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Recent Advancement in the Search of Innovative Antiprotozoal Agents Targeting Trypanothione Metabolism

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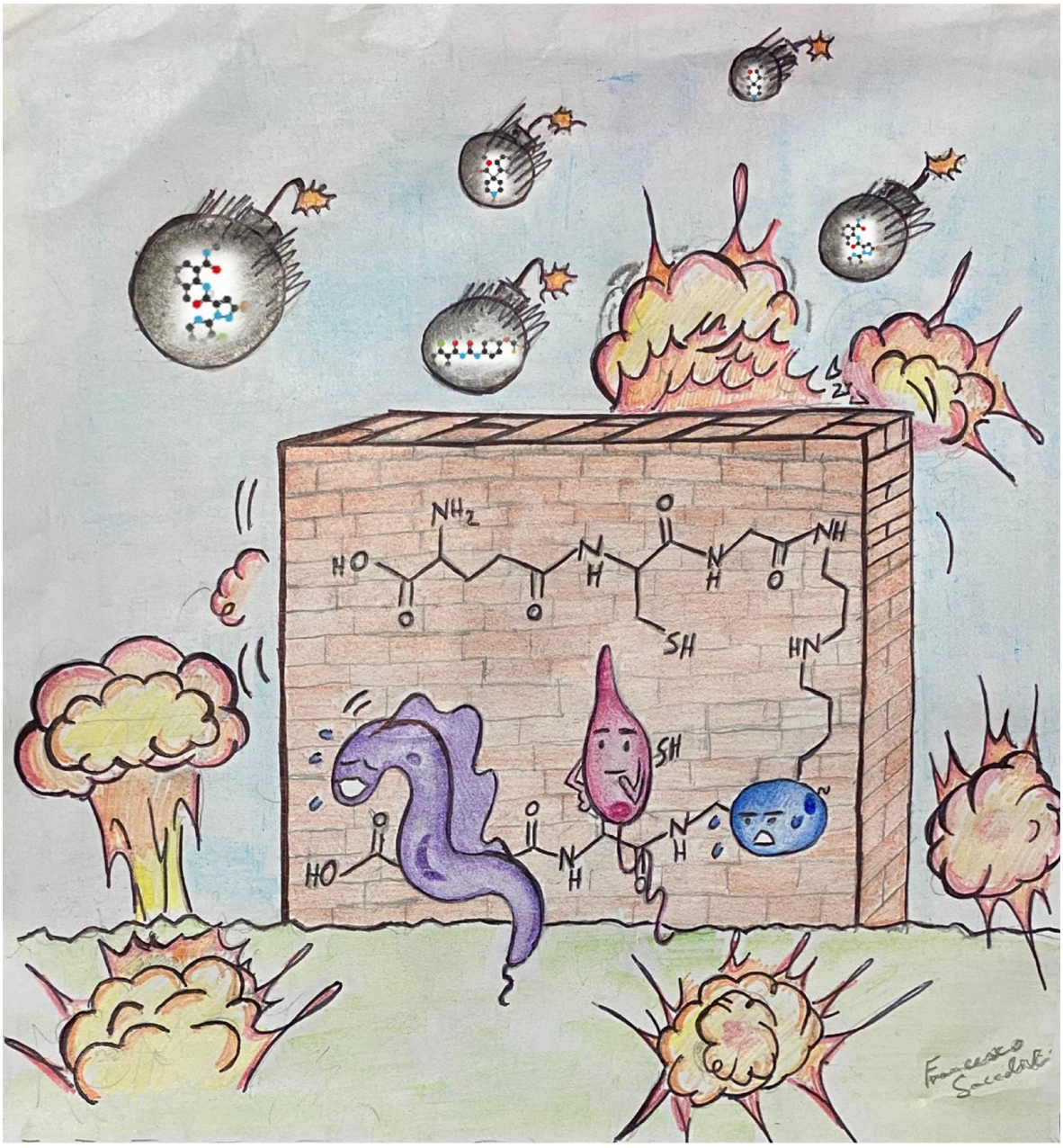

Leishmania and *Trypanosoma* parasites are responsible for the challenging neglected tropical diseases leishmaniases, Chagas disease, and human African trypanosomiasis, which account for up to 40,000 deaths annually mainly in developing countries. Current chemotherapy relies on drugs with significant limitations in efficacy and safety, prompting the urgent need to explore innovative approaches to improve the drug discovery pipeline. The unique trypanothione-based redox pathway, which is absent in human hosts, is vital for all trypanosomatids and offers valuable opportunities to guide the rational development of specific, broad-spectrum and innovative anti-trypanosomatid agents. Major efforts focused on the key metabolic enzymes trypanothione synthetase-amidase and trypanothione reductase, whose inhibition should affect the entire pathway and, finally, parasite survival. Herein, we will report and comment on the most recent studies in the search for enzyme inhibitors, underlining the promising opportunities that have emerged so far to drive the exploration of future successful therapeutic approaches.

1. Introduction

The vector-borne protozoan diseases leishmaniases, Chagas disease (CD) and human African trypanosomiasis (HAT), referred to as trypanosomatid diseases, are leading causes of morbidity and mortality in developing countries accounting for up to 40,000 deaths annually.[1,2,3]

Among the several *Leishmania* species causing different forms of leishmaniasis, *Leishmania infantum* (*L. infantum*) and *Leishmania donovani* (*L. donovani*) parasites are responsible for visceral leishmaniasis, a potentially fatal disease whose co-infection with HIV is an increasing global health concern.[1]

CD is caused by *Trypanosoma cruzi* (*T. cruzi*), which affects up to 7 million people worldwide; the disease is found mainly in endemic areas of 21 continental Latin American countries,[2a] despite being a growing threat for other countries. After acute stage, patients remain chronically infected and may develop life-threatening conditions, including cardiomyopathy, several years after the first infection.[2,4]

Trypanosoma brucei (*T. brucei*) subspecies *gambiense* and *rhodesiense* are responsible for two forms of disease (g-HAT and r-HAT, respectively) in different African countries. Despite its lower prevalence, r-HAT is a more acute and aggressive disease than g-HAT. Major symptoms are detected in the second phase of infection in which trypanosomes reach the central nervous system, causing neurological alterations, coma and death.[3]

Drugs employed for the treatment of these diseases include pentavalent antimonials, amphotericin B, miltefosine and paromomycin for leishmaniases; benznidazole and nifurtimox for CD; suramin, pentamidine, melarsoprol, eflornithine and, more recently, fexinidazole for HAT (Figure 1).[1–5]

However, most of these agents are far from ideal and suffer from major limits of efficacy, toxicity, administration route and drug resistance.[1–4]

In general, despite being competent against the mildest or acute infections, available drugs result less effective in curing the most challenging and life-threatening forms of diseases. This is the case of the antichagasic drugs benznidazole and nifurtimox, whose efficacy against chronic CD in adults is still debated.[2c,4]

Moreover, the long-term use of these drugs has enhanced the onset of resistant parasitic strains; consequently, the efficacy of many of these medications has dramatically dropped over the years and results variable in different geographical regions. The most prominent example is provided by pentavalent antimonials, which are no longer deemed efficacious against *Leishmania* parasites in the state of Bihar (India).[1d,4]

Importantly, many treatments require parenteral administration, long-course regimens and hospitalization for careful monitoring of patients. Indeed, host toxicity is a common and relevant feature of these drugs and side effects occurring during therapy may prompt treatment discontinuation, as happens with melarsoprol (the only choice for second-stage r-HAT), which causes highly lethal reactive encephalopathy.[3,4]

Despite their drawbacks, these drugs currently represent the first-choice options for the treatment of trypanosomatid diseases, reflecting the lack of more suitable alternatives and the limited researches devoted to this field over the last century.[4]

However, the situation dramatically changed in the last two decades and drug discovery efforts in this area have increased.

Indeed, in recent years, various public–private partnerships, academic institutions and pharmaceutical companies become more engaged in the search of new therapeutic options,[4,6] as highlighted by the recent approval of fexinidazole (the first all-oral drug for g-HAT) and pre-clinical development of new candidates.[5,7,8]

According to this impetus, increasing efforts must be done to face the challenge properly.[4,8] Although many related pathological features remain still obscure, increasing understanding of the parasites' biology allowed the identification of peculiar metabolisms of pathogens which can be exploited as drug targets.[4a,b]

In this context, exploration of specific and ubiquitous pathways of trypanosomatids is highly attractive to drive rationally the drug discovery pipeline and exploit the prominent opportunity to affect multiple parasitic diseases.[9]

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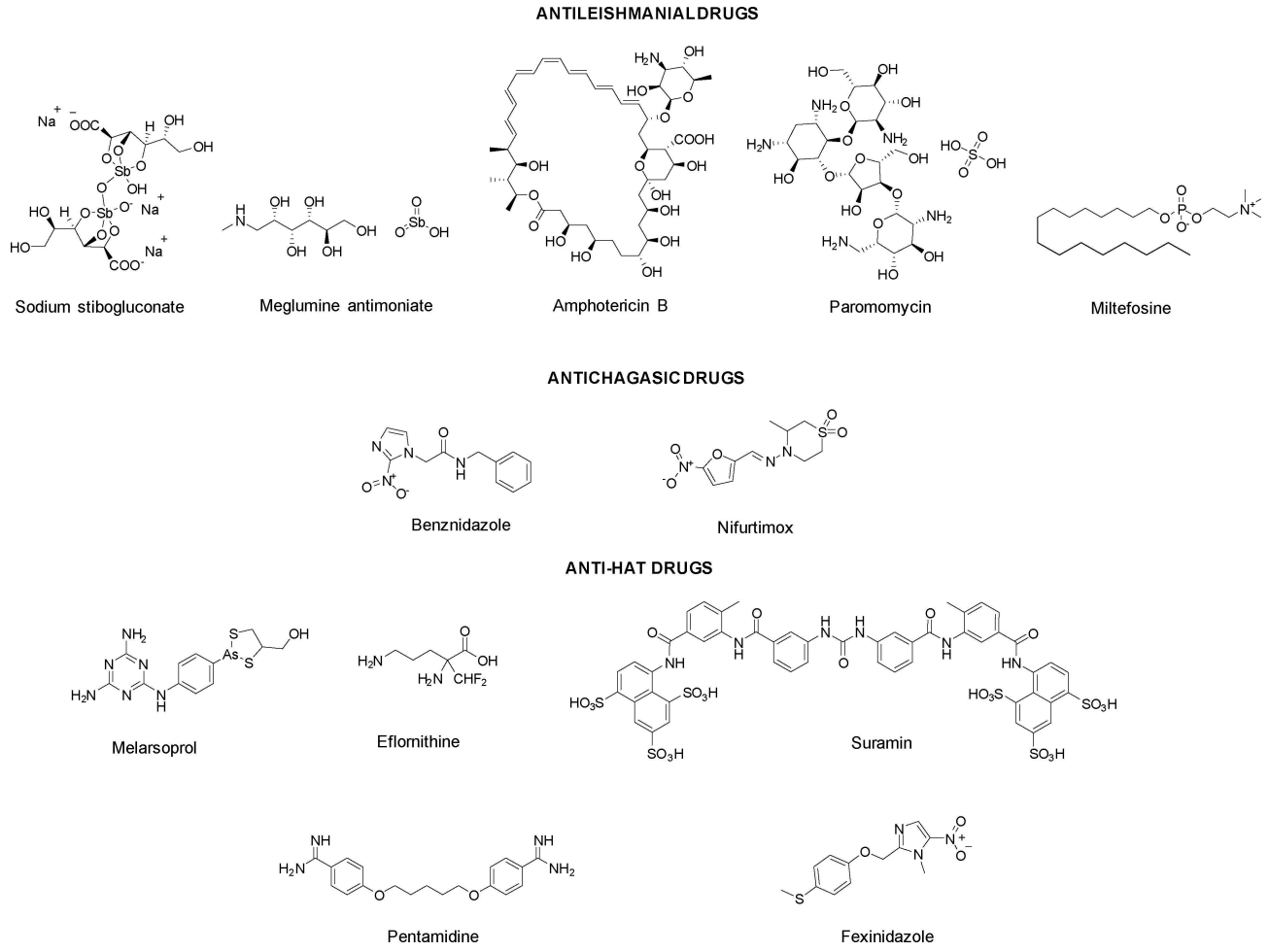
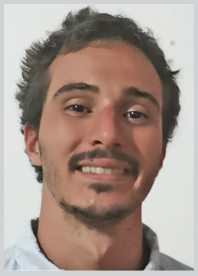


Figure 1. Structures of antileishmanial, antichagasic and anti-HAT drugs in current use.



Francesco Saccoliti graduated in Pharmacy with Honors (2014) and obtained a PhD in Life Sciences with Honors (2019) at University of Rome "La Sapienza". He defended a thesis focused on the design and synthesis of new antiprotozoal agents targeting trypanothione reductase and CYP51 enzymes. He was involved in the design and synthesis of antiviral, anticancer and antimicrobial agents in Prof. Di Santo's group. He currently works at Istituto Italiano di Tecnologia (Genoa) on the design and synthesis of new antitumor agents.

Roberto Di Santo received his MS (Honors) in Chemistry (1986) then in Pharmacy (1994) from University of Rome "La Sapienza". After fellowship from Pasteur Institute in 1988, he started his carrier in "Sapienza" University having a position as researcher (1990) than Associated Professor in 1998 and finally as Full Professor in Medicinal Chemistry in 2016. His research interests are in the areas of medicinal chemistry with focus on drug design and development of antimicrobial, antitumor agents and compounds active on CNS.

Roberta Costi received her MS in Chemistry and Technology of Drug in 1991 (Honors) and in Pharmacy in 1994 (Honors) from University of Rome "Sapienza". She obtained a PhD in 1994 and became Associate Professor (2005) in Medicinal Chemistry at University of Rome "Sapienza". She is the author of more than 100 scientific publications and patents in the field of antimicrobial, antitumor and CNS agents, as well as extraction and purification of natural compounds useful as antimicrobial agents and recovery of food waste and circular bioeconomy.

1.1. Trypanothione metabolism

Differently from humans, trypanosomatids possess a unique thiol-based redox metabolism relying on the glutathione (GSH) analog trypanothione ($T(SH)_2$).

As the major redox reactive metabolite, $T(SH)_2$ takes part in a myriad of enzymatic and non-enzymatic reactions by shuttling electrons to various acceptors involved in key processes, such as peroxide detoxification and DNA synthesis, upon which parasite survival rely. According to these, $T(SH)_2$ is central for all trypanosomatids and plays a key role in defense against oxidative damage, redox homeostasis and replication (Figure 2).

Overall, the $T(SH)_2$ pathway importantly sustains infectivity and survival in host system, while, conversely, disruption of such metabolism should increase susceptibility of parasites toward drugs- and/or host defense-induced oxidative stress.

Owing to critical character in trypanosomatids and absence in humans, the $T(SH)_2$ pathway offers exceptional chances for the development of drugs able to hinder selectively protozoan survival without affecting host. Moreover, as a peculiar system of all trypanosomatids, targeting key enzymes of $T(SH)_2$ metabolism may promote the treatment of many related diseases by acting on a common pathway.[9–11]

Major efforts in this field focused on metabolic enzymes trypanothione synthetase-amidase (TSA) and trypanothione reductase (TR); both are critical proteins involved in synthesis and regeneration of TSH_2 respectively, importantly these contribute to the maintenance of steady-state levels of TSH_2 and the establishment of an intracellular reducing environment. Impairment of TSA and TR, where druggability in pathogenic trypanosomatids has been demonstrated, could affect the entire $T(SH)_2$ system and represents an attractive goal in the search of innovative antiprotozoal agents.[9,11]

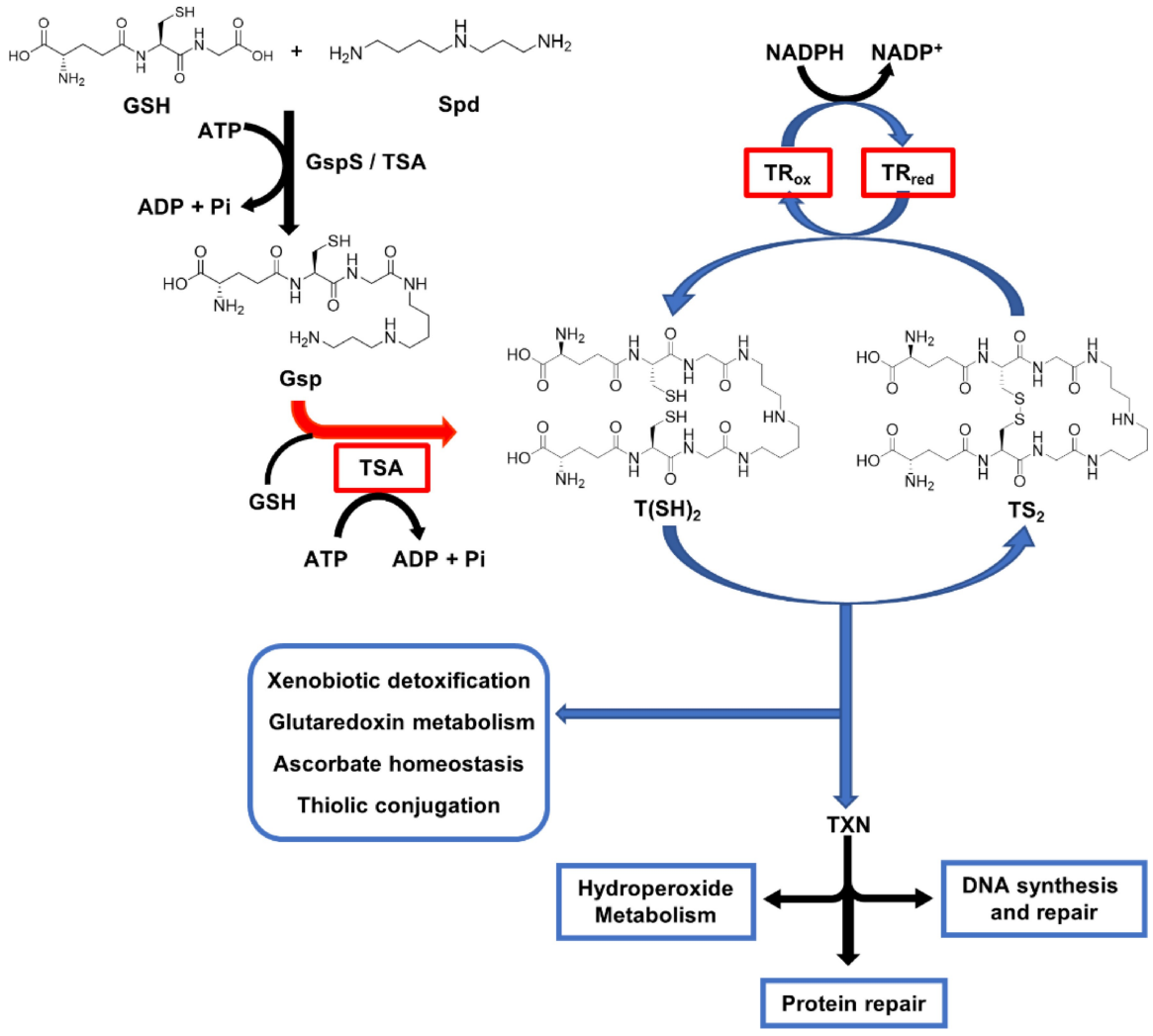


Figure 2. Schematic overview of trypanothione metabolism. Synthesis of trypanothione ($T(SH)_2$) from glutathione (GSH) and spermidine (Spd) via glutathionyl-spermidine (Gsp) intermediate is accomplished in two steps, the first driven by glutathionyl-spermidine synthetase (GspS) or trypanothione synthetase-amidase (TSA) and the second catalyzed exclusively by TSA. $T(SH)_2$ is involved in multiple functions such as detoxification of xenobiotics, DNA synthesis and defense against oxidants, by providing reducing equivalents to different molecular partners (e.g. Tryparedoxin, TXN). Trypanothione reductase (TR) regenerates $T(SH)_2$ from trypanothione disulfide (TS_2) at expenses of NADPH.

$T(SH)_2$ is synthesized in two consecutive ATP-dependent steps involving conjugation of spermidine (Spd) with two molecules of GSH. Ligation of Spd to glycine carboxyl moiety of GSH yields a glutathionylspermidine intermediate (Gsp), which, in turn, reacts with a second molecule of GSH to form $T(SH)_2$ (Figure 2).[10,11]

Depending on trypanosomatid species, the first step can be catalyzed by glutathionylspermidine synthetase (GspS, EC: 6.3.1.8) or TSA (EC: 6.3.1.9), while the second reaction is driven exclusively by TSA. According to these, only TSA is ubiquitous along trypanosomatids, while gene coding GspS is either missing or dysfunctional in some pathogens. Additionally, parasites equipped with both enzymes proved to rely entirely on TSA for $T(SH)_2$ biosynthesis irrespective of the presence of GspS.[10b,c,12]

This is the case of *L. infantum*, for which gene-targeting studies demonstrated that only TSA is essential for survival of both promastigotes and amastigotes, while elimination of gene coding GspS had no relevant impact on parasite.[12]

The critical role of TSA for viability has been demonstrated for pathogenic trypanosomatids *T. brucei*,[13,14] *L. infantum*[12] and *T. cruzi*[15] by means of reverse genetic and chemical inhibition approaches.

The blockage of such enzyme impairs $T(SH)_2$ biosynthesis, causing a decrease in the levels of $T(SH)_2$ and accumulation of GSH. Since GSH is unable to take over $T(SH)_2$ functions in trypanosomatids, these events cause growth arrest, impaired antioxidant capacity and infectivity, and, finally, cell death.[9,10]

The key role played in $T(SH)_2$ synthesis, which cannot be accomplished differently, together with its absence in humans, low-abundance and, finally, dominant role in thiol-based metabolism render TSA a very promising drug target.[9,10]

TSA is a bifunctional enzyme, which, besides acting as synthetase, also harbors amidase activity to convert Gsp and $T(SH)_2$ back to substrates. The two enzymatic functions reside in two distinct domains, a *C*-terminal synthetase domain and an *N*-terminal amidase domain.[16] Among them, the former is the one that predominates[16] and proved indispensable for *T. brucei* survival,[13a] hence attractive in the search of TSA inhibitors (TSAIs).

The X-ray structure of TSA from *Leishmania major* has been solved (*L. major* TSA, *Lm*TSA, PDB ID: 2VPS), which, combined with docking and molecular dynamic studies, provided important biochemical insights into substrate binding and catalysis.[16,17] However, none TSAI has been co-crystallized within *Lm*TSA so far.

Despite the high concentrations of $T(SH)_2$ in parasites, the use of such dithiol as reducing agent causes a decrease in its levels and accumulation of oxidized disulfide form TS_2. Consequently, the maintenance of intracellular pool of $T(SH)_2$ steady relies not only on biosynthesis, but also on a notable system devoted to recycle $T(SH)_2$.

By shuttling reducing equivalents from NADPH to TS_2, trypanothione reductase (EC: 1.8.1.12) drives regeneration of $T(SH)_2$ from its oxidized disulfide form TS_2 (Figure 2).

As the sole enzyme connecting NADPH to thiol-based redox systems of trypanosomatids, TR acts as key enhancer of the whole $T(SH)_2$ metabolism and, thus, represents a prominent target in the search of innovative antiprotozoal agents.[9–11]

TR has been validated as a drug target in *L. donovani* and *T. brucei*. In particular, all attempts to obtain a TR-null mutant in *L. donovani* failed and the mutants, displaying a partial trisomy of TR locus, where two TR alleles were disrupted by gene targeting, showed attenuated infectivity and decreased ability to survive within macrophages.[18a,b]

Similarly, depletion of TR to less than 10% of wild-type level produced *T. brucei* parasites unable to infect mice.[18c]

The role played by TR in parasites' redox pathway resembles that of human homolog glutathione reductase (GR, EC: 1.8.1.7) in keeping glutathione disulfide (GSSG) in its thiol form (GSH). Besides being NADPH-dependent disulfide oxidoreductases, these enzymes display structural analogies and similar catalytic mechanisms involving redox-active cysteine residues and a histidine-glutamate couple (His461'-Glu466' in TR). Both enzymes are two-fold symmetrical homodimers with each monomer bearing one active site and three different domains (FAD-binding domain, NADPH-binding domain and interface domain).[9–11]

Despite these, enzymes display nearly 40% sequence identity and, importantly, mutually exclusive specificity towards their disulfide substrates, which differ in terms of size and charge.

Indeed, while GSSG is a smaller substrate carrying a net charge of -2, TS_2 is a more hydrophobic and bulkier ligand bearing a net charge of $+1$ at physiological pH. These features, in turn, reflect steric and electrostatic differences between respective active sites, which are importantly affected by the replacement of five residues of GR (Ala34, Arg37, Ile113, Asn117 and Arg347) by Glu18, Trp21, Ser109, Met113 and Ala343 in TR (*L. infantum* numbering). In particular, the first four residues constitute a hydrophobic cleft within TR active site in which Glu18 introduces a net negative charge promoting binding of the positively charged substrate.[9,11,19–21]

This hydrophobic groove, which is involved in TS_2 binding, was shown to be occupied by mepacrine, one of the earliest selective TR inhibitors (TRIs) identified and the first that has been co-crystallized within TR. According to these, this cleft was tagged "mepacrine binding site" (MBS) and has been widely targeted in the search of new TRIs.[21–23]

An additional hydrophobic region of TR active site not involved in TS_2 binding has been explored to design inhibitors with improved enzymatic affinity. This cleft, named Z-site, is mainly formed by Phe396', Pro398' and Leu399' (*L. infantum* numbering) and located nearby the central glutamate residues (Figure 3).[20,21,23]

Overall, the active site of TR is much wider, hydrophobic and negatively charged than that of GR, underlining the opportunity to develop selective inhibitors of parasites' enzymes. Moreover, the nearly 100% sequence similarity of active site residues and high to very high overall identity between various parasites' TRs suggests that targeting such protein may represent a viable strategy for the treatment of diverse trypanosomatid diseases.[9b,20,21]

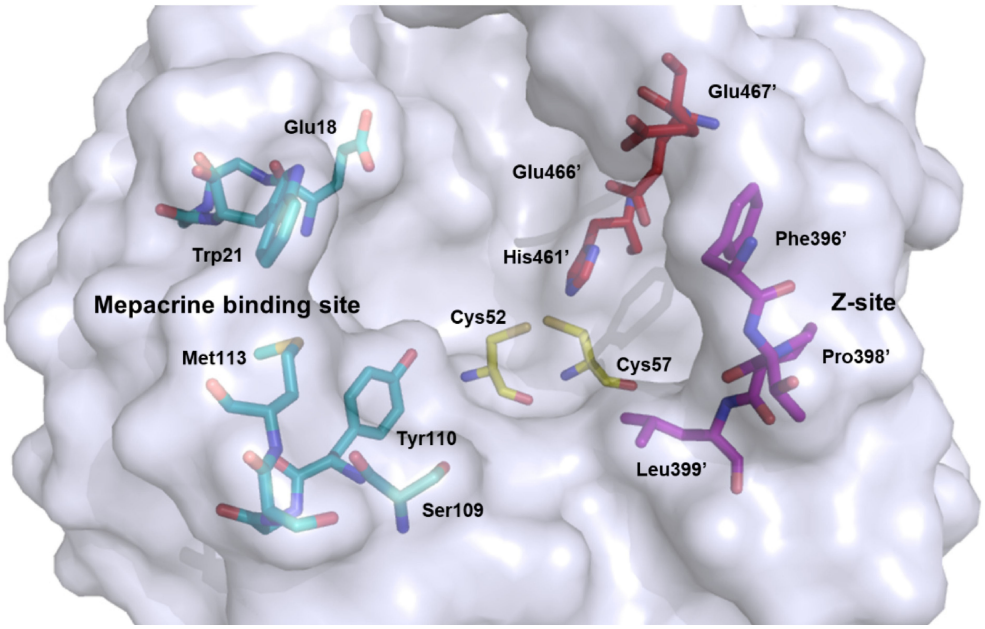


Figure 3. Overview of *Li*TR active site (PDB ID: 2JK6). Residues are represented as sticks and highlighted in cyan (Mepacrine binding site), purple (Z-site), yellow (catalytic cysteines) and red (central glutamates and histidine). The picture was obtained using PyMOL (The PyMOL Molecular Graphics System, Version 2.2.0 Schrödinger, LLC).

The critical role played by TSA and TR within the $T(SH)_2$ metabolism has prompted the search of several enzyme inhibitors as notable tools to disrupt the peculiar thiol-based redox system of parasites and, thus, provide innovative and more suitable anti-trypanosomatid agents.[9–11]

In the following sections we will review the main efforts in the search of TSA and TR inhibitors from a medicinal chemistry point of view, focusing mainly on the advancements and opportunities emerged in recent years.

2. Trypanothione synthetase-amidase inhibitors (TSAIs)

Before structural and functional elucidation of TSA, initial efforts in the search of compounds hindering TSH_2 biosynthesis focused on the design of GSH analogues (peptides and pseudopeptides) or transition state mimics (*e.g.* phosphinic acid analogs of GSH). Most derivatives were assessed against GspS proteins of non-pathogenic trypanosomatid *Crithidia fasciculata* (*C. fasciculata*) and *Escherichia coli*, as more readily available test enzymes. However, despite their valuable potencies against these proteins, compounds displayed lower activities against TSAs of pathogenic trypanosomatids. Moreover, disappointing results were achieved in cellular assays and ascribed to their peptide character, discouraging further developments of this class of derivatives.[10a,11,16]

Similar controversial results were obtained for a set of 7,12-dihydrobenzo[2,3]azepino[4,5-*b*]indol-6(5*H*)-one derivatives (paullones), a class of ATP analogs originally identified as anticancer agents. Indeed, the analogy with human kinase enzymes in terms of ATP-dependence prompted the evaluation of such compounds as potential TSAIs and putative binders of the ATP-cleft of the synthetase domain. However, although derivatives demonstrated nanomolar potency against TSA of *C. fasciculata* (*Cf*TSA), they resulted much less potent against TSAs of pathogenic trypanosomatids.

An explanation for these discrepancies has been provided by docking and molecular modeling studies, which predicted for these compounds additional interactions with a cleft nearby the active site of *Cf*TSA, which, in contrast, is too narrow in TSAs of pathogenic species.[10a,11,16]

These findings underlined the importance of using the authentic target to rationally address drug development.[11] Additionally, they issued a warning about variable susceptibility of trypanosomatid TSAs to inhibition, pointing out the importance of testing enzymes from different pathogens during screening campaigns aimed at detecting putative TSAIs.

Indeed, species-specificity for this target was highlighted by TSAIs reported so far, with potencies differing for various orders of magnitude in diverse trypanosomatids, suggesting relevant structural differences between enzymes.[9b,10b,24,25]

Moreover, discrepancies between high-level enzymatic inhibition and weak antiparasitic activity were reported for some derivatives and ascribed to parasites' ability to survive with low levels of $T(SH)_2$.[11,13b]

However, competent TSAIs and promising antiprotozoal agents were identified so far, providing important insights to guide further optimizations.

In one of earliest study in the search of enzyme inhibitors, some indazole compounds emerged as non-toxic and very

potent *T. brucei* TSAIs from screening campaigns. Two of the most promising derivatives, DDD86243 (**1**) and DDU 86439 (**2**), were used to demonstrate chemical validation of TSA as drug target in *T. brucei.* In particular, when compounds were tested on transgenic cell lines expressing varying amounts of TSA (TSA SKO and TSA-overexpressing *T. brucei* cell lines), changes in the level of the enzyme cells correlated well with their relative sensitivity to compounds.[13a,b,14]

However, although on-target effects were demonstrated, anti-*T. brucei* activities of **1** and **2** were up to two orders of magnitude greater than enzyme IC_{50} values (Figure 4, Table 1).[13a,b,14]

Conversely, a good correlation between enzymatic and antiparasitic activities in the micromolar range was highlighted for the natural products betulin (**3**), tomatine (**4**), uvaol (**5**) and conessine (**6**), the latter being the most potent compound against *Ld*TSA (Figure 4, Table 1).[26]

A similar finding was detected for the promising oxabicyclo [3.3.1] nonanone derivative PS-203 (**7**) which, interestingly, proved to inhibit both *Ld*TSA and *Ld*TR at micromolar concentrations (Figure 4, Table 1).[27]

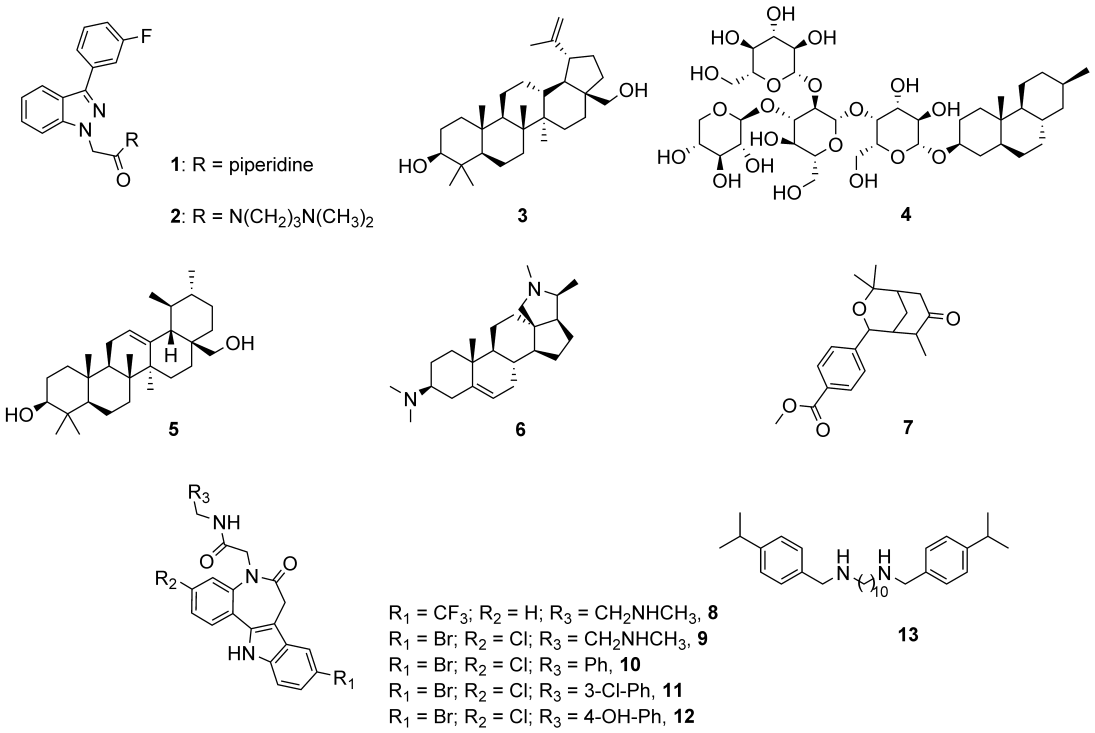


Figure 4. Chemical structures of TSAIs **1–13**.

Table 1. *In vitro* activity profile of TSAIs **1–13**.

Compd	*Leishmania*[a] *L*TSA[d] (IC_{50}, K_i [μM] or %)	EC_{50} [μM]	*T. cruzi*[b] *Tc*TSA[d] (IC_{50} [μM] or %)	EC_{50} [μM]	*T. brucei*[c] *Tb*TSA[d] (IC_{50} [μM] or %)	EC_{50} [μM]	CC_{50}[e] [μM]
1	ND	ND	ND	ND	$IC_{50}=0.140$	5.1	>50 (MRC-5)
2	ND	ND	ND	ND	$IC_{50}=0.045$	7.1 ± 0.2	ND
3	$K_i=6.33\pm0.82$ (*Ld*)	11.71 ± 0.56 (*Ld*)	ND	ND	ND	ND	>50 (HEK293)
4	$K_i=12.54\pm1.22$ (*Ld*)	18.02 ± 0.35 (*Ld*)	ND	ND	ND	ND	>50 (HEK293)
5	$K_i=3.55\pm0.71$ (*Ld*)	11.23 ± 0.48 (*Ld*)	ND	ND	ND	ND	>50 (HEK293)
6	$K_i=3.12\pm0.52$ (*Ld*)	13.42 ± 0.75 (*Ld*)	ND	ND	ND	ND	>50 (HEK293)
7	$K_i=14.2\pm0.8$ (*Ld*)	4.9 ± 0.4 (*Ld*)	ND	ND	ND	ND	>100 (J774 A.1)
8	$IC_{50}=0.35\pm0.05$ (*Li*)	112.3 ± 1.1 (*Li*)	55.5 ± 3.8%	ND	$IC_{50}\sim75$	8.3 ± 0.8	67.38 (J774)
9	$IC_{50}=0.15\pm0.06$ (*Li*)	12.6 ± 1.6 (*Li*)	40.5 ± 5.9%	ND	59.0 ± 6.0%	4.3 ± 0.7	10.32 (J774)
10	50.0 ± 4.0% (*Li*)	ND	27.2 ± 4.0%	ND	54.8 ± 4.1%	0.04 ± 0.01	56.4 (J774)
11	$IC_{50}=17\pm3$ (*Li*)	ND	$IC_{50}=11\pm2$	~30	55.9 ± 6.9%	1.1 ± 0.2	~100 (J774)
12	$IC_{50}=10\pm3$ (*Li*)	ND	$IC_{50}=9\pm3$	~10	58.0 ± 7.2%	0.84 ± 0.2	~200 (J774)
13	47.8 ± 1.8% (*Li*)	ND	53.5 ± 1.3%	ND	51.1 ± 4.2%	0.20 ± 0.02	3 (J774)

[a] Activity profile on *L. donovani (Ld)* and *L. infantum (Li)* parasites; antiparasitic activity on promastigote stage is expressed as EC_{50}. [b] Activity profile on *T. cruzi*; antiparasitic activity on epimastigote stage is expressed as EC_{50}. [c] Activity profile on *T. brucei brucei*; antiparasitic activity on bloodstream form is expressed as EC_{50}. [d] TSA inhibition is expressed as percent inhibition (%) by 30 μM compounds or IC_{50}/K_i values, as specified. [e] Cytotoxicity assessed on mammalian cells indicated in brackets and expressed as CC_{50}. ND: not determined.

According to key role of such enzymes, combined inhibition may represent an innovative strategy to impair the whole T $(SH)_2$ pathway and should be considered in the next future.

Despite the disappointing results highlighted in early studies for this class of compounds, the most recent efforts in the search of TSAIs focused on paullones. Interestingly, N^5-substituted and 4-azapaullones proved selective TSAIs without affecting human kinases and, importantly, some of them were used for chemical validation of TSA.[12,15,24,25,28]

Despite showing a certain cytotoxicity on human cells, N^5-substituted paullone derivatives FS-554 (**8**) and MOL2008 (**9**) proved the most potent *L. infantum* TSAIs reported so far, the latter being a more competent antileishmanial and anti-*T. brucei* agent (Figure 4, Table 1).[12,24]

Basing on docking studies performed on ATP-binding clefts of human GSK-3 and *Lm*TSA, new analogs of **9** were designed to increase potency and selectivity against the putative target by replacing the terminal methylaminomethyl group with arylmethyl moieties (Figure 4). However, although some compounds displayed selective and potent antitrypanosomal effects within the submicromolar-nanomolar range, activities on *T. brucei* TSA were up to three of orders of magnitude greater. A prominent example is provided by compound **10**, which resulted the most active and selective anti-*T. brucei* agent (Figure 4, Table 1). Although **10** resulted one of most potent *T. brucei* TSAIs of the series, the wide difference between antitrypanosomal and TSA inhibitory potency prompted the authors to cast doubts on *Tb*TSA as main target of this derivative and related analogs.[25]

Interestingly, most compounds of the series displayed promising efficacy against *Tc*TSA ($IC_{50} \sim 10\ \mu M$), resulting tolerant to chemical modifications applied and proving more competent *T. cruzi* TSAIs than hit compound. Moreover, despite being less potent on leishmanial enzyme than **9**, some of them showed promising and comparable potencies toward both *Tc*TSA and *Li*TSA.[25]

The representative derivatives KuOrb39 and KuOrb54 (**11**, **12**) were selected to explore the role of TSA in *T. cruzi* (Figure 4, Table 1). Interestingly, besides showing on-target effects, they displayed anti-*Tc*TSA and anti-*T. cruzi* activities at similar concentrations, suggesting that this enzyme could be the main target for such compounds. Additionally, both TSAIs enhanced the efficacy of the antichagasic drugs nifurtimox and benznidazole against *T. cruzi* since co-administration improved >2-fold the IC_{50} values of drugs. These data suggest the involvement of *Tc*TSA in drug-resistance and, importantly, the potential of TSAIs as adjuvant therapy against *T. cruzi*.[15]

Interestingly, the diamine derivative EAP1-47 (**13**) displayed very similar inhibitory activities against multiple TSAs, besides affecting *T. brucei* proliferation at submicromolar concentrations (Figure 4, Table 1). However, although EAP1-47 proved to interfere with TSH_2 biosynthesis of *T. brucei*, the almost two order of magnitude difference between *Tb*TSA inhibition and EC_{50} indicates that compound has also other molecular targets. On the other hand, peculiar behavior of **13** prompts to explore structurally related analogs for the search of multi-TSAIs.[24]

3. Trypanothione reductase inhibitors (TRIs)

The critical role of TR for parasite infectivity and survival prompted the development of a huge number of TRIs. Relevant efforts in the search for new TRIs have been guided by both computational and crystallographic approaches. Indeed, detailed structural details about various TRs have been highlighted by X-ray studies and, additionally, many TR-TRI complexes were solved in recent years.

It is noteworthy that, besides applying rational target-based design, compounds should be optimized for both enzymatic and trypanocidal activities, owing to the parasite ability to survive with very low levels of functional TR.[18]

Although we will mention some older TRIs, interested reader is referred to previous reviews that cover extensively this topic in more detail.[11,21] Owing to space constraints, herein we will focus mainly on recent advancements in the search of TRIs.

In the following sections, TRIs will be classified in different groups based on their common or similar chemical scaffolds, while natural product-based TRIs and compounds with large chemical diversity will be reported separately.

3.1. Tricyclic compounds

Initial efforts in the search of TRIs relied on tricyclic drugs showing selectivity on TR over human counterpart.

They included some neuroleptic, antidepressant drugs and the antimalarial agent mepacrine (**14**), with the latter being the first TRI co-crystallized with *Tc*TR (Figure 5).

X-ray studies showed that this compound occupied the major hydrophobic cleft of TR (MBS) and contacted four residues exclusive of TR via acridine and alkylamino moieties. Besides explaining the selectivity of **14**, these findings suggested the relevance of a hydrophobic core linked to an alkylamino chain as structural features to achieve competent and selective TRIs.[20–22]

Structurally related tricyclic compounds, including chlorpromazine (**15**) and clomipramine (**16**), displayed selectivity on TR and resulted more potent than **14**, prompting the design and

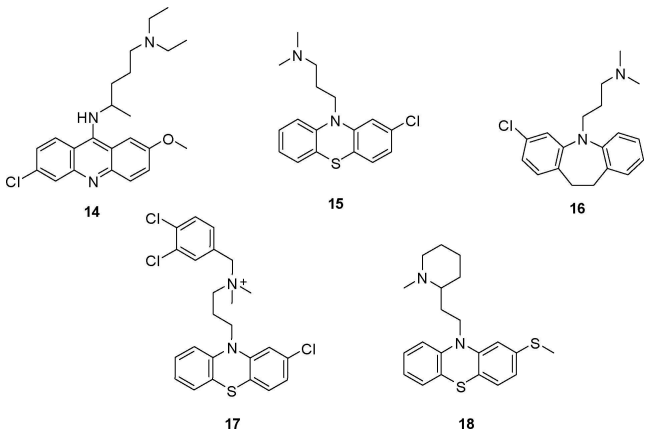


Figure 5. Chemical structures of tricyclic compounds **14–18**.

synthesis of several analogs (Figure 5). For such derivatives, molecular modelling studies suggested an alternative orientation of amino side chains toward central glutamate residues, and, in turn, the opportunity to increase enzymatic affinity by reaching the nearby Z-site. These findings prompted the design of phenothiazine derivatives endowed with additional lipophilic substituents on a quaternized amino side chains. According to a three point mode of attachment, these derivatives (*e.g.* **17**) were predicted to contact both MBS and Z-site via hydrophobic moieties, with ammonium group addressing such interactions by contacting central glutamate residues (Figure 5).[20,21,23]

Besides allowing early efforts in target exploration, this rational target-based approach guided the design and synthesis of potent and selective TRIs. Additionally, via a drug repurposing strategy, the known drugs **16** and thioridazine (**18**) demonstrated promising efficacy in *T. cruzi* mice models of infection (Figure 5).[29]

However, generally weak antiparasitic activities and concerns about neurological side effects restricted researches on tricyclic compounds in the last years.

More recently, a set of phenothiazine-, phenoxazine- and related tricyclics-derived chloroacetamides were reported as antileishmanial agents. Although time-dependent inactivation of reduced *Tb*TR suggested irreversible enzyme inhibition, cytotoxicity was detected on human cells.[30] This finding indicates that, although irreversible inhibition of TR may represent a valuable strategy to strongly impact the $T(SH)_2$ metabolism, higher risk of host toxicity should be taken into account in the development and optimization of such type of inhibitors.

3.2. Diarylsulfide derivatives

In order to eliminate their neuroleptic side effects, the central ring of phenothiazines was opened to yield related aminodiphenylsulfides. Chemical approaches explored for tricyclic compounds were applied also in these derivatives, leading to TR inhibitory potencies within the submicromolar-nanomolar range. Moreover, the diarylsulfide scaffold was coupled to others TRIs (including **14** and quinones) or underwent dimerization to strengthen target inhibition by combining binding mode and/or mechanism of action of single inhibitors.[20,21,31]

Recently, the diarylsulfide RDS 777 (**19**) was identified as promising antileishmanial agent and highly potent *L. infantum* TRI via in-house structure-based screening (Figure 6).[32a] The

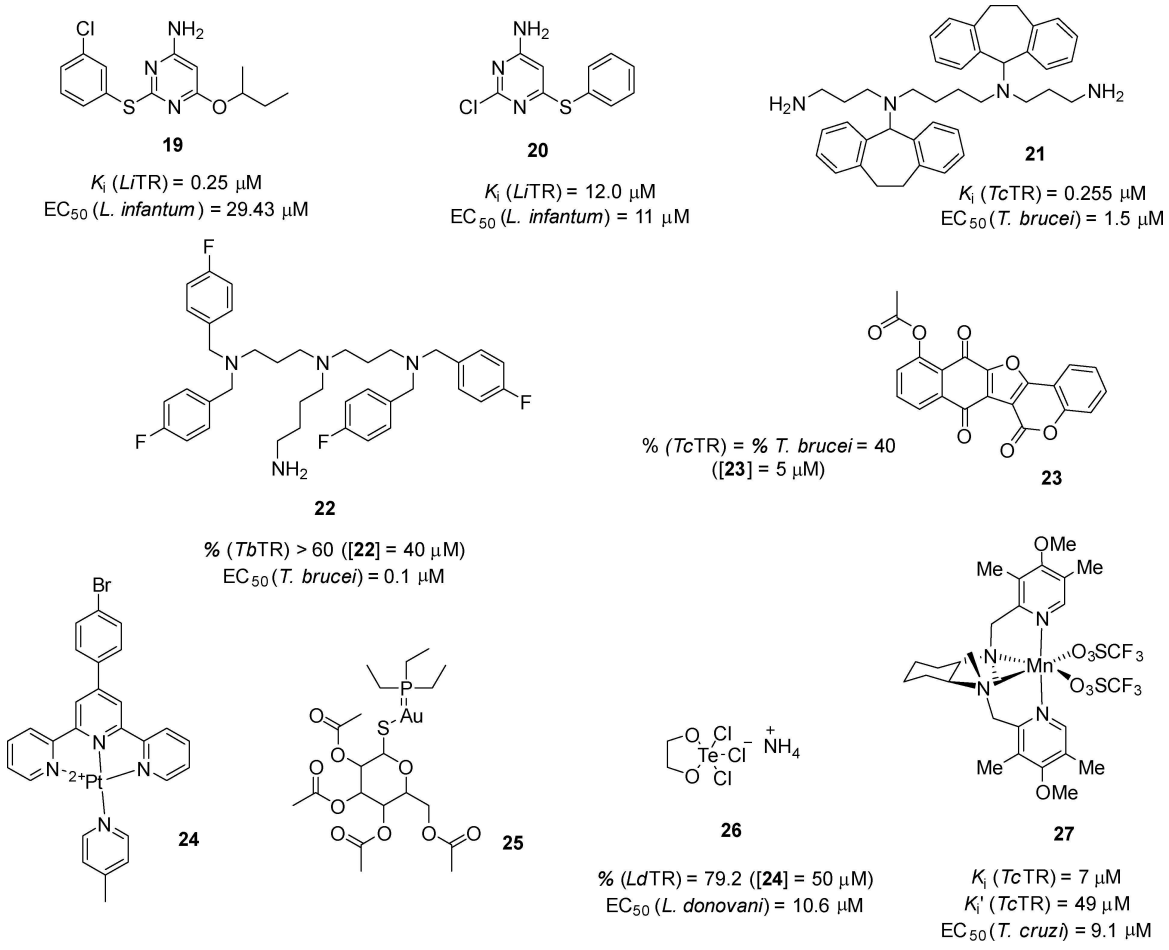


Figure 6. Chemical structures of TRIs **19–27**. Enzymatic activities are reported as K_i, K_i' and percent inhibition (%). Antiparasitic activities are indicated as EC_{50} or parasite growth inhibition (%).

solved X-ray structure of *Li*TR-**19** complex (PDB ID: 5EBK) highlighted

multiple molecules bound to the protein, an effect already observed for other inhibitors and due to the wide dimension of the enzyme.[11,21,22,32a] Interestingly, two molecules of **19** occupied similar clefts in both monomers by interacting with residues more involved in the catalysis, providing important insights about an alternative binding mode in TR active site, which can be exploited in further studies. These findings prompted the identification of structurally related analogs of **19** as more potent antileishmanial agents within the micromolar range. Among them, RDS 562 (**20**) proved one of the most active of the series and, additionally, proved to decrease intracellular TSH_2 pool and showed *Li*TR inhibition with very good correlation with EC_{50} without effect on human GR (Figure 6).[32b]

3.3. Polyamino derivatives

The exclusive affinity of TR toward its spermidine-based substrate prompted the design and synthesis of polyamino compounds as selective TRIs. In general, spermine derivatives proved more potent than spermidine congeners, while insertion of multiple hydrophobic moieties further improved their potency.[11,21]

Similar findings were seen in a set of selective dibenzosuberyl-based polyamino derivatives, for which *Tc*TR inhibition proved affected by length of polyamino chain and number of dibenzosuberyl moieties, with compound **21** resulting the most potent compound (Figure 6). Docking studies predicted occupation of two distinct hydrophobic clefts within *Tc*TR active site, while amino groups interacted with central glutamate residues and a polar cluster nearby MBS, respectively. Despite displaying single-digit micromolar anti-*T. brucei rhodesiense* activity, compounds lacked *in vivo* efficacy in infected mice eliciting toxicity at higher doses.[33]

Similar results were reported for some bis(arylmethyl) spermidine derivatives, which, despite promising submicromolar anti-*T. brucei* activity, caused prominent TR inhibition at concentrations likely to cause toxicity on human cells. Indeed, although compound **22** proved the most active derivative of the series against *T. brucei* in both cellular and enzymatic assays, it displayed also cytotoxicity on macrophages (Figure 6).

Moreover, only few of the most active compounds displayed inhibitory effects against TR and, therefore, further studies are required to determine their mechanism of action and the putative targets.[34]

The lack of *in vivo* efficacy for polyamino derivatives could be ascribed to pharmacokinetic limits (*e.g.* rapid excretion, fast metabolism in mammalian host), whose improvement is critical for further developments.[33]

3.4. Quinone derivatives

The finding that many quinone derivatives act as subversive substrates of TR prompted the development of various quinone-based TRIs and their conjugation with polyamino moieties to improve both activity and selectivity on enzyme.[11,21]

Quinone-coumarin hybrids and, more recently, structurally rigidified crassiflorone analogues were designed to inhibit both TR and glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH).[35] Since these enzymes are involved in pivotal energy and redox metabolisms upon which pathogens rely, their combined inhibition may represent an innovative strategy to impair parasitic survival.

Unfortunately, only few compounds of this set emerged as dual target inhibitors so far. As single targeted inhibitor, the most promising TRI **23** inhibited both recombinant *Tc*TR and *T. brucei* parasite growth by ~40% at low concentration (Figure 6). Moreover, solubility and predicted toxicity issues should be improved in the next generation of derivatives.[35b]

3.5. Metal-based derivatives

According to the ability of cysteine to coordinate metals, various metal-based TRIs were developed to target the critical sulfur-based residues of TR active site via formation of coordination complexes.

Prominent example include the Pt-complexes of terpyridine derivatives (*e.g.* **24**), which proved selective and mainly irreversible TRIs, and the gold-containing drug auranofin (*e.g.* **25**), that demonstrated *in vitro* and *in vivo* antileishmanial efficacy (Figure 6).[11,21,36]

Similar prominent results were recently found for the non-toxic immunomodulator Te-based AS101 (**26**) on *L. donovani*. Moreover, although antileishmanial effects have been ascribed to multiple targets, a strong inhibitory effect on TR was reported, which may contribute to the extensive ROS production observed (Figure 6).[37]

Furthermore, Mn-based compound **27** recently demonstrated *in vitro* and *in vivo* efficacy against acute and chronic models of *T. cruzi* infection. Although compounds showed a much more prominent inhibition of iron superoxide dismutase, mixed-type *Tc*TR inhibition was highlighted (Figure 6).[38] Moreover, combined inhibition of such targets involved in redox homeostasis may represent an alternative attractive strategy to dramatically increase susceptibility of parasite toward oxidative stress and disrupt protozoal survival.

3.6. Nitro-based compounds

Many nitro-substituted compounds were identified as TRIs in recent years.

Interestingly, GlaxoSmithKline HTS diversity set of 1.8 million compounds was screened against *L. donovani*, *T. cruzi* and *T. brucei* in phenotypic assays and resulting lead com-

pounds were clustered in three corresponding anti-trypanosomatid chemical boxes.[39]

Recently, the 192 compounds included in the LeishBox were assessed against *Li*TR and human GR. Seven derivatives displayed IC_{50} lower than 6 μM against TR, with three of them showing selectivity over GR. Interestingly, the most potent TRIs (**28**, **29**, **30**, Figure 7, Table 2) share a *N*-phenyl-5-nitrothiophene-2-carboxamide moiety and were predicted to occupy the same cleft within *Li*TR active site by docking studies. Moreover, computational studies highlighted steric clashes within human GR, providing a possible explanation for the molecular basis of specificity on TR.[40]

Another interesting nitro-substituted derivative (**31**) emerged in a set of chalcone compounds as promising antileishmanial agent endowed with micromolar activities against both promastigote and amastigote forms of *L. donovani* without eliciting toxicity on human cells (Figure 7, Table 2). Besides showing high affinity for TR in SPR-based assay, it was predicted to occupy a hydrophobic pocket nearby catalytic site in *Li*TR by docking studies, which was already shown to be part of **19** binding site in the same enzyme.[41]

Interestingly, the micromolar active compound **32** identified in HTS has been recently co-crystallized within *Li*TR (PDB ID: 6ER5), showing to contact residues located at the entrance of the NADPH binding site and lacking in human homolog (Figure 7, Table 2). This finding can partially explain the selectivity observed on GR and, additionally, may suggest an alternative druggable site within TR.[42]

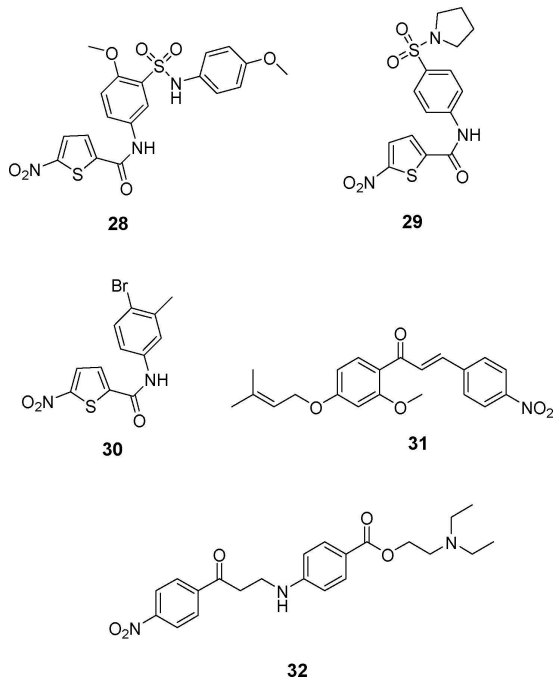


Figure 7. Chemical structures of TRIs **28–32**.

3.7. Natural-product-based derivatives

The finding that various natural products with antiparasitic activities proved TRIs via disparate mechanisms of inhibition prompted the development of related analogs to explore SAR studies and improve drug-like properties.

Prominent examples include the spermine derivative kukoamine A (**33**), quinone compounds (*e.g.* plumbagin, **34**) and the spermidine-based macrocyclic alkaloid lunarine (**35**), the latter causing time-dependent inactivation of reduced TR due to conjugate addition of catalytic cysteine to its α,β-unsaturated amide moiety (Figure 8).[21]

A similar profile was detected for the iso-atriplicolide tiglate ester (**36**), which resulted the most potent TRI of *T. brucei* and *T. cruzi* enzymes within a set of nanomolar potent anti-*T. brucei* agents (Figure 8, Table 3). Despite displaying the highest level of inhibition toward both enzymes, compound **36** resulted the most active anti-*T. brucei* agent, but, conversely, it turned to be the least potent derivative of the series against *T. cruzi* parasite. Interestingly, small differences in the ester moiety conferred different potencies against TR enzymes, prompting the future investigation of alternative iso-atriplicolide-based derivatives to explore SAR studies.

However, although all compounds displayed Michael acceptor moieties, which should be responsible for irreversible enzyme inactivation, only some of them proved TRIs, indicating that the presence of such reactive portion does not automatically imply target inhibition.[43]

Via molecular hybridization of natural product scaffolds with antileishmanial activities, β-carboline-quinazolinone hy-

Table 2. *In vitro* activity profile of nitro-based TRIs **28–32**.

Compd	Leishmania[a] LiTR (IC_{50} or K_i [μM])	EC_{50} [μM]	T. cruzi[b] EC_{50} [μM]	T. brucei[c] EC_{50} [μM]	GR[d] [μM]	CC_{50}[e] [μM]
28	$IC_{50} = 0.52 \pm 0.14$	0.79 (*Ld*, iam)	0.063	0.050	> 25	100 (HepG2)
29	$IC_{50} = 0.22 \pm 0.05$	1.58 (*Ld*, iam)	0.2	0.158	3.2	6.3 (HepG2)
30	$IC_{50} = 0.19 \pm 0.08$	3.16 (*Ld*, iam)	0.251	0.316	> 25	39.8 (HepG2)
31	$K_i = 0.45 \pm 0.11$	3.0 (*Ld*, p) 1.6 (*Li*, p) 14.0 (*Ld*, iam)	ND	ND	ND	600 (THP-1)
32	$IC_{50} = 7.52 \pm 2.53$	12.44 ± 1.09 (*Ld*, p)	ND	ND	> 85	ND

a] Activity profile on *L. donovani (Ld)* and *L. infantum (Li)* parasites; antiparasitic activities on promastigote (p) and intracellular amastigote (iam) stages are expressed as EC_{50} values. [b] Antiparasitic activity on intracellular amastigote stage of *T. cruzi*. [c] Antiparasitic activity on bloodstream form of *T. brucei brucei*. [d] Enzymatic inhibition of human glutathione reductase (GR) expressed as IC_{50} values. [e] Cytotoxicity assessed on mammalian cells indicated in brackets and expressed as CC_{50}. ND: not determined. Enzymatic activities against *Tc*TR and *Tb*TR are not available.

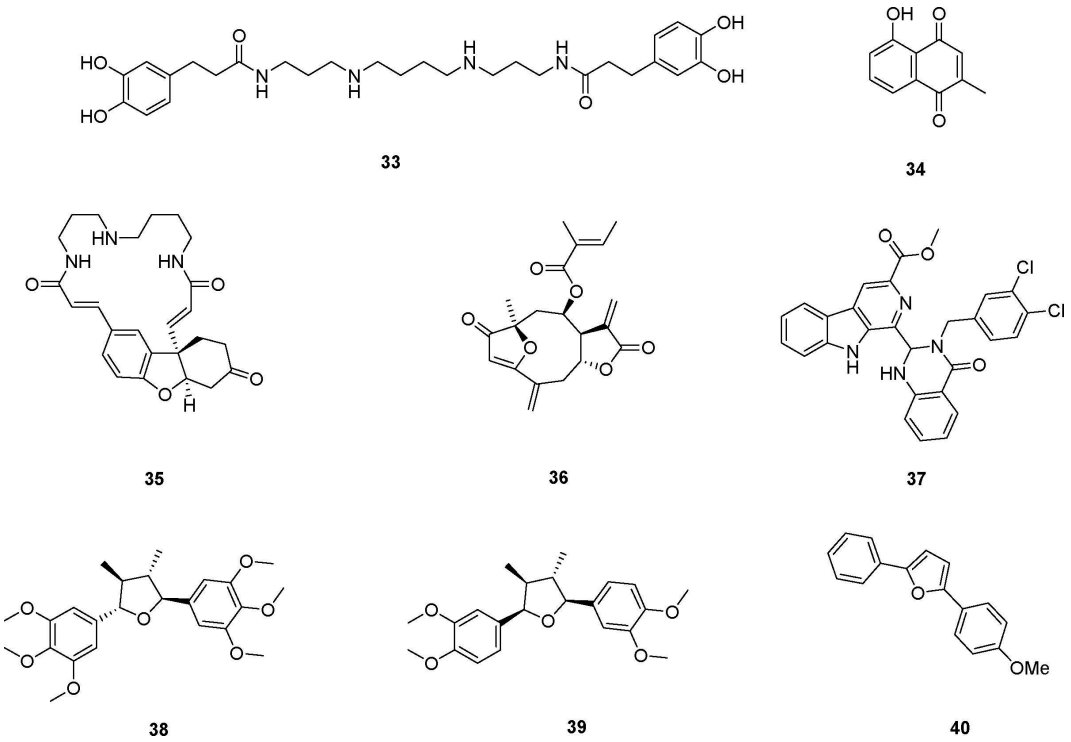


Figure 8. Chemical structures of TRIs **33–40.**

Table 3. *In vitro* activity profile of natural product-based TRIs **36**, **37**, **40**.

Compd	*L. donovani*[a] *Ld*TR[d] (K_i [μM])	EC_{50} [μM]	*T. cruzi*[b] *Tc*TR[d] (IC_{50} [μM] or %)	EC_{50}[e] [μM]	*T. brucei*[c] *Tb*TR[d] (%)	EC_{50} [μM]	CC_{50}[e] [μM]
36	ND	ND	89 ± 1 %	3.74 ± 1.34 (iam)	87 ± 1 %	0.015 ± 0.003	1.15 ± 0.53 (L6)
37	K_i = 3.7 ± 0.7	4.8 ± 0.7 (p) 4.3 ± 0.5 (iam)	ND	ND	ND	ND	103.7 ± 8.7 (Vero)
40	ND	ND	IC_{50} = 7.4	4.75 ± 1.4 (t) 127 ± 4.5 (iam)	ND	ND	> 250 (C57BL/6 spleen cells)

[a] Activity profile on *L. donovani*; antiparasitic activity on promastigote (p) and intracellular amastigote (iam) stages are expressed as EC_{50}. [b] Activity profile on *T. cruzi*; antiparasitic activity on trypomastigote (t) and intracellular amastigote (iam) stages are expressed as EC_{50}. [c] Activity profile on *T. brucei*; antiparasitic activity on bloodstream form of *T. brucei rhodesiense* is expressed as EC_{50}. [d] TR inhibition is expressed as IC_{50} or K_i values; for compound **36**, anti-TR activity was reported as percent inhibition (%) by 100 μM compound after 15 min NADPH pre-incubation. [e] Cytotoxicity assessed on mammalian cells indicated in brackets and expressed as CC_{50}. ND: not determined.

brids were recently designed, synthesized and assessed on TR. Target effects were dependent on amide quinazolone pattern of substitution and, interestingly, some of the most encouraging compounds (*e.g.* **37**) showed micromolar K_i values against *Ld*TR which nicely related with activities against both forms of *L. donovani* (Figure 8, Table 3).[44]

According to their promising antitrypanosomal activities, many recent studies focused on design and synthesis of analogs of the tetrahydrofurane-based neolignans grandisin (**38**) and veraguensin (**39**, Figure 8).[45]

Compared to parent compounds, structurally related methoxy-substituted diphenyl-furane and -tetrahydrofurane derivatives showed improved anti-*T. cruzi* activities within the submicromolar-nanomolar range without toxicity on human cells and were assessed on TR. However, only derivatives bearing a monomethoxy-substituted furane scaffold (*e.g.* **40**) caused promising *Tc*TR inhibition, while the most potent anti-*T. cruzi* compound of the series did not inhibit TR and probably has alternative target (Figure 8, Table 3).[45a]

In contrast, the recently described isoxazole and bis-heterocyclic derivatives designed as bioisosteric analogs of neolignans did not inhibit TR, despite featuring promising antitrypanosomal and antileishmanial potencies.[45b,c]

3.8. Other compounds

Due to their broad structural diversity, heterogeneous compounds will be included in the following section.

According to an innovative approach, some derivatives were recently designed to disrupt protein-protein interactions involved in the formation of TR homodimer.[46] The very

encouraging results highlighted by peptidic disruptors prompted the design of some imidazole-phenyl-thiazole and pyrrolopyrimidine derivatives, which, however, displayed lower effects as TR dimerization inhibitors. Despite showing weak selectivity on parasite, the most promising imidazole-phenyl-thiazole (**41**, Figure 9, Table 4) caused *Li*TR inhibition and antiparasitic effects on both forms of *L. infantum* at similar concentrations, suggesting TR as major target. Unfortunately, attempts to co-crystalize these compounds within *Li*TR failed, while crystallographic studies were performed on a much less potent TRI lacking effect on TR dimerization.[46a]

In a different study, selenourea, heteroaryl-based selecyanate (**42**) and diselenide (**43**) derivatives were evaluated against *L. infantum*. Interesting results were achieved on axenic amastigotes and *Li*TR at similar micromolar-submicromolar concentrations with good selectivity on parasite (Figure 9, Table 4). Despite these, lower activities were detected in infected macrophages, suggesting permeability or intracellular stability issues for these compounds, whose improvement may be very interesting for further developments.[47]

Some of the most prominent and recent efforts in the search of competent TRIs were devoted to the phencyclidine analog BTCP (**44**, Figure 9), a promising but weak TRI identified by HTS (Table 4).[48] In the context of a long and rational research work aided by

both computational and crystallographic studies, the identification of the indole-based BTCP derivative **45** represented a crucial point (Figure 9, Table 4). Indeed, mutational and crystallographic analyses aided to elucidate its binding mode within *T. cruzi* and *T. brucei* TR active sites, highlighting the involvement of MBS and Glu18 in complex stabilization (PDB IDs: 4NEV, 4NEW). Moreover, even though general location of compound

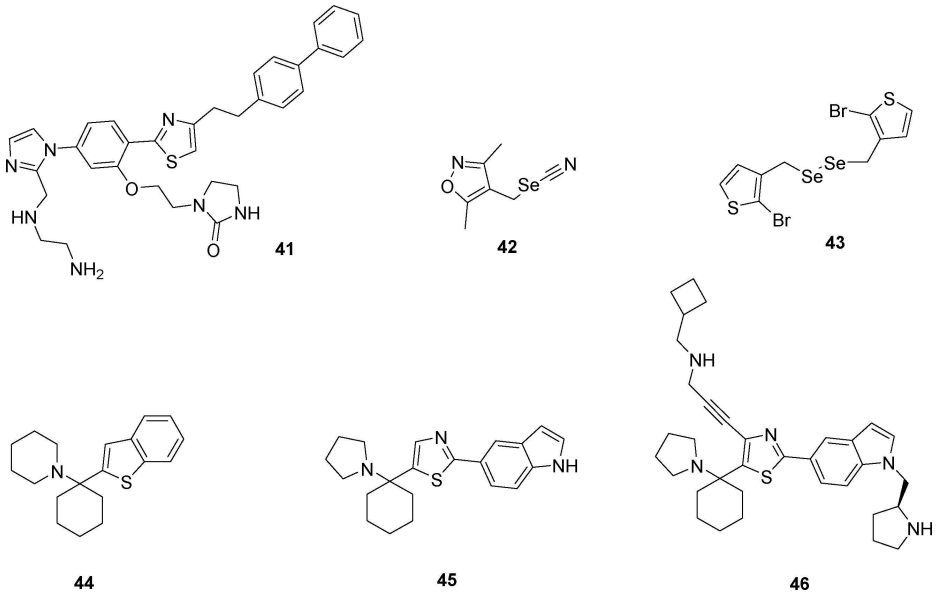


Figure 9. Chemical structures of TRIs **41–46.**

Table 4. *In vitro* activity profile of TRIs **41–46**.

Compd	*Leishmania*[a] *Li*TR[d] (IC_{50} [μM])	EC_{50} [μM]	*T. cruzi*[b] *Tc*TR[d] (IC_{50} or K_i [μM])	EC_{50} [μM]	*T. brucei*[c] *Tb*TR[d] (K_i [μM])	EC_{50} [μM]	GR[e] (IC_{50} [μM] or %)	CC_{50}[f] [μM]
41	8.6 ± 1.4	5.3 ± 0.3 (*Li*, p) 5.3 ± 0.2 (*Li*, ax)	ND	ND	ND	ND	ND	14.2 (THP-1)
42	0.46 ± 0.01	0.73 ± 0.10 (*Li*, ax) 23.2 ± 4.3 (*Li*, iam)	ND	ND	ND	ND	ND	21.82 ± 2.4 (THP-1)
43	6.85 ± 0.49	1.20 ± 0.03 (*Li*, ax) 14 ± 2.1 (*Li*, iam)	ND	ND	ND	ND	ND	30.9 ± 0.02 (THP-1)
44	ND	ND	IC_{50} = 3.7	ND	1.00 ± 0.08	10 (*Tbb*)	IC_{50} > 100	29 (MRC-5)
45	ND	ND	K_i = 4 ± 0.5	19.0	12 ± 2	3.5 (*Tbr*)	12%	28.9 (L6)
46	ND	84 (*Ld*, ax)	ND	3.9	0.073 ± 0.009	0.12 (*Tbr*)	0%	2.4 (L6)

[a] Activity profile on *L. donovani (Ld)* and *L. infantum (Li)* parasites; antiparasitic activity on promastigote (p), axenic amastigote (ax) and intracellular amastigote (iam) stages are expressed as EC_{50}. [b] Activity profile on *T. cruzi*; antiparasitic activity on intracellular amastigote is expressed as EC_{50}. [c] Activity profile on *T. brucei brucei (Tbb) and T. brucei rhodesiense* (*Tbr*); antiparasitic activity on bloodstream forms is expressed as EC_{50}. [d] TR inhibition is expressed as IC_{50} or K_i values, as specified. [e] GR inhibition is expressed as IC_{50} or percent inhibition (%) by 40 μM compounds. [e] Cytotoxicity assessed on mammalian cells indicated in brackets and expressed as CC_{50}. ND: not determined.

was maintained in both TRs, the different ligand orientation observed was ascribed to a difference in a single residue between respective active sites.[48d] Binding to MBS was confirmed in a next set of more potent alkylamino-based indole analogs designed to improve water solubility.[48e]

Chemical modifications aimed to explore nearby enzymatic pockets led to new analogs bearing alternative alkylamino groups on indole moiety and propargylic substituents on thiazole scaffold. Notably, most compounds displayed enzymatic and antitrypanosomal activities at submicromolar-nanomolar concentrations with negligible interference on GR, despite low-to-moderate selectivity on human cells. Noteworthy, showing anti-TR and anti-*T. brucei* activities at nanomolar concentrations, the cyclobutyl derivative **46** (Figure 9, Table 4) resulted not only the most active TRI of the series, but also the most potent *Tb*TRI reported so far, for which the assessment of *in vivo* efficacy in further studies is strongly expected. Moreover, besides maintaining previously observed contacts within MBS, X-ray structure (PDB IDs: 6OEZ) showed extension of propargylamino moiety towards a hydrophobic sub-pocket near the catalytic cysteines of *Tb*TR, while the alkylamino chain interacts with Asp116.[48f]

4. Conclusions and Outlook

As critical and unique system of trypanosomatids, the $T(SH)_2$ metabolism offers valuable opportunities to develop innovative and selective therapeutic agents for the treatment of many related parasitic diseases. In recent years, even new research groups have been engaged in this intriguing field, providing new ideas and developing prominent inhibitors whose exploitation is critical for future developments in the area.

The relevant research works devoted to the search of TRIs over last decades have notably enhanced target exploration and furnished important indications to guide optimization of inhibitor profile. Noteworthy, a prominent contribution in aiding ligands design has been provided by X-ray analyses, which have been exploited to a much greater extent in recent years than in the past for TRIs. A similar approach could be highly useful in driving advancements in the search of TSAIs. Indeed, as reflected by the few inhibitors reported and the fact that only one X-ray structure is currently available for this enzyme with none inhibitor co-crystallized, this issue is only in its early stages and growing efforts are expected. Moreover, structural elucidation of TSAs from various trypanosomatids may be useful to identify possible conserved clefts herein present, whose exploitation could be critical in the search of multi-TSAIs.

Nevertheless, the ability of parasites to survive with very low concentrations of redox active metabolites renders critical the optimization of inhibitors for both their target and phenotypic profile to achieve highly effective antiprotozoal agents. Indeed, while X-ray studies represent a powerful tool to drive rationally improvement of enzymatic potency, this approach should importantly be matched by enhancement of antiparasitic effects to yield *in vivo* efficacy.

Considering that compounds must cross multiple barriers to reach putative target in many parasites, improvement of pharmacokinetic profile and use of more predictive models of infection is very important, besides showing high chemical stability to withstand harsh cellular conditions.

A valuable strategy that has been exploited to some extent in recent years relies on multi-target inhibition. Indeed, this approach could be very promising to strengthen inhibition on multiple diverse or related steps of critical metabolic pathways of trypanosomatids and deeply affect their survival.

Interestingly, application of such strategy to multi-key steps of the $T(SH)_2$ pathway might be very promising to significantly impair redox homeostasis and notably improve antiparasitic efficacy. This approach, which has been only little explored so far, could rely on development of dual inhibitors of TSA and TR or, alternatively, on combination of highly effective single-targeted compounds, whose development is strongly expected in the next future. Nevertheless, in view to disrupt the thiol-redox metabolism in multiple points, other targets could be also considered, which, despite their essential role in parasites, have been much less targeted so far.

In this review, we aimed to provide a general but also critical overview about more recent efforts in this field to, possibly, give some ideas about future development of innovative antiprotozoal agents.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: antiprotozoal agents · drug discovery · inhibitors · redox metabolism · trypanothione.

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