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Chapter

Parasite, Compartments, and Molecules: Trick versus Treatment on Chagas Disease

Marcos André Vannier-Santos, Giselle V. Brunoro, Maria de Nazaré C. Soeiro, Solange L. DeCastro and Rubem F.S. Menna-Barreto

Abstract

Chagas disease, caused by the protozoan *Trypanosoma cruzi*, is endemic to Latin America, standing out as a socio-economic problem for low-income tropical populations. Such disease affects millions of people worldwide and emerges in nonendemic areas due to migration and climate changes. The current chemotherapy is restricted to two nitroderivatives (benznidazole and nifurtimox), which is unsatisfactory due to limited efficacy (particularly in chronic phase) and adverse side effects. *T. cruzi* life cycle is complex, including invertebrate and vertebrate hosts and three developmental forms (epimastigotes, trypomastigotes, and amastigotes). In this chapter, we will discuss promising cellular and molecular targets present in the vertebrate-dwelling forms of the parasite (trypomastigotes and amastigotes). Among the cellular targets, the mitochondrion is the most frequently studied; while among the molecular ones, we highlight squalene synthase, C14α-sterol demethylase, and cysteine proteases. In this scenario, proteomics becomes a valuable tool for the identification of other molecular targets, and some previously identified candidates will be also discussed. Multidisciplinary studies are needed to identify novel key molecules in *T. cruzi* in order to increase trypanocidal activity and reduce mammalian toxicity, ensuring the development of novel drugs for Chagas disease.

Keywords: *Trypanosoma cruzi*, Chagas disease, chemotherapy, drug targets, organelles, proteomics, oxidative metabolism

1. Introduction

Chagas disease, or American trypanosomiasis, was described in 1909 by the Brazilian physician Carlos Chagas, who identified the causative agent—*Trypanosoma cruzi*—the transmission vector, major reservoirs, mechanism of human infection, as well as some clinical manifestations [1]. This disease is primarily transmitted to humans by the feces of hematophagous insects, of the family Reduviidae, subfamily Triatominae. The impressive decrease in Chagas disease prevalence from 16–18 million people by 1990 to 6–8 million people by 2010 was essentially the consequence of the launching of transnational programs in Latin America focused on the elimination of domestic vectors and blood donors screening supported by Pan American

Health Organization/World Health Organization (PAHO/WHO) [2]. Despite these successful control interventions, the Chagas disease prevalence was estimated to reach 1.0–2.4% of the Brazilian population [1]. This disease, classically associated with poor and rural populations, underwent an urbanization process in the 1970s and 1980s to Latin American cities and later on beyond endemic countries, creating new epidemiological, social, and political challenges [3–5].

With the success of vector and blood bank control programs, congenital [6, 7], and oral [8, 9] transmissions have become important sources of new cases of Chagas disease. Congenital infections represent an estimated 22% of new cases in Latin America [2], occurring also in nonendemic countries [10, 11]. The oral route, which is probably the most frequent mechanism among vectors and wild mammals, has recently become relevant, due to environmental changes caused by deforestation [12]. *T. cruzi* DNA was recently shown in 10% among 140 samples of açai-based products marketed in Rio de Janeiro and Pará States in Brazil [13].

Chagas disease results from the establishment of *T. cruzi* in host tissues, involving an initial acute phase followed by a chronic phase, classified as indeterminate, cardiac, and/or digestive syndromes. The acute phase is characterized by detectable parasitemia and is commonly asymptomatic [14]. Without treatment, approximately 5–10% of symptomatic patients die during this phase due to encephalomyelitis or severe cardiac failure and rarely due to cardiac arrest [15]. After 2–3 months, the infection enters the chronic phase, and without successful treatment, it is lifelong. Approximately, two-thirds of infected individuals have the indeterminate form of the chronic phase, which is asymptomatic and defined by the presence of *T. cruzi* antibodies and normal electrocardiographic and radiologic exams. The remaining infected individuals, due to an unbalanced inflammatory response and persistent low parasitism, will develop years or even decades later symptomatic chronic disease with cardiac (20–30%) and/or digestive (15–20%) disorders.

The current etiological treatment for Chagas disease is restricted to two nitroheterocyclic drugs: benznidazole (Bz/LAFEPE, Abarax®, ELEA and Bz/Chemo Research, Exeltis) and nifurtimox (Nif, LAMPIT®, Bayer) (**Figure 1**). Bz has been recently FDA-approved for use in children aged 2–12 years, being the first treatment approved in the United States for Chagas disease [16]. The results obtained with these two nitroderivatives vary according to the phase of Chagas disease, the period, and dose of treatment, as well as the age and geographical origin of the patients [17]. Both drugs have often shown successful results with high parasitological cure rates during the acute phase, but the effectiveness decreases with advance of the infection; therefore, early detection and intervention are crucial for reaching high cure rates [18]. The high incidence of collateral effects, especially for adults, leads to treatment abandonment rates reaching over 30% of the patients [19–21]. In contrast, children have a markedly higher tolerance for treatment [1, 14].

There are significant drawbacks on the use of these drugs, mostly related to the limited efficacy in the chronic phase [22], and so, new alternative therapies

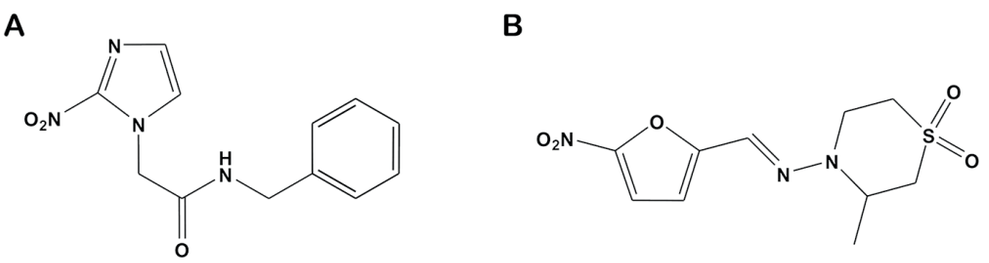

Figure 1.
Clinical drugs for Chagas disease treatment: (A) benznidazole and (B) nifurtimox.

are urgently required. In the last decades, many chemical diversity libraries from several pharmaceutical companies have been screened in the search of novel anti-*T. cruzi* candidates. In these programs, different approaches have been used including target-oriented studies, combination therapies, new formulations for drugs in use, and drug repurposing, and thus, in the present review, some of these points will be addressed.

2. *Trypanosoma cruzi* and drug targets

One important point to be addressed in the search of alternative molecular targets in *T. cruzi* is their presence in parasite forms dwelling in vertebrates. Once the parasite stages present different metabolic profiles [23, 24], the most promising targets are involved in crucial metabolic pathways, such as key enzymes related to antioxidant metabolism or sterol biosynthesis. In this section, we revised some of the most studied targets for drug intervention.

2.1 Mitochondrion, glycosomes, and oxidative metabolism

Mitochondrion plays a pivotal role in the oxidative stress, since the electron leakage from the electron transport chain (ETC particularly from complexes I, III and coenzyme Q) leads to the partial reduction of oxygen, being the main source of reactive oxygen species (ROS) in the cells [25]. During electron leakage, ROS were produced that interfere with different biological processes [26]. Such production leads to the increase in the expression of antioxidant enzymes such as superoxide dismutase (SOD), trypanothione reductase (TR), and peroxidases in response to the oxidative burst, and TR is considered one of the most promising chemotherapy targets in Chagas disease [27]. In trypanosomatids, mitochondrial metabolism is quite similar to that of other eukaryotes. Complex I (NADH: ubiquinone oxidoreductase) is expressed (almost 19 subunits were detected) but its functionality is still controversial [26]. In this way, glucose metabolism results mostly in succinate (complex II substrate) in trypanosomatids, derived from glycosomal and mitochondrial NADH-dependent fumarate reductase activities [28, 29]. Since complex I is not functional, oxidative phosphorylation is exclusively dependent of complex II in these protozoa. On the other hand, complexes III (ubiquinol:cytochrome c oxidoreductase) and IV (cytochrome c oxidase) of high eukaryotes and trypanosomatids display no differences, being complex III considered the major mitochondrial source of ROS production [30]. Many studies pointed to the susceptibility of *T. cruzi* mitochondrion to a great variety of compounds, and such mitochondrial damage (ultrastructural swelling, decreased mitochondrial membrane potential, etc.) may comprise early or late events in trypanocidal agent activity (**Figure 2**) [31].

Glycosomes are organelles crucial for the energetic and antioxidant metabolisms of the parasite, and the compartmentalization of their enzymes (including the majority of the enzymes of the glycolytic pathway) has also been reported to be directly involved in the maintenance of *T. cruzi* viability, indicating this organelle as a potential drug target [32]. Among the glycosomal oxidative, scavengers are SOD isoforms, tryparedoxin, and peroxidases [26]. Up to now, no specific inhibitors of glycosomal enzymes showed promising trypanocidal activity [33].

2.1.1 Mitochondrial ETC

T. cruzi mitochondrion is the most recurrent cellular drug target described in mechanistic studies; however, the exact molecular machinery involved in the

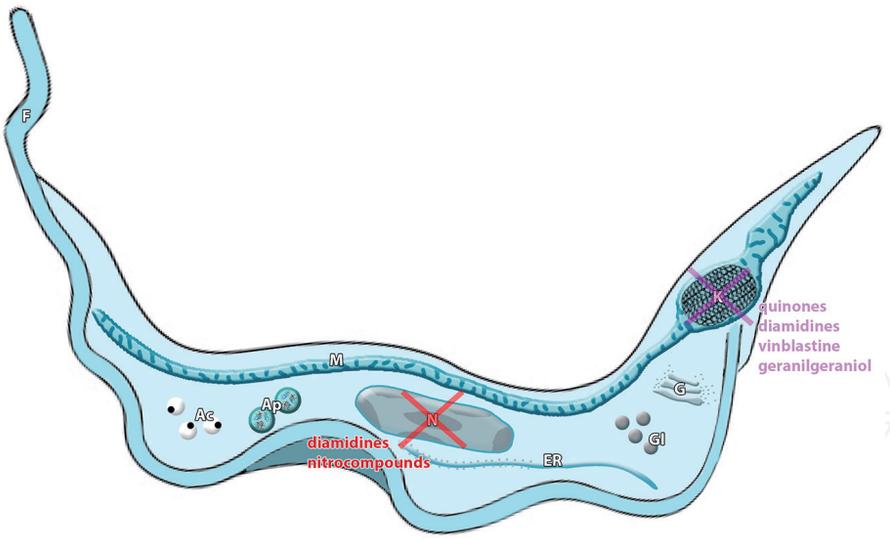

Figure 2.
Most recurrent cellular drug targets in T. cruzi mammalian stages. M: mitochondrion; K: kinetoplast; N: nucleus; F: flagellum; Gl: glycosomes; G: golgi; Ac: acidocalcisomes; ER: endoplasmic reticulum; and Ap: autophagosomes.

susceptibility of this organelle to different classes of compounds is still unclear [31]. No hypothesis about the molecular mechanism involved in the mitochondrial effect of the great majority of trypanocidal drugs was postulated hitherto, and the damage specificity in this organelle is very debatable. Some specific inhibitors of ETC complexes have already been tested on *T. cruzi*. Rotenone, a well-known complex I inhibitor, has a controversial activity in the parasite. However, rotenone at high doses inhibited the activity of *T. cruzi* NADH-dependent enzymes [34]. The existence of complex I activity was not strongly supported by such inhibition and could be caused by nonspecific binding to other electron carriers. On the other hand, depolarization of mitochondrial membrane, ROS production, and apoptotic-like phenotype was detected in parasites after the treatment with inhibitors of complexes III and IV, antimycin A, and potassium cyanide, respectively [24]. Structural and functional similarities between mammalian and trypanosomatidae complexes are suggestive of high toxicity.

2.1.2 Trypanothione reductase

The presence of some antioxidant components that are absent in mammalian cells makes this pathway a promising target of drug intervention in trypanosomatids. The unusual spermidine-glutathione adduct named trypanothione or N1,N8-bis(glutathionyl)spermidine found solely in these parasites functions as an electron donor in many pathways by neutralizing diverse reactive species through redox reactions, also providing reducing equivalents to intermediate molecules in other antioxidant pathways and in biosynthetic pathways such as DNA synthesis [35, 36]. The catalysis of NADPH-dependent reduction of trypanothione disulfide to $T(SH)_2$ is performed by trypanothione reductase (TR), enzyme that has been proposed as a molecular target, based on the specific inhibition of antioxidant defenses of the parasite [37, 38]. The central role of trypanothione makes other enzymes that influence its production also interesting drug targets such as trypanothione synthetase, ornithine decarboxylase (ODC), S-adenosylmethionine (AdoMet) decarboxylase, γ-glutamylcysteine synthetase as well as polyamine transporters [39, 40].

In the last decades, many TR inhibitors were developed, but only a few had a positive correlation between trypanocidal activity and binding to the enzyme demonstrated [41–43]. Recently, a high-throughput screening of 1.8 million compounds

was performed, and specific inhibitors of *Leishmania* TR were identified. Since this enzyme is considered well-conserved among trypanosomatids, this study could represent a critical step for the identification of inhibitors also for *T. cruzi* TR [44]. Up to the moment, the inhibition of trypanothione metabolism of this parasite was poorly assessed in animal models, and no clinical trial has been reported involving this target (**Figure 3**).

2.1.3 ROS inducers

Varying the dose or the time of drug treatment, injury to the mitochondrion usually leads to ROS production [45]. Despite many compounds having induced mitochondrial alterations, generating ROS, the molecular mechanistic action was not elucidated in most studies. In this section, we will discuss only quinones and nitrocompounds, compounds with oxidative mechanisms of action well-characterized.

Quinones: Chemical properties of quinoidal carbonyls lead to the direct ROS generation [46], and trypanocidal effect of natural quinones and derivatives have been assessed [47–50]. In epimastigotes, the oxidative activity of β-lapachone was first reported almost 40 years ago [51, 52], and increase in ROS levels has also been related to the treatment of *T. cruzi* with other naphthoquinones [53, 54]. In 2009, we proposed the trypanocidal mechanism of action of naphthofuranquinones. Such quinones strongly impaired the parasite mitochondrion by the deviation of the electrons from ubiquinone, culminating in this organelle depolarization, loss of respiratory rates, inhibition of complexes I–III activities, and ROS production [54]. Increased levels of ROS were also detected in parasites after the treatment with other classes of compounds such as pyrazyl/pyridylhydrazones and thiosemicarbazones [55–57].

Nitrocompounds: These compounds are usually avoided in medicinal chemistry approaches because the presence of a nitro group creates concerns regarding toxicity issues associated with DNA damage [58]. Regarding Chagas disease, fexinidazole evaluated *in vivo*, led to high cure rates and reduced myocarditis [59]. Subsequently, a phase II clinical trial was performed in chronic chagasic patients in Bolivia using fexinidazole treatment (NCT02498782), and it was observed that parasitemia was cleared; however, after recruiting 47 participants, some safety and tolerability issues arose, and it was decided to conclude the trial without the inclusion of new participants. After a 12 month follow-up, a high efficacy rate was evidenced, without relapses [60]. Therefore, a new proof of concept study was initiated in Spain in 2017, with the results expected in 2019 (EudraCT Number 2016-004905-15).

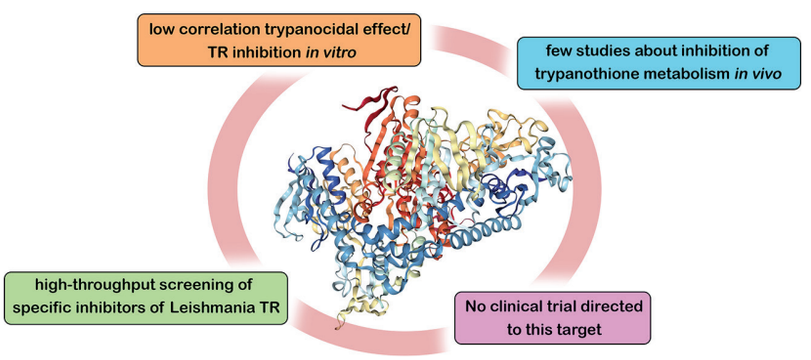

Figure 3.
Landmarks in the investigation of TR as a drug target. Despite many efforts up to now, specific inhibitors of this enzyme presented neither important trypanocidal activity in vitro nor in vivo. TR DPB ID: 1BZL.

Surprisingly, trypanocidal action of Nif and Bz is still controversial. Nif has the oxidative activity demonstrated in the early 1980s, being hydrogen peroxide and superoxide anion production detected, while no reactive species was found after the treatment with Bz [61]. Bz and Nif are considered prodrugs that require activation by nitroreductases—NTR-I, an oxygen insensitive class catalyzing the two-electron reduction of the nitro group and NTR-II, an oxygen-sensitive class catalyzing one-electron reduction [62]. The mechanistic proposal of Nif involves nitroanion radical metabolization by NTR-II, followed by reoxidation by molecular oxygen to form superoxide anion ($\bullet O_2^-$), which is converted to hydrogen peroxide (H_2O_2) under catalysis by SOD (**Figure 4**) [63]. On the other hand, low molecular weight thiol reduction together with no redox cycling in trypanocidal doses supported the hypothesis that oxidative effect was not involved in the parasite killing by Nif [64]. Additionally, NTR-I activity has been related to the trypanocidal effect of Nif and Bz through a two-electron reduction in the nitro group. In an oxygen-independent way, the production of nitroso and hydroxylamine intermediates led to amine generation, using NADH as a cofactor. The cleavage of the Nif furane ring produces a highly reactive unsaturated open chain nitrile [65].

Recently, some highly potent 3-nitro-1H-1,2,4-triazole derivatives emerged as excellent substrates for NTR-I, but the enzymatic activity was not required for the trypanocidal activity [66]. Alternative enzymes have been associated with the reduction of nitro compounds in *T. cruzi*, indicative of a secondary action for these drugs, and further studies about the molecular mechanisms involved must be performed. The high trypanocidal activity together with the identification of exclusive nitroreductases in trypanosomatids supports the hypothesis of selectivity [63].

2.1.4 Polyamines

Polyamines (PA) are ubiquitous organic polycations that play a plethora of ubiquitous biological roles in most cell types, including bacteria, protozoa, and higher organisms [67], with significant metabolic differences, therefore comprising promising drug targets for protozoal diseases [68]. PA metabolism among parasitic protozoa is defective in a number of pathways as compared to mammalian cells. *T. cruzi* parasites lack ODC and so are auxotrophic for the diamine putrescine [69]. Therefore, the protozoan relies on the diamine uptake from the extracellular milieu via surface transporters or permeases [70], and so, these mechanisms comprise targets for chemotherapy agent development [71], and pentamidine was shown to inhibit polyamine transport by *T. cruzi* [72]. Putrescine uptake is required for the massive infection [73] and scape from stress conditions [74]. Spermidine is synthesized by the transfer of an aminopropyl group from decarboxylated S-adenosyl-L-methionine to putrescine and takes part in the biogenesis of T[SH]2 a pivotal adduct in oxidative stress endurance and involved in anti-*T. cruzi* drug

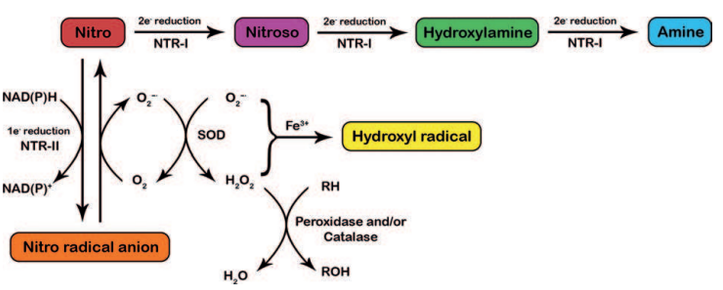

Figure 4.
Metobolization of nitrocompounds by NTR-1 and NTR-II. ROS is generated by reoxidation (one electron route). Two-electron reduction produces hydroxylamine intermediates and reactive nitroso [63].

resistance [75]. In this regard, the putrescine analog DAB (1,4-diamino-2-butanone) promotes oxidative stress in *T. cruzi* [76] and leads to *T. cruzi* mitochondrial destruction [77]. It is noteworthy that DAB not only is involved on reactive species production but also inhibit putrescine synthesis [78] and incorporation [79].

Polyamines may play multiple functions in parasite endurance under oxidative stress conditions, not only for TSH is a spermidine adduct but also because these polycations *per se* may be antioxidant, protecting *T. cruzi* from oxidative stress [80]. Polyamines are also relevant for controlling differentiation, including *T. cruzi* metacyclogenesis [81]. Thus, the enzymes involved in polyamine and TSH metabolism provide important drug targets for potential anti-*T. cruzi* therapy [40].

2.2 Biosynthesis of sterols

Sterols are essential lipid molecules, performing numerous cellular roles associated with membrane and signal functions [82]. Cholesterol is biosynthesized in humans, whereas ergosterol or other 24-alkylated sterols are biosynthesized in opportunistic fungi and parasitic protozoa and such difference is exploited in the drug development [83]. *T. cruzi* and related trypanosomatids have a strict requirement for endogenous sterols (ergosterol and analogs) for survival that cannot be replaced by cholesterol found in the host. Thus, the biosynthesis of sterols is a major target in the drug development for Chagas disease [84]. Among enzymes of the sterol metabolism, squalene synthase (SQS) and C14α-sterol demethylase (CYP51) have been intensively investigated as drug targets (**Figure 5**).

2.2.1 Squalene synthase (SQS)

This enzyme catalyzes the dimerization of two molecules of farnesyl pyrophosphate (FPP) to produce squalene. This enzyme is under study as a possible target for cholesterol-lowering agents in humans [85]. SQS is a membrane-bound enzyme

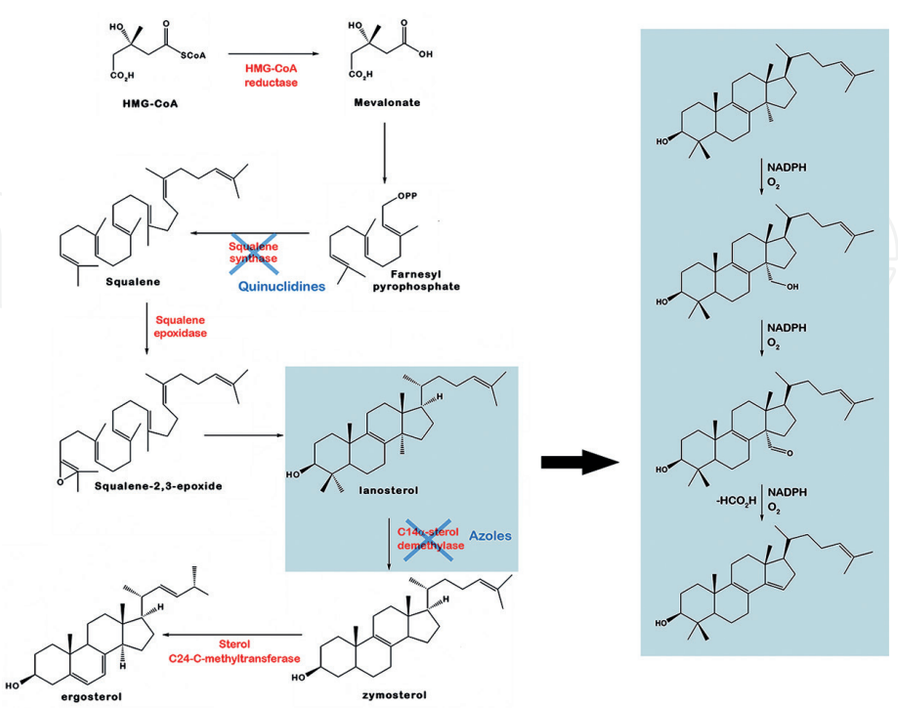

Figure 5.
Ergosterol biosynthesis is an important drug target in T. cruzi. In red, the enzymes described as molecular targets and in blue, the classes of the speciffic inhibitors. Inside gray boxes, the intermediary steps of the conversion of lanosterol into 4,4-dimethyl-5a-cholesta-8,1,4,24-3b-ol by sterol 14-α-demethylase (CYP51).

in *T. cruzi* epimastigotes, being distributed between glycosome and mitochondrial/microsomal vesicles [86]. FPP is a branching point in isoprenoid biosynthesis: conversion to squalene and sterols by SQS or synthesis of other essential isoprenoids. The quinuclidine-based inhibitors of mammalian SQS, 3-(biphenyl-4-yl)-3-hydroxyquinuclidine (BPQ-OH) ER27856, E5700, and ER-119884 were assayed against *T. cruzi*, leading to the *in vitro* inhibition of epimastigote and intracellular amastigote proliferation, depletion of endogenous squalene and sterols, and marked ultrastructural alterations [86, 87] and *in vivo* E5700 led to 100% survival and parasitemia negativation [88]. However, E5700 and ER-119884 have no selectivity toward the parasite enzyme in comparative assays with the recombinant human enzyme [89]. In 2014, the X-ray crystallographic structure of SQS from *T. cruzi* was reported, confirming the binding of the enzyme to distinct classes of inhibitors such as the quinuclidines E5700 and ER119884 and the thiocyanate WC-9 opening possibilities to the development of alternative inhibitors [90].

In a screening of compounds containing the 4-phenoxyphenoxy skeleton, 4-phenoxyphenoxyethyl thiocyanate (WC-9) was highlighted due to the high activity against the proliferative form epimastigotes (low micromolar) and intracellular amastigotes (nanomolar) and a potent inhibitor of the enzymatic activity of both glycosomal and mitochondrial isoforms of SQS [91, 92]. Since then, different series of WC-9 analogs have been developed [91, 93], including seleno-containing analogs resulting in compounds, such as 4-phenoxyphenoxyethyl selenocyanate, with EC_{50} values at low nanomolar level and selectivity index (SI) higher than 900 [94].

2.2.2 C14α-sterol demethylase (CYP51)

This enzyme catalyzes the oxidative removal of the 14α-methyl group from of catalyzing the oxidative removal of the 14α-methyl group from sterol precursors such as lanosterol or eburicol, via a repetitive three-step process that uses NADPH and oxygen to produce 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol [83]. CYP51s are the most conserved cytochrome P450 enzymes [84]. Series of azoles originally developed for the treatment of fungal infections targets this enzyme leading to accumulation of lanosterol and other sterol intermediates and displaying activity *in vitro* and *in vivo* against *T. cruzi* [95–97]. This line of investigation led to the selection of the triazole posaconazole [98, 99] and E1224 (fosravuconazole) [100, 101] for Phase II clinical trials with chronic patients, which, however, led to therapeutic failure as compared to benznidazole, with parasitemia relapses: NCT01162967 (Chagazol) [102], NCT01377480 (Stopchagas) [103], and NCT01489228 (E1224 trial) [104].

VNI, a carboxamide-containing β-phenyl-imidazole, identified from a Novartis collection of azoles, was active in acute and chronic mouse models using Tulahuen strain [105]; whereas in experiments with other parasite strains, no complete parasitological clearance was achieved [106]. In subsequent work, VFV, a fluoro analog of VNI, designed to fill the deepest portion of the CYP51 substrate-binding cavity demonstrated 100% efficacy in experimental infection, displaying favorable oral bioavailability and pharmacokinetics [107]. Comparison between VNI and VFV, in murine models of infection, revealed that regardless of the treatment scheme or delivery vehicle, VFV was more potent in both genders [108]. VT-1161, a 1-tetrazole-based drug undergoing phase II antifungal clinical trials, is active *in vitro* and *in vivo* against *T. cruzi*. It was structurally characterized in a complex with TcCYP51, allowing for the optimization of new tetrazole-based analogs and presents good pharmacokinetic properties and an excellent safety profile [109]. Friggeri et al. [110] synthesized imidazolyl-2-phenylethanol derivatives, and several of them were active against intracellular amastigotes and inhibited TcCYP51. In sequence, eight new derivatives were prepared and assayed against the parasite, and the most active

was a piperazinyl-carbamate derivative at nanomolar range, low cytotoxicity, and good chemical and metabolic stability [111]. Recently, a series of pyrazolo[3,4-e][1,4]thiazepin analogs, novel CYP51 inhibitors, were investigated revealing *in vitro* and *in vivo* activity against *T. cruzi*, with several analogs displaying effect at low micromolar dosis and low host toxicity [112].

2.3 Cysteine proteases

Cysteine proteases are intensively used as molecular targets in trypanosomatid disease drug discovery efforts. Target-based screening, structure-based drug design, and medicinal chemistry approaches targeting cysteine proteases are strategies intensively used in the development of drugs for diseases caused by pathogenic trypanosomatids. *T. cruzi* cysteine protease named cruzipain (or cruzain) is a cathepsin-L-like protease of the papain family and is essential for the intracellular replication, differentiation, and immune evasion of the parasite [113, 114]. Three-dimensional structures of cruzain with different ligands have been reported, allowing the design and synthesis of new hit compounds [115]. Based on the interaction with its active site, enzyme inhibitors have been classified as irreversible, forming covalent bonds with cysteine sulfur, or as reversible, forming 1,2-adducts with cysteine that are generally unstable [116]. Among irreversible peptidyl inhibitors of cruzipain, we highlight diazomethyl ketones, allyl sulfones, vinyl sulfonamides, and vinyl sulfones, including K777 and its arginine variant WRR-483 [117–119]. Among nonpeptidyl inhibitors of cruzipain, we found thiosemicarbazones, thiazolylhydrazones, thiazoles, and oxadiazoles (**Figure 6**) [120].

Another group of compounds that has been studied as cruzipain reversible inhibitors are those containing a nitrile head: purine nitriles [121], nitrile analogs of odanacatib [122, 123], and nonpeptidic nitriles [124]. Salas-Sarduy et al. [125] identified two new cruzipain inhibitory scaffolds from GlaxoSmithKline HAT and Chagas chemical boxes, both containing a nitrile moiety, with major structural differences between them. Benzimidazoles and oxidiazoles have also been explored as noncovalent cruzain inhibitors, using an approach combining high throughput and virtual screenings [126, 127].

Development of cruzipain inhibitors by structure activity relationship (SAR) studies, combinatorial chemistry, HTS, and virtual screening are also employed in repositioning strategies [128]. Bromocriptine (antiparkinson and antidiabetic

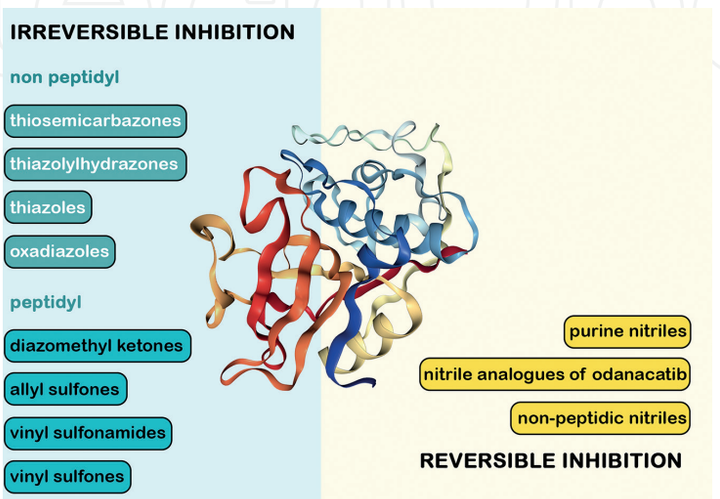

Figure 6.
Cruzipain as a molecular target in T. cruzi. Irreversible and reversible inhibition was demonstrated by different classes of compounds. Cruzipain PDB ID: 3I06.

drug), amiodarone (antiarrhythmic drug), and levothyroxine (hypothyroidism drug) were selected in a screening campaign for cruzain inhibitors of the DrugBank database [129], clofazimine (antileprosy drug) and benidipine (antihypertensive) from the Merck Index 12th database [130, 131], and etofyllin clofibrate (antilipemic drug) and piperacillin, cefoperazone, and flucloxacillin (β-lactam antibiotics) from a collection of 3180 FDA drugs [132].

Calpains are calcium-dependent nonlysosomal cysteine peptidases highly conserved among eukaryotes, but their precise biological function is not completely clear. In mammalian cells, calpains participate in many different calcium processes including proliferation, differentiation, cytoskeletal assembly, cellular signaling, among many others; however, *T. cruzi* calpains do not present a mapped active catalytic site up to now [133]. In man, uncontrolled activity of calpains has been associated with muscular and neurological disorders such as Alzheimer, Parkinson, multiple sclerosis, and arthritis, and the terapeutic effect of specific calpain inhibitors was suggested [134, 135]. Recently, the repurposing of calpain inhibitors was also postulated for neglected tropical diseases, including Chagas disease [133]. In *T. cruzi*, only the inhibitor MDL28170 was tested, and the trypanocidal activity at low micromolar range was shown on all three parasite forms, impairing the ultrastructural architecture of Golgi and reservosomes [136, 137]. Despite the study's scarcity, calpains inhibition has been suggested as an attractive antitrypanosomatid approach even without the confirmation of their proteolytic activity in these parasites.

2.4 Nuclear and kinetoplast DNA

Both *T. cruzi* kinetoplast and nucleus may be targeted by different classes of compounds with antiparasitic activity [138, 139]. Early studies [140] revealed that hydroxystilbamidine led to the disorganization of kinetoplast DNA (kDNA). Later other compounds such as vinblastine, geranylgeraniol, diaminobenzidine, and aromatic diamidines were reported to affect kDNA arrangement causing its fragmentation [138, 141, 142]. Trypanosomatid nucleus and kinetoplast display topoisomerases that show significant structural differences from host orthologs advocating their potential as drug targets [143]. Interestingly, *T. cruzi* topoisomerase I is inactivated by ROS [144], so the oxidative stress induced by both immune response and trypanocidal agents may also affect parasite chromatin organization.

Classic aromatic diamidines have been shown to bind noncovalently and through a nonintercalative manner to the minor groove of DNA; several hypotheses regarding their mode of action were proposed. They could act by complexation with DNA and subsequently lead to a selective inhibition of DNA-dependent enzymes and/or through the direct inhibition of transcription [145]. Thus, evidences suggest that diamidines interfere in the kinetoplast function of trypanosomatids through a selective association to the unique AT-rich regions of kDNA minicircles, perhaps involving DNA-processing enzymes [146]. Medicinal chemistry studies pointed to arylimidamides (AIAs) as the most promising antimicrobial diamidines [106]. DB702, DB786, DB811, and DB889 presented anti-*T. cruzi* activity in the low-micromolar range and led to ultrastructural alterations mainly associated with the nucleus and mitochondrion [147, 148]. On the other hand, recent study with novel bis-AIAs revealed their higher potency and *in silico* analysis showed DNA as the main target, but no DNA ultrastructural alterations were found [149].

On the other hand, enzymes involved in nucleic acid metabolism could be also promising targets. Topoisomerases play a crucial role for the DNA dynamics during the transcription, replication, or even in the repair. Due to their participation in essential cellular processes, interfering with DNA topology, and consequently leading to physiological implications, topoisomerases have been described as molecular

targets for cancer and also parasitic illnesses such as Chagas disease. Up to now, innumerous topoisomerase inhibitors presented antitrypanosomatidae activities such as camptothecin, doxorubicin, etoposide, suramin, among many others [150]. Recently, voacamine and an isobenzofuranone derivative induced important morphological alterations in different trypanosomatids, including *T. cruzi*. In Leishmania parasites, the most affected organelle was mitochondrion (severely swelled presenting membrane depolarization), where the derivative led to kinetoplast network disorganization [151, 152].

3. Proteomic insights for the target identification in the parasite

The evaluation of the proteomic profile in trypanosomatids is particularly interesting because these protozoa exhibit open reading frames in long polycistronic regions, and the regulation of gene expression occurs only post-transcriptionally, justifying the importance on monitoring the protein expression by proteomic approach [153]. This section will focus on proteomic analysis of parasite forms dwelling in mammalian hosts. The first large-scale analysis was performed by Atwood and colleagues in 2005 [23], identifying 1486 proteins of culture-derived trypomastigotes, and 30 trans-sialidases, enzymes that play an important role in parasite host cell invasion, were among the top-scoring proteins exclusively detected in this developmental form. *T. cruzi* surface subproteome and basic proteins analysis confirmed the high distribution of trans-sialidases in this life form [154, 155]. Specific trypomastigote surface analysis also revealed membrane-associated enzymes that are involved in biosynthetic pathway of phospholipid and glycolysis [155]. The evaluation of chromatin fraction of this stage revealed RNA-binding proteins and histones, representing 29% of chromatin protein content [156], providing new insights into gene expression and histone modifications involved in the parasite cycle regulation. Likewise, *T. cruzi* glycoproteome was assessed, and trypomastigote-specific glycoproteins were identified, including mucin family members [157, 158].

In metacyclic trypomastigotes, Atwood and co-workers [23] identified 2339 proteins, and different antioxidant enzymes were among the main proteins detected. The presence of these enzymes in this stage could be related to the parasite adaptation to the oxidative environment inside the vertebrate host circulation and particularly inside the phagocytes. The analysis of metacyclogenesis revealed increased expression of cytoskeletal proteins as well as proteins related to energetic and oxidative metabolisms, suggestive of the morphological and metabolic reorganization [159]. Plasma membrane subproteome pointed to a large repertoire of surface proteins in this parasite stage, including trans-sialidases, mucins, and GP63 protease [160]. Such glycoprotein diversity confers adaptation of the parasite to distinct environmental conditions. Morever, secretome of metacyclic trypomastigotes also demonstrated trans-sialidases and other surface molecules, playing a role in parasite invasion during acute and chronic infections [161, 162]. The blockage of this process could be an interesting strategy in novel drug development.

The first proteomic analysis of bloodstream trypomastigotes was performed by our group in 2015, identifying a total 5901 proteins [163]. In this work, a comparison among the proteomic maps of trypomastigotes (bloodstream, cultured-derived, and metacyclic forms) was also assessed, and 2202 proteins related to the parasite surface, cytoskeleton, redox metabolism, cell signaling, and energetic metabolism were exclusively detected in bloodstream forms. Overall, the proteomic profile of bloodstream form comprises an important tool to discover potential new drug targets and novel antigens for vaccines or diagnostics. The differences in the

trypomastigote proteomic profiles were expected due to their environment, and huge number of stage-specific proteins in bloodstream forms, probably triggered by the exposure to the host immune system reinforces the necessity for drug validation on this developmental form. In relation to proteomic evaluation of trypanocidal action of drugs, β-lapachone-derived naphthoimidazoles induced the increase in the abundance of 27 proteins, involved in stress response, cell structure, energetic metabolism, nucleic and amino acid metabolisms, oxidative metabolism, among other pathways [164]. This large-scale study revealed an important set of proteins belonging to metabolic pathways that play pivotal functions for this parasite form, providing new insights for the understanding of the parasite biology and of potential drugable molecules for the treatment of Chagas disease.

In 2005, 1871 proteins of culture-derived amastigotes were identified, preferably involved in endoplasmic reticulum to Golgi trafficking, suggesting an intense traffic at this stage [23]. The analysis of amastigogenesis evidenced high abundance of glycolytic enzymes in amastigotes as well as the lower abundance of flagellar components, compatible with the morphology of this stage [165]. Later, the surface subproteome of vertebrate-dwelling parasite forms was characterized, displaying molecules involved in cell division, signal transduction, and lipid metabolism, crucial for the parasite intracellular self-maintenance [155].

Another interesting target is the posttranslational modification of parasite proteins. Acetylation at lysine residues exerts important role in both vertebrates and microbial cells. The NAD^+-dependent lysine deacetylases are termed sirtuins. Humans present seven different sirtuins, whereas *T. cruzi*, solely two. TcSIR2RP1 andTcSIR2RP3 are found in the cytosolic and mitochondrial compartments, respectively. Parasites overexpressing TcSIR2RP1 display enhanced metacyclogenesis and host cell infection [166]. The sirtuin antagonist salermide diminishes intracellular parasite proliferation and parasitemia in murine infection [167]. Thus, acetylation of *T. cruzi* proteins may provide useful targets for the development of antiparasitic agents [167, 168]. In addition, mammalian sirtuin targeting may be beneficial in chronic chagasic cardiomyopathy [169].

4. Conclusions

The clinical chemotherapy for Chagas disease (Nif and Bz) led to a parasitological cure in the great number of congenital, adult acute, or early chronic cases [170]. However, undesirable side effects and the resistance of some parasite strains [171], together with the limited efficacy in symptomatic chronic cases, drive the continuous search for novel chemotherapeutic agents [33]. Drug repurposing or even combinations with the current drugs could be options to minimize this problem [59, 120]. In this direction, phenotypic strategy has been considered the most valuable approach for the screening of antiparasitic compounds [172].

High throughput screening complemented by whole-cell phenotypic assays represents the more feasible option in the search for novel anti-*T. cruzi* compounds, also leading to an increment in sensitivity [173]. Preclinical *in silico* combined to *in vitro* assays represents an essential step for the recognition of *T. cruzi* drug targets, generating knowledge about the metabolism, biochemical pathways, or biological processes allowing the target validation. In this way, the identification of selective targets comprises a logical startpoint to the reduction of the side effects in the hosts [153, 163]. Ultrastructural observations can predict the potential primary cellular targets due to their earlier alterations triggered by pharmacologic stimuli, helping the prospection of molecular modes of action of antiparasitic agents [141, 174].

Large-scale proteomics represents an alternative approach for the assessment of molecular mechanisms of trypanocidal drugs. Indeed, despite its potential, such technique is poorly employed in this context. The importance of the use of clinical relevant *T. cruzi* stages in drug screening was evidenced by the remarkable differences found among the proteomic profile of parasite forms [23, 163]. The proteomic analysis of bloodstream trypomastigotes identified a huge variety of proteins from distinct biological processes, pointed to more than 2000 proteins present only in bloodstream forms (not in trypomastigotes from other sources), reinforcing the importance of the chemotherapeutic tests using recent isolated parasites from animals' blood [163].

In the current scenario, the CYP51 still represents one the most promising alternatives. Large-scale screening pointed to the high activity of CYP51 inhibitors *in vitro* (even higher than Bz), but not producing *T. cruzi* elimination [175, 176]. Unfortunately, the clinical trial with posaconazole and E1224 was also unsatisfactory [102]. In Argentina, a drug–drug interaction analysis of the combination Bz and E1224 in healthy volunteers demonstrated no improvement in tolerability or safety parameters [177], and a clinical study using this combination is planned [178]. Also, the use of new scaffolds with the design of inhibitors much more selective toward CYP51 has demonstrated its high trypanocidal activity in association or not with other licensed drugs [108, 179].

Among the parasite antioxidant defenses, the most promising drug target is TR, and the specific inhibitor development has been proposed in the last three decades [36]. However, no active inhibitors of this enzyme were described up to now [180]. The hypothesis that *T. cruzi* is more susceptible to oxidative species than the vertebrate hosts is an old misleading concept, due to the highly efficient scavengers described in the parasite [51, 181]. Another aspect to be considered is the central role of ROS production and the mitochondrion for the trypanocial action of a great variety of preclinical compounds [31]. The mitochondrial swelling is frequently observed in *T. cruzi* after the treatment with different drugs, but this phenotype is rarely associated with a specific molecular mechanism, except for DNA ligands such as aromatic amidines [106]. The molecular mechanistic proposal opens the possibility of the mitochondrial dysfunction to be as a random consequences of indirect effect triggered by the impaired homeostasis, resulting in redox imbalance [31].

Bioinformatic, proteomic, and ultrastructural analyses are pivotal tools in the identification of drug targets; however, the use of specific inhibitors must be validated before the following studies. The absence of the predicted biological activity or even the specific binding to the respective molecular target is not uncommon [178, 182]. To improve the safety, mechanisms of action characterization should be performed in parallel to the high-throughput screening of the trypanocidal activity [183]. In case of obligatory intracellular parasites as *T. cruzi*, the direct analysis in a phenotypic assay is also essential, considering permeability of the host cells besides the therapeutic window related to the mammalian host toxicity aspects [182]. The adequate choice of the experimental design is also crucial. Animal models, parasite strains, and treatment protocols for preclinical assays (*in vitro* and *in vivo*) must be standardized, in order to reach better translation to humans [184].

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Conflict of interest

None to declare.

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Author details

Marcos André Vannier-Santos[1], Giselle V. Brunoro[2], Maria de Nazaré C. Soeiro[3], Solange L. DeCastro[3] and Rubem F.S. Menna-Barreto[3]*

1 Oswaldo Cruz Institute, FIOCRUZ, Rio de Janeiro, Brazil

2 Centre of Excellence in New Target Discovery, Butantan Institute, São Paulo, Brazil

3 Laboratory of Cellular Biology Cellular, Oswaldo Cruz Institute, FIOCRUZ, Rio de Janeiro, Brazil

*Address all correspondence to: rubemsadok@gmail.com

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