Antioxidant defence system as a rational target for Chagas disease and Leishmaniasis chemotherapy

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Chagas disease and leishmaniasis are neglected tropical diseases caused by the protozoan parasites Trypanosoma cruzi and Leishmania spp., respectively. They are among the most important parasitic diseases, affecting millions of people worldwide, being a considerable global challenge. However, there is no human vaccine available against T. cruzi and Leishmania infections, and their control is based mainly on chemotherapy. Treatments for Chagas disease and leishmaniasis have multiple limitations, mainly due to the high toxicity of the available drugs, long-term treatment protocols, and the occurrence of drug-resistant parasite strains. In the case of Chagas disease, there is still the problem of low cure rates in the chronic stage of the disease. Therefore, new therapeutic agents and novel targets for drug development are urgently needed. Antioxidant defence in Trypanosomatidae is a potential target for chemotherapy because the organisms present a unique mechanism for trypanothione-dependent detoxification of peroxides, which differs from that found in vertebrates. Cellular thiol redox homeostasis is maintained by the biosynthesis and reduction of trypanothione, involving different enzymes that act in concert. This study provides an overview of the antioxidant defence focusing on iron superoxide dismutase A, tryparedoxin peroxidase, and ascorbate peroxidase and how the enzymes play an important role in the defence against oxidative stress and their involvement in drug resistance mechanisms in T. cruzi and Leishmania spp.

Key words: Trypanosoma cruzi - Leishmania spp - chemotherapy - antioxidant defence - drug resistance

Chagas disease and leishmaniasis are infectious, parasitic diseases caused by protozoan parasites of the Trypanosomatidae family. Trypanosoma cruzi is the etiological agent of Chagas disease (American trypanosomiasis), affecting 6-7 million people globally. It is endemic to 21 continental Latin American countries, and due to increased migration, the disease has spread across Europe, the United States, Canada, and Japan. Leishmaniasis are a complex of diseases caused by different species of parasites of the genus Leishmania, currently affecting 12 million people globally and presenting an incidence of 0.7-1.0 million new cases annually from nearly 100 endemic countries. The disease can comprise the following main clinical forms: cutaneous leishmaniasis, characterised by cutaneous and mucosal lesions, or visceral leishmaniasis (VL), in which the parasites have tropism for internal organs such as the liver and spleen. VL is the most severe form of the disease and can be lethal if left untreated.

No human vaccine is available for Chagas disease or leishmaniasis, and currently, few drugs are available to treat the diseases. Nifurtimox (5-nitrofuran; NFX) and benznidazole (2-nitroimidazole; BZ) have been used for Chagas disease chemotherapy. Although these drugs have been in use for more than 50 years, they have several drawbacks, including low cure rates in the chronic stage of the disease, significant toxic side effects, and the existence of naturally resistant strains of T. cruzi. Few drugs, including pentavalent antimonials (e.g., sodium stibogluconate and meglumine antimoniate), amphotericin B and formulations, miltefosine, paromomycin sulphate, and pentamidine isethionate are currently available for leishmaniasis treatment. Chemotherapy for leishmaniasis presents several problems, such as high drug toxicity, long treatment protocols, and the occurrence of drug-resistant parasite strains. Therefore, there is a need to understand drug resistance mechanisms and identify new molecular targets for drug development against Chagas disease and leishmaniasis. This article focuses on studies elucidating the importance of antioxidant defence against oxidative stress and its association with drug resistance mechanisms in T. cruzi and Leishmania spp. being considered as a rational target for chemotherapy against the important neglected tropical diseases.

Antioxidant defence - Trypanosomatids are frequently exposed to different reactive oxygen species (ROS), such as superoxide anions, hydrogen peroxide (H₂O₂), and hydroxyl radicals, produced by cellular metabolism and external agents, including products of the immune response of the host and drug metabolism. Since ROS can damage various cellular components, including membrane lipids, nucleic acids, and proteins, all organisms possess defence mechanisms based on antioxidant enzymes. However, trypanosomatid cells lack catalase, selenium-dependent glutathione peroxidase (GPX), glutathione reductase, and thioredoxin reductase. Instead, Trypanosomatids possess a peculiar antioxidant defence mechanism based on the low molecular mass dithiol trypanothione [bis(glutathionyl)spermidine; T(SH)₂]². The trypanothione is a central thiol that
delivers electrons for the synthesis of DNA precursors, the detoxification of hydroperoxides, and other trypa
thione-dependent pathways. Trypanothione directly reduces tryparedoxin, dehydroascorbate, and glutathi
one disulphide by sequential reactions coupled with the reductive detoxification of peroxides and the formation of deoxyribonucleotides (Figure). Trypanothione disulphide (TS₂) is reduced by nicotinamide adenine dinucleotide phosphate to trypanothione (T(SH)), in a reaction catalysed by trypanothione reductase. Thus, cellular thiol redox homeostasis is maintained by biosynthesis and reduction of trypanothione.

The defence machinery in trypanosomatids is composed of many enzymes distributed in diverse cellular compartments that are activated by various oxidants. Iron-superoxide dismutases (FeSODs) detoxify superoxide radicals (O₂⁻), which are converted to oxygen (O₂) and hydrogen peroxide (H₂O₂). Tryparedoxin peroxidases (TXNPx) use tryparedoxin to detoxify H₂O₂, hydroperoxides (ROOH), and peroxynitrites (NOOH). Ascorbate peroxidase (APX) converts H₂O₂ to water (H₂O). Trypanothione reductase (TryR) is an enzyme that utilises NADPH to keep trypanothione in its reduced form [T(SH)₂]. T(SH)₂ converts trypanothione (TXN) to its reduced form, dehydroascorbate (dhAsc) to ascorbate (Asc), and glutathione disulphide (GSSG) to glutathione (GSH). The sequential reactions are coupled to the reductive detoxification of peroxides [H₂O₂, ROOH, and NOOH] and the synthesis of deoxyribonucleotides by ribonucleotide reductase (RR). Resistance to hydro- and lipid-hydroperoxides is conferred by non-selenium glutathione peroxidases-like (GPX-I and GPX-II), which utilise glutathione and/or tryparedoxin as reducing substrates (Figure). Cytosolic and mitochondrial tryparedoxin peroxidase (c- or m-TXNPx) from the 2-cysteine peroxiredoxin family can detoxify peroxynitrite,
H₂O₂, and small-chain organic hydroperoxides using tryparedoxin (Figure). Ascorbate-dependent heme-peroxidase (APX) is located in the endoplasmic reticulum and confers resistance to H₂O₂ challenge using ascorbate as the reducing substrate (Figure).

Other trypanothione-dependent enzymes are related to antioxidant functions, such as enzymes of the glutathione S-transferase class [which in Leishmania act together in elongation factor 1B (eEF1B) in the metabolism of linoleic acid hydroperoxide] and ovotiol A (a mercaptohistidine that works by eliminating H₂O₂ and free radicals).14,17,18

The molecules of the redox system are essential to protect lipids, proteins, and DNA from damage caused by oxidants. A better understanding of the molecular mediators of resistance to oxidative stress enables studying the host-parasite relationships and clarifies the mechanisms of drug resistance in the parasites.

In the next section, we describe studies of FeSODs, TXNPs, and APX, in relation to the important roles they play in defending oxidative stress and their involvement in drug resistance mechanisms in T. cruzi and Leishmania spp.

Iron superoxide dismutase A - FeSOD-A is an important enzyme in the antioxidant defence system that protects parasites against superoxide radicals (O₂⁻), which are converted to oxygen (O₂) and hydrogen peroxide (H₂O₂). H₂O₂ is metabolised by different enzymes with peroxidase activity, such as tryparedoxin peroxidase (TXN), ascorbate peroxidase (APX), peroxiredoxins (PRXs), and glutathione peroxidases (GPxs).14

The metalloenzyme SOD (EC 1.15.1.1) is a key component of the antioxidant defence system of many organisms and contains different metal cofactors at its active site.19 In trypanosomatids, SODs contain iron (Fe) in their structure. They are classified as FeSOD-A expressed in the mitochondria20,21 FeSOD-B1 and FeSOD-B2 are located in the glycosome,22 and FeSOD-C is detected in the mitochondria.20 Because FeSOD is absent in the human host, it can serve as a promising molecular target for drug development against trypanosomatids.

Proteomic and differential expression analyses showed that FeSOD-A is overexpressed in the T. cruzi population with in vitro-induced resistance to benznidazole.23,24 Molecular characterisation of the FeSOD-A gene in 25 different T. cruzi populations and strains showed gene amplification, increased mRNA levels, and protein expression, and FeSOD enzyme activity in a T. cruzi population with in vitro-induced resistance to benznidazole.25 In addition, it has been shown that parasites overexpressing FeSOD-A were more resistant to the programmed cell death stimulus resulting in cytoprotective effects.26

Several studies have shown the role of FeSOD-A in protecting parasites against oxidative stress. FeSOD-deficient L. tropica was shown to be more sensitive to oxidative stress, and FeSOD-deficient L. donovani has a decreased ability to infect murine macrophages.27 L. amazonensis deficient in FeSOD-A was more sensitive to oxidative stress and less effective in producing lesions in mice.28 In addition, mutant L. infantum parasites with lower levels of FeSOD-A were more susceptible to oxidative stress generated by menadione, and their ability to maintain infection in macrophages was decreased.29 It was demonstrated that ROS was needed for parasite infectivity, and the production of H₂O₂ by FeSOD-A was crucial in the process.28

Considering the importance of FeSOD-A for parasites and the potential use of the enzyme as a molecular target for drug development, methodologies have been used to obtain FeSOD-A knockout in Leishmania. Attempts to delete the FeSOD-A enzyme-coding gene using three different methodologies (conventional allelic replacement or two different CRISPR methods) failed because FeSOD-A gene copies were probably retained by aneuploidy or gene amplification, suggesting that the gene plays an essential role in L. infantum.30 Similarly, FeSOD-A could not be deleted in L. amazonensis.28

Several studies have shown that FeSOD-A is associated with SbIII activity and susceptibility to miltefosine. Tessarollo et al.30 reported a higher activity of FeSOD enzymes in L. infantum and L. braziliensis resistant to SbIII. In addition, their study observed that L. infantum and L. braziliensis became more resistant to trivalent antimony and more tolerant to oxidative stress following the overexpression of FeSOD-A.30 Furthermore, L. donovani overexpressing FeSOD-A was more resistant to miltefosine.31 Another study reported that a miltefosine-resistant L. donovani isolate overexpressed FeSOD-A and had increased enzyme activity compared with the susceptible isolate.32

Mutant L. infantum parasites with lower FeSOD-A levels were resistant to trivalent antimony and miltefosine.29 The transcript levels of five FeSODs (FeSOD-B1, FeSOD-B2, and three putative SODs) and six enzymes from the antioxidant defence system (ascorbate peroxidase, tryparedoxin peroxidase, peroxidoxin, non-selenium glutathione peroxidase, and NADH-dependent furmarate reductase) were evaluated to investigate whether other enzymes compensated the decrease in FeSOD-A expression. The transcript level of the enzyme ascorbate peroxidase increased in the two FeSOD-A−/−/− mutants tested.29 In addition, one mutant showed an increase in tryparedoxin peroxidase and SOD putative (LINF_340012900) expression, and the other had an increase in FeSOD putative SODB1, SODB2, and SOD putative (LINF_300033000) expression. The data demonstrate the deregulation of the oxidative stress defence pathways and the ability of the parasite to compensate for the lower FeSOD-A expression.

As FeSOD is not found in mammals and plays an essential role in the defence of the parasite against oxidation, it is a potential target in the development of new chemotherapeutic alternatives. Furthermore, some compounds with inhibitory effects against T. cruzi. Fe-SOD showed remarkable in vitro and in vivo trypanocidal activities.33 Benzo[g]phthalazine and phthalazine derivatives were more active against T. cruzi in vitro and in vivo in the acute and the chronic phase of the infection, less toxic to the host than benznidazole, and showed selective inhibitory effects on T. cruzi Fe-SOD enzyme activity in comparison with human CuZn-SOD.34,35,36
Others compounds such as polyamine macrocycles deri

\[ \text{Tryparedoxin peroxidase - TXNPx belongs to the } \]

\[ \text{2-cysteine peroxiredoxin family and detoxifies peroxynitrite, } \]

\[ \text{H}_2\text{O}_2 \text{ and small-chain organic hydroperoxides using tryparedoxin, a thireodoxin-related protein as } \]

\[ \text{an electron donor, which in turn is reduced by dihydrotrypanothione.} \]

\[ \text{TXNPx can be grouped according to their cytosolic (cTXNPx) or mitochondrial (mTXNPx) compartmentalisation. } \]

\[ \text{An association was found between virulence and the protein levels of both TXNPx enzyme isoforms in several } \]

\[ \text{T. cruzi strains as well as in cTXNPx-overexpressing parasites.} \]

\[ \text{In previous studies carried out by our group using proteomic analysis, TXNPx protein was highly expressed in the } \]

\[ \text{T. cruzi population with } \text{in vitro-induced resistance to } \]

\[ \text{BZ (17LER). } \]

\[ \text{We have extended the results by characterising the two TXNPx enzymes isoforms in nine other strains of } \]

\[ \text{T. cruzi that were either susceptible or naturally resistant to BZ. Our results demonstrated that cTXNPx and } \]

\[ \text{mTXNPx enzymes have an increased expression level in the } \text{in vitro-induced BZ-resistant } \]

\[ \text{T. cruzi population, contrary to what was observed in the } \text{in vivo-selected BZ-resistant and naturally } \]

\[ \text{resistant strains.} \]

\[ \text{Lin et al. reported a concomitant increase in the expression of both enzyme isoforms in several } \]

\[ \text{T. cruzi strains as well as in cTXNPx-overexpressing parasites.} \]

\[ \text{In } \text{T. cruzi, overexpression of cTXNPx or mTXNPx protected the parasite from either hydrogen peroxide or organic peroxide t-butyldihydroperoxide damage. However, parasites overexpressing either enzyme isoform were equally susceptible to NFX and BZ, similar to the parental control. The result may reflect an imbalance in the antioxidant defence of parasites overexpressing only one enzyme involved in the ROS detoxification pathway. } \]

\[ \text{In our previous studies carried using proteomic analysis, seven protein spots corresponding to TXNPx were } \]

\[ \text{2- to 5-fold more abundant in } \text{antimony-resistant } \]

\[ \text{T. brasilienis and } \text{T. cruzi lines.} \]

\[ \text{Furthermore, clones from } \text{T. brasilienis overexpressing cTXNPx were 2-fold more resistant to } \]

\[ \text{SBIII and more tolerant to exogenous } \text{H}_2\text{O}_2 \text{ than } \text{T. cruzi. Previous studies have demonstrated that SBIII perturbs the } \]

\[ \text{thiol redox potential of parasites, leading to the accumulation of } \]

\[ \text{ROS. SBIII decreases the intracellular thiol buffer capacity } \text{by inducing rapid efflux of trypanothione and glutathione, and it increases the intracellular concentration of the disulphide forms of the thiols through inhibition of trypanothione reductase. } \]

\[ \text{The effects of SBIII favour increased ROS levels. Overexpression of } \text{TXNPx confers resistance to } \text{SBIII } \text{by increasing enzyme activity to reduce the } \]

\[ \text{ROS levels induced by exposure to } \text{SBIII. Data from the literature support the results, showing that overexpression of TXNPx in } \text{T. tarentolae causes a significant increase in resistance to } \text{SBIII. Wyllie et al. reported elevated levels of TXNPx in } \]

\[ \text{T. cruzi population with } \text{in vitro induced resistance to BZ are protected against oxidative stress by a mechanism involving the overexpression of tryparedoxin peroxidase, ascorbate peroxidase, and other enzymes associated with } \]

\[ \text{No difference in SBIII susceptibility and a moderate resistance index to } \text{H}_2\text{O}_2 \text{ was observed in } \text{T. infantum clones overexpressing cTXNPx, which could be due to different } \text{antimony-resistance mechanisms between the two } \text{Leishmania species analysed. Moreira et al. demonstrated that the SBIII-resistant } \text{T. brasilienis line presents an increased expression of the MRPA gene and reduced accumulation of } \text{antimony; in contrast, no difference was detected in the SBIII-resistant } \text{T. infantum line compared to their respective SBIII-susceptible lines. } \]

\[ \text{Ascorbate peroxidase - APXs are class I heme-containing enzymes that catalyse } \text{H}_2\text{O}_2-dependent oxidation of ascorbate in photosynthetic microorganisms, plants, and some trypanosomatids such as } \text{Leishmania spp. and } \text{T. cruzi; however, APX is absent in } \text{T. brucei. Since } \text{APX is absent in the human host and presents an important role in the antioxidant defence of the trypanosomatids, the enzyme may be considered a promising drug target for chemotherapy of the parasites.} \]

\[ \text{T. cruzi APX is located in the endoplasmic reticulum and forms part of the antioxidant defence system of the parasite by metabolising } \text{H}_2\text{O}_2 \text{ to water. Furthermore, the amino acid sequence of } \text{T. cruzi APX showed } \text{30-35% similarity to that of plant APXs. Nogueira et al. observed that ascorbate peroxidase levels were enhanced in } \text{T. cruzi populations with } \text{in vitro-induced resistance (17LER) and } \text{in vivo selected (BZK) resistance to benzimidazole. Moreover, the two BZ-resistant populations exhibited higher tolerance to exogenous } \text{H}_2\text{O}_2 \text{ than their susceptible counterparts, and the } \text{TCAPX expression level was modulated by the stress generated by } \text{H}_2\text{O}_2. } \]

\[ \text{APX is an important factor controlling metacyclogenesis and apoptosis in } \text{T. major. Mukherjee et al. observed intra-chromosomal amplification of a subtelomeric locus on chromosome 34, a region coding for APX, in } \text{antimony-resistant } \text{T. major. Overexpression of APX in } \text{T. major confers tolerance to the oxidative stress-mediated oxidation of cardiolipin, consequently protecting cells from damage. Moreira et al. demonstrated that the overexpression of } \text{APX protects } \text{T. braziliensis against the effects of trivalent antimony and } \text{H}_2\text{O}_2. \text{ In addition, susceptibility tests revealed that the APX-overexpressing } \text{T. braziliensis lines were more resistant to isoniazid, an antibacterial agent that interacts with APX. Interestingly, this compound enhanced the antileishmanial SBIII effect, indicating that the combination may be a good strategy for leishmaniasis chemotherapy. The data demonstrate that the APX enzyme is an attractive therapeutic target involved in the antioxidant resistance phenotype of } \text{T. braziliensis, contributing to new strategies for leishmaniasis treatment.} \]

\[ \text{In conclusion - Based on our findings, Trypanosoma cruzi and } \text{Leishmania spp. are protected against oxidative stress by increasing the expression of genes that encode enzymes involved in antioxidant defence. Our previous studies showed that } \text{T. cruzi population with } \text{in vitro induced resistance to BZ are protected against oxidative stress by a mechanism involving the overexpression of tryparedoxin peroxidase, ascorbate peroxidase, and other enzymes associated with anti-} \]
oxidant defence, including iron superoxide dismutase. (25,41,55) The T. cruzi population with in vivo selected resistance to BZ presented a higher expression level of the ascorbate peroxidase protein. However, our findings revealed that the mechanisms involved in natural drug resistance in T. cruzi differ from those involved in induced resistance because drug resistance in T. cruzi is a complex process involving different parasite stages, various metabolic pathways, and the immune system of the host.

Our studies indicated that iron superoxide dismutase-A, tryparedoxin peroxidase, and ascorbate peroxidase play a significant role in antioxidant defence and in maintaining antimony resistance in Leishmania. (30,47,59) Data showed that the mechanism of antimony resistance differs among Leishmania species. The overexpression of iron superoxide dismutase-A is involved in the Sh41-resistance phenotype in L. braziliensis and L. infantum. (30) However, the overexpression of tryparedoxin peroxidase is directly associated with such phenotype in L. braziliensis, but not in L. infantum. (47)

We observed that alterations in the expression levels of enzymes important for drug resistance cause alterations in the levels of other enzymes, which can generate phenotypic compensation. In the case of a decrease in FeSOD-A in L. infantum, other FeSODs, and APX showed an increase in transcript levels, resulting in dysregulation of metabolic pathways related to antimony and miltefosine resistance.

The results of this study contribute to clarifying the regulation of the antioxidant defence pathway and illustrate the complexity of treating Chagas disease and leishmaniasis since the great adaptability of the parasites means that the lack of an enzyme can be overcome through changes in the expression of other enzymes in the same or similar pathways. In addition, the importance of studying the essential genes for parasites and developing new chemotherapeutic strategies using a combination of compounds that inhibit different metabolic pathways of the parasites is evident.

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AUTHORS’ CONTRIBUTION

SMFM and AMMS designed the work, collected data, wrote and revised the manuscript. All authors contributed to the article and approved the submitted version.

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