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Mitochondrial plasticity in trypanosomatids as a stress adaptation mechanism

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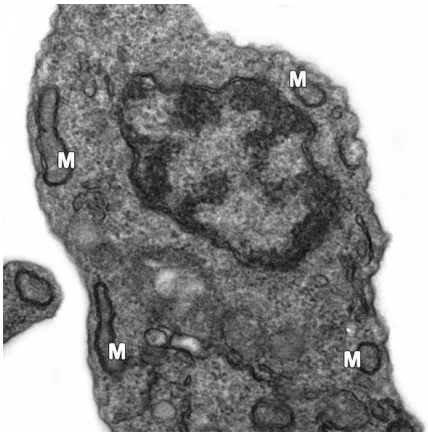
Summary

Trypanosomatids colonize different environments and are submitted to several stress situations in their hosts, which trigger intense metabolic remodeling to ensure the parasites survival in hostile environments. Some trypanosomatids can avoid the host microbicidal mechanisms, exhibiting natural resistance to oxidative and nitrosative stresses, in addition to resistance to current drug treatment. Beyond the classical role in bioenergetics, mitochondria contribute decisively to oxidative stress due to electron leakage from the electron transfer system. Several functional peculiarities made trypanosomatids' organelle an excellent target for drug intervention. Here, we discuss data on mitochondrial susceptibility and adaptative processes obtained by our group in the last 17 years. Different pathways are evaluated associated with metabolic and mitochondrial remodeling during the life cycle of trypanosomatids, and its impact on the interaction with vertebrate and invertebrate hosts. In addition, mechanistic proposals of preclinical drugs are reviewed.



1. Background

The Trypanosomatidae family includes the genera *Trypanosoma* and *Leishmania*, causative agents of important neglected tropical diseases, such as Chagas disease (*Trypanosoma cruzi*), sleeping sickness (*Trypanosoma brucei*) and leishmaniasis (*Leishmania* spp.). These illnesses are directly related to poverty, affecting especially low-income populations in the developing countries (WHO 2022). This scenario worsens due to the unsatisfactory lack of vaccines and chemotherapy (Nussbaum et al 2010; Field et al 2017). Members of the Trypanosomatidae family shelter many non-pathogenic species of protozoa that complete their life cycle in a single invertebrate host (Maslov et al 2013). As parasites of insects, monoxenous trypanosomatids can be as diverse as their hosts, showing exceptional metabolic plasticity (Frolov et al 2021). Interestingly, species from the subfamily Strigomonadinae present a β-proteobacteria in their cytosol, in a well-established mutual dependence where the endosymbiont cell cycle is controlled by the host and the bacteria are unable to grow outside of the protozoan host (Roitman, Camargo 1985; Catta-Preta et al 2015). Biochemical studies revealed that the endosymbiont completes essential metabolic pathways of the host parasite, such as amino acid production and heme biosynthesis (Chang et al 1975; Alves et al 2011, 2013).

Trypanosomatids' mitochondria are promising drug targets due to several morphological and metabolic peculiarities (Fidalgo, Gille 2011). Remarkable ultrastructural differences can be easily observed in comparison to the mammalian organelle; the protozoan shows a single mitochondrion with a ramified and elongated structure, whose form varies depending on the developmental stage and parasite species, besides the nutritional sources available in hosts' environment (de Souza et al 2009). Additionally, there is a specialized region within the mitochondrial matrix named kinetoplast, in which all mitochondrial DNA forms a disk-shaped structure containing a complex network of maxicircles and minicircles (Shapiro, Englund 1995; Liu et al 2005).

Similar to mammalian mitochondria, the electron transfer system (ETS) in trypanosomatids is formed by four integral enzyme Complexes in the mitochondrial cristae; however, other interesting enzymes contribute to electron flow in these parasites. The functionality of the trypanosomatid's NADH:ubiquinone oxidoreductase (Complex I, CI) in these parasites has long been debated. Bioinformatics analysis identified more than 60 subunits in pathogenic trypanosomatids (including core and accessory subunits), and the genomic data indicate that CI is able to perform all bioenergetic activities that have been attributed to it from other organisms, such as NADH oxidation and proton translocation (Opperdoes, Michels 2008; Duarte, Tomás 2014). However, epimastigotes of natural *T. cruzi* mutants, which present deletions in kinetoplast DNA (kDNA) encoding CI subunits, have no alterations in mitochondrial bioenergetics when compared to wild type strains, suggesting a limited function for CI (Carranza et al 2009). In *T. brucei*, the presence of CI has been clearly demonstrated in procyclic and bloodstream forms; however, it appears to be non-essential and possesses limited activity similar to what was demonstrated to *T. cruzi* (Panigrahi et al 2008; Surve et al 2012; Verner et al 2011; Duarte, Tomás 2014). Succinate:ubiquinone oxidoreductase (Complex II, CII) acts as a membrane-bound Krebs cycle enzyme. In *T. cruzi*, Vercesi et al (1991) described the oxygen consumption in digitonin-permeabilized epimastigotes incubated with succinate and ADP, showing that oxidation of this substrate by CII leads to reduction of CIII via ubiquinone, as similarly occurs in other eukaryotes (Vercesi et al 1991). Denicola-Seoane et al (1992) showed that glucose-supported oxygen consumption in *T. cruzi* epimastigotes

was little higher than succinate-supported one; whereas respiration with proline, an abundantly available amino acid in insect hosts, leads to oxygen consumption rates close to those observed with succinate (Denicola-Seoane et al 1992). In *T. brucei*, studies using RNA interference revealed disruption of CII and F_1F_0-ATP synthase activities to be conditionally lethal only when the procyclic form is maintained in a glucose-depleted medium. This phenotype was correlated with the conversion of proline into succinate in the absence of glucose (Coustou et al 2008). Thus, it is possible to conclude that succinate plays an important role in trypanosomatids bioenergetics, especially of *T. cruzi* epimastigotes. Interestingly, incubation of *T. cruzi* epimastigotes with malonate, a competitive inhibitor of succinate dehydrogenase, decreased succinate-dependent oxygen consumption by 50–70 % (Denicola-Seoane et al 1992; Carranza et al 2009), which supports the notion that part of mitochondrial respiration in trypanosomatids is promoted by another substrate. Alternative NADH dehydrogenases (NDH2) contribute to NADH/NAD^+ balance through the transfer of electrons from NADH to ubiquinone without coupled proton translocation. This enzyme was first characterized in the procyclic form of *T. brucei* and reported to have rotenone-insensitive activity (Fang, Beattie 2002). Verner et al (2013) showed that NDH2 in *T. brucei* is an enzyme facing the intermembrane space, and, thus, is not able to compensate the loss of CI (Verner et al 2013). Gene silencing through interference RNA pointed to this enzyme as essential to *T. brucei* procyclic cell growth, as well as to the maintenance of mitochondrial membrane potential ($\Delta\Psi_{mt}$), although the overall oxygen consumption did not change upon enzyme ablation. In contrast to *T. brucei*, NDH2 of *Leishmania infantum* is predominantly a mitochondrial matrix protein. Additionally, overexpression of NDH2 in promastigotes of *L. infantum* leads to significant increases in overall oxygen consumption, even as NADH oxidation increased, suggesting that this enzyme is involved in the electron flow through the ETS. Although these results do not reach statistical significance, curiously, the overexpression of NDH2 in *L. infantum* promastigotes seems to accelerate the succinate-dependent oxygen consumption, probably due to increased metabolic activity of pathways that generate succinate (Duarte et al 2021). Trypanosome alternative oxidase (TAO) was first described in the bloodstream form of *T. brucei* as a mitochondrial non-cytochrome and cyanide-resistant terminal oxidase (Chaudhuri et al 1995; 1998). Interestingly, TAO gene expression is down-regulated during differentiation of the bloodstream form into procyclic trypanosomes (Chaudhuri et al 1998), a phenotype related to the function performed by this enzyme in parasite energy metabolism. *T. brucei*, when proliferating in the mammalian bloodstream, is known to depend on the catabolism of glucose as its energy source. In this context, TAO participates, together with an FAD-dependent glycerol-3-phosphate dehydrogenase and ubiquinone, in the re-oxidation of NADH formed during glycolysis through the reduction of oxygen in a process not coupled to oxidative phosphorylation (Chaudhuri et al 2006; Shiba et al 2013). TAO was not clearly described in other trypanosomatids, and its existence has been only suggested by indirect approaches, which showed a low effect of the TAO inhibitor salicylhydroxamic acid (Santhamma, Bhaduri 1995).

In parallel to its bioenergetic role, mitochondrial metabolism plays a crucial role during oxidative stress. It is well-known that ETS electron leakage triggers the partial reduction of oxygen, culminating in the production of reactive oxygen species (ROS; Venditti et al 2013). There are some sites for the generation and elimination of ROS in trypanosomatids' organelle (Tomás, Castro 2013). In higher eukaryotes, one of the main sites of ROS generation is CI; however, as described above, in trypanosomatids, this

molecule presents partial functionality. Some authors found that neither *T. brucei* procyclic forms nor *T. cruzi* epimastigotes show an increase in superoxide anion ($O_2^{\bullet-}$) production after CI inhibition (Fang, Beattie 2002; Carranza et al 2009). In contrast, treatment of *Leishmania donovani* promastigotes with rotenone increased the generation of ROS (Mehta, Shaha 2004). CII activity in *T. cruzi* epimastigotes and *L. donovani* promastigotes was also associated with oxidative stress, since the treatment of parasites with thenoyltrifluoroacetone, another inhibitor of CII, increased ROS production (Mehta, Shaha 2004; Silva et al 2011). Ubiquinol:cytochrome *c* oxidoreductase (CIII) acts similarly in trypanosomatids and higher eukaryotes. This Complex transfers electrons to cytochrome *c*, which reduces cytochrome *c* oxidase (CIV). As well as in other organisms, Q-cycle hypothesis seems to be real in trypanosomatids, making CIII the major source of mitochondrial ROS. Although this hypothesis should be further investigated, $O_2^{\bullet-}$ formation in *L. donovani* promastigotes and *T. cruzi* epimastigotes was associated with CIII activity after parasites were treated with antimycin A, a classical inhibitor of this enzyme (Mehta, Shaha 2004; Silva et al 2011; Tomás, Castro 2013). Another important site of $O_2^{\bullet-}$ production in trypanosome mitochondria is NDH2. Fang and Beattie (2002) demonstrated that this enzyme is able to produce large amounts of $O_2^{\bullet-}$ in isolated mitochondria of *T. brucei* procyclic form and can be inhibited by diphenyl iodonium, an inhibitor of flavoproteins. These researchers suggest an antioxidant function of TAO, due to increased production of reactive species in the *T. brucei* procyclic form after enzyme inhibition by salicylhydroxamic acid (Fang, Beattie 2003). Thus, in the presence of TAO, the electrons would directly reduce oxygen, without the formation of semiquinone (Fang, Beattie 2003).

Although ROS at basal levels are crucial for signaling pathways and proliferation in trypanosomatids, at higher concentrations these molecules are toxic for the parasites (Nogueira et al 2011). The imbalance in ETS activity directly affects ROS generation and redox homeostasis (Venditti et al 2013), promoting protozoa virulence and disease progression by increased antioxidant environment (Piacenza et al 2013). Today, there are many reports describing that trypanosomatids deal with redox challenge derived from host response, and with the variation of nutrients and/or energetic substrates available during their life cycles (Tielens, Van Hellemond 1998; Gonçalves et al 2011; Bombaça et al 2017, 2020, 2021a; Pedra-Rezende et al 2021; Pinho et al 2020, 2022). In this review, an overall discussion is provided on the main advances about trypanosomatids' mitochondrial plasticity and redox metabolism made over the last 17 years from *in vitro* studies by our group.

2. Mitochondrial plasticity among *T. cruzi* stages

Comparing *T. cruzi* epimastigotes and bloodstream trypomastigotes, the parasite forms found in triatomine insect vector and mammalian host, morphological and functional remodeling are observed in parasites' mitochondria. Despite succinate oxidation supporting ETS and ROS production in both parasite stages, striking differences were detected between their mitochondrial metabolisms. In comparison to epimastigotes, bloodstream trypomastigotes present reduced oxygen consumption supported by succinate and ADP, increased electron leakage and ROS formation, suggestive of mitochondrial impairment. Also, in bloodstream trypomastigotes, an increase in CII-CIII activity facilitates the entry of electrons into the ETS; however, the low CIII gene expression and CIV functionality limits the electrons flow to the downstream

ETS steps and the complete reduction of oxygen. The consequent "electron bottleneck" effect results in high ROS production. On the other hand, epimastigotes have high oxygen consumption rates accompanied by an increase of $\Delta\Psi_{mt}$. Our data evaluating the susceptibility of trypomastigotes and epimatigotes to iodoacetamide (IAA), an inhibitor of glycolytic pathway, as well as antimycin A and hydrogen peroxide (H_2O_2), suggest that the bloodstream form is more fermentative than the invertebrate form — due to its lower LD_{50} value for IAA and higher resistance to ETS inhibition and oxidative stress —, a phenotype consistent with the availability of glucose in the vertebrate´s blood and L-proline in the triatomine's midgut, respectively (Gonçalves et al 2011).

During its life cycle, *T. cruzi* is submitted to different stress conditions that are used as signaling for differentiation. In metacyclogenesis, low nutrient availability and an acidic environment are pivotal features to the transformation of epimastigotes into metacyclic trypomastigotes in triatomine rectum, a phenomenon mimicked *in vitro* by an artificial medium (TAU medium) that has a composition and pH similar to triatomine urine (Contreras et al 1988). Mitochondrial remodeling and nutrient availability are interconnected traits, often mediated by an autophagic pathway; our group studied the relationship between both in epimastigotes subjected to nutritional deprivation and acid stress. In the two conditions, epimastigotes' mitochondria showed strong morphological damage and ETS impairment, which was demonstrated by decreased $\Delta\Psi_{mt}$ and oxygen consumption. Interestingly, only epimastigotes submitted to acid stress condition had high ROS production, which was accompanied by increased expression and activity of trypanothione reductase and tryparedoxin peroxidase, two antioxidant enzymes extremely important for the parasite to deal with oxidative stress situations (Piacenza et al 2013). Nutritional deprivation and acid stress also promoted an exacerbation of autophagy, evidenced by the high number of autophagosomes and overexpression of distinct autophagy-related genes. We showed a direct correlation between autophagy and mitochondrial dysfunction in *T. cruzi* epimastigotes submitted to both stress conditions for 24 h, where the treatment with an antioxidant decreased the number of Atg8+ puncta/parasite (Pedra-Rezende et al 2021). In summary, *T. cruzi* mitochondria show remarkable functional changes during its life cycle, which are crucial for parasite survival in its distinct hosts.

3. Mitochondrial plasticity in *Leishmania* spp.

Similar remodeling can be observed in mitochondria of *Leishmania* spp. A comparative study analyzing the shotgun proteome of *Leishmania braziliensis*, *Leishmania panamensis* and *Leishmania guyanensis* promastigotes allowed us to determine the abundances of proteins involved in the main metabolic processes. The dataset showed that proteins involved in the glycolytic pathway, ETS and oxidative phosphorylation represent 3.7 % of all identified proteins, and other processes involved in ATP production comprising about 7.4 % of proteins detected. The evaluation of cumulative concentration of components related to the glycolytic pathway revealed a significantly higher abundance of these molecules in promastigotes of *L. braziliensis* than in *L. panamensis* and *L. guyanensis* promastigotes, suggesting that this species relies more on glycolysis than the others. In contrast, proteomic data showed significant differences in the abundance of ETS proteins in *L. panamensis* and *L. guyanensis* promastigotes, reinforcing the notion that these species are more dependent on other bioenergetic pathways such as oxidative phosphorylation, amino acid oxidation and fatty acid metabolism. Biochemical assays

corroborated these findings, showing higher levels of 2-NBDG uptake, a fluorescent glucose analogue, in *L. braziliensis* promastigotes; and, conversely, high mitochondrial oxygen consumption in *L. panamensis* and *L. guyanensis* promastigotes (Pinho et al 2020).

The production of cytokines, ROS and nitric oxide ($NO^{\cdot}$) by mammalian cells normally leads to control of trypanosomatid infection; however, some *L. braziliensis* strains have been highlighted due to the existence of a natural $NO^{\cdot}$ resistance. These resistant promastigotes are endowed with specific mechanisms of survival and persistence, causing more lesions and being frequently more resistant to pharmacological treatment with antimonials (Giudice et al 2007; Souza et al 2010). Using proteomic approaches, we demonstrated that *L. braziliensis* NO-resistant promastigotes rapidly modulate their protein content in response to $NO^{\cdot}$ exposure, increasing significatively the total protein levels and the concentration of glutathione pathway's intermediates. It may be a mechanism to maintain the pool of NADPH and the recovery of glutathione levels, protecting parasites from oxidative damages. We also detected an increase of 2-NBDG uptake and abundance of enzymes related to the pentose phosphate pathway, suggesting an increase of glycolytic pathway related to the parasites' antioxidant defenses (Pinho et al 2022). Other groups demonstrated that $NO^{\cdot}$ resistance in *Leishmania infantum* promastigotes is accompanied by the high expression of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Holzmuller et al 2006; Alcolea et al 2016). Despite the higher abundance of enzymes of the glycolytic pathway in NO-resistant promastigotes, the protein concentration of mitochondrial CI, CII and CIV is significantly lower in comparison to NO-susceptible ones. Curiously, after nitrosative challenge the concentration of molecules related to CIII was found to increase in NO-resistant parasites, but was not affected in NO-susceptible promastigotes. In contrast, our functional analysis pointed to low oxygen consumption in the ROUTINE state in NO-susceptible parasites; also, the mitochondrial-independent oxygen consumption was 3.5-fold higher in these cells upon $NaNO_2$ treatment. Together, these data suggest a deficient antioxidant system and elevated ROS production in NO-susceptible epimastigotes, especially in stressful situations (Pinho et al 2022).

Iron is a crucial component in bioenergetics and antioxidant machinery of trypanosomatids, once it is integrated into different Complexes of the ETS (hemoproteins and iron-sulfur clusters) and acts as cofactor of important antioxidant enzymes (Wilkinson et al 2002; Dufernez et al 2006). In *Leishmania* spp. promastigotes, iron is obtained from the extracellular environment by transferrin and heme uptake (Flannery et al 2013) or — as more recently described — by the action of two molecules: a plasma membrane-associated ferric reductase named LFR1 and transmembrane ferrous iron transporter from the ZIP family LIT1 (Jacques et al 2010; Flannery et al 2011). This plant-like system is essential for parasite growth and the development of cutaneous lesions (Huynh et al 2006, 2008; Flannery et al 2011; Mittra et al 2013). Our previous work showed that the iron chelator 2,2-dipyridyl impaired *L. braziliensis* promastigotes' growth, leading to strong disorganization of mitochondrial ultrastructure, in addition to the formation of concentric membrane structures in the organelle matrix and the loss of cristae. The mitochondrial damage was confirmed by $\Delta\Psi_{mt}$ collapse and the negative modulation of the expression of several mitochondrial proteins, such as cytochrome *c* oxidase subunit V (Mesquita-Rodrigues et al 2013). Despite the recent discoveries about the iron and heme uptake in trypanosomatids, its multifactorial responses deserve further analysis.

4. Mitochondrial metabolism and ROS resistance in *Strigomonas culicis*

Among the huge group of monoxenous trypanosomatids, we focused our efforts on *S. culicis,* a symbiont-harboring trypanosomatid that colonizes the midgut of several insects (Novy et al 1907). To analyze the contribution of the endosymbiont to different metabolic processes of the epimastigotes, the proteomic profiles of wild type and aposymbiotic strains were assessed by shotgun approaches. Among the pathways most affected by elimination of the endosymbiont are amino acid synthesis and protein folding; in addition, several molecules involved in glycolysis, gluconeogenesis, pentose phosphate pathway and glutathione metabolism were more abundant in aposymbiotic epimastigotes than in wild type ones. Functional data corroborate the differences concerning protein abundance, which increased activity of glucose 6-phosphate dehydrogenase and 2-NBDG uptake in the epimastigotes without the bacteria (Brunoro et al 2019; Bombaça et al 2020). The symbiont elimination also impaired mitochondrial function in relation to wild type strain, a phenotype observed through the lower activity of CII-CIII and CIV, oxygen consumption and ATP content. In contrast, higher ROS production and remarkable antioxidant response were detected, suggesting the participation of endosymbiotic bacteria in energy metabolism and the maintenance of a reducing environment (Bombaça et al 2017).

Our group induced an artificial ROS resistance in *S. culicis* wild type strain by epimastigote incubation with increasing concentrations of H_2O_2. ROS resistance in *S. culicis* triggered the antioxidant system, increasing thiol-dependent peroxidase activity and decreasing H_2O_2 production and lipid peroxidation. Interestingly, the H_2O_2-resistant strain also showed an increased expression and activity of ascorbate peroxidase in comparison to wild type strain. H_2O_2 resistance was also accompanied by an increase in parasites' mitochondrial functionality, which was observed through higher parasite resistance to ETS inhibitors, elevated oxygen consumption and activity of mitochondrial Complexes and ATP content (Bombaça et al 2017, 2020). Curiously, our unpublished data point out that the H_2O_2-resistant strain has higher concentrations of intracellular iron and heme, suggesting that ROS resistance modulates heme biosynthesis. In addition, the pre-treatment of H_2O_2-resistant parasites with 2,2-dipyridyl decreases mitochondrial and ascorbate peroxidase activities to the same levels detected in wild type strain, demonstrating the importance of iron and heme to maintain energy metabolism and an antioxidant environment during ROS resistance induction (Bombaça et al, unpublished).

S. culicis epimastigotes are part of *Aedes aegypti* microbiota and are submitted to different stress conditions derived from host's metabolism, including ROS production by dual oxidases (DUOXs). Our investigation into the influence of *S. culicis* on mosquitoes' midgut showed the activation of different response mechanisms in the infection by both wild type and H_2O_2-resistant strains. While wild type parasites stimulated the host's mitochondrial metabolism and the consequent production of superoxide radical, the H_2O_2-resistant strain exacerbated DUOX activity and caused a remarkable ROS production. In addition, the infection by both strains compromised mosquitoes' reproductive fitness, decreasing fecundity and fertility of females. These phenotypes can be related to parasite load, once ROS resistance increases the ability of *S. culicis* epimastigotes to infect and persist in pro-oxidant environments (Bombaça et al 2017, 2021a).

5. Preclinical drugs and the mitochondrion

In vitro analysis of parasites treated with different classes of drugs frequently report the mitochondria as the main target during pharmacological treatment. Among the most recurrent phenotypes observed in morphological assays, mitochondrial swelling, cristae disorganization, and matrix electron density impairment are found most commonly (Figure 1); however, this phenotype varies with the drug used, concentration, and time of treatment (Sen, Majumder 2008; Fidalgo, Gille 2011; Silva et al 2011). Indeed, there are many studies in the literature describing mitochondrial damage in treated parasites based on ultrastructural evaluation (Menna-Barreto, de Castro 2014; Vannier-Santos et al 2019).

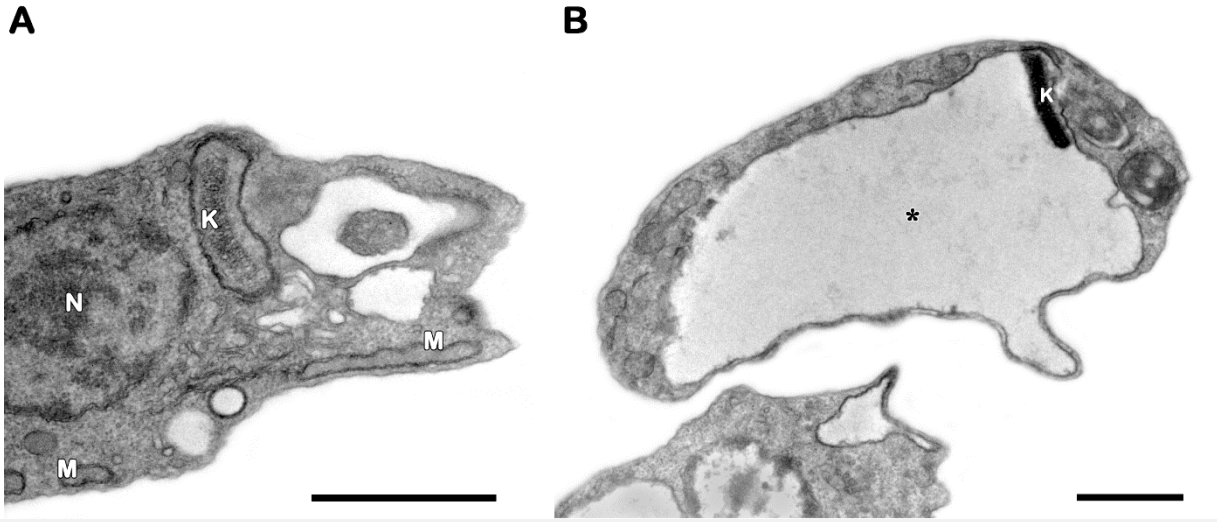


Figure 1. The mitochondrion of trypanosomatids as the most recurrent drug target. **(A)** Control parasite, showing classical morphology of nucleus (N), mitochondrion (M) and kinetoplast (K). **(B)** Treated parasite presenting a strong mitochondrial swelling (asterisk), with a washed-out aspect of the matrix and loss of cristae organization. Bars: 1 µm.

In *T. cruzi*, sesquiterpenoid isolated from the Chilean flora, as well as naphthoquinones and derivatives led to mitochondrial swelling, with $\Delta\Psi_{mt}$ reduction and increased ROS generation in epimastigotes (Menna-Barreto et al 2005, 2007; Salomão et al 2013; Bombaça et al 2018). A similar phenotype was detected in bloodstream trypomastigotes and epimastigotes treated with geranylgeraniol obtained from the Brazilian plant *Pterodon pubescens*or with HIV peptidase inhibitors (Menna-Barreto et al 2008; Sangenito et al 2014, 2018). Such ultrastructural alterations were also observed in treated *Leishmania amazonensis*. Promastigotes incubated with epigallocatechin 3-gallate, the main flavonoid in green tea, or with apigenin, a natural flavone, or even with a metallodrug (zinc complex), showed a dilated aspect and decreased $\Delta\Psi_{mt}$ (Inácio et al 2012; Fonseca-Silva et al 2015; Sangenito et al 2021). Fonseca-Silva et al (2015) demonstrated the protective effect of the antioxidants glutathione and N-acetyl-L-cysteine through the reduction of ROS levels in apigenin-treated parasites and reversal of leishmanicidal activity (Fonseca-Silva et al 2015). Although morphological phenotype and $\Delta\Psi_{mt}$ loss measured by fluorescent probes are interestingly starting points, they do not really assess the mechanistic action of compounds in mitochondrial physiology.

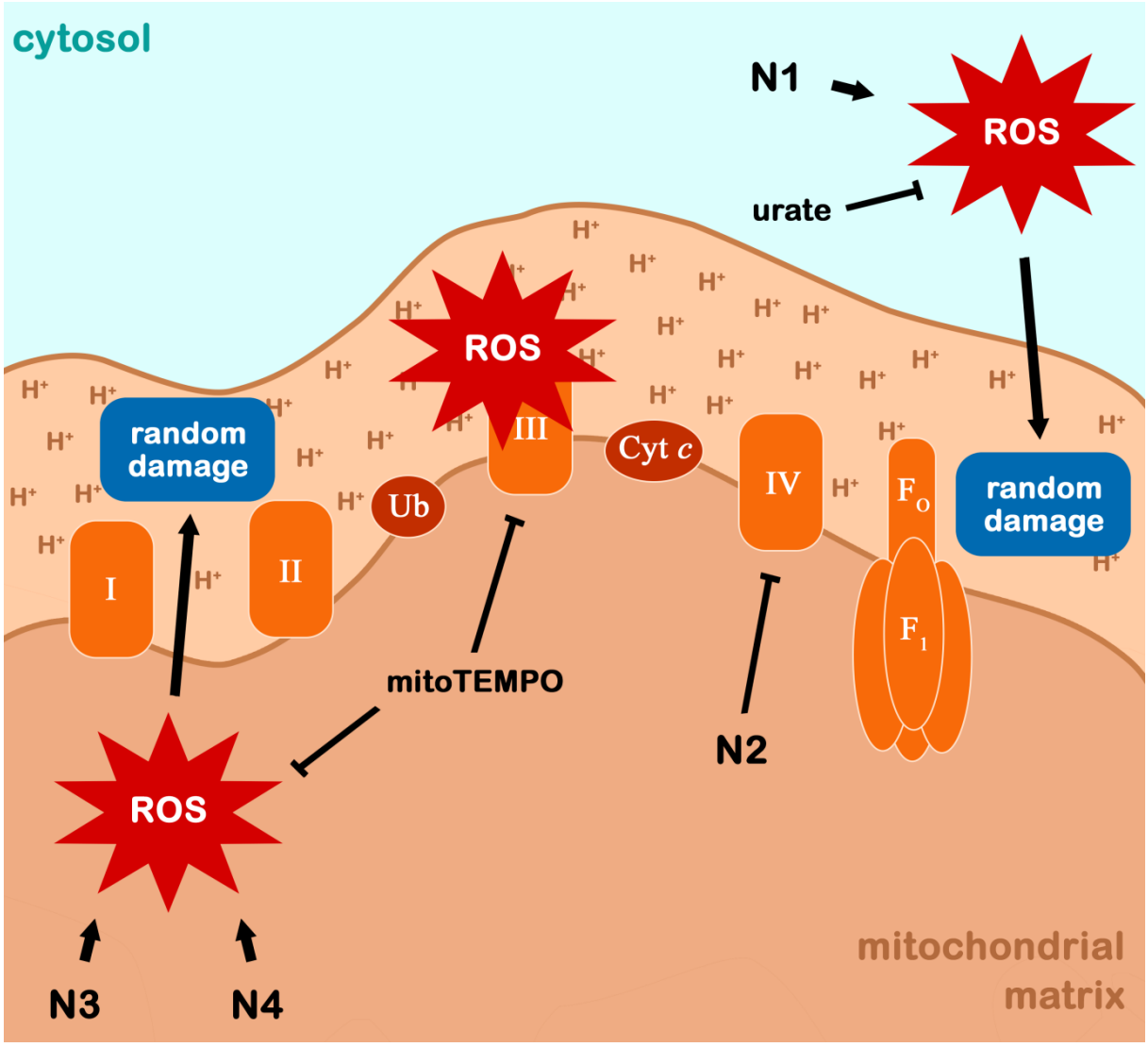


Figure 2. The mechanistic proposal of trypanocidal activity on *T. cruzi* mitochondrion. N1, N3 and N4 induced ROS production, resulting in an unspecific mitochondrial injury. Curiously, N3 and N4 showed the phenotype prevented only by the mitochondrial antioxidant mitoTEMPO. In contrast, N1 led to ROS generation in the cytosol, and the parasite can be protected from its deleterious effect by the incubation with urate. N2 inhibited cytochrome *c* oxidase (Complex IV) activity and, subsequently, induced the increase in mitochondrial ROS levels. Ub: ubiquinone; Cyt *c*: cytochrome *c*.

To further investigate the mitochondria of trypanosomatids as a drug target, our group focused on the mechanisms involved in trypanocidal action of naphthoquinones and derivatives. In 2009, we assessed naphthofuranquinones activity on *T. cruzi* mitochondrial physiology. In both epimastigotes and bloodstream trypomastigotes, the compounds promoted a drastic effect on the organelle, leading to impairment of CI–CIII activity and succinate-induced oxygen consumption. In our mechanistic proposal, we suggested the interference of compounds with mitochondrial electron flow, deviating electrons from ubiquinone (Menna-Barreto et al 2009). On the other hand, proteomic approaches corroborate our electron microscopy data, pointing to the high number of mitochondrial proteins modulated by the treatment with β-lapachone-derived naphthoimidazoles N1, N2 and N3 in both epimastigotes and bloodstream

trypomastigotes of *T. cruzi* (Menna-Barreto et al 2005, 2007, 2010; Brunoro et al 2016). Reinforcing these findings, in a recent work, we described that these three compounds strongly reduced the rates of oxygen consumption and CII-CIII and CIV activities, impairing mitochondrial metabolism. Moreover, ROS production was related to antiparasitic activity, with N2 and N3 reducing ETS electron flux and increasing the mitochondrial ROS levels. Curiously, ROS generation derived from N1 treatment did not derive from mitochondrial injury, probably resulting as a consequence of the inhibition of antioxidant enzymes (Bombaça et al 2019). In 2021, a similar study was performed with a novel naphthoimidazole named N4. Its trypanocidal action is faster than that observed for the other three naphthoimidazoles, increasing ROS levels through CII-CIII impairment in the early hours of treatment (Bombaça et al 2021b). Figure 2 shows the mechanistic proposal of naphthoimidazoles' trypanocidal activity.

6. Concluding remarks

As previously described, the mitochondria of trypanosomatids are commonly affected by numerous classes of drugs. The peculiarities in bioenergetics, such as remarkable differences in ETS, the peculiar antioxidant machinery, as well as the TAO existence and the glycolysis compartmentalization into glycosomes (Tomás, Castro 2013; Michels et al 2021), make these parasites different from their hosts and suggest the energetic and oxidative metabolisms as promising drug targets. Furthermore, during their life cycles, trypanosomatids were challenged to several stress conditions. As an example, the same parasite is submitted to completely different environments inside the invertebrate and vertebrate hosts. Temperature, pH, nutrient availability, among many other factors, can influence the parasite's metabolism and, consequently, success in its life cycle. In this scenario, mitochondrial plasticity plays a crucial role to guarantee their adaptation to the host (Gonçalves et al 2011; Bombaça et al 2017, 2020, 2021a; Pedra-Rezende et al 2021; Pinho et al 2020, 2022). However, despite the recent advances, further studies must be conducted to better characterize the biochemical and molecular mechanisms related to the parasites' adaptative behavior.

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