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RESEARCH ARTICLE

Identification of Novel Chemical Scaffolds Inhibiting Trypanothione Synthetase from Pathogenic Trypanosomatids

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Abstract

Background

The search for novel chemical entities targeting essential and parasite-specific pathways is considered a priority for neglected diseases such as trypanosomiasis and leishmaniasis. The thiol-dependent redox metabolism of trypanosomatids relies on bis-glutathionylspermidine [trypanothione, T(SH)₂], a low molecular mass cosubstrate absent in the host. In pathogenic trypanosomatids, a single enzyme, trypanothione synthetase (TryS), catalyzes trypanothione biosynthesis, which is indispensable for parasite survival. Thus, TryS qualifies as an attractive drug target candidate.

Methodology/Principal Finding

A library composed of 144 compounds from 7 different families and several singletons was screened against TryS from three major pathogen species (*Trypanosoma brucei*, *Trypanosoma cruzi* and *Leishmania infantum*). The screening conditions were adjusted to the TryS' kinetic parameters and intracellular concentration of substrates corresponding to each trypanosomatid species, and/or to avoid assay interference. The screening assay yielded suitable Z' and signal to noise values (≥0.85 and ~3.5, respectively), and high intra-assay reproducibility. Several novel chemical scaffolds were identified as low μM and selective tri-tryp TryS inhibitors. Compounds displaying multi-TryS inhibition (N,N'-bis(3,4-substituted-benzyl) diamine derivatives) and an N⁵-substituted paullone (MOL2008) halted the

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proliferation of infective *Trypanosoma brucei* (EC₅₀ in the nM range) and *Leishmania infantum* promastigotes (EC₅₀ = 12 μM), respectively. A bis-benzyl diamine derivative and MOL2008 depleted intracellular trypanothione in treated parasites, which confirmed the on-target activity of these compounds.

Conclusions/Significance

Novel molecular scaffolds with on-target mode of action were identified as hit candidates for TryS inhibition. Due to the remarkable species-specificity exhibited by tri-trypanothione towards the compounds, future optimization and screening campaigns should aim at designing and detecting, respectively, more potent and broad-range TryS inhibitors.

Author Summary

Parasites from the genus *Trypanosoma* and *Leishmania* are etiologic agents for a group of neglected diseases with high morbidity and mortality rates in the developing world. Inasmuch as vaccine development is hampered by the successful mechanisms employed by the pathogens to evade the host immune response, chemotherapy remains as a safe option to fight these diseases. However, new drugs with better pharmacological performance (i.e. safety, efficacy and ease of administration) than those in current use are urgently needed. The thiol-redox metabolism of trypanosomatids offers an excellent opportunity for the development of more selective and efficacious medicines because it depends on a molecule, trypanothione (a bis-glutathionyl derivative of spermidine), unique and indispensable to the pathogens. Here we report the identification of novel inhibitors of trypanothione synthetase from three major trypanosomatid species of medical and veterinary relevance. Although highly conserved in sequence, trypanothione synthetases display significant species-specificity towards compounds, pointing to structural differences as determinants of ligand selectivity. Most of the active compounds presented two-digit μM inhibitory activity and serve as primary scaffolds to develop more potent inhibitors. Among them, *N,N'*-bis(benzyl)-substituted diamine and paullone derivatives are interesting candidates because of their potent and/or selective anti-trypanosomal and anti-trypanothione synthetase activity.

Introduction

Protozoan parasites from the genus *Trypanosoma* and *Leishmania* are responsible for diseases affecting humans and their livestock. The zoonotic character of these diseases, which involve different insect species as vectors and wild animals as reservoirs, hamper the implementation of successful control strategies [1]. Immuno-prophylaxis is not yet available and for some species, such as *T. brucei* spp. and *T. cruzi*, appears unfeasible due to complex immune-evasion mechanisms [2, 3]. So far, and probably for several decades ahead, chemotherapy remains as the sole choice of treatment. Only a handful of drugs are available to fight Chagas's disease (*T. cruzi*), sleeping sickness (*T. brucei gambiense* and *T. b. rhodesiense*) and the different forms of leishmaniasis (*Leishmania* spp.). Unfortunately, they suffer from several drawbacks encompassing low efficacy, resistance and route of administration [4–7]. Moreover, several of these drugs (e.g. nifurtimox, benznidazole and melarsoprol) present a non-specific mode of action

that accounts for their high toxicity [8, 9]. Thus, the discovery of new chemical entities targeting specific and indispensable components of parasite metabolism is a priority for trypanosomiasis and leishmaniasis.

The thiol-dependent redox metabolism is one of the unique metabolic features that distinguish trypanosomatids from humans and offer reliable molecular targets for selective drug development [10]. An example of genetic hallmarks of trypanosomatids is the lack of genes coding for glutathione reductase and thioredoxin reductase [11–13], which fuel the major redox systems of most living organisms (i.e. the glutathione/glutaredoxin system and the thioredoxin system) with reducing equivalents. Instead, trypanosomatids rely on the low molecular mass thiol N^1 , N^8 -bis(glutathionyl)spermidine [trypanothione, $T(SH)_2$] and the flavoenzyme trypanothione reductase for sustaining the intracellular redox homeostasis (Fig 1). $T(SH)_2$ delivers reducing potential to different redoxin proteins, which, by acting on different targets, control vital functions (for a review see [10]; Fig 1). Proposed functions of $T(SH)_2$ further comprise neutralization of xeno- and endobiotics (e.g. methylglyoxal, iron and nitric oxide), coordination of iron-sulfur complexes and reduction of ascorbate [14, 15].

The biosynthesis of $T(SH)_2$ is achieved in two consecutive steps each involving the ligation of a glutathione (GSH) molecule by its glycine carboxyl group to the free N^1 and N^8 amine groups of spermidine (SP). Both reactions are catalysed by the C-terminal ligase domain of trypanothione synthetase (TryS; EC 6.3.1.9) at the expense of ATP (Fig 1). Some trypanosomatids species harbour (*L. infantum*, *L. donovani*, *L. mexicana* and *T. cruzi*) or express (*Crithidia fasciculata*) a gene coding for glutathionylspermidine synthetase (GspS; EC 6.3.1.8), which synthesizes the reaction intermediate N^8 mono-glutathionylspermidine.

The importance of TryS activity for parasite viability has been demonstrated *in vitro* and *in vivo* for *T. b. brucei* [16–19] and *L. infantum* [20] by means of genetic and pharmacological approaches. In addition, TryS presents several advantages as a drug target candidate: (i) it is encoded by a single copy gene [11–13], (ii) the structure of TryS from *L. major* has been elucidated [21], (iii) TryS has been shown to provide metabolic control to the trypanothione pathway in *T. cruzi* [22], and (iv) kinetic information is available for several TryS [18, 22–27].

At an early state of knowledge, the rational inhibitor design was undertaken using GspS of *C. fasciculata* (CfGspS) and *Escherichia coli* (EcGspS) as test enzymes and compounds isosteric with GSH or related transition state analogues as chemical scaffolds [28–34]. Preliminary studies with GSH analogues identified the γ -glutamyl moiety as critical for molecular recognition [28]. Further work revealed that addition of acidic groups to the *L*- γ -Glu-*L*-Leu dipeptide resulted in CfGspS inhibitors of reasonable potency such as the phosphonic ($K_i \sim 60 \mu\text{M}$) [29], the boronic ($K_i \sim 81 \mu\text{M}$ and $K_i^* \sim 18 \mu\text{M}$) [34] or the diaminopropionic acid derivative ($K_i \sim 7.2 \mu\text{M}$ and $K_i^* \sim 21 \mu\text{M}$) [35].

Transition state mimics, previously identified as potent inhibitors of EcGspS [30–32], proved to be equally active against recombinant CfGspS. Notably, a Gsp-phosphinate derivative was capable to inhibit recombinant TryS from *L. major*, *T. cruzi* and *T. b. brucei* albeit with apparent K_i values 16–40-fold higher than that obtained for CfGspS (K_i of 18.6 nM) [36]. Unfortunately, these compounds displayed null biological activity against pathogenic trypanosomatids at 100 μM . Nevertheless, this phosphinate remained the only compound able to target TryS from three different pathogenic trypanosomatids.

The anti-proliferative activity of GSH derivatives (*N,S*-blocked GSH diesters, *S*-2,4-dinitrophenyl-GSH) [37–39] and GSH-related phosphinopeptides [40] ranked from 35 to 0.2 μM in different trypanosomatids. The poor biological activity exhibited by these inhibitors has been ascribed to their peptidic nature susceptible to hydrolysis by esterases and amidases [37].

Based on the ATP-dependency of TryS, a compound library of protein kinase inhibitors was screened against recombinant CfTryS. This led to the identification of N^5 -acetamide paullones

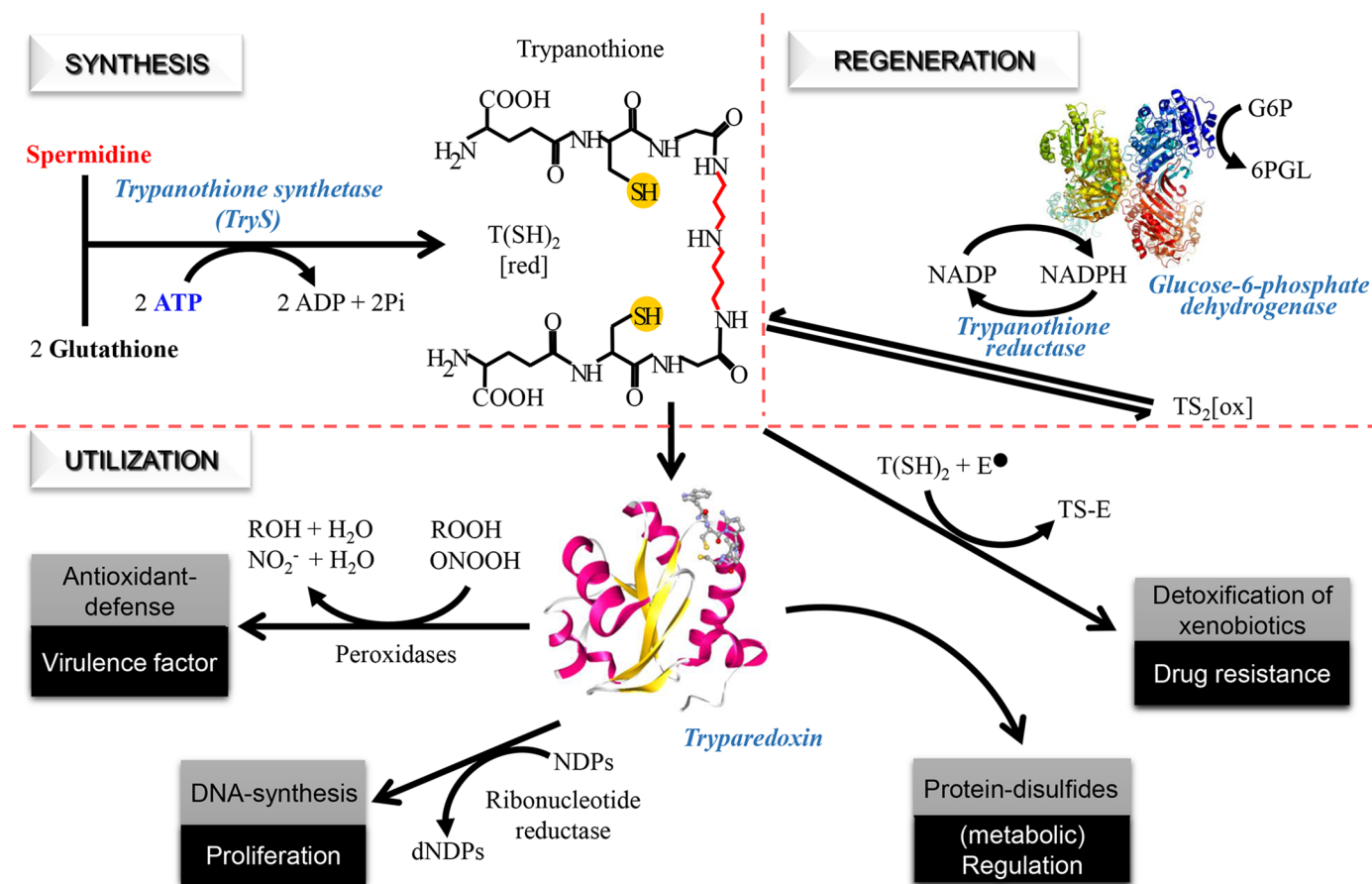


Fig 1. Trypanothione dependent redox metabolism. The chemical structure of trypanothione (*N*¹,*N*⁸-bis(glutathionyl)spermidine; T(SH)₂) is depicted at the center. Synthesis: trypanothione synthetase catalyzes the ligation of two molecules of glutathione to one of spermidine using the energy provided by two ATP molecules. Regeneration: trypanothione reductase maintains trypanothione in the reduced state at expenses of NADPH, which can be supplied by the oxidative phase of the pentose phosphate pathway via glucose 6-phosphate dehydrogenase. Utilization: reduced trypanothione is involved in multiple functions such as the detoxification of xenobiotics, cell proliferation, defense against oxidants and protein thiol-redox homeostasis. The multipurpose oxidoreductase tryparedoxin plays an important role catalyzing electron transfer from T(SH)₂ to different molecular targets (e.g. peroxidases, ribonucleotide reductase and protein disulfides). G6P: glucose-6-phosphate, 6PGL: 6-phosphogluconolactone, T(SH)₂: reduced trypanothione, TS₂[ox]: oxidized trypanothione, NDPs: nucleosides diphosphate, dNDP: deoxynucleosides diphosphate, E•: electrophilic species, TS-E: trypanothione-electrophile adduct, ROOH: hydroperoxide, ONOOH: peroxyntirite, NO₂⁻: nitrite.

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(benzo[2,3]azepino[4,5-*b*]indol-6-ones) as potent inhibitors of CfTryS [10, 41]. Recently, we reported the finding of a related 10-trifluoromethylated acetamide derivative of this paullone as nM inhibitor of LiTryS (IC₅₀ 350 nM) [20]. Information on the activity of these paullones against TryS and parasites from other pathogenic species is lacking.

More recently, a HTS campaign against TbTryS with a library of ~ 62000 compounds identified several hits that upon optimization yielded leaders with inhibitory activity against TbTryS in the nM range (IC₅₀ values of 45, 95 and 140 nM for DDU86439, DDD85811 and DDD86243, respectively) and EC₅₀ values towards infective *T. b. brucei* that ranked from 5 to 10 μM. The compounds allowed the chemical validation of TbTryS as drug target [17, 18].

We here describe the setting-up of a screening technique to detect TryS inhibitors and report the identification and chemical validation of inhibitors from TryS from three major trypanosomatid species (*Trypanosoma brucei brucei*: *T. b. brucei*, *Trypanosoma cruzi*: *T. cruzi* and *Leishmania infantum*: *L. infantum*).

Materials and Methods

Organisms and Reagents

Unless otherwise stated all reagents were of analytical grade and purchased from Sigma-Aldrich, J.T. Baker, Carlo Erba Reagents SA, Gibco, Invitrogen, Life Technologies, Enzo Life Sciences, Roche.

Heterologous Expression and Purification of Recombinant TryS

TryS from different trypanosomatids was produced in recombinant form with an N-terminal His-tag. The constructs pET-15b *Tb*TryS [27], pRSET-B *Tc*TryS and pET-28c(+)*Li*TryS [20] were kind gifts of Alan Fairlamb (Dundee University, Dundee, Scotland), Sergio Guerrero (Universidad Nacional del Litoral, Santa Fe, Argentina) and Helena Castro (Institute for Molecular and Cell Biology, Porto, Portugal), respectively. They were used to express TryS of *T. b. brucei* 427 (MITat1.4, GenBank accession protein id CAC87573.1), *T. cruzi* strain Tulahuen 0 (GenBank accession protein id AAO00722.1) and *L. infantum* JPCM5 (GenBank accession protein id CAM69145.1). *E. coli* strain BL21 (DE3) or Tuner (DE3) (Novagen) served as expression host. For a detailed description of the expression and purification protocols see [S1 Text](#).

Protein concentration was determined using the Bicinchoninic Acid assay with bovine serum albumin as standard. The protocols described above yielded 4–8 mg of recombinant TryS per liter of culture medium with $\geq 95\%$ purity and homogeneous specific activity.

Kinetic Characterization

The kinetic characterization of His-tagged TryS was performed using the LDH/PK assay which couples ATP regeneration to NADH oxidation. The end-point assay based on detection of inorganic phosphate (Pi) by the BIOMOL GREEN reagent was used to estimate the apparent K_i for ADP. All reactions were performed at room temperature (RT, 20–25°C) and a detailed description of both assays is provided in [S1 Text](#). The apparent kinetic parameters (K_M and V_{max}) were calculated by fitting plots of initial velocity (v) vs. substrate concentration ($[S]$), determined at saturating concentration of co-substrates, to the Michaelis Menten equation assisted by the software OriginPro 8. For GSH, the K_M and K_i values were determined using the following equation $v = V_{max} / (1 + K_M / [GSH] + [GSH] / K_i)$, which considers the nonproductive binding of GSH to the substituted enzyme [42]. The apparent K_i for ADP was estimated from linear fitting of the plots $[E]/v$ vs. $[ADP]$ at different concentrations of ATP, where $[E]$ is enzyme concentration.

Compound Library

The compound library involves 144 chemical entities that are clustered by chemical scaffold as follow: (A) 6-arylpyrido[2,3-*d*]pyrimidine-2,7-diamine derivatives (APPDA; [S1 Table](#)) developed as ATP-competitive inhibitors of bacterial D-Alanine:D-Alanine ligase [43] and biotin carboxylase [44]; (B) 1-(benzo[*d*]thiazol-2-yl)-4-benzoyl-3-hydroxy-5-phenyl-1*H*-pyrrol-2(5*H*)-one derivatives (BBHPP; [S2 Table](#)); (C) *N,N'*-bis(3,4-substituted-benzyl) diamine derivatives (BDA; [S3 Table](#)) that display potent anti-malarial or -trypanosomal/leishmanial activity [45, 46] and are simplified derivatives of compounds interfering with the parasite's polyamine metabolism [45, 47–50]; (D) benzofuroxan (BZ; [S4 Table](#)) [51–55]; (E) 4,5-dihydroazepino [4,5-*b*]indol-2(1*H*,3*H*,6*H*)-one derivatives (AI; [S5 Table](#)), some of which with reported anti-TryS or -*T. b. brucei* activity [56]; (F) 1*H*-purine-2,6(3*H*,7*H*)-dione (PD), including the 3-butyl-7-methyl-8-((3-(trifluoromethyl) phenylthio)methyl)3,4,5,7-tetrahydro-1*H*-2,6-dione (kindly provided by Dr. Luise Krauth-Siegel, Heidelberg University, Germany; [S6 Table](#)) that

was reported as inhibitor of the T(SH)₂-dependent oxidoreductase tryparedoxin [57]; (G) 2-aminooxazole-5-carboxamide derivatives (AOCA; S7 Table); (H) and several singletons: *N*, *N*-dibenzyl-1-ethyl-3-methyl-1*H*-pyrazole-5-carboxamide; 2-amino-*N,N*-dibenzyl-4-methyl thiazole-5-carboxamide; prochlorperazine; *tert*-butyl 8-aminooctylcarbamate; *tert*-butyl 12-aminododecylcarbamate; *N*-(8-aminooctyl)acetamide.

TryS Screening Assay Development

General considerations. The BIOMOL GREEN assay was adapted for the screening of potential TryS inhibitors. All assays (screening and interference test) were performed manually (non-automated) in quadruplicates, at RT and using a total reaction volume of 50 μ L in 96-well plates. The colorimetric reaction was initiated by adding 200 μ L BIOMOL GREEN reagent and after 20 min incubation at RT the A₆₅₀ was measured with a MultiScan EX plate reader (Thermo SCIENTIFIC). The interference of reagents (DMSO, NaCl, glycerol, DTT, SP and GSH) with the colorimetric reaction was evaluated individually using 20 μ M K₂HPO₄ as Pi standard. The assay conditions (e.g. reagents, TryS units, time) were adjusted to yield optimal signal detection (20 μ M Pi) taking into account the intracellular concentration reported or estimated for each substrate and/or to avoid interference with the colorimetric reaction.

The linearity range, detection limit (e.g. [Pi] corresponding to $3\sigma^{n-1}$ the A_{650nm} of the blank) and interference was estimated from plots of blank-corrected A_{650nm} vs. [Pi] or the reagent concentration for each condition tested. The plots were prepared and analyzed with OriginPro 8 (OriginLab Corporation). The errors are expressed as $2\sigma^{n-1}$.

Screening assay. For all TryS, ATP was used at 150 μ M because of high background signal at higher concentrations and SP fixed at 2 mM, in agreement with the intracellular levels calculated from data reported in the literature for trypanosomatids [58–60]. GSH was adjusted to 0.05, 0.57 and 0.25 mM for *Tb*TryS, *Tc*TryS and *Li*TryS, respectively, to avoid substrate inhibition or to approach physiological concentrations [14].

A master mix (MM) solution containing all the substrates at 1.25-fold their end concentration in assay was prepared in screening reaction buffer (5 mM DTT, 10 mM MgSO₄, 0.5 mM EDTA, 100 mM HEPES pH 7.4, 9 mM NaCl, 10% v/v DMSO) and kept on ice until use. Microtiter plate wells were loaded with 5 μ L of test compound, DMSO (reaction control) or TryS-specific inhibitor (inhibition control) and 40 μ L of MM. The reactions were then started by adding 5 μ L of TryS (1, 2 and 3 $\times 10^{-5}$ μ mol.min⁻¹.mL⁻¹ for *Tc*TryS, *Li*TryS and *Tb*TryS, respectively) and stopped after 15 min with 200 μ L BIOMOL GREEN reagent.

The compounds used as inhibition control were added at concentrations that inhibited 50% TryS activity: 30 μ M and 150 nM MOL2008 for *Tb*TryS and *Li*TryS, respectively, and 30 μ M J18 for *Tc*TryS. Blanks were prepared for each condition by adding 5 μ L of screening reaction buffer with 150 mM NaCl instead of enzyme.

The TryS activity is calculated as follow: % TryS activity = $\{[(A_{650nm\ Ci} - A_{650nm\ CiB}) / (A_{650nm\ E} - A_{650nm\ EB})] \times 100\}$, where A_{650 nm Ci} is the mean absorbance at 650 nm for the reaction test with compound i, A_{650 nm CiB} is the mean absorbance at 650 nm for the blank control with compound i, A_{650 nm E} is the mean absorbance at A_{650 nm} for the reaction control with DMSO and A_{650 nm EB} is the mean absorbance at A_{650 nm} for the blank control with DMSO. The assays yielded an intra-assay coefficient variation ($CV = \sigma^{n-1}_E / E$) $\leq 2.5\%$, a signal to background coefficient (E/E_B) of ~ 3.5 and a Z' factor [61] ≥ 0.85 .

Screening work-flow. The solubility in DMSO of each compound was evaluated immediately prior to assay. Compounds insoluble at 3 mM in DMSO were excluded from the screening. Soluble compounds were stepwise diluted in DMSO up to 0.3 mM. For the primary screening all compounds were tested (inhibition and blank) at a fixed concentration of 30 μ M.

Compounds that decrease or increase TryS activity $\leq 55\%$ or $\geq 105\%$, respectively, were tested for interference as described in [S1 Text](#).

For compounds that at 30 μM decreased TryS activity $\leq 55\%$, the IC_{50} values were obtained from 10-point dose-response curves ($\% \text{ TryS activity} \pm 2\sigma^{n-1}$ vs. Log nM compound) fitted to a four-parameter sigmoidal equation (Boltzmann model). The reaction conditions were identical to those described for the screening assay and, whenever corresponding, the TryS activity values were corrected by the interference at each compound's concentration.

Biology

Proliferation assays for parasites and macrophages. The biological activity of selected compounds was evaluated in the infective form of *T. b. brucei* strain 427, cell line 514–1313 (WT) and cell line RNAi-TryS [[16](#)], as well as *Leishmania infantum* promastigotes (MHOM MA67ITMAP263; kindly provided by Dr. Helena Castro and Dr. Ana Tomas, IBMC, Portugal). The assay conditions to assess the cytotoxicity of compounds towards parasites and murine macrophages (cell line J774) are described in [S1 Text](#). All assays were performed in duplicates or triplicates.

Western blot analysis of TryS expression. Total cell extracts from the WT and RNAi-TryS cell lines of *T. b. brucei* grown in the presence or absence of oxytetracycline were separated under reducing conditions on an SDS / 10% PAGE. The blotted membranes were incubated with mouse polyclonal antibodies against TbTryS (dilution 1:1000) followed by a horseradish peroxidase-conjugated anti-mouse IgG (dilution 1:10000). Reactive bands were detected by chemiluminescence using ECL plus (GE Healthcare) and quantified by densitometric analysis using the free software ImageJ (<http://imagej.nih.gov/ij/>).

Analysis of thiols. The method is a modified version of that described in [[62](#)]. Briefly, *L. infantum* promastigotes or bloodstream *T. b. brucei* (WT and RNAi-TryS cell lines) treated or not with compounds (MOL2008 or EAP1-47, respectively) for 24 h were harvested by centrifugation (2000g, 10 min at 4°C), washed twice with PBS and resuspended at a density of 10^8 cells/0.1 mL of 40 mM Hepes pH 8.0 and 2 mM mono-bromobimane in methanol. After incubation at 70°C for 3 minutes, the samples were cooled on ice, then added of 100 μL 4 M methanesulfonic acid (adjusted to pH 1.5 with LiOH) and incubated overnight at 4°C. The supernatant containing the derivatized low molecular mass thiols was centrifuged at 13000g 40 min at 0°C. Labeled thiols were separated using solvent A: 0.25% camphorsulfonic acid pH 2.64 and solvent B: 25% 1-propanol in solvent A and the following conditions: 100 μL of the sample, corresponding to 5×10^7 cells, was injected onto a C18, 5 μm , 4.6 x 150 mm column connected to an HPLC (both from Agilent) pre-equilibrated in 12% solvent B followed by a linear gradient from 12–50% solvent B over 36 minutes at a flow of 1 mL/min. Specific thiol concentrations were determined from peak areas relative to different masses of derivatized standards (i.e. 100, 75, 50, 25, 10, 5 and 0.5 nmol) of GSH and T(SH)₂, the last produced according to [[63](#)].

Accession numbers

AAO00722.1; CAC87573.1; CAM69145.1

Results

Kinetic parameters of TryS and its implicancies for enzyme regulation and inhibition

The kinetic studies of TryS employed in this work were performed with three major aims, first to test the quality of the recombinant enzymes and establish optimal assay conditions, second,

Table 1. Kinetic parameters for recombinant tritryp trypanothione synthetase (TryS).

Parasite	<i>T. cruzi</i>			<i>T. b. brucei</i>				<i>L. major</i> [26] Friedlin	<i>L. infantum</i> [20] JPCM5	<i>C. fasciculate</i> [23] US ^b
Strain	Silvio X10 clone 7 [25]	Tulahuen 0	Ninoa [22]	427 (MITat1.4) [27]		427 ^a [24]				
K_M GSH ^c	570 ± 50	140 ± 13	760 ± 210	56.2 ± 10.7 ^d	23.8 ± 2.3 ^e	142 ± 20	34 ± 4	89 ± 7	170 ± 23	1175
K_i GSH	1200 ± 100	1690 ± 172	No ^f	36.5 ± 6.7 ^d	55 ± 6 ^e	254 ± 45	143 ± 20	1000 ± 80	699 ± 75	Yes
K_M SP ^g	625 ± 39	702 ± 70	860 ± 95	37.8 ± 5.0 ^d	45.4 ± 2.0 ^e	238 ± 25	92 ± 25	940 ± 140	1434 ± 135	7424
K_M ATP	53 ± 3	41 ± 6	70 ± 40	7.1 ± 0.4 ^d	8.6 ± 0.6 ^e	19 ± 5	6.6 ± 0.5	63 ± 2	43 ± 7	52
K_i ADP	ND ^h	60 ± 6	ND ^h	ND ^h	ND ^h	40 ± 5	ND ^h	ND ^h	90 ± 8	ND ^h

The apparent K_M and K_i values (μM) were determined using the lactate dehydrogenase (LDH)/pyruvate kinase (PK) assay and the K_i ADP was assessed using the end-point assay. Whenever available, the values are expressed as the mean ± S.D.. The parameters determined for the N-terminal His-tagged tritryp TryS studied in this work are shown in bold letters and the references to data published earlier in brackets. Please, refer to [S8 Table](#) for details about the conditions employed in the kinetic characterization of each enzyme.

^a *Trypanosoma brucei brucei*, strain 427, cell line 449.

^b US, unknown strain.

^c GSH, glutathione

^d Kinetic parameters determined for untagged *Trypanosoma brucei* TryS (*TbTryS*) using the PK/LDH coupled assay [27].

^e Kinetic parameters determined for untagged *TbTryS* using the end-point assay [18].

^f No, denotes lack of GSH inhibition at 4.5 mM.

^g SP, spermidine.

^h ND, not determined.

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to disclose kinetic data previously not addressed for TryS from *T. cruzi* strain Tulahuen 0 and *L. infantum* (strain JPCM5) and a reaction product (ADP), and third, to compare the kinetic behavior of different TryS and its implication for enzyme regulation and inhibition. The kinetic data available for TryS from earlier [18; 22–27] and the present work are presented in [Table 1](#) and [S1 Fig](#).

LiTryS presented apparent K_M values of 166 ± 62 μM for GSH, 1335 ± 167 μM for SP and 42 ± 10 μM for ATP, and a apparent K_i for GSH of 680 ± 160 μM, all of which are in the same order of magnitude as those reported for non-tagged TryS from the related species *L. major* [26].

The apparent kinetic parameters of *TcTryS* strain Tulahuen 0 (K_M GSH = 123 ± 23 μM, K_M SP = 685 ± 105 μM, K_M ATP = 41 ± 6 μM and K_i GSH = 1600 ± 230 μM) are similar to those published for *TcTryS* strain Silvio X10 clone 7 [25] and strain Ninoa [22], except that inhibition by GSH was not observed for TryS from the last strain [22].

The apparent K_M values for substrates and K_i for GSH obtained for His-tagged *TbTryS* (K_M GSH = 135 ± 43 μM, K_M SP = 238 ± 51 μM, K_M ATP = 18 ± 6 μM and K_i GSH = 242 ± 102 μM) were almost 3- to 7-fold higher than those reported for the untagged version of this protein (K_M GSH = 23.8–56.2 μM, K_M SP = 37.8–92 μM, K_M ATP = 6.6–8.6 μM and K_i GSH = 36.5–143 μM) by different laboratories [24, 27], which may be ascribed to different assay conditions (summarized in [S8 Table](#)) as highlighted in previous publications [23, 24]. Nonetheless, all values determined here for His-tagged tritryp TryS differed in less than one order of magnitude from those reported for tag-free versions from identical or homologue proteins, hence the recombinant form of the enzymes were rated as suitable for the screening assay.

Inhibition by ADP has been reported to occur for *CfGspS* with a K_i of 80 μM [64] but information is lacking for related enzymes from pathogenic trypanosomatids. As shown here, ADP competed for the ATP-binding site of *TbTryS*, *LiTryS* and *TcTryS* ([S1 Fig](#)) with apparent K_i

values of $40 \pm 5 \mu\text{M}$, $90 \pm 8 \mu\text{M}$ and $60 \pm 6 \mu\text{M}$, respectively (Table 1). It is worth to note that these K_i values represent overall estimates of product inhibition because the assay conditions used do not allow distinguishing a preferential inhibition of the first or second biosynthetic step catalyzed by TryS.

Comparison of the kinetic parameters for TryS from three major trypanosomatid species obtained under similar experimental conditions (this study), shows that the enzyme from African trypanosomes presents K_M values for ATP and SP that are remarkable lower (2.3–5.6-fold) than those for TryS from *L. infantum* and *T. cruzi* (Table 1). A similar conclusion can be drawn from studies carried out for related TryS in other laboratories [18, 22, 24–27]. At variance with *Leishmania* spp. and *T. cruzi*, *T. brucei* spp. is an extracellular pathogen that fully relies on *de novo* synthesis of polyamines and glycolysis to fulfil its metabolic and energetic needs [58, 65]. Thus, TryS from African trypanosomes presents kinetic parameters that guarantee the production of the indispensable metabolite trypanothione [16, 17] under conditions of restricted supply of ATP and SP that the parasite may face during its complex life cycle (e.g. differentiation, which entails drastic reprogramming of energetic metabolism, and different nutrient availability in vector and host). All three tritryp TryS displayed similar K_M values for GSH, which contrast with earlier studies reporting a higher affinity of *T. b. brucei* TryS for this substrate [18, 22, 24, 27]. As previously reported [18, 23–27], tritryp TryS were susceptible to substrate inhibition by GSH, although the inhibitory efficiency varied between species as follows: *T. b. brucei* (apparent K_i/K_M GSH = 1.8) > *L. infantum* (apparent K_i/K_M GSH = 4.1) > *T. cruzi* (apparent K_i/K_M GSH = 13.0). This suggests that the *T. b. brucei* enzyme is particularly sensitive to inhibition by the substrate GSH. However, the physiological role of this inhibition mechanism, if any, remains questionable because substrate accumulation will further enhance TryS inhibition and GSH cannot surrogate T(SH)₂ functions in *T. brucei* [16]. For the leishmanial and *T. cruzi* TryS, substrate inhibition will become relevant only at high concentrations of GSH (e.g. > 1.5 mM), which, except for a few examples of *L. donovani* parasites grown to mid-log phase (e.g. 2.27 mM GSH for axenic amastigotes from the strain BOB and 1.68 mM for promastigotes from the strain LV9) [14], appears to be a non-physiological condition. Indeed, *L. infantum* parasites harbouring a single *trys* allele and with a TryS content about 50% lower than that of wildtype cells show no phenotype *in vitro* and in infected animals [20].

The K_i ADP / K_M ATP ratios for tri-tryp TryS range from 1.5 to 2.2-fold, suggesting that ADP may be a physiological modulator of TryS activity. However, taking into account that the intracellular concentration of ATP for trypanosomatids (e.g. 2–4 mM and 0.58 mM for infective *T. b. brucei* [65, 66] and *T. cruzi* [67], respectively, and 0.87 mM for *L. donovani* promastigotes [68]) is > 14-fold in excess with respect to the respective K_M values for tritryp TryS (e.g. 18–60 μM ; Table 1) and that the ATP/ADP ratio reported or estimated by us for trypanosomatids is between 3 to 10 [65, 66, 69–71], such regulatory role of ADP in T(SH)₂ biosynthesis may be questioned. A recent kinetic analysis of *TbTryS* assisted by computational modeling of the enzymatic mechanism [24] highlighted that the activated enzyme is particularly sensitive to inhibition by GSH and T(SH)₂. Because the experimental setup employed in this study did not consider inhibition by ADP and excluded ADP as an integral component of the enzyme activated complex, the real contribution of this product to TryS inhibition remains to be addressed.

Screening

Assay development and validation. Under reaction conditions resembling the screening assay and using KH_2PO_4 as Pi standard, the detection limit and linearity range of the colorimetric reaction with BIOMOL GREEN was ~ 1.9 μM Pi and 2–50 μM Pi, respectively (S2 Fig). DTT and NaCl (salt present in enzyme buffer) added up to 5 mM and 30 mM, respectively, do not affect

assay's readout (S3A and S3D Fig). DMSO (compound solvent) and glycerol (cryoprotective agent added to TryS) interfered with BIOMOL GREEN signal in an additive fashion. For instance, 10% v/v DMSO (e.g. concentration used in the assay) and 4% v/v glycerol reduced assay sensitivity $8.4 \pm 1.5\%$ ($n = 8$) and $\sim 12\%$ (S3C Fig), respectively, whereas addition of both reagents (DMSO 10% v/v and glycerol 4% v/v) decreased $\sim 25\%$ assay sensitivity (S3D Fig). In order to reduce the signal interference, assays were performed at glycerol concentrations $< 1\%$ v/v.

Assay linearity is affected at ATP concentrations $> 150 \mu\text{M}$ due to increase background signal and ADP-mediated inhibition of TryS (S1 Fig; see previous section). Thus, for practical reasons ATP was adjusted to $150 \mu\text{M}$, a concentration that is 4–27-fold lower than the estimated physiological concentration of the substrate in different trypanosomatids [66–70] but ~ 3.5 and 8-fold higher than the K_M values for *TcTryS* and *LiTryS*, and *TbTryS*, respectively (Table 1).

GSH added up to 2.5 mM does not interfere with the colorimetric signal. For assays with *TcTryS*, GSH was used at $570 \mu\text{M}$, a value within the physiological concentrations reported for infective forms of *T. cruzi* strains (i.e. 340 to $1180 \mu\text{M}$) [14]. For the screening involving *LiTryS*, GSH was employed at a concentration of $250 \mu\text{M}$, which is near the intracellular content estimated for amastigote forms of *L. donovani* ($250 \mu\text{M}$) and *L. major* ($310 \mu\text{M}$) isolated from hamsters and mice, respectively [14]. In order to avoid substrate inhibition of *TbTryS*, GSH was adjusted to $50 \mu\text{M}$ a value close to the corresponding K_M (Table 1) and 5-fold lower than the intracellular concentration (i.e. 230 – $260 \mu\text{M}$) reported for this substrate in trypanosomes isolated from infected rats [14], respectively.

Irrespective of the TryS species, SP, a substrate not interfering with the colorimetric reagent (S3B Fig), was fixed to 2 mM for the screening assays. This concentration is 8.4-, 2.9- and 1.5-fold higher than the K_M values for *TbTryS*, *TcTryS* and *LiTryS*, respectively and close to that we estimated for the clinically relevant forms of *T. b. brucei* (SP $\sim 2 \text{ mM}$ for bloodstream stage), *T. cruzi* ($\sim 3 \text{ mM}$ for amastigotes and trypomastigotes) and *L. mexicana* ($\sim 7 \text{ mM}$ for amastigotes) according to information available on polyamine content [58–60], cell volumes and/or total protein content per cell for each parasite species [14, 71, 72].

The linearity $A_{650\text{nm}} \text{ corr}$ vs. time was evaluated for each TryS at substrates concentrations used in the screening assay (see Experimental Procedures, S4 Fig). For *LiTryS* ($2.3 \times 10^{-5} \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mL}^{-1}$), the assay was linear for at least 30 min whereas the production of Pi by *TcTryS* ($3.5 \times 10^{-6} \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mL}^{-1}$) and *TbTryS* ($1.5 \times 10^{-5} \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mL}^{-1}$) displayed a linear relation up to 15 min. As indicated above, the trypanosomal TryS were more sensitive than *LiTryS* to inhibition by ADP, which may explain the lower turnover of the former at longer reaction times. Therefore, a reaction end-point of 15 min was selected for the screening assays, which corresponds to the formation of $20 \mu\text{M}$ Pi and ADP for all three enzymes.

The assay conditions reported above for each enzyme yielded a Z' factor [61] of ≥ 0.85 , a signal background coefficient (E/EB) of ~ 3.5 and a CV (σ^{n-1}_E/E) intra-assay $\leq 2.5\%$, which validate the screening test as highly robust and reproducible.

Compound screening at near physiological concentrations of substrates. The screening work flow is shown in Fig 2 and all data here reported correspond to assays that fulfilled the quality control parameters indicated above.

The initial screening performed with 144 compounds belonging to 7 different families (Fig 3 and S1–S7 Tables) and tested at $30 \mu\text{M}$ against tritryp TryS, allowed the identification of 22 compounds that lowered Pi signal to $\leq 55\%$. An assay interference counter-screen confirmed 15 of them as true TryS inhibitors (Table 2) and the remaining 7, all being BDA, as false positive readouts (S3 Table).

There was no correlation between the rate of hit identification for each enzyme (i.e. 5.6% for *TbTryS* and 4.2% for *TcTryS* and *LiTryS*), and the potency of the compounds (i.e. 2 out of 6 were nM inhibitors of *LiTryS* whereas all compounds targeting the trypanosomal TryS were two

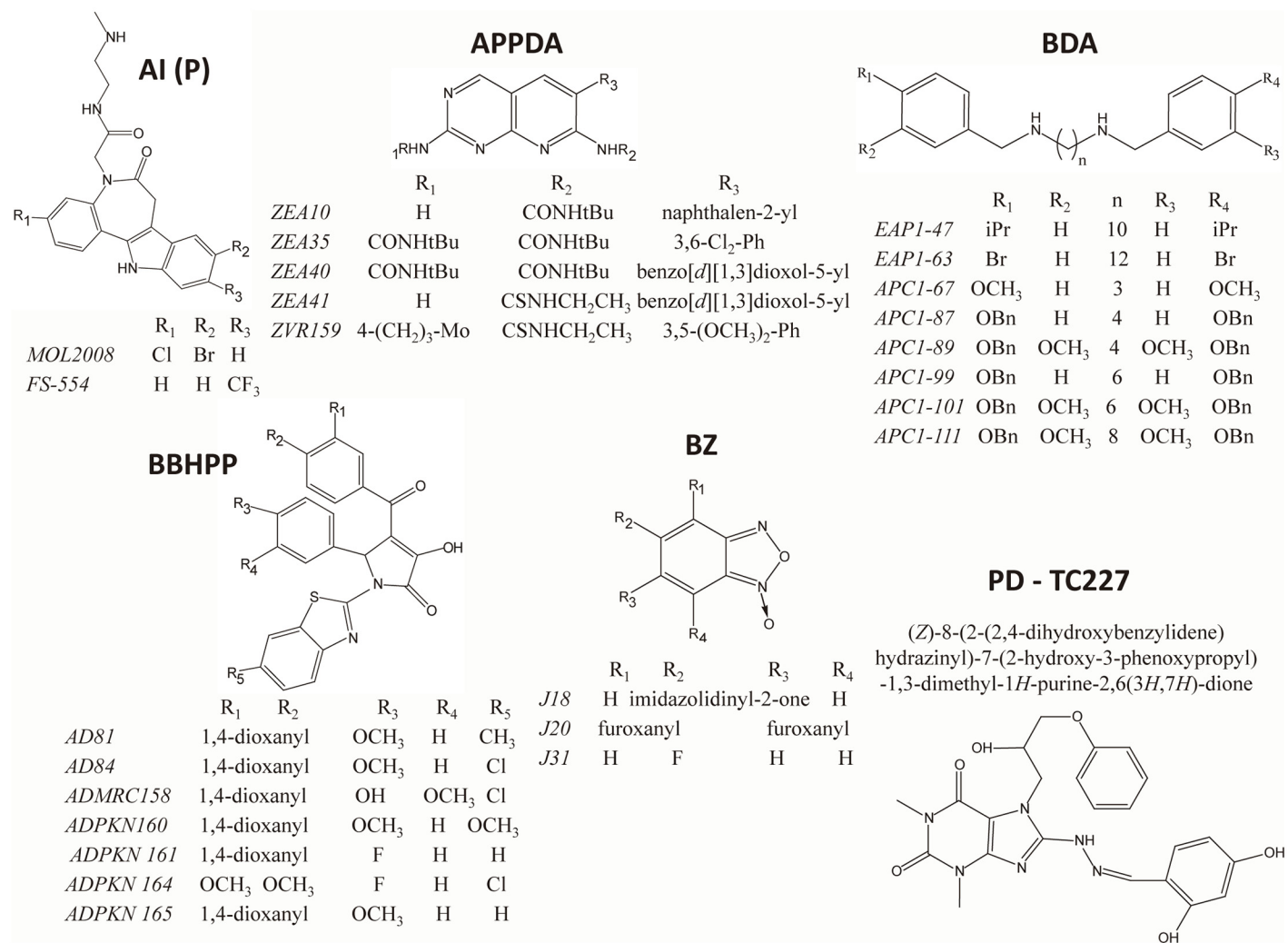


Fig 2. Structure of compounds affecting tritryp trypanothione synthetase activity. AI (P), 4,5-dihydroazepino[4,5-b]indol-2(1H,3H,6H)-one derivatives, paullones derivatives, (FS-554 and MOL2008), five APPDA, 6-arylpyrido[2,3-d]pyrimidine-2,7-diamine derivatives (ZEA10, ZEA35, ZEA40, ZEA41 and ZVR159), eight BDA, N,N'-bis(3,4-substituted-benzyl) diamine derivatives (EAP1-47, EAP1-63, APC1-67, APC1-87, APC1-89, APC1-99, APC1-101 and APC1-111), seven BBHPP, 1-(benzo[d]thiazol-2-yl)-4-benzoyl-3-hydroxy-5-phenyl-1H-pyrrol-2(5H)-one derivatives (AD81, AD84, ADMRC158, ADKPN160, ADKPN161, ADKPN164 and ADKPN165), three BZ, benzofuroxan derivatives (J18, J20 and J31) and one PD, 1H-purine-2,6(3H,7H)-dione derivatives [(Z)-8-(2-(2,4-dihydroxybenzylidene)hydrazinyl)-7-(2-hydroxy-3-phenoxy propyl)-1,3-dimethyl-1H-purine-2,6(3H,7H)-dione, TC227]. iPr, tBu, OBn, Mo and Ph, correspond to an isopropyl, tert-butyl, O-benzyl, 4-morpholinyl and phenyl substitution, respectively.

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digit μ M inhibitors, see Table 2). Supporting the existence of species-specific differences between TryS, the individual enzymes were preferentially targeted: *Li*TryS by AI; *Tb*TryS by BDA and *Tc*TryS by APPDA. Only 4 compounds out of 15, each two BDA and AI, were able to target multiple TryS, though to different extent. EAP1-47 [*N*¹,*N*¹⁰-bis(4-isopropylbenzyl)decane-1,10-diamine] and APC1-111 [*N*¹,*N*⁸-bis(4-(benzyloxy)-3-methoxybenzyl)octane-1,8-diamine] at 30 μ M halved activity of all three TryS or *Tb*TryS and *Li*TryS, respectively; MOL2008 and FS-554 were two digits nM inhibitors of leishmanial TryS (Fig 4) that, to a remarkable minor degree (~ 50% inhibition at 30 μ M), also targeted both trypanosomal enzymes or *Tc*TryS, respectively.

Early investigations had shown that the *N*⁵-substituted paullone derivatives MOL2008 and FS-554 are potent inhibitors of TryS from *C. fasciculata* (IC₅₀ = 30 nM) [10] and *L. infantum*

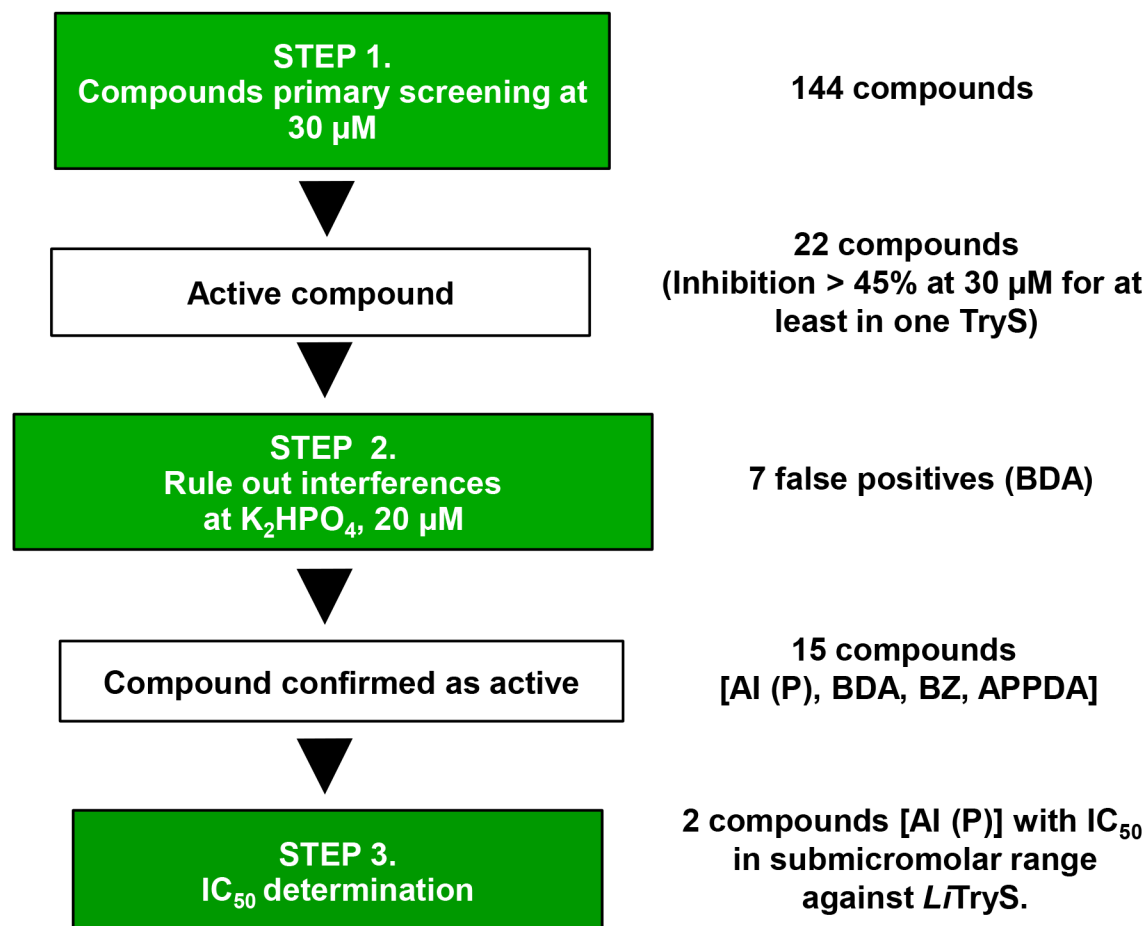


Fig 3. Screening work flow. The different steps, the most relevant assay conditions and the go/no-go criteria of the screening campaign are indicated in boxes. The figures on the right refer to the number of compounds screened and that subsequently advanced during the campaign. From 144 compounds, 22 compounds lowered assay signal $\geq 45\%$ for at least one TryS. From these 22, 7 BDA were false positive and the remaining 15 compounds were confirmed as enzyme inhibitors. Two of them are **AI** with potency in the submicromolar range against *L*TryS. **AI (P)**, 4,5-dihydroazepino[4,5-*b*]indol-2(1*H*,3*H*,6*H*)-one derivatives (P, paullone); **APPDA**, 6-arylpyrido[2,3-*d*]pyrimidine-2,7-diamine derivatives; **BZ**, benzofuroxan derivatives; **BDA**, *N,N'*-bis(3,4-substituted-benzyl) diamine derivatives.

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(IC₅₀ = 350 nM) [20], respectively, but information on their activity towards the homologue enzyme from related trypanosomatids was missing. MOL2008 was the most potent inhibitor of *L*TryS (IC₅₀ = 0.15 ± 0.06 µM) identified in the screening that displayed a moderate activity against *Tb*TryS (IC₅₀ ~ 30 µM) and *Tc*TryS (40% TryS inhibition at 30 µM; see Table 2). On the other hand, FS-554 showed moderate activity against *Tc*TryS (~50% inhibition at 30 µM) and a weak activity against *Tb*TryS (~50% inhibition at 75 µM) (Table 2). Substitution of position *N*⁵ appears key to confer anti-*L*TryS activity to **AI** since the inhibitory activity of unsubstituted derivatives was almost abrogated (compound 11a and 11b in S5 Table). Given the amine nature of the *N*⁵ substituent, a potential SP-competitive mechanism of MOL2008 on *L*TryS was investigated. Assays performed at fixed concentration of co-substrates (250 µM GSH and 150 µM ATP) and variable concentrations of SP (0.2 to 16 mM) show that enzyme inhibition was inversely proportional to polyamine concentration (S9 Table) and confirmed the proposed inhibition mechanism. A collection of 4-azapaullones substituted in position 9 and 11 was recently assayed for their anti-*T. b. brucei* and *Tb*TryS activity [56]. Only derivatives containing in position 9 an α,β -unsaturated carbonyl substituted with a phenyl, phenyl

Table 2. Inhibitors of tritryp trypanothione synthetase (TryS) identified in this work and their biological activity.

Compound	Structure ^a	TcTryS	LiTryS	TbTryS	<i>T. b. Brucei</i> EC ₅₀ (μM) ^b	<i>L. infantum</i> EC ₅₀ (μM) ^c	SI ^d
MOL2008	AI (P)	40.5 ± 5.9%; (1.00)	0.15 ± 0.06 μM ^e ; (0.99); 0.35 ± 0.03 ^f	59.0 ± 6.0%; (0.97)	4.3 ± 0.7	12.6 ± 1.6	2.4 (0.8)
FS-554	AI (P)	55.5 ± 3.8%; (1.02)	0.35 ± 0.05 μM ^e ; (0.99); 0.36 ± 0.04 ^f	~75 μM; (0.94 at 300 μM)	ND	112.3 ± 1.1 ^g	ND (0.6)
ZEA10	APPDA	32.3 ± 4.0%	32.1 ± 6.4%	48.7 ± 2.8%; (1.41)	0.28 ± 0.08	ND	>4
ZEA35	APPDA	61.3 ± 7.6%; (1.01)	13.8 ± 9.7%	24.3 ± 6.1%	ND	ND	ND
ZEA40	APPDA	52.2 ± 1.4%; (1.02)	15.3 ± 8.3%	25.3 ± 5.2%	ND	ND	ND
ZEA41	APPDA	65.4 ± 1.5%; (0.99)	13.6 ± 3.4%	20.9 ± 4.4%	ND	ND	ND
EAP1-47	BDA	53.5 ± 1.3%; (1.48)	47.8 ± 1.8%; (1.34)	51.1 ± 4.2%; (1.49)	0.20 ± 0.02	ND	15
EAP1-63	BDA	30.8 ± 3.8%	25.9 ± 9.3%	47.5 ± 2.8%; (1.30)	0.090 ± 0.007	ND	124
EAP1-67	BDA	23.8 ± 6.6%; (1.34)	51.5 ± 5.3%; (1.35)	42.4 ± 1.6%; (1.33)	ND	ND	ND
APC1-87	BDA	26.6 ± 4.1%	56.7 ± 7.9%; (1.26)	37.9 ± 4.0%	ND	ND	ND
APC1-89	BDA	25.6 ± 1.4%	23.4 ± 2.7%; (1.32)	50.9 ± 1.5%; (1.31)	0.061 ± 0.001	ND	164
APC1-99	BDA	33.4 ± 4.1%	39.1 ± 3.4%	48.9 ± 1.7%; (1.05)	0.015 ± 0.001	ND	67
APC1-101	BDA	17.0 ± 2.1%; (1.40)	26.3 ± 10.1%; (1.36)	61.2 ± 1.8%; (1.33)	0.28 ± 0.09	ND	10
APC1-111	BDA	28.9 ± 4.1%; (1.57)	49.6 ± 5.7%; (1.51)	55.6 ± 3.2%; (1.52)	0.040 ± 0.001	ND	67
J18	BZ	63.5 ± 1.5%; (1.00)	41.3 ± 3.6%; (1.00)	7.3 ± 5.9%	ND	ND	ND

In vitro and *in vivo* (cell) activity profile of most promising TryS inhibitors. Enzyme inhibition is expressed as % TryS inhibition ± 2σⁿ⁻¹ and IC₅₀ values in μM concentration ± 2σⁿ⁻¹. For compounds affecting BIOMOL GREEN signal, the interference factor used to correct TryS activity is provided in brackets (see S1 Text). All values reported stem from at least duplicates. ND, not determined.

^a the chemical scaffolds are: AI (P), 4,5-dihydroazepino[4,5-*b*]indol-2(1*H*,3*H*,6*H*)-one derivatives, paullone derivatives; APPDA, 6-arylpyrido[2,3-*d*]pyrimidine-2,7-diamine derivatives; BDA, *N,N'*-bis(3,4-substituted-benzyl) diamine derivatives; BZ, benzofuroxan derivatives.

^b Cytotoxicity against bloodstream *Trypanosoma brucei brucei* expressed as effective concentration 50: EC₅₀.

^c Cytotoxicity against *Leishmania infantum* promastigotes is expressed as effective concentration 50: EC₅₀.

^d Selectivity index calculated as the ratio: EC₅₀ for murine macrophages (cell line J774) vs. EC₅₀ for *T. b. brucei* or EC₅₀ for *L. infantum* (value in brackets).

^e IC₅₀ value calculated from 3 independent experiments and expressed as mean ± 2 S.D.

^f Slope from IC₅₀ plots calculated from 3 independent experiments and expressed as mean ± 2 S.D.

^g EC₅₀ value reported by [20]

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(amino) or benzylamino group showed a minor anti-*TbTryS* activity (30–40% inhibition at 30 μM). Given the species-specificity displayed by tri-tryp TryS, the complete set of 4-azapaul-lones was tested against the *L. infantum* and *T. cruzi* enzyme (S5 Table). None of the com-pounds inhibited *TcTryS* to a significant level (enzyme inhibition ≤ 17% at 30 μM compound) whereas *LiTryS* showed again to be more susceptible to inhibition by the paullone scaffold, with 4-chloro-phenyl > furan-2-yl > 4-methoxy-phenyl analogues in 9-position reducing 32–44% enzyme activity at 30 μM. Identical substitutions (i.e. 4-methoxy-phenyl and 4-chloro-phenyl) in 11-position of 4-azapaul-lones rendered analogues with similar inhibitory activity (30–35%) towards *LiTryS*.

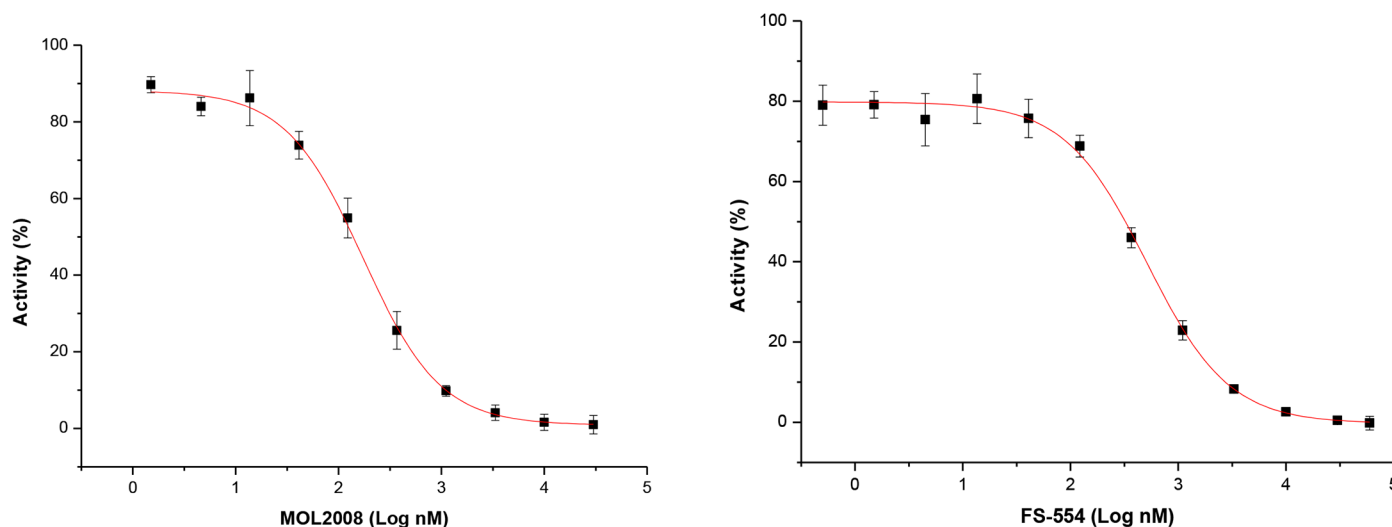


Fig 4. Inhibition plot of *Leishmania infantum* trypanothione synthetase (TryS) by paullones. The data are presented as mean TryS activity (%) $\pm$ 2 S.D. ($n = 4$) as function of Log_{10} concentration (nM) of compound adjusted to a four-parameter Boltzmann sigmoidal equation. Representative dose-response plot obtained for MOL2008 ($\text{IC}_{50} = 0.14 \pm 0.03 \mu\text{M}$; slope plot 0.38 ± 0.03) and for FS-554 ($\text{IC}_{50} = 0.32 \pm 0.73 \mu\text{M}$; slope plot 0.37 ± 0.03).

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The **BDA** (S5 Fig, S3 Table) exerted a low to moderate inhibition of TryS that allows performing a SAR analysis. For all TryS, the inclusion of bulky substitutions ($R^1 = R^4 = \text{OBn}$: APC1-87, APC1-99 and APC1-109, or in addition $R^2 = R^3 = \text{OMe}$: APC1-111, APC1-101 and APC1-89) at the benzyl rings yields derivatives with higher activity (40–60% inhibition) than the unsubstituted congeners and analogues containing less bulky groups ($R^2 = R^3 = \text{OMe}$, $R^1 = R^4 = \text{OH}$) (S5A Fig). In contrast to *Li*TryS, the trypanosomal enzymes were particularly sensitive to the length of the linker, with derivatives bridged by 3- or 10-carbon being less active. This suggests a substituent additive effect to the length of the carbon chain with a possible steric fulfillment demand of the interacting pocket. For the halogenated **BDA**, the length of the linker appears to be a common determinant of activity against all TryS, whereas each enzyme displayed certain specificity for the nature of the halogen atom (S5B Fig). Thus, derivatives with a 12-carbon linker are more potent TryS inhibitors than those consisting of shorter chains and the halogen substitution yielding the highest activity (~50% inhibition at 30 μM) for this scaffold is F (EAP1-69), Cl (EAP1-67) and Br (EAP1-63) for *Tc*TryS, *Li*TryS and *Tb*TryS, respectively. On the other hand, the inclusion of an isopropyl group at position $R_1 = R_4$ of **BDA** with a 10-carbon linker (EAP1-47) improved up to 40% tri-tryp TryS inhibition when compared to the unmodified compound EAP1-9 and produced the best multi-TryS inhibitor here identified (50% inhibition at 30 μM).

From a group of 35 **APPDA** tested, three derivatives containing a substituted phenyl or benzyl group in position R3 (i.e. ZEA35, ZEA 40 and ZEA 41) showed selectivity and a moderate potency towards *Tc*TryS (50% inhibition at 30 μM ; Table 3). Replacing the R3 benzyl of ZEA41 by a naphthalen group (ZEA10) increased the activity against *Tb*TryS (50% inhibition at 30 μM) and *Li*TryS (17% increased inhibition) but was detrimental to *Tc*TryS inhibition (18% lower inhibitory activity). *Tc*TryS was also target of selective inhibition by several **BZ** with J18 being the most active ($\text{IC}_{50} \sim 30 \mu\text{M}$), and the derivatives J20 and J21 reducing about 40% enzyme activity when tested at 30 μM . J18 produced a similar degree of inhibition of *Li*TryS, whereas *Tb*TryS was almost refractory to inhibition by the **BZ** (S4 Table).

Table 3. Compounds enhancing tritryp trypanothione synthetase (TryS) activity.

Compound	Core structure ^a	TcTryS	LiTryS	TbTryS
ZVR159	APPDA	168.6 ± 4.1; (1.10)	100.6 ± 5.8	81.3 ± 1.6
AD81	BBHPP	109.7 ± 8.9	83.9 ± 4.2	128.2 ± 3.6; (0.93)
AD84	BBHPP	98.7 ± 5.1	82.1 ± 4.0	119.0 ± 6.3; (0.95)
ADMRC158	BBHPP	97.4 ± 6.1	85.3 ± 8.7	119.1 ± 4.6; (0.88)
ADPKN160	BBHPP	97.6 ± 3.7	77.9 ± 3.6	116.4 ± 4.2; (0.87)
ADPKN161	BBHPP	90.8 ± 5.8	83.5 ± 9.2	126.7 ± 0.8; (0.86)
ADPKN164	BBHPP	99.6 ± 5.7	66.0 ± 3.4	123.2 ± 7.0; (0.83)
ADPKN165	BBHPP	107.1 ± 4.5	83.5 ± 6.0	166.3 ± 5.5; (0.79)
J20	BZ	64.4 ± 3.8	111.5 ± 14.5; (0.97)	118.8 ± 2.4; (0.97)
J31	BZ	118.8 ± 6.1; (1.00)	84.1 ± 0.001	96.2 ± 3.7
TC227	PD	161.6 ± 5.3; (0.98)	88.5 ± 7.1	102.0 ± 2.3

Enzyme activity is expressed as % TryS activity ± 2σⁿ⁻¹ and in brackets is provided the interference factor used to correct TryS activity of compounds affecting BIOMOL GREEN signal (see [S1 Text](#)). All values reported stem from at least triplicates. TcTryS, TbTryS and LiTryS are the trypanothione synthetase of *Trypanosoma cruzi*, *Trypanosoma brucei* and *Leishmania infantum*, respectively. ND, not determined.

^a the chemical scaffolds are: APPDA, 6-arylpyrido[2,3-*d*]pyrimidine-2,7-diamine derivative; BBHPP, 1-(benzo[*d*]thiazol-2-yl)-4-benzoyl-3-hydroxy-5-phenyl-1*H*-pyrrol-2(5*H*)-one derivatives; BZ, benzofuroxan derivatives; PD, 1*H*-purine-2,6(3*H*,7*H*)-dione derivative.

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Certain **BBHPP** (ADMRC150, ADMRC154, ADMRC159 and ADPKN164) showed specificity for targeting LiTryS with an average inhibitory activity of 35% at 30 μM and marginal or, even, enhancing activity of trypanosomal TryS ([Table 3](#) and [S2 Table](#)).

Under the assay conditions used for the screening, none of the **PD**, **AOCA** and singletons inhibited tri-tryp TryS to a significant level ([S6](#) and [S7 Tables](#)). In this respect, prochlorperazine has been early reported as a SP-competitive inhibitor of TbTryS with an IC₅₀ of 19 μM, when assayed at a subsaturating concentration of the competing substrate (i.e. 25 μM SP) [[18](#)]. To investigate the potential of prochlorperazine to target TryS from other species and validate their mode of inhibition, we tested the activity of this compound against different TryS and at different SP concentrations. At 2 mM SP, the estimated intracellular concentration of this polyamine in trypanosomatids [[58–60](#)], none of the TryS was inhibited by prochlorperazine added at 30 and 300 μM. Lowering the SP concentration in the assay to 0.24 mM SP, 300 μM prochlorperazine reduces the activity of TbTryS, TcTryS and LiTryS by 58%, 34% and 0%, respectively. On the one hand, this result show that high intracellular concentrations of prochlorperazine have to be reached to impair T(SH)₂ synthesis to a significant level and, on the other hand, highlights that more stringent assay conditions, as those representing nearly physiological substrate concentrations, will avoid the detection of hits with low pharmacological potential.

The screening also allowed the identification of compounds that increased 16–69% the activity of the trypanosomal TryS (i.e. for TbTryS: 7 **BBHPP** and 2 **BZ** and for TcTryS the **APPDA** ZVR159 and the **PD** TC227) ([Table 3](#)). Strikingly, none of the compounds tested here produced a similar stimulatory effect on the activity of the leishmanial enzyme. In order to shed light on the mechanism underlying the activation of TryS, the most active compounds were tested against trypanosomal TryS in the presence of different combinations of co-substrates. As shown in [S10 Table](#), enhancement of TryS ATPase activity by non-chemically related compounds (ZVR159, TC227 and ADPKN165) was detected only when both co-substrates, GSH and SP, were present in the reaction mixture. These results are suggestive of an allosteric mechanism of TryS activation.

Biological activity of compounds inhibiting TryS from African trypanosomes and *L. infantum*

The biological activity of the most active inhibitors of *TbTryS* (i.e. $IC_{50} \leq 30 \mu M$; MOL2008, ZEA10, EAP1-47, EAP1-63, APC1-89, APC1-99, APC1-101, APC1-111) and *LiTryS* (i.e. $IC_{50} < 1 \mu M$; MOL2008 and FS-554) was evaluated against the infective form of *T. b. brucei* or promastigotes of *L. infantum* and murine macrophages. Except for the paullone MOL2008 ($EC_{50} 4.3 \pm 0.7 \mu M$), all other *TbTryS* inhibitors presented anti-*T. b. brucei* activity in the nM range. The most active being the **BDA** APC1-99 ($EC_{50} 15 \pm 1$ nM), APC1-111 ($EC_{50} 40 \pm 1$ nM), APC1-89 ($EC_{50} 61 \pm 1$ nM) and EAP1-63 ($EC_{50} 90 \pm 7$ nM), followed by ZEA10, EAP1-47 and APC1-101 with EC_{50} between 200–280 nM (Table 2). In comparison to paullone FS-554 ($EC_{50} 112.3 \pm 1.1 \mu M$) [20], MOL2008 displayed a 10-fold higher anti-leishmanial activity ($EC_{50} 12.6 \pm 1.6 \mu M$).

The selectivity of the anti-parasitic effect was assessed using murine macrophages. Most compounds, except for MOL2008, FS-554 and ZEA 10, presented a selectivity index (SI) ≥ 10 with EAP1-63 (SI = 124) and APC1-89 (SI = 164) having the highest selectivity towards blood-stream *T. b. brucei* (Table 2).

In order to get an insight into the on-target activity of promising compounds, the intracellular thiol content of WT parasites exposed for 24 h to EAP1-47 and MOL2008 was determined. For bloodstream *T. b. brucei* treated with 50 nM EAP1-47, $T(SH)_2$ content decreases by 28% while GSH level increases by 39% with respect to untreated cells, which overall resembles the metabolite changes observed for parasites with 48 h RNAi-downregulated expression of TryS (S11 Table and [16]). A compound acting on $T(SH)_2$ metabolism is expected to display an increased cytotoxicity against parasites depleted in TryS. The cytotoxic effect of 100 nM EAP1-47 towards trypanosomes with a TryS content that is 1/3 of that corresponding to non-induced RNAi or wild-type cells was slightly increased (1.3-fold; Fig 5). For comparison, nifurtimox, a drug inducing thiol depletion [73], added at 5 μM (EC_{50} determined for WT cells) displayed a 1.8-fold increased potency towards TryS-depleted parasites. Strikingly, identical assays performed with the chemically-related and trypanosome-selective compounds EAP1-63 and APC1-99 did not show differences in the potency of these compounds towards WT or TryS-depleted parasites. Taking together, these results shows that EAP1-47 is interfering with $T(SH)_2$ biosynthesis, although the almost two order of magnitude difference between *TbTryS* inhibition and EC_{50} indicates that the compound has also other molecular targets *in vivo*. In contrast, the lack of enhanced cytotoxicity displayed by EAP1-63 and APC1-99 towards the TryS-RNAi induced cell line, is an strong indication that their potent trypanocidal activity is unrelated to interference with $T(SH)_2$ metabolism.

On the other hand, *L. infantum* promastigotes in the log growth phase exposed to MOL2008 at its EC_{50} (12 μM) for 24 h presented a marked decrease in the intracellular pool of thiols. From two independent experiments, $T(SH)_2$ and GSH content decreases $\geq 90\%$ and $> 30\%$, respectively, upon MOL2008 treatment (S11 Table). Interestingly and at variance with the metabolic changes observed for the genetic (RNAi) or chemical (EAP1-47) silencing of TryS in *T. b. brucei*, inhibition of TryS by MOL2008 did not lead to accumulation of the substrate GSH in *L. infantum*. Further experiments are required to establish whether the reduction on GSH level is caused by paullone-mediated inhibition of any of the two ATP-dependent enzymes in charge of glutathione synthesis or is a consequence of a species-specific regulatory mechanism triggered by $T(SH)_2$ depletion.

Discussion

The screening of a compound library consisting of 7 major chemical scaffolds and several singletons against TryS from three major pathogenic species of trypanosomatids at near

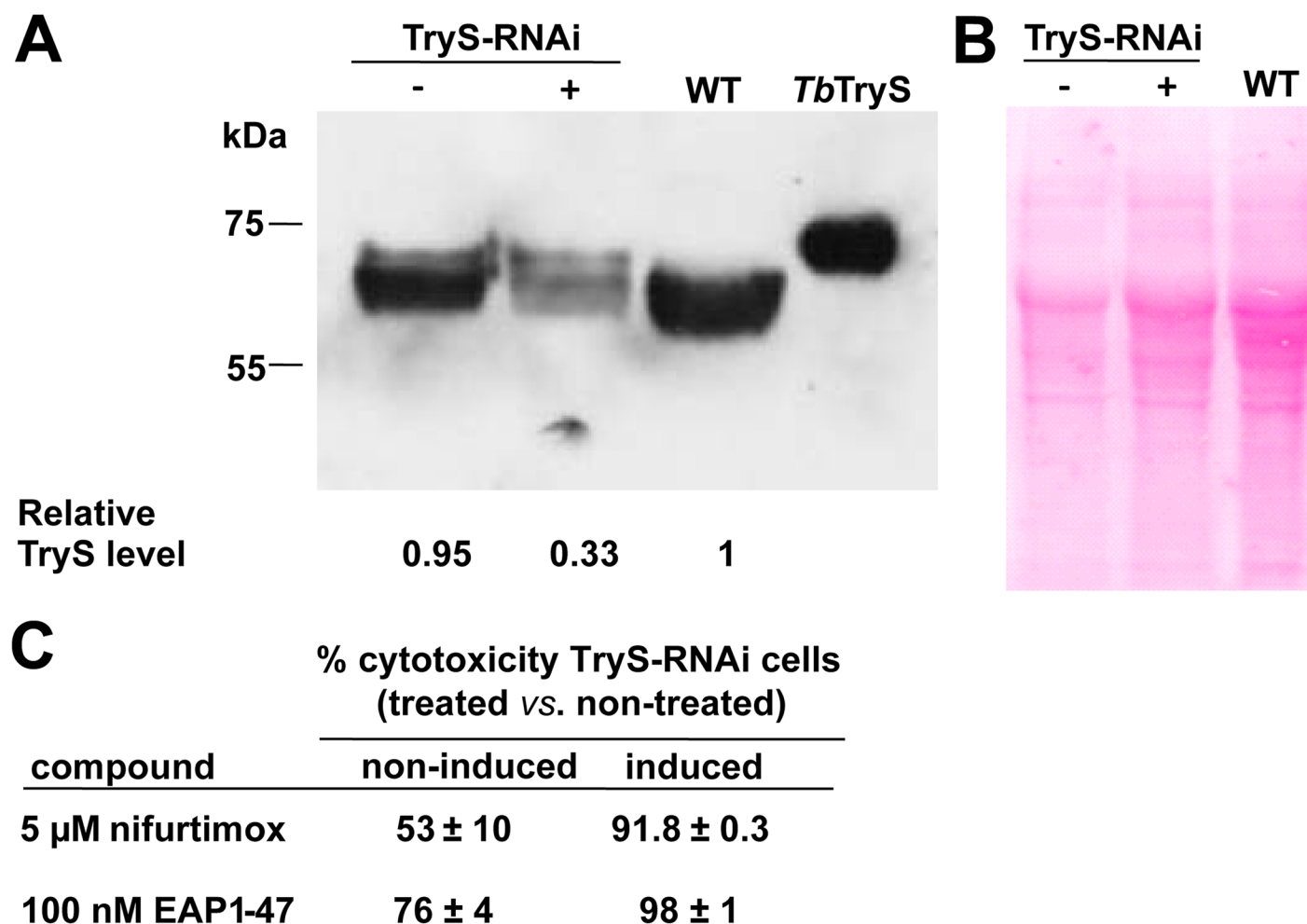


Fig 5. Biological activity of compounds against infective *Trypanosoma brucei brucei* with downregulated expression of trypanothione synthetase (TryS). **A)** Western blot analysis of cell extracts from 2×10^7 *T. b. brucei* from the wildtype (WT), 48 h tetracycline-induced (+) and non-induced (-) TryS-RNAi cell line. Two hundred ng of recombinant TbTryS was loaded as control. Bands from the molecular weight marker are indicated on left. The picture at the bottom shows the abundance of TryS for each condition as estimated by densitometry and expressed relative to the level of the WT cell line. **B)** Ponceau staining of the Western blot membrane that served as normalization control of protein load for each condition. **C)** Cytotoxicity (%) $\pm$ S.D. (n = 2) for tetracycline-induced (+) and non-induced (-) TryS-RNAi *T. b. brucei* treated with 5 μ M nifurtimox or 100 nM EAP1-47.

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physiological concentration of substrates led to the identification of 15 inhibitor molecules with μ M (APPDA, BDA, BZ, AI) and sub- μ M (AI) potency and a remarkable species-specificity for the molecular target. Despite the high sequence identity between tri-tryp TryS (e.g. the amino acid sequence identity is 71.6% for TbTryS/TcTryS, 64.7% for TbTryS/LiTryS and 63.4% for TcTryS/LiTryS) and the almost strict conservation of residues involved in substrate binding [20, 21], only 4 compounds were able to target TryS from different species, with only one of them (a BDA) inhibiting all three TryS with moderate potency (50% at 30 μ M). This behavior highlights the existence of structural differences between tritryp TryS that determine their specificity for ligands. In support of this observation, single amino acid substitutions in the homologue enzyme from *C. fasciculata* were previously reported to produce drastic changes in enzyme activity [23, 36] and our comparative analysis of the kinetic parameters of tritryp TryS obtained under similar assay conditions reveals considerable differences between species.

Early investigations have shown that substitutions at different positions of the paullone scaffold yield nM inhibitors of TryS from *C. fasciculata* or *L. infantum* (N^5 -substituted **AI**) [10, 20] and potent anti-*T. b. brucei* agents with moderate inhibition towards *TbTryS* (11-substituted 4-azapaullone) [56]. By extending the analysis of **AI** activity towards tritryp TryS, we have confirmed that the inclusion of a *N*-[2-(methylamino)ethyl] acetamide in position N^5 confers SP-competitive inhibition of *LiTryS* activity, since compounds lacking this substitution were almost inactive. From the tritryp TryS evaluated here, *LiTryS* presented the higher K_M for SP, which indicates the enzyme binds less efficiently this substrate probably due to an unfavorable conformation of the polyamine binding site. However, the shape adopted by the SP-binding site of *LiTryS* appears suitable for accommodating the N^5 -substituent present in MOL2008 and FS554. In support of this hypothesis, the trypanosomal TryS displayed a 2- to 6-fold lower K_M values for SP and a marked refractoriness to inhibition by N^5 -substituted paullones (> 200 -fold higher IC_{50}). The opposite behavior was observed for prochlorperazine, a SP-competitive inhibitor of *TbTryS* [18]. In this case, the degree of enzyme inhibition was inversely proportional to the SP K_M value for each TryS, with *TbTryS* being the most sensitive to inactivation followed by *TcTryS*, whereas *LiTryS* was refractory to inactivation (300 μ M prochlorperazine) even at a sub- K_M concentration of SP (240 μ M vs. K_M of 1335 μ M). Altogether, these data strongly point to the existence of remarkable differences in the polyamine binding site of tritryp TryS that should be carefully considered for the design of new **AI** with multi-species activity. In this respect, our study also demonstrated that in spite of the potent anti-*T. b. brucei* activity [56], **AI** substituted with α,β -unsaturated carbonyl chains in position 9 or 11 should be disregarded as ligands for future optimization because of their null to marginal anti-TryS activity.

Both N^5 -substituted paullones identified as potent *LiTryS* inhibitors displayed an IC_{50} slope (Hill coefficient) below the unity (~ 0.35), which suggests binding of the inhibitor to non-equivalent binding pockets or to partitioning of the compounds into an inactive, less potent or inaccessible form at higher concentrations [74]. Since both paullones displayed good solubility in the concentration range tested (~ 1 nM–50 μ M) and produced 100% *LiTryS* inhibition, we ruled out insolubility or aggregation phenomena as responsible of this behavior. On the other hand, the dose-response plots do not show the presence of a second inflection point that would be indicative of a second binding pocket for paullones. TryS are monomeric but mechanistically complex (trisubstrate) enzymes [23, 24] whose substrate binding sites display large conformational changes during catalysis [21, 75, 76], hence, it is possible that the low Hill slopes reflect an equilibrium between two or more forms of the enzyme that interact differentially with the paullones. Further mechanistic studies are needed to shed light on this issue.

From this screening, MOL2008 was the most potent inhibitor identified for *LiTryS* that, in addition, shows potential for further optimization of its anti-trypanosomal TryS activity ($IC_{50} \sim 30$ μ M). The related paullone FS554 was chemically validated in a previous study using transgenic cell lines of *L. infantum* [20]. Here we show that MOL2008 is almost one order of magnitude more potent towards *L. infantum* promastigotes than FS554 and, more importantly, that it targets *in vivo* trypanothione biosynthesis. The future design of MOL2008 analogues should also aim at improving its biological properties, which according to our data are far from optimal (EC_{50} in the μ M range and $SI \leq 2$) and suggest off-target effects in host cells.

Precedent studies demonstrated that disubstituted polyamines are potent antiproliferative agents that interfere with the polyamine metabolism of Plasmodium and African trypanosomes [47–50]. On the basis that the substituted diamine moiety may eventually be recognized as ligand by TryS, a collection of simplified derivatives was screened for their anti-synthetase activity. Two **BDA** (EAP1-47 and APC1-111) displayed inhibitory activity, albeit moderate ($IC_{50} \sim 30$ μ M), against multiple TryS. SAR analysis revealed the need to fulfill a specific steric

demand on the enzyme interacting pocket. A rational optimization of these compounds seems difficult, since the screening performed here included a wide diversity of derivatives (mono- and di-substituted, with bulky groups or halogen atoms, and with linker of different length) that yielded only weak TryS inhibitors. Nevertheless, as in a fragment-based approach, certain moieties of the most active **BDA** can be selected as substituents of novel or known TryS inhibitors.

With few exceptions, most APC derivatives tested here were previously shown to display low to sub- μ M potency towards infective *T. b. brucei* [46]. Our study confirmed and disclosed the potent (EC_{50} 15–280 nM) and selective (SI = 10–164) anti-parasitic activity of APC analogues and of two halogenated **BDA** from the EAP series, respectively, all of which exerted a moderate inhibition of *TbTryS* (IC_{50} ~ 30 μ M). Interestingly, EAP1-47 induced changes in the intracellular pool of low molecular thiols that overall resembled those triggered by RNAi-mediated silencing of TryS [16] and displayed a moderate increase in its cytotoxicity towards TryS-deficient cells that was not paralleled by the more potent and selective molecules APC1-99 and EAP1-63. Nonetheless, as pointed out before, the higher *in vivo* (anti-trypansomal activity) vs. *in vitro* (*TbTryS* inhibition) activity of EAP1-47 suggests that this compound is targeting other essential molecular target(s) in addition to TryS.

The positive loop of substrate inhibition of TryS by GSH has a direct impact on the design of inhibitors. On the one hand, it suggests that groups competing with GSH can be excluded from the ligand scaffold to avoid unnecessary substrate competition and to reduce the molecular mass of the inhibitor. On the other hand, it shows that full inhibition of TryS is not required because the phenomenon of substrate inhibition will amplify *in vivo* the inactivation of the enzyme, which represents a pharmacological advantage of this molecular target.

The four **APPDA** identified as moderate inhibitors of, preferentially, the *T. cruzi* TryS, are structurally related to compounds originally designed as inhibitors of bacterial D-Ala:D-Ala ligase. Interestingly, these derivatives presented a 10-fold lower IC_{50} towards the trypanosomal enzyme than the analogue compounds against the corresponding bacterial target (best IC_{50} = 260 μ M) [43]. The **APPDA** may serve as primary scaffolds to develop analogues with improved anti-TryS activity and, consequently, superior selectivity index. *TcTryS*, and to minor extent *LiTryS*, was target of inhibition by a **BZ** substituted with an imidazolone. A **BZ** with an additional imidazolone group retained activity against *TcTryS* but enhanced ATP hydrolysis by *LiTryS* and *TbTryS*, indicating that these ligands present a molecular pattern recognized by the active site of the enzymes that should be further investigated.

Our work also disclosed several compounds enhancing ATP consumption by trypanosomal TryS. Adding value to the existence of distinctive structural features between trityp TryS, *TbTryS* was targeted specifically by large heterocyclic compounds from the **BBHPP** whereas *TcTryS* was the most promiscuous enzyme, being activated by a **PD**, an **APPDA** and the **BZ** mentioned above. A single compound, a bis-imidazolone **BZ**, increased *LiTryS* activity. According to our results, the activation of ATPase activity in trypanosomal TryS by these compounds takes place only when both co-substrates, namely GSH and SP, are present in the reaction. More detailed studies are required to establish whether such allosteric activation of TryS is paralleled by an increased production of T(SH)₂. Compounds promoting the non-productive consumption of ATP are attractive candidates for biological evaluation, because the metabolic outcome of their action should be similar to that of true enzyme inhibitors.

In summary, this study: (i) led to the identification and on-target validation of a potent inhibitor of leishmanial TryS and of chemical scaffolds that can be further developed into inhibitors of wide spectrum against TryS from different trypanosomatid species, (ii) highlights the existence of remarkable kinetic and structural differences between trityp TryS that prompt to obtain 3D structures of TryS from different trypanosomatids in order to guide a structure-

based rationale design of multi-TryS inhibitors; iii) demonstrates the relevance of running a HTS under near physiological concentration of substrates to minimize the possibility to detect weak competitive inhibitors with low potential to act *in vivo*

Supporting Information

S1 Fig. Kinetic characterization of tritryp trypanothione synthetase (TryS). The plots of velocity (v) / $[E](s^{-1})$ vs. different substrate or product concentrations (μM) are shown for: **A)** *Trypanosoma cruzi* trypanothione synthetase (*TcTryS*) with a.1) spermidine (SP), a.2) ATP, a.3) glutathione (GSH) and a.4) ADP; **B)** *Trypanosoma brucei* trypanothione synthetase (*TbTryS*) with b.1) SP; b.2) ATP; b.3) GSH, and b.4) ADP; **C)** *Leishmania infantum* trypanothione synthetase (*LiTryS*) with c.1) SP, c.2) ATP, c.3) GSH, and c.4) ADP. See [Materials and Methods](#), [S1 Text](#) and [S8 Table](#) for details about assay conditions. (TIF)

S2 Fig. Sensitivity and linearity of the end-point trypanothione synthetase (TryS) assay. In a total volume of 50 μL containing all assay reagents [150 μM ATP, 2 mM spermidine (SP), 250 μM glutathione (GSH), 5 mM DTT, 10 mM $MgSO_4$, 0.5 mM EDTA, 100 mM HEPES pH 7.4, 9 mM NaCl and 10% v/v DMSO] except TryS, K_2HPO_4 was added at concentrations ranging from 1.25 to 140 μM . Two hundred μL BIOMOL GREEN reagent were added per well and the colorimetric reaction was allowed to develop for 20 min. The absorbance at 650 nm was measured in each well using a MultiScan EX plate reader (Thermo SCIENTIFIC). The mean $A_{650\text{ nm corr}}$ values $\pm$ 2 S.D. BIOMOL GREEN signal is plotted against $[K_2HPO_4]$. A linear regression model based on the least square method was fitted for K_2HPO_4 concentrations from 0 to 50 μM . (TIF)

S3 Fig. Evaluation of interference with colorimetric reaction by assay' reagents. The corrected absorbance of BIOMOL GREEN at 650 nm ($A_{650\text{ nm corr}}$) is plotted against different concentrations of: **A)** DTT (mM) for a reaction containing 20 μM K_2HPO_4 in 150 μM ATP, 2 mM spermidine (SP), 250 μM glutathione (GSH), 10% v/v DMSO and 9 mM NaCl. The $A_{650\text{ nm corr}}$ is expressed as percentage relative to $A_{650\text{ nm corr}}$ at 5 mM DTT; **B)** SP (mM) for a reaction containing 20 μM K_2HPO_4 , 150 μM ATP, 2 mM SP, 250 μM GSH, 5 mM DTT, 10% v/v DMSO and 9 mM NaCl. The $A_{650\text{ nm corr}}$ is expressed as percentage relative to $A_{650\text{ nm corr}}$ at SP 2 mM; **C)** glycerol (%) for a reaction containing 20 μM K_2HPO_4 , 150 μM ATP, 2 mM SP, GSH 250 μM , 5 mM DTT and 9 mM NaCl. The $A_{650\text{ nm corr}}$ is expressed as percentage relative to $A_{650\text{ nm corr}}$ at 0% v/v glycerol; **D)** GSH (mM) for a reaction containing 20 μM K_2HPO_4 , 150 μM ATP, DTT 5 mM, 10% v/v DMSO, 9 or 30 mM NaCl, and 0 or 4% v/v glycerol. The $A_{650\text{ nm corr}}$ is expressed as percentage relative to $A_{650\text{ nm corr}}$ at 0% v/v glycerol, 0 mM NaCl and 2.5 mM GSH (black squares), 4% v/v glycerol and 0 mM NaCl (red circles), 4% v/v glycerol and 30 mM NaCl (blue triangle) and 4% v/v glycerol and 9 mM NaCl (green triangle). (TIF)

S4 Fig. Time-dependent linearity of the screening assay. Pi production ($A_{650\text{ nm corr}}$) was monitored at different time points (min) for recombinant *Leishmania infantum* trypanothione synthetase, *LiTryS* (a, b), *Trypanosoma cruzi* trypanothione synthetase, *TcTryS* (c, d) and *Trypanosoma brucei* trypanothione synthetase, *TbTryS* (f, g). The assays were conducted at RT in a total reaction volume of 50 μL containing 150 μM ATP, 2 mM SP, 5 mM DTT, 10 mM $MgSO_4$, 0.5 mM EDTA, 100 mM HEPES pH 7.4, 10% v/v DMSO and 9 mM NaCl, with variable concentrations of glutathione (GSH) and enzyme units according to the TryS species: 250 μM GSH and $2.3 \times 10^{-5} \mu mol.min^{-1}.mL^{-1}$ for *LiTryS*, 570 μM GSH and $3.5 \times 10^{-6} \mu mol.min^{-1}.mL^{-1}$ for *TcTryS*, and 50 μM GSH and $1.5 \times 10^{-5} \mu mol.min^{-1}.mL^{-1}$ for *TbTryS*. Blanks lacking enzyme

were prepared for each condition. The TryS reaction was stopped at different time points by adding 200 μ L BIOMOL GREEN reagent. The plates were incubated for 20 min at RT and $A_{650\text{ nm}}$ measured using a MultiScan EX plate reader (Thermo SCIENTIFIC). The mean $A_{650\text{ nm corr}} \pm 2$ S.D. of BIOMOL GREEN signal is plotted vs. time. A linear regression model based on the least square method was applied to each plot to estimate the linearity range.

(TIF)

S5 Fig. Activity profile for *N,N'*-bis(3,4-substituted-benzyl) diamine derivatives (BDA). All compounds were evaluated at 30 μ M against *Trypanosoma cruzi* trypanothione synthetase, *TcTryS* (*Tc*), *Leishmania infantum* trypanothione synthetase, *LiTryS* (*Li*) and *Trypanosoma brucei* trypanothione synthetase, *TbTryS* (*Tb*). TryS activity is expressed as percentage (see [S3 Table](#)). “n” denotes the number of carbons in the linker between both nitrogens. **A)** Oxygen substituted (OMe: methoxy, OBn: O-benzyl, OH: hydroxyl) **BDA** with the number indicating the position of the substitution in the phenyl ring. **B)** Halogenated (F, Cl and Br) and alkylated (iPr: isopropyl) **BDA**.

(TIF)

S1 Table. APPDA, 6-arylpyrido[2,3-*d*]pyrimidine-2,7-diamine derivatives.

(DOCX)

S2 Table. BBHPP, 1-(benzo[*d*]thiazol-2-yl)-4-benzoyl-3-hydroxy-5-phenyl-1*H*-pyrrol-2(5*H*)-one derivatives.

(DOCX)

S3 Table. BDA, *N,N'*-bis(3,4-substituted-benzyl)diamine derivatives.

(DOCX)

S4 Table. BZ, benzofuroxan derivatives.

(DOCX)

S5 Table. AI, 4,5-dihydroazepino[4,5-*b*]indol-2(1*H*,3*H*,6*H*)-one derivatives, 4-azapaullones derivatives.

(DOCX)

S6 Table. PD, 1*H*-purine-2,6(3*H*,7*H*)-dione derivatives.

(DOCX)

S7 Table. AOCA, 2-aminooxazole-5-carboxamide derivatives.

(DOCX)

S8 Table. Kinetic characterization conditions for recombinant TryS.

(DOCX)

S9 Table. SP-competitive inhibition of *LiTryS* by MOL2008.

(DOCX)

S10 Table. Characterization of compounds enhancing tritryp trypanothione synthetase (TryS) activity.

(DOCX)

S11 Table. Analysis of intracellular thiols in bloodstream *T. b. brucei* and *L. infantum* promastigotes.

(DOCX)

S1 Text. Supporting Information.

(DOCX)

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Author Contributions

Conceived and designed the experiments: MAC DB. Performed the experiments: DB LFi AM. Analyzed the data: MAC DB GRL. Contributed reagents/materials/analysis tools: EAPZ FM KCP MR TC AD TJ JŠ LPM CK GRL LFI. Wrote the paper: MAC DB.

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