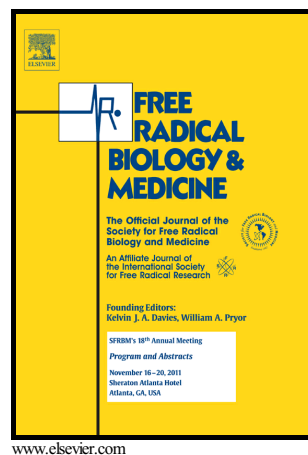# Author's Accepted Manuscript

Trypanothione synthetase confers growth, survival advantage and resistance to anti-protozoal drugs in *Trypanosoma cruzi*

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Trypanothione synthetase confers growth, survival advantage and resistance to anti-protozoal drugs in *Trypanosoma cruzi*

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ABSTRACT

Background:

Chagas cardiomyopathy, caused by *Trypanosoma cruzi* infection, continues to be a neglected illness, and has a major impact on global health. The parasite undergoes several stages of morphological and biochemical changes during its life cycle, and utilizes an elaborated antioxidant network to overcome the oxidants barrier and establish infection in vector and mammalian hosts. Trypanothione synthetase (TryS) catalyzes the biosynthesis of glutathione-spermidine adduct trypanothione $(T(SH)_2)$ that is the principal intracellular thiol-redox metabolite in trypanosomatids.

Methods and Results:

We utilized genetic overexpression (TryS[hi]) and pharmacological inhibition approaches to examine the role of TryS in *T. cruzi* proliferation, tolerance to oxidative stress and resistance to anti-protozoal drugs. Our data showed the expression and activity of TryS was increased in all morphological stages of TryS[hi] (*vs.* control) parasites. In comparison to controls, the TryS[hi] epimastigotes (insect stage) recorded shorter doubling time, and both epimastigotes and infective trypomastigotes of TryS[hi] exhibited 36-71% higher resistance to H_2O_2 (50-1000 μM) and heavy metal (1-500 μM) toxicity. Treatment with TryS inhibitors (5-30 μM) abolished the proliferation and survival advantages against H_2O_2 pressure in a dose-dependent manner in both TryS[hi] and control parasites. Further, epimastigote and trypomastigote forms of TryS[hi] (*vs.* control) *T. cruzi* tolerated higher doses of benznidazole and nifurtimox, the drugs currently administered for acute Chagas disease treatment.

Conclusions:

TryS is essential for proliferation and survival of *T. cruzi* under normal and oxidant stress conditions, and provides an advantage to the parasite to develop resistance against currently used anti-trypanosomal drugs. TryS indispensability has been chemically validated with inhibitors that may be useful for drug combination therapy against Chagas disease.

Graphical abstract

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ABBREVIATIONS

BZ, Benznidazole, GSH, Glutathione, NFX, Nifurtimox, TryS, Trypanothione synthetase, TryS[hi], TryS-overexpressing parasites, T(SH)$_2$, Trypanothione

KEYWORDS: *Trypanosoma cruzi*, Chagas disease, trypanothione synthetase, small molecule inhibitors, anti-parasite drugs, paullones.

INTRODUCTION

Chagas cardiomyopathy, caused by a hemoflagellate protozoan *Trypanosoma cruzi* (*T. cruzi* or *Tc*)*,* is a major health concern in Latin America and is an emerging infectious disease in the United States, Europe, Japan, and other countries [1]. The World Health Organization estimates that ~6-7 million people are infected worldwide, and ~10000 deaths per year are caused by this illness [2]. Indeed, the global health impact of *T. cruzi* infection and Chagas disease might be underestimated due to a lack of access to health system and inaccurate diagnosis in the endemic regions. The majority of individuals infected with *T. cruzi* control the acute parasitemia and remain clinically asymptomatic for the rest of their life. However, 30-40% of the infected individuals develop clinical symptoms that progress from cardiac hypertrophic remodeling (wall thickening) to dilated cardiomyopathy and ultimately result in cardiac arrest and death. Up to 10% of the patients may also develop digestive, neurological or mixed alterations.

From the beginning of 1970s, two drugs, benznidazole (BZ) and to a lesser extent nifurtimox (NFX), are administered for the treatment of acute *T. cruzi* infection [3]. However, these drugs are well tolerated by children only, and even then exhibit varying efficacy against different parasite isolates belonging to discrete typing units (DTU) I-VI. Limited-to-no-efficacy of BZ and NFX is observed in chronically infected adult patients, and currently recommended dosages of BZ and NFX produce adverse reactions including abdominal pain, nausea, vomiting, rash, decreased appetite and headache in adults, resulting in discontinuation of treatment [4,5]. Adjuvant therapies that can decrease the efficacious dosages of BZ and NFX against *T. cruzi* infection will improve the tolerance and completion of anti-protozoal treatment.

Trypanothione synthetase (TryS) is a trypanosomatid-specific enzyme that catalyzes the two-step synthesis of trypanothione (T(SH)$_2$; N^1,N^8-bis(glutathionyl)spermidine), from two molecules of glutathione (GSH; L-γ-Glu-Cys-Gly) and a spermidine core along with ATP consumption [6]. The N-terminal domain of TryS possesses weak amidase activity and can hydrolyze T(SH)$_2$. The T(SH)$_2$, together with its associated enzyme trypanothione reductase (TR), can carry out many of the antioxidant and metabolic functions of the glutathione / glutathione reductase and thioredoxin / thioredoxin reductase systems in other organisms [7]. TryS is shown to be essential for the survival of *T. brucei* and *Leishmania* species [8,9]. Nevertheless, the role of TryS in *T. cruzi* biology is not delineated.

In this study, we aimed to investigate the role of TryS in various life cycle stages of *T. cruzi*. For this, we developed genetically-modified TryS-overexpressing parasites (referred as TryS^{hi}) and repressed TryS activity with small enzyme inhibitors [10]. We evaluated the growth and survival of epimastigote (insect, replicative) and trypomastigote (infective) forms of TryS^{hi} and control parasites in the absence and presence of oxidative stress and TryS inhibitors. We also determined the effect of

TryS overexpression and TryS inhibition on the efficacy of anti-protozoal drugs (NFX and BZ) against *T. cruzi.* We discuss the biological significance of TryS in *T. cruzi* development, and the pharmacological potential that selective inhibition of TryS offers in a single or combined therapeutic approach with drugs in clinical use.

MATERIALS AND METHODS

Parasites, plasmids, and transfection

T. cruzi SylvioX10/4 strain (ATCC 50823) was maintained and propagated as epimastigote axenic culture in liver infusion-tryptose medium (LIT) supplemented with heat-inactivated, 10% fetal bovine serum (FBS). To obtain metacyclic trypomastigotes, epimastigotes were pelleted by centrifugation, re-suspended (5 x 10^8 cell/ml) in Triatomine Artificial Urine (TAU) medium (190 mM NaCl, 17 mM KCl, 2 mM $CaCl_2$, 8 mM phosphate buffer, 2 mM $MgCl_2$, pH 6.8) and incubated at 28°C for 3 h. Thereafter, parasites were transferred to TAU3AAG medium (TAU supplemented with 0.035% sodium bicarbonate, 10 mM L-proline, 50 mM sodium glutamate, 2 mM sodium L-aspartate and 10 mM glucose) and incubated at 28°C for 72-96 h [11]. The mouse myoblast cell line C2C12 was purchased from the American Type Culture Collection and maintained in complete RPMI 1640 medium. The metacyclic trypomastigotes were used to infect C2C12 cells, and culture derived trypomastigotes were maintained and propagated by continuous *in vitro* passage in C2C12 cells.

For metacyclogenesis assay, epimastigotes in exponential growth were synchronized by incubating in culture media without FBS for 24 h and then changed to LIT supplemented with 10% triatomine gut homogenate [12] at 28°C. The number of metacyclic and epimastigote forms were recorded on alternative days for 10 days by light microscopy. The difference between parasites stages was established according to its form and movement. Results are expressed as percentage of differentiated parasites.

The pTREX plasmid is designed to allow integration of the gene of interest in the ribosomal locus of *T. cruzi* [13]. To construct pTREX.TryS, forward and reverse oligonucleotides were designed using the GenBank AF311782.1 sequence to amplify full-length *T. cruzi TryS* gene with *EcoRI* and *HindIII* restriction sites for directional cloning. Total DNA was purified from 1×10^7 epimastigotes by phenol/chloroform extraction/ethanol precipitation method. Total DNA (100 ng) was used as template with *TryS*-specific oligonucleotides under standard PCR conditions performed on a Verity thermal cycler (Thermo Fisher Scientific, Waltham MA). The PCR product was cloned at the *EcoRI/HindIII* restriction sites in the pTREX expression vector. The pTREX.TryS plasmid was transformed into *Escherichia coli* DH5α competent cells, grown in LB Broth containing 100 µg/ml ampicillin, and purified with the Qiagen Plasmid Maxi Kit (#12162, Chatsworth, CA) according to the manufacturer's specifications. The recombinant plasmid clones were confirmed by restriction analysis and sequencing at the Cerela-CONICET facility (Tucumán, Argentina).

For transfection, *T. cruzi* epimastigotes (1.5×10^8 per ml) in exponential growth phase were suspended in HBS solution (21 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM glucose, pH 7), and transfected with 20 µg of pTREX or pTREX.TryS plasmids by electroporation (2 pulses of 1500 V, 25 µF) by using a BTX ECM 630 electroporator (MA, USA). The transfectants were selected in LIT complete medium with 300 µg/ml G418 sulfate (Corning, Corning NY) during 30 days, and then cloned by seeding in Difco blood (Usmaru) agar plates [14] supplemented with 300 µg/ml G418. Colonies were recovered after 30 days.

To confirm genomic integration of pTREX-TryS, total DNA was isolated from clonal populations of wild type (WT), and pTREX- or pTREX.TryS-transfected epimastigotes (1×10^7 each), as above.

Total DNA (100 ng) was subjected to standard PCR reaction with oligonucleotides complementary to the neomycin resistance gene. A second set of oligonucleotides (*T7-F* and *RIB-R*) was used to check integration of the recombinant plasmid at the ribosomal locus in *T. cruzi* [15]. All oligonucleotides used in this study are listed in Supplemental S1 Table.

*Tc*TryS antisera

The TryS protein sequence was analyzed by Kolaskar & Tongaonkar algorithm available at the ExPASy platform of the Swiss Institute of Bioinformatics (Lausanne, Switzerland), and the N-terminal peptide sequence (LQSLAVPFGCVQGY) predicted to exhibit maximum antigenicity was selected. The TryS peptide was synthetized at GenScript (Piscataway, NJ, USA) and conjugated to bovine serum albumin (BSA) by glutaraldehyde method. Polyclonal sera against TryS was raised by inoculating a rabbit by intradermal injection with 400 μg of BSA-conjugated TryS peptide in Freund´s complete adjuvant followed by two booster doses of 200 μg of TryS peptide with incomplete adjuvant at 30 days and 50 days after the first immunization. The serum was collected 15 days after the last immunization. To absorb the resulting anti-TryS antibodies, serum (5 ml) was incubated (1 h at room temperature followed by 12 h at 4°C) with 800 μg of recombinant TryS peptide coated on to nitrocellulose membrane. The antigen-bound antibody was eluted in 0.1 M glycine-HCl buffer (pH 2.7), and neutralized with 1/10$^{\text{th}}$ volume of 2 M Tris-HCl, pH 8.0.

Western blotting

T. cruzi epimastigotes and trypomastigotes (3×10^7 parasites) were frozen overnight and homogenized in 100 μl lysis buffer (50 mM HEPES, 200 mM NaCl, and 1% NP-40, pH 7.4) containing a protease inhibitor cocktail. The parasite lysates (60 μg protein per well) were resolved on 10% polyacrylamide gel under reducing conditions, and transferred to Immobilon-P PVDF membrane

(Millipore, Burlington MA). The membranes were blocked with 5% non-fat dry milk in 50 mM Tris pH 7.0, 150 mM NaCl (TBS) for 1 h, washed thrice with TBS - 0.05% Tween 20 (TBST), and then incubated for 2 h with affinity-purified anti-TryS (1:200-dilution in TBST) or anti-GAPDH (kindly provided by Dr. C. Labriola, 1:1500 dilution) antisera. Membranes were washed with TBST and TBS, and incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit secondary antibody (Sigma–Aldrich, St. Louis, MO, 1:5000 dilution). The signal was developed and visualized by using an enhanced chemiluminescent detection reagent and hyperfilms (GE Healthcare Life Sciences, Pittsburgh, MA).

Indirect immunofluorescence assay

Epimastigotes were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS), spotted on to poly-L-lysine-treated glass slides, and permeabilized with 0.1% Triton-X 100 in PBS. Slides were washed with PBS, and incubated at room temperature for 2 h with anti-TryS antisera (1:200 dilutions in TBST). After washing with PBS thrice, slides were incubated for 1 h with Alexa fluor 488-conjugated goat anti-rabbit secondary antibody (Thermo Fisher Scientific, 1:2000 dilution) and DNA was stained with DAPI. Slides were mounted in 70% glycerol, and examined with an Axio Imager 2 fluorescence microscope (Zeiss, San Diego, CA).

Trypanothione synthetase activity

The TryS activity was determined by using the malachite green reagent that measures the amount of inorganic phosphate released from ATP during catalysis [16]. Briefly, 50-µl reaction buffer (50 mM HEPES pH 8.0, 5 mM MgCl$_2$, 1 mM DTT) containing 0.5 mM GSH, 10 mM spermidine, and 1.5 mM ATP substrates was added to 96-well plates. The reagent was freshly prepared by mixing 0.5 mM aqueous solution of malachite green (M6880, Sigma), 0.034 M ammonium molybdate in 4 M HCl, and 2% Tween 20 (3.0: 1.0: 0.6, v/v/v). Parasite homogenates were prepared by using 1.5-5x10^7 total cells. The reaction was initiated by adding epimastigote or trypomasatigote homogenates to the wells, and after 15 min incubation at 37°C, reaction was stopped with 200 µl of malachite green reagent.

The colorimetric signal was allowed to develop for 30 min, and the change in absorbance was recorded at 650 nm on a Thermo Electron Multiskan Ascent Microplate Reader (Woonsocket, RI). Total proteins were measured by Bradford colorimetric assay (Bio-Rad). All reactions were tested in triplicate and the enzymatic activity was expressed as mU per mg protein. One unit (U) of activity is defined as the amount of enzyme that catalyzes the hydrolysis of 1 μmol of ATP per minute under the conditions specified in each case.

Trypanothione (T(SH)$_2$) and glutathione (GSH) level

Epimastigotes and trypomastigotes (pTREX- and pTREX.TryS-transfected) were resuspended in 4% (w/v) trichloroacetic acid (1 x 10^7 per 50 μl) and incubated at 4 °C for 10 min. The lysates were centrifuged at 21,000 x g for 30 min at 4°C, and supernatants were then lyophilized, and resuspended in 30 µl of Milli-Q water to start enzymatic T(SH)$_2$ determination. Samples (5 µl) were mixed in a 1:10 ratio with reaction buffer (400 µM NADPH, 5 nM recombinant trypanothione reductase, 100 mM Tris-HCl pH 7.5, 2 mM EDTA, 1 mM DTNB). T(SH)$_2$-dependent DTNB reduction was measured in kinetic mode at 405 nm. GSH content was determined by a kinetic method, as described previously [17].

Growth curve, doubling time, and trypanocidal effect of TryS small molecule inhibitors

For growth curves, non-clonal and clonal populations of pTREX- and pTREX.TryS-transfected epimastigotes were synchronized by incubating them in LIT medium without FBS for 48 h. Epimastigotes were seeded in 24-well plates (5x10^5 parasites/well in 1 ml complete LIT medium), and parasite number was quantified daily for 12 days by using a Neubauer chamber and light microscope equipped with 40X objective. The growth velocity and doubling time were calculated by the Exponential growth equation tool in GraphPad Prism 5 (La Jolla CA, USA).

To confirm the doubling time, synchronized epimastigotes (5×10^6 per sample) were incubated in 1 ml PBS with 5 µM CellTrace CFSE (cat# C34554, Thermo Fisher Scientific) for 20 min, washed thrice, resuspended in 1 ml complete LIT media, and incubated again at 28°C for 3 days. Then, epimastigotes were centrifuged at 3000 RPM for 5 min, and incubated with Fixable Viability Dye eFluor 780 (1:1000 dilution in PBS, Invitrogen) at 4°C for 30 min. Thereafter, parasites were washed with PBS, and fixed with 4% formaldehyde for 20 min. Samples were visualized on a LSRII Fortessa Cell Analyzer, acquiring 100,000 events in a live cell gate, and further analysis was performed by using the FlowJo software (version 7.6.5, Tree-Star, San Carlo, CA).

The TryS chemical inhibitors, KuOrb39 and KuOrb54 (S1 Figure), were synthesized as described by Orban *et al* [10] and are referred as 3d and 3h. Synchronized, clonal parasite cultures (pTREX- and pTREX.TryS transfectants) were seeded in 96-well flat-bottom cell culture plates, and KuOrb39 or KuOrb54 (0, 5, 10, or 30 µM) added. Epimastigotes (1×10^4/well/100 µl) were incubated at 28°C for 24 h and trypomastigotes (2.5×10^5/well/100 µl) were incubated at 37°C / 5% CO_2 for 6 h. The growth rate of epimastigotes and survival of trypomastigotes was monitored by quantifying the motile parasites by light microscopy.

Resistance to reactive oxygen species (ROS) and anti-parasitic drugs

For this, wild type (WT), pTREX- and pTREX.TryS transfectants were seeded in 96-well flat bottom cell culture plates as above. Parasites were incubated in triplicate with H_2O_2 (50-1000 µM), cadmium acetate (1-500 µM), nifurtimox (NFX, 1-400 µM), or benznidazole (BZ, 5-500 µM). In some experiments, parasites were co-incubated with 10-30 µM KuOrb54 or KuOrb39 to examine the effect of TryS inhibition on parasite resistance to H_2O_2 and anti-parasitic drugs. In all experiments,

epimastigotes (1×10^4/well/100 µl complete LIT medium) were incubated at 28°C for 24 h and trypomastigotes (2.5×10^5/well/100 µl RPMI containing 10% FBS) were incubated at 37°C / 5% CO_2 for 6 h, and viable (motile) parasites were counted by light microscopy.

Data analysis

All data were analyzed by using GraphPad Prism 5 statistical package (La Jolla, CA). Data were acquired in at least two independent experiments performed on different days (triplicate observations per experiment), and expressed as mean value ±SEM. Data were analyzed by using a Student t test and One-way analysis of variance (ANOVA), and p values < 0.05 were considered significant. The drug activities were calculated by sigmoidal regression analysis (dose-response – inhibition model), and expressed as the concentration needed to inhibit epimastigote growth by 50% or to obtain a 50% mortality of non-replicative trypomastigotes (IC_{50}). IC_{50} values are plotted as mean with a 95% confidence interval (95% CI).

RESULTS

Transfection, overexpression, and activity of TryS in *T. cruzi*

As a first step towards the study of TryS associated functions in the context of *T. cruzi* biology, we generated parasites that constitutively overexpress this enzyme. The insertion of the *TryS*-encoding sequence in the pTREX vector was confirmed by restriction analysis and sequencing of the *TryS* open reading frame. *T. cruzi* SylvioX10 strain belonging to TcI DTU was transfected with pTREX.TryS and control (empty pTREX) plasmids and transfectants were selected with G418. We confirmed the insertion of pTREX.TryS in the parasite's genome by PCR amplification of the 320 bp

fragment encoding the neomycin gene selection marker with *Neo-F* and *Neo-R* oligonucleotides, and a 900 bp DNA fragment encompassing sequences of pTREX vector and the 18S ribosomal locus of the parasite genome by using the *T7-F* and *RIB-R* oligonucleotides (Fig.1A). The PCR amplification of these fragments in the transgenic but not in the WT parasites demonstrated that the pTREX ($\pm$ TryS) was stably integrated into the ribosomal locus of *T. cruzi* (Fig.1B&1C). The transfectants were cloned and all studies from this point onward were conducted in clonal populations of the transgenic cell lines.

Next, we evaluated the expression and activity of TryS in the transfectants. Western blotting showed that expression of TryS (74.5 kDa) was increased by >4-fold and >2.5-fold in epimastigote and trypomastigote stages, respectively, of *T. cruzi* clones transfected with pTREX.TryS (referred as TryS^{hi}) when compared to the content in control transfectants (referred as pTREX) (Fig.2A&2B). No differences in GAPDH levels were noted in controls and TryS^{hi} parasites. Immunofluorescence staining with TryS antisera showed normal distribution of TryS in cytoplasm with some punctated dispersion pattern in all transfectants. The TryS^{hi} (*vs.* control) transfectants exhibited a substantial increase in cytoplasmic TryS level (Fig.2C, compare panel b and panel d). The enzymatic activity of TryS was determined by an end-point assay based on reaction of the inorganic phosphate released during TryS catalysis with malachite green reagent. The overall levels of TryS specific enzymatic activity (SEA) and the trypanothione (T(SH)$_2$) content (produced by TryS enzymatic activity) were higher in trypomastigote stage compared to epimastigote stage (Fig.3A&3B). The overexpression of TryS (TryS^{hi}) resulted in an increase in enzymatic activity in epimastigote and trypomastigote forms (2-fold and 1.7-fold, respectively), as compared to that noted in control pTREX parasites (Fig.3A, p<0.05). No significant differences were observed in intracellular T(SH)$_2$ content at epimastigote stage of both studied clones (pTREX and TryS^{hi}). In comparison, T(SH)$_2$ metabolite level was increased by 2.3-fold in TryS^{hi} (*vs.* pTREX) trypomastigotes (Fig.3B, p<0.05). The content of GSH, a T(SH)$_2$ precursor, was higher in the epimastigotes when compared to trypomastigotes of both clones (TryS^{hi}

and pTREX) (Fig.3C). In contrast, GSH contents in TryS[hi] trypomastigotes were ~24% lower than that estimated in pTREX parasites, indicating its higher consumption for $T(SH)_2$ synthesis. Together, the results presented in Fig.2 and Fig.3 suggest that epimastigote and trypomastigote stages of *T. cruzi* stably transfected with pTREX.TryS overexpress a functionally active form of TryS.

Role of TryS in replication, differentiation and survival of *T. cruzi*

In *T. brucei,* the $T(SH)_2$-system provides reducing power to sustain ribonucleotide reductase activity [18], the enzyme responsible for deoxyribonucleotide synthesis, which are the building blocks of DNA. Thus, with the aim to analyze the effects of TryS on *T. cruzi* proliferation, we plotted growth curves of synchronized clonal cultures of TryS[hi] and control epimastigote form in complete LIT medium. The TryS[hi] epimastigotes exhibited a significant increase in replication than was observed for the pTREX-transfected controls (doubling time: 1.77 and 2.05 days, respectively, Fig.4A&4B), and this difference became particularly evident in the late-log phase of the growth curve. The uncloned TryS[hi] epimastigotes also exhibited a significantly higher rate of replication than the pTREX control epimastigotes (doubling time: 1.58 and 1.83 days, respectively, S2 Figure) at five days after seeding. Parasite proliferation was also assessed by a flow cytometry approach relying on the fluorescence loss of CFSE label after cell division. Three days after CFSE pulse labeling, 93% and 78% of the TryS[hi] and pTREX epimastigotes exhibited cell division, respectively (Fig.4C). In both groups, ~99% epimastigotes were live-stained and viable (Fig.4D).

Metacyclogenesis, i.e., differentiation of epimastigotes to metacyclic, infective trypomastigote form in the insect gut, involves the up-regulation of antioxidant defense genes, such as TryS [19,20]. We, therefore, evaluated if the TryS overexpression confers differentiation advantage to the epimastigotes. For this, TryS[hi] and pTREX epimastigotes were incubated in presence of triatomine gut

homogenate, resembling the natural differentiation process, and the number of parasites exhibiting differentiated morphologies were recorded for 10 days. The TryS[hi] epimastigotes displayed a higher differentiation capacity upon stimulus as they achieved a 53% of differentiated forms out of the total population, whereas only 15% of the pTREX-transfected epimastigotes achieved metacyclic differentiation during 10 days (S3 Figure).

Further evidence of the role of TryS in replication and survival of epimastigote and infective trypomastigte forms, respectively, was obtained by incubating the transgenic clones with paullone-derived TryS-specific inhibitors [10]. The TryS inhibitors affected the proliferation of TryS[hi] and control epimastigotes in a dose-dependent manner, though KuOrb39 was more effective than KuOrb54 in impairing epimastigote growth (Fig.5A). The growth of TryS[hi] and pTREX-transfected epimastigotes was decreased in presence of 30 µM KuOrb39 by 28% and 54%, respectively ($p < 0.05$). Likewise, TryS inhibitors affected the survival of infective, non-replicative trypomastigote forms of TryS[hi] and pTREX parasites in a dose-dependent manner; and in this case, KuOrb54 was found to be more effective. For instance, exposure to 10 µM and 30 µM KuOrb54 resulted in a relative cell death of 25% and 52%, respectively, for TryS[hi] trypomastigotes and of 50% and 75%, respectively, for wild type trypomastigotes (Fig.5B). Together, the results presented in Fig.4, Fig.5, and S3 Figure suggest that a) TryS is an essential enzyme required for replication of epimastigotes and survival of insect and mammalian stages of *T. cruzi*; and b) increased expression of TryS favors a shorter doubling time of epimastigote forms as well as their increased differentiation to the infective metacyclic form of *T. cruzi*.

TryS increases tolerance to oxidative stress in *T. cruzi* life cycle

Because $T(SH)_2$ can scavenge ROS or neutralize them *via* the $T(SH)_2$-dependent antioxidant machinery of the parasite, we next examined the effect of TryS overexpression on *T. cruzi*'s ability to

survive under oxidative stress conditions. H_2O_2 is known to be a ubiquitous reactive oxygen molecule produced by the insect as well as the mammalian host immune system [21–23]. We incubated the insect and infective forms of pTREX, and TryS[hi] parasites with 50-1000 µM H_2O_2, and evaluated the percent inhibition of growth and survival. The TryS[hi] (*vs.* control) epimastigotes exhibited a ~1.7-fold increase in H_2O_2 tolerance, evident by 50% growth inhibition of TryS[hi] and pTREX epimastigotes at 169.6 µM and 99.1 µM H_2O_2, respectively (Fig.6A&6B, $p<0.05$). To verify that the increased resistance to H_2O_2 occurred in a TryS-dependent manner, we incubated the epimastigotes with 100 µM H_2O_2 (~IC_{50} for pTREX-transfectants) in the presence of TryS inhibitors. We observed no additive effects of 10 µM KuOrb39 on H_2O_2-dependent inhibition of TryS[hi] and control epimastigotes' growth. However, increasing KuOrb39 concentration to 30 µM abolished the protective effects against H_2O_2 observed for TryS[hi] (*vs.* control) epimastigotes (Fig.6C). The data on survival of trypomastigotes upon challenge with increasing oxidant doses are presented in Fig.6D-6F. As noted for the epimastigote stage, the TryS[hi] (*vs.* pTREX) trypomastigotes exhibited a 1.4-fold increase in H_2O_2 tolerance, that was evident by IC_{50} values of 87.7 µM and 64.6 µM H_2O_2 for TryS[hi] and pTREX trypomastigotes, respectively (Fig.6D&6E). Co-incubation with 10 µM KuOrb54 had a significant effect on H_2O_2-dependent killing of pTREX trypomastigotes, which was more clearly noticed after co-incubation with 30 µM of the inhibitor. In comparison, TryS[hi] trypomastigotes exhibited significantly higher capacity to resist oxidative stress even in presence of KuOrb54 (Fig.6F).

It is known that metals may lead to an increase in ROS level and exert toxicity against *T. cruzi* [24]. We, therefore, investigated the effects of cadmium as an alternative oxidative stressor. The TryS[hi] epimastigotes exhibited a >6-fold increase in Cd^{2+} tolerance when compared to pTREX with IC_{50} values of 31.4 µM and 5.1 µM, respectively (S4 Figure). In summary, the results presented in Fig.6 and S4 Figure suggest that: a) despite the low endogenous expression, TryS contributes to H_2O_2 management in epimastigote form; b) TryS and other antioxidant enzymes are up-regulated in trypomastigote (*vs.* epimastigote) form that also appears to be more sensitive to oxidants; and c) TryS

provides protection from oxidative stress in both epimastigote and trypomastigote forms of *T. cruzi*. Further, d) TryS displays a pivotal role in heavy metal detoxification via $T(SH)_2$ in *T. cruzi*. We surmise that up-regulation of TryS can potentially increase parasites' tolerance to oxidative stress in the vector as well as in the mammalian host.

TryS increases *T. cruzi* resistance to anti-parasite drug therapies

Nifurtimox (NFX) and benznidazole (BZ) are drugs of choice for the treatment of *T. cruzi* infection. NFX is a nitrofurane derivative, and it is believed to exert its biological activity through formation of a highly reactive and cytotoxic unsaturated open-chain nitrile [25] and, secondly, through thiol depletion [26]. To analyze the effect of TryS overexpression on parasite resistance to NFX, we incubated the pTREX and TryS^{hi} parasites with 1-400 µM NFX, and determined the inhibition percentage of proliferation of the replicative epimastigotes and survival of the non-replicative trypomastigotes. The TryS^{hi} epimastigotes showed >3-fold increase in NFX tolerance, with IC_{50} values of 5.93 µM and 1.84 µM for TryS^{hi} and pTREX epimastigotes, respectively (Fig.7A&7B, p<0.0001). Notably, the infective TryS^{hi} trypomastigotes showed a 7.5-fold higher resistance towards NFX than the pTREX cells. This was evidenced by IC_{50} values of 16.53 µM and 2.19 µM NFX for TryS^{hi} and pTREX trypomastigotes, respectively (Fig.7C&7D, p<0.0001). Independently of the genetic background, co-incubation with KuOrb54 significantly decreased trypomastigote resistance to NFX. Nonetheless, the effect of the TryS inhibitor was significantly less marked for TryS^{hi} than for pTREX trypomastigotes. This was evidenced by finding of 50%, 59% and 69% cell death of pTREX trypomastigotes; and 27%, 34%, and 55% cell death of TryS^{hi} trypomastigotes in the presence of 2.5 µM NFX only, NFX plus 10 µM, and 30 µM KuOrb54, respectively (Fig.7E, all p<0.05).

It has been already demonstrated that benznidazole, a nitroimidazole, has a pleiotropic effect by depleting low molecular mass thiols through the formation of adducts and thereby enhancing

oxidative stress [27]. The TryS[hi] epimastigotes showed >4.5-fold increase in BZ tolerance, with IC_{50} value of 26.1 µM and 5.3 µM for TryS[hi] and pTREX epimastigotes, respectively (Fig.8A&8B, p<0.0001). Similar to the behavior against NFX, the TryS[hi] trypomastigotes showed a 1.7-fold increase in BZ tolerance with IC_{50} values of 120.2 µM and 68.6 µM BZ for TryS[hi] and pTREX trypomastigotes, respectively (Fig.8C&8D, p<0.0001). Furthermore, co-incubation with KuOrb54 significantly enhanced the toxicity of BZ against trypomastigote form independently of the genetic background. This was evidenced by finding of 50%, 64%, and 86% cell death of pTREX trypomastigotes; and 31%, 48% and 74% death of TryS[hi] trypomastigotes in the presence of 60 µM BZ only, BZ with 10 µM and 30 µM KuOrb54, respectively (Fig.8E, all *p*<0.05). Altogether, the results presented in Fig.7 & Fig.8 suggest that TryS confers NFX and BZ resistance to *T. cruzi* epimastigote and trypomastigote stages and that the paullone inhibitors offer potential as adjuvant therapy for enhancing nitroheterocyclic drug efficacy against *T. cruzi.*

DISCUSSION

Herein, we describe the generation of recombinant *T. cruzi* with genomic insertion of an extra copy of the *TryS* gene. The aim of this approach was to elucidate the role of TryS enzyme in the context of *T. cruzi* biology. The TryS[hi] parasites, as compared to controls, exhibited increased levels of TryS protein in all stages of parasite development analyzed (Figures 1-3). The increased expression and activity of TryS was particularly evident in infective form when recombinant TryS led to increased $T(SH)_2$ synthesis from spermidine and GSH substrates. The overexpression of TryS enhanced the replication and survival of *T. cruzi* under physiological conditions as well as in the presence of oxidative and heavy metal stress (Figures 4-6 and S4 Figure). Further, overexpression of TryS conferred an advantage to the parasite to develop resistance to currently used anti-trypanosomal drugs and TryS small molecule inhibitors were efficacious in improving the efficacy of NFX and BZ against *T. cruzi* (Figure 7 and Figure 8). The important role of TryS in parasite

proliferation, infectivity and resistance against oxidative stress have previously been shown in *T. brucei* [18,28] and *L. infantum* [29] that are related pathogenic trypanosomatids. To the best of our knowledge, this is the first study in which we have employed the genetic overexpression and small molecule inhibitors to demonstrate the biological significance of TryS in *T. cruzi* development, survival and resistance to oxidative stress and anti-parasite therapies. Our data allow us to propose that TryS small molecule inhibitors offer adjuvant therapy for lowering the efficacious dose of anti-parasite drugs in killing *T. cruzi*.

The TryS enzyme produces $T(SH)_2$ and the latter is maintained in its reduced state by the NADPH-dependent flavoenzyme trypanothione reductase (TR) [7]. $T(SH)_2$ (reduced form) transfers electrons to the multipurpose thiol/disulfide oxidoreductase tryparedoxin (TXN). TXN plays a central role as biological catalyst, reducing a large diversity of molecular targets such as different types of peroxidases and ribonucleotide reductase [30]. Metabolic flux studies performed in *T. cruzi* highlighted that $TryS/T(SH)_2$, γ-glutamylcysteine synthetase, the first enzyme of the GSH biosynthetic pathway, and TXN are the rate-limiting steps in the antioxidant network of the parasite [31]. In the *T. cruzi* epimastigote form that replicates and undergoes metacyclogenesis in insects, trypanothione antioxidant network was not very active. This was evidenced by the finding of low expression of TryS (Figures2&3) and other proteins of the antioxidant network in epimastigote form of *T. cruzi* [20,32,33]. Further, the GSH level in epimastigotes was >2.5-fold higher than that observed in the infective stage (Fig.3C). Overexpression of TryS in epimastigotes was clearly insufficient to cause an increase in the $T(SH)_2$ content. These findings suggest that either the attained level of enzyme was yet the rate limiting step or that additional component(s) of the system (i.e. polyamine uptake) exert a higher metabolic control of the pathway. Regardless of the reasons for this metabolic output and the observed significance of GSH being the predominant low molecular weight thiol in epimastigotes (TryS[hi] or pTREX), our results indicate that $TryS/T(SH)_2$ supported growth, survival, and higher replication rate in TryS[hi] (*vs.* control) epimastigotes, a phenotype that was abolished upon selective

inhibition of the enzyme (Figure 4, Figure 5, and Figure 6). Such advantageous outcomes may be associated to the fact that the $T(SH)_2$-system supported the synthesis of DNA precursors [18] and the replication of the parasite mitochondrial DNA [34,35]. Interestingly, the axenic epimastigotes displayed a marked increase in their proliferation rate during the late phase of the growth curve, when the culture milieu undergoes an important shift in the ratio of nutrients to metabolic products. Although with a lower efficiency than that occurring in the insect gut, this metabolic change triggers the differentiation of axenic epimastigotes into the highly infective and non-replicative metacyclic trypomastigotes [36,37]. It is, thus, tempting to speculate that up-regulation of TryS under *in vivo* conditions may be beneficial to achieve a larger amount of epimastigotes that may undergo metacyclogenesis and establish the infective inoculum. Indeed differentiation process seems to be enhanced in TryS-overexpressing parasites (S3 Figure) when compared to the wild type clone. One possible explanation could be related to the higher content of antioxidant defenses that could be somehow monitored by the parasite as a metacyclogenesis driving force.

Trypomastigotes are released from host cells and swim in interstitial fluid and bloodstream until they invade a new cell, such as macrophages. Both the extracellular medium and the invasion of activated macrophages represent an oxidative challenge for trypomastigotes. This may explain the need for a higher content of $TryS/T(SH)_2$ in this parasite stage. In agreement with this proposal and the almost absolute $T(SH)_2$-dependency of the hydroperoxide metabolism of the parasite [38–40], overexpression of TryS in both epimastigotes and trypomastigotes conferred protection against H_2O_2, a physiological oxidant produced by macrophages as part of the host immune response. The slight, but significant ($p<0.001$, Figure 6D), difference observed between TryS[hi] and pTREX trypomastigotes suggests that reactions downstream to $T(SH)_2$ synthesis may limit the capacity of the system to neutralize or repair the damage caused by H_2O_2. In this regard, it is important to recall that the reaction between $T(SH)_2$ and TXN has been pointed to be the rate limiting step in the hydroperoxide metabolism of trypanosomatids [31,41].

Paullones are a group of 7,12-dihydroindolo[3,2-*d*][1]benzazepin-6(5*H*)-ones that were initially described for their inhibitory activity against mammalian protein kinases, among other effects. Orban and co-workers developed novel *N*5 aryl-substituted derivatives of 3-chlorokenpaullone that displayed significant inhibitory activity against the recombinant *Tc*TryS protein [10]. From this group of compounds, the on-target effect of KuOrb39 (referred as 3d in [10]) and KuOrb54 (referred as 3 h in [10]) was tested for the first time in *T. cruzi* in this study. Both compounds exerted a remarkable cytotoxic effect against both life stages of the parasite that were subject of our study. Specifically, IC_{50} concentration of KuOrb54 (~10 µM) against *T. cruzi* parasite was similar to the levels needed to observe 50% inhibition of the recombinant enzyme [10]. The apparent higher sensitivity of trypomastigotes to the cytotoxic action of the paullones is likely explained by the fact that this stage is not replicative. In almost all assays, except for that conducted against epimastigotes at 30 µM compounds, the 4-hydroxy phenyl derivative KuOrb54 exhibited a higher cytotoxicity than the 3-chloro phenyl analogue KuOrb39. The observed plateau in anti-epimastigote activity of KuOrb54 when added at 30 µM may likely be due to an impaired uptake and/or bioavailability of this molecule. Another important finding is that KuOrb54 displayed potent anti-trypanosomal activity against a clinically relevant form of the parasite (trypomastigotes) at concentrations below (50% and 75% cell death at 10 µM and 30 µM, respectively) to that observed for the drug BZ (IC_{50} = 69 µM). Conversely, KuOrb54 was comparatively less active than the potent drug NFX (IC_{50} = 2 µM).

As commented previously, the mechanism of action of both anti-protozoal drugs leads to depletion of low molecular weight thiols [26,27,42]. The involvement of trypanothione metabolism in neutralization of nitro-drugs has been demonstrated for different trypanosome species. In *T. brucei*, RNAi-mediated downregulation of *TryS* sensitized the parasite against the action of NFX [43]. In *T. cruzi*, treatment with buthionine sulfoximine, an inhibitor of γ-glutamylcysteine synthetase, enhanced the anti-trypanosomal activity of NFX and/or BZ *in vitro* and *in vivo* (unpublished observations). Here, we provide conclusive evidence for the direct implication of TryS/T(SH)$_2$ in the management of BZ-

and NFX-induced stress in *T. cruzi*. The role of TryS was particularly noticeable in NFX detoxification, as the IC_{50} value of NFX was estimated to be ~7.5-fold higher for TryS^{hi} parasites than that observed for pTREX trypomastigotes (Figure 7C). Importantly, chemical inhibition of TryS increased parasite sensitivity towards NFX and BZ (Figure 7E&8E), as noted by IC_{50} values for these drugs that were >2-fold lower than those obtained in cells not exposed to paullones. Overall, these results suggest that TryS is a resistance gene for current anti-chagasic drugs, and lead us to speculate that its endogenous up-regulation – observed in some virulent parasite strains [33] – is, at least in part, associated to cases of drug failure.

SUMMARY

By employing reverse genetics and chemical inhibition approaches, we analyzed the importance of trypanothione synthetase in *T. cruzi* biology. TryS proved relevant for the proliferation of the insect stage (epimastigote) of the parasite under starvation conditions, which may favor metacyclogenesis to infective trypomastigotes form. The higher expression of TryS conferred to the parasite an advantage to deal with the oxidative stress produced by different sources (e.g. H_2O_2 and cadmium), in the replicative epimastigote as well as in the infective trypomastigotes forms of the parasite. Furthermore, *T. cruzi* overexpressing TryS tolerated higher dosages of anti-protozoal drugs, benznidazole and nifurtimox, suggesting that the protein may contribute to the drug failure cases. Specific TryS inhibitors proved beneficial in increasing parasite's sensitivity to anti-parasitic drugs and should be considered in the design of new treatment strategies.

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Conflict of interest:

The authors declare no financial conflict of interest.

SUPPLEMENTARY DATA

S1 Table: Oligonucleotides used in this study. Oligonucleotides 1 and 2 were designed for *TryS* amplification and directional cloning into the pTREX vector. Oligonucleotides 1-4 were used to confirm the sequence of TryS open reading frame in pTREX.TryS vector. Total DNA isolated from WT and transfectants (pTREX and pTREX.TryS) was used as template with *Neo-F* and *Neo-R* oligonucleotides for PCR amplification of neomycin-encoding gene (Neo in pTREX) and with *T7-F* and *RIB-R* oligonucleotides to amplify a DNA fragment encompassing sequence from pTREX and 18S ribosomal locus in *T. cruzi* genome.

S1 Figure: Chemical structure of small molecule inhibitors of *Trypanosoma cruzi* trypanothione synthetase. The TryS inhibitors KuOrb39 and KuOrb54 are referred as 3c and 3d in Orban *et al* [10].

S2 Figure: Growth curve of non-clonal populations of TryS-overexpressing *T. cruzi*. (A) Epimastigotes were transfected with pTREX or pTREX.TryS (TryS^{hi}) plasmids, and incubated under G418 selection pressure for 30 days. Parasite cultures were seeded at 5 x 10^5 epimastigotes/ml complete LIT medium in 24 well plates, and parasite growth was monitored up to 12 days by light microscopy. The data are representative of three independent experiments (three determinations per sample per experiment), and plotted as mean value ± SEM. **(B)** The doubling times of TryS^{hi} and control epimastigote populations were calculated by using a GraphPad Prims 5 software. Rate constant *k* fitted for an exponential growth curve differed significantly between the two datasets ($p <$ 0.05).

S3 Figure: TryS-overexpression favors metacyclogenesis. Epimastigotes of pTREX– and pTREX.TryS-transfected clones in exponential growth phase were synchronized by incubating in culture media without FBS for 24 h, and then were transferred to complete LIT medium supplemented with 10% triatomine gut homogenate. The differentiation of epimastigotes to metacyclic forms was recorded by light microscopy. The data are representative of ≥ 3 independent experiments (three determinations per sample per experiment) and plotted as mean value ± SEM (* and *** correspond to $p < 0.05$ and $p < 0.001$, respectively).

S4 Figure: TryS overexpression confers cadmium tolerance. Clonal epimastigote cultures of TryS^{hi} and control *T. cruzi* were incubated for 48 h with 0-500 μM cadmium acetate. The viable, motile epimastigotes were visually quantitated by light microscopy. **(A)** IC$_{50}$ concentration of cadmium that resulted in 50% inhibition of TryS^{hi} (vs. control) parasites. **(B)** Parasite growth inhibition curve fitted after incubation with increasing concentrations of cadmium acetate. All experiments were conducted thrice (three determinations per sample per experiment), and data are plotted as mean value ± SEM (*** $p < 0.001$).

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Figure 1: Stable integration of pTREX.TryS expression vector in *T. cruzi*. (A) Scheme of pTREX.TryS and pTREX expression vectors integration into the ribosomal locus of *T. cruzi* genome. Full-length *TryS* coding sequence (1960 bp) was amplified by using the *Tc* SylvioX10/c4 DNA as template in a PCR reaction, and cloned downstream of HX1 intergenic region (HX1) at EcoRI/HindIII sites in pTREX plasmid. (**B&C)** Sylvio X10/c4 epimastigotes were electroporated with pTREX or pTREX.TryS plasmids and transfectants were selected in presence of G418. Representative PCR amplification of neomycin resistance gene (Neo, *B*) and T7 – ribosomal locus **(C)** in pTREX and pTREX.TryS (TryS[hi]) transfectants are shown. Note that no amplification of pTREX-derived sequences was observed in wild-type (WT) parasites.

Figure 2: TryS enzyme is over-expressed in pTREX.TryS-transfected *T. cruzi*. The pTREX and pTREX.TryS (TryS[hi]) transfectants were cloned as described in Materials and Methods. **(A&B)** Representative Western blot images are shown for the levels of *Tc*TryS and *Tc*GAPDH in clonal populations of epimastigote *(A)* and trypomastigotes *(B)* forms. Detection of GAPDH (housekeeping protein) was used as loading control. Ponceau staining of the gel is also shown as an evidence for equal loading of epimastigote lysates. **(C)** Shown are representative bright field (a&c) and fluorescence (b&d) images of pTREX-transfected (a&b) and pTREX.TryS-transfected (c&d) epimastigotes. For immunofluorescence, parasites were stained with anti-TryS antisera and Alexa-488-conjugated secondary antibody (green) together with DAPI (blue, DNA marker).

Figure 3: Recombinant TryS is produced as an active enzyme in epimastigote and trypomastigote forms of *T. cruzi*. Clonal epimastigote (Epi) and trypomastigote (Tryp) forms of pTREX- and pTREX.TryS-transfectants were expanded as described in Materials and Methods. Bar

graphs present the **(A)** specific enzymatic activity (SEA) of TryS, **(B)** total trypanothione $(T(SH)_2)$ content by an enzymatic recycling method, and **(C)** content of total GSH. The data in bar graphs are representative of ≥ 2 independent experiments (three determinations per sample per experiment) and plotted as mean value ± SEM (* and *** correspond to $p < 0.05$ and $p < 0.001$, respectively, *ns* stands for non significant).

Figure 4: Effect of TryS overexpression on parasite growth. (A) The growth of synchronized epimastigote cultures of pTREX and pTREX.TryS (TryS[hi]) transfectants was monitored by cell counting under a light microscope. Data are representative of three independent experiments (three determinations per sample per experiment), and plotted as mean value ± SEM. **(B)** The doubling time of TryS[hi] and control epimastigotes was calculated in GraphPad Prims 5. Rate constant *k* fitted for an exponential growth curve differed significantly between the two datasets ($p < 0.05$). **(C)** pTREX and TryS[hi] clonal epimastigote populations were pulse labeled with CFSE and examined 3 days later by flow cytometry. The decline in CFSE fluorescence intensity corresponds to cell division. **(D)** A fixable viability dye was used to gate CFSE^{+} viable cells. For both pTREX and TryS[hi] populations, 99% of the analyzed parasites were viable.

Figure 5: **Effect of TryS inhibitors on *T. cruzi* replication and viability.** Clonal cultures of TryS[hi] and control epimastigote and trypomastigote forms of *T. cruzi* were incubated in presence of varying concentrations of TryS inhibitors (0, 5, 10, and 30 µM of KuOrb39 or KuOrb54). **(A)** The percentage (%) of growth inhibition of epimastigotes at 24 h post-incubation was determined by counting live, motile parasites by light microscopy. **(B)** Live motile trypomastigotes were quantitated by light microscopy, and percentage (%) of cell death calculated at 6 h post-incubation. Data are representative of two independent experiments (three determinations per sample per experiment), and plotted as mean value ± SEM (* $p < 0.05$).

Figure 6: Role of TryS in *T. cruzi* resistance to oxidative stress. *T. cruzi* transfectants, pTREX and pTREX.TryS (TryS[hi]), were cloned and expanded as described in Materials and Methods. Cloned
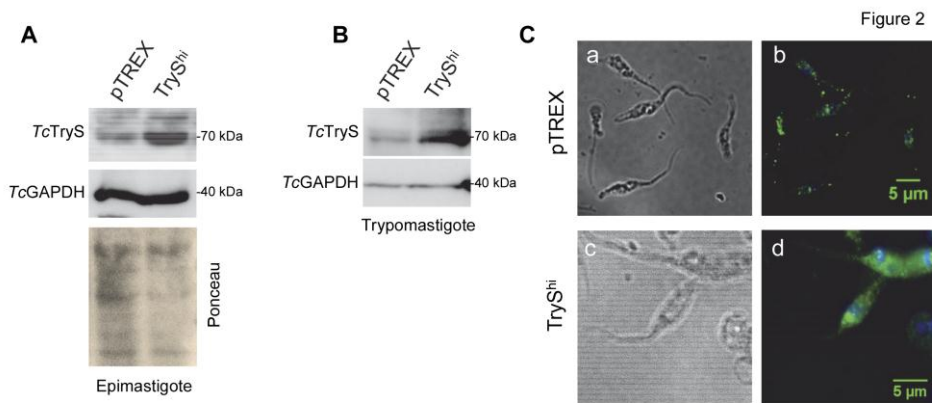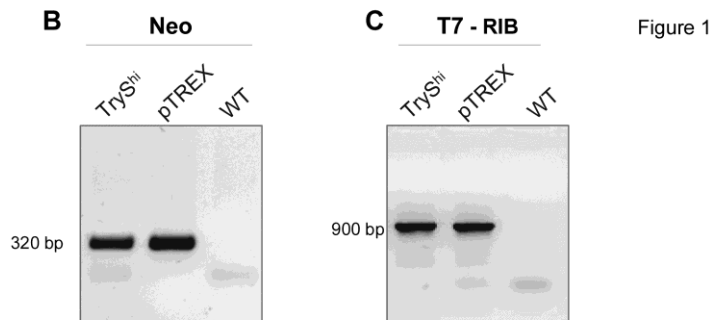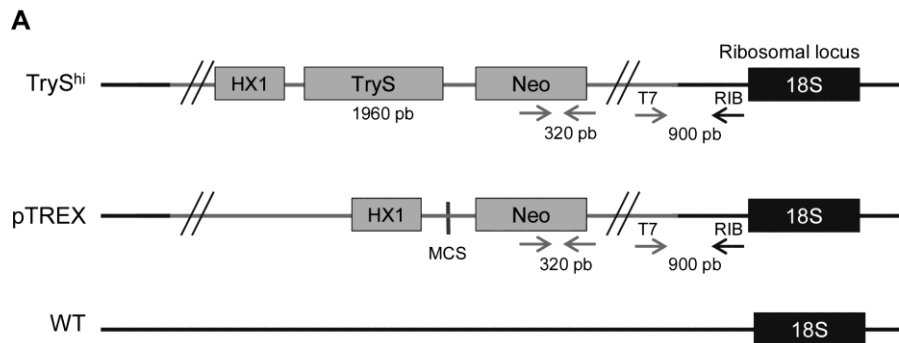
epimastigote **(A-C)** and trypomastigote **(D-F)** populations were incubated for 48 h and 6 h respectively, in presence of 0-1000 µM H_2O_2 and 0-30 µM of TryS inhibitors KuOrb39 or KuOrb54. **(A&D)** IC_{50} concentration of H_2O_2 that resulted in 50% inhibition of $TryS^{hi}$ (*vs.* control) parasites. **(B&E)** Parasite growth or survival inhibition curves fitted after incubation with increasing H_2O_2 concentrations. **(C&F)** Percentage (%) inhibition of growth or survival of $TryS^{hi}$ (*vs.* control parasites) in presence of H_2O_2 at its ~IC_{50} value and varying concentrations of TryS antagonists. For all experiments, viable, motile parasites were visually quantitated by light microscopy. All experiments were conducted at least twice (three determinations per sample per experiment), and data are plotted as mean value ± SEM (* $p < 0.05$).

Figure 7: TryS inhibition is detrimental to parasite resistance to nifurtimox. Clonal cultures of $TryS^{hi}$ and control epimastigotes **(A&B)** and trypomastigotes **(C-D)** of *T. cruzi* were incubated for 48 h or 6 h respectively, with 0-400 µM nifurtimox (NFX) in presence or absence of KuOrb54 (10 µM or 30 µM). **(A&C)** IC_{50} concentration of NFX that resulted in 50% inhibition of $TryS^{hi}$ (*vs.* control) parasite forms. **(B&D)** Parasite growth or survival inhibition curves fitted after incubation with increasing concentrations of NFX**. (E)** Percentage (%) inhibition of trypomastigotes' survival in presence of 2.5 µM NFX (IC_{50} value) and KuOrb 54 (10 and 30 µM). All experiments were conducted twice (three determinations per sample per experiment), and data are plotted as mean value ± SEM (* $p < 0.05$).

Figure 8: TryS inhibitors increase *T. cruzi* susceptibility to benznidazole. Clonal cultures of pTREX and $TryS^{hi}$ epimastigote **(A-B)** and trypomastigote **(C-D)** forms of *T. cruzi* were incubated for 48 h and 6 h respectively, with benznidazole (BZ, 0-500 µM) in presence or absence of KuOrb54 (10 µM and 30 µM). **(A&C)** IC_{50} concentration of BZ that resulted in 50% inhibition of $TryS^{hi}$ (*vs.* control) epimastigote and trypomastigote forms of *T. cruzi*. **(B&D)** Parasite growth or survival inhibition curves fitted after incubation with increasing concentrations of benznidazole. **(E)** Percentage (%) inhibition of $TryS^{hi}$ (*vs.* control) trypomastigotes' survival in presence of 60 µM BZ (IC_{50} value) and TryS antagonist KuOrb 54 (10 and 30 µM). All experiments were conducted twice (three determinations per sample per experiment), and data are plotted as mean value ± SEM (* $p < 0.05$).

HIGHLIGHTS

- Trypanothione synthetase (TryS) is an essential enzyme for *T. cruzi.*

- TryS-overexpression confers higher replication rate to *T. cruzi* epimastigotes.

- TryS provides protection against oxidative stress to different parasite stages.

- TryS confers resistance to current treatment drugs, benznidazole and nifurtimox.
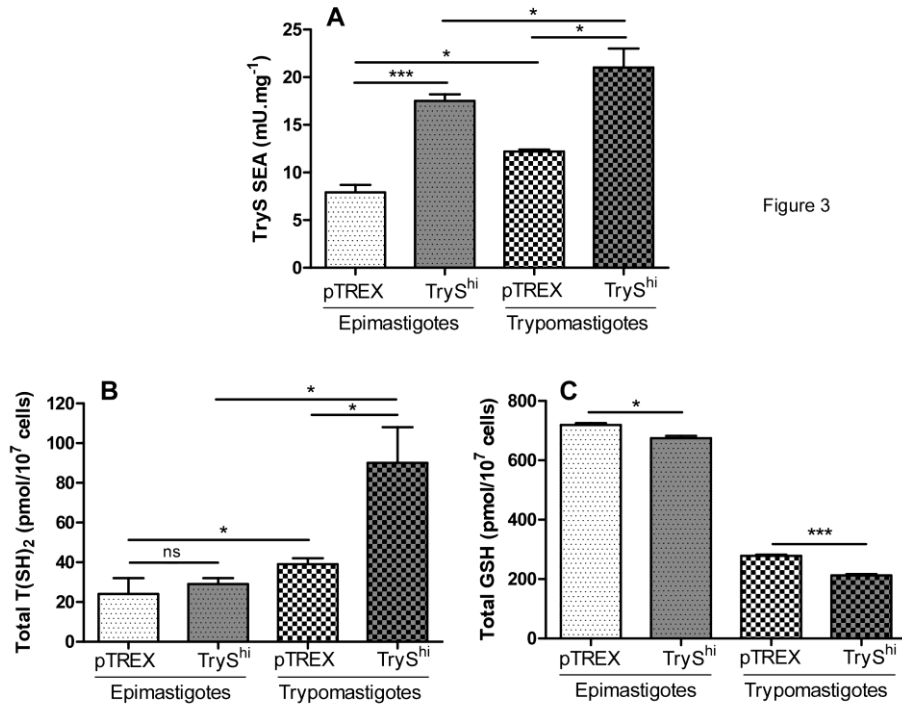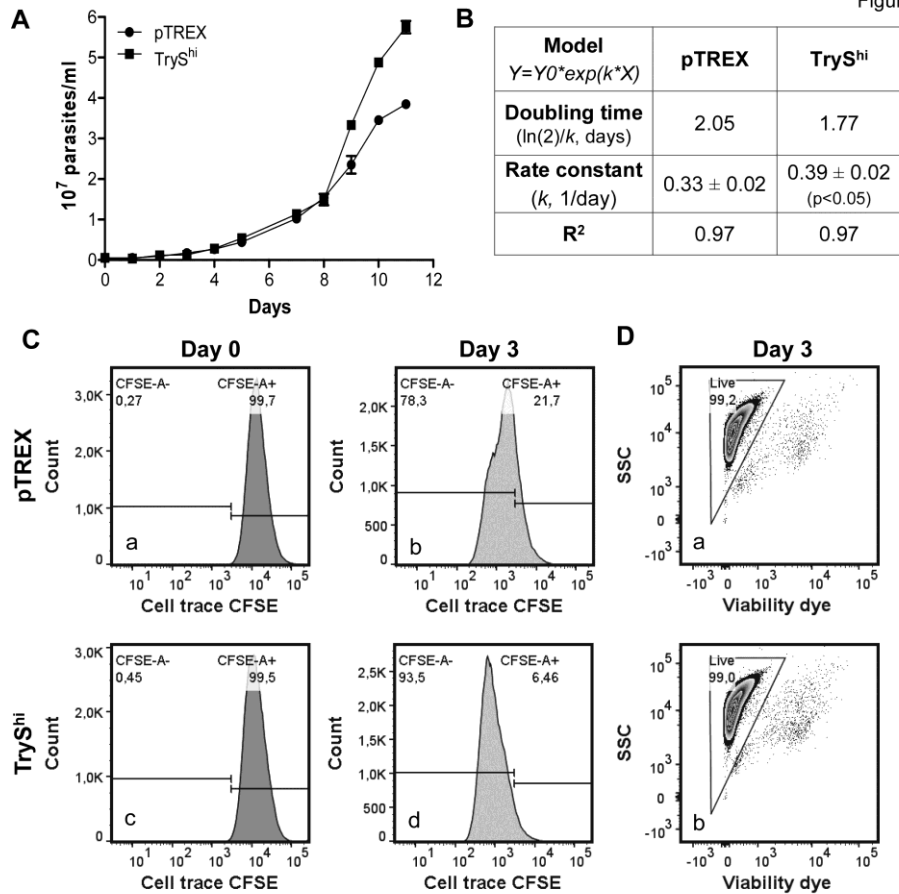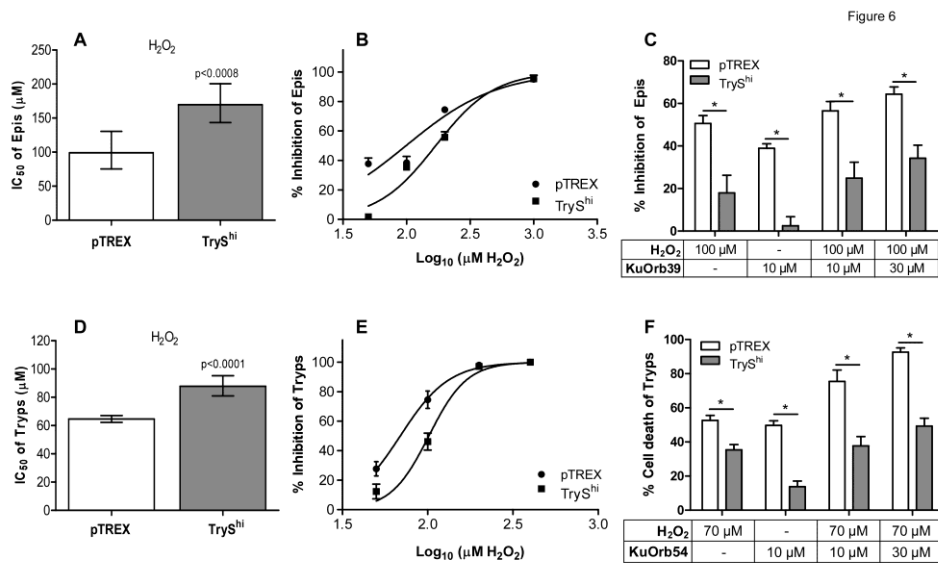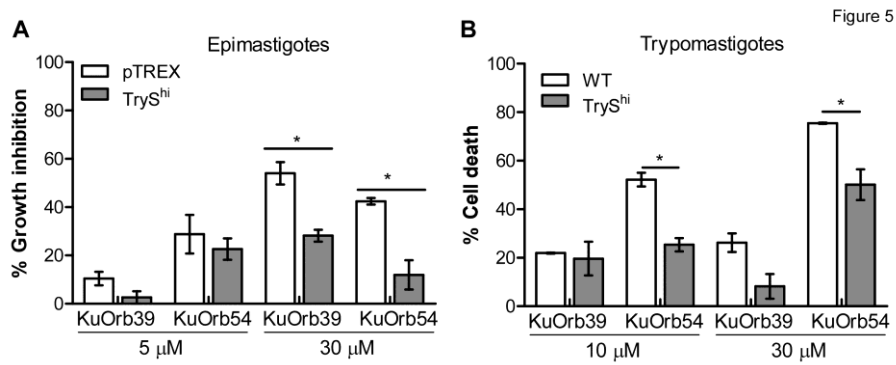

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

KuOrb39/54

Graphical abstract

2 ATP

2 GSH + Spermidine —TryS→ Trypanothione

2 ADP + 2 Pi

T. cruzi
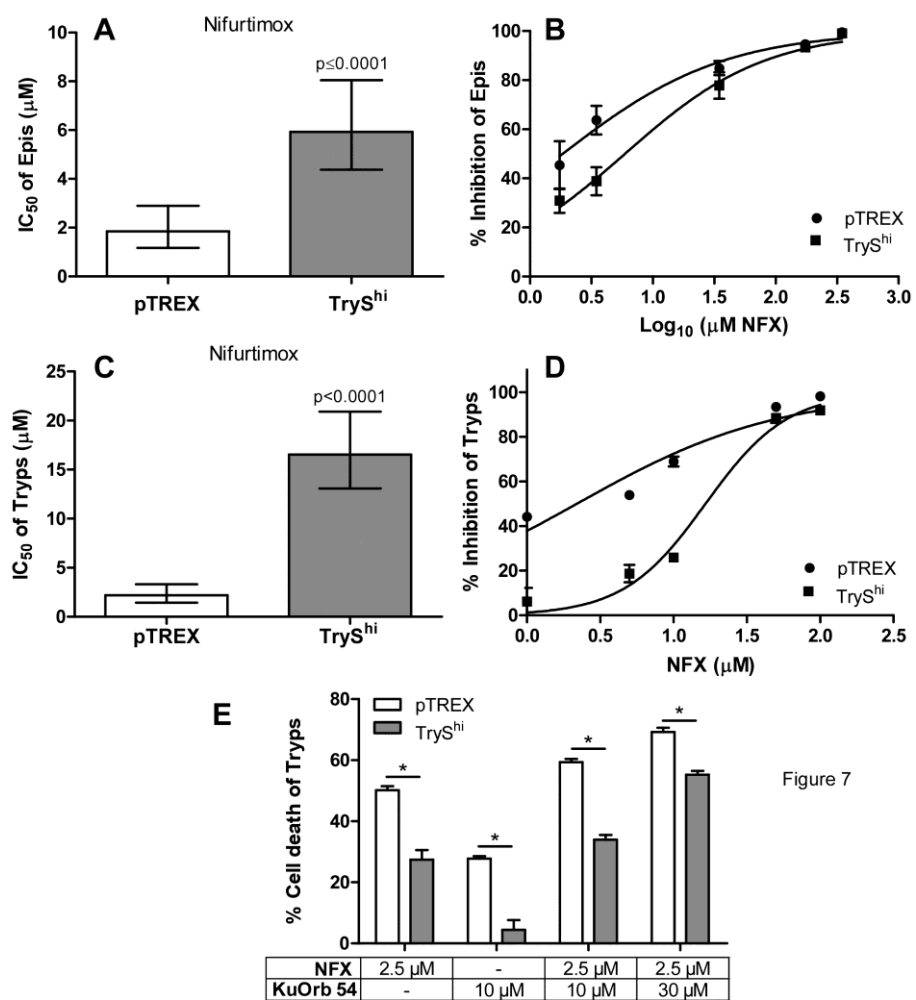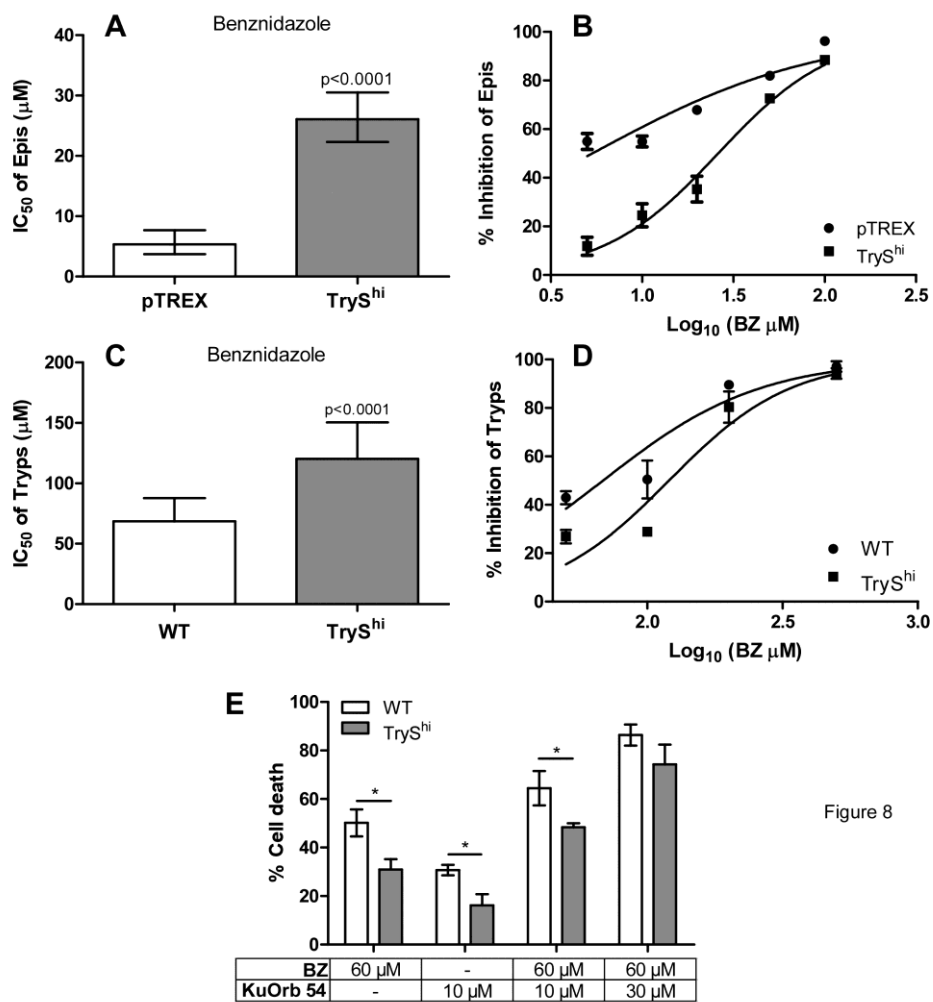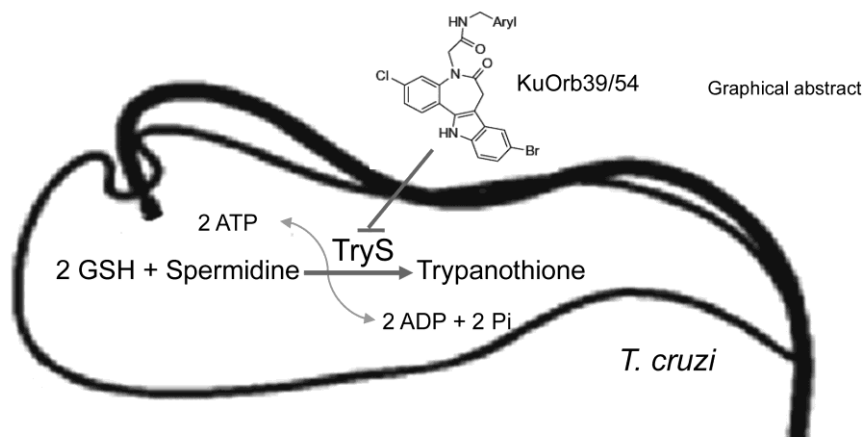
Parameters	WT	WT + inhibitor	TryS^{hi}	TryS^{hi} + inhibitor
Replication (epimastigote/insect form) Survival (trypomastigote/infective form)	+	−	++	+
Survival under stress (H$_2$O$_2$, heavy metal)	−	− −	++	+
Resistance to anti-protozoal drugs (nifurtimox & benznidazole)	−	− −	++	+

TryS^{hi}: Trypanothione synthetase-overexpressing *T. cruzi*