong electrophiles, quinones are easily reduced. In *T. cruzi*, the trypanocidal effect of β-lapachone and other naphthoquinones is associated with ROS generation [27, 34 35], with more than 90% of H_2O_2 produced in epimastigotes after treatment with β-lapachone located in the mitochondrion [36].

In spite of the several studies on the biological activity of naphthoquinones, data on naphthoimidazolic derivatives are scarce. Recently, the anti-inflammatory and antimicrobial effects of naphthoimidazoles were reported [37-39]. Since 1997, our group has investigated the trypanocidal activity of naphthoimidazoles synthesized from β-lapachone [13-15]. N1, N2 and N3are the most active derivatives in the three life stages of *T. cruzi*, blocking crucial cellular processes, such as metacyclogenesis and cytokinesis. Electron microscopy demonstrated that these derivatives led to alterations in the Golgi, reservosomes, kinetoplast, and autophagic phenotype, depending on the parasite form; however, the most prominent ultrastructural effect of these derivatives was observed in the mitochondrion. An accentuated reduction in the mitochondrial membrane potential was also detected by flow cytometry and fluorescence microscopy techniques and together with the decreased cytochrome c reductase activity reinforced that this organelle is part of the mode of action of these three naphthoimidazoles [16, 17]. To assess the molecular mechanisms involved in the trypanocidal activity of these compounds, different proteomic approaches were employed. In both epimastigotes and bloodstream trypomastigotes, mitochondrial proteins were predominantly modulated by treatment with these compounds, confirming that this organelle is a target of these derivatives [22, 23]. Surprisingly, proteomic evidence also showed high levels of trypanothione synthetase in treated epimastigotes, suggesting the participation of this antioxidant pathway in naphthoimidazole detoxification [22]. These data motivated us to evaluate whether oxidative metabolism is involved in the anti-*T. cruzi* effect of N1, N2 and N3 as well as its previously observed correlation with mitochondrial damage.

In this work, α-tocopherol and urate protected epimastigotes and bloodstream trypomastigotes against the effect of the naphthoimidazoles, increasing parasite viability, as well as preventing ROS production in epimastigotes. In parallel, the anti-*T. cruzi* effects of N1, N2 and N3were potentialized after preincubation with sublethal doses of pro-oxidant agents (H_2O_2 and menadione). These results clearly demonstrate that the trypanocidal activity of these compounds depends on oxidative stress and show that an increase in the compound's effect triggers the oxidative burst. These results motivated us to investigate whether naphthoimidazoles increased ROS production. Surprisingly, the three compounds increased oxidative stress by up to 4-fold in treated parasites. It was plausible that mitochondrial swelling and morphological injury were mediated by ROS generation during naphthoimidazole treatment. Our morphological analysis showed that preincubation with urate (the antioxidant that was most effective during naphthoimidazole treatment) abolished the mitochondrial swelling induced by the three naphthoimidazolic derivatives, one of the most recurrent forms of damage in treated parasites. This result indicates that ROS production in N1-, N2- and N3-

treated parasites is the main cause of the previously described ultrastructural phenotypes [16,17].

As expected for the naphthoimidazoles, their heteroaromatic character precludes their reduction at the potentials observed for quinones, which makes them difficult to reduce. The irreversible nature observed in electrochemical analysis is possibly due to the dimerization or protonation of the electron-generation of anion radicals, involving residual water or proton capture from ammonium salt. Compared to the original quinone β-lapachone, the difference in electrochemical behavior is clear. Due to their very negative reduction potentials, direct ROS generation by N1, N2 and N3 is very unlikely. Thus, in one of the proposed mechanisms, naphthoimidazoles could return to β-lapachone and the respective aldehyde inside the parasite, with the oxidative activity derived from the naphthoquinone effect. The higher activities of N1, N2 and N3 in relation to the original naphthoquinone [13-15] suggested that a reverse reaction could occur in the protozoa, but an additive effect is required. The trypanocidal activity of the three original aldehydes was evaluated, and no effects on epimastigotes and trypomastigotes were observed (data not shown). This proposed mechanism was discarded because the presence of β-lapachone and aldehydes could not explain the increase in the trypanocidal effect of the three naphthoimidazoles, and therefore, further study to determine the mode of action of N1, N2 and N3 is needed.

Because mitochondrial metabolism is one of the main ROS sources in eukaryotic cells [40] and because the results of our group showed that mitochondrial metabolism is part of the mode of action of the naphthoimidazoles, we analyzed the mitochondrion function in treated parasites. The three compounds compromised mitochondrion metabolism, decreasing O_2 consumption and complexes II-III and IV activities. In N1-treated parasites, we observed that urate and MitoTEMPO were able to reverse the effect of this compound in all of the analyses performed. However, the prevention of oxidative stress was increased in the presence of urate, which suggests cytosolic ROS generation occurred after treatment with N1. Corroborating this hypothesis, the mitochondrial-targeted antioxidant did not revert ROS production. By contrast, ROS production by N2 and N3was only prevented by MitoTEMPO, pointing to the mitochondrial electron transport system as the primary target of these two naphthoimidazoles. Using a proteomic approach, Menna-Barreto et al. [22] showed a decrease in the subunit IV of cytochrome c oxidase (complex IV) levels in N2-treated epimastigotes, reinforcing our data. MitoTEMPO did not protect complex IV activity from the inhibition induced by naphthoimidazole, and this enzyme may be an important target for the action of N2.

Our data demonstrated that ROS production by N1 did not result from mitochondrial damage; however, this organelle is not the exclusive ROS source in *T. cruzi*. A redox imbalance also occurs when endogenous antioxidants fail to cope with excessive ROS, triggering oxidative stress. Some compounds, called redox-active antiparasitic drugs, are used to treat parasitic diseases. These compounds have a redox center or affect redox biology (inhibiting vital redox reactions) and promote oxidative stress in parasites [41]. Doxorubicin and mitomycin C are quinone derivatives that have anti-leishmanial activity. In *Leishmania donovani*, these drugs inhibit trypanothione reductase, an important antioxidant enzyme, acting as subversive substrates that converting it to a pro-oxidant molecule and generate ROS [42, 43]. To determine whetherN1 and the other naphthoimidazoles could compromise the parasite antioxidant defenses, we evaluated the trypanothione reductase activity during treatment. In trypanosomatids, ROS detoxification is mediated by numerous enzymes, such as tryparedoxin peroxidases, nonselenium glutathione peroxidases, ascorbate peroxidases, and trypanothione-related enzymes [24, 44]. Here, a significant increase in trypanothione reductase activity was observed after treatment with N1 and N3, corroborating the previously detected overexpression

of trypanothione synthetase [22]. This increase could be explained by the large amounts of reactive species produced during treatment with these two compounds, leading to increased expression of antioxidant defenses. Similar results were shown in *Leishmania braziliensis* promastigotes, in which the protein expression and activity of cysteine synthase and cystathionine β-synthase, two important molecules in the cysteine biosynthesis pathway, were increased after challenge with H_2O_2 [45]. Cysteine biosynthesis is a crucial pathway in trypanosomatids because it supports the requirement of the thiol pool, especially for antioxidant synthesis [46]. Similar results have been shown in *T. cruzi* epimastigotes treated with a hemin analogue (pro-oxidant), leading to an increase in superoxide dismutase and ascorbate peroxidase activities [47]. In addition to the antioxidant activity results, our docking analysis showed that N1 and N3 could enter the cavities of trypanothione reductase; however, the distances found were not long enough to be suggestive of strong binding. Proteins are dynamic molecules, and their flexibility represents a significant challenge for the docking of potential ligands because the binding site can take different forms. Many commonly used molecular docking algorithms allow only the ligand to be flexible and keep the protein rigid. Correct evaluation of the binding form of a ligand in a cavity can be impaired by the stiffness of the protein, preventing the ligand from fitting into a correct pose in the recipient cavity. Further ensemble docking analysis will be performed to reduce the problems caused by the rigidity of the protein and will identify other cavities that can be exploited since various receptor structures are used, and the results will be merged later. Different receptor states can be obtained either by experimental (NMR, X-ray crystallographic and CryoEM) or computational (normal mode and molecular dynamics analysis) approaches.

In conclusion, the cytoplasmic ROS production by N1 led to a morphological injury in treated parasites and affected the trypanothione reductase activity, compromising the antioxidant balance and oxidative burst. On the other hand, the direct impairment of the mitochondrial electron transport system by N2 and N3culminated in a loss of oxidative balance, exacerbating ROS production and leading to parasite death.

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Table 1 Major electrochemical parameters in cyclic voltammetry of the N1, N2 and N3 (c = 1 mmol.L^{-1})

Compound	*E*pIc (V)	*E*pIIc (V)
N1	-2.340	-2.479
N2	-2.421	-2.485
N3	-2.366	-2.443

Table 2 Blind docking of naphthoimidazoles to *T. cruzi* anti-oxidant enzymes.

Protein name	score[a]			total energy			interaction energy		
	N1	N2	N3	N1	N2	N3	N1	N2	N3
Trypanothione synthetase	-7.61	-7.24	-7.55	51.81	45.62	49.40	-16.32	-17.02	-15.93
Trypanothione reductase	-8.14	-7.26	-7.51	54.24	47.46	51.67	-13.83	-15.00	-13.66
Tryparedoxin peroxidase[b]	-7.22	-6.84	-7.53	54.39	47.68	51.84	-13.70	-14.81	-13.52
Tryparedoxin peroxidase[c]	-7.48	-7.04	-6.82	55.03	47.12	52.68	-13.04	-15.37	-12.71

[a] Represents a scoring function for Dockthor docking.
[b] Mitocondrial isoform.
[c] Cytosolic isoform.

Fig. 1: Chemical structures of β-lapachone-derived naphthoimidazoles N1, N2 and N3.

Fig. 2: Antioxidants prevented the trypanocidal effect of the naphthoimidazoles. Evaluation of trypanocidal activity of naphthoimidazoles (IC_{50}/24h) in *T. cruzi* epimastigotes (A,B) and bloodstream trypomastigotes (C,D) pre-treated with antioxidants. The pre-incubation with urate (A,C) or α-tocopherol (B,D) reverted completely N1, N2 or N3 activity at IC_{50}/24h doses. The graphs show mean and standard deviations of at least three independent experiments. Significant differences: * $p \leq 0.05$; ** $p \leq 0.03$.

Fig. 3: Naphthoimidazoles induced ROS production in *T. cruzi* epimastigotes. ROS production by the treatment with naphthoimidazoles (IC_{50}/24h) in *T. cruzi* epimastigotes was evaluated by Amplex Red method. N1, N2 and N3 induced ROS generation, reverted by the pre-treatment with α-tocopherol (80 μM) and urate (100 μM). Menadione (4 μM) was used as a positive control. The graphs show mean and standard deviations of at least three independent experiments. Significant differences: * $p \leq 0.05$.

Fig. 4: Urate abolished the mitochondrial swelling in naphthoimidazoles-treated epimastigotes. Transmission electron microscopy analysis of *T. cruzi* epimastigotes control (A,B) or treated with N1 (C,D), N2 (E,F) or N3 (G,H) (IC_{50}/24h). The untreated epimastigotes in the absence (A) or presence (B) of urate (100 μM) preserved typical ultrastructural aspects of the mitochondrion (M). The treatment with the three compounds (C, E, G) led to a mitochondrial swelling (black and white stars). In N2-treated parasites (E), the loss of electron density of the mitochondrial matrix could be also detected (black star). The pre-incubation with urate (D, F, H) abolished completely the damage in mitochondria induced by the naphthoimidazoles. Nucleus (N), mitochondrion (M), kinetoplast (K), Golgi (G) and flagellum (F). Bars = 0.5 μm.

Fig. 5: Naphthoimidazoles impaired mitochondrial metabolism in *T. cruzi* epimastigotes. The oxygen uptake (A) and activity of complex II-III (B) and IV (C) were evaluated in *T. cruzi* epimastigotes pre-incubated with urate (100 μM) following the treatment with naphthoimidazoles (IC_{50}/24h). (A) N1, N2 and N3 induced an important reduction in the parasite oxygen consumption during routine stage (open bars). The pre-incubation with the antioxidant protected the protozoa from respiratory collapse after the treatment with N1. AA (2 μM) was added (ROX state) to confirm that O_2 uptake was mitochondrial (dashed bars). (B) Naphthoimidazoles treated epimastigotes showed a decrease in complex II-III activity, while the antioxidant protects the N1 damage. (C) The complex IV activity was affected by the three compounds, being the functionality restored in the pre-treated conditions. The graphs show mean and standard deviations of at least three independent experiments. Significant differences: * $p \leq 0.05$; ** $p \leq 0.03$.

Fig. 6: MitoTEMPO restored the mitochondrial functionality in N2 and N3-treatement. The ROS production (A), the oxygen uptake (B), activity of complex II-III (C) and IV (D) were evaluated in *T. cruzi* epimastigotes pre-incubated with MitoTEMPO (10 nM) following the treatment with naphthoimidazoles (IC_{50}/24h). (A) N1, N2 and N3 induced ROS generation, reverted in N2 and N3-treatment by the pre-incubation with the antioxidant. Menadione (4 μM) was used as a positive control. (B) N1, N2 and N3 induced an important reduction in the parasite oxygen consumption during routine stage (open bars). The pre-incubation with the antioxidant protected the protozoa from respiratory collapse after the treatment with the three naphthoimidazoles. AA (2 μM) was added (ROX state) to confirm that O_2 uptake was mitochondrial (dashed bars). (C) Naphthoimidazoles treated epimastigotes showed a decrease in complex II-III activity, while the antioxidant protects the parasites against de damages. (D) The complex IV activity was affected by the three compounds, being the functionality restored in the N1 and N3 pre-treated conditions. The graphs show mean and standard deviations of at least three independent experiments. Significant differences: * $p \leq 0.05$; ** $p \leq 0.03$.

Fig. 7: N1 and N3 promoted an increase in trypanothione reductase activity in *T. cruzi* epimastigotes. The trypanothione reductase activity was evaluated in *T. cruzi* epimastigotes treated with the three naphthoimidazoles (IC_{50}/24h). N1 and N3 treated parasites showed an increase in the enzyme activity. The graphs show mean and standard deviations of at least three independent experiments. Significant differences: * $p \leq 0.05$.

Fig. 8: N1 and N3 positioned in *T. cruzi* trypanothione reductase pockets. Three-dimensional structure of trypanothione reductase with the best Dockthor score of the N1, N2 and N3 compounds after the blind docking. Chains A, B, C and D are shown in brown, turquoise, green

and orange respectively. Highlighted are the side chains of the residues positioned up to 5 Å of the compounds N1 (purple square), N2 (blue square) and N3 (gray square). Ligands are represented as ball and stick with carbons colored in gray, nitrogen in blue and oxygen in red. Side chains of residues are represented as thin sticks. The surface of the protein cavities is also shown with a transparent surface.

Legends to supplementary figures

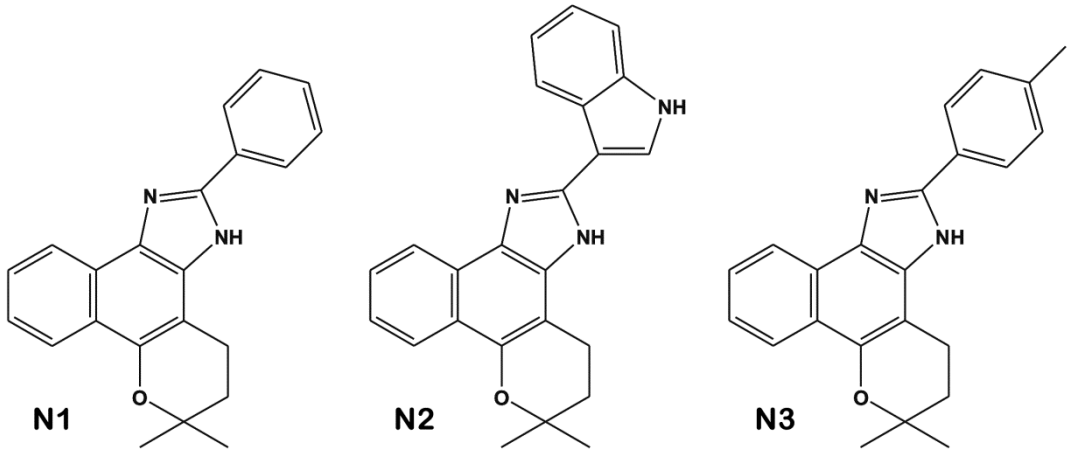
Fig. S1: H_2O_2 and menadione improved the trypanocidal activity of the naphthoimidazoles. Evaluation of trypanocidal activity of naphthoimidazoles in *T. cruzi* epimastigotes (A,B) and bloodstream trypomastigotes (C,D) pre-treated with pro-oxidants. The preincubation with 175 and 360 μM H_2O_2 (A,C) or 1.0 and 1.5 μM menadione (B,D) increased the N1, N2 or N3 trypanocidal effect at IC_{50}/24h doses. The graphs show mean and standard deviations of at least three independent experiments. Significant differences: * $p \leq 0.05$.

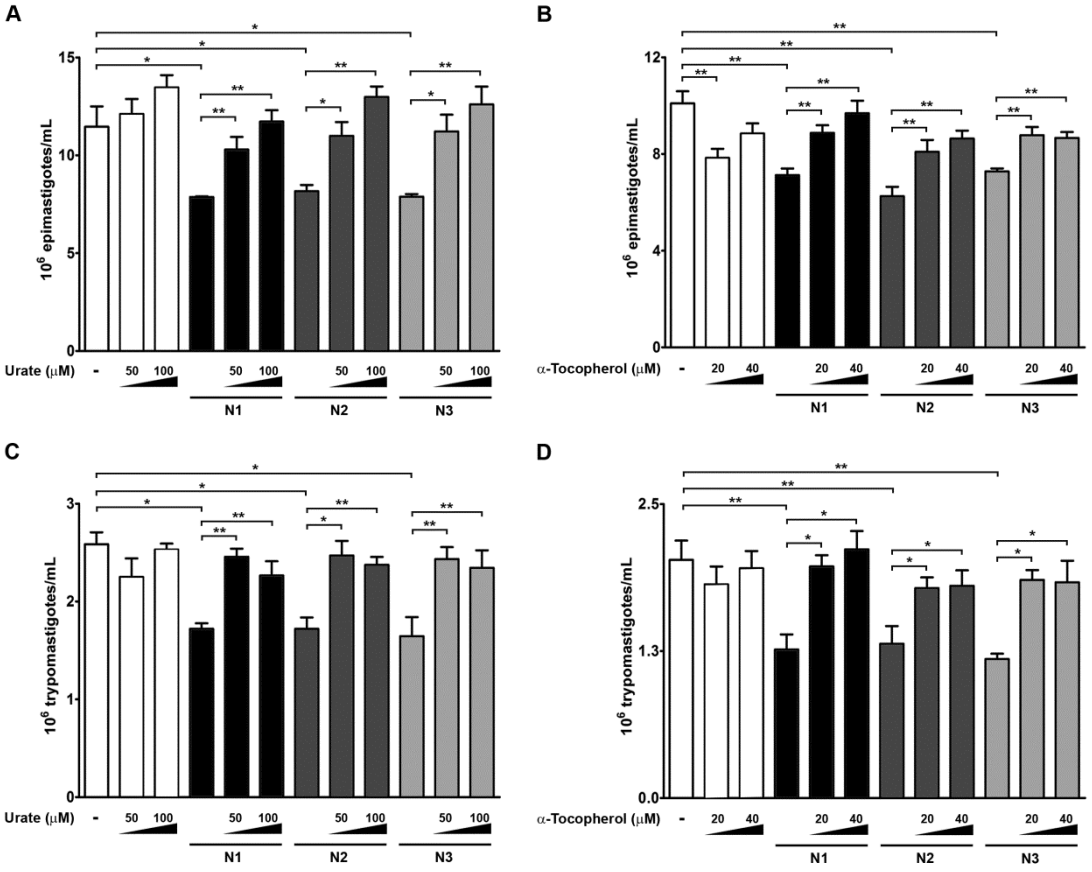
Fig. S2: ROS production in naphthoimidazoles treated *T. cruzi* epimastigotes was stable. ROS production by the treatment with naphthoimidazoles (IC_{50}/24h) in *T. cruzi* epimastigotes was evaluated by Amplex Red method. N1, N2 and N3 induced ROS generation, reverted by the pre-treatment with α-tocopherol (80 μM) and urate (100 μM). Menadione (4 μM) was used as a positive control. Representative graph of four independent experiments.

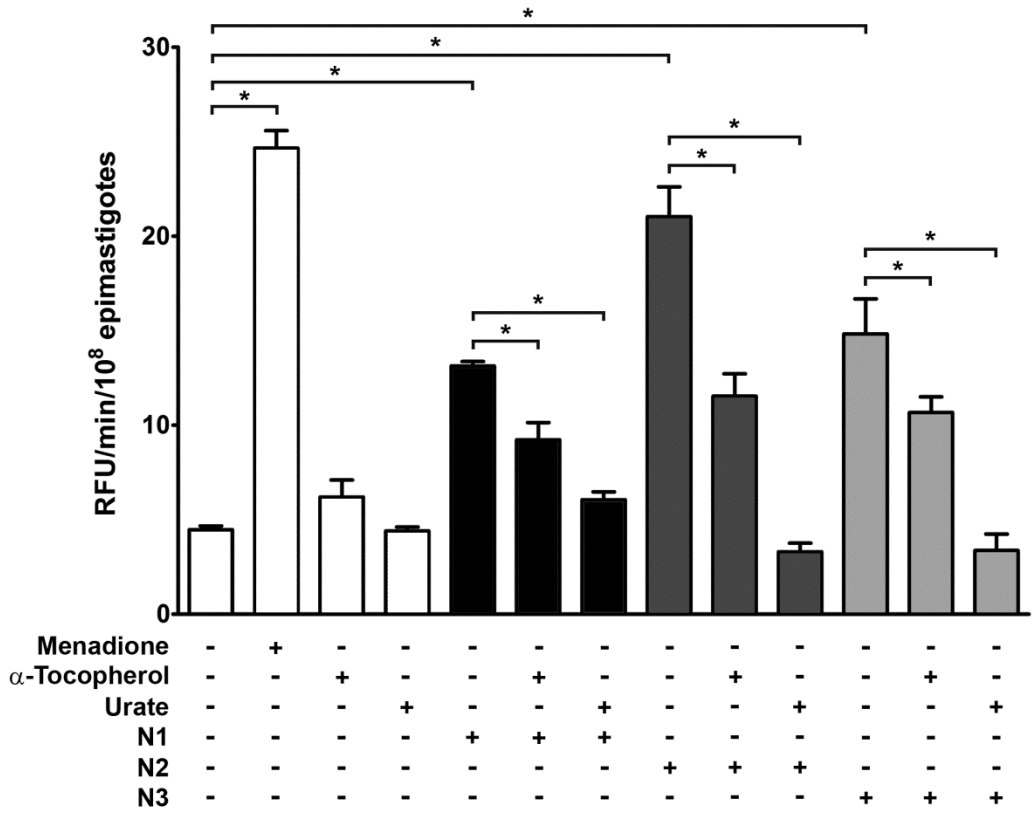
Fig. S3: Naphthoimidazoles presented very negative redox potential. The redox potential of the three naphthoimidazoles was evaluated. The CV of N1 (A), N2 (B) and N3 (C) (2 $mmol.L^{-1}$) in DMF + $TBAPF_6$ (0.1 M), glassy carbon electrode, cathodic direction, ν = 0.1 V s^{-1}. Left column: successive CVs; black line – scan 1 and red line – scan 2. Right column: several inversion potentials.

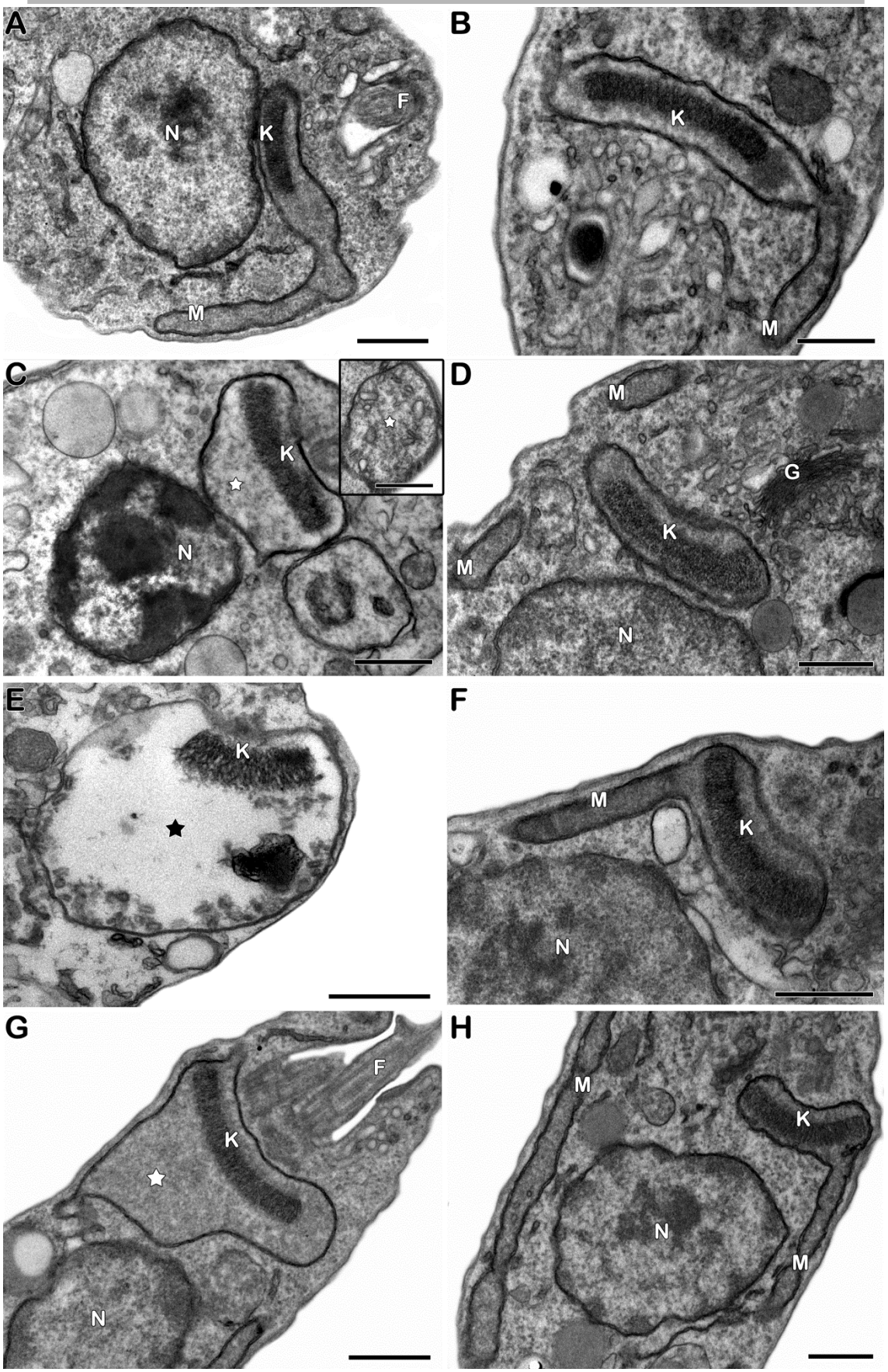
Highlights:

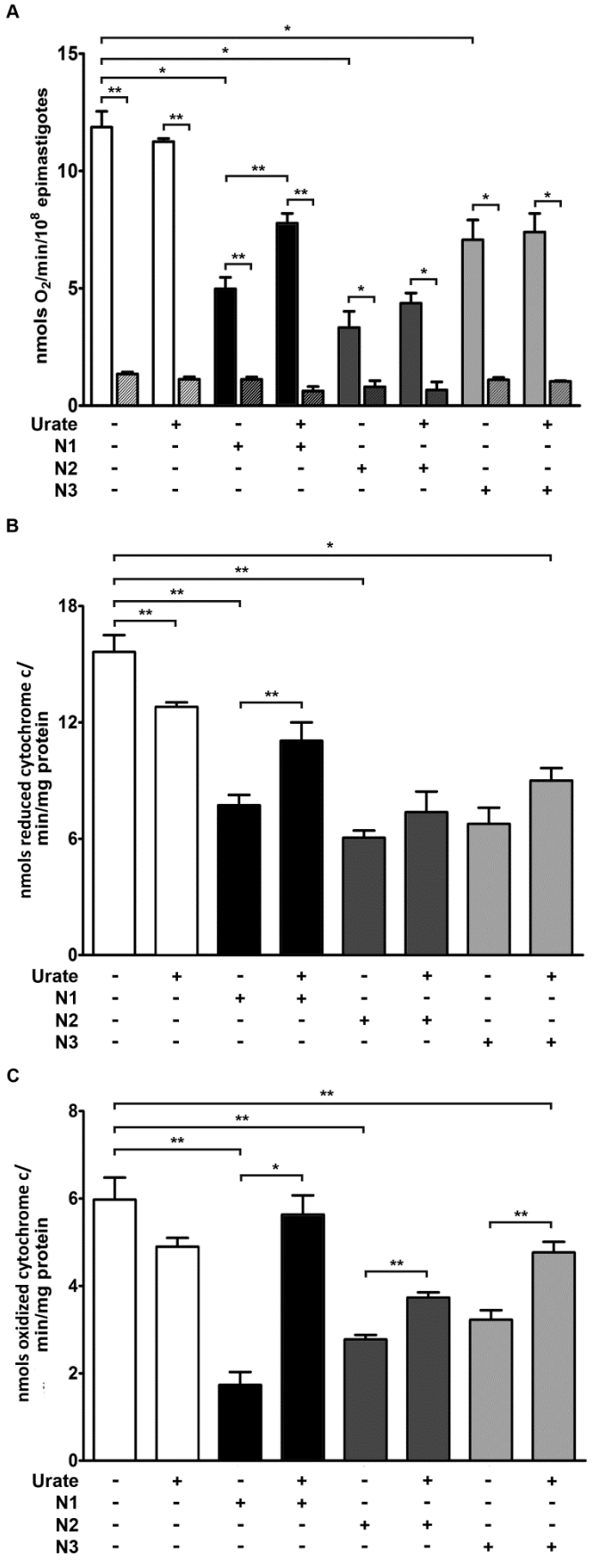
- Naphthoimidazoles promote ROS production, mitochondrial respiration impairment and trypanothione reductase activity in *T. cruzi* epimastigotes.
- Pre-incubation with antioxidants led to ROS production reversion, mitochondrial oxygen consumption restore, together with total reversion of swelling ultrastructural phenotype, and the abolishment of trypanocidal effect of the naphthoimidazoles.

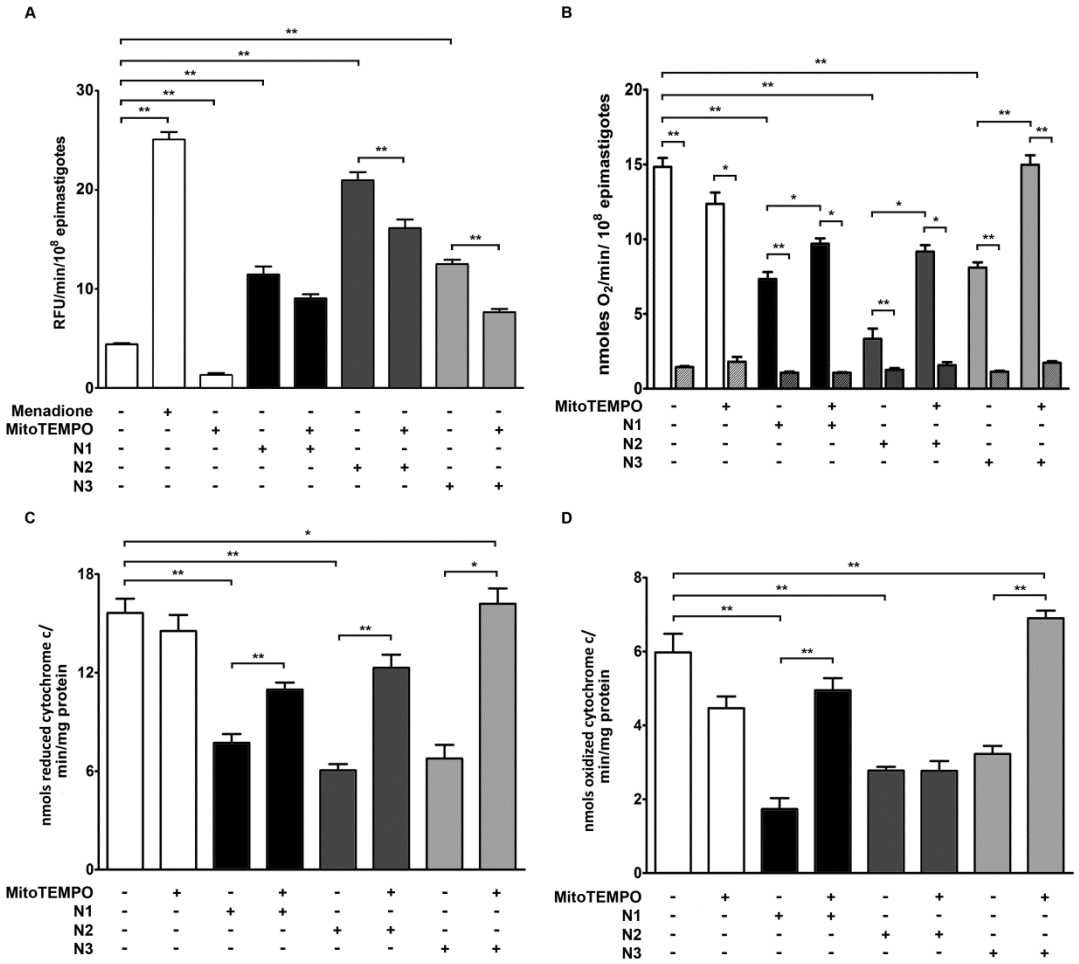

N1
N2
N3
N
NH
O

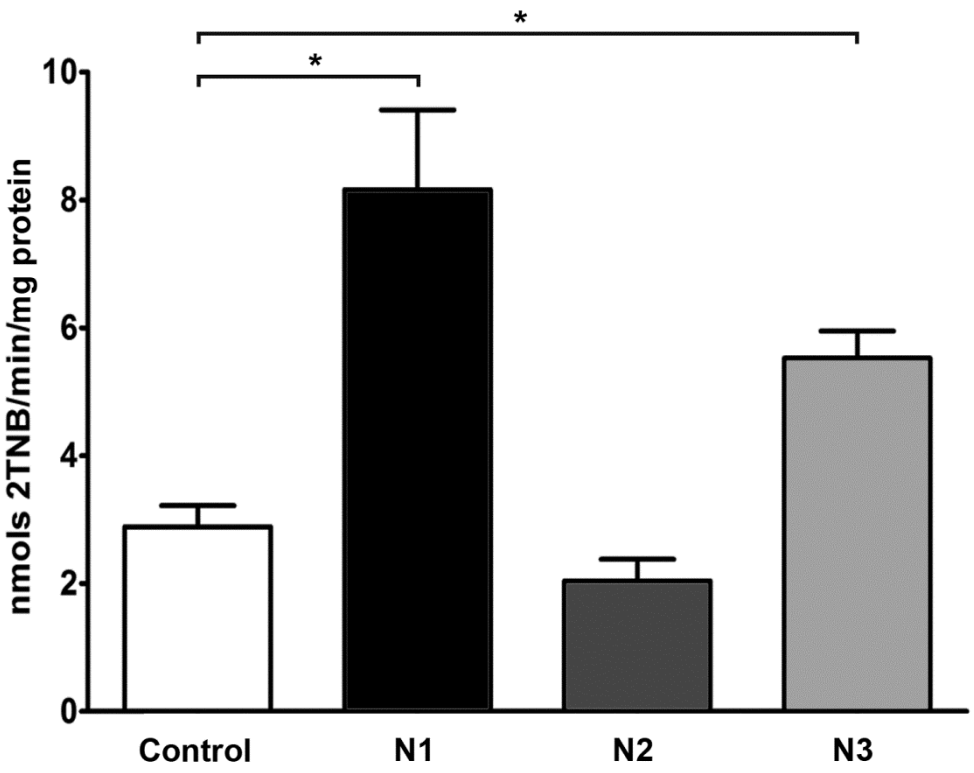

A
B
C
D
10⁶ epimastigotes/mL
10⁶ trypomastigotes/mL
Urate (μM)
α-Tocopherol (μM)
N1
N2
N3

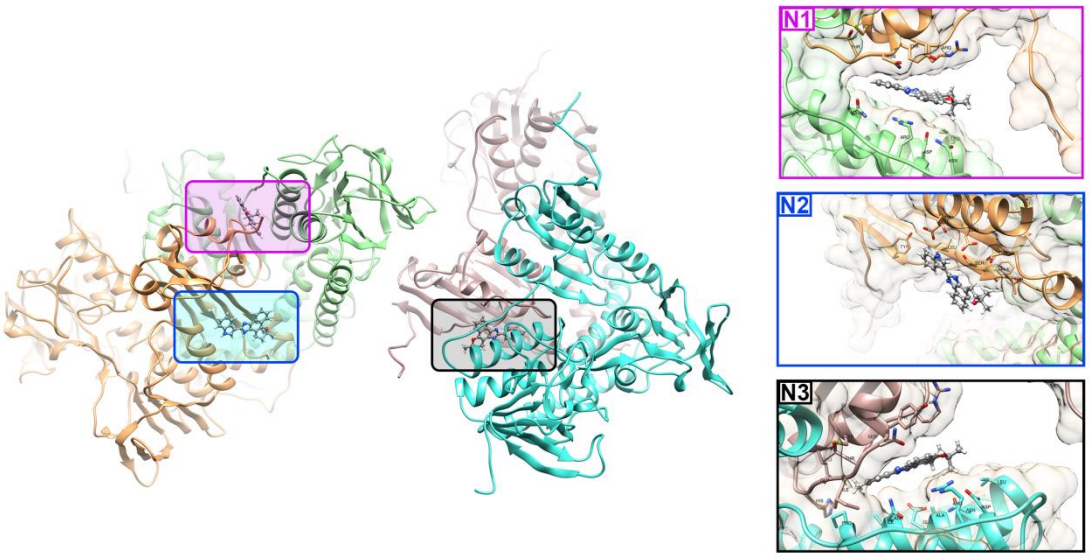





A
nmols O_2/min/10^8 epimastigotes
15
10
5
0
Urate - + - + - + - +
N1 - - + + - - - -
N2 - - - - + + - -
N3 - - - - - - + +
B
nmols reduced cytochrome c/ min/mg protein
18
12
6
0
Urate - + - + - + - +
N1 - - + + - - - -
N2 - - - - + + - -
N3 - - - - - - + +
C
nmols oxidized cytochrome c/ min/mg protein
8
6
4
2
0
Urate - + - + - + - +
N1 - - + + - - - -
N2 - - - - + + - -
N3 - - - - - - + +


A
RFU/min/10^8 epimastigotes
Menadione
MitoTEMPO
N1
N2
N3
B
nmoles O2/min/ 10^8 epimastigotes
MitoTEMPO
N1
N2
N3
C
nmols reduced cytochrome c/ min/mg protein
MitoTEMPO
N1
N2
N3
D
nmols oxidized cytochrome c/ min/mg protein
MitoTEMPO
N1
N2
N3


*
*
10
8
6
4
2
0
nmols 2TNB/min/mg protein
Control
N1
N2
N3


N1
N2
N3