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Differential expression on mitochondrial tryparedoxin peroxidase (mTcTXNPx) in *Trypanosoma cruzi* after ferrocenyl diamine hydrochlorides treatments

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ABSTRACT

Resistance to benznidazole in certain strains of *Trypanosoma cruzi* may be caused by the increased production of enzymes that act on the oxidative metabolism, such as mitochondrial tryparedoxin peroxidase which catalyses the reduction of peroxides. This work presents cytotoxicity assays performed with ferrocenyl diamine hydrochlorides in six different strains of *T. cruzi* epimastigote forms (Y, Bolivia, SI1, SI8, QMII, and SIGR3). The last four strains have been recently isolated from triatominae and mammalian host (domestic cat). The expression of mitochondrial tryparedoxin peroxidase was analyzed by the Western blotting technique using polyclonal antibody anti mitochondrial tryparedoxin peroxidase obtained from a rabbit immunized with the mitochondrial tryparedoxin peroxidase recombinant protein. All the tested ferrocenyl diamine hydrochlorides were more cytotoxic than benznidazole. The expression of the 25.5 kDa polypeptide of mitochondrial tryparedoxin peroxidase did not increase in strains that were more resistant to the ferrocenyl compounds (SI8 and SIGR3). In addition, a 58 kDa polypeptide was also recognized in all strains. Ferrocenyl diamine hydrochlorides showed trypanocidal activity and the expression of 25.5 kDa mitochondrial tryparedoxin peroxidase is not necessarily increased in some *T. cruzi* strains. Most likely, other mechanisms, in addition to the over expression of this antioxidative enzyme, should be involved in the escape of parasites from cytotoxic oxidant agents.

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Introduction

Chagas disease is caused by the protozoan parasite *Trypanosoma cruzi*, transmitted to humans by domestic and sylvatic insects of the subfamily Triatominae (Hemiptera, Reduviidae), the kissing bug, and endemic in the Americas from US to Argentina. Migratory movements have brought *T. cruzi* to other regions, such as Europe, Japan, and Australia. In these regions, transmission occurs by blood transfusion, from mother to child, and by organ transplantation.[1]

The only prescription drugs to treat the disease are nifurtimox [(*RS*)-3-methyl-*N*-[(1E)-(5-nitro-2-furyl) methylene] thiomorpholin-4-amine 1,1-dioxide], and benznidazole [*N*-benzyl-2-(2-nitro-1H-imidazol-1-yl)]. These drugs have limited tissue penetration and relatively short half-lives, and therefore, they present low activity during the chronic stage because the parasites are located in deep tissues. The major limitation of nifurtimox (NF) and benznidazole (BZ) is their low antiparasitic activity in the chronic phase of the disease. Both drugs have significant adverse effects that can lead to treatment discontinuation. Some effects of NF include anorexia, nausea and vomiting causing severe weight loss, insomnia, irritability, and less commonly peripheral polyneuropathy. As for BZ the most common adverse effects are allergic dermopathy and gastrointestinal syndromes; less frequently bone marrow depression, thrombocytopenic purpura and agranulocytosis, polyneuropathy, paresthesia and peripheral polyneuritis.[2]

An important factor underlying the low cure rate of cases that are to nitro derivatives is the high genetic variability of *T. cruzi*.[3] *T. cruzi* parasites are classified based on multilocus genotyping, with six distinct DTUs (discrete typing units) according to their genetic similarity. DTU is formed by two major groups, DTU I (TcI) and DTU II (TcIV, TcII, TcIII, TcV and TcVI, also known as IIa, IIb, IIc, IId, and IIe, respectively).[3,4]

Knowledge of the mechanisms used by *T. cruzi* to manage reactive oxygen species (ROS) will help to identify novel targets and develop more specific chemotherapies.[5] For this reason, identifying genes that are expressed differentially in *T. cruzi*-susceptible and -resistant populations is also important.[6] Studies with epimastigotes have shown increased expression of tryparedoxin peroxidase (TXNPx) in resistant strains treated with benznidazole (BZ),[7,8] peroxides[9] or hydrogen peroxide (H_2O_2).[10]

TXNPx can be found in the cytosol (cTcTXNPx) and in the mitochondria (mTcTXNPx) in trypanosomatids.[8,9] TXNPx is a peroxidase that uses tryparedoxin as an electron donor. In trypanosomatids TXNPx exhibits peroxidase activity and catalyzes the reduction of hydrogen peroxide (H_2O_2) or small chain organic hydroperoxides to water and alcohol, respectively. In addition, it also displays peroxynitrite reductase activity.[11]

Incorporation of the ferrocenyl (Fc) group into standard drugs has proven a successful strategy to improve their activity and reverse drug resistance in a number of cases.[12,13] Interestingly, the development of ferroquine, an analogue of chloroquine with an Fc group in the lateral chain, showed particularly good *in vitro* and *in vivo* activity against chloroquine-resistant malaria parasite strains.[14] Considering the success of this approach and because alkyl diamines had been recently proposed as leading molecules for the development of new antiparasitic drugs,[15] our team decided to investigate the activity of novel Fc diamine hydrochlorides against *T. cruzi* and *T. brucei*.[16] The results revealed that the Fc derivatives were toxic either to *T. cruzi* and *T. brucei*, but not toxic to HepG2 cells, a model of mammalian cells.[16]

Although there is controversy among authors regarding the use of epimastigotes for these trials, it is important to note that previous studies demonstrated a correlation between epimastigote, trypomastigote, and amastigote forms of *T. cruzi*[17,18]; then, we understand that such data would be important as an initial screening in different strains, particularly those recently isolated from triatominae and mammalian host.[19–21] Herein we have examined the differences in susceptibility of epimastigote forms of six *T. cruzi* strains to the Fc diamine hydrochlorides and also evaluated the differential expression of mTcTXNPx related to resistance to oxidative agents. In addition, as a control, the treatment with BZ has also been analyzed.

Materials and methods

T. cruzi strains and culture

The following epimastigote forms of *T. cruzi* strains were used in this study: Y,[22] Bolivia,[23] Santo Inácio 1,[19] Quarai II,[20] and Santo Inácio 3 and 8.[21] SI1, SIGR3 and SI8 strains were recently isolated in the district of Santo Inacio located in Bahia state, Brazil. All strains were grown at 28 °C in liver infusion tryptose (LIT) medium[24] supplemented with 10% inactivated fetal bovine serum (Invitrogen).

Evaluation of the ferrocenyl diamine hydrochlorides and BZ toxicity

The compounds *N*-(ferrocenylmethyl)-*N*′-(2-methoxybenzyl) ethane-1,2-diamine dihydrochloride (**4**), *N*-(ferrocenylmethyl)-*N*′-(pyridyl)ethane-1,2-diamine trihydrochloride (**7**), and *N*-(7-chloroquinolin-4-yl)-*N*′-ferrocenylmethyl-1,2-diamine dihydrochloride (**11**) were synthesized as described elsewhere.[16] Their identity was confirmed by ^{1}H NMR (Varian VNMRS 300 MHz spectrometer) and their purity (≥95%) by elemental analysis (Perkin–Elmer CHN 2400 micro analyzer at Central Analítica IQ-USP, SP, Brazil) and melting point measurement (Digital Melting Point IA9100, ThermoFischer Scientific-USA apparatus). The cytotoxicity assay was performed according to Cotinguiba et al.[25] using tetrazolium MTT, 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide (Sigma): seven-day culture after treatment for 72 h with the compounds were treated with a solution of MTT/PMS, and in viable cells mitochondria MTT salt was reduced to formazan by the action of succinate dehydrogenase; then a new treatment with HCl/SDS solution dissolves the formazan crystals. This assay was employed to evaluate *T. cruzi* susceptibility to the compounds **4**, **7**, and **11** dissolved in dimethylsulfoxide (DMSO – Sigma) at different concentrations. The cytotoxicity index (IC_{50}) was calculated using Origin 7.0 program[26] and the probit analysis for statistical