Send Orders for Reprints to reprints@benthamscience.ae

Recent Patents on Anti-Infective Drug Discovery, 2016, *11*, 74-173

REVIEW ARTICLE

Targets and Patented Drugs for Chemotherapy of Chagas Disease in the Last 15 Years-Period

Vilma G. Duschak*

Instituto Nacional de Parasitología "Dr. Mario Fatala Chaben", ANLIS-Malbrán, Ministerio de Salud de la Nación. Av. Paseo Colón 568 (1063), Buenos Aires, Argentina

ARTICLE HISTORY

Received: April 4, 2016
Revised: September 6, 2016
Accepted: October 24, 2016

DOI: 10.2174/1574891X11666161024165304

Abstract:

Background: The American trypanosomiasis, Chagas disease, is a parasitic infection typically spread by triatomine vectors affecting millions of people all over Latin America. Existing chemotherapy is centered on the nitroaromatic compounds benznidazole and nifurtimox that provide unsatisfactory results and substantial side effects. So, the finding and exploration of novel ways to challenge this neglected disease is a main priority.

Methods: The biologic and biochemical progress in the scientific knowledge of *Trypanosoma cruzi* in the period comprising last 15-years has increased the identification of multiple targets for Chagas´ disease chemotherapy. In the middle of the best encouraging targets for trypanocidal drugs, ergosterol biosynthesis pathway and cruzipain, a key cysteine protease (CP) of *T. cruzi*, have been pointed out. Unfortunately, recent clinical trials investigating the administration of pozoconazole and ravuconazole to chronic indeterminate Chagas disease patients revealed their inferiority compared to the standard drug Benznidazole.

Results: In view of the information gained in the preceding years, a reasonable approach for the fast development of novel anti-*T. cruzi* chemotherapy would be focused on K777, the cysteine proteinase inhibitor (CPI) near to enter to clinical trials, and founded on the clinical evaluation of combination of known drugs with existing trypanocidal agents to obtain more efficiency and less secondary effects.

Top series of xanthine have been recently identified as clinical candidate for Chagas disease. In addition, trypanothione biosynthesis, thiol-dependant redox and polyamine metabolism, the glycolytic, glyconeogenic, pentose phosphate, lipidic and polyisoprenoid biosynthetic pathways, and the enzymes from biosynthetic glycoconjugates pathways have been studied. Several specific enzymes from these particular biosynthetic pathways such as hypoxanthine-guanine-phosphoribosyl-transferase and farnesyl-pyrophosphate synthase, among others, have also been broadly studied in *T. cruzi*. Novel synthesized anti-*T. cruzi* compounds with or without specific single or multi-target assigned are also described in detail.

Conclusion: In summary, loans on anti-Chagas disease agents focused to specific parasite targets as their metabolic pathways or specific enzymes will be summarized. Targets will also be specifically discussed. Patent literature collected and published from 2000 to 2015, alleging inhibitors for specific *T. cruzi* targets or trypanocidal activity was achieved over the search database from Delphion Research intellectual property network including international patents and the European patent office, Espacenet.

Keywords: Chagas disease, *Trypanosoma cruzi*, drug targets, patents.

1. INTRODUCTION

American Trypanosomiasis, a main health problem caused by the parasitic protozoan *Trypanosoma cruzi* is endemically spread in vast areas of Latin America. The complex life cycle of *T. cruzi* contains proliferative stages in the vector (epimastigotes) and in the vertebrate (intracellular amastigotes), in addition to non-proliferative infectious stages (trypomastigotes) in both hosts. Even though Chagas disease transmission has been dramatically decreased in several countries by control of the domestic triatomine vector, sero-

*Address correspondence to this author at the Area de Bioquímica de Proteínas y Glicobiología de Parásitos. Departamento de Investigación. Instituto Nacional de Parasitología "Dr. Mario Fatala Chaben", ANLIS-Malbrán, Ministerio de Salud de la Nación. Av. Paseo Colón 568 (1063), Buenos Aires, Argentina; Tel: + 541143314010; Fax: +541143317142; E-mail: vduschak@conicet.gov.ar

2212-4071/16 $100.00+.00 © 2016 Bentham Science Publishers

Recent Patents on Anti-InfectiveDrug Discovery

logical screening of blood donors and insecticide spraying [1, 2], the parasite remains usually being transmitted by infected triatomine bugs. Transmission also ensues by organ transplantation or blood transfusion, from mother to newborn, and seldom due to eating or drinking contaminated aliments [3-7]. Although the control of natural transmission by insect vectors has been achieved in several countries, natural transmission persists in certain areas of Latin America. Currently, the quantity of infected people globally estimated by the World Health Organization (WHO) amounts to 7 to 8 million and more than 10,000 deaths are thought to occur annually [8]. The disease has also emerged as a public health problem elsewhere in non-endemic countries of the word due to transmission of *T. cruzi* by people migration [9, 10].

Three clinical phases, acute, indeterminate and chronic characterize the disease. In the acute phase, just in the place where metacyclic trypomastigotes enter, a local inflammatory lesion appears, while the parasites spread through the host organism. During the acute phase of this infection, parasitemia is frequently high and diagnosis can be made by the microscopic examination of blood, searching for trypomastigotes blood-forms of the parasite. The indeterminate phase includes a period generally symptomless, between the acute and chronic phases that may last from ten to twenty years. By contrast, the presence of myocarditis and/or pathological disturbances in the peripheral nervous and gastrointestinal systems is the characteristics of the chronic phase. The percentage of chronic infected individuals that develop cardiac abnormalities is comprised between 30 to 40% and around 10% in those that develop digestive tract disease [11]. The most relevant clinical manifestation is chronic chagasic cardiomyopathy, causing sudden death and heart failure [12].

A remarkable heterogeneity has been detected among the results obtained using different methods when searching for the presence of *T. cruzi*-specific immunoglobulin G (IgG), during the chronic stage of Chagas disease. Therefore, diagnosis can be serologically performed by means of enzyme-linked immunosorbent assay, indirect immunofluorescence, indirect hemagglutination, or immunochromatography techniques. Then, at least, two different kinds of serological tests are used to analyze each potentially infected subject or sample. It is worth noting that there is substantial risk of finding false positive results with individual tests due to cross-reactivity of anti-*T. cruzi* with antigens of strictly related species of trypanosomatids [13-16]. By using antigenic carefully purified samples prior to examination and choosing tests with the maximum specificity available can diminish the possibility of obtaining false positive results with serological assays. Additional diagnostic tool for *T. cruzi* infection is the polymerase chain reaction (PCR) used in order to evaluate the presence of *T. cruzi* DNA. Although auspicious results for operational use of PCR in the diagnosis of *T. cruzi* were considered, in those cases showing that serological diagnosis was particularly limited, such as HIV co-infected patients and in neonates with low parasitemia, the favorite method for diagnosis is still a combination of serological methods due to higher sensitivity, commercial availability, and lower heterogeneity [13-15, 17].

Only benznidazole (Bz) and nifurtimox (Nx) have been plenty effective to deserve extensive use in the treatment of Chagas disease. To some extent, Bz, a 2-nitroimidazole pro-drug that requires nitroreduction to become active, induces the creation of free radicals and other metabolites capable to bind to the nuclear and mitochondrial DNA of the parasite producing lethal DNA strand breaks [18]. Nx also takes advantage of the parasite's susceptibility to oxygen radicals by inhibiting the enzyme function required by the parasite for detoxifying this kind of compounds [19]. Existing chemotherapy of Chagas disease centered on these nitroaromatic compounds is debatable because it is responsible for unacceptable results, undergoes significant side effects and is effective only for acute and indeterminate stages of the infection. During the chronic phase of the disease, its usefulness is controversial and resistance to Nx has been accepted. However, some information related to the treatment of chronically infected patients has been early reported to improve cardiac grade [20, 21]. Even though the existing agents display severe toxicities, treatment of chronic patients has been considered suitable during the last years as it frequently reduces clinical pathology [22-24]. The use of Bz may determine a minor risk to heart function than Nx in cases than any cardiac dysfunction is present [25, 26]. Existing treatment protocols are statistically curatives in about 60% of all acute Chagas cases, but only 10 to 20% for symptomatic chronic Chagas disease cases have been successful. Treatment with Bz is noticeably

more effective and better tolerated in *T. cruzi*-infected children than in adults. The general rate of curative therapy has been informed as 71% for acute cases of Chagas disease in children, with more than 90% cure rates described in cases of congenital infection when treatment was administered within the first year of life [4, 27-30]. A stimulating alternative for optimizing the paediatric treatment of Chagas disease was the obtainment of a paediatric oral liquid Bz suspension [31]. In children up to 14 years old, the cure rate of recent chronic Chagas disease has been stated as 58%. However, this percentage signifies a minority of all chronic Chagas patients, as the preponderance of chronic Chagas patients is between 15 and 69 years old [28, 32, 33]. Taking into account that Nx and Bz are distant from the requests to consider them ideal as trypanocidal drugs (very safe, very effective, very stable and inexpensive) in conjunction with the results obtained in trials with allopurinol that in the last decade did not show good results [34], searching for new agents with anti-*T. cruzi* activity, with low toxicities and improved efficacies during the indeterminate and chronic phases, is still under development. In 2014, members of the NHEPACHA network ("Nuevas Herramientas para el Diagnóstico y la Evaluación del Paciente con Enfermedad de Chagas/New Tools for the Diagnosis and Evaluation of Chagas Disease Patients"), based on clinical and immunological evidence, have considered that the etiological treatment should be obligatory for all adult chronic Chagas disease patients, arguing in favor of antiparasitic treatment for all chronic patients [35]. In addition, WHO also recommended specific antiparasite treatment for all chronic-phase *T. cruzi*-infected individuals. This consensus seemed to be an important scientific advance that requires a careful evaluation of the security and effectiveness of currently available drugs (Bz and Nx) as well as of new trypanocidal drug candidates in chronic patients, who had been previously excluded from such treatment. While Bz is efficient in reducing the levels of circulating parasites in most of these patients, side effects can determine treatment discontinuation between 10-20% of cases. Therefore, prevention of chronic chagasic cardiomyopathy by treating infected populations with trypanocidal therapy remains a challenge. Despite the evidences obtained by treatment therapy, discrepancy of the data for patient-important outcomes must be treated with carefulness. Geographically diverse controlled trials testing newer forms of treatment therapy have been necessary in order to estimate efficacy more precisely, explore factors potentially responsible for the heterogeneity of results and increase knowledge on the equilibrium between efficacy and tolerance of conventional treatment therapy [36]. In this sense, the final report of a multicenter randomized study involving more than 2500 patients with Chagas' cardiomyopathy treated with Bz or placebo for up to 80 days and followed about a lustrum, concluded that trypanocidal therapy with Bz in patients with established Chagas' cardiomyopathy significantly reduced serum parasite detection but did not significantly reduce cardiac clinical deterioration through 5 years of follow-up [37]. Unfortunately, the role of trypanocidal therapy in patients with established Chagas' cardiomyopathy is unproven yet.

The identification of novel trypanocidal agents may be founded on rational drug design and natural products screening [38, 39]. However, there is a crucial necessity to identify either specific enzymes or metabolic pathways in the parasite valuable as potential targets for drug expansion. On the other hand, as a consequence of the parasite genome sequencing project [40], the chance of identifying new specific pathways and novel drug targets has been released since 2005. In the preceding 15-years period, new progresses in the biologic and biochemical knowledge on *T. cruzi* have allowed the identification of novel targets for Chagas disease chemotherapy. Among auspicious targets for antiparasitic agents, proteinases, in particular cysteine proteases (CPs), ergosterol biosynthesis, thiol-dependent redox metabolism and isoprenoids biosynthetic pathways are well-known. In addition, polyamine metabolism and transport pathways; the enzymes of the glycolytic, glyconeogenic and pentose phosphate biosynthetic pathways; lipidic (alkyl-lysophospholipids, glycosphingolipids) biosynthesis, sialic acid transference and purine salvage pathways including nucleotide synthesis, proline pathway (proline racemase), and enzymes from biosynthetic glycoconjugates pathways; as well as some specific parasite enzymes including farnesyl-pyrophosphate synthase, hexokinase, enzymes of trypanothione synthesis and redox metabolism, hypoxanthine-guanine phosphoribosyl- transferase (an essential enzyme from purine salvage), arginine kinase, protein kinases and protein tyrosine phosphatases have been also extensively studied in *T.*

cruzi and related organisms. Additionally, some organelles roles including DNA modulation in nucleus and kinetoplast, involving topoisomerases, as well as the exchanger Na^+/H^+ mechanism from acidocalcisomes, some membrane components, such as receptors, contractile vacuole complex and osmoregulation, the glycosome and vitamin C synthesis and mitochondrial components have been also reflected as promising targets for antiparasitic agents. Recently, cellular cycle and programmed cell death (PCD) in *T. cruzi* have been also taken into account as potential targets. Thus, herein, the novel biotargets defined in the last years and the groups of novel anti-Chagas disease compounds with or without specific single or multi-target assigned will be also described in detail. The most studied targets for antiparasitic drugs are the ergosterol biosynthesis pathway and the main cysteine protease (CP) of *T. cruzi*, cruzipain. Unfortunately, recent clinical trials investigating the treatment of chronic indeterminate Chagas disease with the ergosterol biosynthetic inhibitors (EBIs) pozoconazole and ravuconazole revealed their inferiority compared to the current standard drug Bz.Taking into account the results obtained in the last 15 years, a reasonable approach for the prompt development of new anti-*T. cruzi* chemotherapy would be focused on K777, the cysteine proteinase inhibitor (CPI) near to enter to clinical trials, and based on the proven evaluation of drugs combination with existing trypanocidal agents in order to obtain more efficiency and less secondary effects. Interestingly, lead series of xanthine have been recently identified as clinical candidate for Chagas disease.

In summary, this review aims to present a whole view comprising most advances on anti-Chagas disease agents directed to specific parasite targets and patents referring to their specific drug targets. Metabolic pathways and specific enzymes used as targets and patents will be also argued in detail. The search strategy of patent literature claiming for trypanocidal activity against *T. cruzi* was completed using the search database from Delphion Research intellectual property network including international patents and Espacenet, the European patent office (2000- 2015).

2. TRYPANOSOMA CRUZI TARGETS: ENZYMES, ORGANELLES, AND PROCESSES

Uncommon biochemical pathways, different in numerous aspects from those of mammalian cells are present in *T. cruzi.* This point may offer selective targets for drug development, essentially, metabolic pathways or specific enzymes. To some extent, rational approach to chemotherapy requires target validation as an indispensable step. The desirable characteristics in a drug target should be essentiality, druggability (interaction between drug-like molecules and the target), assayability, importance in life cycle stages of the pathogen relevant to human health, and specificity (absence of the target, or considerably differences with the present in the host).

2.1. Proteinases

These kinds of enzymes have been associated in multiple roles making them striking potential targets for the appearance of new drugs against Chagas disease [41]. Once *T. cruzi* clone CL Brener genome was completely sequenced, seventy cysteine peptidases (CPs), forty serine peptidases (SPs), about two hundred and fifty metallopeptidases (MPs), twenty five threonine peptidases, and only two aspartic peptidases (APs) have been predicted [40]. Among parasite proteinases, in addition to cruzipain, the major CP, expressed as a mixture of isoforms, two metacaspases, two autophagins and a 30 kDa cathepsin B-like enzyme, have been reported not only as digestive enzymes and virulence factors but also as intermediaries in autophagy and PCD processes [42].

2.1.1. Cysteine Proteinases (CPs)

CPs participate in the regulation of a wide range of biological effects. Regarding that the survival mechanisms of the parasite may be potentially impaired by specific inhibition of this kind of enzymes, they are promising targets for chemotherapy. Cruzipain (Cz), also named as cruzain and GP57/51 [43-45], is expressed by the developmental forms of the parasite cycle having microheterogeneities [46]. Although the bulk of the enzyme is lysosomal, parasite reservosomes also contain it. Besides, some plasma membrane bound isoforms [47] and Cz released forms into the medium [48] have been reported. In *T. cruzi*, the enzyme bears a catalytic domain and a peculiar C-terminal extention (C-T) [49]. This immunodominant antigen in human chronic Chagas disease was associated with virulence [50], the interaction between plasma membrane-bound isoforms with alpha-macroglobulins was described [51] and the humoral immune response to Cz was associated with

the severity of chronic disease [52]. Knowing that sialylation is a surface reaction in *T. cruzi,* the detection of membrane bound isoforms of Cz, turned interesting to identify the presence of sialic acid in the C-T of Cz. In parallel, N-acetyl-D-glucosamine has been also determined in O-glycosidic linkages [53]. These findings might help to elucidate the migratory route followed by Cz. Strikingly, we have provided evidence indicating O-GlcNAc moieties constitute a common epitope between Cz and either myosin or other cardiac O-GlcNAc-containing proteins, as a new interesting feature into the molecular immunepathogenesis of Chagas heart disease [54]. Later, we have progressed in the structural characterization of the oligosaccharide chains of this glycoprotein, revealing the presence of sulfated residues [55], essential for Cz recognition by IgG antibodies from sera of Chagas disease patients [56]. We have also demonstrated that sulfated epitopes are shared between Cz and *T. cruzi* sulfatides and that IgG2 antibody levels specific for sulfated groups, inversely correlate with disease severity in chronic Chagas disease patients [57]. On top of this, we have evidenced by immune assays performed with Chagas disease sera that the structure of synthetic GlcNAc6S mimics the N-glycan-linked sulfated epitope displayed in Cz [58]. All the aspects related to the major CP of *T. cruzi* studied so far include the advances as proteinase, antigen and glycoprotein [59]. Furthermore, results suggesting that *T. cruzi* sulfation occurs via PAPS, and that Cz sulfates are involved in the infection process by *T. cruzi* were reported [60]. Finally, the involvement of sulfates from Cz in the interaction with the sialic acid-binding immunoglobulin like lectin-E (Siglec-E) was demonstrated [61]. This molecule is crucial for the survival and propagation of *T. cruzi* and it was demonstrated that inhibitors of CPs capable to block steps from parasite life cycle killed the parasite and cured infected mice, validating to Cz as a very promising target for developing new drugs against Chagas disease [39, 49]. On the other hand, some minor and highly specific CPs might also be involved in the inhibition of the parasite life cycle. In this sense, we have described the presence of *Tc*CPmet, a proteinase secreted by metacyclic trypomastigotes showing a different nature with Cz [48]. In addition, a minor sub-class of atypical Cz molecules (NACrI) containing differences in both oligosaccharide pattern and preference of chromogenic substrates has been identified [62]. Moreover, among cathepsin B-like CPs in *T. cruzi*, a 30 kDa enzyme has been studied [63, 64]. Minor CPs may constitute new targets for the development of novel inhibitors. However there is no data available yet. The development of novel and more specific Cz inhibitors is based on studies related with the crystal structure and specificity of cruzain bound to various inhibitors [65, 66]. Studies on animal models with these type of agents established that an effective chemotherapy of the American Trypanosomiasis based on CP inhibitors (CPIs) is relevant. The emerging role of CPs in a wide array of parasitic diseases is highlighted with the vision that CPIs could convert in the β-lactams of anti-parasitic treatments in the coming decades. New CPIs research will optimize intra- and extracellular enzyme targeting, for a better understanding of pharmacokinetic-pharmacodynamic interactions searching for compounds with improved efficacy and viability for clinical therapies [67]. An overview of the development of small molecule CPIs with anti-parasite activity and the current background on natural peptidase inhibitors in trypanosomatids was reported [68]. Two types of CPIs, peptidyl and non-peptidyl containing inhibitors have been synthesized.

2.1.1.1. Peptidic CPIs

Among peptidic compounds, groups of irreversible or reversible inhibitors will be described (Table **1**):

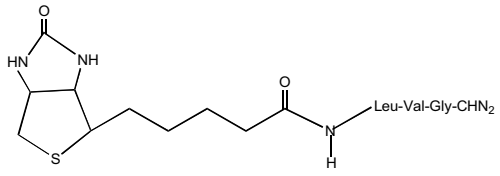
2.1.1.1.1. Irreversible Peptidic CPIs

a. Peptidyl diazomethane inhibitors. Based on the N-terminal segment of the natural protein inhibitor of CPs, cystatin C, biotin-labelled peptidyl diazomethane CPIs were synthesized. At difference with the mammalian equivalents, a strong reaction between Cz and these inhibitors was observed when the inhibitor included a spacer arm, containing a segment of the sequence of cystatin. These results may be probably due to differences in the topologies of the binding site [69].

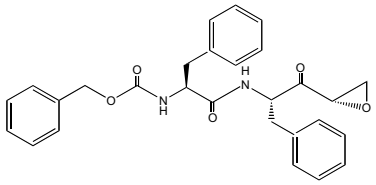
b. Peptidyl ketone based inhibitors. A variety of peptidyl fluoromethylketones, powerful irreversible Cz inhibitors, were designed and synthesized, revealing that dipeptidyl alpha', beta'-epoxy ketones were Cz inhibitors more effective than the selective irreversible CPI E-64. Aditionally, two aminoacidic substitutions replaced the L-Leu residue of this compound, obtaining D-Phe- and D-Tyr containing epoxysuccinate derivatives from

Table 1. Chemical structures of peptide-based cysteine proteinase inhibitors (CPIs). Representative compounds

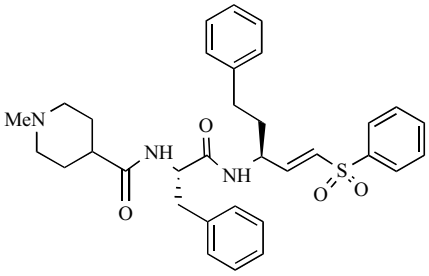
A. Irreversible



a-Peptidyl diazomethane [69]

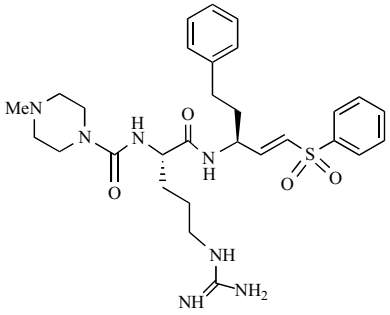


b-Peptidyl ketone based [70, 71]

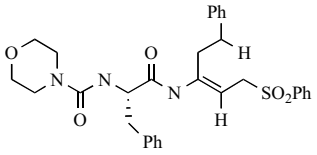


K11777

c1-Peptidyl vinyl sulphone [72-85]



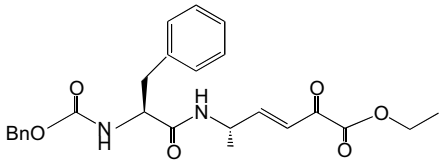
WRR-483



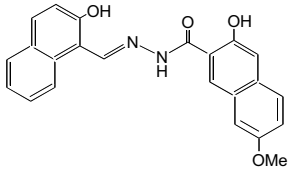
(E)-Mu-Phe-Hfe-AS-Ph

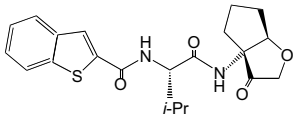
c2-Peptidyl allyl sulphone [86]

B. Reversible

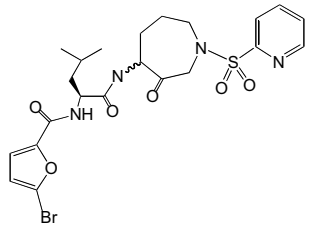


d-Bis-arylacylhydrazides [87, 88]

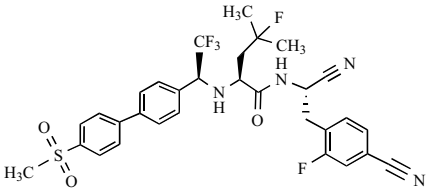




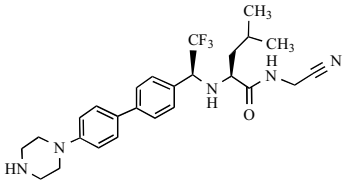
Ketone based (cyclic structures) [89-100]



Azepanone based [101-105]



Cz007



Cz008

Nitrile based [106-113]

the peptidyl-epoxysuccinate E-64, demonstrating to be potent irreversible Cz inhibitors but showing low efficacy when tested in *T. cruzi* cultures [70]. Finally, two different irreversible peptidyl halomethyl ketone inhibitors have been studied by hybrid quantum mechanics/molecular mechanics-molecular dynamics simulations in order to obtain a complete representation of the possible free energy reaction paths of the mechanism of cruzain inhibition. This process involving the formation of a protonated thiohemiketal, with participation of His159 as a proton donor, seemed to be feasible despite showing high free energy barriers [71].

c. Peptidyl vinyl/allyl sulphone inhibitors. The potential toxicity related to the use of irreversible inhibitors determined the initiation of the screening of compounds including vinyl sulphones. The trypanocidal activity involved to vinyl sulphone derivatized dipeptides in *in vivo* assays showing that these compounds efficiently saved mice from an acute lethal *T. cruzi* inoculation for the first time, displaying a significant reduction of parasites number in blood of infected animals [72]. Particularly, two vinyl sulphones, morpholinourea-FhF-vinyl sulphone phenyl and morpholinourea-FhF-fluoromethylketone produced parasite death by arresting epimastigotes growth [73]. These compounds inhibit Cz. Therefore, Cz was early identified as a promising chemotherapeutic target in the treatment of Chagas disease [74]. Also, a second generation of potent N-alkoxyvinylsulfonamide inhibitors of Cz was established. One of them, named 13, was highly effective against *T. cruzi* trypomastigotes in tissue culture [75]. Besides, dipeptidyl allyl sulphones were shown to be powerful than dipeptidyl vinyl sulphones [76]. Georgia Tech Research Corporation disclosed two patents related to these compounds (Table **2**), providing the procedures for synthesizing peptidyl allyl sulfones, as well as *in vivo* and *in vitro* methods of using these protease inhibitors [77]. Doyle *et al*., 2007 [78] have studied the effects of the dipeptidic inhibitor N-methyl-Pip-F-homo-F-vinyl-sulfonylurea phenyl (K777) in the course of infection in immunodeficient and normal *T. cruzi* infected mice, finding that immunodeficient mice treated with this inhibitor were rescued from lethal infection, showed increased survival, negative PCR, and histopathologically normal tissues. The vinyl sulfone inhibitor K777, is a clinical candidate. So, the preclinical assessment compliance for filing as an Investigational New Drug with the United States Food and Drug Administration (FDA) has been presented, and an outline of potential clinical trial has been given [79]. The current state of clinical trial will be explained in section 6 of this manuscript. On the other hand, the synthesis of vinyl sulphone-containing macrocycles via olefin ring-closing metathesis was performed to evaluate conformationally constrained inhibitors. Unluckily, they were considerably less active as inhibitors of cruzain and other CPs in comparison with the acyclic vinyl sulphone K777 [80]. In order to study the mechanism of CP inhibition by vinyl sulfones, the binding specificity of these proteases and the capacity of vinyl sulfones as antiparasitic agents, the crystal structures of cruzain, falcipain-3, and rhodesain in complex with vinyl sulfones, and comparative inhibition kinetics, were reported [81]. Additionally, the treatment of dogs with the Cz inhibitor K777, abrogated myocardial damage produced by *T. cruzi* [82]. Later, the compound WRR-483, analog of K777, was synthesized, tested as cruzain inhibitor and evaluated against *T. cruzi* proliferation in cell culture demonstrating a respectable potency on cruzain and a high efficacy in the cell culture assay. The mode of action was confirmed by crystallographic analysis, by targeting the active site of cruzain. WRR-483 has also eradicated parasite infection in a mouse model of acute Chagas disease, indicating to this compound as an effective CPI with trypanocidal activity in cell culture and animal model. The comparable efficacy to K777 suggests that WRR-483 showed potential to be developed as candidate for the treatment of Chagas disease [83]. Kinetic analyses of a series of synthesized oxyguanidine analogues of WRR-483 showed comparable potency to previously prepared vinyl sulfone cruzain inhibitors. A rare example of noncovalent inhibition of a CP by a vinyl sulfone inhibitor against cruzain was described. Depending on the aryl moiety of the P1' inhibitor subunit, co-crystal structures of two oxyguanidine analogues bound to cruzain revealed differences in binding interactions with cruzain, demonstrating that the analogue WRR-669 is specifically noncovalently bound in the crystal structure [84]. By using circular dichroism and NMR spectroscopy, the solution-state structural dynamics of the enzyme in complex with a covalently bound vinyl sulfone inhibitor (K777) was also examined. Chemical shift perturbation mapping verified that six out of eight compounds tested bind to cruzain at the active site. Three different binding modes (covalent, noncovalent, and noninteracting) were delineated for the compounds [85].The synthesis and inhibitory potency of a series of new dipeptidyl allyl sulfones as clan CA CPIs were reported. The inhibitors structure consisted of an R(1)-Phe-R(2)-AS-Ph scaffold (AS=allyl sulfone). R(1) and R(2) were varied with benzyloxycarbonyl, morpholinocarbonyl, or N-methylpiperazinocarbonyl substituents and Phe of Hfe residues, respectively. The inhibitors were assayed with different CPs such as cruzain, cathepsin B and calpain I, and with the SP trypsin. (E)-Mu-Phe- Hfe-AS-Ph was

the most potent inhibitor for cruzain, representing at least 10-fold selectivity over CPs cathepsin B and calpain I [86].

2.1.1.1.2. Reversible Peptidic CPIs

a. Bis-arylacylhydrazides and aryl ureas. Centered on the structure of the active site of Cz, some novel classes of reversible CPIs, have been designed and synthesized comprising a family of bis-arylacylhydrazides [87] and some aryl ureas [88].

b. Ketone based inhibitors. The use of solid-phase parallel synthesis allowed the development of some potent ketone based peptides, reversible against Cz. Thus, the formation of hemithioacetal complexes with CPs inhibited the enzyme in the nM range [89]. Crystal structures of ketone-based reversible inhibitors of Cz were analyzed [66]. Innovative series of alpha-ketoamide-, alpha-ketoacid-, alpha-ketoester-, and aldehyde-based inhibitors of Cz were synthesized. *In vitro* assays showed that some of them displayed potency in the picomolar range. In cells culture, anti-trypanosomal activity was demonstrated by using three different alpha-keto-based inhibitor scaffolds [90]. Searching for protease inhibitors patents, scientists from Glaxo Smith Kline Corp described a series of cyclic ketone compounds capable to form a hemithioacetal with the Cys 25 residue preserving oral bioavailability and improving pharmacokinetics. However, these compounds have not been tested against Cz [91]. Regarding the epimerization problem in the ketone based inhibitors, a series of substituted amides and 2 acylamide-bicyclic ketone derivatives were synthesized by Medivir UK Ltd, Genzyme Corp. In the first patent dealing with substituted amides [92], the synthesis of these CPIs and its potential use in infectious diseases including Chagas disease was reported. Although a tetrahydropyran-3-one derivative was used as cathepsin S inhibitor, no biological data were presented. In a similar way, Incenta has also described a series of peptide mimics 2-acylamino bicyclic ketone derivatives, claiming that tetrahydrofuran-3-one derivatives were more potent inhibitors of Cz than those of the previous series described. Also, Amura patented inhibitors of Cz and other CPs [93-96]. This company disclosed a series of pirrole compounds, with activity on Cz and different cathepsins, useful for the *in vivo* chemotherapy of CP-implicated diseases [97]. Other peptide based CPIs, claimed by Corvas International Inc. as useful antiparasitic agents, and were tested as effective against Cz showing IC50 values lower than 50 nM. However, no specific biological data are available [98]. On the other hand, some amides that inhibit Cz more effectively than mammalian CPs, have been also patented by Amura Therapeutics Ltd. [99], as well as inhibitor compounds of Cz and other CPs suitable as therapeutic agents for Chagas disease [100] (Table **2**).

c. Azepanone based inhibitors. Smith Kline Beecham Corp published a number of patents describing the synthesis and use of peptidomimetics based on azepine or thiazepane [101, 102]. Although they were tested as cathepsin K inhibitors, and claimed to be useful against different parasitic diseases including trypanosomiasis, only two patents reported biological data [103-105]. Only one of them disclosed the inhibition by 4-aminoazepan-3 one derivatives of seven parasitic proteases including Cz. 43 out of 222 compounds tested, showed Ki values lower than 5 nM against Cz. 1-(pyridine2-ylsulfonyl) Azepan-3 one derivatives resulted the most potent CPIs against Cz [103] (Table **2**).

d. Nitrile based inhibitors. Novartis has patented a series of peptidic heteroaryl nitrile derivatives [106] for the treatment of osteoporosis and several parasitic diseases.The patent describes that the compound would be useful in the prevention and treatment of several parasitic diseases including Chagas disease. A series of alpha-amino-carbonitrile-derived inhibitors of human dipeptidyl peptidase and cathepsin B, H and L, claiming that can be used for Chagas disease were further disclosed by The Combio Company [107]. However, data related to their efficacy against parasitic diseases have not been reported. Later, Boehringer Ingelheim Pharmaceuticals Inc disclosed 404 nitrile compounds claiming their suitability as reversible inhibitors for the treatment of CPs-mediated diseases treatment but no detailed experimental results were shown [106, 107]. Ten compounds showing Ki values ranging from 0.09 to 20 μM were tested against Cz [108]. It is worth noting that biological data for these nitrile based inhibitors regarding their efficacy on parasitic diseases are also absent (Table **2**).

An *in vitro* search for reversible strong Cz inhibitors for the chemotherapy of Chagas disease,

Table 2. Patent protected drug targets and tripanocidal compounds.

Target	Inhibitor/Function	Patent Number *
CPs	**CP inhibitors**	**2001:** WO0195911 [91]. **2002:** WO024046A2/A3 [92]; WO02057246A2/A3 [93]; WO02057248A2/A3 [94]; WO02057249A1 [94]; WO02057270A1 [94]; CA2436462AA [94]; WO00217924A1 [103]; WO02100849A2/A3 [106]; WO02100849A3 [107]. **2003:** EP1362052A1 [95]; NO20033220/A/A0 [95]; WO0248097A1/B1 [98]; WO0248097C2 [98] WO03053331 A2/A3 [101]; WO03103574 A2/A3 [102]; WO03104257A2/A3 [102]; WO03097593 A2/A3 [102]; WO03097664A2/A3 [108]. **2004:** CN1486320A [96]; MX3006224A [96]; ZA0305259A [96]; NZ0526913A [96]; WO04007501A1 [97]; WO04020441A1 [104]; WO04110988A1 [105]. **2005:** 6958358 [99]; US6897240 [121]; US7521427 [77]. **2006:** US6982263 [107]. **2008:** US7425562 [100]. **2009:** US7521427 [77]; US7495023 [121]. **2010**: CA20100305056 [153]; WO2010059418 A1 [152]. **2012**-: US20120101053 A1 [151]; EP2445872A1/A4 [151]; WO2010148488A1 [151]. **2013:** CA 20130244962 [153]. **2014:** WO2014019044 (A1) [134]; CA 8642799 [153].
Ergosterol Biosynthesis	**OSC inhibitors** **C14 demethylase**	**2000:** WO0076316A1 [247]. **2003:** WO03006012A1; CA 2453396AA [263, 264]. **2004:** BR0211098A [265].
Synthesis of Poliisoprenoids	**PFT inhibitors**	**2001:** WO00105384A3 [282]. **2003:** US03134846A1 [283].
Redox metabolism	**TR inhibitors** **G S S inhibitors**	**2000:** WO0050431A1 [318]. **2001:** US6291217 [366].
Proline Race-mase	**Parasite B cell mitogen**	**2007:** US7262015 [430]. **2009:** US7556939 [430]; US7585656 [431]. **2010:** US7732563; US7851603 [433]. **2011:** EP2272510A1; EP2451450A1; EP2451450B1; WO2011004323A1; WO2011004323A9 [434].
Protein Kinase	**TrK inhibitors**	**2013:** WO2013161919 [443]. **2015:** JP20150111865 [443].
Polyamine		**2005:** US6949679 [450].
DNAnucleotide synthesis	**DHFR inhibitors**	**2001:** WO0153276A1 [477]; WO0114401A1 [479]. **2013:** WO 2013068551 A1 [481]. **2014:** EP 2776410 A1 [481].
Acidocalcisome	**Exch. Na^+/H^+ inhibitors**	**2000:** US6114393 [522].
Nucleus	**DNA binder, antimitotic drugs, topoisomerase II, NADH analogs.**	**2002:** WO02057224 A2/A3 [499]. **2003:** WO03090678 A2/A3 [505]. **2005:** US6967205 [495]; US6906076 [519].
Sialic Acid trans-ference	**Neuraminidase / sialidase inhibitors**	**1999:** WO9906369A1 [574]. **2000:** US6114386 [575].

Table (2) contd…..

Anti-Chagas Compounds	Groups	Patent Number *
	-Imidazo[4,5- c][1,2,6] thidiadiazine 2,2 oxides	**2012:** EP2392577A4, EP2392577 [600]. US20120035160 A1 [600]. **2014**: WO2010086481A1; US8815846 B2 [600].
	-Hybrid furoxany N-acylhydrazone derivatives	**2007:** MX2007013128 (A) [603]. **2009:** MX2007013128 (A) [603].
	-5-R1-2[(N-R2)- Furfuryliden carbazones and thiasemicarbazones	**2013:** WO2013059898A1 [607].
	Diaminophenothiazinium Compounds	**2013:** AU2013200264A1 [610].
	-Imido-substituted 1,4- Naphthoquinone	**2015:** US2015073177(A1) [612].
	-Squaramide Compounds	**2014:** WO2014184416 (A1) [616].
	-Pradimicin derivatives	**2015:** WO2015051422 (A1) [618].
	-QDO compounds	**2012:** WO2012096556 (A1) [625]; WO 2012096556 A1 [626].
	-Quaternary n-(halomethyl) ammonium salts	**2014:** US 0194640 A1 [630].
	-Naphthoquinone- and phthalimide-based lipocations	**2013:** WO2013036766 (A1) [632].
	-Scorpion-tail-like macrocyclic compounds	**2013:** WO 2013087965 A1 [636].
	-Bis and mono spiro-indo- le derivatives	**2012:** NZ588217 (A) [641].
	-Thiazolidine and imidazolidine Compounds	**2012:** WO2012119212 A1 [644]**.** **2011:** JP2011098968 (A) [646].
	5.Ephenylethenylbenzofuroxan derivatives	**2011:** CN102026989 (A); EP2269996A1; EP2269996A4; EP2269996B1; US8288374; US20110021450; WO2009113569A1 [646].

*the first two letters in the Patent number corresponds to PCT (Patent Corporation Treaty) contracting states: BR, Brazil; CA, Canada; CN, China; EP, Europe; MX, Mexico; NO, Norway; US, United States of America; WO, World Intellectual Property Organization; ZA, South Africa. CP, cysteine proteinase; OSC, oxidosqualene cyclase; PFT, protein farnesyl transferase; TR, trypanothione reductase; DHFR, dihydrofolate reductase; nd, non determined.

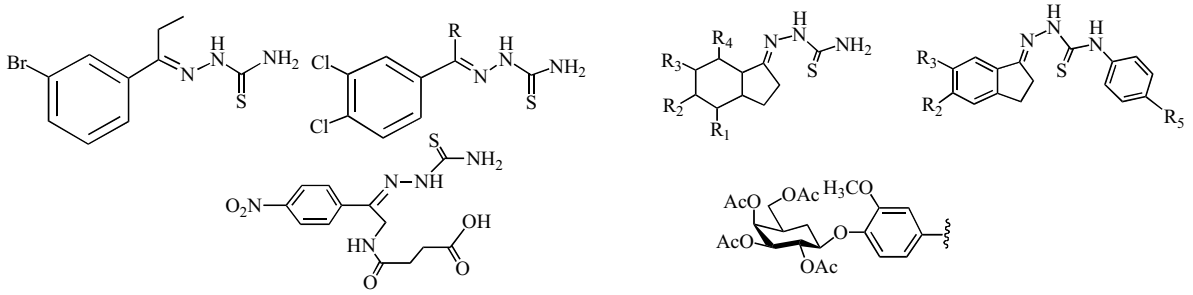
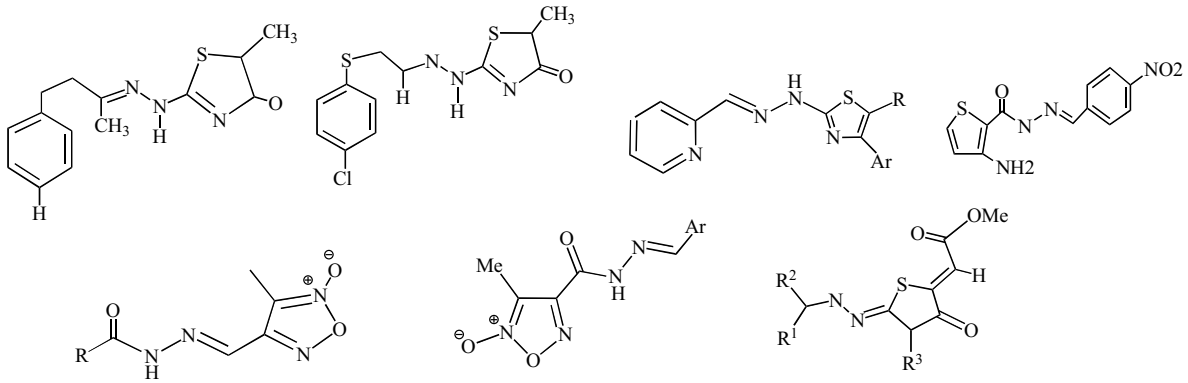
showed inhibitors bearing an amino nitrile warhead in P1 position that exhibited low nM potency against this enzyme. Added structure activity relationship (SAR) in P2 portion offered novel opportunities for safer treatment through the identification of some compounds, showing selectivity profile against other CPs [109]. Additionally, triazine nitrile compounds, known inhibitors of other CPs, were identified by a quantitative high-throughput screen of cruzain as reversible enzyme inhibitors. The *in vitro* potency against cruzain was highly increased (350-fold) by modifying the structural core scaffold from triazine to purine [110]. Molecules with a nitrile moiety, a group susceptible to a nucleophilic attack by CPs, have been identified as good inhibitors. It is known that the nitrile group binds covalently to Cys25. Thus, in order to investigate the molecular recognition of CPs by nitrile-containing molecules, density functional theory and quantum semi-empirical analysis were conducted, reporting an interaction that starts with a nucleophilic attack from the Cys25 to the inhibitor followed by a proton transfer from His162, in addition to the detection of a transition state [111]. Reversible nitrile containing Cz inhibitors were synthesized showing IC50s in the nM range in a baculovirus generated Cz enzyme test. Although in *in vitro* assays using epimastigotes and amastigotes, the most potent compounds displayed antiparasitic behavior with IC50 values ranging from 5 to 10 μM, trypomastigotes production was inhibited 90 to 95% at 2 μM. The key compounds Cz007 and Cz008, showed IC50 values of 1.1 and 1.8 nM, respectively, against the recombinant enzyme when tested in a murine model of acute *T. cruzi* infection. Based on negative quantitative PCR results that discard the presence of the parasite in blood and mice organs, the surviving animals showed cure rates values of 90% for Cz007 and 78% for Cz008, in comparison with Bz (71%) [112]. The design, synthesis and evaluation of a series of compounds based on the dipeptidyl nitriles scaffold confirmed their inhibitory activity against cruzain. SARs were proven using three, eleven and twelve variants at the P1, P2 and P3 position, respectively. The most potent inhibitor showed a Ki value of 16 nM reflecting a degree of non-additivity in the SAR. Also, the trypanocidal effects of some of the 33 inhibitors evaluated showed EC50 values in the μM order. Further optimization transferable to the design of cruzain inhibitors based on weapons other than nitrile as well as alternative scaffolds was suggested [113].

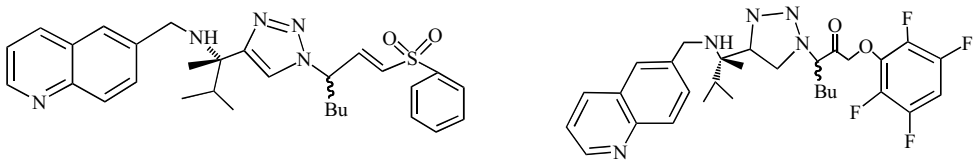
2.1.1.2. Non Peptidic CPIs

SARs for non peptidic inhibitors of Cz based on different scaffolds are reported (Table **3**).

a. Thiosemicarbazones. Non-peptidic inhibitors based on the thiosemicarbazone lead were reported as active Cz inhibitors at the nM range; a lot of them presenting small size and low cost showed trypanocidal activity against intracellular amastigotes *in vivo* turning them in interesting candidates for drug development [114-117]. The presence of parasite populations resistant to some of these inhibitors was described. Although the phenotypically stable *T. cruzi* cell line R-Dm28, displayed an increased resistance to the irreversible CPI Z-(SBz) Cys-Phe-CHN 2, capable to preferentially inactivate cathepsin L-like enzymes, constituting a limitation for CPs as targets for chemotherapy [65], further assays with non-stable cell lines, showed that the phenotype was reversed upon elimination of the inhibitor from the culture media [118]. In order to identify novel drug-like non-peptidic parasitic CPIs, a virtual screening was performed by using the ChemBridge database. A number of non-peptidic inhibitors avoided the hydrolysis of proteases in living systems, retaining *in vivo* activity and selectivity [119]. Lead optimization libraries of thiosemicarbazone inhibitors were designed. Some of these compounds were tested on different CPs and on their respective parasites showing that they were able to kill several species of protozoan parasites through the inhibition of CPs and other novel targets [120]. Two patents related to thiosemicarbazone and semicarbazone CPIs were presented by Researchers of the University of California in conjunction with methods of using such compounds to treat protozoan infections [121] (Table **2**). A series of novel thiosemicarbazone derivatives was designed by combining a potent Cz-inhibitor and an oxidative stress promoter in the same molecule, the thiosemicarbazone moiety and the 5-nitrofuryl group, respectively. Some of the derivatives resulted 1.5-1.7-fold more active than Nx against epimastigotes [122]. The bioreductive mode of action of antitrypanosomal 5-nitrofuryl containing thiosemicarbazones was analyzed by electronspin resonance spectra of radicals generated in *T. cruzi*

Table 3. Chemical structures of non peptidic-based cysteine proteinase inhibitors (CPIs).

a- Thiosemicarbazones and cyclic analogues

[65, 114-128]
b- Aminoacylthiazolidones and hydrazone derivatives.

[129-136]
c-Ethenylbenzofuroxan derivatives

[137]
d-Inhibitors with cysteine protease mechanism-based pharmacophores.

[138, 139]
e-Oxorhenium (v) and Palladium (II) metal complexes / **f- Alkyldiamine cryptolepine**

R1,R2,R3: alkyldiamine side chains
[140, 141] / **[142]**

by bioreduction, finding three different patterns of signals produced by the diverse compounds tested according to the *T. cruzi*-oxygen uptake changes [123]. A series of thiosemicarbazones derived from 1-indanones were synthetized. Most of them displayed remarkable trypanocidal activity. Among these compounds, in particular, thiosemicarbazones 12 and 24, dispayed good inhibition of Cz [124]. A series of 12 aryl thiosemicarbazones were synthesized, biologically assayed and docking studies were directed towards the two parasite enzymes cruzain and trypanothione reductase. In *in vitro* assays, three p-nitroaromatic thiosemicarbazones showed high activity against *T. cruzi* showing IC50 values lower than 57 μM. Unfortunately, although the *in vitro* inhibition test showed that eight compounds inhibited cruzain activity, no correlation was found between cruzain inhibition and trypanocidal activity [125].

The structural development, the synthesis and the trypanocidal evaluation of new aryl thiosemicarbazones (9a-x), designed as more conformationally restricted compounds was described. An increased trypanocidal activity was shown by including a spacer group to aryl thiosemicarbazones. By varying substituents attached to the phenyl ring, they were observed to retain, enhance or greatly increase the activity anti-*T. cruzi*, compared to the non-substituted derivatives. In most cases, hydrophobic and bulky substituents, such as bromo, biphenyl and phenoxyl groups, greatly increased antiparasitic activity. Specifically, thiosemicarbazones, toxic for trypomastigotes without affecting mouse splenocytes viability, and capable to inhibit the epimastigotes proliferation, were identified. When the most potent anti-*T. cruzi* thiosemicarbazones were evaluated against cruzain, no enzyme inhibition was observed, leading to the identification of new potent anti-*T. cruzi* agents, such as compounds (9h) and (9r), which greatly inhibited epimastigote proliferation, and demonstrated toxicity for trypomastigotes, but not for splenocytes. Mechanistically, these compounds did not inhibit to cruzain, but induced *T. cruzi* cell death by an apoptotic process [126]. Mainly, thiosemicarbazones and heterocyclic analogues have shown both trypanocidal and inhibitory activity against parasite CPs.

Searching for better drugs, a series of compounds were synthesized, biochemical assays were used to evaluate against cruzain potency and SAR leading to the discovery of 4 cruzain inhibitors with IC50s lower than 10 μM. *In vitro* evaluation of compounds against *T. cruzi* revealed active compounds among this series. Some modifications as the removal of thiosemicarbazone or its replacement by semicarbazone resulted in virtually inactive compounds. Other modifications in the sugar also resulted in decreased potency [127]. Recently, by using a rigidification strategy of the iminic bond, aryloxy/aryl thiosemicarbazone-based conformationally constrained analogs of thiosemicarbazones have been developed as potential inhibitors of cruzain. A SAR analysis was performed in substituents attached in both aryl and aryloxy rings. Apolar substituents or halogen atom substitution at the aryl position improved cruzain inhibition and antiparasitic activity as compared to unsubstituted thiosemicarbazone. Two of them showed strong inhibitory anti-*T. cruzi* activity by inhibiting cruzain and so were able to diminish the parasite burden in infected cells causing parasite cell death through necrosis. These findings have shown to conformational restriction as a valuable strategy in the advance of antiparasitic thiosemicarbazones [128].

b. Aminoacylthiazolidones and hydrazone derivatives. A series of aminoacyl thiazolidones derivatives were synthesized. Some of them were capable to inhibit *T. cruzi* growth in concentrations non-toxic for mammalian cells [129]. Studies with aryl-4-oxothiazolylhydrazone derivatives against *T. cruzi* were performed showing potency comparable with reference drugs and strong activity at non-cytotoxic concentrations in *in vitro* assays with mammalian cells [130]. Trypanocidal promising cruzain inhibitors based on thiazolylhydrazones were identified and synthesized. Thiazolylhydrazones 3b and 3j, exhibiting IC50 values between 200 and 400 nM were identified as potent cruzain inhibitors. Lead compounds presenting *in vitro* activity against both *T. cruzi* epimastigote and trypomastigote forms were recognized and *in vivo* toxicity analysis was achieved. The relevance of the thiocarbonyl carbon attached to the thiazolyl ring and a comparison between thiazolylhydrazones and thiosemicarbazones was reported [131]. Moreover, the synthesis of a series of 18 novel 2-hydrazolyl-4-thiazolidinones-5-carboxylic acids, amides and 5,6- α,β-unsaturated esters was described, and their *in vitro* activity on Cz and *T. cruzi* epimastigotes was evaluated. Some compounds showed activity by enzyme assays in the

micromolar order. Computational tools and docking were used to correlate the biological response with the physicochemical parameters of the compounds and their Cz inhibitory effects [132]. Anti-*T. cruzi* activities and docking studies of a series of synthesized 2-(pyridin-2-yl)-1, 3-thiazoles derived from 2-pyridine thiosemicarbazone were studied. The majority of these compounds showed to be potent cruzain inhibitors, displaying excellent inhibition on trypomastigotes. In addition, the resulting SARs have been analyzed. Altogether, these data presented a novel series of thiazolyl hydrazones with potential effects against Chagas disease proposing that they could be important leads against Chagas disease [133]. Researchers from the University of Alagoas and Federal University of Rio de Janeiro, Brazil, studied a method using intermediate compounds for producing hydrazide-N-acylhydrazone compounds for the chemotherapy of trypanosomatid diseases including Chagas disease, and their pharmaceutical compositions [134]. Moreover, hybrid bioisoster derivatives from N-acylhydrazones and furoxan groups were designed in order to obtain at least a double mechanism of action: cruzain inhibition and nitric oxide (NO) releasing activity. The synthesis of fifteen compounds included variations in the substitution in N-acylhydrazone as well as in the furoxan group. These compounds showed trypanocidal activity in amastigote forms, NO releasing capacity and inhibitory cruzain activity. Two compounds resulted the most active in parasite amastigotes and showed to be less cytotoxic than the reference drug Bz. In addition to activity, the most promising showed good permeability and selectivity index, higher than the reference drug [135]. Finally, 3-aminothiophene-2-acylhydrazones were described as non-toxic, analgesic and anti-inflammatory lead-candidates [136].

c. 5-Ethenylbenzofuroxan derivatives. These compounds were developed and evaluated as anti-proliferative *T. cruzi* drugs displaying notable *in vitro* activities against different strains and were able to reduce the parasite load in animals with established *T. cruzi* infections. By additional binding interactions introduced in the S3 pocket of cruzain, substrates were optimized and were converted to inhibitors. CP mechanism-based pharmacophores were used. One of them showed to be reversible even after the incorporation of the vinyl sulphone pharmacophore showing to be well documented as irreversible cruzain peptidic inhibitor [137].

d. Acyl and aryl-oxymethyl ketones. A previously unexplored beta-chloro vinyl sulphone pharmacophore led to the development of potent irreversible acyl- and aryl-oxymethyl ketone cruzain inhibitors. Among them, 2, 3, 5, 6-tetrafluorophenoxymethyl ketone was identified as one of the most potent inhibitor against this enzyme with capacity to eradicate the parasite from mammalian cell cultures entirely [138]. Moreover, this nonpeptidic tetrafluorophenoxymethyl ketone cruzain inhibitor was capable to substantially ameliorate symptoms of acute Chagas disease in a mouse model with no apparent toxicity. The mode of action was studied by using a high-resolution crystal structure revealing key binding interactions of this novel inhibitor class, obtaining inhibitor analogues with improved potency and enhanced activity in cell cultures suggesting that non-peptidic tetrafluorophenoxymethyl ketone cruzain inhibitors had the potential to satisfy the need for improved Chagas disease chemotherapy [139].

e. Oxorhenium (V) and paladium (II), and ruthenium metal complexes. When the activity of gold (III), palladium (II) cyclometallated complexes, and oxorhenium (V) complexes against mammalian and parasitic CPs was studied, six complexes were tested against the parasite CPs, cruzain from *T. cruzi*, and CPB from *L. major* resulting two rhenium complexes the most potent inhibitors. Preliminary results showed that two oxorhenium (V) compounds and the palladium compound 11 inhibited *T. cruzi* intracellular growth suggesting that metal complexes targeted parasite CPs showing promise for the treatment of both Chagas disease and Leishmaniasis [140]. Regarding ruthenium complexes, cis-[RuCl(NO_2)(dppb)(5'-mebipy)], cis-[Ru(NO_2)2(-dppb)(5'-mebipy)], ct-[RuCl(NO)(dppb)(5'-mebipy)](PF6)2, and cc-[RuCl(NO)(dppb)(5'mebipy)](PF6) (complex 1 to 4, respectively), where 5'-mebipy is 5'-dimethyl-2'-bipyridine and dppb is 1,4-bis(diphenylphosphino)butane, were synthesized and characterized. All complexes exhibited a higher trypanocidal activity than the control Bz. Complex 3 was the most potent against trypomastigotes and amastigotes, exhibiting low toxicity to macrophages. It was observed that the nitrosyl complex 3, but not its analog lacking the nitrosyl group, released nitric oxide into parasite cells. This release

diminished the effect on cruzain and induced substantial parasite autophagy resulting in parasite cell death. Orally administered complex 3 reduced blood parasitemia in infected mice and increased the mice survival rate. The *in vitro* activity of complex 3 against trypomastigotes was synergic with Bz. These findings supported that drug combination enhanced efficacy in infected mice, suggesting that ruthenium-nitrosyl complexes seem to be potential constituents for drug combinations [141].

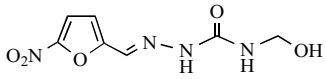
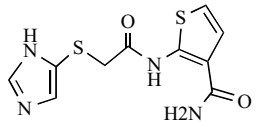
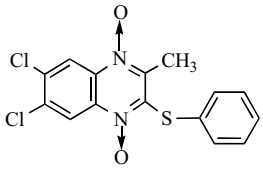
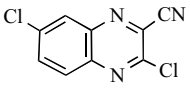
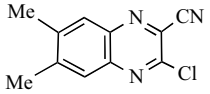
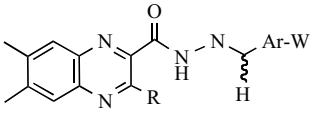
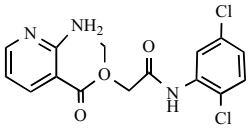
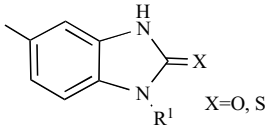
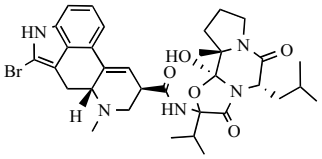
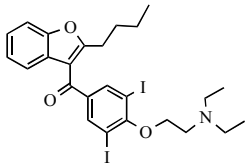
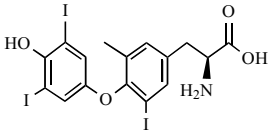
f. Alkyldiamine cryptolepine derivatives. Early, trypanocidal activity was exhibited by 2-bromo, 2-nitro-, and 2-methoxy-9-cyanoneocryptolepine in the micromolar range in the absence of cytotoxicity. Further assays described that cryptolepine derivatives containing alkyldiamine side-chains, showed potent inhibitory activity and selectivity against *T. brucei brucei* when compared to the lead compound. They were also potent inhibitors of cruzain, which could, at least in part, explain their trypanocidal activity providing another starting point for the rational design of novel and effective anti-trypanosomal drugs [142].

2.1.1.3. Synthetic Derivatives or Approved Drugs Active on Cz

The chemical structures of the compounds discussed in this item are displayed in Table (**4**).

a. Nitrofurazones (NFs). NFs and its derivative hydroxymethylnitrofurazone (NFOH) have shown anti-Chagas disease activity due to TR inhibitory activity. In addition to this activity, *in vitro* cruzain inhibition assays were developed

Table 4. Synthetic derivatives or approved drugs with probed activity on Cz.

Nitrofurazone derivatives	2-acetamidothiophene-3-carboxamides
	
[143]	compound Nequimed 176 [148]
Quinoxaline and quinoxaline-N-acylhydrazon derivatives	
   	
Compound 13 19 [144] 20 [145]	
Oxadiazoles	**Benzimidazole**
	
Compound 1 [146]	Compound 2 [147]
Drug repositioning of known drugs	
  	
Bromocriptine Amiodarone [149, 150] Levotiroxine	

for both compounds, showing IC50 values in the μM range. In addition, AM1 semi-empirical molecular modelling studies supported cruzain inhibitory activity [143].

c. Quinoxaline and acylhidrazones derivatives. *In vitro* assays of some synthetic quinoxaline derivatives showed similar inhibitor growth activity than Nx. The quinoxaline N' N'-dioxide derivative compound 13, and the reduced derivatives 19 and 20 were the most cytotoxic compounds against the protozoan [144]. New quinoxaline-N-acylhydrazon derivatives, evaluated as cruzain inhibitor candidates were designed, synthesized, studied by docking analysis and tested for trypanocidal activity. Two salicylaldehyde N-acylhydrazones presented IC50 values of the same magnitude order than the standard drug Nx when tested *in vitro* against epimastigotes of *T. cruzi* resulting non-toxic at the highest assayed doses in macrophages [145].

d. Oxadiazole derivatives. A series of oxadiazoles, reversible and noncovalent inhibitors of cruzain were identified by docking screen and chemically optimizated, having interpretable SAR and high potencies. Many members of the oxadiazole class act via divergent modes of inhibition, depending on the assay conditions tested [146].

e. Benzimidazole derivatives. Thirty three novel 1,2,5-tri-substituted benzimidazole derivatives were obtained by solid-phase synthesis and their *in vitro* activity on Cz and *T. cruzi* epimastigotes was reported. Seven compounds showed potent inhibition of *T. cruzi* growth with IC50 values in the μM range. Docking studies revealed the binding orientation of the ligands in the active site of Cz providing a guide for the design of better inhibitors. A promising hit compound named 2a for novel anti-*T. cruzi* agents showed that the benzimidazole scaffold might represent an interesting therapeutic alternative for the treatment of Chagas disease [147].

Promising cruzain inhibitors retaining trypanocidal activity for SAR studies were selected. When the most potent inhibitors were assayed against *T. cruzi*-infected cells, two compounds showed trypanocidal activity. A SAR was established using the compound Nequimed42 as precursor, identifying the group 2-acetamidothiophene-3-carboxamide as crucial for enzyme and parasite inhibition activities. This compound was ten-fold more active against trypomastigotes than Bz. In addition, a smaller compound derived from Nequimed42, named Nequimed176, was highlighted as a novel non-peptidic, non-covalent cruzain inhibitor and a trypanocidal drug candidate [148].

f. Clinical and investigationally approved drugs. A 2D-classifier capable of identifying Cz inhibitors was useful in a virtual screening on the DrugBank database, compiling FDA-approved and investigational drugs. Fifty-four approved drugs were selected as candidates, four of which were acquired and tested on Cz and *T. cruzi* epimastigotes. Among them, the antiparkinsonian and antidiabetic drug bromocriptine and the antiarrhythmic amiodarone showed dose-dependent inhibition of Cz and antiproliferative activity on the parasite [149]. An 8-descriptor conformation-independent model was applied to select potential anti-Chagas disease drugs from the DrugBank database, three candidates were experimentally tested in enzymatic and inhibitory assays. Among them, levothyroxine (traditionally indicated for patients with hypothyroidism) showed a dose dependent inhibition on Cz activity. After a screening, among approved drugs, using Bz-Pro-Phe-Arg-*p*NA as chromogenic substrate, levothyroxine displayed a significant dose dependent inhibition on *T. cruzi* Cz activity. *Trypanosoma cruzi* epimastigotes proliferation was affected by levothyroxine in a time and dose-dependent manner. This study showed the potential of computer-aided drug repositioning in the search of novel agents for neglected diseases [150]. A patent presented compounds containing the capability of inhibiting and/or reducing the cathepsins activity, with the aim to treat and/or prevent CPs and papain-like CPs associated diseases, also including Chagas disease among them [151]. Researchers from Secretary of Health and Human Services, Department government, USA, presented a patent describing novel substituted triazine and purine compounds, inhibition methods of cruzain and rhodesain, and methods for the treatment and prevention of Chagas disease and African trypanosomiasis [152]. In the last years, researchers from Merck Canada Ltd described CPs dependant diseases in mammals caused by numerous parasites and inhibitors that can be valuable in the treatment and/or prevention of various parasitic diseases comprising Chagas disease, among others [153].

2.1.1.4. CPIs Obtained from Natural Compounds

A Kunitz-type inhibitor, BbCI, obtained from *Bauhinia bauhinioides*, capable to inactivate Cz was studied by its antiproliferative properties. The

endothelial proliferation, the intracellular Ca^{2+} concentration and the membrane potential were significantly influenced by BbCI [154]. An oxyran derivative of alpha-lapachone, the epoxy-alpha-lapachone, selected among natural naphthoquinones, showed a high inhibitory activity against *T. cruzi* and a low toxicity [155]. Active naphthoquinone-compounds were evaluated as specific inhibitors of *T. cruzi* CPs and SPs activities and different targets were revealed. A theoretical analysis confirmed that beta-lapachone and E-64 interact with the active site of cruzain, whereas epoxy-alpha-lapachone exhibited no significant interactions. The results obtained inferred that beta-lapachone and epoxy-alpha-lapachone compounds might inhibit *T. cruzi* epimastigotes growth by acting on different *T. cruzi* proteinases. Natural naphthoquinones have been described as proteinase inhibitors models for design and development of anti-Chagas disease compounds [156]. Searching for novel lead compounds have extended over SAR studies integrating high-throughput, virtual screens and drug development strategies for a correct analysis of new anti-Chagas disease agents. Progress on the development of cruzain inhibitors emphasized on structural aspects of the ligand-cruzain recognition process has recently been reviewed [157].

2.1.1.5. Calpains

These intracellular calcium-dependent CPs, described in some bacteria and most eukaryotes have been associated with varied biological processes. At neutral pH, calpains exhibited partial proteolytic activity on proteic substrates transforming their structures and biological activities in order to modulate them [158]. After in silico analysis of the Tritryps genome, it was first reported that calpain-like proteins probably play important roles in cell functions of trypanosomatids [159, 160]. The calpain inhibitor MDL28170 produced a strong growth rate reduction at 70 μM. Calpain-like molecules were detected in *T. cruzi* epimastigotes [161]. Calpains were considered in some reports as promising targets to treat Chagas disease. In this sense, the *in vitro* evaluation of MDL28170 activity on the relevant clinical forms of *T. cruzi* produced a significant reduction on bloodstream trypomastigote viability. In order to study the functions of *T. cruzi* calpains, the inhibitor MDL28170 was tested in crucial steps of the parasite life cycle, in parasite viability and morphology [162, 163]. Regarding that in *T. cruzi* H49, the CP catalytic triad is partially conserved, it was classified as a calpain-like protein. Its locatization at the zone where the flagellum is attached to the parasite suggested that this protein and calpains could have a structural role, connecting the subpellicular microtubule array to the parasite body [164]. A large family of calpain-related proteins was identified among numerous trypanosomatids genes with interesting chemotherapeutic characteristics. In this case, 40 genes were assigned as calpains in *T. cruzi*. On top of this, an overview describing the biochemical and biological studies performed on calpain inhibitors in trypanosomatids was presented [165].

2.1.2. Serine Peptidases (SPs)

Proteinases display key functions for the metabolism and infectivity of pathogens. Parasite SPs described include oligopeptidase B, a secreted prolyl endopeptidase (*Tc*80), and a lysosomal serine-carboxypeptidase. On one hand, oligopeptidase B was involved in Ca^{2+} signaling during cell invasion in mammals [166, 167]. On the other hand, *Tc*80 was purified from *T. cruzi* and displayed collagenolytic activity [168]. The enzyme ability to hydrolyze peptide bonds at the carboxyl side of Pro residues suggested that it is a prolyl endopeptidase also belonging to the S9ASP family, but different from the oligopeptidase B. Selective inhibitors of the enzyme have been synthesized, showing Ki values in a low nM range and capacity to block the entrance of the parasite into the host cells [169-171]. Therefore, this SP was shown as a very auspicious target for the development of new agents against this neglected disease. Moreover, a 75 kDa *T. cruzi* serine oligopeptidase, secreted from the parasite was purified and subcellularly localized into intracellular structures, including the flagellar pocket, plasma membrane and reservosomes [172]. Prolylprolylisoxazoles and prolyl-prolylisoxazolines, two synthetic strong inhibitors of human and *Trypanosoma* prolyloligopeptidase (POP), were capable to inhibit *T. cruzi* and *T. brucei* in *in vitro* systems showing ED50 values in the low μM range [173]. Most assays performed with novel inhibitors on rPOP *Tc*80 resulted efficient, presenting Ki values lower than 1.52 nM. Infective parasites treated with these specific POP*Tc*80 inhibitors were capable to attach to the surface of host cells in mammals, but unable of infecting them. Furthermost properties of these

enzymes and the development of their prospective as targets for drugs against Chagas disease, leishmaniasis and African trypanosomiasis have been described in detail [174, 175].

2.1.3. Metalloproteinases

Enzymes with homology to the leishmanial gp63 are also present in *T. cruzi* [49, 176]. MPs belonging to the M32 family of peptidases are lacking in eukaryotic genomes with the notable exception of those present in these trypanosomatids. As mentioned before, the genome of the CL Brener clone of *T. cruzi*, encodes two MPs, named *Tc*MCP-1 and *Tc*MCP-2, presenting 64% homology between them. The structure of *Tc*MCP-1 showed powerful topological similarity with archaeal, bacterial and mammalian MPs. Trypanosomatids are so far the only group of eukaryotes encoding M32 MPs, making these enzymes an attractive potential promising target for trypanocidal drug development [177-179]. On the other hand, LAP*Tc*, a 330-kDa homohexameric metalloaminopeptidase with leucyl aminopeptidolytic activity was isolated from *T. cruzi* epimastigotes and expressed by all the parasite stages. As biosynthetic pathways for vital amino acids, such as leucine, are lacking in *T. cruzi*, LAP*Tc* could have a function in nutritional supply [180]. The mechanisms displayed by parasite-derived products alter host expression of matrix MPs and endogenous metalloproteinases inhibitors (MPIs) are capable to reduce collateral tissue damage, as has been reported for tetracyclines as MP regulators in parasite infections [181]. Studies related with MPIs have not been still reported in *T. cruzi*.

2.1.4. Aspartyl Proteinases (APs)

APs are present in numerous organisms like vertebrates, plants, fungi, protozoa and some retroviruses, participating in relevant metabolic processes and playing main roles in infectious diseases. *Trypanosoma cruzi* genome predicted the presence of two APs [40] cruzipsin-I and cruzipsin-II, identified and isolated from *T. cruzi* epimastigotes. The cathepsin D substrate Phe-Ala-Ala-Phe (4-NO2)-Phe-Val-Leu-O4MP was hydrolyzed by APs but serine and other proteinase substrates were not. The AP inhibitors (APIs) pepstatin-A, and the 1, 2-epoxy-3-(phenyl-nitrophenoxy) propane strongly inhibited the activities of both proteinases indicating that the latter are novel *Tc*APs [182,183]. A few reports have been performed related to APs in trypanosomatids. However, some advantageous properties of APIs have been described on vital biological processes of these parasites. In *T. cruzi,* both APs activities and parasite proliferation were significantly inhibited with pepstatin-A, indicating that AP might be favorable targets in trypanosomatids and APIs could act as helpful therapeutic agents against these human flagellates [184, 185]. When the effects of pepstatin-A on *T. cruzi* were explored, the proliferation of *T. cruzi* epimastigotes was arrested in a time- and dose-dependent way showing an IC50 value in the μM order. The hydrolysis of a substrate specific for cathepsin was inhibited (65%) by pepstatin-A using an extract of *T. cruzi* epimastigotes, signifying that an AP might be the intracellular target of this inhibitor. *In vitro* findings suggested that APs might participate in the parasite physiology, and be considered as promising targets for the treatment of this neglected disease [186].

2.1.5. Threonine Proteinases (Proteasome)

A structure formed by intracellular complexes named proteasome controls protein degradation from Archaebacteria to humans, participating in cellular differentiation and replication in protozoan parasites. So, it could constitute a promising target [187]. The presence of a proteasome with similar characteristics to those of other eukaryotes was early reported in *T. cruzi* [188]. On the other hand, while lactacystin inhibition blocked some differentiation steps in the parasite life cycle, an inactive analogue of lactacystin, clasto-lactacystin, did not show effect. By using proteasome inhibitors the accumulation of ubiquitinated proteins was demonstrated and the fact that flagellum associated cytoskeleton proteins are targets of the ubiquitin-proteasome pathway was early shown [189]. The inhibition of the ubiquitin-proteasome pathway in *T. cruzi* epimastigotes by lactacystin determined the blocking of parasite growth but no of adhesion, interrupted cell division and affected factors causing differentiation [190]. A patent of Nereus Pharmaceuticals, Inc. [191] claimed the use of analogue compounds to the heterocyclic compound salinosporamide A, as proteasome inhibitor for the treatment of neoplasm, inflammation and microbial infection. Despite the known *in vitro* capacity of proteasome inhibitors against trypanosomes [49, 192], no biological data of trypanocidal activity were divulged. *Trypanosoma cruzi* is a

eukaryote rare example containing genes for the two threonine proteases known as 20S proteasome and HslVU complex. The latter is an ATP-dependent protease comprising multimeric components. In order to study the possibility that a proteasome inhibitor could modulate these components and proteasome, *T. cruzi* epimastigotes were grown with the classical proteasome inhibitor PSI, which induced no effect or rise in the level of expression of proteasome components in different strains, suggesting that the protease HslVU and 20S proteasome coexist in *T. cruzi*, and non-lysosomal degradation vias are relevant for *T. cruzi* biology [193].

An old patent was presented by researchers from University of New York, USA, claiming methods for treating parasitic diseases with lactacystin, MG-132, N-methoxysuccinyl-glu-val-lys-phe-modified compounds among others, selected as effective agents as proteasome and ubiquitin pathway inhibitors inhibitors on malaria, Chagas disease and other neglected diseases [194].

2.1.6. Other Peculiar Proteases

Regarding that peptidases are common drug targets, the identification of pathogens peptidases that show no homology in their hosts could be useful for selecting the most promising ones. Some peculiar protease families, absent in metazoans, have been described in *T. cruzi*. Among them, two eukaryote homologues of carboxypeptidases Taq, and Atg4 proteinases can be mentioned. Due to their particular phylogenetic distribution, the carboxypeptidases Taq were proposed as promising targets [195]. On the other hand, Atg4, CPs commonly named autophagins, involved in the regulation of the conserved degradative autophagic pathway, result important for parasite survival under nutritional stress conditions and differentiation. There are no specific inhibitors for these families so far, however, the increasing knowledge of their biochemical properties and biological functions, is a crucial step for the development of inhibitors [196]. Researchers from the University of Florida presented a patent containing a method for treating a parasitic infection which pretends to establish an intracellular niche for survival and replication by administering a composition comprising N-2-pyridinyl-2-pyridinecarbothioamide and/or cambendazole to an infected individual [197].

2.2. Ergosterol Biosynthesis Pathway (EBI)

In mammals, sterols are vital structural constituents of cellular membranes acting as predecessors of steroid hormones and vitamin D. Also, they serve as growth and development modulators in unicellular organisms. In trypanosomatids, sterols are localized in plasma, inner mitochondrial and glycosomal membranes [198]. Contrasting human hosts, the key sterol in *T. cruzi* metabolism is ergosterol as a substitute of cholesterol. This discovery caused an exhaustive search for the identification and possible effect of inhibitors of ergosterol biosynthesis (EBIs).

Sterol biosynthesis pathway has been determined as a promising target for drug therapy against *T. cruzi*. This depends on i) the peculiarity of this pathway in kinetoplastids, ii) the requirement of *T. cruzi* for specific endogenous sterols, similarly to fungi and yeast, for cell viability and proliferation in all stages of parasite life cycle, and to iii) the *in vitro* and *in vivo* susceptibility to sterol biosynthesis inhibitors (SBIs). Chemically, the ergosterol biosynthesis pathway has been validated *in vitro* at several different steps. The effects shown by the commercially available EBIs, successful for the treatment of fungal diseases, have been suppressive but not curative against human or experimental *T. cruzi* infections, resulting unable to stop the progression of the disease [199, 200]. The sterol metabolic pathway Fig. (**1**) contains several enzymes shown as potential drug targets of this biosynthesic via for combating Chagas disease:

2.2.1. C14-Alpha-Lanosterol Demethylase (CYP51)

In eukaryotes, the sterol C14 α-demethylases are essential enzymes in sterol biosynthesis as well as drug targets in antifungal therapy. They catalyze the oxidative removal of the C14-α-methyl group from the precursors of postsqualene sterol Fig. (**1**) and have been reported in trypanosomatids. Early, despite it was only a very low percentage of amino-acid homology through the biological kingdoms, the orthologous enzymes from bacteria to mammals keep strict catalytic region- and stereo-specificity showing a very limited range of substrates [201]. All of them catalyze basically the three-steps required for the oxidative removal of the C14-α-methyl group from the lanostane structure. In the sterol biosynthesis of pathogenic microbes, this reaction is a requisite. Also, the specific inhibition of

protozoan CYP51 could possibly cure human trypanosomiases [202]. *Tc*CYP51 catalytically strictly related to animal/fungi-like CYP51, showed preference for C4 dimethylsterols. On the contrary, *T. brucei* ortholog enzyme, similarly to plant CYP51 necessitates C4-monomethylated sterol substrates. Interestingly, the CYP51 family is a matter for central P450 structure/function studies as well as an important clinical drug target [203]. In *T. cruzi*, the effects of the SBIs simvistatin, zaragosic acid, terbinafine, ketoconazole, and others on the modulation of diverse sterol biosynthesis genes and their proteins have shown a possible specific regulation of the CYP51 gene expression [204]. Crystal structures of *Tc*CYP51 and *Tb*CYP51 complexed with fluconazole and posaconazole permitted the prediction of essential chemical features, thus offering a starting point for rational trypanocidal drugs design. The structural data showed the molecular background of CYP51 inhibition facilitating the design of novel, selective and more potent agents [205, 206]. The sterol 14-α-methylenecyclopropyl-Δ7-24, 25-dihydrolanosterol (MCP) binds tightly to all protozoan CYP51s and has a strong mechanism-based inhibitory effect on *Tc*CYP51. The crystal structure of *Tb*CYP51 complexed with this substrate analog was presented showing to inflexibility as a typical feature of the CYP51 substrate binding cavity, ratifying this enzyme as an outstanding aspirant for design of novel drugs, comprising the mechanism-based in substrate analog inhibitors [207]. Alike therapeutic targets to CYP51 were identified. Among them, *Tc*CYP51 active site was probed with a library of synthetic small molecules. Whereas the screening allowed the reduction of the library number from about 104,000 to 185 clue compounds showing KD values in the nanomolar order, the cross-validation against *T. cruzi*-infected cells generated 57 active key compounds. The best one showed *T. cruzi* inhibition with EC50 at 17 nM and trypanocidal effect at 40 nM. The structural diversity of the selected key compounds, indicated that CYP51 is a relatively permissive enzyme target for small molecules [208].

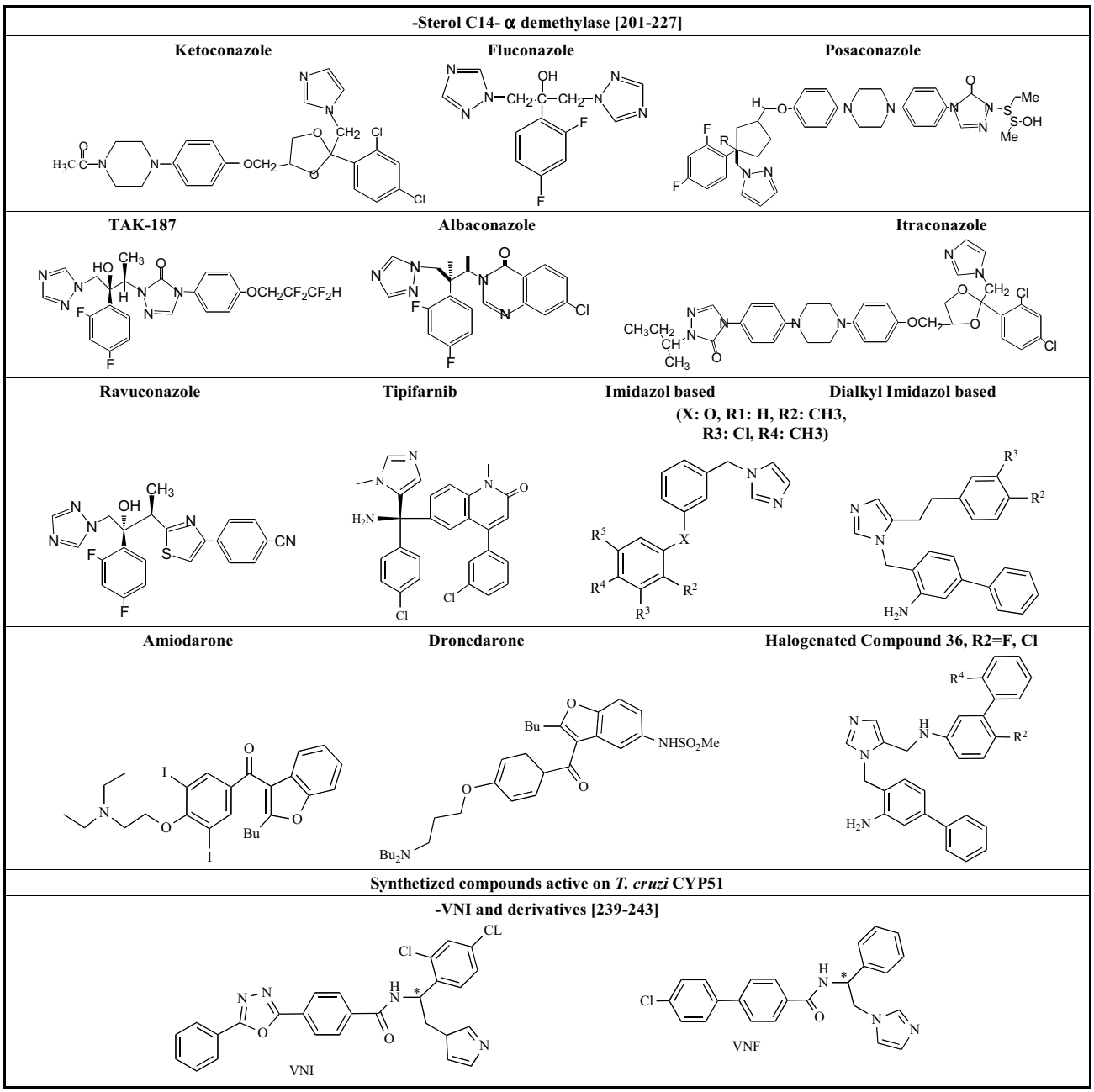
2.2.1.1. Azolic Inhibitors

The chemical structures of the compounds discussed in this item are displayed in Table (**5**).

Among azolic drugs, ketoconazole and itraconazole, target the enzyme CYP51 (Table **5**), producing both the accumulation of 14 α-methylsterols and the declining production of ergosterol. The triazole derivatives, posaconazole, (SCH56592, Schering-Plough Research Institute), D0870, the D(+) isomer of fluconazole (Astra-Zeneca Pharmaceuticals), and TAK-187 (Takeda Chemical Company), known inhibitors of fungal CYP51, are capable of inducing parasitological cure in mice models of both acute and chronic Chagas' disease in absence of toxic side effects [209-211]. The *in vivo* antiparasitic activities displayed by D0870 and posaconazole were attributed to the grouping of unusual pharmacokinetic properties and their strong and selective inhibitory effect on the proliferation of intracellular amastigotes at nanomolar range [199, 211-213]. Noticeably, itraconazole and fluconazole prevented or diminished chronic phase [214]. One of the most potent EBIs tested against *T. cruzi* was albaconazole (UR-9825; Uriach & Company, Barcelona, Spain) [130, 212, 215]. Whereas ketoconazole was no capable to eliminate *T. cruzi* from experimental or natural infection [199], ravuconazole (RAV, Eisai Co., Tokyo, Japan and BMS-207147, Bristol-Myers Squibb) showed to be one of the most progressive contenders for clinical assays [213]. Nevertheless, the cross-resistance between ketoconazole, miconazole and itraconazole exposed in *in vitro* assays, in conjunction with the induced resistance in *T. cruzi* to some azoles, determined the quizzing of the chemotherapeutic use of these compounds [216-218].

Amiodarone, an antiarrhythmic compound, frequently used in symptomatic treatment of Chagas disease infected people, showed activity against *T. cruzi*, both *in vitro* and *in vivo*, by acting synergistically with posaconazole [219] and itraconazole [220]. These results have pointed out the possibility of promising novel approaches using combination chemotherapy for the treatment of Chagas disease by using existing approved drugs [221]. As previously described, some azole derivatives upon long-term treatment frequently cause resistence constituting a severe difficulty [216]. The only azolic compounds that entered clinical trials for the treatment of the chronic form of *T. cruzi* infection have been posaconazole and ravuconazole. The availability of the crystal structure of *Tc*CYP51, studied in complexes with numerous protozoa-specific CYP51 inhibitors, the validation and efficacy of antifungals as *T. cruzi*- specific CYP51 inhibitors could permit the treatment for this infection, alone or combining with the existing drugs [222, 223]. The evolution of clinical process will be discussed in section 6 of this manuscript.

Table 5. Azole inhibitors of CYP51.



The new antifungal drug candidate, the 1-tetrazole-based agent VT-1161, currently in two phase 2b clinical trials, [(R)-2-(2,4-difluorophenyl)-1,1-difluoro-3-(1H-tetrazol-1-yl)-1-{5-[4-(2,2,2tri-fluoroethoxy)phenyl]pyridine-2-yl}propan-2-ol], showed to be a strong inhibitor of *Tc*CYP51. Additionally, VT-1161 revealed a high level of anti-parasitic activity against amastigotes and caused >99.8% *in vivo* suppression of peak parasitemia by using the drug-resistant Y strain in a mouse model of infection. The structural characterization of *T. cruzi* CYP51, complexed with VT-1161 supported the potential utility of this novel tetrazole-based inhibitory chemotype in the treatment of Chagas disease [224].

a. Imidazole-based compounds. Three sets of CYP51 inhibitors including azoles, non-azole compounds, and substrate analogs of the CYP51 reaction were *in vitro* and *in vivo* assayed. The

compound 32-methylene cyclopropyl lanost-7-enol revealed selectivity to *T. cruzi* showing 50% cell proliferation inhibition at 3 μM [218]. On the-other hand, compounds 4c (NEU321) and 4j (NEU704) showed strong activities in contrast to *in vitro T. cruzi* cultures, 160-fold superior than those on host cells. Drug metabolism and properties of compound 4c were *in vitro* studied showing that this chemical agent inhibited *Tc*CYP51. Compound 4c displayed an enhanced ligand efficacy and a smaller synthetic route in comparison with preliminar disclosed CYP51 inhibitors, showing that this compound should be after reflected as a hopeful agent after optimization [225].

b. Dialkyl imidazole derivatives. Inhibitors containing structures simpler than that of posaconazole, were tested as *T*cCYP51 inhibitors, displaying *in vitro* strength for killing *T. cruzi* amastigotes with EC50 values in the nM range. By using a murine model of acute Chagas disease, two compounds were capable to reduce blood parasitemia to undetectable levels. The production of these compounds, considerably less expensive than that of posaconazole, proposed them as clinical candidates for further development of anti-Chagas disease agents. Thus, the most strong and selective inhibitor against parasite cultures showing high efficiency in the acute disease model was identified as compound 2. The latter showed high hydrophobicity and high molecular weight (MW), therefore, methodical modifications were performed on different scaffold positions, identifying inhibitors with the highly potent values of EC50 lower than 1 nM against *T. cruzi* cultures. Halogenated derivatives (Table **5**) displayed excellent activity against *T. cruzi* amastigotes, with reduced molecular weight and lipophilicity, exhibiting suitable physicochemical properties for an oral drug candidate as potential anti-*T. cruzi* agent [226, 227].

2.2.1.2. Non Azolic Inhibitors

The chemical structures of the non-azolic class CYP51 of inhibitors are displayed in Table (**6A**) and the inhibitors of other enzymes of the ergosterol biosynthesis on Table (**6B)**.

a. Tipifarnib analogues. Tipifarnib (R115777), a known inhibitor of human protein farnesyltransferase (PFT), strongly inhibited *T. cruzi* proliferation (ED50=4nM). Unexpectedly, this fact was ascribed to the inhibition of the mentioned CYP51 [228]. Additionaly, a lot of compounds capable to kill *T. cruzi* parasites in a subnanomolar order, lacking PFT inhibition, and beneficial on behalf of other lanosterol CYP51 inhibitors showed low potency inhibition of the cytochrome P450 human enzyme. As tipifarnib exhibited high oral bioavailability and suitable pharmacokinetic properties, the lately discovered tipifarnib analogues showed to be models for the development of drugs to treat this parasitic disease. In a structure-guided fashion, performing changes in the substituents linked to the phenyl group located at the 4-position of the quinoline ring of tipifarnib as well as by replacing the amino group by O-me [229]. In order to diminish the PFT-inhibitory activity and augment the CYP51 inhibition, tipifarnib analogs were developed. The efficacy of the lead tipifarnib analog as compared to that of posaconazole in a mice model of *T. cruzi* infection showed strong blocking activity on parasitemia but no success at curing mice. Crystal structure of *Tc*CYP51 bound to a tipifarnib analog was reported providing a novel structure-based drug design for better tripanocidal compounds [230].

b. LP10. This non-azolic CYP51 inhibitor containing an N-[4-pyridyl]-formamide scaffold with ability to bind to the active site of *Tc*CYP51, has been studied in an acute murine model of *T. cruzi* infection. Similarly to the results reported for posaconazole, the curative effect of this compound was observed. A treatment of mice with LP10 during 30 days, initiated 24 h post-infection, showed no signs of acute disease and tissues remained histologically unaffected after 6 months. The analysis of sterol composition confirmed that LP10 blocked the C14-alpha demethylation step, showing severe ultrastructural membrane abnormalities and amastigotes death. The analysis of sterol composition confirmed the alterations observed [231].

c. Indomethacin-amide derivatives. Searching for *Tc*CYP51 inhibitors, a set of indomethacin-amides displaying a structure with similarities to a class of cyclooxygenase-2-selective inhibitors was obtained. These derivatives bind to *Tc*CYP51, *in vitro* inhibiting the enzyme activity and the parasite sterol biosynthetic pathway producing strong trypanocidal effects in parasite cultures [232].

d. 4-Aminopyridyl-based derivatives. The use of structure-based drug design and SAR analyses allowed the development of a series of 4-aminopyridyl-based lead inhibitors capable to tar-

get *Tc*CYP51. The key leader of this screening was LP10 (EC50=0.65 μM), which was optimized for giving other hit compounds also with high binding affinity to *Tc*CYP51 and substantial activity on amastigotes. Many of the improved compounds resulted selective against human metabolic CYPs enzymes showing an IC50 value in the μM order [233].

e. d-Tryptophan-derived inhibitors. Tryptophan-derived potent and selective inhibitors of *Tc* CYP51 were developed. The co-crystal structure information obtained from CYP51 and (R)-2, which resulted more than 1000-fold strong than its enantiomer (S)-1, was used to design additional analogues. *In vitro* data obtained for compounds (R)-2-(R)-8, in conjunction with pharmacokinetic data suggested that this new CYP51 inhibitor scaffold series has potential to deliver drug candidates for treatment of *T. cruzi* infections [234].

f. N-indolyl-oxopyridinyl-4-aminopropanyl-based scaffold. The SAR of N-indolyl-oxopyridinyl-4-aminopropanyl-based scaffold identified in a target-based screening was explored in order to optimize specific drug candidates for *Tc*CYP51. This scaffold advanced by medicinal chemistry yielding oral lead compounds with strong *in vivo* trypanocidal activity. An animal model of infection using a transgenic *T. cruzi* Y luc strain that expresses firefly luciferase, allowed the selection of biaryl and N-arylpiperazine analogues. Crystal structure analysis allowed the characterization of drug-target complexes for both scaffold alternatives. The optimization of the binding and pharmacokinetic characteristics of these compounds might bid strong inhibitors against experimental *T. cruzi* infection [235]. In addition, the SAR of an N-arylpiperazine series of N-indolyloxopyridinyl-4-aminopropanyl-based inhibitors designed in order to test the effect of substituents in the terminal N-phenyl ring on binding type, selectivity and potency was studied. Two different ring binding types, hidden and solvent-exposed have been detected depending on the substituents at C-4, by X-ray structure analysis. Two 5-chloro-substituted analogs, 9 and 10, with no substituent at C-4 proved enhanced selectivity and potency, reducing more than 99.8% blood parasites in mice [236].

g. Vinylsulfone K777 analogues. Recent SAR aimed at addressing potential liabilities of the *T. cruzi* CPI have generated the compound N-[(2S)-1-[[(E,3S)-1-(benzenesulfonyl)-5-phenylpent-1-en-3-yl]amino]-3-(4-methylphenyl)-1-oxopropan-2-yl]pyridine-4-carboxamide (4), showing a trypanocidal effect at ten-fold lower concentrations than K777. Anti-*T. cruzi* activity of the carboxamide analogue depends firstly from the *Tc*CYP51 inhibition. Also, this new compound inhibited CYP isoforms in mammals but showed trypanocidal activity at concentrations under those required to *in vitro* inhibit mammalian CYPs significantly. The evaluation of computational docking studies and truncated analogues of this novel class of inhibitors revealed structural determinants responsible for *Tc*CYP51 binding [237].

h. Benzamide derivative. The compound (R)-N-(1-(3,4'-difluorobiphenyl-4-yl)-2-(1H-imidazol-1-yl)ethyl)-4-(5-phenyl-1,3,4-oxadiazol-2-yl)benzamide, VFV, designed to fill the deepest portion of the CYP51 substrate-binding cavity, revealed a strong antiparasitic activity in cellular experiments and cured the experimental Chagas disease with 100% efficacy. Studies on oral bioavailability, target activity, satisfactory pharmacokinetics and tissue distribution characterized VFV as an auspicious new drug candidate [238].

i. VNI and derivatives. The first enantioselective chemical synthesized compound showed to be a strong experimental *Tc*CYP51 inhibitor [239]. This small molecule, non-toxic and with high selectivity, exhibits favorable pharmacokinetics and once administered by oral via to mice achieved a parasitological cure, including acure rate (100%) and survival (100%), in both stages of *T. cruzi* infection [240]. An exploration of the potential of VNI by nitro-derivative-resistant *T. cruzi* strains, using strict protocols of acute infection showed high anti-*T. cruzi* efficacy of VNI and its derivative VNF against *T. cruzi* bloodstream trypomastigotes and amastigotes. VNI showed no mutagenic potential. In *T. cruzi*, cell ultrastructure analysis showed that the damage induced by VNI presented an autophagic phenotype. Thus, VNI was described as a very auspicious anti-*T. cruzi* drug candidate for Chagas disease therapy [241]. Comparative structural analysis of CYP51 complexes with the inhibitor VNI and two derivatives suggested that broad-spectrum CYP51 inhibitors are likely to be preferable as anti-Chagas disease drug candidates [242]. Finally, the main steps of the sterol biosynthetic pathway has been recently reviewed showing each enzyme involved in the

steps, as well as the antiproliferative, physiological, biochemical, and ultrastructural effects of the main known inhibitors (Table **5**) [243].

2.2.2. Oxidosqualene Cyclase (OSC)

OSC, also named lanosterol synthase, is a clue enzyme in sterol biosynthetic pathway, converting 2, 3-oxidosqualene to lanosterol, a tetracyclic product Fig. (**1**). In the production of mature sterols, the synthesis of lanosterol constitutes an essential step. In yeast and higher eukaryotes, OSC catalyzes the synthesis of lanosterol from 2, 3-oxidosqualene by a complex cyclization-rearrangement reaction involving the formation of a total of six new carbon carbon bonds by a single enzyme. OSCs from trypanosomes and hosts use different catalytic motifs, which could lead to the development of specific inhibitors for this enzyme [244]. This was done in the following years but with non-azolic compounds.

a. Pyridinium ion based inhibitors. N-Alkyl- and N-prenylpyridinium ions showed potent anti-*T. cruzi* activities and inhibited the sterol biosynthesis in these organisms. Specific non-azolic inhibitors such as N-(4E, 8E)-5, 9, 13-trimethyl-4, 8, 12- tetradecatrien-1-ylpyridinium and a series of compounds were designed to inhibit OSC and tested against mammalian parasite stages, resulting twelve of them highly active in the nM range against trypomastigotes [245] (Table **6B**).

b. Phenylthiovinyl derivatives. Nineteen inhibitors such as aza, methylidene, vinyl sulfide, and conjugated vinyl sulfide derivatives of oxidosqualene and squalene were tested by using a recombinant *T. cruzi* OSC. Some phenylthiovinyl derivatives resulted 10 to 100 times more effective on the *T. cruzi* OSC than on the control enzymes [246]. OSC inhibitors used to treat fungal, bacterial and parasite infections including trypanosomatids based on the drug induced blockage of sterol biosynthesis were reported by members of the University of Utah Research Foundation [247] (Tables **2** and **6B**).

c. Cyclohexylamine based inhibitors. A series of 25 inhibitor compounds of human liver OSC, were tested as inhibitors of *Saccharomyces cerevisiae*, *T. cruzi*, *Pneumocystis carinii* and *Arabidopsis thaliana* OSCs expressed in a *S. cerevisiae* OSC-defective strain. Remarkably, two derivatives identified by screening, the cyclohexylamine substituted compounds 9 and 20, showed an activity lower than 60 μM, similarly to the analog of 5-phenyl-benzo[d]isothiazol compound 11. In particular, these compounds resulted auspicious for the development of new anti-*T. cruzi* agents [248] (Table **6B**).

2.2.3. Squalene Epoxidase (SQO)

SQO catalyzes the transformation of squalene to (3S) 2, 3-oxidosqualene Fig. (**1**). In vertebrates, it is a non-metallic, flavoprotein monooxygenase enzyme which serves as a potential target for the design of chemotherapeutic drugs for different pathogenic organisms [249].

a. Allylamine based inhibitors. In the sterol biosynthesis, the allylamine terbinafine, among antifungal drugs, is capable to inhibit squalene epoxidase pathway and resulted to be synergistic with ketoconazole against *T. cruzi* [250] (Table **6B**).

b. 5-Nitrofuranes and 5-nitrothiophenes derivatives. Among 5-nitrofurane derivatives, the synthesized heteroallyl-containing 5-nitrofuranes exhibited a dual mechanism of action based in oxidative stress and inhibition of membrane sterol biosynthesis as potential anti-*T. cruzi* agents. Some of the derivatives displayed high and selective activity against the proliferative parasite forms, showing IC50 values against the amastigote forms in the low μM to sub-mM range. The *in vitro* activity of these novel compounds was higher than those of nitrofurane and terbinafine. In addition, novel 5-nitrofuranes and the thia-analogues displayed outstanding effects on *T. cruzi* viability. Free sterols analysis from parasite incubated with these compounds showed squalene accumulation in comparison with Nx, proposing the inhibition of parasite squalene epoxidase activity. SAR studies showed relevant features for the design of novel derivatives. In trypanosomatids, altogether, these findings pointed to a general effect of 5-nitrofuranes and 5-nitrothiophenes, considering them as latent chemotherapeutic agents for parasitic diseases [251, 252] (Table **6B**).

2.2.4. Squalene Synthase (SQS)

SQS catalyzes the first step in sterol biosynthesis by a head-to-head reductive dimerization of two molecules of farnesyl pyrophosphate (FPP) in a reaction of two steps to produce squalene Fig. (**1**). At present, this enzyme is extensively studied as a possible target for cholesterol-lowering

agents, and interestingly described as a promising target for antiparasitic chemotherapy [253, 254].

a. Thiocyanate derivatives. The 4-phenoxyphenoxyethyl thiocyanate (WC-9) was shown to be an effective and strong agent against epimastigote proliferation capable to produce the accumulation of low MW metabolites from mevalonate to squalene [255]. WC-9 induced *T. cruzi* epimastigotes growth inhibition associated with a decrease in parasite endogenous sterols content owed to a specific blockage of their *de novo* synthesis at the level of squalene synthase enzyme [256] (Table **6B**). Also some aryloxyethyl thiocyanate derivatives, structurally related WC-9 were reported, some of them with trypanocidal activity comparable with ketoconazole. Moreover, one of them was shown to be an effective anti-Chagas disease agent with auspicious future as a lead drug for further *in vivo* studies [257].

b. Quinuclidine based inhibitors. Among quinuclidine inhibitors synthesized, 3-(biphenyl-4-yl)-3 hydroxyquinuclidine (BPQ-OH) resulted a powerful non-competitive inhibitor of *T. cruzi* SQS, showing a Ki value in the nM range (Table **6B**). BPQ-OH showed the ability to eliminate intracellular *T. cruzi* amastigotes from culture cells

Table 6A. Non-azole inhibitors of CYP51.

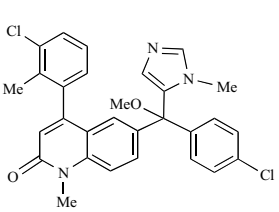
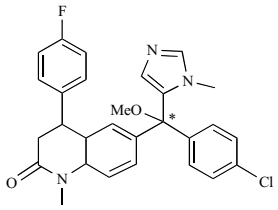
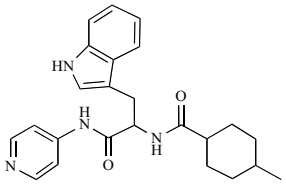
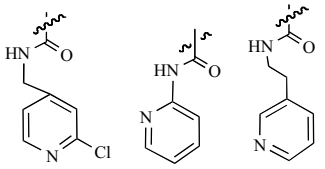
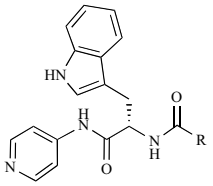
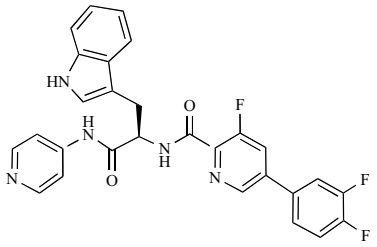
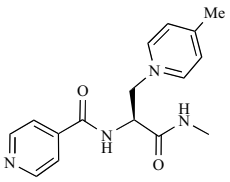
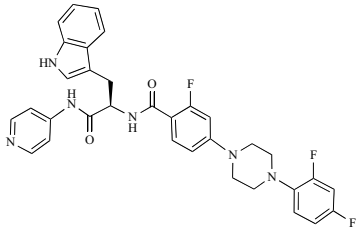
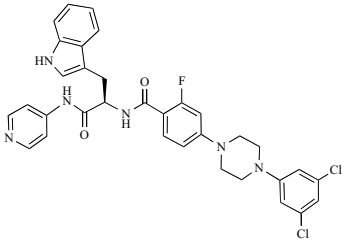
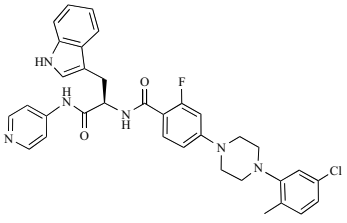
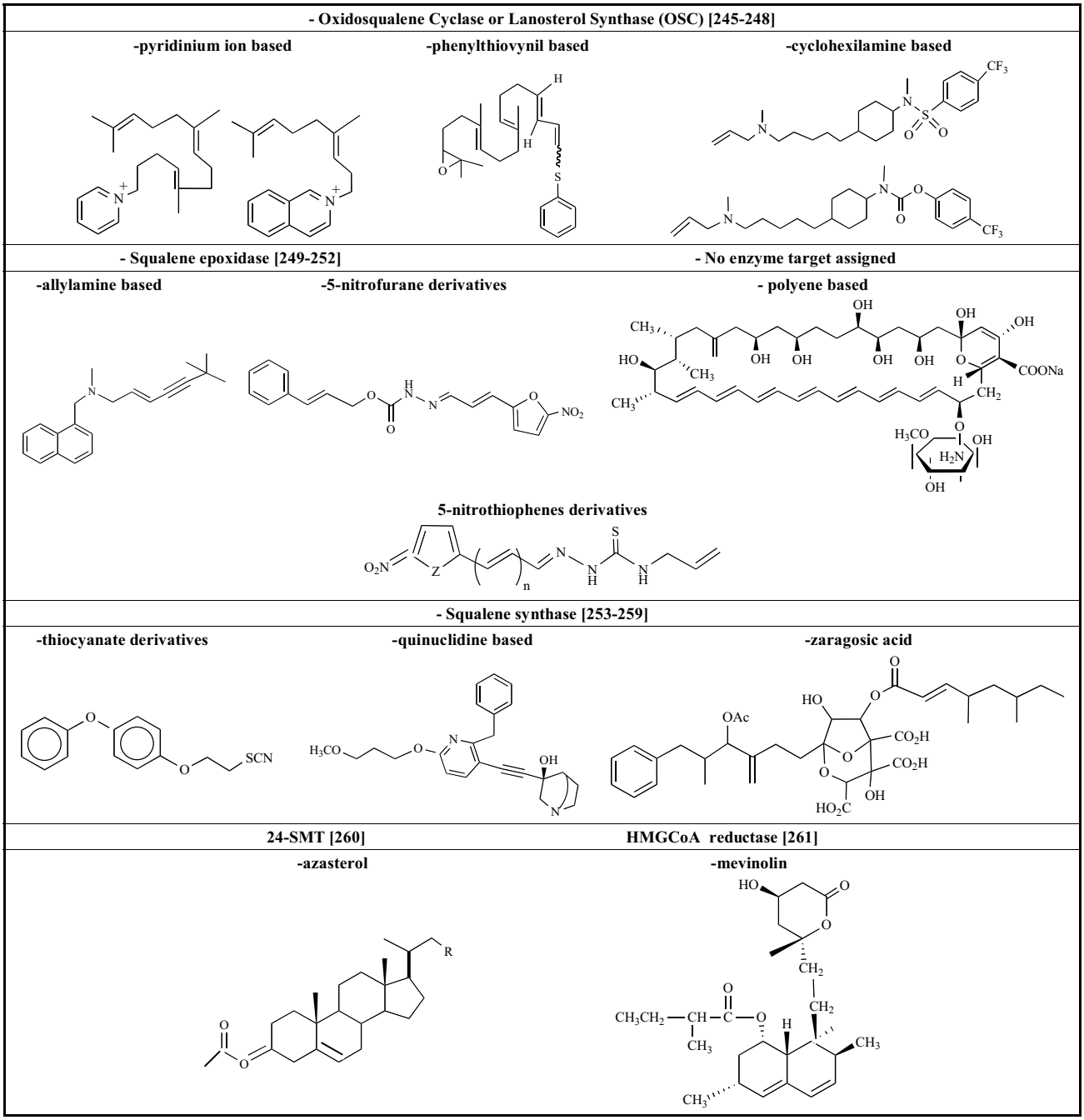
- C14α-lanosterol demethylase (CYP51) [228-238]	
Tipifarnib analogs [228-231]	
 	 **LP10 (ChemDiv C155-0123)**
Indomethacin-amide derivatives	**4-Aminopyridyl-based derivatives**
 **[232]**	 **[233]**
d-Tryptophan-derived inhibitors.	**Vinylsulfone K777 analogues**
 **[234]**	 **[237]**
N-indolyl-oxopyridinyl-4-aminopropanyl-based scaffold	
 **[235]**	 **Compound 9**  **Compound 10** **[236]**

Table 6B. Inhibitors of other enzymes of the ergosterol biosynthesis.



producing no side effects on the host cells [199, 258]. Aditionally, E5700 and ER-119884 were reported as strong non-competitive or mixed-class inhibitors of *T. cruzi* SQS with Ki values in the low nM to sub nM range. E5700, by oral administration, was capable of providing complete protection in *in vivo* studies [254]. SQS inhibitors were screened against a recombinant leishmanial SQS, against *L. mexicana* promastigotes, and *T. cruzi* intracellular amastigotes. An association between compounds that inhibited the enzyme and the reduction in the levels of steroids caused proliferation inhibition of *L. mexicana* promastigotes [259].

2.2.5. Delta 24 (25)-Methyltransferase (24-SMT)

As shown in the biosynthetic pathway, this enzyme is essential for the biosynthesis of ergosterol, but not required for the biosynthesis of cholesterol

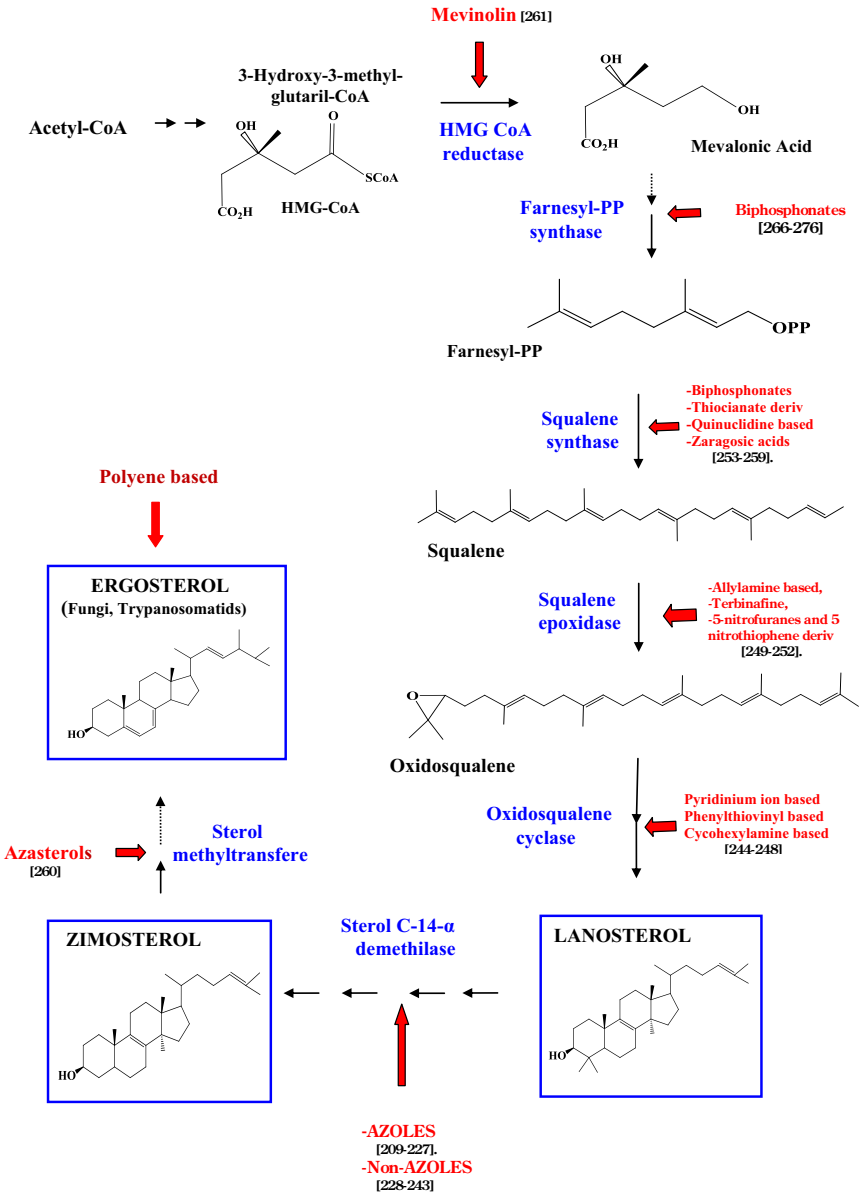


Fig. **(1).** Metabolic pathway of ergosterol biosynthesis in *Trypanosoma cruzi* and enzyme inhibitors.

Fig. **(1)**. The design, synthesis and evaluation of a series of potential transition state analogues of 24-SMT against the recombinant *L. major* 24-SMT and the parasites *L. donovani* and *T. cruzi in vitro* were carried out. Some of the compounds showed inhibition of the recombinant *L. major* 24-SMT and inhibited parasite proliferation. Other analogs, although did not show enzyme inhibition, presented anti-*T. cruzi* activity [260] (Table **6B**).

2.2.6. 3-Hydroxy-3-Methyl-Glutaryl-Coenzyme A-(HMGCoA) Reductase

Mevinolin, an inhibitor of HMGCoA, showed antiproliferative effects when tested on *T. cruzi* by *in vivo* and *in vitro* assays (Fig. **1**; Table **6B**). Besides, the evaluation of the ability of ketoconazole, terbinafine, and other specific EBIs, was performed. The combination of mevinolin with ketoconazole, terbinafine, and other specific EBIs revealed a synergic action against *T. cruzi* proliferative forms [261]. Different Amphotericin B-lipid formulations assayed *in vitro* and *in vivo* displayed strong trypanocidal activities [262]. It is worth mentioning that the low number of disclosed patents does not reflect the increasing multiple clues related to enzymes of this biosynthetic pathway. Scientist of the Yale University presented the synthesis of CYP51 inhibitors including 5-amino-1-

benzylimidazole derivatives, showing antibacterial, antifungal and anti-trypanosomal activity. These agents showed no host cells toxicity and a notable IC50 ranging from μM to pM values against *T. cruzi* amastigotes. The analysis of *in vivo* experimental mice tests suggested both the responsability of a phenylbenzylimidazole moiety for the enzyme inhibition and anti-*T. cruzi* activity. However, no data on enzymatic inhibition was described [263-265] (Table **2**).

2.3. Biosynthesis of Polyisoprenoids

The enzymes of the isoprenoid pathway have been consigned to different compartments in eukaryotes, counting trypanosomatids. The chemical structures of the compounds discussed in this item are displayed in Table (**7**).

2.3.1. Farnesylpyrophosphate Synthase (FPPS)

FPPS is the enzyme in charge for the formation of farnesylpyrophosphate, marking the branching point in the synthesis of a diversity of sterols and other indispensable isoprenoids in these pathogenic protozoa. The condensation of the diphosphates of C5 alcohols to form C10 and C15 diphosphates (geranyl and farnesyl) is performed by FPPS. In *T. cruzi*, the gene codifiying for *Tc*FPPS was cloned, sequenced, expressed and characterized as a crucial enzyme for parasite survival. *Tc*FPPS confines to the cytoplasm and is absent in other organelles such as the mitochondria and glycosomes [266].

Bisphophonates, metabolically inert inorganic PP analogues compounds that inhibit FPPS, can block the above mentioned pathways [267]. Human bone resorption disorders implicate bisphosphonates as good candidates with potential innocuousness to control tropical diseases. The nitrogen-containing bisphosphonates risedronate and pamidronate inhibited to the recombinant FPPS by both reducing parasitemia in infected mice and inhibiting intracellular replication of amastigotes in *in vitro* assays [199, 213]. By contrast, etidronate, another non-nitrogen-containing bisphosphonate did not affect parasite proliferation [268]. Sterols analysis performed in treated parasites showed that risedronate inhibited epimastigotes proliferation and sterol biosynthesis at a presqualene level, associating these results with FPPS inhibition, appearing as a positive lead compound for the development of novel agents against *T. cruzi* [269-271]. Although some fatty acids-derived bisphosphonate agents showed to be strong growth inhibitors of amastigotes at low μM level, none of them resulted effective against epimastigotes [272, 273]. The selective action exhibited by bisphophonates against *T. cruzi* was attributed to the drug accumulation in parasite acidocalcisomes [274]. The structures of the *Tc*FPPS alone and in complexes with substrates and inhibitors were analyzed revealing that the enzyme undergoes conformational changes after binding, enabling the enzyme to bind to a bisphosphonate inhibitor [275]. 2-Alkylmercaptoethyl-1,1-bisphosphonate derivatives were synthesized and tested against *T. cruzi*, and several of them resulted strong inhibitors on parasite amastigotes, targeting *Tc*FPPS. A remarkable emergence of long chain length sulfur-containing bisphosphonates as relevant antiparasitic agents occurred. The representative members of this class of drugs, shown as compounds 37, 38, and 39, exhibited ED50 values against *T. cruzi* amastigotes and IC50 for *Tc*FPPS inhibition in the μM order [276] (Table **7**).

2.3.2. Protein Farnesyltransferase (PFT)

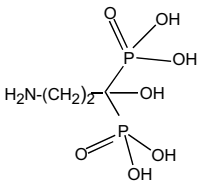
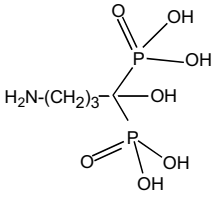
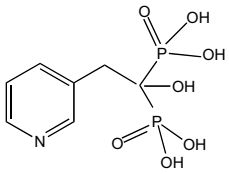
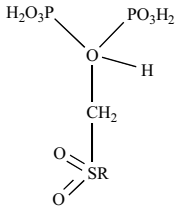
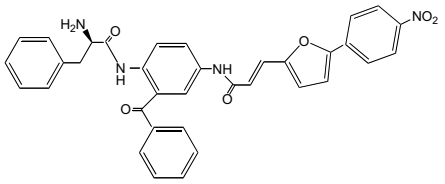
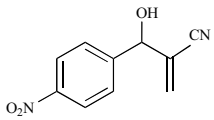
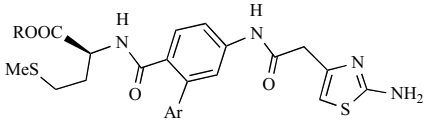
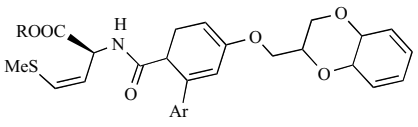
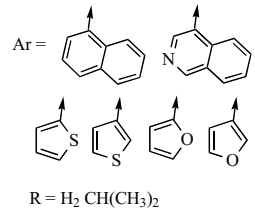
PFT is the enzyme responsible for the transference of a farnesyl residue from farnesylpyrophosphate to the thiol of a cysteine side chain of proteins carrying the so called CaaX-sequence at the C-terminus. Protein prenylation defined as the attachment of polyisoprenoids to specific proteins, is involved in signal transduction and anchorage of protein to cell membranes [277, 278]. Prenylation was demonstrated in trypanosomatids. When PFT of *T. cruzi* was cloned, differences with its mammalian counterpart were found, validating the use of PFT as target for a trypanocidal drug [279]. Some PFT inhibitors (PFTIs) have been assayed in the context of phase III clinical assays in treatment of human cancer [280]. The improvement of PFTIs development is visibly directed to non-thiol PFTIs because side drug effects were associated to free thiols. Numerous non-thiol benzophenone-scaffold-based PFTIs were examined in *in vitro* and *in vivo* assays with *T. cruzi*. The structural feature shared by all inhibitors was an amino function capable to be protonated. Thus, R-phenylalanine and N-propylpiperazinyl derivatives showed no cytotoxicity to cells, displaying the best *in vitro* activity with IC50 values in the nM range. On the other hand, *in vivo* testing of the survival rates of infected animals ranged from 60 to 80% at day 115 post infection. As mentioned above, tipifarnib, the PFTI in anticancer clinical trials, was able to

kill *T. cruzi* by blocking the CYP51. The development of tipifarnib analogues displaying reduced affinity for human PFT in order to reduce toxicity while increasing affinity against *Tc*CYP51 was performed including reasonable modifcations, resulting efficient in an acute-Chagas disease-mouse model [281]. Mark Field patented the use of PFTIs, including manumycin A, a natural antibiotic and other synthetic compounds such as cyclic hexenones, used for the treatment of parasitic diseases [282]. Schering Corp also disclosed twenty one piperazine or piperidine scaffold based-PFTIs used for *T. brucei* infection treatment [283]. However, no patents connected with *T. cruzi* PFT inhibitors have been reported yet (Table **2**). The Morita-Baylis-Hillman adduct (MBHA) (Compound 3), 3-hydroxy-2-methylene-3-(4-nitrophenyl)-propanenitrile) was synthesized, strongly inhibited epimastigotes proliferation, caused intense trypomastigotes lysis and morphological ultrastructural changes in both parasite forms. Docking calculations of compounds 1, 2 and 3 proposed the possibility of 3 mechanism of action on *Tc*FPPS inhibition [284] (Table **7**).

2.3.3. Protein Geranylgeranyltransferase Type I (PGGT-I)

PGGT-I, similarly to PFT, occurs in many eukaryotic cells. It consists of two subunits, a common alpha subunit and a different beta subunit. In *T. cruzi* gene database, a protein showing 20% amino acid sequence identity to the PGGT-I beta of other species was identified [285]. The cloning and characterization of a recombinant ortholog of *Tc*PGGT-I showed PGGT activity with distinct specificity toward protein substrates when compared to that of the mammalian PGGT-I and *Tc*PFT. Interestingly, it was shown that cytosol

Table 7. Isoprenoid metabolism inhibitors.

-farnesyl PP synthase inhibitors [266-276]
Bisphosphonates
Pamidronate  **Alendronate**  **Risedronate** 
Sulfur containing Bisphosphonates [276]  **37 (R: n-octyl), 38 (R: n-nonyl), 39 (R: n-decyl)**
-farnesyl transferase inhibitors [277-284]
Benzophenone based  **MBHA (3-Hydroxy-2-methylene-3-(4-nitrophenylpropanenitrile) Compound 3** 
-Protein Geranylgeranyltransferase Type I inhibitors [285-288]
Terminal CaaX tetrapeptide mimetics    R = H_2 $CH(CH_3)_2$

fractions obtained from trypomastigotes and epimastigotes contained 100-fold lower levels of PGGT-I activity compared with PFT activity. Although the CaaX motif mimetics PGGT-I inhibitors exhibited very low potency on *Tc*PGGT-I compared to the mammalian enzyme, it was suggested as a potential target to develop selective inhibitors [286, 287]. Mimetics of terminal CAAX tetrapeptide of Ras protein containing different biaryl cores in place of AA were prepared and studied as PFTI [288] (Table **7**).

2.4. Thiol-Dependent Redox Metabolism

A unique thiol-dependant redox metabolism, based on a low molecular weight thiol-polyamine conjugate, N1, N8 bis (glutationyl), trypanothione T(H)2, an unusual spermidine-glutathione conjugate, is exclusively found in flagellates of the order Kinetoplastida replacing the ubiquitous glutathione reductase (GR) [289]. The parasite antioxidant defense contains a key enzyme, TR, that catalyses the NADPH-dependent reduction of T(H)2. The presence of the trypanothione system was proven by PCR and homology cloning. In *T. cruzi*, the synthesis of glutathione begins with the consecutive action of ɣ-glutamyl cysteine synthase and glutathion synthase by an ATP-dependant reaction. Thus, trypanothione synthesis requires the conjugation of two molecules of glutathione with spermidine. The enzyme ɣ-glutamylcysteine synthase is the clue limitant enzyme of this pattern and buthionine sulfoximine (BSO) has been described as its inhibitor Fig. (**2**). BSO and other inhibitors from trypanothione metabolism resulted to be potential candidates as agents against *T. cruzi,* alone or in conjunction with with free radical-producing drugs such as the existing used Nx and Bz [290]. The absence of trypanothione in the mammalian host in addition to the sensitivity of trypanosomatids concerning oxidative stress and, the unability to synthesize T(SH)2, validate the enzymes of the trypanothione metabolism as drug-targets [291, 292]. X-ray crystallography analysis was early performed on the three-dimensional structure of the purified TR in free form, in complex with substrates and/ or in the presence of inhibitors.

The proximal reductant of tryparedoxin is responsible for substitution of thioredoxin-, glutaredoxin- and glutathione-dependent reactions. Finally, the heterologous expression, the functional characterization and the crystallization of some components of this recombinant system enabled the design of inhibitors based on rational structure [293]. In this biosynthetic pathway, there are three key points that are susceptible to be attacked pharmacologically. These are: the activity of the TR, the function of glutamate-cysteine ligase (GCL) and polyamine transport in *T. cruzi,* in contrast, to GCL and the polyamine uptake system [294]. On the other hand, notably, the trypanothione system is capable to sustain several cellular functions mediated by thiol-dependent (redox) processes. Novel functions have emerged for trypanothione as a potential cofactor in iron metabolism. The minimalist thiol-redox system, developed by trypanosomatids, is an example of metabolic fitness driven by the remarkable physicochemical properties of a glutathione derivative [295, 296].

Many trypanocidal agents have been involved in the trypanothione metabolism, some of them

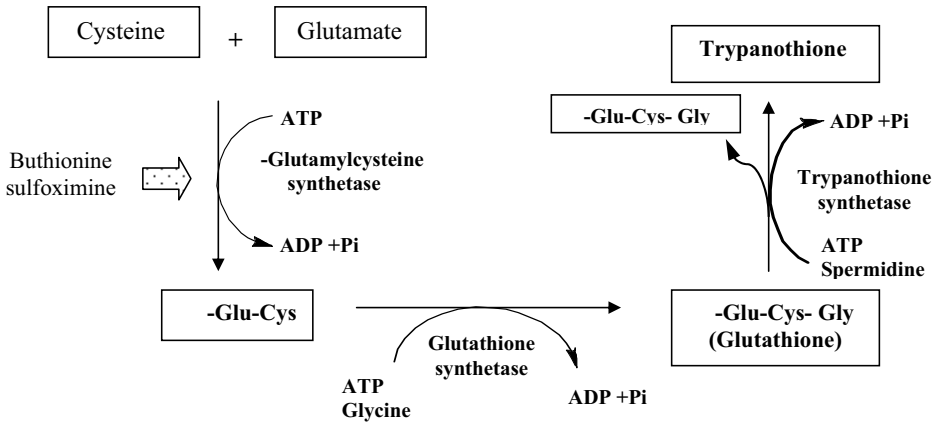


Fig. (2). Trypanothione synthesis requires the conjugation of two molecules of glutathione with spermidine. The enzyme ɣ-glutamylcysteine synthase is the clue limitant enzyme of this pattern and buthionine sulfoximine (BSO), the inhibitor of this step.

inhibiting *T. cruzi* TR [297]. The chemical structures of the compounds discussed in this item are displayed in Table (**8**).

2.4.1.1. TR Irreversible Inhibitors

a. Subversive substrates or sabotage inhibitors. Molecules capable to convert an antioxidative disulfide reductase into a pro-oxidative enzyme are usually known as sabotage inhibitors. Characteristic subversive substrates are reduced in single-electron steps to the respective radicals that further react with molecular oxygen yielding superoxide anion radicals, thus increasing the consequences of oxidative stress. The subversive process may occur avoiding the regeneration of T (SH)2, in case that the acting reductase is TR [298]. Among compounds that can act subversive substrates of TR and other flavoenzymes it can be considered nitrofurans and naphthoquinones (Table **8**) [297, 299]. Nitrofuran compounds, moderate inhibitors of TR and GR and the compounds nifuroxime, nifuroxazide, and nifurprazine, showed to be the most promising derivatives, since they were redox-cycled by the *T. cruzi* flavoenzymes lipoamidedehidrogenase (LipDH) and TR having pronounced antiparasitic effects on *T. cruzi* cultures, suggesting that these nitrofuran derivatives as trypanocidal drugs [299]. The bactericidal nitrofuran derivative chinifur is an inhibitor and subversive substrate of TR that interacts weakly to some structurally related antioxidant enzymes [300]. However, a series of nitrofurazones and nitrothienyl analogues derivatives, proposed as TR inhibitors, in which the semicarbazide moiety consisted of different alkyl and aromatic chains aiming at mimicking the spermidine part of trypanothione were not found to be significantly better inhibitors of *T. cruzi* in *in vitro* growth [301]. When hydroxymethylnitrofurazone (NFOH), generated by the addition of a hydroxymethyl group to nitrofurazone was tested in a murine model of Chagas' disease, a dramatic reduction of parasitemia levels similar to that obtained with Bz was observed. The percent of mortality were 16%, 33% and 66% espectively for NFOH, Bz and placebo groups [302]. On the other hand, nitrofurazone was highly toxic, leading to an overall rate of mortality near to 75%, requiring the interruption of the treatment. Naphthoquinones readily suffer redox cycling in aerobic conditions. Several natural naphthoquinones and synthetic derivatives, have been tested as trypanocidal agents [297, 303, 304]. Among them, menadione, plumbagin, and lapachol although presenting trypanocidal activities, interacted with *Tc*TR as well as *h*GR [305]. A series of menadione, plumbagin, and juglone derivatives have been synthesized in order to obtain trypanocidal compounds specific for *Tc*TR [297]. Two 1, 4-naphthoquinone moieties linked by a polyamine spacer were contained in two of the most strong derivatives [297]. A significant trypanocidal activity is not obtained by inhibiting TR alone but the combination of both inhibition of T(SH)2 reduction and redox cycling is required to render the parasite more susceptible to free radical species detrimental effects [303].

Later, knowing that methylene blue display trypanocidal activity, when this phenothiazine drug was tested on a quantity of specific molecules of the parasite antioxidant metabolism, disulfide reductases and its thiol products, *Tc*TR inhibition was detected [306]. Also, the diaryl sulfide-based TR inhibitors (TRIs) were investigated as subversive substrates with anti-*T. cruzi* properties [307].

b. Ajoene. The spontaneous degradation product of the major sulfur garlic-derived natural compound allicin, ((E,Z)-4,5,9-trithiadodeca-1,6,11-triene-9-oxide), is known for its antifungal, antiviral, anti-trypanosomal, and antimalarial activity. Additionally, it is a covalent inhibitor and subversive substrate of both human GR and *T. cruzi* TR. The study of a crystal structure of GR inhibited by (E)-ajoene evidenced a mixed disulfide between the active site Cys58 of the enzyme with a specific moiety of ajoene. The flavoenzymes and ajoene interact increasing the cellular oxidative stress. Part of the antiparasitic and cytostatic actions of ajoene may at least be due to the numerous effects on key enzymes of the antioxidant thiol metabolism (Table **8**) [308].

c. Organ metallic complexes. Some platinum II organometallic complexes are extensively used in therapy of cancer. They are also irreversible ligands of *Tc*TR, but not of GR, with both *in vivo* and *in vitro* trypanocidal activities assays [309, 310]. On the other hand, it was reported that complexation of the known antiparasitic drug ketoconazol with ruthenium II or III and rhodium II enhanced the activity of the parental drugs disabling primary and secondary drug resistance [311]. Synthesized copper (II) and gold (I) clotrimazole and ketoconazole complexes exhibited higher inhibitory activity on *T. cruzi* proliferation

than their own parental compounds [312]. Isis Innovation Ltd presented a patent claiming that some (2, 2 ’6 ’2 ’’terpyridine) platinum II complexes were useful as antitumoral and antiprotozoal agents [313] (Table **2**). Nearly forty complexes including pyridine-2-thiolate-(4-chloro-2, 2’, 6’, 2’’terpyridine) platinum (II), were synthesized, inhibited the TR reduced form and were active on *T. cruzi* and other trypanosomatids. The Mannich bases, unsaturated irreversibly compounds inactivated *Tc*TR and exposed a divinyl ketone as the active compound responsible for the enzyme inactivation by structural studies [314]. Among sixteen palladium (II) complexes with some bioactive nitrofuran-containing thiosemicarbazones as ligands, many of them showed higher *in vitro* growth inhibition against *T. cruzi* than Nx and potent DNA binding but the main mechanism of action seems to be due to the oxidative stress as a result of their bioreduction and extensive redox cycling. Additionally, the complexes were shown to be irreversible TRI [315] (Table **8**).

2.4.1.2. Reversible Inhibitors (Table 8)

a. Tricyclic compounds. Tricyclic neuroleptic compounds have been considered as an auspicious class of TRIs [316]. Trypanocidal effects on epimastigote and trypomastigote forms are exerted by clomipramine, trifluopherazine combined with thioridazine, and prometazine. Also, clomipramine and thioridazine resulted effective in *in vivo* models [317] (Table **8**). Clomipramine led to 70% mice survival after more than 2 years, preventing the evolution to fibrosis of the inflammatory infiltrates [318, 319]. However, the psychotropic activity of clomipramine rules out its use as chemotherapeutic agent. Analogs of this drug containing a tricyclic dibenzosuberyl moiety and a series of polyamines substituted with N-dibenzosuberyl structure were prepared. The analogs of clomipramine were poor TRIs, while the polyamine derivatives were effective with the strongest compound, N(4),N(8)-bis(dibenzosuberyl)spermine(7), having a Ki value=0.26 mM [320].The transmission of Chagas disease by blood transfusion could be prevented by the acridine derivative mepacrine, which similarly to phenothiazines, acts as TR reversible competitive inhibitor but not of GR. Finally, a crystallographic TR-inhibitor complex was obtained coupling mepacrine to the active site of *T. cruzi* TR [321]. Furthermore, studies in conjuction of biological activities, mutation studies, and virtual ligand docking simulations performed with a new series of TR small-molecule inhibitors led to the prediction of a binding mode that was confirmed by crystal structure analysis. The binding conformation and potency of the inhibitors resulted wide-ranged between TR from *T. brucei* and *T. cruzi* [322]. A synopsis of the diverse tricyclic compounds, strong TRIs displaying inhibitory activities against parasites has been recently reported [323].

b. Aminodiphenylsulfides. Some compounds with lesser neuroleptic activity than phenothiazines corresponding to a series of 2-amino diphenylsulfides were synthesized resulting strong TRIs, demonstrating that extremely bulky ligands provided easy accommodation to the enzyme active site (Table **8**) [324, 325].

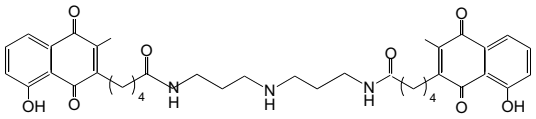
c. Polyamine derivatives. The analysis of numerous synthesized polyamine-based inhibitor compounds showed that many of the spermine derivatives were significantly more effective than the equivalent spermidines [326] (Table **8**). A library of spermidine-peptide conjugate was screened exposing that N 1, N 1, N 4, N 8, N 12-penta (3-phenylpropyl) spermine was the greatest effective competitive inhibitor of *Tc*TR. The compounds of this series resulted potent trypanocides, but a clear correlation between enzyme inhibition and anti-*T. cruzi* activity was not demonstrated. Numerous synthesized polyamine derivatives showed to be strong competitive inhibitors of *Tc*TR. Among them, compound 12 showed to be the most effective inhibitor studied [327]. The natural spermine derivative from the root bark of *Lycium chinense,* kukoamine A, a mixed-type inhibitor of TR, showed no significant inhibition of human GR [328]. The synthesis of benzofuranyl 3, 5-bis-polyamine derivatives was reported, resulting as time-dependent inhibitors of *Tc*TR. In addition, the design of the spermidine-bridged macrocyclic alkaloid lunarine 1, showed to be a known time-dependent TRI. In the series of the bis-polyaminoacrylamide derivatives, the compounds 2 and 4 were shown to be competitive inhibitors, but only the bis-4-methyl-piperazin-1-yl-propylacrylamide derivative 4 (Table **8**) displayed time-dependent activity [329].

d. Bisbenzylisoquinoline alkaloids. Daphnoline and cepharanthine showed to be TRIs, the first one exhibited parasitological cure rate (70%) similar to Bz in an acute mice model (Table **8**) [330].

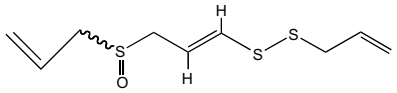
Table 8. Trypanothione reductase inhibitors. Representative compounds.

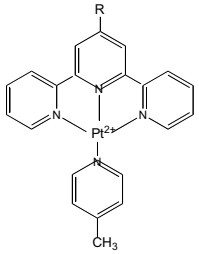
I-Irreversible Inhibitors

a- Subversive substrates or sabotage inhibitors [301-307]



b- Ajoene [308]

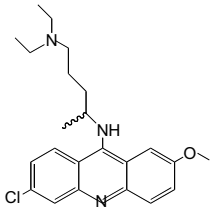




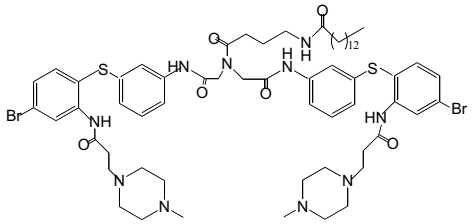
c- Organo-metallic complexes [309-315]

II-Reversible Inhibitors

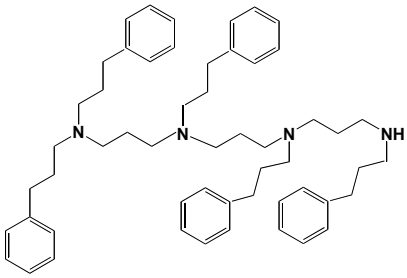
a- Tricyclic compounds [316-323]

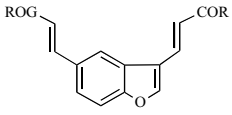


b- Aminodiphenylsulfides [324, 325]

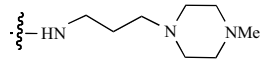


c- Polyamine derivatives [326-329]



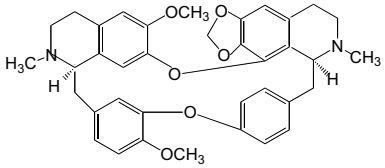


R:

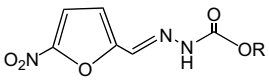


Compound 4

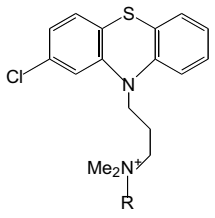
d- Bisbenzylisoquinoline alkaloids [330]



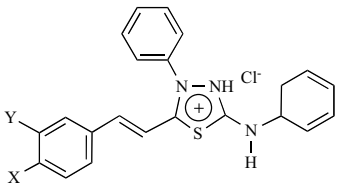
e- Quaternary arylalkylammonium phenothiazines [331-334]



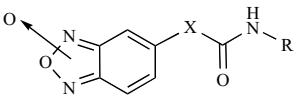
f- Nitrofuryl derivatives [335-337]



g- Mesoionic 1,3,4-thiadiazolium-2-aminidederivatives [338]



h- 5-nitro-2-furaldehyde semicarbazones and thiophene derivatives [339, 340]



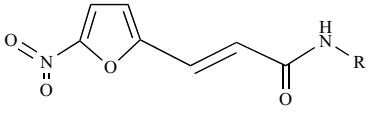
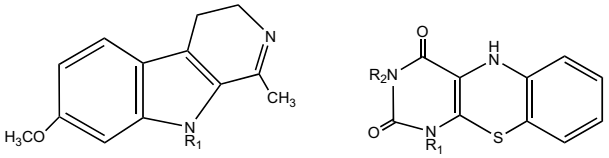
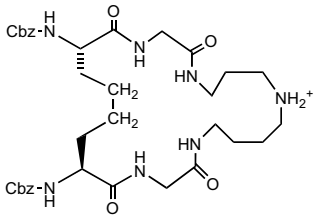


Table (8) contd….

1- Natural products scaffolds [341-343]



j- Analogues of Dethiotrypanothione [344]



e. Quaternary arylalkylammonium phenothiazines. In order to introduce a permanent positive charge into inhibitor molecules, a series of substituted benzyl [3-(2-chloro-phenothiazine-10-yl) propyl] dimethylammonium salts were synthesized by quaternization of the tertiary alkylamine omega-nitrogen atom of chlorpromazine, resulting linear competitive inhibitors of recombinant *Tc*TR, with either trypanothione disulfide or N-benzyloxycarbonyl-L-cysteinylglycyl 3-dimethylaminopropylamide disulfide as substrate. The most potent inhibitor of this series showed about two orders of magnitude more inhibitory than the parent chlorpromazine rendering a Ki value of 0.12 μM [331]. The quaternization of the nitrogen atom of 2-amino-4-chlorophenyl phenyl sulfide analogues of chlorpromazine enhanced the inhibition of *Tc*TR nearly forty-fold times with a linear competitive Ki value in the μM range [332]. A new Receptor-Dependent LQTA-QSAR approach was proposed as a new 4D-QSAR method to carry out molecular dynamics simulations in order to generate a conformational profile based on a group of 38 phenothiazine derivatives acting as specific competitive TRIs. A combination of molecular docking and molecular dynamics simulations was used to evaluate the binding mode of the phenotiazine derivatives and the descriptors could assist to design novel inhibitors [333]. One of the most potent phenothiazines capable to inhibit TR irreversibly is thioridazine (TDZ). The effect of TDZ on mice infected with different *T. cruzi* strains and treated at the acute or chronic phase was studied. The treatment strongly reduced the mortality rates, the cardiac histological and electrocardiographical abnormalities, and modified the natural evolution of the experimental infection (Table **8**) [334].

f. Nitrofuryl derivatives. In a series of novel 5-nitrofuryl derivatives designed to contain in the same molecule the nitro group, an oxidative stress promoter, and lateral chains that could interact with TR, 75% of them were more active on epimastigotes than Nx [335, 336]. The antimicrobial chlorhexidine linear competitive inhibitor {1, 1'-hexamethylenebis [5-(4-chlorophenyl) biguanide]}, and a piperidine derivative acting as mixed inhibitor are two structurally novel types of TRIs but not of GR. These compounds did not exert an improved inhibitory activity when compared to chlorhexidine, but the change from competitive to mixed-type inhibition resulted beneficial, since substrate accumulation did not overcome inhibition [337].

g. Mesoionic 1,3,4-thiadiazolium-2-aminide derivatives. Studies of a series of mesoionic 1,3,4-thiadiazolium-2-aminide derivatives evidenced to (MI-HH; MI-3-OCH(3); MI-4-OCH(3) and MI-4-NO(2)) as the most active compounds determining their effect on TR activity in *Leishmania sp*. and *T. cruzi.* Only compound MI-4-NO(2) caused enzyme inhibition effect on extracts from parasites cultures, which was confirmed by using the recombinants *Tc*TR and *L. infantum* TR [338].

h. 5-Nitro-2-furaldehyde semicarbazones and 1[(5R1thiophene derivatives. MDL28302 and MDL29431, two N,N'-thiophene-substituted polyamine analogs inhibited trypomastigotes infectivity in mammalian host cells and this effect was reversible. Their effects were abrogated by MDL72527, known inhibitor of polyamine oxidase (PAO). The PAO competitive substrates -N1-acetylspermine and N1-acetylspermidine also abolished the effect induced by MDL28302 or MDL29431. These compounds when added to infected cultures caused a marked reduction in intracellular parasite proliferation [339]. *In vitro* and *in vivo* trypanocidal activity of some nitrofurazones (5-nitro-2-furaldehyde semicarbazones) and thiophene analogues against *T. cruzi* were studied by applying the SIMCA methodology to the electrostatic and steric fields (CoMFA fields) of the

molecules, 3D-QSAR models were obtained. When nitrofurazones bearing N4 substituents were tested, a complete survival was observed in infected mice. The *in vitro* model allowed larger N4 substituents than the survival model, but positive centres in the region from the nitro group were not tolerated. Likewise, the *in vitro* model was in line with the active site of TR. Both models could be used in the design of new drugs containing an amide-like group at a distance of 7-9 A from a definitely reducible group [340].

i. Natural product scaffolds. The harmaline 10-thiaisoalloxazine and aspidospermine frameworks were identified as the base of *T. cruzi* TRIs, showing linear competitive inhibition with Ki values in the mM range [341]. In a screening using *Tc*TR, the crude extract of the the fungus *Lentinus strigosus* was able to strongly inhibit the enzyme. By using a bioassay guided fractionation of this extract, the triquinane sesquiterpenoid hypnophilin, and the panepoxydol derivative panepoxydone inhibited TR. Hypnophilin also killed intracellular amastigotes [342]. An organic extract from a culture of the endophytic fungus *Alternaria sp*, isolated from the plant *Trixis vauthieri*, inhibited almost 99% TR, and its fractionation led to altenusin, a biphenyl derivative showing also with high activity against the enzyme [343].

j. Dethiotrypanothione analogues. The synthesis of these macrocycles feature ring-closing olefin metathesis reactions rendered a derivative number 4 as the most potent inhibitor of *T. cruzi* TR obtained with a Ki=16 μM (Table **8**) [344].

k. Aryl β-aminocarbonyl derivatives. Three QSAR models were built and validated using different alignments based on docking with the *Tc*TR crystal structure, pharmacophore, and molecular interaction fields. The models obtained assured that this second generation of GRIND descriptors was able to detect the most important TR residues for binding the aryl β-aminocarbonyl derivatives, rationalizating distances among them. Finally, a revised binding mode has been proposed for these inhibitors, allowing further optimization of the lead compounds with such combined structure- and ligand-based approaches in the fight against the Chagas disease [345].

l. Substrate analogue compounds. These type of compounds were synthesized and evaluated showing to be low micromolar *T. cruzi* TRIs. Two of them were designed as potential irreversible inhibitors. However, they in conjunction with a third one, displayed reversible competitive inhibition. Only one of them was displayed as the most potent inhibitor with a Ki value in the μM order [346].

2.4.2. Other Enzymes of the Trypanothione Metabolism

This metabolism contains other key enzymes, besides TR, without counterparts in the mammalian host, which could be also mentioned as prospective drug targets [287,347]. They are γ-glutamylcysteine synthetase, trypanothione synthetase, ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (S-ADC), similarly to the proposed for polyamine transported [348, 349]. Peroxide detoxification in this parasite is achieved by ascorbate peroxidase and different thiol-dependent peroxidases.

In order to study the efficiency of targeting each pathway enzyme, different characteristics were considered: (i) the kinetic properties of the enzyme and the antioxidant metabolite concentrations and (ii) the existing knowledge and investigational methods for the study of the control of fluxes and intermediary concentrations in these metabolic pathways. The enzyme kinetic parameters were determined under near-physiological conditions, including glutathione synthetase. Then, based on these parameters, the enzyme activities, the metabolite concentrations and the fluxes measured in the parasite under control and oxidizing conditions, a kinetic model of T(SH)2 metabolism was constructed in *T. cruzi* and the contribution of each enzyme was quantified. The concentration of reduced T(SH)2 was controlled by TR and oxidative stress; however, γECS and TryS also controlled the cellular level of T(SH)2 when more than 70% of inhibition was found. The model predicted that in order to diminish the T(SH)2 synthesis flux by 50%, while it was necessary to inhibit γECS or TryS by 58 or 63%, respectively, or both by 50%, more than 98% inhibition was required for TR. For this reason, a concurrent and reasonable inhibition of γECS and TryS appeared to be an auspicious multi-target therapeutic strategy. By contrast, the use of highly strong and specific TRIs and the antioxidant machinery are necessary to affect the parasites antioxidant capabilities [350].

Ascorbate-dependent hemoperoxidase (*Tc*APx): A plant-like ER-localized ascorbate-dependent hemoperoxidase, *Tc*APX, was discovered in *T. cruzi*. This enzyme belongs to the para-

site oxidative defense system and is involved in the reduction of the parasite-specific thiol trypanothione by ascorbate in a redox pathway involving non-enzymatic interactions [351]. To evaluate the relevance of *Tc*APx in protecting *T. cruzi* from oxidative stress and to determine if it is critical for virulence, null mutants have been generated by targeted gene disruption. However, *Tc*APx was not essential for parasite viability within the mammalian host and did not show a significant role in establishment or maintenance of chronic infections, therefore, this enzyme should not be considered a main concern for drug design [352].

Glutathione cycle: It is well-known that glutaredoxins play a main role in different cellular functions. The gene corresponding to a putative dithiol glutaredoxin, encoded in the *T. cruzi* genome was cloned and the recombinant protein expressed resulted a characteristic thioltransferase. The variable activity was dependent on the nature of the reducer and/or oxidant agent used. The epimastigote extracts displayed a similar activity, suggesting that the enzyme was present in the parasite. A redox scenario, involving glutaredoxin mainly in reduction of glutathione disulfide as well as in deglutathionylation process of target proteins was supported in *T. cruzi.* Thus, this cycle could be a recognized target for the design of specific inhibitors with antiparasitic properties [353].

a. Phosphinopeptides Structurally Related to Glutathione. A series of phosphinopeptides structurally related to glutathione was developed, two of them showing a strong inhibitory effect on parasite amastigotes [354].

b. Phosphonate and Phosphinate Analogues of Glutathionyl-Spermidine. These compounds were reported as potent inhibitors of glutathionylspermidine synthetase (GspS) from *E. coli*, showing to be similarly strong against *C. fasciculata* GspS (*Cf*GspS). The recombinant TryS from *C. fasciculata*, *L. major*, *T. cruzi* and *T. brucei* were inhibited by phosphinate analogues with Ki apparent values 20-40-fold greater than that of *Cf*GspS. This phosphinate analogue remained to be the strongest enzyme inhibitor identified yet, representing a good starting point for drug discovery for these tropical diseases [355].

c. 5-Nitrofuran derivatives. In the last years, several potential antiprotozoal containing thiosemicarbazone and carbamate nitrofurans have been studied by cyclic voltammetry and electron spin resonance techniques. A self-protonation process involving the nitro group was observed. Cyclic voltammetry studies have demonstrated that glutathione could react with radical species from 5-nitrofuryl system. The nitrofuran-free radicals were characterized by electron spin resonance. The dependence between both the thiosemicarbazone or carbamate substructure and the length of the linker, furyl- or furylpropenyl-spacer was observed. A selective anti-trypanosomal activity of these derivatives was found. All the derivatives resulted active in a dose-dependent way on epimastigotes and trypomastigotes [356].

Tryparedoxin: The tryparedoxin-dependent peroxide detoxification pathway is the main oxidative-stress defense in *T. cruzi*. It is constituted by the enzymes TR, tryparedoxin (TXN), tryparedoxin peroxidase (TXNPx) and tryparedoxin-dependent glutathione peroxidase A (GPxA). TXNs, are unique multipurpose oxidoreductases, members of the thioredoxin superfamily from trypanosomatids, responsible for the transference of reducing equivalents from trypanothione to dithiol proteins as sulfur-dependent peroxidases. In trypanosomes, TXNs but no thioredoxins are the oxido-reductases of peroxiredoxins. Two genes codifying for TXN-like proteins have been identified in *T. cruzi*. They are TXNI, characterized as a cytoplasmic protein capable to intervene in the electron transfer between trypanothione and peroxiredoxins, and TXNII, a putative tail-anchored membrane protein [357]. *Tc*TXN1 interacting-proteins were discovered, increasing the knowledge on *T. cruzi* redox interactome. Studies on this interactome included the design of an active site mutant protein lacking the resolving cysteine, and the validation of the *in vitro* complex between the mutated *Tc*TXN1 and the cytosolic peroxiredoxin. This mutant protein was expressed, heterodisulfide complexes were isolated and identified by 2-DE/MS. Thus, fifteen *Tc*TXN1 proteins involved in oxidative metabolism and protein synthesis and degradation were identified [358]. The gene region coding for the soluble version of TXNII was cloned, expressed and showed TXN activity. It was also able to transfer reducing equivalents from trypanothione, glutathione, or dihydrolipoamide to various acceptors, including methionine sulfoxide reductases and peroxiredoxins supporting the occurrence and functionality of a second tryparedoxin, as a new component in *T. cruzi* redox scenario [359]. Metabolic Control Analysis estab-

lished that while 10% control was attained by TR, the TXN-TXNPx and tXN-GPxA redox pairs controlled the pathway flux by 90 to 100%. Quantitative kinetic and metabolic analyses pointed out to TXN as a convenient drug target due to its low catalytic efficiency, high control on the flux of peroxide detoxification and role as supplier of reducing equivalents to the two parasite major peroxidases [360]. These oxidoreductases mediators of electron transfer between trypanothione and peroxiredoxins constitute a difference with the host cells, in which these activities are mediated by thioredoxins. These differences mark TXNs an interesting target for the improvement of therapeutic agents. The interactions of *Tc*TXNII were investigated. An active mutant with the absence of the resolving cysteine was designed, expressed and incubated with *T. cruzi* proteins. The hetero-disulfide complexes were purified by affinity chromatography and identified by electrophoresis followed by MS. Sixteen *Tc*TXNII interacting proteins involved in relevant cellular processes were thus identified [361].

2.5. Glyoxalase System

The glyoxalase system, involving two metalloenzymes glyoxalase I (GLO1) and glyoxalase II (GLO2), is a nearly ubiquitous metabolic pathway linked to the detoxification of extremely reactive aldehydes such as the glycolytic byproduct methylglyoxal to D-lactate, by using glutathione as a cofactor. Methylglyoxal is a toxic by-product of glycolysis and other metabolic pathways. In mammals, the main route for detoxification of this reactive metabolite is via the glutathione-dependent glyoxalase pathway forming D-Lactate, containing lactoylglutathione lyase and hydroxyacylglutathione hydrolase. Recent studies have revealed a single requirement upon the thiol trypanothione as a cofactor in trypanosomatids, suggesting that the trypanothione-dependent glyoxalase system may be a striking target for rational parasite drug design. The enzymes in *T. cruzi* and *Leishmania* spp. have shown more than 200-fold selectivity for both glutathionylspermidine and trypanothione over glutathione. Thus, they are lactoylglutathionylspermidine lyases and hydroxyacylglutathionylspermidine hydrolases. The strict substrate specificity of the parasite GLO enzymes can be directly attributed to their unusual active site architecture. Unconventional routes of methylglyoxal detoxification have been studied almost two decades ago in order to exploit trypanosomatid glyoxalase enzymes as targets for chemotherapy [362, 363]. *Trypanosoma cruzi* GLO1 was cloned, expressed and characterized, showing to be able to isomerise hemithio-acetal adducts of trypanothione more than 2400 times more competently than glutathione adducts, and being methylglyoxal adducts 2-3-fold improved substrates than the comparable phenylglyoxal ones. However, glutathionylspermidine hemithioacetal adducts were isomerised in a more efficient way [364]. Methylglyoxal metabolism was compared in trypanosomatids showing the major differences in *T. cruzi* compared to those present in *L. major* and *T. brucei* [365]. An enzyme presenting glutathionylspermidine synthetase activity, showed differences in several physicochemical parameters as compared to known enzymes with similar activities. It was early patented by a research german group, the new procedure to isolate this enzyme from *C. fasciculata*, the way for produce it in genetically transformed organisms, and its use as a target for the discovery of trypanocidal drugs [366] (Table **2**).

Protozoan parasites showed marked deviations in the GLO pathway. Among them, the functional replacement of glutathione by trypanothione as a characteristic of trypanosomatids can be mentioned. These differences in the methylglyoxal metabolism in protozoan parasites indicate their potential therapeutic value. GLO enzymes characterization, vital features of the GLO pathway in the main human protozoan parasites, particularly in those from *P. falciparum*, *T. brucei*, *T. cruzi*, and *Leishmania* spp., in addition to the genes codifying for GLO I and II in *Entamoeba histolytica*, *Toxoplasma gondii*, and *Giardia lamblia* have been reviewed a few years ago [367].

S-4- Bromobenzylglutathionylspermidine, a glutathionylspermidine-based inhibitor was found to inhibit the *T. cruzi* enzyme in a potent linear competitive mode with a Ki near to 5 μM. The prediction algorithms, in conjunction with subcellular fractionation, suggested that *Tc*GLO1 in epimastigotes is located in the cytosol and the mitochondrion. The opposing substrate specificities of human and trypanosomatid GLO enzymes suggested this system might be another striking drug target [365].

2.6. Glycolysis and Glyconeogenesis

The metabolic processes named glycolysis and glyconeogenesis play essential roles as ATP

source and synthesis of glycoconjugates, relevant for the viability and virulence, respectively, of the human-pathogenic forms of trypanosomatids. Therefore, the two pathways are targets for anti-parasitic drugs. In *T. cruzi* amastigotes, it was described that possibly the energy entirely derives from glycolysis [368]. The glycolytic pathway has been first recognized as the unique mechanism for ATP generation in the infective stage of these organisms, and many glycolytic enzymes have been identified and studied as potential drug targets:

Phosphofructokinase (PFK). The gene that codifies for *T. cruzi* CL Brener phosphofructokinase (PFK) and the recombinant enzyme have been characterized. In contrast to previous reports, kinetic properties and size of the PFK genes in the *T. cruzi* strains studied resulted similar to those of *L. mexicana* and *T. brucei* homologs. Regarding the comparison of sequences from genes of different eukaryotes, it was shown that, although being an ATP-dependent enzyme, *T. cruzi* PFK showed a substantial sequence matching with inorganic pyrophosphate-dependent PFKs [369].

ML251 is a novel potent inhibitor of *T. brucei* and *T. cruzi* PFKs, active in the nM order, and the SAR within the series have been described [370].

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The comparison between the glycosomal GAPDH (gGAPDH) and that present in the mammalian counterpart showed structural differences favoring the improvement of specific inhibitors. Although adenosine was found to be a very unfortunate inhibitor, the obtainment of disubstituted nucleosides by addition of substituents to the 2' position of ribose and the N6-position of adenosine l, showed that the adenosine derivative [N6-(1 naphthalenemethyl)- 2'-(3-chlorobenzamido) adenosine] inhibited amastigotes growth but no effect was observed on the corresponding GAPDH human enzyme. It has been suggested that a tight binding competitive inhibitor of this glycolytic pathway enzyme might block the energy production in trypanosomatids [371, 372].

T. cruzi and *T. brucei* gGAPDHs showed high homology (> 95 %). However, some specific interactions identified could be useful to design selective irreversible inhibitors against *T. cruzi* gGAPDH. The use of two different approaches resulted in the identification of three hits chemical classes with moderate inhibitory activity (high micromolar range) against this enzyme [373]. GAPDH is also involved in nuclear functions, including transcriptional control, and maintenance of telomere structure, among others. It was proposed that the balance in the NAD+/NADH ratio during *T. cruzi* life cycle homeostatically controls GAPDH telomere association, suggesting that redox status locally modulates the association of GAPDH enzyme with telomeric DNA [374].

Coumarin derivatives. The design of a series of 3-piperonylcoumarins derivatives as inhibitors of *T. cruzi* gGAPDH was focused on the structures of previously identified natural products. Regarding the biological activity, the molecules could be clustered in different groups according to chemical substitutions, finding that the best active synthesized derivatives contained heterocyclic rings at position 6. When molecular modeling studies of these derivatives by docking assays were compared to natural hit chalepin, suggested a different mode of binding [375]. In order to develop a dual target enzyme inhibitor against the parasites *T. brucei* and *T. cruzi*, a series of quinone-coumarin hybrids against GAPDH/TR was designed and developed. The synthesized molecules were characterized by enzyme assays and in *in vitro* parasite cultures showing good inhibition parameters at the μM range both against *Tb*GAPDH and *Tc*TR. Strikingly, 2-{4-[6-(2-dimethylaminoethoxy)-2-oxo-2H-chromen-3-yl]phenoxy}anthracene-1,4-dione displayed a remarkable EC50 value for *T. brucei* parasites (0.026 μM) combined with very low cytotoxicity toward mammalian cells, at least partially due to the fact that it does not inhibit human GR [376].

Cis and trans-methylpluviatolides. The SAR for racemic mixtures of cis- and trans-methylpluviatolides was evaluated *in vitro* by using trypomastigotes and an enzymatic assay with *T. cruzi* gGAPDH. A trypanocidal activity with IC50 value in the μmolar range was exhibited by the combination of the trans-stereoisomers. Only the (-) enantiomer was active and despite being inactive the (+) enantiomer acted as an antagonistic competitor. In addition, at the evaluated concentrations trans-methylpluviatolide displayed low toxicity, and neither inhibited gGAPDH activity nor hindered peroxide and NO production [377].

"Bi-Substrate" analogues. A series analogues was synthesized as potential inhibitors of the GAPDH, and only one compound was identified as capable to inhibit this enzyme with good affinity and a 50-fold high specificity [378].

Anacardic acids, glucosylxanthon and flavonoid derivatives. Seven natural products were identified as novel inhibitors of *T. cruzi* GAPDH by combination of structure and ligand-based virtual screening techniques, including anacardic acids, flavonoid derivatives, and one glucosylxanthon. The structural diversity of this series might be considered for future medicinal chemistry [379]. Also, the evaluation of the inhibitory effects of a library of natural and synthetic anacardic acid derivatives against GAPDH showed among the best potent inhibitors the compounds 6-n-pentadecyl- and 6-n-dodecylsalicilic acids. *Trypanosoma cruzi* GAPDH-catalyzed reaction analysis showed that these derivatives were non-competitive inhibitors with respect to both substrate and cofactor [380]. Novel ruthenium compounds, NO donors, cis-[Ru (NO)(bpy)(2)L]X(n)) showed inhibitory effect on this enzyme, exhibiting strong *in vitro* and *in vivo* activities at doses up to 1000-fold lower than Bz. One of the mode of action of these compounds occurs via the S-nitrosylation of Cys166 of *T. cruzi* GAPDH [381].

Hexose-phosphorylating enzymes. In *T. cruzi*, the essential substrate glucose is intracellularly phosphorylated to glucose-6-phosphate. In most organisms, hexokinase (HK) is the first enzyme that participates in the glycolysis. Unlike to the human enzyme, the corresponding enzyme in *T. cruzi* presented an unusual inhibition by inorganic diphosphate (PPi) [382]. Additionally, an ATP-dependent glucokinase (GlcK) displaying a ten-fold inferior substrate affinity in comparison with the hooknose was further identified in *T. cruzi.* The two enzymes, from the same family but belonging to very different groups, are located in glycosomes of kinetoplastids which contain the first seven glycolytic steps as well as enzymes of the oxidative branch of the penthose phosphate pathway (PPP). After cloning and sequencing and expression of GlcK genes of *T. cruzi* and *L. major*, the recombinant soluble and active enzymes, *Tc*GlcK and *Lm*jGlcK were purified and kinetically characterized. It was found that no inhibition was exerted either by glucose-6-phosphate or by PPi. The absence of inhibition by PPi is in contrast to the observed for the *T. cruzi* and *L. mexicana* hooknoses [383]. The analysis of the crystal structure of *Tc*GlcK exposed the oligomerization and anomer specificity of hexose-phosphorylating enzymes. The fact that *Tc*HK and GlcK showed preference for distinct anomers suggested that these enzymes are not directly competing for the same substrate and perhaps exert different physiological functions [384].

Bisphosphonates These non-hydrolysable analogues of PPi are strong*Tc*HK inhibitors. Analysing a series of 42 bisphosphantes, the most active compound against *Tc*HK displayed IC50 value versus intracellular amastigotes in the μM range [382]. The effect of the three more active compounds of this series was analyzed on purified *Tc*HK, on the enzyme in purified intact glycosomes, on glucose consumption by intact and digitonin-treated parasite epimastigotes, and on the parasite growth [385]. They were non-competitive or mixed *Tc*HK inhibitors, resulted several orders more active than PPi, were able to block the use of glucose by the epimastigotes, and did not alter the sterol composition, demonstrating that they were not inhibitors of FPPS and suggesting that these bisphosphonates acted formerly as specific *Tc*HK inhibitors and might signify a new class of selective trypanocidal agents [386].

Glucose-6-phosphate dehydrogenase (G6PDH). In *T. cruzi*, G6PDH, a key enzyme in the parasite glycolytic pathway catalyzes the oxidative phosphorylation of D-glyceraldehyde-3-phosphate to 1, 3-bisphosphoglycerate associated to the reduction of oxidized NAD to NADH. The cloning, sequencing, expression of *Tc*GAPDH was followed by purification and characterization of the recombinant protein. Response surface methodology allowed further determining the region of optimal conditions for the enzyme [387].

Human steroids. Steroids such as dehydroepiandrosterone (DHEA) and epiandrosterone (EA) exert multiple effects in mammals including the inhibition of G6PDH. Although this inhibition was initially considered specific for the mammalian enzyme, it was later shown that DHEA and EA also inhibited *T. cruzi* G6PDHs but not the enzyme from *Leishmania* species. In addition, *in vitro* studies on these steroids showed that they were capable to kill trypanosomes. In contrast to wild type trypanosomes, mutant forms expressing *L. mexicana* G6PDH were not susceptible to the steroids, indicating that G6PDH is the *in situ* target. In addition, the use of bromo-derivatives of the steroids showed 50-100 fold lower Ki' values for G6PDH displaying an increased trypanocidal strength [388].

Triosephosphate isomerase (TIM). The single gene that encodes for *Tc*TIM was cloned and sequenced. The native form of the enzyme was early purified from epimastigotes. The crystal structure analysis showed two hexanes localized at <4 A from residues that form the dimer interface, near to a site considered as potential target for drug design [389]. In *T. cruzi* glycolytic pathway, *Tc*TIM exhibits high catalytic rates of glyceraldehyde-3-phosphate- and dihydroxyacetone-phosphate-isomerization only in its dimeric form. Thus, to become in selective anti-Chagas disease drugs, compounds must be able to specifically disrupt *Tc*TIM but not human TIM (hTIM) dimer interface. It is an essential *T. cruzi* enzyme and one of the potential drug targets for Chagas disease. The impact of E104D, the most frequent mutation in the autosomal "TPI deficiencies" in humans, was studied in TIMs from different organisms including *T. cruzi*. The mutation affected the rate and extent of formation of active dimers from unfolded monomers differentially and produced considerable changes in activation energy in *Tc*TIM, indicating that the role of a conserved non-catalytic residue is extremely dependent on its molecular background [390].

Benzothiazoles. The compounds 3-(2-benzothiazolylthio)-propanesulfonic acid, 2-(p-aminophenyl)-6-methylbenzothiazole-7-sulfonic acid, and 2-(2-4(4-aminophenyl) benzothiazole-6-methylbenzothiazole-7-sulfonic acid were among the strongest inhibitors of *Tc*TIM. The half-maximal inactivation was shown with 33, 56, and 8 microM, respectively; in comparison with human TIM, which required 422 microM, 3.3 mM, and 1.6 mM. In *Tc*TIM, the effect of the benzothiazoles decreased as the enzyme concentration increased. *Tc*TIM and human TIM differ in the specific amino acid cysteine localized in dimer interface of the parasitic enzyme. All the findings suggested that the benzothiazoles perturb the interactions between the two subunits of *Tc*TIM and that the interface cysteine is central in their harmful action [391]. The possible binding sites at the interface of *Tc*TIM showed that fully flexible benzothiazoles were docked onto the dimer interface, purposing that the dimer disruption was not via Cys 15, as presented in last studies, but it could be carried out through the unstabilization of pi-pi interactions of two aromatic clusters present in the interface, enabling a novel alternative for rational SAR for drug design [392]. In addition, 3-(2-benzothiazolylthio)-propanesulfonic acid (BTS) has been described as a powerful and selective inhibitor of *Tc*TIM [393].

Enzyme-dimer-interface-irreversible inhibitors. Taking into account that *Tc*TIM exhibits high catalytic rates of glyceraldehyde-3-phosphate- and dihydroxyacetone-phosphate-isomerization only in its dimeric form, as above described, the enzyme was screened against an in-house chemical library containing about 230 compounds fitting to different chemotypes. After a second screening, 26 compounds corresponding to 8 different chemotypes were positives. In addition to the *in vitro* activity displayed against *T. cruzi*, 4 compounds exhibited selectivity for *Tc*TIM over hTIM [388].

Phenazine, thiadiazole and thiadiazine derivatives. The mechanism of inhibition of *Tc*TIM by 1,2,4-thiadiazol, phenazine and 1,2,6-thiadiazine derivatives was investigated. To this aim, biochemical and molecular docking studies combined with molecular dynamics simulations were performed in order to study their interaction with wild-type and mutant *Tc*TIM. It was shown that phenazine and 1,2,6-thiadiazine derivatives, 2 and 3, act as dimer-disrupting inhibitors of *Tc*TIM having also allosteric effects in the conformation of the active site. These compounds induced a high selective irreversible inactivation of this parasitic enzyme at low mM concentrations. Simulations obtained by molecular docking indicated the possible interference between the phenazine derivative and the association of the two monomers of the dimeric enzyme by localizing at the dimer interface, while 1,2,6-thiadiazine could act as an inhibitor binding to a region surrounding Cys-118 [394]. On the other hand, the binding of 1,2,4-thiadiazol derivative 1 into the active site caused a significant decrease in enzyme mobility in both monomers. The loss of conformational flexibility upon compound 1 binding suggests that this inhibitor could be preventing enzyme optimal activity. The elucidation of the mode of action of this kind of inhibitors could help future rational novel drug design [395].

Bis-thiazole and 3H-[1,2] dithioles derivatives. Some bisbenzothiazoles had been described as irreversible inhibitors of this druggable *Tc*TIM and novel bioactive furane-containing thiazoles had been reported as excellent *in vivo* anti-*T. cruzi* agents. Therefore, new bis-thiazoles were designed and developed. The bis-thiazol 5, 3,3'-allyl-2,2'-

bis[3-(2-furyl)-2-propenylidenehydrazono]-2,2',3,3'-tetrahydro-4,4'-bisthiazole, showed the best *in vitro* anti-*T. cruzi* profile with a higher selectivity index than Nx and Bz against parasite amastigotes. Although this derivative displayed marginal activity against *Tc*TIM, the bis-thiazol 14, 3-allyl-2-[3-(2-furyl)2-propenylidenehydrazono]-3'-phenyl-2'-(3-phenyl-2-propenylidenehydrazono]-2,2',3,3'-tetrahydro-4,4'-bisthiazole, showed to be an excellent inhibitor of *Tc*TIM. The authors stated that the activity of bis-thiazol 5 *in vivo*, together with the lack of *in vitro* mutagenic and *in vivo* toxicity effects, suggest an auspicious future for this compound as promising trypanocidal drug exceeding the "hit-to-lead" step in the drug development process [396]. Additionally, 3H-[1,2] dithioles derivatives also showed the ability to inhibit *Tc*TIM. This chemotype was structurally modifyed in order to analyze the influence of volume, lipophilicity and electronic properties in the trypanomicidal activity in the different structures. In addition, the selectivity of parasites vs. mammalian cells was also examined. With the aim to guess a possible mechanism of action, not only the inhibition of purified *Tc*TIM activity was analyzed. Inhibition of Cz, membrane sterol biosynthesis and excreted metabolites, using the whole parasite, were achieved, considering this structural framework as interesting for the generation of new drugs for Chagas disease [397]. The known *Tc*TIM inhibitors behave poorly or have shown low activity in the parasite.

Isocitrate dehydrogenases (IDHs). *Trypanosoma cruzi* exhibit two putative IDHs. The cloning of both IDH genes was carried out and the recombinant enzymes were expressed in *E. coli. Trypanosoma cruzi* IDHs are strictly dependent on NADP+ and displayed apparent affinities towards isocitrate and the coenzyme in the low micromolar range. In *T. cruzi*, IDHs are cytosolic and mitochondrial enzymes, and there is no evidence for the typical Krebs cycle-related NAD-dependent IDH. Hence, like in *T. brucei*, the Krebs cycle is not a canonical route in *T. cruzi*. However, the citrate produced in the mitochondrion could be isomerized into isocitrate in the cytosol and the mitochondrion by means of the putative aconitase, which would provide the substrate for both IDHs. The cytosolic IDH is significantly more abundant in amastigotes, cell- derived and metacyclic trypomastigotes than in epimastigotes, accordingly with with the expected oxidative burst that this pathogen has to face when infecting the mammalian host [398].

Enolase. This glycolytic/gluconeogenic enzyme is commonly extremely conserved, with comparable overall fold and matching catalytic residues in all organisms. However, significant differences exist between the trypanosomatid and host enzymes, with three unique, reactive residues close to the active site of the parasite enzyme that might be useful for the improvement of novel trypanocidal drugs [399].

Phosphoenolpyruvate carboxykinase (PEPCK). ATP-dependent PEPCK is a key enzyme that participates in *T. cruzi* catabolism of glucose and amino acids. The significant differences in the amino acid sequence and substrate specificity of the human PEPCK (GTP-dependent) in comparison with those of *Tc*PEPCK determine it as good target for the development of anti-Chagas disease drugs. The crystal structure of the recombinant *Tc*PEPCK was solved up to 2.0 A resolution. Although the structure of the *Tc*PEPCK was presented in 2001 providing a good basis for the modelling of new trypanocidal drug leads. However, no specific inhibitors have been described up to date [400].

Fructose-1,6-bisphosphatase. This gluconeogenic enzyme is also present. It was described many years ago but no inhibitors have been described yet. Details on glucose metabolism can be found in expert revision [401].

2.7. Pentose Phosphate Pathway

In the last decade, results concerning the pentose phosphate pathway (PPP) have been described in *T. cruzi*. All the enzymes of this pathway are present in the main developmental forms of the parasite cycle [402]. The cloning and sequencing of the seven enzymes of PPP were carried out and the expression as active proteins was developed. 6PGDH controls glucose flux through the pathway by its response to the NADP/NADPH ratio. It is encoded by a number of genes per haploid genome. Although the recombinant form of *Tc*6PGDH showed kinetic parameters identical to the values reported for from mammalian counterparts, Km reported for NADP was significantly lower than the value reported for the human enzyme, and closer to that for the *T. brucei* enzyme. These results suggested that inhibitors of *Tb*6PGDH might be effective for the chemotherapy of Chagas disease. The behaviour of the enzyme showed to be similar to the redox regulated

6PGDHs from chloroplasts and cyanobacteria. In addition, a considerable 6PGDH increase was observed in metacyclic trypomastigotes under oxidative stress conditions, suggesting that the enzyme might play a noticeable role in the defense mechanisms against oxidative stress becoming an important target for chemotherapy [403]. In the CL Brener clone, the genes encoding for 6-phosphogluconolactonase, 6-phosphogluconate dehydrogenase (6PGD), transaldolase and transketolase are present as a single copy per haploid genome. Taking into account that 6PGD is not very stable; the enzyme stabilization was acquired by introducing two salt bridges using site-directed mutagenesis. Ribose-5-phosphate isomerase (Rpi) belongs to Type B enzymes; genes encoding Type A enzymes, present in mammals, are absent. Two genes codify for ribulose-5-phosphate epimerase. Several PPP enzymes have a major cytosolic component, secondary glycosomal localization and also minor localizations in other organelles [404].

This functional PPP is probably indispensable in *T. cruzi*, for protection against oxidative stress and also for ribose 5-phosphate (R5P) production for nucleotide biosynthesis. The recombinant RpiB catalyzes the isomerization of R5P to ribulose 5-phosphate (Ru5P) showing Km values of 4 mM (R5P) and 1.4 mM (Ru5P). An analogue of the reaction intermediate, 4-phospho-D-erythrono-hydroxamic acid, when the Rpi acts via a mechanism involving the formation of a 1,2-cis-enediol, was capable to inhibit the enzyme competitively. Moreover, the lack of RpiBs in genomes of higher animals also marks this enzyme as a feasible target for the treatment of Chagas disease [405]. Crystallographic and kinetic studies of the *Tc*RpiB were reported. The structures of the wild-type and main mutant enzyme, alone or bound to phosphate, D-R5P, or the inhibitors 4-phospho-D-erythrono-hydroxamic acid and D-allose-6-phosphate, have emphasized some topography points of the active site, showing that small conformational changes are linked to binding. The kinetic studies have confirmed that *Tc*RpiB is capable to isomerize D-R5P effectively, but not the 6-carbon sugar D-allose-6-phosphate; in its place, this sugar acts as an enzymatic inhibitor. The results obtained provided discernments into the action of RpiB enzymes and potential future work in drug design [406]. It is worth noting that *T. cruzi* trypanothione-dependent antioxidant system requires the supply of NADPH, provided by G6PD and 6PGD enzymes to work correctly. Different patterns of G6PD and 6PGD activities were observed among strains along the growth curve and when cells were challenged with H_2O_2 reinforcing the heterogeneity within *T. cruzi* populations and the significance of G6PD in protecting the parasite against ROS [407].

a. Dehydroepiandrosterone (DHEA) and 16BrEA. The administration of DHEA to infected rats reduced blood parasite levels in the acute and chronic *T. cruzi* infection [408]. 16α-Bromoepiandrosterone (16BrEA) inhibited the proliferation of epimasigotes and was also a strong *Tc*G6PDH inhibitor [409].

b. Steroidal halogenated compounds derivatives. These compounds derived from DHEA are the best characterized inhibitors of *Tc*G6PDH, but also noble inhibitors of the homologue enzyme in humans. Therefore, the absence of target selectivity might determine partial inhibition of human G6PDH in red blood cells resulting in hemolytic side effects. In addition, the treatment of Chagas disease patients with steroidal drugs might also produce unwanted androgenic side effects. Novel *Tc*G6PDH inhibitors were identified among thienopyrimidine and quinazolinone derivatives by a target-based high-throughput screening against a commercial library. SAR for the identified hits and structural features contributing for the enzyme selectivity indicated quinazolinones as promising leads for further optimization [410].

2.8. Enzymes From Biosynthetic Glycoconjugates Pathways

2.8.1. B-D-Galactofuranose (Galf) Biosynthetic Enzymes

Galf is a constituent of parasite glycoconjugates located on the cell surface of *Leishmania* Spp. and *T. cruzi*, but is not present in the host mammals. Then, it was early considered a respectable chemical therapeutic target for inhibitors design. Therefore, alkyl, benzyl and aryl 1-thio-β-D-galactofuranosides were synthesized. The best inhibitor was the compound 4-aminophenyl-1-thio-β-D-galactofuranoside obtained by catalytic hydrogenation of the nitrophenyl derivative, constituting an adequate ligand for the preparation of an affinity phase capable to isolate β-D-galactofuranosidases from different sources. Also, the inhibitory activity of D-galactono-1, 4-lactone was shown. In addition, the presence of exo β-D-

galactofuranosidase was evidenced in *T. cruzi* [411, 412].The relevance of the β-Galf-containing glycans has been described in parasite-cell interaction and protection against oxidative stress. Recent studies on UDP-galactose 4' epimerase (GalE), UDP-galactopyranose mutase (UGM), and UDP-galactofuranosyl transferase (GalfT) enzymes and on the role of β-Galf in the disease pathogenesis were developed. The principal role in Galf formation, its exclusive chemical mechanism, and the lack of a homologous enzyme in humans recognize UGM as the best striking drug target in the β-Galf-biosynthetic pathway of protozoan parasites [413].

D-galactofuranosidase inhibitors. D-galactofuranosyl nucleoside analogues, derived by the addition of nucleophiles to perbenzoylated-D-galactofuranosyl isothiocyanate were firstly prepared. Among them: N-D-galactofuranosyl-O-ethylthiourethane, N-D-galactofuranosyl-4-oxoimidazolidine-2-thione, N-D-galactofuranosyl-4-imidazoline-2-thione, and N-D-galactofuranosyl-4-methoxyimidazolidine-2-thione can be mentioned. Biological assays were performed showing that imidazoline and imidazolidine-2-thione derivatives acted as a new type of exo-D-galactofuranosidase inhibitors. On the other hand, the synthesis of oligosaccharides, glycoconjugates, and mimetics of D-Galf requires specific methods for the preparation of galactose derivatives in the furanosic configuration, the synthesis of appropriate acceptors, and efficient glycosylation methods for the construction of α- and β-D-Galf linkages [414].

2.8.2. UDP-Galactopyranose Mutase (UGM)

In *T. cruzi,* as well as in other pathogens, the flavoenzyme UGM catalyzes the conversion of UDP-galactopyranose to the precursor of the cell surface β-galactofuranose (UDP-galactofuranose) involved in the virulence of the pathogen. Galf, key for virulence and absent in humans, makes its biosynthetic pathway as another interesting target for the development of novel anti-*T. cruzi* drugs [415]. The crystal structure of the biosynthetic *Tc*UGM was reported. The absence of galactofuranose in humans in addition to the fact that UGM is an essential component of key glycoconjugates in trypanosomatids, determine that it is an attractive target for drug design. Novel information about the UGM biochemistry suggested a combined strategy for the design of UGM inhibitors from eukaryotic pathogens [416]. The lack of UGM in humans makes this enzyme inhibition a good approach in the design of new Chagas therapeutics. A series of computer simulations of *Tc*UGM with or without an active site ligand and the molecular details of the mechanism that controls the uptake of the substrate was addressed, suggesting a modular mechanism in which each moiety of the substrate controled the flexibility of a different protein loop. Furthermore, the calculations indicated that interactions with the substrate diphosphate moiety resulted especially important for stabilizing the closed active site. Kinetics measurements of site directed mutants of *Tc*UGM supported this hyphotesis. These findings offer new alternatives for the design of drugs [417].

2.9. Arginine Kinase (AK) AND Arginase

The use of creatine kinase (CK) for the storage of ATP in the form of phosphocreatine, in vertebrates, allows maintaining ATP homeostasis during muscle contraction. *Trypanosoma cruzi* and *T. brucei*, have an alternative pathway that usages as the catalyst for arginine phosphorilation to the AK enzyme, producing the analogous phosphagen, phosphoarginine. When the production of ATP is required, the high-energy phosphate is prepared to be transferred to adenosine diphosphate ADP. To this aim, phosphagens, posphoarginine and phosphocreatine, play a critical role as energy reserve. In addition, the molecular and biochemical characterization of AKs in trypanosomes have been reported [418]. Also, this pathway is widespread through invertebrates, comprising a great diversity of phosphagens other than arginine, but not creatine. CK and AK are homologous proteins that fit to the family of the conserved proteins guanidino kinases (GKs), with phosphotransferase activity. A near relationship exists between the energy requirements within the cell and the activity of GKs. *Tc*AK relevance is acquired during the vertebrate stage of the parasite life cycle, attributed to discrepancies in the insect feeding conditions. Thus, phosphoarginine results a rapid source of energy under starvation stress conditions or during bursts of cellular activity allowing the parasite to adaptate either to environmental changes or stress conditions [419]. The overexpression of AK in parasites was studied showing a significantly increased survival during hydrogen peroxide exposure suggesting that AK participates in oxidative stress response systems [420]. In addition, crystal structure was reported [421], the subcellular local-

ization after digitonin extraction pattern of AK resulted similar to the cytosolic indicator and the immunofluorescence analysis revealed that although AK is localized mainly in unidentified punctuated structures, and also in the cytosol, it did not co-localize with any of the subcellular markers [422]. Moreover, some reports showed that AK inhibition caused parasite growth inhibition in culture. The arginine analogs agmatine, canavanine, nitroarginine also inhibited to the AK enzyme. Among them, canavanine resulted a strong AK inhibitor. The green tea catechins showed trypanocidal action against two different developmental stages of *T. cruzi*. In addition, the recombinant *Tc*AK was inhibited by the polyphenols catechin, gallate or gallocatechin gallate [423]. However, patents related to catechins compounds in the last years described that they presented anticancer activity. It is worth noting that amino acid metabolic routes as possible therapeutic targets against Chagas disease have been properly described in detail [424].

Small-molecule arginase inhibitors.Taking into account that pathogens are capable to synthesize their own arginase to evade the immune reaction, small-molecule arginase inhibitors were reported as auspicious therapeutics for the treatment of several diseases, such as allergic asthma, cardiovascular diseases, cancer, immune disorders and diseases associated with pathogens including *T. cruzi*. Interestingly, data about arginase inhibitors and their properties was discussed [425].

2.10. Proline Pathway

L-Proline display several roles in trypanosomatids. In *T. cruzi*, proline participates in energy metabolism, in differentiation procedures and resistance to osmotic stress.

2.10.1. Proline Racemace (PRAC)

This enzyme, initially found in *Clostridium sticklandii*, is responsible for the interconversion of L- and D-proline enantiomers, bears cysteine residues in the active site and does not necessitate neither co-factors nor other known coenzymes. After isolation and cloning of the gene, the first eukaryotic amino acid (proline) racemase was identified in *T. cruzi* (*Tc*PRAC). Two paralogous genes per parasite haploid genome codify for *Tc*PRACA and *Tc*PRACB, respectively, giving rise to intracellular protein and secreted isoforms. Remarkably, the secreted form of PRAC is a strong host B-cell mitogen supporting parasite evasion of specific immune responses. The intracellular or secreted forms of the enzyme exhibited different kinetic properties that might be important for their relative catalytic efficiency. Studies with an enzyme-specific inhibitor and abolition of enzymatic activity by site-directed mutagenesis of the active site Cys330 residue encouraged the possibility to use PRAC as a new target for Chagas disease drug chemotherapy On the other hand, the overexpression of *Tc*PRAC headed to an increase in parasites differentiation into infective forms and its consequent penetration into host cells. Additionally, parasite viability was weakened in functional knock-down parasites emphasizing to *Tc*PRAC as a latent target for drug design and immunomodulation of parasite-induced B-cell polyclonal activation [426, 427].

In this homodimeric enzyme, each monomer folded in two symmetric alpha/beta subunits is divided by a profound cleft. The crystal structure of *Tc*PRAC in complex with a transition-state analog, pyrrole-2-carboxylic acid, have demonstrated the existence of a reaction center per monomer, with two Cys residues optimally located to carry out the acid/base catalysis. The mutation of the catalytic Cys residues abolished the enzymatic activity, preserving the ptotein mitogenic properties. By contrast, the inhibitor binding stimulates the closing of the interdomain crevice abrogating B cell proliferation and suggesting that the *Tc*PRAC mitogenic properties depend on the ligand-free-enzyme exposure of temporary epitopes [428]. Through the use of wild-type parasites and parasites overexpressing *Tc*PRAC genes, the relative contribution of *Tc*PRAC to the disposal of D-proline followed by further assembly into peptides was estimated, suggesting that D-proline-containing peptides might be benefitial for *T. cruzi* by providing resistance against the proteolytic mechanisms of the host, similarly to the mucopeptide layer of bacterial cell wall [429]. The identification and characterization of racemase, particularly PRAC, was disclosed in a patent by researchers from Institute Pasteur and Centre National de la Recherche Scientifique (CNRS), Paris, France. It is related to the methods and kits for detecting racemases, the peptides consisting of the motifs and the antibodies directed to these peptides but no specific inhibitors for the chemotherapy of the trypanosomiasis have been included [430]. Moreover, crystallographic structure of *Tc*PRACA and methods of modelling

identified drugs that treat or prevent infection by *T. cruzi* were also provided [431]. Interestingly, PRAC was investigated showing to be an effective mitogen for B cells that contributes to the parasite's immune evasion and persistence in the human host. The recombinant epimastigotes overexpressing *Tc*PRAC genes presented an increased capacity to differentiate into metacyclic infective forms and afterwards *in vitro* penetrate into host-cells. It was demonstrated that *T. cruzi* antibodies specific for PRAC and the specific PRAC inhibitor pyrrole-2-carboxylic acid were used in *in vitro* assays affecting significantly the parasite infection of Vero cells, demonstrating that this inhibitor is capable to impede *T. cruzi* intracellular differentiation [432]. Finally, a patent related with *Tc*PRAC inhibitors was presented by researchers from Institute Pasteur, Paris, France. The invention concerns a novel class of inhibitor of this essential enzyme present in several important organisms, particularly, *Tc*PRAC (Table **2**). PRAC inhibitors for the treatment of *Trypanosoma* spp. infection were also patented [433, 434].

2.10.1. Proline Racemace (PRAC)

This enzyme, initially found in *Clostridium sticklandii*, is responsible for the interconversion of L- and D-proline enantiomers, bears cysteine residues in the active site and does necessitate neither co-factors nor other known coenzymes. After isolation and cloning of the gene, the first eukaryotic amino acid (proline) racemase was identified in *T. cruzi* (*Tc*PRAC). Two paralogous genes per parasite haploid genome codify for *Tc*PRACA and *Tc*PRACB, respectively, giving rise to intracellular protein and secreted isoforms. Remarkably, the secreted form of PRAC is a strong host B-cell mitogen supporting parasite evasion of specific immune responses. The intracellular or secreted forms of the enzyme exhibited different kinetic properties that might be important for their relative catalytic efficiency. Studies with an enzyme-specific inhibitor and abolition of enzymatic activity by site-directed mutagenesis of the active site Cys330 residue encouraged the possibility to use PRAC as a new target for Chagas disease drug chemotherapy. On the other hand, the overexpression of *Tc*PRAC headed to an increase in parasites differentiation into infective forms and its consequent penetration into host cells. Additionally, parasite viability was weakened in functional knock-down parasites emphasizing to *Tc*PRAC as a latent target for drug design and immunomodulation of parasite-induced B-cell polyclonal activation [426, 427].

In this homodimeric enzyme, each monomer folded in two symmetric alpha/beta subunits is divided by a profound cleft. The crystal structure of *Tc*PRAC in complex with a transition-state analog, pyrrole-2-carboxylic acid, have demonstrated the existence of a reaction center per monomer, with two Cys residues optimally located to carry out the acid/base catalysis. The mutation of the catalytic Cys residues abolished the enzymatic activity, preserving the ptotein mitogenic properties. By contrast, the inhibitor binding stimulates the closing of the interdomain crevice abrogating B cell proliferation and suggesting that the *Tc*PRAC mitogenic properties depend on the ligand-free-enzyme exposure of temporary epitopes [428]. Through the use of wild-type parasites and parasites overexpressing *Tc*PRAC genes, the relative contribution of *Tc*PRAC to the disposal of D-proline followed by further assembly into peptides was estimated, suggesting that D-proline-containing peptides might be benefitial for *T. cruzi* by providing resistance against the proteolytic mechanisms of the host, similarly to the mucopeptide layer of bacterial cell wall [429]. The identification and characterization of racemase, particularly PRAC, was disclosed in a patent by researchers from Institute Pasteur and Centre National de la Recherche Scientifique (CNRS), Paris, France. It is related to the methods and kits for detecting racemases, the peptides consisting of the motifs and the antibodies directed to these peptides but no specific inhibitors for the chemotherapy of the trypanosomiasis have been included [430]. Moreover, crystallographic structure of *Tc*PRACA and methods of modelling identified drugs that treat or prevent infection by *T. cruzi* were also provided [431]. Interestingly, PRAC was investigated showing to be an effective mitogen for B cells that contributes to the parasite's immune evasion and persistence in the human host. The recombinant epimastigotes overexpressing *Tc*PRAC genes presented an increased capacity to differentiate into metacyclic infective forms and afterwards *in vitro* penetrate into host-cells. It was demonstrated that *T. cruzi* antibodies specific for PRAC and the specific PRAC inhibitor pyrrole-2-carboxylic acid were used in *in vitro* assays affecting significantly the parasite infection of Vero cells, demonstrating that this inhibitor is capable to impede *T. cruzi* intracellular differentia-

tion [432]. Finally, a patent related with *Tc*PRAC inhibitors was presented by researchers from Institute Pasteur, Paris, France. The invention concerns a novel class of inhibitor of this essential enzyme of several important organisms, particularly, *Tc*PRAC (Table **2**). PRAC inhibitors for the treatment of *Trypanosoma* spp. infection were also patented [435, 436].

2.11. Protein Kinases (PKs) /Protein Tyrosine Phosphatase (PTP)

Among promising drug targets for mumerous human and animal diseases comprising trypanosomiasis and leishmaniasis, protein kinases (PKs) were described. The completion of the genome sequences of *L. major*, *T. brucei* and *T. cruzi* has defined the eukaryotic PKs or kinome for each parasite representing a third part of the human complement. In order to develop novel anti-parasitic chemotherapeutic agents, the analysis of the kinome analysis allowed exploiting differences between parasite and mammalian PKs [437]. On the other hand, cyclic AMP-PKA (PKA) signalling is substantial for *T. cruzi* growth and differentiation. In this sense, immunofluorescence assays proposed that PKA can associate with trypomastigotes plasma membrane, probably by interaction of the PKA regulatory subunit with some P-type ATPases capable to anchor PKA to the *T. cruzi* plasma membrane [438]. Likewise, by using the specific PKC inhibitors Ro 32-0432 and Rottlerin, a possible correlation between *T. cruzi* metacyclogenesis induced by oleic acid and the activation of a particular PKC isoenzyme was investigated. The mentioned compounds abrogated epimastigote differentiation and membrane translocation of PKC beta, gamma, and delta, thus supporting a crucial role for classical and novel PKC isoenzymes in *T. cruzi* signalling pathways involved in oleic acid induced-metacyclogenesis [439].

a. Staurosporine, genistein, wortmannin. In order to evaluate PKs as drug target these Ser/Thr, Tyr and phosphatidylinositol 3' (PI3) PK inhibitors (PKIs), respectively, were evaluated on *T. cruzi* epimastigotes and amastigotes. Staurosporine was the most effective after 24 h of treatment and genistein produced a potent inhibition during the whole treatment (60-70% inhibition) whereas wortmannin showed a low IC50 value in the mM range. Also, these PKIs showed strong ultrastructural effects on the epimastigotes, but without interference either with the division of intracellular amastigotes or with their differentiation to trypomastigotes. Nevertheless, considering that trypanosomes have kinomes that comprise a great set of PKs and phosphatases, PKs should not be ignored as an important drug target [440].

b. 1-O-Hexadecylphosphocoline. This compound, commonly named miltefosine, is capable to inhibit the *Tc*PKC activity in *T. cruzi* through a Na^+-ATPase-independent way, indicating that the inhibition of parasite´s growth, at least in part, is due to the inhibition of the both enzymes [441]. Recently, the role in health and disease of the nervous system tropomyosin-related TK receptors (Trk), known as a family of growth factor receptors essential for neuronal survival, has been discussed [442]. Regarding these receptors, Ono Pharmaceutical Co., Ltd., presented a Trk-inhibiting compound in different presentations forms, beneficial for the prevention and/or treatment of numerous diseases including Chagas disease. These drugs were described as heterocyclic carbon compounds containing a hetero ring having chalcogen such as O, S, Se or Te, or nitrogen as the only ring hetero atoms [443].

Recently, the *Tc*PTP1 has been implicated in the cellular differentiation and infectivity of the parasite. Consequently, it is a hopeful target for the design of new anti-parasitic drugs. The crystal structure of *Tc*PTP1 obtained at a resolution of 2.18 Å provide structural insights into the active site environment to start structure-based drug design by different strategies in order to develop specific *Tc*PTP1 inhibitors [444].

c. Aromatic 6-substituted thienopyrimidines. Due to the recent discovery of a lapatinib-derived analog 2 with excellent potency against *T. brucei* in the nM order and selectivity over human host cells, other classes of human TKI scaffolds have been explored aimed to expand the range of pursued chemotypes. Two analogs corresponding to the group 4 with sub-micromolar potencies for *T. cruzi* were discovered, confirming the value of concurrent screening of a chemical library against different protozoan parasites [445].

2.12. Polyamine Metabolism and Transport Pathways

Polyamines participate in numerous cellular processes, including chromatin condensation, sta-

bilization of tRNA´s structure, DNA conformational transitions and post-translational modification (PTM) of proteins, among others [446]. Polyamine metabolism has been considered as a promising chemotherapeutic target in parasite infections, taking into account that they are essential requirements for parasite cell growth and differentiation. ODC is a key enzyme of the polyamine biosynthesis pathway and it is usually inhibited by difluormethylornithine (DFMO), however, *T. cruzi* has not been affected by this inhibitor and ODC has not been detected in any stage of its life cycle. In adding, *T. cruzi* was found to be susceptible to an ODC-related compound, difluoromethylarginine (DFMA), which is supposed to inhibit arginine decarboxylase (ADC), but this enzyme activity was only found in trypomastigotes at almost undetectable levels [447].

Knowing that in *T. cruzi* genome ODC gene is missing, the trypanosomatid was transformed with a recombinant plasmid bearing the complete coding region of ODC gene. The transgenic parasites acquired the capacity to synthesize putrescine and became susceptible to DFMO, irreversible inhibitor of ODC. The appearance of DFMO-resistant *T. cruzi* after a first step selection of ODC-transformed parasites grown in the presence of high drug levels were reported in parasites transfected with ODC gene [448].

Putrescine analogues. 1,4-Diamino-2-butanone (DAB) was capable to inhibit epimastigotes proliferation producing notable signs of oxidative stress. Aditionally, the measurement of thiobarbituric-acid-reactive substances was performed in order to evaluate lipid peroxidation. The finding of a dose-dependent response indicated that putrescine uptake by this diamine auxotrophic parasite might be vital for epimastigote growth in axenic medium and cellular organization [449]. The design, synthesis and therapeutic use of diverse inhibitors of polyamine transport to prevent polyamines salvage in tumoral cells was included into a patent from Laval University, Canada [450]. Despite the results obtained, the significance of polyamines in cell survival and the complete information of the synthetic pathways in *T. cruzi* still needs additional investigation.

Polyamine biosynthetic enzyme inhibitors. Considering that the targeting of the polyamine pathway enzymes might offer a new therapy approach capable to inhibit the deoxyhypusine hydroxylase and in order to identify new lead compounds, piperidines were synthesized and biologically evaluated [451]. Different inhibitors were tested considering that the enzymes involved in spermidine synthesis are auspicious targets for drug development to therapy of many tropical diseases including Chagas disease [452].

Amino acid and polyamine exclusive transporters from trypanosomatids, members from the Amino Acid/Auxin Permeases (AAAP) family, were investigated, considering that these permeases are absent in mammals and could be then evaluated as a potential target against *T. cruzi* [453]. In addition, some exclusive properties of the metabolism of polyamines in trypanosomatids have been reviewed, including the prozyme S-ADC regulation activity, the ODC co-expression, and the formation of trypanothione, a unique compound linking polyamine and thiol metabolism in trypanosomatids. In particular, remarkable points within polyamine metabolism are emphasized for their probable use in selective therapeutic strategies [454].

Pentamidine. This aromatic diamidine is capable to block a polyamine transporter present in *L. major*, decreased the viability of *T. cruzi* trypomastigotes and the parasite burden of infected cells; diminished the inflammation and parasite burden in hearts from infected mice, and decreased parasitemia, increasing the survival rate. In addition, transport of putrescine and spermidine in *T. cruzi* epimastigotes and amastigotes was potently inhibited by pentamidine. In this sense, the extensive use of pentamidine in human could be an advantage over newly synthesized molecules which require more trials prior to their clinical use to be used as a new agents against Chagas disease [455].

2.13. Purine Salvage Pathway and Nucleotide Synthesis

In mammals, nucleotides are synthesized de novo and also salvaged from recycled purine bases. However, most parasites are obligate purine auxotrophs. So, they must salvage purines from their host and they have developed patterns to transport, internalize and metabolize the constituents of nucleic acids and ATP. Thus, *T. cruzi* nucleotide synthesis is dependant on the scavenging of exogenous purines. Among enzymes from trypanosomatids that participate in the scavenging of purines from the host can be mentioned:

2.13.1. Purine (Hipoxantine/Guanine)-Phosphoribosyltransferase (HGPRT)

The transference of a phosphoribosyl moiety on the nucleobase hypoxanthine or guanine for converting purine bases to ribonucleotides is catalyzed by HGPRT. This enzyme is responsible for the initiation of the metabolism of certain cytotoxic purine base analogues (allopurinol) in the parasite. Then, both inhibitors and substrates of HGPRT are considered virtuous targets for effective and selective chemotherapeutic agents. The cloning, sequencing and overexpression of hgprt genes from *T. cruzi* and other pathogenic trypanosomatids were addressed, and all the recombinant proteins have been purified and characterized [456]. The purine (3'-azido-3'deoxyinosine, 3'-deoxyadenosine) and pyrimidine (3'-azido-3'-deoxythymidine) analogues inhibited the proliferation of amastigotes in culture cell lines [457]. Also, allopurinol (4-hidroxy-pyrazol-(3,4d)-pyrimidine) has been used in humans for the treatment of gout. In vertebrates, it is transformed in oxypurinol, a strong inhibitor of xanthine oxidase (XO). The compound acts as a purine analogue and is incorporated via HGRPT into DNA disrupting the synthesis of RNA and proteins in XO-deficient-trypanosomatids. Allopurinol was active in mice models of acute Chagas disease [458], despite differences in susceptibilities among *T. cruzi* strains and some conflicting reports related to its efficacy in humans were shown. The drug showed no *in vivo* activity due to low incorporation in *T. cruzi* vertebrate stages and perhaps to inadequate pharmacokinetic properties. The interaction between purine analogues with the *Tc*HGPRTs and its human counterpart was assayed, and some of them showed affinity for the trypanosomal enzyme [459]. A structure-based docking method identified a number of possible *Tc*HRPT inhibitors. The compounds (2,4,7-trinitro-9-fluorenyl-idenemalononitrite,3-(2- fluorophenyl)-5-(phenoxy)-1,2, 4-triazolo (4,3-C)-quinazoline and [6-(2,2- dichloroaceta mido)chrysene] proved to be strong inhibitors [460]. The design of a mechanism-based inhibitor of *Tc*HPRT based on kinetic parameter analysis was developed [461].

2.13.2. Dihydrofolate Reductase (DHFR)

Among enzymes involved in DNA nucleotide synthesis, DHFR and thymidylate synthase (ThyS) are well-known. Both constitute a bifunctional protein present in different species of protozoa and has been productively used as a drug target in chemotherapy of cancer, malaria and also infectious diseases. The gene coding for the *Tc*DHFR was cloned and expressed [462]. By using a structure-based approach, several derivatives of methotrexate, the inhibitor of the human enzyme, were designed and synthesized, showing some of them higher selectivity for the parasite enzyme than for the human counterpart. The design, synthesis and screening of another group of compounds as inhibitors of DHFR of trypanosomatids showed weak activity in *in vitro* assays using *T. cruzi* amastigotes [463]. On the other hand, a structure-based three-dimensional quantitative SAR (3D-QSAR) tactic was used in order to produce a library of selective lead *Tc*DHFR-ThyS inhibitors for additional development as antiparasitic agents. The 3D-QSAR models obtained for *Tc*DHFR-ThyS and human DHFR showed a worthy agreement between experimental and predicted enzyme inhibition data [464]. Aimed to study the interactions between DHFR enzyme and classical and novel inhibitors, *Tc*DHFR-ThyS was crystallized complexed with the dihydrotriazine-based or quinazoline-based antifolates C-448, cycloguanil and Q-8 [465]. Also, six strong inhibitors of this parasitic enzyme were synthesized, characterized, and the inhibitory activity of each compound against *T. cruzi* and human DHFR was tested. Among these compounds, compound 6b, ethyl 4-(5-[(2,4-diamino-6- quinazolinyl) methyl] amino-2 methoxyphenoxy) butanoate, was co-crystallized with the bifunctional *Tc*DHFR-ThyS enzyme and the crystal structure of the ternary enzyme:cofactor:inhibitor complex was determined. The potential interactions of all inhibitors with *Tc*DHFR and hDHFR were analyzed by molecular docking [466]. DHFR inibitors include:

a. 2, 4-Diaminopyrimidines derivatives. 5-Benzyl-2, 4-diaminopyrimidines were reported as selective inhibitors of the trypanosomal and leishmanial enzymes. Numerous compounds with alkyl/aryl substitution on the 6-position of the pyrimidine ring were prepared and evaluated against the recombinant enzymes and the complete organisms finding that the presence of a substituent did not enhance the inhibitor activity neither against the enzyme nor whole parasites compared to unsubstituted compounds [467]. On the other hand, synthesized 4'-substituted and 3', 4'-disubstituted 5-benzyl-2,4-diaminopyrimidines were tested against the recombinant parasite and human

DHFRs. Some of them showed a respectable *in vitro* activity against *T. cruzi*. Those compounds which bound within the enzyme pocket of trypanosomatid enzymes presented the highest selectivity as shown by molecular modeling [468, 469].

b. 2, 4-Diaminoquinazolines. In different trypanosomatids, a series of 2, 4-diaminoquinazolines were designed, synthesized and evaluated as DHFR inhibitors. Some of them displayed strong activity against *T. cruzi* [470]. Also a set of quinazoline-2,4,6-triamine derivatives (1-9) was rationalized basing on docking studies of the DHFR structures from trypanosomatids and Plasmodium, and three compounds out of nine were the most effective against *T. cruzi* than Nx and Bz, and were endowed with redox properties, representing a good start for drug development [471].

c. Antifolate drugs. The FDA-approved drug for the treatment of *Pneumocystis carini* infection in AIDS patients, the lipophilic trimetrexate (TMQ), showed to be a strong inhibitor of *Tc*DHFR activity and was also very effective in killing the clinical forms of *T. cruzi*. Unfortunately, TMQ also showed to be a noble inhibitor of human enzyme [472].

2.13.3. Pteridine Reductase (PTR)

Reduced pteridines are required in many important cellular functions. Unlike their mammalian host, trypanosomatids are pteridine auxotrophs and salvage the precursor pteridines from the host reducing them to the respective biologically active tetrahydro-forms using parasite enzymes which may be useful as drug targets. The enzyme pteridine reductase 1 (PTR1), first related with reduction of unconjugated pteridines, was only found in trypanosomatids and plant pathogens. PTR1 catalyzes the reduction of folate to dihydrofolate and tetrahydrofolate mediating in the salvage of oxidized pteridines showing lower sensitivity to methotrexate than DHFR, interfering in the efficiency of antifolate drugs targeting DHFR [473]. In addition, PTR2 was identified and expressed *in T. cruzi*, it can reduce dihydropterin and dihydrofolate substrates but is not capable to reduce oxidized pteridines [474]. A set of pteridine analogues at the active site of PTR2 were analized by a docking study finding better results than that of methotrexate [475]. The crystal structures of PTR2 complexed to methotrexate and dihydrofolate was reported [476]. Triazine derivatives were patented as novel DHFR inhibitors useful for parasitic infections including Chagas disease by Isis Innovation Ltd, 2001 [477]. Disclosed patents on purine analogues have been related to antiviral and/or anticancer activity [478], only a few of them claimed their effects on parasitic diseases. Among ATP analogues, nucleoside pirophosphate and triphosphate analogues were useful against infectious diseases caused by some protozoans including Chagas disease. However, no experimental evidences were described [479], (Table **2**). The identification of *Tc*PTR1 inhibitors was done by a rapid screening approach using a folate-based library with structure-based design. Folate dependent enzymes included PTR1, DHFR, and ThyS. The affinity profile determined selectivity and specificity of a series of quinoxaline and 2, 4-diaminopteridine derivatives. Nine compounds showed superior activity against parasite enzymes compared to the human enzymes. In *T. cruzi*, selected inhibitors were evaluated on wildtype and overexpressing PTR1 lines, as a model for PTR1-driven antifolate drug resistance. When PTR1 inhibitors were used in combination with known DHFR inhibitors, an additive profile and a reduction in the treatment toxicity was observed compared to the administration of the DHFR inhibitor alone. The combination of antifolates targeting two enzymes showed to be successful in the improvement of new antiparasitic drugs [480].

Triazine dimers. Researchers from the University of Antwerpen, Belgium have patented disubstituted triazine dimers for the treatment and/or prevention of infectious diseases, including Chagas disease. The new compounds contain two disubstituted triazine rings covalently linked by an organic linker, thereby creating dimers [481].

2.13.4. Dihydroorotate Dehydrogenase (DHOD)

The fourth enzyme of the pathway that catalizes the transformation from dihydroorotate to orotate in *T. cruzi* diverges from the human enzyme. Searching for strong inhibitors against *T. cruzi* DHOD activity, this activity was inhibited by the extracts from the brown algae *Fucus evanescens* and *Pelvetia babingtonii*. Besides, these extracts resulted effective against both the protozoan infection and the proliferation in mammalian cells [482]. On the other hand, a recombinant *Tc*DHOD, complexed to orotate was recently crystallized [483], opening the possibility for inhibitors design. Genetic studies have demonstrated that *Tc*DHOD is vital for parasite survival validating it as promis-

ing target for antichagasic agent's development. Thus, the crystal structure analysis of *Tc*DHOD complexes has allowed proposing possible sites for structure-based specific inhibitors design and studying mechanisms of fumarate reduction and dihydroorotate oxidation [484, 485]. The identification of new*Tc*DHOD inhibitors was allowed by combination of virtual screening and structural analysis. Among the investigated class 1A inhibitors, the strongest compound was able to inhibit *Tc*DHOD enzyme with a Kiapp value in the μM order. The compounds were considered promising hits for further drug development [486]. Purine salvage pathway has been meticulously investigated over the last twenty years. However, the existence of by-pass mechanisms by other enzymes and transporter systems could be suggested recently, based on the published genomic data of *Trypanosoma, Leishmania* and *Plasmodium*. Hence, the inhibition of a single salvage enzyme could be able or not to cause parasite death or growth arrest [487].

2.14. Organelles, Structures and Processes as Targets

2.14.1. DNA Modulation/Replication, Nucleus and Kinetoplast

Different classes of molecules were assayed aiming to interfere with processes involving DNA, including interference with topoisomerases, use of DNA binders, antimitotic agents and NADH analogues.

Topoisomerases are ubiquitous and indispensable for nucleic acid biosynthesis and cell survival by modifying DNA topology. They are responsible for solving the torsional tensions produced during replication and transcription, and are in control of maintaining the genomic stability during the recombination of DNA. Top are involved in the kinetoplastids metabolism of both nuclear and mitochondrial (kinetoplast) DNA. Molecular and cellular biology studies aiming for antiparasitic chemotherapy have focused on parasites DNA Top, considering them as target. Particularly, Top II, essential for kinetoplast replication. Some inhibitors of bacterial DNA Top II resulted active against *T. cruzi*, generating damage to epimastigotes nucleus and/or kinetoplast as well as inhibiting proliferation and differentiation, proposing both organelles as putative drug targets [488].

The Top activities of relaxation and decatenation are based on a common nicking-closing cycle which includes one or both DNA strands and have been considered as an auspicious drug target. Interestingly, the comparison between Tops from members of the Trypanosomatidae family and those from the host showed significant structural differences, making them motivating for the design of specific inhibitors capable to bind to the interface of DNA-Top complexes, stabilizing Top-mediated transient DNA breaks [489, 490]. Regarding the complex II (succinate: ubiquinone reductase), it often plays a pivotal role in the adaptation of parasites into host organisms and could be a possible target for the development of new drugs. In this sense, when *T. cruzi* complex II was studied, it was found to be composed of differents hydrophilic and hydrophobic nucleus encoded subunits (SDHs). In trypanosomatids unusual features were discovered, making complex II a target for new chemotherapeutic agents [490]. The trypanosoma pre-replication machinery component Orc1/Cdc6 is different from the Orc1 and Cdc6 mammal proteins. It was proposed as a possible target for drug development. Likewise, studies in T. *brucei* showed that the RNAi-mediated silencing of trypanosoma Orc1/Cdc6 expression reduced cell survival, demonstrating that Orc1/Cdc6 is critical for trypanosoma subsistence [491].

a. Cryptolepine derivatives. 2-Bromo-, 2-nitro-, and 2-methoxy-9-cyanoneocryptolepine compounds revealed activity against *T. cruzi* and *T. brucei* in the μm range, in absence of toxicity to mammalian cells [492].

b. Substituted N-benzene and naphthalenesulfonamides. In order to evaluate the *in vitro* effectiveness of a series of six amine N-benzenesulfonamides substituted aromatic rings, against *T. cruzi*, the interaction of a selection of sulfonamides with pUC18 plasmid DNA was examined by using nuclease activity assays. Data revealed the participation of a redox mechanism. Three sulfonamide derivatives evidenced the formation of ROS in charge for DNA strand scission. The analysis of the parasites treated with these compounds by transmission electron microscopic exhibited mainly a complete cellular disorganization mostly directed to DNA bearing structures. The *in vitro* screening of a new series of fifteen N-substituted benzene and naphthalenesulfonamides against *T. cruzi* was performed and three of them

exhibited notable *in vitro* activity and selectivity towards epimastigotes and amastigotes, and one of them also decreased parasitemia levels in a mice model of acute Chagas disease [493, 494]

c. Quinolone derivatives. ***Trypanosoma*** *cruzi* is sensitive to quinolone derivatives possibly due to the inhibition of DNA Top II. New Pharma Research Sweden AB has presented a patent on these derivatives considering them as useful agents in the treatment of bacterial and parasitic diseases comprising those caused by trypanosomatids. However, no specific data were reported [495] (Table **2**).

d. Pyrazole and propenone quinoxaline derivatives. Although the pyrazole quinoxaline series resulted inactive against *T. cruzi*, the compounds fitting to the propenone quinoxaline series showed a moderate activity. The compound (2E)-1-(7-Fluoro-3-methyl-quinoxalin-2-yl)-3-(3,4,5-trimethoxy-phenyl)-propenone was active against intracellular amastigotes of *T. cruzi* with no toxicity to mice peritoneal macrophages. Docking studies revealed the possible interaction of the compounds of the second series with the *T. cruzi* poly-(ADP-ribose)-polymerase-protein [496].

e. Dicationic guanidine and amidine derivatives. Aromatic diamidines were described as DNA minor groove-binding ligands, such as pentamidine, present an extensive spectrum of activities against human and veterinary pathogens. Twenty dicationic molecules comprising either diguanidino or reversed amidine cationic groups were *in vitro* assayed vs *T. cruzi*. Among the most active DNA modulating agents, belonging to the reversed amidine series, six displayed IC50 values lower than 1 μM and were described as auspicious agents for the treatment of trypanosomiasis [497, 498]. The synthesis of compounds with strong DNA binding affinities such as the dicationic reversed amidines, in particular new 2, 5-bisalkyl (or aryl) imino aminophenyl furanes and thiophenes was described by scientist from University of North Carolina at Chapel Hill. The presented patent claimed them as mycobacterial, fungal and protozoal useful compounds for infections including *T. cruzi* [499] (Table **2**). The diarylthiophenediamidine DB1362, four diamidines DB811, DB889, DB786, DB702 and the diguanidine DB711 showed a strong *in vitro* activity against intracellular amastigotes and bloodstream trypomastigotes and no toxicity to host cells [500, 501]. Considering that despite their microbicidal activity, they had presented rather poor bioavailability and high toxicity, several analogues and derivatives were synthesized and *in vitro* and *in vivo* screened in order to improve their selectivity and pharmacological properties. Thus, structure optimization of novel aromatic dicationic compounds was proposed for future design of new antiparasitic drug candidates [502]. Although ten novel structurally related amidines were active against *T. cruzi* trypomastigote forms and DB2247 was 6-fold more effective than Bz displaying very low toxicity, none presented superior trypanocidal effect against intracellular amastigotes as compared with the reference drug. These results might be attributed to dissimilarities in mechanisms and cellular targets between bloodstream and amastigote forms. The design of novel amidines might provide promising activity against *T. cruzi* [503, 504].

f. Dinitroaniline sulfonamide derivatives. Scientist from Ohio State University disclosed these antimitotic compounds with activity against tubulin as useful for the treatment of diseases caused by parasitic protozoa. Although these compounds exhibited good *in vitro* activity, they were unsuccessful to cure parasite infected mice. The toxicity of compounds containing nitroaromatic groups has not been still addressed [505]. Recently, mononuclear complexes composed of (5-chloro-2-hydroxybenzylidene) aminobenzenesulfonamides (L1-3) of general formula (L2(M)2H2O, where M is Co, Cu, Zn, Ni or Mn) reduced epimastigote proliferation and were found trypanocidal for trypomastigotes of *T. cruzi* Y strain. Complexes C5 and C11 showed similar IC50 values in the μM order for trypomastigotes, than the positive control Nx. While none of these complexes inhibited TR, some degree of DNA binding was observed, albeit less pronounced than the experimented for cisplatin in this assay. Unfortunately, most of these complexes were toxic for mouse splenocytes [506].

g. Vanadium mixed-ligand complexes. The synthesis, characterization and evaluation of four new mixed-vanadyl ligand complexes, [VIVO(L(2)-2H)(L(1))], containing a bidentate polypyridyl DNA intercalator L(1), and a tridentate salycylaldehide semicarbazone derivative L(2) as ligand were developed, displaying a similar activity on *T. cruzi* epimastigotes as Nx. When DNA was studied as possible parasite target, the results obtained by electrophoretic analysis suggested that

the DNA interactions of these complexes could be involved in their mode of action [507]. The inclusion of the lipophilic 3,4,7,8-tetramethyl-1,10-phenanthroline NN ligand and seven tridentate salicylaldehyde semicarbazone derivatives (L1-L7) formed part of the development of a new series of heteroleptic [VIVO (L-2H)(NN)] compounds. The complexes exposed more *in vitro* activity against *T. cruzi* than Nx and a great part of them displayed more activity than that previously described for [VIVO (L-2H)(NN)] complexes of other NN co-ligands. The great activity and selectivity shown by L2, L4, L5 and L7 complexes allowed them to be considered as new leads for drug development. Unfortunately, although all of them interacted well with DNA, with binding modes and strength altered in different extents by the NN and semicarbazone co-ligands, molecular docking studies suggested that the anti-*T. cruzi* activity displayed could not be explained only by intercalation of DNA as mechanism of action [508].

h. Ruthenium complexes. Searching for novel metal-based agents acting against diseases produced by trypanosomatids, four organoruthenium (II) compounds [Ru2(p-cymene)2(L)2]X2, being L= bioactive 5-nitrofuryl-containing thiosemicarbazones and X=Cl or PF6 were evaluated on *T. cruzi*. Two of them, [Ru2(p-cymene)2(L4)2]Cl2 and [Ru2(p-cymene)2(L1)2]Cl2 presented substantial *in vitro* activity against trypomastigotes showing IC50 values in the μM order On the other hand, the compounds HL4=5-nitrofuryl-N-phenyl-thiosemicarbazone and HL1=5 nitrofurylthiosemicarbazone, displayed fairly good selectivities toward trypanosomes compared to mammalian cells. The studies on trypanomicidal action of the organoruthenium agents showed their ability to produce toxic free radicals by bioreduction and also to interact with the two possible parasite targets, DNA and Cz, suggesting that these complexes seem to display a "multi-target" mechanism of trypanosocidal action [509]. Otherwise, the synthesis of the novel ruthenium complexes [RuCl2(HL)(HPTA)2]Cl2 with HL=bioactive 5-nitrofuryl containing thiosemicarbazones and PTA=1,3,5-triaza-7-phosphaadamantane was developed including PTA as co-ligand with the aim to modulate the aqueous solubility of the complexes. Once water soluble complexes were obtained, activity against *T. cruzi* was evaluated *in vitro* and the [RuCl2(HL4)(HPTA)2]Cl2 complex, with HL4=N-phenyl-5-nitrofuryl-thiosemicarbazone, was the most active compound. Oxidative stress plus the interaction with parasite DNA could participate as possible targets in the potential mode of action of these ruthenium complexes. The exploration of metal compounds was extended to activity against several parasites [510].

i. Diphenylamine derivatives. Selective and strong anti-trypanosomal agents, in particular 4,4'-bis(imidazolinylamino)- and 4,4'-bis(guanidine) diphenylamine compounds, CD27 and CD25, respectively, were discovered, searching for DNA-binding small molecules. The trypanocidal properties of these symmetric diphenylamine compounds in addition to the crystal structure analysis of the detailed interaction of these compounds with DNA, suggests the start for studying the mechanism of trypanocidal activity of these compounds [511].

j. Bisbenzimidazole derivatives. Six derivatives characterized by a 3, 4-ethylenedioxy-extension of thiophene core, exposed a pronounced affinity to bisbenzimidazole, and a potent thermal stabilization effect toward ds-DNA, and one of them inhibited *T. cruzi* epimastigotes growth [512]. Such inhibition was produced by the specific interaction between parasite tubulin and ligands such as benzimidazoles, colchicine and vinblastine. Thus, in kinetoplastids, tubulin has been projected as a possible target [513]. Fifteen years ago, selective hit compounds against kinetoplastid tubulin have been identified, suggesting to this protein as target for the development of novel drugs [514].

k. Ribavirin. This 1, 2, 4-triazole-3-carboxamide riboside is a well-known antiviral drug, capable to inhibit human S-adenosyl- L-homocysteine hydrolase (hSAHH), catalyzing the conversion of S-adenosyl-L-homocysteine to adenosine and homocysteine. The drug structure, similar to that of adenosine, caused inactivation of hSAHH and *Tc*SAHH, dependent on time. Ribavirin is capable to bind to the adenosine-binding site of the two SAHHs enzymes and to reduce the NAD (+) cofactor to NADH. The binding step of ribavirin to hSAHH and *Tc*SAHH is reversible and presents similar Ki values. However, the slow inactivation step is 5-fold more rapidly with *Tc*SAHH, offering a structural lead for the design of selective *Tc*SAHH inhibitors as potential antiparasitic drugs [515].

l. NADH analogues. To date, no significant results have been obtained headed for the design

of selective inhibitors against *Tc*SAHH targeting the substrate binding site. A validation for the design of anti-parasitic drugs directed toward cofactor-binding sites was provided by kinetic and thermodynamic studies related with the association and dissociation of NAD/H with *Tc*SAHH and hSAHH. Analogues of NAD and the reduced NADH have shown a substantial selective inactivation of *Tc*SAHH, approving this rational design approach [516]. The fact that hSAHHs bind to the cofactor NAD (+) more tightly than several parasitic SAHHs about 1000-fold proposes to the cofactor binding site of this essential enzyme as a potential anti-parasitic target against *Tc*SAHH. The determination of kinetic parameters for NAD (+)/NADH analogues suggested that NADH analogues resulted the most auspicious for *Tc*SAHH selective inhibition [517]. Based on the crystal structures of SAHHs, the free energy simulations were useful for the prediction of residues clue for the differential cofactor binding properties between human and trypanosomal SAHHs, showing specific potential sites for mutagenesis [518]. Scientist from Bradley Cytokine Pharmasciences, Inc, have described a patent on compounds and methods for infectious diseases treatment. Some of them, targeting specific nuclear location, signal blocking ingress of specific proteins or nuclear molecular complex claim their use for the treatment or prevention of parasitic and viral diseases [519], (Table **2**).

2.14.2. Acidocalcisomes and Exchanger Na^+/H^+ Mechanism

Another unusual feature of *T. cruzi* in comparison with mammalian cells is the storage of calcium in acidocalcisomes, parasite specialized acidic organelles. These structures participate in polyphosphate and calcium storage in addition to the adaptation to environmental oxidative stress [520]. 3,5-Dibutylhydroxytoluene inhibits Ca^{2+} discharge via the acidocalcisomal exchanger Na^+/H^+, a mechanism involved in $Ca^{2+}$and pH homeostasis exclusive of trypanosomatids. The drug also showed controversial effects on the vacuolas H^+-ATPase, depending on the princubation times and were obtained in concentrations used for this drug as antioxidant agent, indicating that care must be taken when evaluating this drug as trypanocidal agent [521].

a. Guanidine derivative compounds. The use of Na^+/H^+ exchange inhibitors for the treatment of protozoal infections comprising Chagas disease was claimed by Hoechst Marion Russel Deutchland CmbH. Although these compounds were described, neither synthesis nor characterization was reported [522], (Table **2**).

b. β-Lapachone-derived naphthoimidazoles. Forty five semi-synthetic naphthoquinones derivatives were obtained from lapachol and β-lapachone extracted from *Tabebuia sp* and the naphthoimidazole 4,5-dihydro-6,6-dimethyl-6H-2-(phenyl)-pyran [betha-4,3] naphth[1,2-d]imidazole (N1) were considered among the more active compounds against *T. cruzi*. The effect of N1 against epimastigotes proposed that reservosomes, mitochondrion, and nucleus as targets, while in trypomastigotes, in which reservosomes are missing, mitochondrion, nucleus and acidocalcisomes were the compartiments affected by the compound [523].

2.14.3. Membrane Components, Receptors, Contractile Vacuole and Osmoregulation

Almost a decade ago, parasite membrane components, nutrients and metabolites transport proteins localized in the parasite-host interface have got into focus as novel drug targets.

a. Aquaporins (AQPs). In the protozoan genomes, the genes codifying for AQP water and solute channels have been identified. The cloning and functional characterization of six protozoan AQPs was developed. The permeability properties were attributed to specific protein features. Also, AQPs were considered responsible for probable physiological roles in osmotic protection and metabolism. The presence of *Tc*AQP was described in the parasite acidocalcisomes and contractile vacuole complex [524]. The prospective use of protozoan AQPs as a target for chemotherapeutic agents was reviewed some years ago [525]. Likewise, a contractile vacuole complex is involved in *T. cruzi* osmoregulation [526]. *Trypanosoma cruzi* evolution allowed the ability to transit between diverse hosts and the replication in hostile environments. The aquaporin gene *Tc*AQP1 was knocked down or overexpressed and experiments with inhibitors, revealed its significance for the cellular response to hyperosmotic stress. The analysis of a genome-wide transcriptional of stressed parasites revealed down-regulation of genes belonging to diverse functional categories and up-regulation of genes encoding trans-sialidase-like and ribosomal proteins. The analysis of the sequences from 3'UTRs of up- and down-

regulated genes allowed the identification of conserved structural RNA motifs enriched in each group, proposing that specific ribonucleoprotein complexes could be of great significance in the adaptation of this parasite to diverse environments through regulation of transcript abundance [527]. The identification of compounds capable to interfere with the uptake function of a subset of transporters, which are indispensable for parasite viability, could be useful as targets for new drug chemical therapies [528].

b. Phosphatidylinositol kinases (PIKs) *Tc*Vps34, a *T. cruzi* phosphatidylinositol-3-kinase (PI3K) plays a noticeable role in vital processes for parasite survival comprising osmoregulation, acidification, and vesicular trafficking [529]. In *T. cruzi* epimastigotes, enzymatic parameters of the classes PI4K, PIPK and PI3K and detailed responses to the confirmed kinase inhibitors adenosine, sodium deoxycholate, wortmannin and LY294002, as well as the activators Ca^{2+}, phosphatidic acid, spermine and heparin were evaluated suggested that could participate in signaling pathways. The three classes were cloned and expressed and their product was tested for kinase activity, showing that the phosphatidylinositol metabolism through PIKs activities plays a central role in signaling pathways [530].

c. Exopolyphosphatase (PPX). The exopolyphosphatase from *T. cruzi* (*Tc*PPX) was cloned, expressed, purifyed, and characterized. *Tc*PPX and most exopolyphosphatases differ in their preference for the short-chain polyphosphate poly P. *Tc*PPX overexpression occasioned an important reduction in total short-chain poly P and partial diminution in long-chain poly P, together with a late regulatory volume reduction after hyposmotic stress supporting the role of poly P in the osmoregulation of the parasite [531].

d. Ecto-enzymes, precursors of surface molecules, organelle membranes, transporters. Ecto-enzymes are present in parasite membrane; contain active site facing towards the external medium rather than to the citoplasm. A chromium (III) adenosine 5'-triphosphate complex (Cr-ATP) emerged as a novel inhibitor of ecto-ATPases in trypanosomatids with the aim to obtain a better understanding of properties and role of these ATPases in the biology of parasites. DIDS (4, 4 diisothiocyanatostilbene 2,2' disulfonic acid), suramin and ADP showed to be effective inhibitors. Only ADP presented no additive inhibition pattern with Cr-ATP [532]. Inositol is known as the precursor for most *T. cruzi* surface molecules, comprising phosphoinositides, glycosylinositolphospholipids andg lycosylphosphatidylinositol anchors. Regarding that the parasite is an inositol auxotroph, the inositol transport system might constitute a probable target for novel trypanocidal agents, as some of its properties are different from its mammalian counterpart. In *T. cruzi e*pimastigotes, the myo-inositol transport system is inhibited by PKA and stimulated by PKC effectors [533].

The synthesis of novel copper (II) complexes of two triazolo-pyrimidine derivatives (1,2,4-triazolo-[1,5-a]pyrimidine, and 5,7-dimethyl 1,2,4-triazolo-[1,5-a]pyrimidine, showed anti-proliferative effect against *T. cruzi*. Copper (II) complexes C24b, C49 and C35, operated both on the parasites energy metabolism at the level of the NAD(+)/NADH balance and at the level of the organelle membranes, producing damage and cell death [534]. At difference with Arg transporters in higher eukaryotes, trypanosomatids arginine transporter genes were characterized showing that they also transport Lys. However, these parasite transporters only translocate Arg. *Trypanosoma cruzi* encodes for the Lys-specific permease *Tc*AAP7, a member of the large amino acid/auxin permease family. The deletion of the alleles coding for these permeases failed suggesting that genes encoding for Lys permeases should be essential [535].

e. Phosphodiesterases (PDEs). ***Trypanosoma cruzi*** codifies for four different families. PDEC2, characterized as a FYVE domain containing protein [536], has been involved in *T. cruzi normal* osmoregulation. The cells that overexpress this enzyme were unaffected by PDEC2 inhibitors. In *T. cruzi*, the localization of PDEC2 shows a strong labelling in the contractile vacuole complex. Moreover, the significance of this domain in the *Tc*PDEC2 localization and activity was evidenced when transgenic parasites overexpressing a truncated version of *Tc*PDEC2 without the FYVE domain showed a miscarriage in its targeting to the contractile vacuole complex and a manifest reduction in PDE activity [537]. *Tc*PDEC2 was proposed for the development of PDE inhibitors as lead compounds for trypanocidal drugs [538]. A new and uncommon *Tc*PDEC, capable to hydrolyze cyclic GMP, despite it prefers cyclic AMP, has a FYVE-type domain in its N-terminal region.

*Tc*PDEC showed homology to the mammalian PDE4 family members. PDE4 inhibitors are at present under development for the treatment of inflammatory diseases. However, different compounds have been identified as probable *Tc*PDEC inhibitors through virtual screening by homology modeling and were tested against amastigotes growth and recombinant *Tc*PDEC activity, resulting strong inhibitors. The best inhibitors were found to rise the cAMP cellular concentration, in line with their *in vitro Tc*PDEC inhibitory activity, providing chemical validation of this target. The mentioned compounds could be useful tools in the study of *T. cruzi* osmoregulation and in the development of novel drugs against Chagas' disease and other trypanosomiases [539]. In trypanosomatids, although cAMP is produced during the life cycle stages, its signaling pathways are very different from those of mammals. The identification of downstream cAMP Response Proteins (CARPs) were discovered in trypanosomatids. CARPs expression levels correlated with sensitivity to PDE inhibitors, suggesting a complex signaling cascade. The relationship between the roles of these novel CARPs and the signaling pathway on cell division and differentiation are relevant for cell biology and represent a new architype in cAMP signal transduction, and credible targets for trypanosomatid-specific cAMP pathway-based therapeutics [540].

2.14.4. Glycosome and Vitamin C Synthesis

Glycosomes, unique single-membrane peroxisome-related organelles are found in all the trypanosomatids forms studied. They contain a glycolytic pathway that catalyzees the aerobic fermentation of glucose to succinate. Aditionally, these organelles contain enzymes for many other routes including gluconeogenesis, the PPP, beta-oxidation of fatty acids, purine salvage, and biosynthetic pathways for pyrimidines, ether-lipids and squalenes. The enzymatic content of glycosomes is quickly transformed during differentiation of bloodstream mammalian forms to those living in the insect midgut. Autophagy seems to play a central role in trypanosomatids differentiation by degrading old glycosomes, while a population of new organelles comprising diverse enzymes is synthesized [541]. On the other hand, it was verified that *T. brucei* and *T. cruzi* are capable to synthesize vitamin C and that the reaction takes place in the parasite glycosomes. Regarding that the ability to synthesize vitamin C or ascorbate is widespread in eukaryotes but is absent from humans, this feature set up another possible chemotherapeutic drug target [542]. A variety of glycosomal enzymes contributing to diverse biological routes have been certified as drug targets by genetic or chemical means. The inhibitors used for several of these enzymes have been got by different methodologies such as by using compound libraries screening or design followed by synthesis. A number of inhibitors caused growth inhibition of the clinically relevant stages of trypanosomatid species and in some cases exerted therapeutic effects in infected animals. On the other hand, three isoenzymes of phosphoglycerate kinase (PGK) were simultaneously expressed in *T. cruzi*, the cytosolic isoenzyme PGKB and two glycosomal enzymes, PGKA and PGKC. In *T. cruzi* epimastigotes, PGKA is associated to the glycosomal membrane and responsible for about 23% of the glycosomal PGK activity in contrast to the 77% soluble activity mainly attributed to PGKC. Results performed with antibodies specific for PGKA almost completely blocked the epimastigotes glucose consumption indicating that PGKA is the predominant isoenzyme for sustaining glycolysis through parasite glycosomes. The glycosomes integrity and the correct compartmentalization of at least some matrix enzymes are critical for parasites viability, for participating proteins in the assembly of the organelles and transmembrane route of substrates and products of glycosomal metabolism, offer also potential as drug targets [543, 544].

2.14.5. Mitochondrion

The exclusive characteristics of *T. cruzi* mitochondria and mitochondrial metabolism in conjunction with the possibility of taking advantage of these particularities to target novel drugs against this parasite has been extensively discussed [545, 546].

a. C-allyl lawsone-derived naphthofuranquinones. These napththoquinones were active against trypomastigotes and epimastigotes, showing at the ultrastructural level alterations on the parasite mitochondrion, which looked severely swollen. Aditionally, these compounds caused a collapse in the mitochondrial membrane potential, reduced specifically the activity of the mitochondrial complex I-III, and induced oxygen consumption indicating an association between trypanocidal action of these compounds and mitochondrial

dysfunction, thus augmenting ROS generation and parasite death [547].

b. Cyclopalladate complex: [Pd2 (S(-)C_2,N-DMPA)$_2$ (m-DPPE)]Cl_2 was more active *in vitro* and *in vivo* against *T. cruzi* than Bz, showing low toxicity to mammalian cells. An apoptosis-like death in *T. cruzi* trypomastigote forms and causes mitochondrion disruption seen by electron microscopy. This complex applies an apoptosis-like death in *T. cruzi* trypomastigotes causing ultrastructural mitochondrion disruption [548].

c. Compounds related to furoxans and alkylnitrates. Among over a hundred furoxans, alkylnitrates and related compounds examined, a furoxan 3-sulfonyl derivative, named compound 4 was as lead compound inhibiting *T. cruzi* trypomastigotes and amastigotes, ascribing the effect to activity on mitochondrial dehydrogenases [549].

d. Diamidines and arylimidamides (AIAs). The *in vitro* and *in vivo* effects of DB766 against *T. cruzi* showed a potent activity on bloodstream trypomastigotes and amastigotes and low toxicity to mammalian cells. Fluorescent and transmission electron microscopy showed the localization of this AIA in DNA-enriched compartments inducing mitochondrial damage. *In vivo* DB766 efficiently decreased the blood and cardiac tissue parasite load and presented efficacy similar to that of Bz [550].The circular dichroism (CD) spectra using the whole kDNA was similar to those found for the conserved sequence and revealed minor groove binding. Some of the compounds displayed the maximum trypanocidal activities, such as DB766, producing low or no variation in the thermal denaturation (Tm) measurements. Nevertheless, they induced deep alterations of kDNA topology. Others, like the AIA DB1831, despite effective, showed no altered Tm and CD measurements, suggesting that the potent affinity of amidines with kDNA per se is not sufficient to cause their trypanocidal activity. This AIA and the diamidine DB1965 exhibited a strong trypanocidal *in vitro* effect trypomastigotes and amastiogtes, displaying a high selectivity index. DB1965 showed *in vivo* activity in an acute mice model similar to Bz. After treating with 20 daily consecutive doses, a combined dosage of DB1965 with Bz, although no parasitological cure was observed, resulted in parasitaemia clearance and 100% animal survival. These findings confirmed that AIAs represent encouraging new chemical entities against *T. cruzi* in therapeutic schemes using the AIA alone or in combination with other drugs, like Bz [551, 552].

e. Trifluoromethylquinoxaline N, N'-dioxides. The analysis of some derivatives containing optimal requirements for *in vitro* activity against *T. cruzi* showed that those having electron-withdrawing substituents in the positions 2-, 3-, 6-, and 7- were the most active compounds. Accordingly, mutagenicity and *in vivo* analyses and study of mechanism of action were performed with the best derivatives, demonstrating that mitochondrial dehydrogenases are involved in the anti-*T. cruzi* activity [553].

f. (2E)-N-(1,3-benzothiazol-2-yl)-3-(2,5-dimethoxyphenyl)-2-propenamide (CAD-1). This cinnamic acid derivative induced 58% of *T. cruzi* epimastigotes death; principally by apoptosis. The decrease in the transmembrane mitochondrial electrical potential in conjunction with the increase in the intracellular ROS accumulation, suggested the parasites mitochondria as the key target for CAD-1-induced death. Despite the concentration of CAD-1 used is not sufficiently low to consider it as a strong trypanocydal agent, altogether, the novel mechanism that induces *T. cruzi* death and the originality of its chemical structure, signed to CAD-1 as a lead compound that could serve as a template to obtain novel agents [554].

g. Berenil. Mitochondrial DNA (kDNA) is formed by interlocked molecules: minicircles and maxicircles. kDNA is a sign of kinetoplastids, constituting a valuable target in chemotherapeutic and biological studies. The effects of this minor-groove-binding agent that acts preferentially at the kDNA level, thus affecting ultrastructure and mitochondrial activity of *T. cruzi* epimastigotes promoted a reduction on parasite growth, but cell viability was not affected. While nuclear ultrastructure was not modified, this compound produced substantial changes in kDNA organization. It was shown that berenil prevented the minicircle decatenation of the network, thus avoiding DNA replication and ending in the presence of dyskinetoplastic cells. Moreover, berenil-treated parasites presented high levels of ROS and a slight reduction in the oxygen consumption and in the mitochondrial membrane potential. These findings revealed that this DNA-binding drug essentially affects kDNA topology and replication, reinforcing the idea that the kinetoplast symbolizes a prospective target for chemotherapy against trypanosomatids [555].

2.15. Programmed Cell Death (PCD) in *T. cruzi*.

Natural PCD occurs in *T. cruzi* epimastigotes maintained in axenic cultures [556]. Under suitable stimuli, *T. cruzi* undergoes PCD, offering interesting new therapeutic targets. It is worth mentioning that the mechanisms of the proccess has not been established yet, but the identification and modulation of molecular targets inducing PCD in *T. cruzi* may lead to new potential therapeutic approaches [557]. The cellular process known as autophagy helps to reutilize the cytoplasmic components and aged or damaged organelles, in normal conditions. The autophagic pathway has been associated in various physiological and pathological circumstances, even throughout the course of infection caused by intracellular pathogens. A lot of compounds are presently used for modulating the autophagic response. During host cell invasion, *T. cruzi*, interacts with autophagic compartments and the pre-activation of autophagy considerably rises the parasite colonization of the host cell.

a. Diterpene 5-epi-icetexone. This drug was tested on parasites synchronized with hydroxyurea at different periods of time after removal of the nucleotide in order to study the effect of this agent on growth and morphology. When the diterpene was added at 12 h after removal of hydroxyurea, the agent affected parasites growth, slightly inhibited the incorporation of thymidine, perhaps on the transition S/G2 of the cellular cycle and induced a delay in the progression of cell division as shown by transmission electron microscopy. At cytostatic dose, the compound affected *T. cruzi* cell cycle, feasibly in the transition S/G2 phase and cell division. Indeed, 5-epi-icetexone targets have not been still identified [558].

b. Ruthenium nitrosyl complexes and nitric oxide. Particularly, ruthenium (III) and (II) amines are among coordination compounds, good NO-captors-deliverers. Numerous ruthenium amine complexes were studied as NO-carriers *in vitro* and *in vivo* and their chemical and photochemical properties were evaluated. Interestingly, these nitrosyl complexes, effective in different models of cancer, can also control *T. cruzi* infection. The activation of these complexes may be chemical or photochemical, and the presence of NO in the compound can be responsible for the observed biological effects. The efficiencies of these compounds have been explained based on the specific rate constant for NO liberation from the [RuNO](2+) moiety, the quantum yield of NO release, and the [Ru(II)NO(+)](3+)/[Ru(II)NO(0)](2+) reduction potential [559].

c. Eupomatenoid-5. The neolignan isolated from the leaves of *Piper regnellii var. pallescens,* eupomatenoid-5, showed trypanocidal activity, inducing oxidative imbalance in the three parasitic forms, especially trypomastigotes. This was reflected by a decrease in the activity of TR and increase in the formation of ROS. In addition, a reduction of mitochondrial membrane potential was triggered, thus impairing the cell redox system through the production of more ROS and reactive nitrogen species. All these effects led to oxidative stress, characterized by lipid peroxidation and DNA fragmentation. These alterations are key events in the induction of parasite death through apoptosis, necrosis, and autophagy [560].

d. Iron superoxide dismutase (FeSODA). *Trypanosoma cruzi* antioxidant network composed by cytosolic and mitochondrial peroxiredoxins and TS, act as a virulence factor for strains causing Chagas disease. An augmented resistance of *T. cruzi* peroxirredoxins overexpressers to *in vivo* or *in vitro* nitroxidative stress conditions was observed. Additionally, the regulation of the levels of mitochondrial superoxide radical by iron superoxide dismutase (FeSODA) influences parasite PCD, highlighting this enzyme role in parasite survival. The role of FeSODs in *T. cruzi* PCD in Chagas disease chronic infection is under investigation yet. The possible use of the antioxidant enzymes as therapeutic targets requires further investigation [561].

e. Natural sesquiterpene lactones (STLs). At difference with the reference drugs, the two natural STLs, induced PCD in both epimastigotes and trypomastigotes. A combination of dehydroleucodine (DhL) and either Bz or Nx showed an increased effect of natural compounds and synthetic drugs on the decrease of parasite viability. DhL and helenalin (Hln) induced PCD in *T. cruzi* epimastigote and trypomastigote forms, by a different mechanism of action than the conventional drugs to kill the parasite, proposing to DhL and Hln as an interesting option for Chagas disease treatment, alone or in combination with conventional drugs [562].

f. N-oxide-containing heterocycles. The selection of these compounds was performed with *T. cruzi* epimastigotes according to the mechanism of

action and type of death. Most of the N-oxides evaluated were capable of decreasing the release of succinate and the shedding of acetate, probably by acting on mitochondria. Quinoxalines and nitrofuranes exhibited substantial mitochondrial dehydrogenase inhibition. None of the selected compounds showed a positive TUNEL assay at low concentration. However one furoxan and one benzofuroxan, showed a necrotic effect at high concentrations by microscopic assays. The use of a protease inhibitor (3-methyladenine) and studies of transmission electron microscopy suggested an autophagic phenotype for the benzofuroxan Bfx1 and the nitrofurane Nf1 and a 'BigEye' phenotype for the furoxan Fx1 [563].

g. Morita-Baylis-Hillman adducts 3-hydroxy-2-methylene-3-(4-nitrophenylpropanenitrile). 3-Hydroxy-2-methylene-3-(4-nitrophenylpropanenitrile) (MBHA3), derived from the Morita-Baylis-Hillman reaction, effectively caused a loss of viability in both epimastigote and trypomastigote forms. However, the mechanisms of parasite death elicited by MBHA3 remain unknown. *Trypanosoma cruzi* treatment with lower concentrations of MBHA3 led to alterations in the mitochondrial membrane potential and acridine orange labeling, but did not decrease the viability of epimastigotes forms, as determined by the calcein-AM/ethidium homodimer and annexin-V/propidium iodide assays. In contrast, treatment with higher concentrations of MBHA3 led to extensive plasma membrane damage, loss of mitochondrion membrane potential and DNA fragmentation suggesting that MBHA3 induced death by necrosis in a mitochondrion-dependent manner at the highest concentrations tested [564].

h. Memantine. The effect of memantine, antagonist of the glutamate receptor in the CNS of mammals, was evaluated on *T. cruzi* epimastigotes exhibiting trypanocidal effect. Besides, this compound reduced both metacyclogenesis and the parasite energy metabolism. Aditionally, memantine activated different mechanisms that drove to the apoptosis-like cell death of epimastigotes. Likewise, the interference of memantine in intracellular amastigote displayed with an IC50 value five times lower than the obtained in epimastigotes [565].

i. Difluoromethylornithine (DFMO). The complete depletion of intracellular polyamines by inhibiting ODC with DFMO blocked the induction of autophagy both in response to starvation or rapamycin treatment. This effect was linked with a reduction in the levels of two proteins essential for autophagosome formation. DFMO, acting as host cell autophagy inhibitor, blocked *T. cruzi* colonization, demonstrating that polyamines and autophagy enable parasite infection. Among autophagy inhibitors, wortmannin and 3-methyladenine, are non-specific and possibly toxic, however, DFMO is a FDA-approved drug that may be appreciated in restricting autophagy and the propagation of Chagas disease infection [566].

j. Thiazolidinones derivatives. Among a series of designed thiazolidinones, 2-[2-Phenoxy-1-(4-bromophenyl) ethylidene) hydrazono]-5-ethylthiazolidin-4-one (4h) and 2-[2-phenoxy-1-(4-phenylphenyl) ethylidene) hydrazono]-5-ethylthiazolidin-4-one (4l) were the most potent against epimastigote proliferation and trypomastigotes, while did not display host cell toxicity. Thiazolidinone 4 h was able to reduce the *in vitro* parasite burden and the blood parasitemia in mice with similar potency to Bz. These thiazolidinones did not inhibit cruzain activity. However, they exhibited strong antiparasitic activity by acting as parasiticidal agents and inducing a necrotic parasite cell death [567].

k. β-Carboline-3-carboxamide derivative. The synthetic compound N-butyl-1-(4-dimethylamino) phenyl-1,2,3,4-tetrahydro-β-carboline-3-carboxamide (C4) showed activity against epimastigotes and trypomastigotes Alterations in mitochondrial membrane potential and in the cell membrane integrity, an increase in the formation of reactive oxygen species and in phosphatidylserine exposure, a reduction of cell volume, DNA fragmentation, and the formation of lipid inclusions, suggests that mitochondria are a target of C4, and its dysfunction can lead to different pathways of cell death [568].

l. Dibenzylideneacetones. The dibenzylideneacetones A3K2A1 and A3K2A3 have strong activity against *T. cruzi*. An increased oxidant species production and a depletion of the endogenous antioxidant system revealed that A3K2A1 and A3K2A3 induced oxidative stress in the three parasitic forms, especially trypomastigotes. Parasite lipid peroxidation and DNA fragmentation, proved that the produced oxidative imbalance determined the final damage in essential *T. cruzi* structures. So, A3K2A1 and A3K2A3 in-

duced vital alterations in *T. cruzi*, leading to parasite death through the three pathways, apoptosis, autophagy, and necrosis [569].

2.16. Sialic Acids Transference

Despite trypanosomes are not capable to synthesize sialic acids, they can scavenge them from its hosts via an exclusive neuraminidase that contains trans-sialidase (TS) activity. This peculiar enzyme, developmentally regulated, is responsible for the transference of sialic acid molecules from host glycoconjugates to mucin-like acceptors located in the surface membrane from the parasite and appeared to be essential for the parasite survival and the cell invasion in the mammalian host [570, 571]. Also, TS inhibitors are considered potential trypanocidal therapeutic agents. The crystal structures of *Tc*TS alone or complexed with substrates and sialidase inhibitors showed that a significant number of amino acid residues within the active site of *Tc*TS is conserved and shared by all identified sialidases. However, crucial amino acid residue discrepancies between mammalian sialidases and the parasite TS offer an explanation for the peculiar glycotransfer enzymatic activity of *Tc*TS [572]. Some target synthetic sialylmimetics-cyclohexenephosphonate monoester compounds have exhibited auspicious inhibitory properties when assayed with parasitic or bacterial sialidases [573]. Horenstein and Parr from the University of Florida patented novel N-substituted piperidines claiming that these compounds with neuraminidase inhibitory activity could be useful for the treatment of bacterial, viral and parasitic infections caused by trypanosomes [574]. Besides, scientists from University of Alabama disclosed a bacterial sialidase inhibitor and methods of preventing and treating bacterial or trypanosomal infections [575], (Table **2**). Nevertheless, no data on the activity against the parasite enzymes was presented in both patents. Also, TS was examined as probable drug target. To this purpose, a library of substrate analogues centered on 1,4- disubstituted 1,2,3-triazole derivatives of galactose modified in the C-1 or C-6 positions were synthesized and evaluated, demonstrating to be TS acceptor substrates, despite the sugar triazoles displayed low inhibition towards *in vitro* *Tc*TS-catalyzed hydrolysis of 2'-(4-methylumbelliferyl)-alpha-d-N-acetylneuraminic acid. However, *in vitro* trypanocidal activity assays against the *T. cruzi* trypomastigotes, exposed many compounds active in a low μM range [576]. Moreover, in order to evade the infection from the host's immune system, TS transfers sialic acids from mammalian host cells to parasitic cell constituting an auspicious target for the improvement of new therapeutics to treat the disease. To begin the elucidation of the catalytic mechanism of this reaction, the formation of a long-lived covalent intermediate could be estimated and a Tyr/Glu pair could be identified as an uncommon catalytic nucleophile [577]. The characterization of a labeled TS by UV-MALDI-TOF-MS revealed that the inactivation of the enzyme happens through the establishment of a covalent bond between the Arg245 and Asp247 and the inhibitor aglycone. The contribution of Asp247 in the catalytic mechanism was demonstrated with the construction of a mutant (TSD247A) that only contained residual activity permitted by the obtainment of a more open catalytic cleft. Regarding that NeuNAcFNP is the mechanism-based inhibitor of a TS enzyme described for the first time, it represents a novel template for drug design that opens new possibilities for chemotherapy of Chagas' disease [578]. Therefore, in order to obtain ligands for *Tc*TS, a lot of α-configured C-sialosides were developed and their affinities to the immobilised enzyme were measured by surface plasmon resonance. Since Tyr (119) and Trp (312) constitute the acceptor binding region in the enzyme active site, the results showed the significance of the side chains of these residues of *Tc*TS in target oriented ligand synthesis [579].

Regarding inhibitors, the 1,2,3-triazole linked sialic acid-6-O-galactose and the sialic acid-galactopyranoside displayed elevated *Tc*TS inhibitory activity, whereas only the former exhibited significant trypanocidal activity. The former was highlighted as a prototype for further design of novel neoglycoconjugates against Chagas' disease [580]. In contrast to the general hydrolases, *Tc*TS showed outstanding regio- and stereoselectivity as well as high yields in transfer reactions. In detail, the preparative use by chemoenzymatic syntheses with *Tc*TS are outlined on the design of modified donor and acceptor substrates. On the other hand, activities to develop *Tc*TS inhibitors are based on donor- and acceptor- modifications and on some completely different structures [581].

a. Macrocyclic inhibitor for neuraminidases (NAs). A macrocyclic mechanism-based inhibitor for NAs composed by a 2 difluoromethylphenyl

aglycone and a linker between the aglycone and C-9 positions of sialic acid was synthesized. The macrocyclic structure was deliberately designed to keep the aglycone moiety in the active site of the NA after cleavage of the glycoside bond. While studies with some NAs treated with a similar acyclic derivative, showed that the irreversible inhibition was restricted, this new macrocyclic compound proceeded as an irreversible inhibitor for the same NAs. *Trypanosoma cruzi*, human, and numerous NAs, were inhibited irreversibly by this macrocyclic compound. This inhibition resulted inversely proportional to the k (cat) of the target NA, in contrast to common k (cat) inhibitors [582]. TS presents no analogy with the human counterpart converting to this enzyme in an exciting drug target to fight against the parasite. Although several compounds have been checked against TS activity, no potent inhibitors have been still recognized [583].

b. Glycosyl diketopiperazines (DKPs). Glycosyl DKPs cyclo [Asp-(αGalNAc) Ser] 3 and cyclo [Asp-(αGalNAc) Thr] 4 were synthesized and biologically evaluated for the improvement of new trypanocidal agents and in particular, *T. cruzi* inhibitors. While the DKPs 3 and 4 revealed significant trypanocidal effects with IC50 values in the μM order, glycosyl amino acids 1 and 2 exhibited healthier inhibition of TS than the equivalent DKPs [584].

2.17. Biosynthesis of Lipids

a. Alkyl-Lysophospholipids (ALPs). ALPs constitute another set of auspicious compounds with activity against *in vitro* and *in vivo T. cruzi* proliferation and differentiation. First, these analogues of lisophospholipids were designed and synthesized as potential immunomodulator agents, progressing as antitumoral and antileukaemial drugs [585]. In contrast with the conditions in the vertebrate host, where the CDP-choline pathway preponderates, ALPs anti-*T. cruzi* activity has been associated with a selective blockage of parasite phosphatidyl-choline (PC) biosynthesis including the transmethylation pathway. However, the mechanism related with antiparasitic activity is not known yet. ALPs showed respectable oral activity and low toxicity [586]. Besides, lysophospholipid analogues (LPAs) formerly established as anti-cancer agents, have also presented substantial *in vitro* and *in vivo* activity against *T. cruzi.* Although the lipid synthesis in *T. cruzi* and cancer cells is inhibited by LPAs, the activity showed to be about 20-fold superior against the parasite [587]. A report described the antiproliferative synergy of LPAs with ketoconazole against *T. cruzi* epimastigotes and amastigotes. Similarly to the use of edelfosine or ketoconazole alone which induced morphological alterations in parasites plasma membrane and reservosomes, the combination of the drugs, probably by interfering with lipid metabolism also produced severe mitochondrial damage, and formation of multinucleated structures [588]. The three developmental stages of the parasite suffered alterations in the plasma membrane when were treated with LPAs. In epimastigotes, the effects of these compounds affected the mitochondrion proposing to these organelles as potential targets of these analogues [589]. In addition, the interference of LPAs in the lipid biosynthesis alter the quantity of phospholipids and sterols, and so the membrane physical properties [590]. On the other hand, taking into account that the natural antibiotic thiolactomycin (TLM) is an inhibitor of Type II fatty acid synthase but not of Type I fatty acid synthase in mammals, a series of analogues of TLM have been evaluated showing similar or higher inhibition than TLM and some of them displayed activity against *T. cruzi* [591].

b. Glycosphingolipids (GSLs). In the last years, studies on lipid metabolism have focused not only in basic biology but also in using them for chemotherapeutic purposes. In eukaryotic cells, glycosphingolipids (GSLs) are ubiquitous. However, very little information about their role in parasites has been reported. Posaconazole, which acts as described in section 2.2.1. by blocking sterol biosynthesis, is the best advanced novel anti-Chagas disease drug prototype, however, did not completely confirm its original potential in a Phase II clinical trial for chronic Chagas' disease. *In vitro* assays, demonstrated that posaconazole is extremely active against *T. cruzi* in addition to be very well tolerated in clinical trials. Thus, combination therapies might provide a point of view categorically promising. In this sense, evidences suggested the *in vivo* functional interactions between sterols and sphingolipids, proposing that the combination of sterol and sphingolipid biosynthesis inhibitors might allow advancing in drug development against Chagas' disease [592].

c. Glycosylphosphatidylinositol (GPI) biosynthetic pathway. A number of proteins are anchored to the membrane through GPI molecules that are crucial for parasite virulence and participate in host immune responses. Thus, *T. cruzi* genes related to the biosynthesis of GPI anchors represent probable novel targets for the progress of improved therapies against Chagas disease. *In silico* analysis of the *T. cruzi* genome allowed the identification of eighteen genes codifying for proteins of the GPI biosynthetic pathway and the inositolphosphorylceramide (IPC) synthase gene. The two genes were constitutively expressed in all the parasite stages. *Trypanosoma cruzi* genes *Tc*DPM1, *Tc*GPI10 and *Tc*GPI12 complemented yeast mutants in GPI biosynthesis. Unsuccessfully, efforts for generating *T. cruzi* knockouts for the three genes failed, suggesting that GPI might be an essential parasite constituent. *Trypanosoma cruzi* sequences codifying for components of the GPI biosynthetic pathway were analyzed indicating that these are essential genes participating in crucial characteristics of host-parasite interactions [593].

d. Fatty acid acylation. The addition of the fatty acid moieties myristate and palmitate to proteins is essential for the survival, growth, and infectivity of Trypanosomatids. Myristoylation and palmitoylation processes are critical for parasite growth, targeting, localization, and specific functions of some proteins. A single N-myristoyltransferase (NMT) and multiple palmitoyl acyltransferases are present in trypanosomatids, and these enzymes and their protein targets have not been still fully characterized. Total inhibition of each process drives to cell death in trypanosomatids, and the genetic ablation of NMT compromise parasite virulence. Likewise, NMT inhibitors efficiently cure *T. brucei* infection in rodents. Consequently, protein acylation signifies a promising target for novel trypanocidal drugs [594].

3. ANTI-*T. CRUZI* COMPOUNDS WITHOUT ASSIGNED SPECIFIC TARGET

In relation to the chemical strucutres of the compounds mentioned in this item please refer to Table (**9**).

a. Megazol derivatives. From an *in vitro* screening of a series of 1,3,4-thiadiazole-2-arylhydrazone derivatives of megazol against trypomastigotes, eight compounds were chosen for *in vivo* studies. The two compounds derived from the reaction of megazol with 4-hydroxybenzaldehyde and 4-bromobenzaldehyde showed a significant decrease in the measured levels of parasitemia and in the mortality rates (Table **9**). The dihydroxybenzaldehyde derivative, although be 2-fold more strong *in vitro* than megazol, showed no *in vivo* effect [595].

b. Fenarimol and analogs. The non-toxic fungicide fenarimol was activity *in vivo* in a murine model, inhibiting blood parasitemia to almost undetectable levels [596]. Optimization of this drug led to the synthesis of 1-[phenyl(pyridin-3-yl)methyl] piperazinyl analogues containing amide, sulfonamide, carbamate/carbonate and aryl moieties, several of them displaying low nM activities on *T. cruzi*. Later, two analogues showed a high curative rate in a mouse model of acute *T. cruz*i infection (Table **9**) [597].

c. Benzoxaboroles. Benzoxaboroles were firstly reported as antifungal, antibacterial and anti-inflammatory activities, determining that boron-based drugs were capable to display striking properties and activities contrary to parasites responsible for neglected tropical diseases. Many biotechnology companies have effectively identifyed to this new type of boron-based drugs, as talented for the mentioned diseases. Among exclusive properties of these agents, its capacity to reversibly interact with biochemical targets through an empty p-orbital, is central for their triumph as prototypes. Main features essential for oral absorption; metabolic stability and low toxicity are required for their progression to clinical trials in line with the physicochemical and pharmacokinetic properties shown by these compounds [598]**.** The compound concentration and incubation time needed to achieve maximum efficacy *in vitro* between the oxaborole AN4169 was compared with the broad class of nitroherocyclic compounds: benznidazole, nifurtimox, and fexinidazole sulfone, and four ergosterol biosynthesis inhibitors – posaconazole, ravuconazole, EPL-BS967 and EPL-BS1246. The oxaborole and the nitroheterocyclics, although less potents, showed broad efficacy and variable responses against all *T. cruzi* tested and were rapidly trypanocidal, whereas ergosterol biosynthesis inhibitors showed variable activity that was both compound- and strain-specific, and were unable to eradicate intracellular

infection at the most efficacious concentrations [599].

d. Imidazo[4,5-c][1,2,6]thiadiazine 2,2-dioxides derivatives. Researchers from the Consejo Superior de Investigación, University of Uruguay, have patented a new family of anti-Chagas disease derivatives from imidazo[4,5-c][1,2,6] thiadiazine 2,2-dioxides (Table **9**) to be used as medicament or pharmaceutical composition for the treatment of parasitic diseases, particularly Chagas disease [600].

e. Hybrid furoxanyl N-acylhydrazone derivatives. Several prototypes and drug candidates for neglected diseases contain a repeated functional N-acylhydrazone moiety. In parallel, the furoxan system has been investigated as pharmacophore Chagas disease. Forty hybrid furoxanyl N-acylhydrazones and their activity on *T. cruzi* were designed and synthetized. Among them, four derivatives exhibited excellent to good selectivity indexes against the *T. cruzi* and other microorganisms. The derivative furoxanylN-acylhydrazone (E)-2-methyl-N'-(4-phenyl-3-furoxanylmethylidene)-4H-imidazo[1,2-a]pyridine-3-carbohydrazide 15 resulted ten-fold more strong against *T. cruzi* amastigotes than Nx. The satisfactory stability, in a virtual biological system (Table **9**) and the lack of mutagenicity of some derivatives allowed the authors proposing them as prototypes for future pre-clinical studies [601].

f. Nitro-heterocyclic derivatives.

f.1. Nitrofuroxazide derivatives. The *in vitro* anti-*T. cruzi* activity assays of nifuroxazide analogues, such as 5-nitro-2-furfuryliden and 5-nitro-2-theniliden derivatives, were performed and compared to Bz. A molecular modeling approach was also carried out to relate the lipophilicity potential property with the biological activity data. The 5-nitro-2-furfuryliden derivatives presented better pharmacological profile than the 5-nitro-2-theniliden analogues and the design of new analogues is required for achieving optimum lipophilicity [602]. In this sense, 5-R1-2[(NR2)-furfuryliden, toleniliden) semicarbazones and thiosemicarbazone and its derivatives, the obtention methods and their use for producing compounds for treatment of Chagas disease were patented by researchers from the Universidad Nacional Autónoma de México [603].

f.2. 3-Nitro-1H-1,2,4-triazole-based derivatives. A series of novel nitro (triazole/imidazole)-based heteroarylamides/sulfonamides, (including 3-, 2- and 4-nitroimidazoles-based compounds) was synthesized and the 2-nitroimidazoles showed a discrete active against *T. cruzi*, while the 4-nitroimidazoles were mostly inactive. The strongest compound generated IC50 values lower than 1 μM and up to more than 1400-fold selectivity towards the parasite (Table **9**). From the detailed SARs, the 3-nitrotriazole-based chlorinated thiophene/benzothiophene sulfonamides/amides were the best active trypanocidal compounds, displaying up to 14-fold greater potency against *T. cruzi* as compared to Bz [604] and presented excellent performance in an acute model of infection [605].

g. Sesquiterpene lactone type furan-heliangolides. Dichloromethane and methanol crude extracts obtained from leaves plus inflorescences *of Lychnophora pohlii* showed activity against *T. cruzi* trypomastigotes. The bioassay-guided fractionation of the extracts yielded seven active compounds: the sesquiterpene lactones lychnopholide, centratherin, goyazensolide and 15-desoxygoyazensolide in the dichloromethane extract, and caffeic acid and the flavonoids luteolin and vicenin-2 in the methanol extract. One active caffeoyl quinic acid derivative was isolated from the inactive hydroalcoholic extract. Chemically, this type of plant contains sesquiterpene lactone type furan heliangolides among other derivatives [606]. Researchers from Universidad Federal from Ouro Preto and Fundacao Fapemig (Brazil) patented pharmaceutical compositions containing sesquiterpene lactones, type furan heliangolides. This patent disclosed compounds with pharmacological activity and effectiveness for pharmaceutical using for humans and animals, for treating diseases caused by naturally resistant parasites, such as *T. cruzi* among others. Furan heliangolide sesquiterpenoid compounds activity has been significantly *in vivo* and *in vitro* tested. Lichnofolide (LYC) presented the best results for the treatment of infection by *T. cruzi* of mice infected with different strains, evidencing for the first time the potential of this compound for *in vivo* treatment of parasite strains with different sensitivity profiles to drugs usually used for Chagas disease treatment [607]. *In vivo* activity and the effects against *T. cruzi* of the new drug LYC, loaded in nanocapsules (NC), were compared with those from Bz. Treatment with free LYC, LYC-poly-ε-

caprolactone, and LYC-poly(lactic acid)-co-polyethylene glycol of infected mice in the acute phase or Bz solution were carried out. As expected, although there were no mice cure, free LYC decreased the parasitemia and enhanced mice survival, confirming LYC as a potential novel agent for Chagas disease treatment [608].

h. Diaminophenothiazinium compounds. Studies based on Alzheimer's disease and related tauopathies established that the diaminophenothiazine compounds, such as methylthioninium were proposed as potential chemotherapeutic agents [609]. Interestingly, a patent pertaining in general to the chemical synthesis and purification area, and specifically to the methods of synthesis and/or purification of some 3,7-diamino phenothiazin-5-ium compounds as diaminophenothiazinium compounds, including methylthioninium chloride, commonly known as methylene blue, was presented by Wista Lab Ltd. In addition, the description of the purification method, compositions, their use in methods for inactivating pathogens and those of medical treatment, prophylaxis, diagnosis of tauopathies, different types of infections including Chagas' disease among them, were presented [610].

i. Imido-substituted 1,4-naphthoquinone derivatives. Eleven imido-naphthoquinone analogs IMDNQ1-IMDNQ11 showed stronger activity on *T. cruzi* in comparison with Nx. Studies in a murine cell line exposed that IMDNQ1, IMDNQ2, IMDNQ3, and IMDNQ10 exhibited higher selectivity than Nx [611]. Researchers from Howard University, USA, have recently provided methods capable to inhibit *T. cruzi* proliferation with novel imido-substituted-1,4-naphthoquinones, proposing that their administration could offer prophylaxis or treatment of Chagas disease [612].

j. Aryloxi compounds

j.1. Aryloxi-quinones. Twenty seven novel synthetic aryloxy-quinones were evaluated against *T cruzi* epimastigotes, and compared to Nx. 2-Phenoxy-naphthoquinone (Table **9** - **3b**) exhibited a significant inhibitory activity and a great selectivity. The most active compounds of each series showed redox properties as determined by cyclic voltammetry commonly used to obtain electrochemical parameters for biological studies with quinones. No correlation was found between the potential of reduction (Epc) calculated and the trypanocidal effect. The design of a pharmacophore recognized the strongest and more selective compounds and the 3D-QSAR equation could be useful for the prediction of new designed trypanocidal compounds [613].

j.2. Aryloxy-indole-4,9-diones. The synthesis and *in vitro* evaluation against *T. cruzi* epimastigotes of novel indole-4, 9-dione and their phenoxy derivatives presented them as selective as and extremely stronger than Nx. It was interesting that phenoxyindole-4, 9-dione 9d displayed excellent nM inhibitory activity and high selectivity. *In silico* studies using MOE program were addressed to generate a preliminary pharmacophore model [614].

k. N, N'-Squaramides. When combined with amine and carboxylic groups, squaramide compounds have increased solubility and therefore make suitable therapeutic agents. A group of Lipinski's rules of five compliant squaramides as candidates for treating Chagas disease was introduced. The *in vivo* studies confirmed the positive expectations arising from the preliminary *in vitro* studies, revealing compound 17 (Table **9**) to be the most effective for both acute and chronic phases. Activity, stability, low cost of starting materials, and straightforward synthesis turn amino squaramides in appropriate molecules for the development of an affordable anti-Chagas disease agent [615]. Researchers from the Universities of Granada and Illes Balears, Spain have patented antiparasitic activity of squaramide. The synthesis and use of squaramide for the treatment of diseases of parasitic origin, such as Chagas disease, have been related [616].

l. Pradimicin derivatives. Pradimicins are a single type of non-peptidic carbohydrate-binding compounds capable of blocking HIV infection due to an efficient binding to glycans of the HIV-1 envelope gp120 in the presence of Ca^{2+}. The pradimicin-cation complexes are inept to further coordinate with the gp120 glycans. Accordingly, in order to acquire antiviral activity, a few cations are able to bind to pradimicin to form a dimeric complex and consequently coordinate the interaction between pradimicin/cation and the glycans of gp120 [617]. The mechanism of action of the anti-HIV antibiotic, pradimicin A, has been early studied using cell-free and cell to cell HIV infection systems. Researchers from the University of Leuven Kath, Belgium and the Consejo Superior de Investigación, Japan, Toyama Prefecture have patented new pradimicin derivatives for the diseases

caused by kinetoplastids treatment. The patent described new pradimicin derivatives for the treatment of deseases caused by Kinetoplastids, including Chagas disease [618].

m. Arjunolic acid and derivatives. The triterpene arjunolic acid reduced the *in vitro T. cruzi* epimastigote proliferation. Electron microscopy analysis revealed remarkable effects on the parasite surface and architecture, mainly restricted or originated at the parasite peripheral cytoplasm, including the cytoskeleton membrane linkage, inferring that the compound targeted primarily the lipid bilayer. Therefore, a synthetic modification was performed to increase the molecule lipophilicity and thus the membrane permeability. The methyl ester of arjulonic acid and the tri-acetylated derivatives had potentiated trypanocidal activity. Both derivatives were able to produce remarkable parasite ultrastructural alterations in Golgi apparatus and the endocytic/autophagic pathway [619].

n. Abietic acid derivatives. The effectiveness of a group of chemically stable and well characterised abietic acid derivatives was assayed *in vitro* and *in vivo*. *In vitro* results showed that some compounds showed low toxicity against Vero cells combined with high efficacy against *T. cruzi*. Further *in vivo* studies on mice models confirmed the expectations of improvements in infected mice. The morphological alterations found in treated parasites confirmed broad damage and energetic metabolism disturbances. The demonstrated *in vivo* activity and low toxicity, the lack of synthetic complexity, placed these abietic acid derivatives as candidates for the treatment of Chagas disease [620].

o. Quinoxaline derivatives. Forty novel quinoxaline 1, 4-di-N-oxide derivatives assayed and two carboxylic acid quinoxaline 4-di-N-oxides (CAQDOs), compounds 12 and 22 (Table **9**) exhibited IC50 values for epimastigotes in the same order as Nx [621]. Aza-thiaheterocycles, analogues of naftifine, were investigated as trypanocidal agents, evidencing that these pharmacophores act through oxidative stress. Novel quinoxaline 1,4-dioxides were evaluated as possible SBIs [622]. Two compounds (22 and 23) (Table **9**) revealed outstanding parasite/mammal selectivity indexes. The analysis of the free sterols from parasites incubated with these compounds showed that they were capable to accumulate squalene, suggesting that the inhibition of sterol biosynthesis is not involved in the anti-*T. cruzi* mechanism of action. In another work, 3-Chloro-7-methoxy-2-(methylsulfonyl) quinoxaline showed auspicious *in vitro* effect against epimastigotes and trypomastigotes, alone or combined with Bz. The ultrastrucutural alterations observed suggest an autophagic-like cell death [623]. The synthesis and antitrypanosomal properties of 46 novel 2,3-disubstituted quinoxaline derivatives were assayed in with the three evolutive forms of *T. cruzi*. Several compounds led to IC50 values at low micromolar range, and their activity was directly associated to the methylsulfoxyl, methylsulfonyl, and amine groups as well as to the presence of chlorine or bromine in these quinoxaline derivatives (Table **9**) [624].

Quinoxaline derivatives which are selective against *T. cruzi* and do not cause mutagenic effects were patented. Particularly, derivatives of 3-trifluoromethylquinoxaline 1,4-dioxide with ester or amide functionality in the 2 position, presenting a selective mechanism of action showed to be different to the drugs used in therapy. Their lipophilicity is suitable for use in any pharmaceutical formulation. The methods for preparing alleged compounds by reacting a benzofuroxan and a trifluoromethyl were recorded. The invention also related to pharmaceutical formulations for treating patients with Chagas disease [625, 626].

p. Clofazimine, benidipine and saquinavir. The approved drugs clofazimine, benidipine and saquinavir (Table **9**) were identified by computer-aided protocol as potential trypanocidal compounds and their effects at biochemical and cellular levels on the different parasite forms were tested. The potential of computer-guided drug relocating to connect and improve drug discovery and preclinical development was used; rational rules to choice which among repositioned prototypes should progress to be an investigational drug and provide a new vision on clofazimine and benidipine as candidate for the treatment of Chagas disease was evaluated. The repositioning of these permitted drugs as potential novel agents for Chagas disease treatment, integrated computer-aided drug screening and biochemical, cellular and preclinical tests. Finding other therapeutic indications for known drugs, and reports on drug repositioning oriented to Chagas disease, with a focus on computer-guided drug repositioning campaigns were reviewed [627, 628].

q. N'-[(5-nitrofuran-2-yl) methylene] substituted hydrazides. Focused on the nitroheterocyclic drug nifuroxazide (NF), a set of twenty one compounds were designed in order to improve activity against *T. cruzi*. Aimed to support drug-likeness, the designing Lipinski's rules were taken into account. The group of N'-[(5-nitrofuran-2-yl) methylene] substituted hydrazides were tested against the three lineages *Tc*I, *Tc*II and, *Tc*V, which are prevalent in human patients. Most of the derivatives, exhibited improved trypanocidal activity against the three strains in comparison with Bz. In the Y strain (TcI), the compounds showed to be 62% more active than Nx. N'-((5-nitrofuran-2-yl) methylene) biphenyl-4-carbohydrazide (C20), was the most active compound for the three strains tested (Table **9**). Several compounds showed high selectivity indices in cytotoxicity assays. Mainly, studies on the topological, steric and electronic properties of the set of investigated compounds, helped to the authors in the designing and synthesis of hopeful drugs against Chagas disease [629].

r. Quaternary n-(halomethyl) ammonium salts. The use of quaternary halogenated ammonium salts for treating parasitic diseases was described in patents from researcher of the University of Caldas (US), Trustees of Illinois State University Board of Colombia and University of Antioquia. These compounds, in particular those with a terminal arylalkenyl or diarylalkenyl moiety, are shown to inhibit the growth of *L. pamensis* parasites, a known causative agent of leishmaniasis disease. In addition, this series of compounds are also applied against Chagas disease and other parasitic diseases [630].

s. Small molecules naphthoquinone- and phthalimide-based lipocations. The synthesis and evaluation of cations of hydroxy-substituted 1,4-naphthoquinones were first tested as antiplasmodial agents. The atovaquone analogues resulted inactive as antagonists of parasite growth, due to the ionization of the acidic hydroxyl moiety. In further studies, the synthesis and evaluation of phosphonium lipocations for inhibiting *T. cruzi* development were performed. In *in vitro* studies, phthalimides and 1, 4-naphthoquinone-based lipocations were active at low μM concentrations against *T. cruzi* [631]. A patent related with small molecules naphthoquinone- and phthalimide-based lipocations, claiming that these compounds were useful for the treatment or prevention of anti-parasitic diseases including Chagas disease was presented by a researcher from the Universty of Georgia, USA [632].

t. Macrocycles derivatives. A library focused on C2-substituted-1,4,7,10-tetraazacyclododecanes was synthesized and the compounds were assayed for their trypanocidal activity. Numerous C2-substituted polyazamacrocycles compounds showed important *in vitro* activity and were selectively active against the parasites as compared to human cells [633]. Three pyrazole-containing macrobicyclic polyamines 1, 2 (N-methyl-substituted) and 3 (N-benzyl-substituted) (Table **9**) were active *in vitro* and less toxic to mammalian cells than Bz. *In vivo* compound 1 was effective in the chronic phase. In addition, compound 2 was a strong inhibitor of Fe-SOD in trypanosomatids, while its effect on human CuZn-SOD was reduced. Molecular modelling suggested that compound 2 could inactivate Fe-SOD due to a sterically favoured improved capacity to interact with the H-bonding net that supports the enzyme`s antioxidant characteristics [634]. Ten derivatives of a family of aza-scorpiand-like macrocycles were active *in vitro* and less toxic to mammalian cells than Bz. *In vivo* compound 4 (Table **9**) was the most active, mainly in the chronic phase. All these compounds showed a remarkable inhibition of *Tc*Fe-SOD while a negligible effect on of human CuZn-SOD was observed [635]. Researchers from the University of Granada and Valencia, Spain, contributed with a patent regarding to scorpion-tail-like macrocyclic compounds and to the use as antiparasitic drugs. This invention describes to these compounds that are so named owing to the particular form of the chemical structure formed by a macrocyclic body and a hanging arm where various radicals can be substituted. The present invention considers their use as drugs, particularly in the treatment of parasitic diseases, preferably Chagas disease [636]. Many macrocycles exhibiting biological activities as antitrypanosomal and/or antileishmanial were isolated, synthetized, biologically evaluated and detailed in the last decade [637].

u. 3-Phenylthio-nor-β-lapachone derivatives. A one-step reaction, simple and efficient, synthesized novel nor-β-lapachone derivatives tethered with phenylthio groups at position 3 of the furan ring. Searching for a novel prototype with high trypanocidal activity, the compounds were

screened on bloodstream trypomastigotes of *T. cruzi*. A broad range of activity was displayed by the novel compounds showing IC50 values in the microM order, being four of them superior than Bz. Compound 13b (Table **9**) was the the most active, being 11 times more active than Bz and the less toxic derivative to heart muscle cells [638].

v. Bis and mono spiro-indole derivatives. The synthesis of a series of new spiro[indole thiazolidine]spiro[indole-pyran] derivatives was developed from N-(bromoalkyl)indol-2,3-diones through monospiro-bisindole intermediates, being the two indole nuclei connected via a N-(CH(2))(n)-N linker [639]. The spiro-indole-pyrrolidine ring system is a structural moiety frequently found in a lot of biologically and pharmacologically key alkaloids. The derivatives of spirooxindole ring systems are used as antimicrobial and antitumour agents. The recently discovered small-molecule MDM2 inhibitor MI-219 and its analogues are in advanced preclinical development as cancer therapeutics [640]. It is worth noting that the discovery, presented by Novartis AG, relates spiro-indole derivatives for the prevention and treatment of infections caused trypanosomatids, including *T. cruzi*. The patent also relates to pharmaceutical compound compositions, as well as processes for their preparation [641].

w. Thiazolidine and imidazolidine compounds. A series of compounds was modified at the thiazolidinic ring and the 2-iminothiazolidin-4-one derivative (18) (Table **9**) was identified as a potent inhibitor of Cz, of epimastigotes and trypomastigotes, without toxicity to mammalian cells. In sequence the thiazolidinic ring was substituted by a thiazolic one, and it was observed that the news compound did not inhibit the enzyme, but they exhibited trypanocidal effects [642]. Another study showed that thiazolidine LPSF SF29 was capable to inhibit epimastigotes and amastigotes growth, produce *T. cruzi* trypomastigote lysis, conducing to protozoan death, inducing the appearance of autophagosomes among the ultrastructural abnormalities [643]. Regarding these compounds, a patent related with thiazolidine and the proline analogue imidazolidine derivatives compounds has been presented by researchers from Federal University from Pernambuco (Brazil). The synthesis and structural identification of new thiazolidine and imidazolidine molecules with trypanocidal activity and the therapeutic use as drugs against Chagas disease is revealed in the present invention [644].

x. 5-Ephenylethenylbenzofuroxan derivatives. Among 5- ephenylethenylbenzofuroxan derivatives, although its oral bioavailability was affected by the crystallization process, 5PhEBfx was described as an excellent anti-Chagas drug candidate. Both solid forms, a thin yellow powder (5PhEBfx-Y) and orange needles (5PhEBfx-O) showed variable *in vivo* activity. In order to correlate the solid-state properties with the variable bioavailability of 5PhEBfx, vibrational spectroscopy, X-ray powder diffraction, differential scanning calorimetry, optical and electron scanning microscopies showed that 5PhEBfx-Y presented and consequently higher bioavailability when compared with 5PhEBfx-O [645].

z. Benzo[a]phenoxanthin compound. A medicinal composition which contains benzo[a] phenoxanthin compounds as the active component for prevention or treatment of protozoal diseases may be considered as agent for treating and/or preventing protozoan infections including Chagas' disease among others. A patent was presented by researchers from Hoshi University; Synstar Japan Co Ltd. [646].

aa. Amide-contaning compounds

*aa.1. Amide containing thiazoles***.** Novel amide-containing thiazoles were evaluated against *T. cruzi*. The lead amide-containing thiazol derivative (Table **9**) showed strong *in vitro* activity on the three parasite forms, absence of both *in vitro* mutagenic and *in vivo* clastogenic effects, as well as *in vivo* tolerance. This compound exhibited low mortality and blocked mice parasitemia similarly to Bz [647].

aa.2. Arylimidamides. The activity of new AIAs against *T. cruzi* infection was *in vitro* and *in vivo* evaluated. By using up to 32 microM, none of the compounds induced a loss in cellular viability. Two of them, 18SAB075 (Table **9**) and 16DAP002A, displayed respectable *in vitro* activity against different parasite strains against amastigotes and trypomastigotes. Particularly, 18SAB075 decreased parasitemia levels up to 50 % and showed 40% protection against mortality, being however less effective than Bz [648].

ab. Thiosemicarbazones derivatives

ab.1.5-[(Trifluoromethyl) phenylthio]-2-furaldehyde derivatives. Thiosemicarbazones deriva-

tives of 5-[(trifluoromethyl) phenylthio]-2-furaldehyde compounds showed a strong effect on epimastigotes, similar to Nx and Bz, and compound 4 (Table **9**) showed the highest activity. The presence of the nitro group in the molecule seemed to be responsible for the improved activity [649]. In order to facilitate a better understanding of the likenesses and dissimilarities concerning apicomplexa and trypanosomatids organisms, the Malaria Box screening and triaging of the identified hits against some kinetoplastids was performed. The characteristics and *in vitro* and *in vivo* drug assays of the most promising active compounds were described [650].

ab.2. Metal complexes with thiosemicarbazone derivatives. The complexes: (1) [Sb (2Ac4oClPh) Cl2], (2) [Sb(2Ac4oFPh)Cl2], (3) [Sb(2Ac4*o*NO-2Ph)Cl2], (4) [Sb(2Bz4oClPh)Cl2], (5) [Sb(2Bz-4oFP)Cl2] and (6) [Sb(2Bz4oNO2Ph)Cl2] were obtained with 2-acetylpyridine-N(4)-orthochlorophenyl thiosemicarbazone (H2Ac4oClPh) and its analogues: N(4)-ortho-fluor (H2Ac4oFPh), N(4)-ortho-nitro (H2Ac4oNO2Ph), and with the resultant 2-benzoylpyridine-derived thiosemicarbazones Sb(H2Bz4oClPh, H2Bz4oFPh, H2Bz4oNO2Ph). The obtained compounds were described as outstanding *T. cruzi* growth inhibitors. H2Bz4oClPh and the complexes 1 and 4 showed the best trypanocidal activity. Although after coordination of H2Ac4oClPh to antimony (III) in compound 1, the best values of therapeutic index were found for H2Bz4oClPh and H2Ac4oNO2Ph. The cytotoxicities and physico-chemical properties were correlated. However, no association between the anti-*T. cruzi* activity and the physico-chemical parameters was got by SAR studies [651, 652].

a.c. Terpenoid derivatives. The activity against *T. cruzi* of four terpenoid derivatives was evaluated on trypomastigotes and intracellular amastigotes. Two of them were very active, even-more effective than Bz with low toxicity for the host cells. The ultrastructural analysis of terpenoid-treated-epimastigotes displayed ultrastructural abnormalities and some metabolic changes occurred probably in succinate and acetate production, feasibly produced by the alteration of the enzymes from mitochondrial sugar metabolism. The use of these compounds showed a significant low parasitic load with respect to Bz and the diminishing of the antibodies specific for *T. cruzi* levels during the chronic stage [653].

a.d. Allopurinol derivatives. C6-alkyl (2a-c) and N-acyl (3a-c) derivatives of allopurinol were synthesized in good yields and their structures were characterized. Only 2a, 2b and 3c showed inhibitory activity against epimastigotes, with 3c (Table **9**) exhibiting an IC50 value similar to that of allopurinol and no toxicity for mammalian cells. Pharmaceutical physicochemical properties were determined by using Lipinski's rules, polar surface area and molecular rigidity. The results demonstrated that the studied derivatives had optimal properties for bioavailability and oral absorption. Micellar Liquid Chromatography was used as the analytical method, resulting fully validated according to the FDA guidelines and shown to be a suitable, sensitive and simple method for routine analysis of derivatives stability [654].

4. NOVEL ANTI *T. CRUZI* ACTIVITIES WITH KNOWN SINGLE OR MULTI TARGET

In relation to the chemical strucutres of the compounds mentioned in this item please refer to Table (**10**).

a. Indazoles

a.1. Nitroindazoles. The indazoles resulted to be strong *T. cruzi* growth inhibitors, capable to lead ROS formation and TR inhibition. The trypanocidal properties of indazoles, their involvement in the biological properties and the mode of action were reported [655]. Among a series of 5-nitroindazoles many of them were active against epimastigotes and trypomastigotes with low toxicity on macrophages. Three derivatives, 1, 10 and 12, displayed an oxidative stress-mediated mechanism of action on the proliferative form of *T. cruzi*. Two compounds (1 and 2) were submitted to *in vivo* assays in an acute model of Chagas' disease, with survival of all the treated animals [656]. 3-Alkoxy-1-alkyl- and 3-alkoxy-1-(ω-aminoalkyl)-5-nitroindazoles exhibited *in vitro* activities against the three parasite forms comparable or higher than those of Bz, with low cytotoxicity to host cells. *In vivo* two derivatives (1 and 2) were assayed in models of acute and chronic phase, reducing parasitemia and levels of anti-*T. cruzi* antibodies, respectively [657].

a.2. Fused tri- and tetracyclic indazoles and analogues. A series of fused tri- and tetracyclic

Table 9. Anti-Chagas disease compounds without assigned specific target.

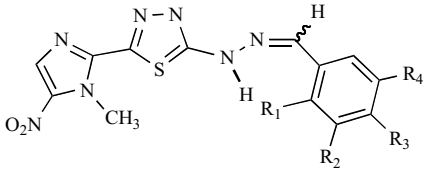
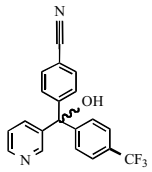
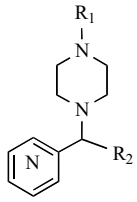
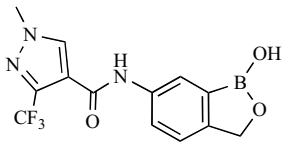
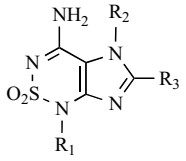
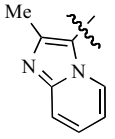
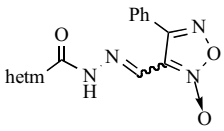
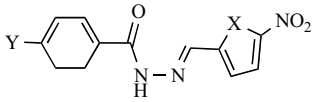
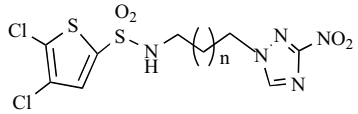
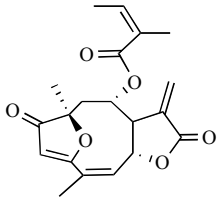
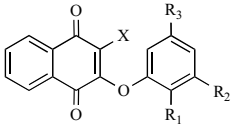
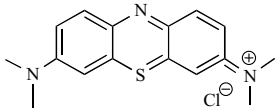
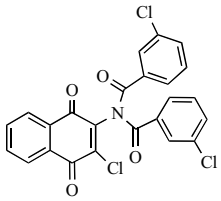
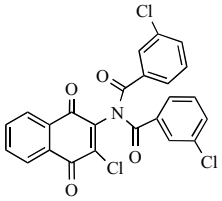
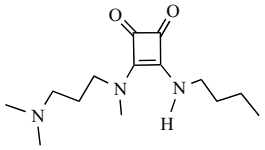
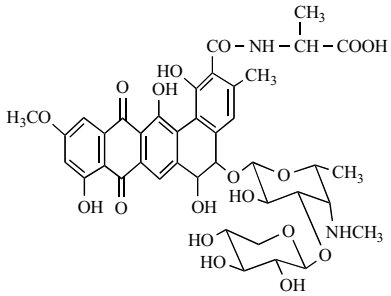
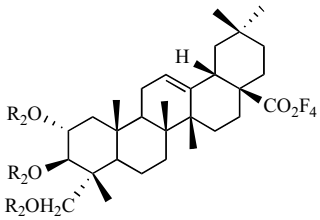
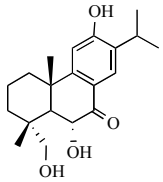
a-Megazol derivatives.	**b-Fenarimol analogs**	**c-Benzoxaboroles**
	 	
[595]	**[596, 597]**	**[598]**
d-Imidazo[4,5-c][1,2,6] thiadiazine 2,2-dioxides	**e-Hybrid furoxanyl N-acylhydrazone derivatives**	**f-.1- 5-Nitrofuroxazide derivatives**
	 	
	Hetm **Compound 15**	**X=O, furfuryliden deriv.** **X=S, thiophyliden derv.**
[600]	**[601]**	**[602, 603]**
f-2.-ii)-3-Nitro-1H-1,2,4-triazole-based arylamides and arylsulfonamides.	**g-Sesquiterpene lactone type furanoheliangolides**	**h-Diaminophenothiazinium compounds**
		
Compound b X=H; R1=R3=H; R2=NO_2		**MTC**
[604, 605]	**[606-608]**	**[609, 610]**
i-Imido-substituted 1,4-naphthoquinone derivatives	**j.1-Aryloxy-quinones** **j.2- Aryloxy-indole-4,9-diones**	**k-N,N'-Squaramides**
	 	
IMDNQ10	**j.1- Compound 3b** **j.2- Compound 9d, R: OMe**	**Compound 17**
[611, 612]	**[613]** **[614]**	**[615, 616]**
l-Pradimicins derivatives.	**m-Arjunolic acid and derivatives**	**n-Abietic acid derivatives**
		
Pradmicine-A	**R1=R2=AA; R1=Me, R2=AA methyl ester** **R1=H; R2=H_3CCO 3Ac AA**	**Phen 4**
[617, 618]	**[619]**	**[620]**

Table (9) contd….

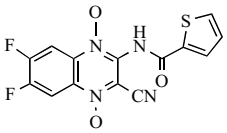
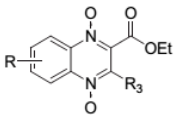
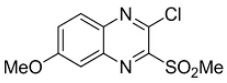
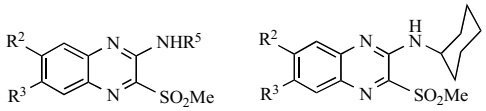
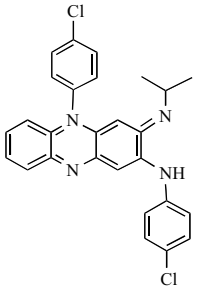
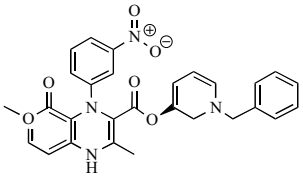
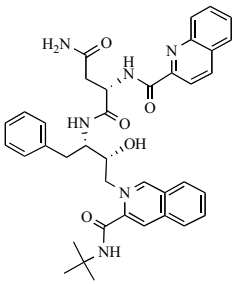
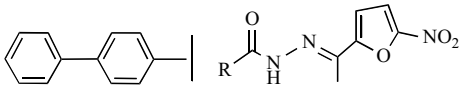
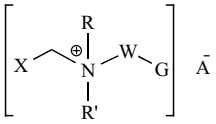
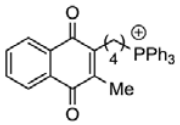
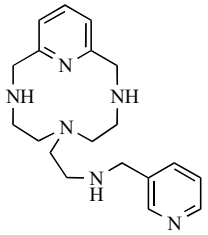
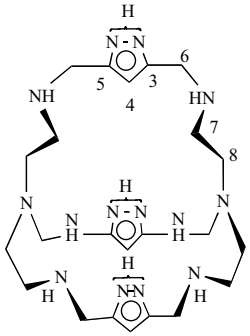
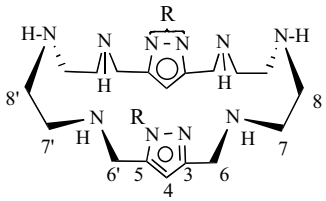
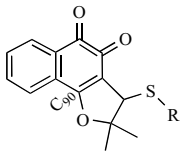
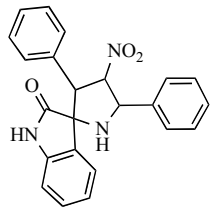
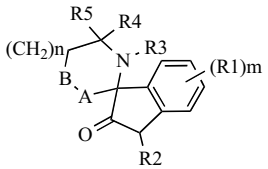
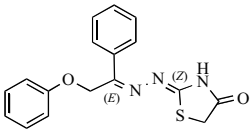
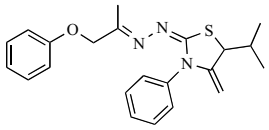
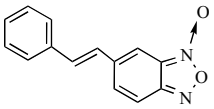
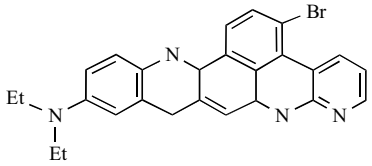
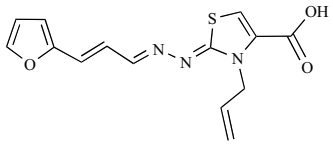
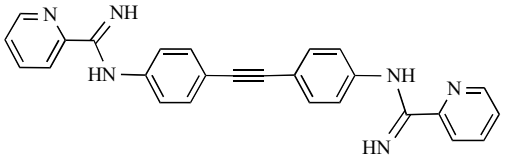
o. Quinoxaline derivatives.			
			
CAQDO	**R3; CH3 or Ph**	**Quinox 4**	**Compound 12, 13**
		[621-626]	
p. Repositioning of approved drugs			
			
Clofazimine	**Benidipine**	**Saquinavir**	
	[627, 628]		
q. N'-[(5-nitrofuran-2-yl) methylene] and substituted hydrazides	**r. Quaternary n-(halomethyl) ammonium salts**	**s. Small molecules naphthoquinone-phthalimide-based lipocations**	
R; 			
Compound 20	**G: CH3, OH, etc**	**Compound 9o**	
[629]	**[630]**	**[631, 632]**	
t. Macrocycles derivatives			
Scorpian-like azamacrocycle	**Pyrazole containing- macrobicyclic polyamine**	**N-Methyl substituted monocyclic polyamines**	
			
Compound 4	**Compound L1**	**Compound 2: R1=Me, R2= H**	
	[633-637]		
u. 3-Phenylthio-nor-β-lapachone derivatives		**v. Bis and mono spiro-indol derivatives**	
		 	
Compound 13b, pCl-C_6H_4			
[638]		**[639-641]**	

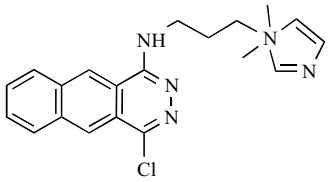
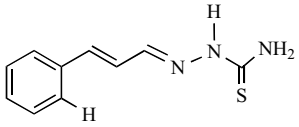
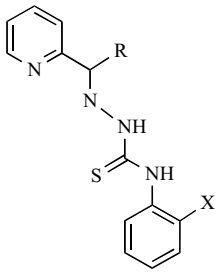
Table (9) contd….

w. Thiazolidine and imidazolidine compounds	**x. Benzo[a]phenoxanthin derivatives**	**z-5Ephenylethenylbenzofuroxan compound**
 **Thiazolidinone 4q** **[642-644]**	 **Iminothiazolidine 18**	 **5PhEBfx** **[645]**
 **Compound 1F** **[646]**		

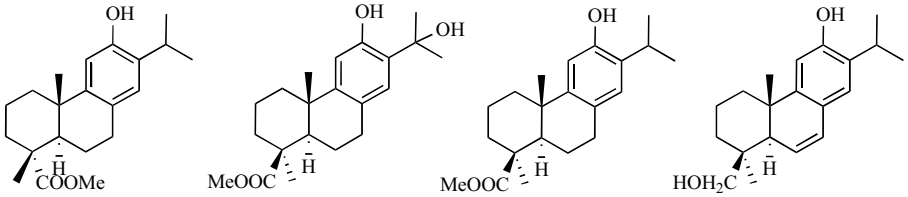
aa. Amide containing compounds

aa1. Amide containing thiazole dervatives	**aa.2. Arylimidamide derivatives**
 **Compound 3** **[647]**	 **18SA8075** **[648]**

ab. Thiosemicarbazones derivatives of 5-[(trifluoromethyl) phenylthio]-2-furaldehyde

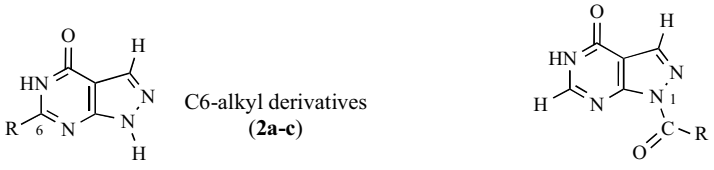
 **Imidazol based-Compound 4** **[649]**	 **[650]**	 **R=CH3; C6H5; X=Cl; F, NO2** **[651, 652]**

ac. Terpenoid derivative structures



[653]

ad. Allopurinol derivatives



R: CH_3(a), C_2H_5 (b), C_3H_7 (c)

[654]

indazoles and analogues compounds presenting potential parasiticidal effects were studied by voltamperometric and spectroscopic techniques. In addition, the interaction between radical species generated from nitroindazole derivatives and glutathione was examined. Finally, spin trapping and molecular modeling studies were performed in order to elucidate the potentials action mechanisms involved in the trypanocidal activity and the target/s involved [658].

b. Synthetic thiosemicarbazones and semicarbazones. Among these compounds, the 4-N-(2'-methoxy styryl)-thiosemicarbazone showed a strong anti-*T. cruzi* against amastigotes internalized in human macrophages. A signifcant reduction of nitric oxide synthase activity was detected [659]. A series of 12 aryl thiosemicarbazones were tested on cruzain and TR. Among them, three p-nitroaromatic thiosemicarbazones showed high *in vitro* activity against *T. cruzi* without a mutagenic profile. However, no correlation was found between cruzain inhibition and trypanocidal activity [125].

c. Quinoxaline 1,4-di-N-oxide derivatives. Eighteen novel quinoxaline 1,4-di-N-oxide derivatives exhibited admirable *in vitro* activity against *T. cruzi*. The most active and selective was compound 17 (Table **10**) containing fluoro groups at the 6- and 7-positions of the quinoxaline ring. The most active were those substituted by electron-withdrawing groups, showing the best easiness of reduction of the N-oxide groups by an electrochemical study [660]. Sb (III) complexes of 3-aminoquinoxaline-2-carbonitrile 1,4-dioxides were synthesized aiming to improve the trypanocidal activity of aminoquinoxaline ligands, it was observed a 2- to 12-fold activity increase of these ligands by metal coordination to antimony [661]. A novel series of 33 methyl and ethyl quinoxaline-7-carboxylate 1,4-di-N-oxide derivatives were evaluated *T. cruzi* and three compounds showed high activity against trypomastigotes than Nx and Bz. *In silico* molecular docking simulations of TR suggested that compound M2 (Table **10**) is a potential TRI [662].

d. Aziridinyl nitrobenzamides and nitrobenzylphosphoramide mustards. Trypanosomes expressing the luciferase reporter gene were generated for the development of a *T. cruzi* drug screening. Against amastigotes of *T. cruzi* and trypomastigotes of *T. brucei*, the the most potent trypanocidal compounds corresponded to the structures previously shown to be substrates for a type I nitroreductase (NTR1) described in trypanosomes and absent in mammalian cells [663]. The activation of nitroheterocyclic prodrugs by NTR1 has permitted the screening of compounds that specially target the parasite, identificating to nitrobenzylphosphoramide mustards and aziridinylnitrobenzamides as two novel types of trypanocidal agents [664].

e. Heteroleptic oxidovanadium (IV) compounds with phenanthroline-derived co-ligands. A series of 10 mixed-ligand oxidovanadium (IV) complexes, [VIVO (L-2H)(NN)], wherever L is a tridentate salicylaldehyde semicarbazone derivative (L1-L5) and NN is either 5-amine-1,10-phenanthroline (aminophen) or 5,6-epoxy-5,6-dihydro-1,10-phenanthroline (epoxyphen), developed in the solid state and in solution, were tested as potential metal-based drugs for the Chagas disease treatment. The activity against *T. cruzi* of all the complexes was higher than Nx and than the earlier described [VIVO (L-2H)(NN)] complexes. The formation of complexes has increased selectivities headed for the parasite. The variations in the biochemical pathways produced by two of the most active and most selective complexes were investigated by determination of some excreted metabolites by ^{1}H NMR spectroscopy, suggesting to mitochondrion as target. In addition, it is worth noting that DNA had been earlier evaluated as a potential target for these compounds [665].

f. 2-Oxidovanadium (IV) and dioxidovanadium (V) complexes of tridentate salicylaldehyde semicarbazones. In order to study SAR of [VIVO (L-2H)(NN)] tripanocidal complexes (where NN is a bidentate polypyridyl DNA intercalato and L is a tridentate salicylaldehyde semicarbazone derivative) and identify the relevant species for biological activity, new [VVO2 (L-2H)] and [VIVO (L-2H)(NN)] complexes comprising bipy or dppz (dipyrido[3,2-a:2′,3′-c]phenazine) co-ligands were synthesized and characterized both in the solid state and in solution. The novel [VIVO (L-2H) (dppz)] complexes showed a toxicity for *T. cruzi* near 10 to 15 times higher than the bipy analogues. The interaction with DNA was shown, supporting that this biomolecule might be the parasite target. Globally these data suggested that [VIVO (L-2H) (NN)] compounds were relevant species for biological activity depending on the NN moiety, but not on the substitution on the salicylaldehyde semicarbazone motif. An optimal relationship between lipophilicity and biological response was determined

proposing them for future development of trypanocidal compounds [666].

g. MCl_2(thiosemicarbazone)] complexes (Pt/Pd). In order to confirm data reported about anti-*T. cruzi* activity of 5-nitrofurylthiosemicarbazones-containing complexes, a comparative characterization of physicochemical properties and pharmacological activities of sixteen Pt(II)/Pd(II) compounds and the corresponding 5-nitrofurylthiosemicarbazone ligands (L) was performed. The authors proposed that the activity of these complexes might occur depending on the nature of the metal and the ligand via double or multiple modes of action. Likewise, data obtained excluded the possibility of DNA binding *in vivo*, suggesting an earlier biotransformation, previous reaching to cellular nuclear DNA. The inhibitory strength and the mode of binding of selected metal complexes and ligands with two *T. cruzi* enzymes, Cz and TR, were analyzed by modeling through molecular docking too [667].

h. 4-Substituted and 1,4-disubstituted 7-nitroquinoxalin-2-ones. The reduction mechanisms of 4-substituted and 1,4-disubstituted 7-nitroquinoxalin-2-ones were characterized by cyclic voltammetry, electrochemical and ESR studies. The first reduction mechanism found for compounds bearing labile hydrogen involved a self-protonation mechanism, while the second, for compounds lacking labile hydrogen, was based on the nitroheterocycles-typical electrochemical reduction mechanism. Four novel compounds were selected based on the high activity against trypomastigotes and epimastigotes, *T. cruzi* TR inhibition might be one of the potential mechanisms involved in the trypanocidal action as suggested by molecular modeling [668].

i. Coumarin and coumarin-chalcones derivatives. Coumarin goes to a group as benzopyrones, which consists of a benzene ring connected to a pyrone moiety, compounds that cover immense interest due to their diverse pharmacological properties. A series of six, isosters of quercetin, the 4-hydroxycoumarin derivatives was evaluated. Although the compounds have been demonstrated to be good antioxidants, they have exposed moderate trypanocidal activity. Particularly, compound 7 (Table **10**) was shown to be the best antioxidant and was considered a possible candidate to be used by its free radicals overproduction [669]. Another series of coumarin derivatives was prepared and all the compounds confirmed a moderate activity in epimastigotes and trypomastigotes. One of them presented the higher activity than Nx, an antioxidant profile and lack of cytotoxicity. Based on these results, the researchers proposed the optimization of this scaffold [670]. Five hybrid compounds, created on coumarin and chalcone scaffolds were synthesized, and the second one was more active against trypomastigotes and epimastigotes than Nx, but presented cytotoxic effects in the mammalian cells tested. A SAR study suggested that methoxy substitution at positions 2' and 5', present in compound 5 (Table **10**) in the designed scaffold seemed to be a key feature for the trypanocidal activity [671].

j. Imidazo-azine scaffold. A chemical database of 30 representative imidazo-azines was built and screened against important tropical disease targets by computational docking. After three rounds of screening, an interaction profile was generated and analyzed. Based on binding energy and ligand efficiency, it was considered that imidazo-azine scaffold has a potential of being selective and simultaneous inhibitor against the five receptors Pf-DHFR, Pf-enoyl acyl carrier protein reductase (EACPR), Pf-PK7 and Mt-pantothenate synthetase (PS). Interestingly, two compounds 2-(4-chlorophenyl)-N-cyclohexyl-6-methylH-imidazo [1,2-a]pyridine-3-amine (MCL011) and N-cyclohexyl-2-(4-methoxyphenyl)-6-methylH-imidazo [1,2-a]pyridine-3-amine (MCL017) showed the highest binding energy against four targets namely Pf-DHFR, Pf-EACPR, Pf-PK7 and Mt-PS [672].

k. Xanthine analogs. A scaffold derived from xanthine was identified as trypanocidal and developed as lead series to identify a clinical candidate for Chagas disease [673].

l. 2-Alkylaminoethyl-1-hydroxy-1,1-bisphosphonic acids. A series of 2-alkylaminoethyl-1-hydroxy-1,1- bisphosphonic acids were studied. Surprisingly, while a hydroxyl group at the C-1 position is present in most pharmacologically active bisphosphonates, the additional presence of an amino group at C-3 resulted in decreased activity towards the parasite and the enzyme. Although these compounds were devoid of activity against *T. cruzi* cells and *Tc*FPPS at different with those mentioned in sub item 2.3.1., they were efficient growth inhibitors of tachyzoites of *T. gondii* [674].

m. Lanthanide complexes. The synthesis and physical properties of seven new lanthanide complexes [Ln(mtpO)3(H2O)6]·9H2O (Ln=La (III), Nd (III), Eu (III), Gd (III), Tb (III), Dy (III) and Er (III)) with the anionic form of the bioactive ligand 5-methyl-1,2,4-triazolo[1,5-a]pyrimidin-7(4H)-one

(HmtpO) were reported. The *in vitro* high activity against *T. cruzi* and their low cytotoxicity to host cells suggest their potential as drug candidates [675].

n. Triazolopyrimidine compounds containing first-row transition metals. Six complexes including 5,7-dimethyl-1,2,4-triazolo[1,5-a]pyrimidine (dmtp) as bioactive molecule were reported. The characterization was performed by X-ray, spectroscopic and thermal methods. The *in vitro* and *in vivo* activity on *T. cruzi* revealed a strong potential of three compounds (Table **10**) as novel drug candidates [676].

o. Benzyl ester of N-propyl oxamate. When inhibitors of α-hydroxy acid dehydrogenase (HADH)-II were analyzed, they exhibited trypanocidal activity. Thus, it was reported that N-propyl oxamate (NPOx) inhibited specifically the HADH-II, and its non-polar ethyl ester (Et-NPOx) was cytotoxic to *T. cruzi*. A new NPOx derivative with higher trypanocidal activity has been developed. In contrast to NPOx, the hydrophobic ester B-NPOx exhibited *in vitro* and *in vivo* trypanocidal activity higher than the polar compound Et-NPOx, Bz and Nx towards five *T. cruzi* strains. The increased activity of B-NPOx was attributed to its hydrolysis inside the parasites to give NPOx and benzyl alcohol, with trypanocidal effects. The high lipophilicity from B-NPOx compared to Et-NPOx, facilitates a better penetration into *T. cruzi*, resulting in the enzymatic cleavage of B-NPOx and the releasing of NPOx and benzyl alcohol as potential trypanocidal agents. These results in conjunction with its low toxicity, suggest that B-NPOx could be used as a potent prodrug for the treatment of Chagas disease [677].

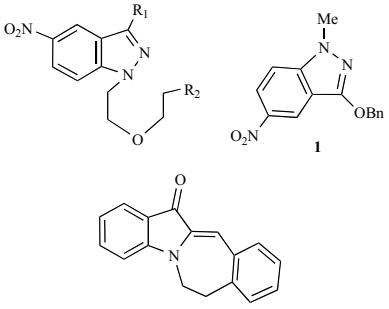
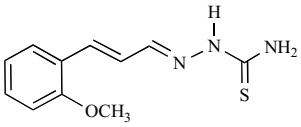
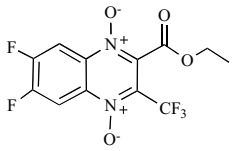
p. Hydroxyphthalazine derivatives. These compounds were chemically modified including a hydroxyl group in the main ring, increasing the solubility of the molecule. Those with *in vitro* activity were tested *in vivo*. Ultrastructural analysis and enzymatic study of inhibition over the iron superoxide dismutase (Fe-SOD) were performed. Compound 2 (Table **10**) showed to be a great inhibitor of Fe-SOD. The high *T. cruzi* activity and low toxicity together with the production economic costs make this compound a proper molecule for further investigation as a trypanocidal agent [678].

q. Enzymes of ascorbate biosynthesis. As described in section 2.14.4, *T. cruzi* is not capable to salvage l-ascorbate (vitamin C) from its environment and depends on the *de novo* synthesis for its survival. The capacity to synthesize ascorbate in humans is missing, therefore *T. cruzi* ascorbate biosynthesis enzymes have transformed in remarkable targets for drug chemotherapy. Flavin-dependent aldonolactone oxidoreductases, belonging to the vanillyl-alcohol oxidase (VAO) protein family, catalyze the last step of the ascorbate biosynthesis. Recombinant *T. cruzi* galactonolactone oxidoreductase (*Tc*GAL) was purified and characterized showing activity with l-galactono-1,4-lactone and d-arabinono-1,4-lactone [679].

r. A-type methionine sulfoxide reductase (MSR). The amino acid methionine is susceptible to being oxidized to methionine sulfoxide (MetSO). The enzyme MSR, present in almost all organisms catalyses the reduction of MetSO to methionine. In trypanosomatids, studies of the antioxidant systems have been essentially based on the participation of trypanothione. Nevertheless, there is no information concerning the parasites mechanisms for repairing oxidized proteins, relevant for the survival of the differential stages of these pathogens life cycle. Three genes codifying for a putative A-type MSR in trypanosomatids were cloned. The corresponding recombinant proteins were expressed, purified and functionally characterized. Then, using *Tc*TXN I as the reducing substrate, the enzymes were specific for the reduction of L-Met(S)SO. The *in vivo* immunological detection of these enzymes in the replicative *T. cruzi* stages supports the existence of a metabolic pathway responsible of repairing oxidized macromolecules. This pathway and/or MSR could be considered a potential novel trypanocidal target [680].

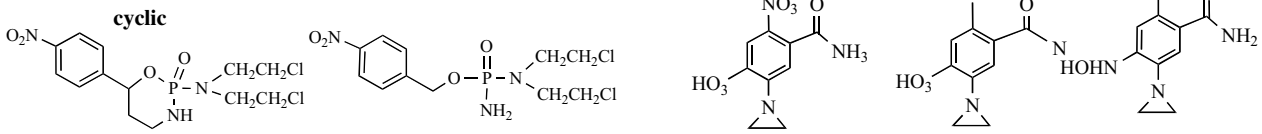
s. Symmetrical diarylideneketones derivatives. A novel TIM enzymatic inhibitor showed trypanocidal activity without inhibition on the mammalian enzyme. Also, these compounds affected Cz. This dual activity is vital to evade resistance problems. In addition to non-specific toxicity to mammalian cells, the compounds were tested *in vitro* against epimastigotes (Table **10**). Three of the best derivatives were also assayed *in vivo*. Some of these derivatives showed higher *in vitro* anti-*T. cruzi* activity than Nx or Bz and were effective in protecting infected mice [681].

Table 10. Novel anti-*T.cruzi* activities with or without known single or multi-target.

ai)-5-nitroindazoles. **aii) Fused tri-/tetracyclic indazoles**	**b-Synthetic thiosemicarbazones and semicarbazones.**	**c-Quinoxaline 1,4-di-N-oxide derivatives.**
 **1** **[657-658]**	 ***4-N-(2´-methoxy styryl)-thiosemicarbazone*** **[659, 125]**	 **[660-662]**

d-Aziridinyl nitrobenzamides and nitrobenzylphosphoramide mustards

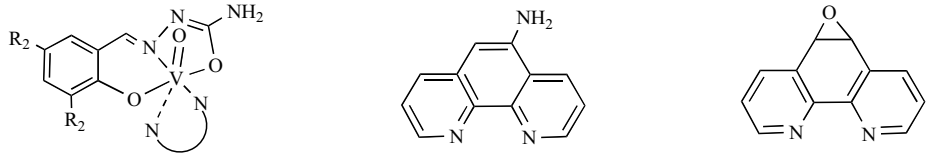
cyclic **acyclic** **NFB mustards**



CB1954 **2 and 4 hidroxilamine derivatives**

[663] **[664]**

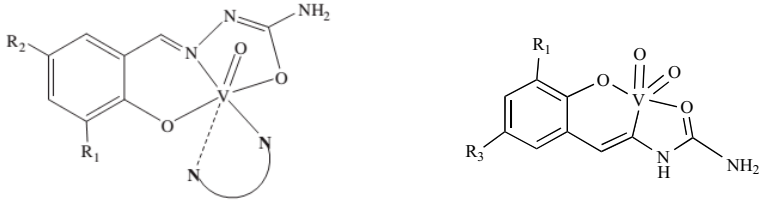
e-heteroleptic oxidovanadium (IV) compounds with phenanthroline-derived co-ligands



NN: aminophen/epoxyphen compounds **5-amine, 1, 10-phenantroine** **5, 6 epoxy-5,6-dihydro-1,10 phenantroline**

[665]

f- Oxidovanadium (IV) and dioxidovanadium (V) complexes of tridentate salicylaldehyde semicarbazone



[666]

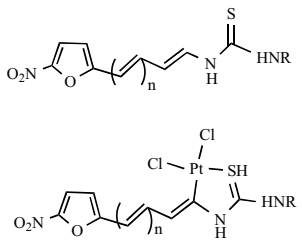
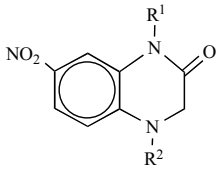
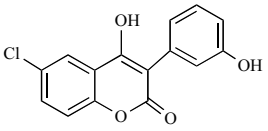
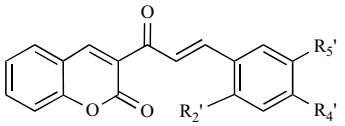
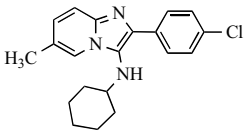
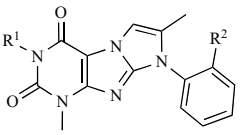
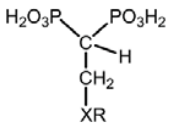
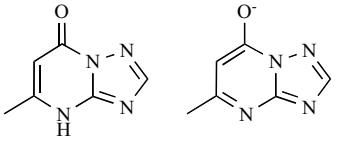
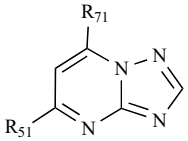
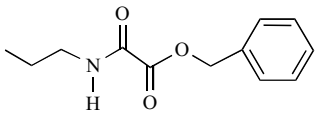
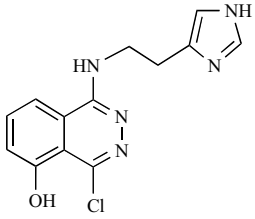
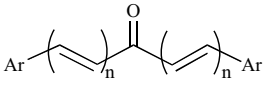
g. MCl_2(thiosemicarbazone) complexes (Pt/Pd)	**h. 4-substituted and 1,4-disubstituted 7-nitroquinoxalin-2-ones**
 **MCl2L** **[667]**	 **1a R1=H,Me R2= Me** **1b: R1=H,Me, R2=H, (CH2)y; y, 2, 4, 6 etc** **[668]**

Table (10) contd….

i-Coumarin and coumarin-chalcones derivatives	
	
Compound 7	**5R2′=R5=OMe; R4=H**
[669-671]	
j-Imidazo-azine scaffold	**k: xanthine analogs**
	
MCLO11 **MCLO17**	
[672]	**[673]**
l-2-alkylaminoethyl-1-hydroxy-1,1-biphosphonic acids	**m-Lanthanide complexes**
	
Compounds 12, 13, 14 **(X=NH R=n-hexyl, ,heptyl, dodecyl)**	**HmtpO** **mtpO-**
[674]	**[675]**
n-Triazolopyrimidine compounds	**o-benzyl ester of N-propyl oxamate:** **p-hydroxyphthalazine derivatives**
	 
$[Cu(dmtp)_4(H2O)_2](ClO4)_2.2H_2O$ (5) **R=CH3, dmtp**	**B-NPOx** **Fe-SOD-Inhibitor 2**
[676]	**[677]** **[678]**
s-Symmetrical diarylideneketones derivatives.	
	
n: 1, 2, 3 Ar: phenyl, thienyl, furyl	
[681]	

5. IDENTIFICATION OF NOVEL BIOTARGETS IN THE LAST YEARS

5.1. Biochemical Metabolism of *T. Cruzi.*

5.1.1. Catabolism Aromatic Amino Acids

Unlike in the mammalian host, the metabolism of aromatic amino acids is a very simple pathway in these parasites. *Trypanosoma cruzi* is able to transaminate the three aromatic amino acids, the resulting 2-oxo acids being reduced to the corresponding lactate derivatives and excreted. In *T. cruzi*, two enzymes are involved in this process: TAT, a tyrosine aminotransferase that although shows great sequence homology with the mammal's enzyme, presents differental substrate specificity; and AHADH, an aromatic L-2-HADH, that despite belongs to the subfamily of the cytosolic

malate dehydrogenases (MDHs), do not exhibit MDH activity. In *T. cruzi* AHADH, the substitution of Ala102 for Arg enables AHADH to reduce oxaloacetate. *Trypanosoma cruzi* does not possess a cytosolic MDH but contains a mitochondrial and a glycosomal MDH [682].

5.1.2. Proteins Involved in the T. cruzi Metabolism

FPPS, TS, Cz, TR, G6PDH, glyceraldehyde-3-phosphate-dehydrogenase and the aromatic AHADH are among the enzymes evaluated as probable targets for further designing of novel drugs for Chagas disease [683].

5.2. RNA Editing

Mitochondrial RNA editing, catalyzed by multi-protein complexes known as editosomes, has provided a chance for development of efficient and specific chemotherapeutic targets against trypanosomatid pathogens including *T. cruzi*. Methods for discovery of RNA editing inhibitors through creative virtual and high throughput screening in addition to their use as agents that can block or perturb one or more steps of this process have been reported. The inhibitors mentioned in the revision can potentially be used to study the dynamic processing and assembly of the editosome proteins. However, the possibility of drug development against trypanosomatids in a near future still requires further studies [684].

5.3. Sirtuins (SIRs)

SIRs displayed antiparasitic activity against infections caused by trypanosomatids. Thus, might be considered as drug targets. Based on the C pocket of *T. cruzi* SIR 2, a virtual screening was performed. The top ligand found was nicotinamide. In *T. cruzi*, the trypanocidal activity of nicotinamide was confirmed by *in vitro* tests developed on epimastigotes and trypomastigotes and the treatment of *T. cruzi*-infected macrophages with nicotinamide produced a substantial decrease in amastigotes number. The fine analysis of the complex between SIR 2 and nicotinamide indicated a potential use of *Tc*SIR 2 as a target for trypanocidal chemotherapeutic agent's discovery [685]. On the other hand, homology modelling, docking and molecular dynamics simulations have generated 3D models of SIR2rp3, the NAD^+-dependent deacetylases from trypanosomatids. Molecular docking of known inhibitors revealed strong analogies with the mitochondrial human SIR 5 in terms of binding mode and interaction strength. In addition, a detailed analysis showed differential regions between host and parasitic targets, resulting useful for future selective drug design [686].

5.4. Sumoylation

In eukaryotes proteins, this PTM is very important. The C-terminal of proteolytically activated SUMO, small ubiquitin-like modifier, is covalently linked to a Lys residue of the target protein by an isopeptide bond. It occurs by a mechanism comprising an E1-activating enzyme, an E2-conjugating enzyme, and transference to the target, occasionally assisted by a ligase. A protease is responsible for the reversion of this modification as well as for SUMO maturation. Numerous proteins have been recognized as SUMO targets, involved in the modulation of different nuclear cellular functions. In *T. cruzi*, the presence of orthologous genes from the SUMOylation pathway has been reported. Transfectant *T. cruzi* epimastigotes expressing a double-tagged *T. cruzi* SUMO were generated in order to identify SUMOylation targets and learn about their physiological roles. 236 proteins with varied biological functions were identified as potential *T. cruzi* SUMO targets. Among them, metacaspase-3 was validated as a genuine SUMOylation substrate. However, proteomic analysis of probable orthologs of *T. cruzi* SUMOylated proteins have demonstrated conserved functions for protein SUMOylation in *T. cruzi*. Further studies will allow investigating this pathway and/or the enzymes involved as potential trypanocidal target [687].

5.5. CA^{2+} Homeostasis

In all eukaryotes, from mammals to parasites, the Ca^{2+} ions have been mostly identifyed as an indispensable messenger. Disruption of Ca^{2+} homeostasis frequently pushes to lethal effects causing cell death by apoptosis or necrosis. Regarding to regulation of intracellular Ca^{2+}, trypanosomatids possess a single mitochondrion capable to accumulate big quantities of Ca^{2+}. Also, the endoplasmic reticulum participates in Ca^{2+} regulation. In addition, these organisms accumulate vast extents of polyphosphates with Ca^{2+} ions in the acidocalcisomes (described in section 2.14.2), and contain large amounts of calmodulin. The mentioned protein is conserved in vertebrates and there is 89% amino acid sequence homology between calmodulin from *T. cruzi* and mammals.

Amiodarone, an antiarrhytmic compound, commonly used in chronic Chagas' patients with heart problems, is capable to cause trypanocidal effect. The single large mitochondrion and the acidocalcisomes are the intracellular compartments responsible for the rise in the intracellular Ca^{2+} concentration after the adding of amiodarone. This drug is also able to block OSC, a key enzyme in the biosynthesis of ergosterol. Amiodarone displayed an effect highly synergistic with a known strong inhibitor of the synthesis of ergosterol, posaconazole. In humans, amiodarone in combination with itraconazole was reported to induce the cure of a Chagas' disease patient. It was reported that pozaconazole is capable to determine the increase of the intracellular Ca^{2+} concentration and that miltefosine produce the disruption of the parasite's intracellular Ca^{2+} homeostasis. Altogether the findings suggested that the modification of the intracellular Ca^{2+} homeostasis of these parasites seemed to be a talented target of novel and repurposed old-known drugs [688, 689].

5.6. Vitamin B(6) Dependent Enzymatic Reactions

Vitamin B(6) or pyridoxal 5'-phosphate contains chemical properties exclusively suitable to be extensively used as cofactors in decarboxylations and transaminations reactions. Enzymatic reactions that depends from vitamin B(6) were also explored as drug targets. Among these enzymes a number of prospective targets based on diseases originated by parasitic protozoan might be of interest in the development of parasiticidal drugs [690].

5.7. Peptide Deformylase (PDF)

The N-terminal methionine excision in bacterial and eukaryotic organelles requires the sequential action of two main activities, first a peptide deformylase (PDF), that is responsible for methodically remove the N-formyl group existing in all nascent polypeptides and second the methionine aminopeptidase (MAP), capable to specifically exscind methionine depending on the preceding elimination of the N-formyl group. Two genes codifying for bacterial PDF homologues have been identified in *T. cruzi* (*Tc*PDF-1 and *Tc*PDF-2). A soluble version of *Tc*PDF-1 without the hydrophobic N-terminal domain, by truncation, with activity on the bacterial PDF substrate formyl-methionyl-alanyl-serine was not inhibited by actinonin, at difference with other PDFs. *Tc*PDF displays exclusive features, then, constituting a novel target for the design of possible inhibitors for the treatment of diseases caused by trypanosomatids [691].

5.8. Specific Receptors for Platelet-Activating Factor (PAF)

The understanding of the biological processes occurring in the interface between the pathogenic trypanosomatid and host cell during interaction is essential for the characterization of virulence factors which may be considered as targets for the progress of selected inhibitors for effective chemotherapy. Accordingly, evidences suggesting the existence of specific receptors for PAF in *T. cruzi* were reported. Therefore, the role of PAF on the control of parasite differentiation and the potential of exploring these presumed receptors were proposed as new targets for Chagas disease chemotherapy [692].

5.9. Alpha-Class Carbonic Anhydrase

Trypanosoma cruzi encodes an alpha-class carbonic anhydrase, *Tc*CA, enzyme reported as essential for parasite life cycle. Thiols constitute a type of potent inhibitors of *Tc*CA, which were also exposed to block the *in vitro* pathogen growth. In addition, the *Tc*CA inhibition by complex and inorganic anions and other molecules such as sulfamide, sulfamic acid, phenylboronic/arsonic acids, capable to interact with zinc proteins, were reported. Sulfamic acid was considerable less effective than sulfamide. Iodide, cyanate, thiocyanate, hydrogensulfide and trithiocarbonate inhibited *Tc*CA in the low μM range but diethyldithiocarbamate resulted to be the top inhibitor, and might be helpful to identify leads for evolving trypanocidal agents with varied modes of action in comparison with Bz and Nx [693].

5.10. PEPX, X-Prolyl Dipeptidyl Aminopeptidase of S15 Family

This serine exopeptidase, counterpart of the mammalian DDP-4 cleaves dipeptides from the N-terminus of polypeptides having a proline or alanine residue at the second position that has been suggested to be an auspicious target against trypanosomes. Searching for specific inhibitors, docking simulations on the whole surface of PepX from a protein type of the S15 family revealed a new putative binding site in connection with the active site and involving the C-terminal domain. Accordingly to computational results the two peptidomimetics valinephenylpiperazine and valine-isopropylpiperazine that can accommodate to this putative binding

site were synthesized. The experiments exposed that the valine-phenylpiperazine was an uncompetitive inhibitor, while the valine-isopropylpiperazine showed to be an enzyme activator. The results pointed out that C-terminal domain should control the access to the enzymes active site of the S15 family, like PepX, and could have applications in human health giving new perspectives to struggle against trypanosomes by designing inhibitors specific to the S15 enzymes family [694].

5.11. CYTOCHROME B (CYT b)

The *T. cruzi* inhibitor GNF7686, discovered in a parasite growth inhibition high throughput screen, targets CYT b. A GNF7686-resistant culture of *T. cruzi* epimastigotes was evolved. The L197F mutation, coding for a substitution of leucine by phenylalanine, confers resistance to GNF7686 in both parasite cell growth and biochemical CYT b assays, as well as resistance to antimycin A. GNF7686 represents a promising starting point for drug development as it inhibited growth of intracellular *T. cruzi* amastigotes, and is highly specific for *Tc*CYT-b. The use of an approach combining *T. cruzi* chemical genetics with biochemical target validation was suggested to be mostly used for the development of novel drug for Chagas disease treatment [695].

5.12. Spermidine Synthase (Spdsyn)

The transfer of an aminopropyl group from the decarboxylated S-adenosylmethionine (dcSAM) to putrescine contains SpdSyn as a key pathway enzyme. Searching for potent inhibitors of *Tc*SpdSyn was conducted by fragment-based drug discovery. The analysis of the crystal structure of dcSAM complexed with *Tc*SpdSyn showed that dcSAM stabilizes the conformation of the `gatekeeping' loop forming the putrescine-binding pocket and revealed the presence of two fragment-binding sites. Probably, the dynamic structural changes occurring in *Tc*SpdSyn by inhibitor binding might facilitate the development of more selective and potent inhibitors [696].

5.13. Kinetoplastid Targets

Recently, a pharma compound collection against kinetoplastids was published including the first high throughput screening. The Glaxo Smith Kline high-throughput screening variety set of 1.8 million compounds was screened against the most relevant kinetoplastids for human disease, including *T. cruzi* among them. The hypothetical biological target space covered by these diversity sets was investigated through bioinformatical methodologies. Thus, three anti-kinetoplastid chemical boxes of ~200 compounds each were assembled. Functional analyses of these compounds suggest a wide array of potential modes of action against kinetoplastid kinases, proteases and cytochromes as well as potential host-pathogen interactive molecules [697].

6. TRYPANOCIDAL AGENTS IN/OR ENTERING IN CLINICAL TRIALS

Despite the numerous types of natural and synthetic compounds tested for anti-*T. cruzi* activity, since Bz and Nf were introduced, merely a small number of drugs, such as allopurinol and a few EBIs or CPIs have moved or are ready to enter in clinical trials, respectively. Ravuconazole prodrug E1224 (NCT01489228) [698] and posaconazole (NCT01162967; Chagaszol) [699], have been evaluated in phase II clinical trials for the treatment of chronic patients. Both drugs were capable to clear parasites from the blood at the culmination of the treatment, however afterwards parasitemia rebounded. In this sense, the experts explained that among EBIs, posaconazole and ravuconazole are better tolerated but their efficacy at the doses and treatment duration used in the initial studies was significantly low; attributing such results to suboptimal exposure and/or treatment duration. Although it is currently considered that combination therapies are a promising perspective, the lack of certified biomarkers of response to etiological treatment and eventual parasitological cures in chronic patients remains a serious challenge [700]. Unfortunately, recent reports of treatment failure in the clinic for both posaconazole and ravuconazole indicate the urgence for drug treatments having a different mode of action to CYP51. Therefore, a high throughput fluorescence based functional inhibition assay using recombinant *Tc*CYP51 is considered valuable on *T. cruzi* active series arising from a phenotypic screening where the principal mode of action is expected not to act via inhibition of CYP51 [701].

Recently, infected mice were treated with posaconazole or Bz in order to identify a dose up regimen capable to led to parasitological, defined by absence of parasitemia recrudescence after immunosuppression. Bz occasioned a dose-dependent antiparasitic activity rise, with 100% cure at 100 mg/kg of body weight/day. By contrast, posaconazole treatment (10 to 100 mg/kg/day) led to cure of only 25%. Differences between both drugs were

also observed parasite burden in the heart [702]. These findings agree with the conclusions of recent phase 2 trials with posaconazole.

In the last lustrum, two methodologies heading for addressing the challenge of developing novel drugs for Chagas disease treatment have been reported. One of them, a target-based drug discovery includes cytochrome P450 CYP51 and Cz, considering the vinyl sulfone inhibitor K777 as clinical applicant. The second tactic is related to parasite phenotypic screens in culture leaning towards the identification of novel drugs [79, 703]. Regarding the first approach, K777 originally identified by *in vitro* screening of CPIs against *T. cruzi* was also effective in both mice and dogs models of acute and chronic Chagas disease. K777 is non-mutagenic and well tolerated in animal safety studies. In 2009, FDA formally reviewed the proposed Investigational new drug (IND) package for K777 in order to allow the initiation of human clinical trials [704]. Regarding the second approach, a public-private partnership has been established by Mc Kerrow and co-workers between the University of California, San Francisco and the Genomics Institute of the Novartis Research Foundation (GNF) with the aim of delivering clinical prototypes for Chagas disease treatment. Two screening methods were used, one of them included a colorimetric-based assay by using a *T. cruzi* expressing β-galactosidase enzyme and the other one, an image-based, high-content screening (HCS) assay using a *T. cruzi* strain. In both cases, the assays were used to notify SARs for pharmacokinetics and mainly antiparasitic efficacy. A novel trypanocidal xanthine scaffold derivative was interestingly evidenced and developed as lead series to identify a clinical candidate for Chagas disease [673].

7. CURRENT & FUTURE DEVELOPMENTS

Currently, the number of infected people globally estimated by the WHO amounts to 7 to 8 million and more than 10,000 deaths are thought to occur annually [8]. On top of this, Chagas disease, the first cause of cardiac morbidity and mortality in poor rustic and suburban zones of Latin America and the prevalent parasitic disease load in the continent, has also emerged as a public health problematic in non-endemic countries due to transmission of *T. cruzi* by people migration around the world [9, 10]. Despite Chagas disease cannot be eliminated due to the proved presence of infected wild triatomines in perpetual contact with domestic cycles contributing to the incidence of novel cases, it could be likely to interject *T. cruzi* transmission in a huge region and to eradicate this disease as a public health problem with a intense decrease in the burden of Chagas disease [705]. In this environment, search for novel chemotherapeutic anti-*T. cruzi* targets is of vital urgency, regarding that there is no effective vaccine currently in practice to combat this neglected disease. Furthermore, the existing drugs for the disease treatment are not suitable, are expensive and produce toxic side effects. In addition, currently, once the disease has progressed to the chronic stage there is no effective drug. Moroever, in the last years, children treatment was difficult due to the absence of these drugs in pediatric version. However, solid dispersions have been recently presented as alternative drug delivery system to improve the chemotherapy of Chagas disease and paediatric oral liquid suspension containing Bz (1%) was easily prepared starting from commercial tablets, being an interesting alternative for optimising the paediatric treatment of the disease [706, 707].

During the last 15 years-period, parasite biology and biochemistry has been extensively studied. Aditionally, since 2005 as a consequence of the accessibility to parasite genome sequences [40], the probability of detecting novel new drug targets has augmented. A detailed description of auspicious newly validated biochemical targets, its dissimilarities respect to the host, the understanding of their mechanism of action and inhibitors of biosynthetic pathways and/or enzymes exclusively found in trypanosomatids have been compiled for the development of anti-*T. cruzi* drugs. Herein, novel targets have been included in section 6 of this manuscript [682-697]. In addition, in section 3 anti-Chagas disease compounds without assigned specific target have been extensively described [595-654] and in section 4, those anti-*T. cruzi* activities with known single or multi-target were reported [655-681]. To this aim, the experimental evaluations together with bioinformatic tools can be included for the selection of prototype trypanosomatid drug targets [708], and give examples of signaling and metabolic pathways in the protozoan parasites that can be exploited for rational drug design [709]. Currently, despite some progress in preclinical studies, there is no yet an ideal drug or formulation for human treatment. A major problem in the evaluation of potential Chagas disease therapeutics is the lack of tools availability. Indeed, there is still an urgent need to discover a better biomarker capabe to determine the efficacy of potential chemotherapeutics in treated patients [710]. The Drugs for Neglected Diseases initiative

(DNDi) has defined and implemented an early discovery strategy over the last few years. This strategy consists in a medium- to high- throughput phenotypic assay to accelerate the screening of compound libraries against kinetoplastids in collaboration with partners from the pharmaceutical industry, in order to identify a new class series for further development into preclinical candidates [711].

Unfortunalely, trypanocidal therapy with Bz in patients with established Chagas' cardiomyopathy significantly reduced serum parasite detection but did not significantly reduce cardiac clinical deterioration through 5 years of follow-up [37]. Additionaly, controversial findings were detected between these clinical studies and data from a group of patients treated with amiodarone, showing some evidences that the antiarrhythmic drug produced anti-*T. cruzi* activity, causing ultrastructural damage [712]. Prevention of chronic chagasic cardiomyopathy by treating infected populations with trypanocidal therapy remains a challenge and discrepancy of the information for patient-important outcomes must be treated with caution. More geographically varied randomised controlled trials testing newer forms of trypanocidal therapy shoud be merited in order to evaluate efficacy more accurately, to investigate factors possibly responsible for the heterogeneity of results and to intensificate evidences on the efficacy/tolerance equilibrium of traditional trypanocidal therapy [36]. Although the identification of new anti-Chagas disease agents is not only focused on target-based drug design and its derivatives and on synthetic or natural products screening [713] but also in old ones rediscovered as new drugs against Chagas disease [148, 149, 458, 290], despite all the new data available, an appropriate drug has not been still identified. In addition, the synergism of drugs such as Nx and buthionine sulfoximine has been demonstrated by *in vitro* and *in vivo* experimentations [290]. However, human investigations are required to ratify these results. Likewise, potential use of amiodarone and dronedarone was proposed [222]. Similar results could be predictable with the combination of itraconazole and allopurinol [34], Bz and posaconazole [714], Bz and itraconazole [715], and Bz and allopurinol. The results obtained emphasized the importance of exploring the potential of the combination of treatments with currently available compounds to specifically treat Chagas disease. Regarding the combined drug treatment of Bz and allopurinol, studies have been performed in the last years in murine model and in chronically infected patients. The effect of combined chemotherapy in murine experimental models of *T. cruzi* infection, showed the highest beneficial effect, not only by reducing parasitaemia but also by lowering the degree of inflammation and fibrosis In *T. cruzi* chronically infeted subjects, the association of Bz and allopurinol was capable to induce substantial changes in T and B cell responses indicating a decrease in parasite burden [716, 717].

During 2000-2015 period, a search over the patent literature related with specific target-based trypanocidal drugs was done. However, some of them claim compounds valued for the treatment of human diseases such as various cancers, bone diseases or antiviral activity and also report potential trypanocidal activity. Among them, those related with CPIs, organometalic complexes and purine analogues can be mentioned. Compounds with specific protozoan, parasitic or trypanocidal activity as a central claim, including Chagas disease have been also revealed [328, 499, 522]. Noticeably, an abrupt upsurge about the knowledge on the parasite biochemistry has not been reflected in the number of disclosed patents, likewise a small number among the analyzed patents displayed specific data related with anti-*T. cruzi* activity. Additionally, only a few significant *in vivo* results were reported, while most patents set up are associated with parasitic targets revealing *in vitro* anti-*T. cruzi* activity. Particularly, patents include those inhibitors based on the drug induced blockage of specific enzymes participating in sterol biosynthesis specially, CYP51 [229-243] and OSC [244-248]. Patents related with CPIs showed to be the most represented among those claiming for potential therapeutic agents against *T. cruzi*, comprising peptidyl allyl sulfone compounds for inhibiting proteases [77], azapanone based inhibitors [101-105], thiosemicarbazone and semicarbazone inhibitors [121], hydrazideNacylhydrazone compounds [134] or synthetic derivatives or approved drugs with probed activity on Cz [151-153]; tropomyosin-related TK receptors [444]; polyamine transport inhibitors [450] and dihidrofolate reductase [477, 479, 481], among others. In addition, different anti-Chagas disease series of synthetic compounds and its derivatives were patented and detailed in this review (Table **2**). Unfortunately, recent clinical trials investigating treatment of chronic indeterminate Chagas disease with the two re-purposed azole anti-fungal drugs, revealed their inferiority to the current standard drug Bz, showing the failure of the existing pre-clinical testing model for this disease [700]. Regarding all the data

achieved in the last years, a rational approach for the fast advance of novel trypanocidal chemotherapy would be focused on drugs ready to enter to clinical trials [704], on new scaffolds [673] and on the clinical evaluation of drug association with existing trypanocidal agents to get extra effectiveness and fewer secondary effects.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

Part of this work was supported by grants from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET); and I thank to the Instituto Nacional de Parasitología (INP), "Dr Mario Fatala Chaben", ANLIS-Malbrán, Ministerio de Salud de la Nación, Argentina. V.G.D. is a Research Member of CONICET from Argentina.

REFERENCES

[1] Samuels AM, Clark EH, Galdos-Cardenas G, Wiegand RE, Ferrufino L, Menacho S, *et al*. Epidemiology of and impact of insecticide spraying on chagas disease in communities in the Bolivian Chaco. PLoS Neg Trop Dis 2013; 7: e2358.

[2] Schmunis GA, Cruz JR. Safety of the blood supply in Latin America. Clin Microbiol Rev 2005; 18(1): 12-29.

[3] Benchimol Barbosa PR. The oral transmission of Chagas disease: An acute form of infection responsible for regional outbreaks. Int J Cardiol 2006; 112:132-3.

[4] Bern C, Montgomery SP, Herwaldt BL, Rassi A Jr, Marin-Neto JA, Dantas RO, *et al*. Evaluation and treatment of Chagas disease in the United States: A systematic review. JAMA 2007; 298: 2171-81.

[5] Rassi A Jr., Rassi A, Marcondes de Rezende J. American trypanosomiasis (Chagas Disease). Infect Dis Clin North Am 2012; 26: 275-91.

[6] Sesti-Costa R, Silva JS, Gutierrez FR. Congenital Chagas disease: Time to screen pregnant women? Expert Rev Anti Infect Ther 2012; 10:1279-82.

[7] Kransdorf EP, Zakowski PC, Kobashigawa JA. Chagas disease in solid organ and heart transplantation. Curr Opin Infect Dis 2014; 27(5): 418-24.

[8] World Health Organization. WHO Fact Sheet No 340. http://www.who.int/mediacentre/factsheets/fs340/en/ (accessed May 29, 2014).

[9] Gascon J, Bern C, Pinazo MJ. Chagas disease in Spain, the United States and other non-endemic countries. Acta Trop 2010; 115(1-2): 22-7.

[10] Pérez-Molina JA, Norman F, López-Vélez R. Chagas disease in non-endemic countries: Epidemiology, clinical presentation and treatment. Curr Infect Dis Rep 2012; 14(3): 263-74.

[11] Panamerican Health Organization Status of Chagas disease in the region of Americas. Epidemiologic bulletin. Pan American Health Organization, Washington, DC 1984.

[12] Moolani Y, Bukhman G, Hotez PJ. Neglected tropical diseases as hidden causes of cardiovascular disease. PLoS Negl Trop Dis 2012; 6(6): e1499.

[13] Ramirez JD, Guhl F, Umezawa ES, Morillo CA, Rosas F, Marin-Neto JA, *et al*. Evaluation of adult chronic Chagas' heart disease diagnosis by molecular and serological methods. J Clin Microbiol 2009; 47(12): 3945-51

[14] Brasil PE, De Castro L, Hasslocher-Moreno AM, Sangenis LH, Braga JU. ELISA versus PCR for diagnosis of chronic Chagas disease: Systematic review and meta-analysis. BMC Infectious Disease 2010; 10: 337-54.

[15] Souza RM, Amato Neto V. Discrepancies and consequences of indirect hemagglutination, indirect immunofluorescence and ELISA tests for the diagnosis of Chagas disease. Rev do Inst Med Trop de Sao Paulo 2012; 54(3): 141-3.

[16] Gilber SR, Alban SM, Gobor L, Bescrovaine J de O, Myiazaki MI, Thomaz-Soccol V. Comparison of conventional serology and PCR methods for the routine diagnosis of *Trypanosoma cruzi* infection. Rev Soc Bras Med Trop 2013; 46(3): 310-15.

[17] De Winne K, Buscher P, Luquetti AO, Tavares SB, Oliveira RA, Solari A, *et al*. The *Trypanosoma cruzi* satellite DNA OligoC-TesT and *Trypanosoma cruzi* Kinetoplast DNA OligoC-TesT for diagnosis of Chagas disease: A multi-cohort comparative evaluation study. PLoS Negl Trop Dis 2014; 8(1): e2633.

[18] Rajao MA, Furtado C, Alves CL, Passos-Silva DG, de Moura MB, Schamber-Reis BL, *et al*. Unveiling Benznidazole's mechanism of action through overexpression of DNA repair proteins in *Trypanosoma cruzi.* Environ Mol Mutagen 2014; 55(4): 309-21.

[19] Docampo R, Moreno SN. Free radical metabolism of antiparasitic agents. Fed Proc 1986; 45(10): 2471-6.

[20] de Andrade AL, Zicker F, de Oliveira RM, Almeida Silva S, Luquetti A, Travassos LR, *et al.* Randomised trial of efficacy of ben-Randomised trial of efficacy of benznidazole in treatment of early *Trypanosoma cruzi* infection. Lancet 1996; 348: 1407-13.

[21] Sosa Estani S. Treatment of *Trypanosoma cruzi* infection in the undetermined phase. Experience and guidelines of treatment in Argentina. Mem Inst Oswaldo Cruz 1999; 94 (Suppl. I): 363-5.

[22] Rodriques Coura J, de Castro SL. A critical review on Chagas disease chemotherapy. Mem Inst Oswaldo Cruz. 2002; 97 (1): 3-24.

[23] Streiger ML, del Barco ML, Fabbro DL, Arias ED, Amicone NA. Longitudinal study and specific chemotherapy in children with chronic Chagas disease, residing in a low endemicity area of Argentina. Rev Soc Bras Med Trop 2004; 37: 365-75.

[24] Fabbro DL, Streiger ML, Arias ED, Bizai ML, del Barco M, Amicone NA. Trypanocide treatment among adults with chronic Chagas disease living in Santa Fe city (Argentina), over a mean follow-up of 21 years: parasitological, serological and clinical evolution. Rev Soc Bras Med Trop 2007; 40: 1-10.

[25] Garcia S, Ramos CO, Senra JF, Vilas-Boas F, Rodrigues MM, Campos-de-Carvalho AC, *et al*. Treatment with benznidazole during the chronic phase of experimental Chagas disease decreases cardiac alterations. Antimicrob Agents Chemother 2005; 49(4): 1521-8.

[26] de Mecca MM, Bartel LC, de Castro RC, Castro JA. Benznidazole biotransformation in rat heart microsomal fraction without observable ultrastructural alterations: comparison to nifurtimox-induced cardiac effects. Mem Inst Oswaldo Cruz, Rio Janeiro 2008; 103(6): 549-53.

[27] Bern C. Antitrypanosomal therapy for chronic Chagas' disease. New Eng J Med 2011; 364(26): 2527-34.

[28] Altcheh J, Biancardi M, Lapena A, Ballering G, Freilij H. Congenital Chagas disease: Experience in the Hospital de Niños, Ricardo Gutierrez, Buenos Aires, Argentina. Rev Soc Bras Med Trop 2005; 38(Suppl. 2): 41-4

[29] Altcheh J, Moscatelli G, Moroni S, Garcia-Bournissen F, Freilij H. Adverse events after the use of benznidazole in infants and children with Chagas disease. Pediatrics 2011; 127(1): e212-e218.

[30] Carlier Y, Torrico F, Sosa-Estani S, Russomando G, Luquetti A, Freilij H, *et al*. Congenital Chagas disease: Recommendations for diagnosis, treatment and control of newborns, siblings and pregnant women. PLoS Neg Trop Dis 2011; 5(10): e1250.

[31] García MC, Manzo RH, Jimenez-Kairuz AF. Extemporaneous benznidazole oral suspension prepared from commercially available tablets for treatment of Chagas disease in paediatric patients. Trop Med Int Health 2015; 20(7): 864-70.
[32] Guedes PM, Silva GK, Gutierrez FR, Silva JS. Current status of Chagas disease chemotherapy. Exp Rev Anti Inf Ther 2011; 9(5): 609-20.
[33] Matta Guedes PM, Gutierrez FR, Nascimento MS, Do-ValleMatta MA, Silva JS. Antiparasitical chemotherapy in Chagas' disease cardiomyopathy: Current evidence. Trop Med Int Health 2012; 17(9): 1057-65.
[34] Apt W, Arribada A, Zulantay I, Solari A, Sánchez G, Mundaca K, *et al.* Itraconazole or allopurinol in the treatment of chronic American trypanosomiasis: The results of clinical and parasitological examinations 11 years post-treatment. Ann Trop Med Parasitol 2005; 99(8): 733-41.
[35] Viotti R, Alarcón de Noya B, Araujo-Jorge T, Grijalva MJ, Guhl F, López MC, *et al*. On behalf of the Latin American Network for Chagas Disease, NHEPACHA.Towards a paradigm shift in the treatment of chronic Chagas Disease. Antimic Agents Chemother 2014; 58(2): 635-9.
[36] Villar JC, Perez JG, Cortes OL, Riarte A, Pepper M, Marin-Neto JA, *et al*. Trypanocidal drugs for chronic asymptomatic *Trypanosoma cruzi* infection. Cochrane Database Syst Rev 2014; 5:CD003463.
[37] Morillo CA, Marin-Neto JA, Avezum A, Sosa-Estani S, Rassi A Jr, Rosas F. Randomized trial of benznidazole for chronic Chagas' cardiomyopathy. N Engl J Med 2015; 373(14): 1295-1306.
[38] Duschak VG, Couto AS. In: Atta-ur-Rahman, Choudhry I, Eds. Frontiers in Anti-Infective Drug Discovery. Sharjah: Bentham Science Publishers 2010.
[39] Duschak VG. A decade of targets and patented drugs for chemotherapy of Chagas disease. Recent Pat Antiinfect Drug Discov 2011; 6: 216-59.
[40] El-Sayed NM, Myler PJ, Bartholomeu DC, Nilsson D, Aggarwal G, Tran AN, *et al.* The genome sequence of *Trypanosoma cruzi*, etiologic agent of Chagas disease. Science 2005; 309(5733): 409-15.
[41] Cazzulo JJ, Stoka V, Turk V. The major cysteine proteinase of *Trypanosoma cruzi*: A valid target for chemotherapy of Chagas disease. Curr Pharm Des 2001; 7(12): 1143-56.
[42] Alvarez VE, Niemirowicz GT, Cazzulo JJ. The peptidases of *Trypanosoma cruzi*: digestive enzymes, virulence factors, and mediators of autophagy and programmed cell death. Biochim Biophys Acta 2012; 1824(1): 195-206.
[43] Cazzulo JJ, Cazzulo Franke BM, Martinez J, Franke de Cazzulo BM. Some kinetic properties of a cysteine proteinase (cruzipain) from *Trypanosoma cruzi*. Biochim Byophys Acta 1990; 1037(2): 186-91.
[44] Eakin AE, Mills AA, Harth G, McKerrow JH, Craik CS. The sequence, organization, and expression of the major cysteine proteinase (cruzain) from *Trypanosoma cruzi.* J Biol Chem 1992; 267: 7411-20.
[45] Murta ACM, Persechini PM, de Souto Padrón T, de Souza W, Guimaraes JA, Scharfstein J. Structural and functional identification GP57/51 antigen of *Trypanosoma cruzi* as a cysteine proteinase. Mol Biochem Parasitol 1990; 43: 27-38.
[46] Cazzulo JJ, Labriola C, Parussini F, Duschak VG, Martinez J, de Cazzulo FBM. Cysteine proteinases in *Trypanosoma cruzi* and other Trypanosomatid parasites. Acta Chim Slov 1995; 42: 409-18.
[47] Parusini F, Duschak VG, Cazzulo JJ. Membrane-bound cysteine proteinase isoforms in different developmental stages of *Trypanosoma cruzi*. Cell Mol Biol 1998; 44(3): 513-9.
[48] Duschak VG, Barboza M, Garcia GA, Lammel EM, Couto AS, Isola EL. Novel cysteine proteinase in *Trypanosoma cruzi* metacyclogenesis. Parasitology 2006; 132(3): 345-55.
[49] Cazzulo JJ. Proteinases of *Trypanosoma cruzi*: Potential targets for the chemotherapy of Chagas disease. Curr Top Med Chem 2002; 2(11): 1261-71.
[50] Duschak VG, Ciaccio M, Nassert JR, Basombrio MA. Enzymatic activity, protein expression, and gene sequence of cruzipain in virulent and attenuated *Trypanosoma cruzi* strains. J Parasitol 2001; 87(5): 1016-22.
[51] Ramos AM, Duschak VG, Gerez de Burgos NM, Barboza M, Remedi MS, Vides MA, *et al*. *Trypanosoma cruzi*: Cruzipain and membranebound cysteine proteinase isoform(s) interacts with human alpha (2)-macroglobulin and pregnancy zone protein. Exp Parasitol 2002; 100(2): 121-30.
[52] Duschak VG, Riarte A, Segura EL, Laucella SA. Humoral immune response to cruzipain and cardiac dysfunction in chronic Chagas disease. Immunol Lett 2001; 78(3): 135-42.
[53] Barboza M, Duschak VG, Cazzulo JJ, Lederkremer RM, Couto AS. Presence of sialic acid in N-linked oligosaccharide chains and O-linked N-acetylglucosamine in cruzipain, the major cysteine proteinase of *Trypanosoma cruzi.* Mol Biochem Parasitol 2003; 126: 293-6.
[54] Acosta DM, Soprano LL, Ferrero M, Landoni M, Esteva MI, Couto AS, *et al*. A striking common O-linked N-acetylglucosaminyl moiety between cruzipain and myosin. Parasite Immunol 2011; 33(7): 363-70.
[55] Barboza M, Duschak VG, Fukuyama Y, Nonami H, Erra-Balsells R, Cazzulo JJ, *et al*. Structural analysis of the N-glycans of the major cysteine proteinase of *Trypanosoma cruzi.* Identification of sulfated high-mannose type oligosaccharides. FEBS J 2005; 272(15): 3803-15.
[56] Acosta DM, Arnaiz MR, Esteva MI, Barboza M, Stivale D, Orlando UD, *et al*. Sulfates are main targets of immune responses to cruzipain and are involved in heart damage in BALB/c immunized mice. Int Immunol 2008; 20: 461-70.
[57] Acosta DM, Soprano LL, Ferrero MR, Esteva MI, Riarte A, Couto AS, Duschak VG. Structural and immunological characterization of sulfatides: Relevance of sulphate moieties in *Trypanosoma cruzi* glycoconjugates. Parasite Immunol 2012; 34(11): 499-510.
[58] Couto AS, Soprano LL, Landoni M, Pourcelot M, Acosta DM, Bultel L, *et al*. An anionic synthetic sugar containing 6-SO3 -NAcGlc mimics the sulfated cruzipain epitope that plays a central role in immune recognition. FEBS J 2012; 279(19): 3665-79.
[59] Duschak VG, Couto AS. Cruzipain, the major cysteine protease of *Trypanosoma cruzi*: A sulfated glycoprotein antigen as relevant candidate for vaccine development and drug target: A review. Curr Med Chem 2009; 16: 3174-3202.
[60] Ferrero MR, Soprano LL, Acosta DM, García GA, Esteva MI, Couto AS, *et al*. Effects of chlorate on the sulfation process of *Trypanosoma cruzi* glycoconjugates. Implication of parasite sulfates in cellular invasion. Acta Trop. 2014; 161-73.
[61] Ferrero MR, Heins AM, Soprano LL, Acosta DM, Esteva MI, Jacobs T, *et al*. Involvement of sulfates from cruzipain, a major antigen of *Trypanosoma cruzi*, in the interaction with immunomodulatory molecule Siglec-E. Med Microbiol Immunol 2016; 205(1): 21-35.
[62] Duschak VG, Barboza M, Couto AS. *Trypanosoma cruzi:* partial characterization of minor cruzipain isoforms non-adsorbed to Concanavalin A-Sepharose. Exp Parasitol 2003; 104(3-4): 122-30.
[63] Yong V, Schmidtz V, Vannier-Santos MA, de Lima AP, Lalmanach G, Juliano L, *et al*. Altered expression of cruzipain and a cathepsin B-like target in a *Trypanosoma cruzi* cell line displaying resistance to synthetic inhibitors of cysteine-proteinases. Mol Biochem Parasitol 2000; 109(1): 47-59.
[64] García MP, Nóbrega, OT, Teixeira, ARL, Sousa MV, Santana, JM. Characterization of a *Trypanosoma cruzi* acidic 30 kDa cysteine protease. Mol Biochem Parasitol 1998; 91: 263-72.
[65] McGrath ME, Eakin AE, Engel JC, McKerrow JH, Craik CS, Fletterick RJ. The crystal structure of cruzain: A therapeutic target for Chagas disease. J Mol Biol 1995; 247(2): 251-9.
[66] Huang L, Brinen LS, Ellman JA. Crystal structures of reversible ketone-based inhibitors of the cysteine protease cruzain. Bioorg Med Chem 2003; 11(1): 21-9.

[67] Nicoll-Griffith DA. Use of cysteine-reactive small molecules in drug discovery for trypanosomal disease. Expert Opin Drug Discov 2012; 7(4): 353-66.
[68] Lima AP, Reis FC, Costa TF. Cysteine peptidase inhibitors in trypanosomatid parasites. Curr Med Chem 2013; 20(25): 3152-73.
[69] Lalmanach G, Mayer R, Serveau C, Scharfstein J, Gauthier F. Biotin-labelled peptidyl diazomethane inhibitors derived from the substrate-like sequence of cystatin: Targeting of the active site of cruzipain, the major cysteine proteinase of *Trypanosoma cruzi*. Biochem J 1996; 318: 395-9.
[70] Hernandez AA, Roush WR. Recent advances in the synthesis, design and selection of cysteine protease inhibitors. Curr Opin Chem Biol 2002; 6(4): 459-65.
[71] Arafet K, Ferrer S, Moliner V. First quantum mechanics/molecular mechanics studies of the inhibition mechanism of cruzain by peptidyl halomethyl ketones. Biochemistry 2015; 54(21): 3381-91.
[72] Engel JC, Doyle PS, Hsieh Y, McKerrow JH. Cysteine protease inhibitors cure an experimental *Trypanosoma cruzi* infection. J Exp Med 1998; 88(4): 725-34.
[73] Engel JC, Doyle PS, Palmer J, Bainton DF, Hsieh I, McKerrow JH. Cysteine protease inhibitors alter Golgi complex ultrastructure and function in *Trypanosoma cruzi*. J Cell Sci 1998; 11: 597-606.
[74] McKerrow JH. Development of cysteine protease inhibitors as chemotherapy for parasitic diseases: Insights on safety, target validation, and mechanism of action. Int J Parasitol 1999; 29(6): 833-37.
[75] Roush WR, Cheng J, Knapp-Reed B, Alvarez-Hernandez A, McKerrow JH, Hansell E, *et al*. Potent second generation vinyl sulfonamide inhibitors of the Trypanosomal cysteine protease cruzain. Bioorg Med Chem Lett 2001; 11: 2759-62.
[76] Gotz MG, Caffrey CR, Hansell E, Mc Kerrow JH, Powers JC. Peptidyl allyl sulfones: A new class of inhibitors for clan CA cysteine proteases. Bioorg Med Chem 2004; 12(19): 5203-11.
[77] Powers, J.C., Gotz, M.G. Peptidyl allyl sulfones. US7521427B2 **(2009)**.
[78] Doyle PS, Zhou YM, Engel JC, McKerrow JH. A cysteine protease inhibitor cures Chagas disease in an immunodeficient-mouse model of infection. Antimicrob Agents Chemother 2007; 51(11): 3932-9.
[79] McKerrow JH, Doyle PS, Engel JC, Podust LM, Robertson SA, Ferreira R, *et al*. Two approaches to discovering and developing new drugs for Chagas disease. Mem Inst O. Cruz 2009; 104 Suppl 1: 263-9.
[80] Chen YT, Lira R, Hansell E, McKerrow JH, Roush WR. Synthesis of macrocyclic Trypanosomal cysteine protease inhibitors. Bioorg Med Chem Lett 2008; 18(22): 5860-3.
[81] Kerr ID, Lee JH, Farady CJ, Marion R, Rickert M, Sajid M, *et al*. Vinyl sulfones as antiparasitic agents and a structural basis for drug design. J Biol Chem 2009; 284(38): 25697-703.
[82] Barr SC, Warner KL, Kornreic BG, Piscitelli J, Wolfe A, Benet L, *et al*. A cysteine protease inhibitor protects dogs from cardiac damage during infection by *Trypanosoma cruzi*. Antimicrob Agents Chemother 2005; 49(12): 5160-1.
[83] Chen YT, Brinen LS, Kerr ID, Hansell E, Doyle PS, McKerrow JH, *et al*. *In vitro* and *in vivo* studies of the trypanocidal properties of WRR-483 against *Trypanosoma cruzi.* Plos Negl Trop Dis 2010; 4(9): pii: e825.
[84] Jones BD, Tochowicz A, Tang Y, Cameron MD, McCall LI, Hirata K, *et al*. Synthesis and evaluation of oxyguanidine analogues of the cysteine protease inhibitor WRR-483 against Cruzain. ACS Med Chem Lett 2015; 7(1):77-82.
[85] Lee GM, Balouch E, Goetz DH, Lazic A, McKerrow JH, Craik CS. Mapping inhibitor binding modes on an active cysteine protease via nuclear magnetic resonance spectroscopy. Biochemistry 2012; 51(50): 10087-98.
[86] Fennell BD, Warren JM, Chung KK, Main HL, Arend AB, Tochowicz A, *et al*. Optimization of peptidyl allyl sulfones as clan CA cysteine protease inhibitors. J Enzyme Inhib Med Chem 2013; 28(3): 468-78.
[87] Li R, Chen X, Gong B, Selzer PM, Li Z, Davidson E, Kurzban G, *et al*. Structure-based design of parasitic protease inhibitors. Bioorg Med Chem 1996; 4(9): 1421-7.
[88] Du X, Hansell E, Engel JC, Caffrey CR, Cohen FE, McKerrow JH. Aryl ureas represent a new class of antitrypanosomal agents. Chem Biol 2000; 7(9): 733-42.
[89] Huang L, Lee A, Ellman JA. Identification of potent and selective mechanism-based inhibitors of the cysteine protease cruzain using solid-phase parallel synthesis. J Med Chem 2002; 45(3): 676-84.
[90] Choe Y, Brinen LS, Price MS, Engel JC, Lange M, Grisostomi C, *et al*. Development of alpha-keto-based inhibitors of cruzain, a cysteine protease implicated in Chagas disease. Bioorg Med Chem 2005; 13(6): 2141-56.
[91] Marquis, R.W., Ru, Y., Veber, D.F., Cummings, M., Thompson, S.K., Yamashita, D. Protease inhibitors. WO0195911 **(2001)**.
[92] Quibell, M., Taylor, S., Grabowska, U., Nilsson, M., Morrison, V. Cysteine protease inhibitors. WO0240462A2 and WO0240462A3 (**2002**).
[93] Quibell, M. Ramjee, M.K. Inhibitors of cruzipain and other cysteine proteases. WO02057246A2 **(2002)** and WO02057246A3 **(2002)**.
[94] Quibell, M. Inhibitors of cruzipain and other cysteine proteases WO02057248A2/A3 **(2002)** WO02057270A1 **(2002)**, CA2436462AA **(2002)**; Cyclic 2-carbonylaminoketones as inhibitors of cruzipain and other cysteine proteases. WO02057249A1 **(2002)**.
[95] Quibell, M. Inhibitors of cruzipain and other cysteine proteases. EP1362052A1 **(2003),** NO20033220A **(2003),** NO20033220A0 **(2003)**.
[96] Quibell, M. Inhibitors of cruzipain and other cysteine proteases. CN1486320A **(2004),** MX3006224A **(2004),** ZA0305259A **(2004)**. NZ0526913A **(2004)**.
[97] Quibell, M. Ray, P.C., Watts, J.P. Inhibitors of cruzipain and other cysteine proteases. WO04007501A1 **(2004)**.
[98] Lim-Wilb, M., Semple, J.E., Araldi, G.L., Goldman, E.A., Weinhouse, M.I. Compounds, compositions and methods for treatment of parasitic diseases. WO0248097A1 **(2003)**, WO0248097B1 **(2003)**, WO0248097C2 **(2003)**.
[99] Quibell, M. Ramjee, M.K. Inhibitors of cruzipain and other cysteine proteases. US20056958358 **(2005)**.
[100] Quibell, M. Inhibitors of cruzipain and other cysteine proteases. US7425562 **(2008)**.
[101] Marquis, R.W., Jr; Veber, D.F.; Yamashita, D.S.: Protease inhibitors. WO03053331A2/A3 **(2003)**.
[102] Jeong, J.U.; Yamashita, D.S.: Protease inhibitors. WO03103574A2/A3 **(2003)**, WO03104257A2/A3 **(2003)**, WO03097593A2/A3 **(2003)**.
[103] Tew, D.G., Thompson, S.K., Veber, D.F. Protease inhibitors. WO00217924A1 **(2002)**.
[104] Altman, E., Betschart, C., Hayakawa, K., Osamu, I., Junichi, S., Genji, I., René, L., Martin, M., Naoki, T. Hetroaryl nitrile derivatives. WO04020441A1 **(2004)**.
[105] Bondebjerg, J. Fuglsang, H., Naerum, L. Protease inhibitors. WO04110988A1 **(2004)**.
[106] Hickey, E.R., Bekkali, Y., Patel, U.R., Spero, D.M., Thomson, D.S., Young, E.R. Novel nitriles useful as reversible inhibitors of cysteine proteases. WO02100849A2/A3 **(2002)**.
[107] Hickey, E.R., Bekkali, Y., Patel, U.R., Spero, D.M., Thomson, D.S., Young, E.R. Nitriles useful as reversible inhibitors of cysteine proteases. WO02100849A3 **(2002)**, US6982263 **(2006)**.
[108] Sulea, T., Purisima, E.O., Menard, R., Wang, J. Non-covalent inhibitors of cysteine proteases with a tripeptide backbone. WO03097664A2/A3 **(2003)**.
[109] Beaulieu C, Isabel E, Fortier A, Massé F, Mellon C, Méthot N, *et al*. Identification of potent and reversible cruzipain inhibitors for the treatment of Chagas disease. Bioorg Med Chem 2010; 20(24): 7444-9.
[110] Mott BT, Ferreira RS, Simeonov A, Jadhav A, Ang KK, Leister W, *et al*. Identification and optimization of inhibitors of Trypanosomal cysteine proteases: Cruzain, rhodesain, and TbCatB. J Med Chem 2010; 53(1): 52-60.

[111] Méndez-Lucio O, Romo-Mancillas A, Medina-Franco JL, Castillo R. Computational study on the inhibition mechanism of cruzain by nitrile-containing molecules. J Mol Graph Model 2012; 35: 28-35.

[112] Ndao M, Beaulieu C, Black WC, Isabel E, Vasquez-Camargo F, Nath-Chowdhury M, *et al*. Reversible cysteine protease inhibitors show promise for a Chagas disease cure. Antimicrob Agents Chemother 2014; 58(2): 1167-78.

[113] Avelar LA, Camilo CD, de Albuquerque S, Fernandes WB3, Gonçalez C2, Kenny PW, *et al*. Molecular design, synthesis and trypanocidal activity of dipeptidyl nitriles as cruzain inhibitors. PLoS Negl Trop Dis 2015; 9(7): e0003916.

[114] Du X, Guo C, Hansell E, Doyle PS, Caffrey CR, Holler TP, *et al*. Synthesis and structure-activity relationship study of potent trypanocidal thio semicarbazone inhibitors of the Trypanosomal cysteine protease cruzain. J Med Chem 2002; 45(13): 2695-707.

[115] Chibale K, Musonda CC. The synthesis of parasitic cysteine protease and trypanothione reductase inhibitors. Curr Med Chem 2003; 10(18): 1863-89.

[116] Chiyanzu I, Hansell E, Gut J, Roshenthal PJ, McKerrow JH, Chibale K. Synthesis and evaluation of isatins and thiosemicarbazone derivatives against cruzain, falcipain-2 and rhodesain. Bioorg Med Chem Lett 2003; 13(20): 3527-30.

[117] Fujii N, Mallari JP, Hansell EJ, Mackey Z, Doyle P, Zhou YM, *et al*. Discovery of potent thiosemicarbazone inhibitors of rhodesain and cruzain. Bioorg Med Chem Lett 2005; 15(1): 121-3.

[118] Engel JC, Garcia CT, Hsieh I, Doyle PS, McKerrow JH. Upregulation of the secretory pathway in cysteine protease inhibitor-Resistant *Trypanosoma cruzi*. J Cell Sci 2000; 113: 1345-54.

[119] Desai PV, Patny A, Sabnis Y, Tekwani B, Gut J, Rosenthal P, *et al*. Identification of novel parasitic cysteine protease inhibitors using virtual screening 1. The ChemBridge database. J Med Chem 2004; 47(26): 6609-15.

[120] Greenbaum DC, Mackey Z, Hansell E, Doyle P, Gut J, Caffrey CR, Lehrman J, *et al*. Synthesis and structure-activity relationships of parasiticidal thiosemicarbazone cysteine protease inhibitors against *Plasmodium falciparum, Trypanosoma brucei,* and *Trypanosoma cruzi.* J Med Chem 2004; 47(12): 3212-9.

[121] Cohen, F.E., Du, X., Guo, Ch., McKerrow, J.H. Thio semicarbazone and semicarbazone inhibitors of cysteine proteases and methods of their use. US6897240 **(2005),** US7495023 **(2009)**.

[122] Aguirre G, Boiani L, Cerecetto H, Fernández M, González M, Denicola A, *et al*. *In vitro* activity and mechanism of action against the protozoan parasite *Trypanosoma cruzi* of 5-nitrofuryl containing thiosemicarbazones. Bioorg Med Chem 2004; 12(18): 4885-93.

[123] Otero L, Maya JD, Morello A. Insight into the bioreductive mode of action of anti-trypanosomal 5-nitrofuryl containing thiosemicarbazones. Med Chem 2008; 4(1): 11-7.

[124] Caputto ME, Fabian LE, Benítez D, Merlino A, Ríos N, Cerecetto H, *et al*. Thiosemicarbazones derived from 1-indanones as new anti-*Trypanosoma cruzi* agents. Bioorg Med Chem 2011; 19(22): 6818-26.

[125] Blau L, Menegon RF, Trossini GH, Molino JV, Vital DG, Cicarelli RM, *et al*. Design, synthesis and biological evaluation of new aryl thiosemicarbazone as antichagasic candidates. Eur J Med Chem 2013; 67: 142-51.

[126] Magalhaes Moreira DR, de Oliveira AD, Teixeira de Moraes Gomes PA, de Simone CA, Villela FS, Ferreira RS, *et al*. Conformational restriction of aryl thiosemicarbazones produces potent and selective anti-*Trypanosoma cruzi* compounds which induce apoptotic parasite death. Eur J Med Chem 2014; 75: 467-78.

[127] Fonseca NC, da Cruz LF, da Silva Villela F, do Nascimento Pereira GA, de Siqueira-Neto JL, Kellar D, *et al*. Synthesis of a sugar-based thiosemicarbazone series and structure-activity relationship versus the parasite cysteine proteases *Rhodesain, Cruzain*, and *Schistosoma mansoni Cathepsin* B1. Antimicrob Agents Chemother 2015; 59(5): 2666-77.

[128] Espíndola JW, Cardoso MV, Filho GB, Oliveira E Silva DA, Moreira DR. TM- Synthesis and structure-activity relationship study of a new series of antiparasitic aryloxyl thiosemicarbazones inhibiting *Trypanosoma cruzi* cruzain. Eur J Med Chem 2015; 101: 818-35.

[129] Leite AC, de Lima RS, Moreira DR, Cardoso MV, Gouveia de Brito AC, Farias Dos Santos LM, *et al*. Synthesis, docking, and *in vitro* activity of thiosemicarbazones, aminoacyl-thiosemicarbazides and acyl-thiazolidones against *T. cruzi*. Bioorg Med Chem 2006; 14(11): 3749-57.

[130] Leite AC, Moreira DR, Cardoso MV, Hernandes MZ, Alves Pereira VR, Silva RO, *et al*. Synthesis, cruzain docking, and *in vitro* studies of aryl-4-oxothiazolylhydrazones against *Trypanosoma cruzi*. Chem Med Chem 2007; 2(9): 1339-45.

[131] Hernandes MZ, Rabello MM, Leite AC, Cardoso MV, Moreira DR, Brondani DJ, *et al*. Studies toward the structural optimization of novel thiazolylhydrazone-based potent antitrypanosomal agents. Bioorg Med Chem 2010; 18(22): 7826-35.

[132] Pizzo C, Saiz C, Talevi A, Gavernet L, Palestro P, Bellera C, B, *et al*. Synthesis of 2-hydrazolyl-4-thiazolidinones based on multicomponent reactions and biological evaluation against *Trypanosoma cruzi*. Chem Biol Drug Des 2011; 77(3): 166-72.

[133] Cardoso MV, de Siqueira LR, da Silva EB, Costa LB, Hernandes MZ, Rabello MM, *et al*. 2-Pyridyl thiazoles as novel anti-*Trypanosoma cruzi* agents: Structural design, synthesis and pharmacological evaluation. Eur J Med Chem 2014; 86: 48-59.

[134] Moreira L.L., De lacerda B.E. J., Amaral A.M. Hydrazide-n-acylhydrazone compounds, method for producing hydrazide-n-acylhydrazone compounds, use of intermediate compounds for producing hydrazide-n-acylhydrazones fof the treatment of leishmaniasis and Chagas disease, and thus obtained pharmaceutical compositions. WO2014019044A1 **(2014)**.

[135] Massarico Serafim RA, Gonçalves JE, de Souza FP, de Melo Loureiro AP, Storpirtis S, Krogh R, *et al*. Design, synthesis and biological evaluation of hybrid bioisoster derivatives of N-acylhydrazone and furoxan groups with potential and selective anti-*Trypanosoma cruzi* activity. Eur J Med Chem. 2014; 82: 418-25.

[136] Cupertino da Silva JK, Moreno Reyes CT, Rivera G, Alves MA, Barreiro EJ, Moreira MS, *et al*. 3-Aminothiophene-2-acylhydrazones: Non-toxic, analgesic and anti-inflammatory lead-candidates. Molecules 2014; 19(6): 8456-71.

[137] Porcal W, Hernández P, Boiani M, Aguirre G, Boiani L, Chidichimo A, *et al*. *In vivo* anti-Chagas vinylthio, vinylsulfinyl-, and vinylsulfonylbenzofuroxan derivatives. J Med Chem 2007; 50(24): 6004-15.

[138] Brak K, Doyle PS, McKerrow JH, Ellman JA. Identification of a new class of nonpeptidic inhibitors of cruzain. J Am Chem Soc 2008; 130(20): 6404-10.

[139] Brak K, Kerr ID, Barrett KT, Fuchi N, Debnath M, Ang K, *et al*. Nonpeptidic tetrafluorophenoxymethyl ketone cruzain inhibitors as promising new leads for Chagas disease chemotherapy. J Med Chem 2010; 53(4): 1763-73.

[140] Fricker SP, Mosi RM, Cameron BR, Baird I, Zhu Y, Anastassov V, *et al*. Metal compounds for the treatment of parasitic diseases. J Inorg Biochem 2009; 103(5): 869-75.

[141] Bastos TM, Barbosa MI, da Silva MM, da C Júnior JW, Meira CS, Guimaraes ET, *et al*. Nitro/nitrosyl-ruthenium complexes are potent and selective anti-*Trypanosoma cruzi* agents causing autophagy and necrotic parasite death. Antimicrob Agents Chemother 2014; 58(10): 6044-55.

[142] Lavrado J, Mackey Z, Hansell E, McKerrow JH, Paulo A, Moreira R. Antitrypanosomal and cysteine protease inhibitory activities of alkyldiamine cryptolepine derivatives. Bioorg Med Chem Lett 2012; 22(19): 6256-60.

[143] Trossini GH, Malvezzi A, T-do Amaral A, Rangel-Yagui CO, Izidoro MA, Cezari MH, *et al*. Cruzain inhibition by hydroxymethylnitrofurazone and nitrofurazone: investigation of a new target in *Trypanosoma cruzi.* J Enzyme Inhib Med Chem 2010; 25(1): 62-7.

[144] Aguirre G, Cerecetto H, Di Maio R. Quinoxaline N, N'-dioxide derivatives and related compounds as growth inhibi-

tors of *T. cruzi*. Structure-activity relationships. Bioorg Med Chem 2004; 14(14): 3835-9.

[145] Romeiro NC, Aguirre G, Hernández P, González M, Cerecetto H, Aldana I, *et al*. Synthesis, trypanocidal activity and docking studies of novelquinoxaline-N-acylhydrazones, designed as cruzain inhibitors candidates. Bioorg Med Chem 2009; 17(2): 641-52

[146] Ferreira RS, Bryant C, Ang KK, McKerrow JH, Shoichet BK. Renslo AR Divergent modes of enzyme inhibition in a homologous structure-activity series. J Med Chem 2009; 52(16): 5005-8.

[147] Ríos N, Varela J, Birriel E, González M, Cerecetto H, Merlino A, *et al*. Identification of novel benzimidazole derivatives as anti-*Trypanosoma cruzi* agents: Solid-phase synthesis, structure-activity relationships and molecular docking studies. Future Med Chem 2013; 5(15): 1719-32.

[148] Wiggers HJ, Rocha JR, Fernandes WB, Sesti-Costa R, Carneiro ZA, Cheleski J, *et al*. Non-peptidic cruzain inhibitors with trypanocidal activity discovered by virtual screening and *in vitro* assay. PLoS Negl Trop Dis 2013; 7(8):e2370.

[149] Bellera CL, Balcazar DE, Alberca L, Labriola CA, Talevi A, Carrillo C. Application of computer-aided drug repurposing in the search of new cruzipain inhibitors: Discovery of amiodarone and bromocriptine inhibitory effects. J Chem Inf Model 2013; 53(9): 2402-8.

[150] Bellera CL, Balcazar DE, Alberca L, Labriola CA, Talevi A, Carrillo C. Identification of levothyroxine antichagasic activity through computer-aided drug repurposing. Scientific World J 2014; 30; 2014: 279618.

[151] Black C., Beaulieu C., Cathepsin cysteine protease inhibitors for the treatment of various diseases. US20120101053A1, EP2445872A1, EP2445872A4, WO2010148488A1 **(2012)**.

[152] Craig, T., Mott B.T., Maloney, D.J., Simeonov A., Jadhav, A. Substituted triazine and purine compounds, methods of inhibiting Cruzain and Rhodesain and methods of treating chagas disease and African trypanosomiasis. WO2010059418**A1 (2010)**.

[153] Elise I., Mellon C., Beaulieu C. Cysteine protease inhibitors for the treatment of parasitic disease. EliseCA 20100305056 **(2010)**, CA20130244962 **(2013)**, CA8642799 **(2014)**.

[154] Bilgin M, Neuhof C, Doerr O, Benscheid U, Andrade SS, Most A, *et al*. *Bauhinia bauhinioides* cruzipain inhibitor reduces endothelial proliferation and induces an increase of the intracellular Ca2+ concentration. J Physiol Biochem 2010; 66(4): 283-90.

[155] Bourguignon SC, Castro HC, Santos DO, Alves CR, Ferreira VF, Gama IL, *et al*. *Trypanosoma cruzi*: *In vitro* activity of Epoxy-alpha-Lap, a derivative of alpha-lapachone, on trypomastigote and amastigote forms. Exp Parasitol 2009; 122(2): 91-6.

[156] Bourguignon SC, Cavalcanti DF, de Souza AM. *Trypanosoma cruzi*: Insights into naphthoquinone effects on growth and proteinase activity. Exp Parasitol 2011; 127(1): 160-6.

[157] Martinez-Mayorga K, Byler KG, Ramirez-Hernandez AI, Terrazas-Alvares DE. Cruzain inhibitors: Efforts made current leads and a structural outlook of new hits. Drug Discov Today 2015; 20(7): 890-8.

[158] Sorimachi H, Hata S, Ono Y. Expanding members and roles of the calpain superfamily and their genetically modified animals. Exp Anim 2010; 59(5): 549-56.

[159] Giese V, Dallagiovanna B, Mancini FK. *Trypanosoma cruzi*: A stage-specific calpain-like protein is induced after various kinds of stress. Mem Inst O Cruz 2008; 103(6): 598-601.

[160] Kosec G, Alvarez V, Cazzulo JJ. Cysteine proteinases of *Trypanosoma cruzi*: From digestive enzymes to programmed cell death mediators. Biocell 2006; 30(3): 479-90.

[161] Sangenito LS, Ennes-Vidal V, Marinho FA, Da Mota FF, Santos AL, D'Avila-Levy CM, *et al*. Arrested growth of *Trypanosoma cruzi* by the calpain inhibitor MDL28170 and detection of calpain homologues in epimastigote forms. Parasitology 2009; 136(4): 433-41.

[162] Ennes-Vidal V, Menna Barreto RF, Santos AL. Effects of the calpain inhibitor MDL28170 on the clinically relevant forms of *Trypanosoma cruzi in vitro*. J Antimicrob Chemother 2010; 65(7): 1395-8.

[163] Ennes-Vidal V, Menna-Barreto RF, Santos AL, Branquinha MH, d'Avila-Levy CM. MDL28170, a calpain inhibitor, affects *Trypanosoma cruzi* metacyclogenesis, ultrastructure and attachment to Rhodnius prolixus midgut. PLoS One 2011; 6(4): e18371.

[164] Galetović A, Souza RT, Santos MR, Cordero EM, Bastos IM, Santana JM, *et al*. The repetitive cytoskeletal protein H49 of *Trypanosoma cruzi* is a calpain-like protein located at the flagellum attachment zone. PLoS One 2011; 6(11): e27634.

[165] Branquinha MH, Marinho FA, Sangenito LS, Oliveira SS, Goncalves KC, Ennes-Vidal V, *et al*. Calpains: Potential targets for alternative chemotherapeutic intervention against human pathogenic trypanosomatids. Curr Med Chem 2013; 20(25): 3174-85.

[166] Burleigh BA, Andrews N. A 120-kDa alkaline peptidase from *Trypanosoma cruzi* is involved in the generation of a novel Ca2+-signaling factor for mammalian cells. J Biol Chem 1995; 270(10): 5172-80.

[167] Burleigh BA, Caler EV, Webster P, Andrews NW. A cytosolic serine endopeptidase from *Trypanosoma cruzi* is required for the generation of Ca2+ signaling in mammalian cells. J Cell Biol 1997; 136(3): 609- 20.

[168] Santana JM, Grellier P, Schrevel J, Teixeira AR. A *Trypanosoma cruzi*-secreted 80 kDa proteinase with specificity for human collagen types I and IV. Biochem J 1997; 325(1): 129-37.

[169] Vendeville S, Bourel L, Davioud-Charvet E, Grellier P, Deprez B, Sergheraert C. Automated parallel synthesis of a tetrahydroisoquinolin-based library: Potential prolyl endopeptidase inhibitors. Bioorg Med Chem Lett 1999; 9: 437-42.

[170] Joyeau R, Maoulida C, Guillet C, Frappier F, Teixeira AR, Schrével J, *et al*. Synthesis and activity of pyrrolidinyl- and thiazolinidyl-dipeptide derivatives as inhibitors of the Tc80 prolyl oligopeptidase from *Trypanosoma cruzi*. Eur J Med Chem 1999; 35: 257-66.

[171] Grellier P, Vendeville S, Joyeau R, Bastos IM, Drobecq H, Frappier F, *et al*. *Trypanosoma cruzi* prolyl oligopeptidase *Tc*80 is involved in nonphagocytic mammalian cell invasion by trypomastigotes. J Biol Chem 2001; 276: 47078-86.

[172] Silva-Lopez RE, Morgado-Díaz JA, dos Santos PT, Giovanni-De-Simone S. Purification and subcellular localization of a secreted 75 kDa *Trypanosoma cruzi* serine oligopeptidase. Acta Trop 2008; 107(2): 159-67.

[173] Bal G, Van der Veken P, Antonov D, Lambeir AM, Grellier P, Croft SL, *et al*. Prolylisoxazoles: Potent inhibitors of prolyloligopeptidase with anti-trypanosomal activity. Bioorg Med Chem Lett 2003; 13(17): 2875-8.

[174] Bastos IM, Grellier P, Martins NF, Cadavid-Restrepo G, de Souza-Ault MR, Augustyns K, *et al*. Molecular, functional and structural properties of the prolyl oligopeptidase of *Trypanosoma cruzi* (POP *Tc*80), which is required for parasite entry into mammalian cells. Biochem J 2005; 388(1): 29-38.

[175] Bastos IM, Motta FN, Grellier P, Santana JM. Parasite prolyl oligopeptidases and the challenge of designing chemotherapeuticals for Chagas disease, leishmaniasis and African trypanosomiasis. Curr Med Chem 2013; 20(25): 3103-15.

[176] Cuevas IC, Cazzulo JJ, Sanchez D. gp63 homologues in *Trypanosoma cruzi*: surface antigens with metalloprotease activity and a possible role in host cell infection. Infect Immun 2003; 71(10): 5739-49.

[177] Niemirowicz G, Parussini F, Agüero F, Cazzulo JJ. Two metallocarboxypeptidases from the protozoan *Trypanosoma cruzi* belong to the M32 family, found so far only in prokaryotes. Biochem J 2007; 401(2): 399-410.

[178] Niemirowicz G, Fernández D, Solà M, Cazzulo JJ, Avilés FX, Gomis-Rüth FX. The molecular analysis of *Trypanosoma cruzi* metallocarboxypeptidase 1 provides insight into fold and substrate specificity. Mol Microbiol 2008; 70(4): 853-66.

[179] Frasch AP, Carmona AK, Juliano L, Cazzulo JJ, Niemirowicz GT. Characterization of the M32 metallocarboxypeptidase of *Trypanosoma brucei*: Differences and similarities

with its orthologue in *Trypanosoma cruzi.* Mol Biochem Parasitol 2012; 184(2): 63-70.

[180] Cadavid-Restrepo G, Gastardelo TS, Faudry E, de Almeida H, Bastos IM, Negreiros RS, *et al*. The major leucyl aminopeptidase of *Trypanosoma cruzi* (LAPTc) assembles into a homohexamer and belongs to the M17 family of metallopeptidases. BMC Biochem 2011; 12: 46-61.

[181] Geurts N, Opdenakker G, Van den Steen PE. Matrix metalloproteinases as therapeutic targets in protozoan parasitic infections. Pharmacol Ther 2012; 133(3): 257-79.

[182] Pinho RT, Beltramini LM, Alves CR, De-Simone SG. *Trypanosoma cruzi*: isolation and characterization of aspartyl proteases. Exp Parasitol 2009; 122(2): 128-33.

[183] Pinho RT, Beltramini LM, Alves CR, De-Simone SG. *Trypanosoma cruzi*: Isolation and characterization of aspartyl proteases. Curr Med Chem 2013; 20(25): 3116-33.

[184] Santos LO, Garcia-Gomes AS, Catanho M, Sodre CL, Santos AL, Branquinha MH, *et al*. Aspartic peptidases of human pathogenic trypanosomatids: Perspectives and trends for chemotherapy. Exp Parasitol 2009; 122(2): 128-33.

[185] Santos LO, Garcia-Gomes AS, Catanho M, Sodre CL, Santos AL, Branquinha MH, *et al*. Aspartic peptidases of human pathogenic trypanosomatids: Perspectives and trends for chemotherapy. Curr Med Chem 2013; 20(25): 3116-33.

[186] Sangenito LS, Gonçalves KC, Abi-Chacra EA, Sodré CL, d'Avila-Levy CM, Branquinha MH, *et al*. Multiple effects of pepstatin-A on*Trypanosoma cruzi* epimastigote forms. Parasitol Res 2012; 110(6): 2533-40.

[187] Paugam A, Bulteau AL, Dupuoy-Camet J, Creuzet C, Friguet B. Characterization and role of protozoan parasite proteasomes. Trends Parasitol 2003; 19(2): 55-9.

[188] González J, Ramalho-Pinto FJ, Frevert U, Ghiso J, Tomlinson S, Scharfstein J, *et al*. Proteasome activity is required for the stage-specific transformation of a protozoan parasite. J Exp Med 1996; 184(5): 1909-18.

[189] de Diego JL, Katz JM, Marshall P, Gutiérrez B, Manning JE, Nussenzweig V, *et al*. The ubiquitin-proteasome pathway plays an essential role in proteolysis during *Trypanosoma cruzi* remodeling. Biochemistry 2001; 40(4): 1053-62.

[190] Cardoso J, Soares MJ, Menna-Barreto RF, Le Bloas R, Sotomaior V, Goldenberg S, *et al*. Inhibition of proteasome activity blocks *Trypanosoma cruzi* growth and metacyclogenesis. Parasitol Res 2008; 103(4): 941-51.

[191] Palladino, M., Neuteboom, S.T.C., Macherla, V.R.R., Potts, B.C. Use of [3.2.0] heterocyclic compounds and analogs thereof for the treatment of cancer, inflammation and infectious diseases. WO2005002572A2/A3 **(2005)**.

[192] Steverding D, Spackman RW, Royle HJ, Glenn RJ. Trypanocidal activities of trileucine methyl vinyl sulfone proteasome inhibitors. Parasitol Res 2005; 95(1): 73-6.

[193] Barboza NR, Cardoso J, de Paula Lima CV, Soares MJ, Gradia DF, Hangai NS, *et al*. Expression profile and subcellular localization of HslV, the proteasome related protease from *Trypanosoma cruzi.* Exp Parasitol 2012; 130(2): 171-7.

[194] Eichinger, D., Gonzalez, J., Nussenzweig, V. Sinnis Photini Method for treating parasitic diseases with proteasome inhibitors. WO1998010779A1 **(1998)**.

[195] Rawlings ND. Unusual phyletic distribution of peptidases as a tool for identifying potential drug targets. Biochem J 2007; 401(2): e5-7.

[196] Alvarez VE, Niemirowicz GT, Cazzulo JJ. Metacaspases, autophagins and metallocarboxypeptidases: Potential new targets for chemotherapy of the trypanosomiases. Curr Med Chem 2013; 20(25): 3069-77.

[197] Dunn W.A., JR., Akin D.E., Progulske-Fox A.D.A., Methods and compositions for the treatment of cancers and pathogenic infections. WO2009089147A2 **(2009),** WO2009089147A3 **(2009),** US8545841 B2 **(2013)**.

[198] Quinones W, Urbina JA, Dubourdieu M, Luis Concepcion J. The glycosome membrane of *Trypanosoma cruzi* epimastigotes: protein and lipid composition. Exp Parasitol 2004; 106: 135-49.

[199] Urbina JA. Chemotherapy of Chagas disease. Curr Pharm Des 2002; 8(4): 287-95.

[200] Urbina JA, Docampo R. Specific chemotherapy of Chagas disease: Controversies and advances. Trends Parasitol 2003a; 19(11): 495-501.

[201] Lepesheva GI, Waterman, MR. CYP51-the omnipotent P450. Mol Cell Endocrinol 2004; 215: 165-70.

[202] Lepesheva GI, Zaitseva NG, Nes WD, *et al*. CYP51 from *Trypanosoma cruzi*: A phyla-specific residue in the B' helix defines substrate preferences of sterol 14alpha-demethylase. J Biol Chem 2006; 281(6): 3577-85.

[203] Lepesheva GI, Waterman MR. Sterol 14alpha-demethylase cytochrome P450 (CYP51), a P450 in all 09biological kingdoms. Biochim Biophys Acta 2007; 1770 (3): 467-77.

[204] Hanskins EG, Gillespie JR, Aikenhead K, Buckner FS. Upregulation of sterol C14-demethylase expression in *Trypanosoma cruzi* treated with sterol biosynthesis inhibitors. Mol Biochem Parasitol 2005; 144 (1): 68-75.

[205] Chen CK, Leung SS, Guilbert C, Jacobson MP, McKerrow JH, Podust LM. Structural characterization of CYP51 from *Trypanosoma cruzi* and *Trypanosoma brucei* bound to the antifungal drugs posaconazole and fluconazole. Plos Negl. Trop dis 2010b; 4(4): e651.

[206] Lepesheva GI, Hargrove TY, Anderson S, Kleshchenko Y, Furtak V, Wawrzak Z, *et al*. Structural insights into inhibition of sterol 14alpha-demethylase in the human pathogen *Trypanosoma cruzi*. J Biol Chem 2010; 285(33): 25582-90.

[207] Hargrove TY, Wawrzak Z, Liu J, Waterman MR, Nes WD, Lepesheva GI. Structural complex of sterol 14α-demethylase (CYP51) with 14α-methylenecyclopropyl-Delta7-24, 25-dihydrolanosterol. J Lipid Res 2012; 53(2): 311-20.

[208] Gunatilleke SS, Calvet CM, Johnston JB, Chen CK, Erenburg G, Gut J, *et al*. Diverse inhibitor chemotypes targeting *Trypanosoma cruzi* CYP51. PLoS Negl Trop Dis 2012; 6(7): e1736.

[209] Urbina JA, Payares G, Molina JT, Sanoja C, Liendo A, Lazardi K, *et al*. Cure of short-and long-term experimental Chagas disease using D0870. Science 1996; 273: 969-71.

[210] Urbina JA, Payares G, Contreras LM. Antiproliferative effects and mechanism of action of SCH56592 against *Trypanosoma cruzi*: *In vitro* and *in vivo* studies. Antimicrob Agents Chemother 1998; 42: 1771-7.

[211] Urbina JA, Payares C, Sanoja, Molina J, Lira R, Brener Z, *et al*. Parasitological cure of acute and chronic experimental Chagas disease using a long-acting experimental triazole TAK-187. Activity against drug-resistant *Trypanosoma cruzi* strains. Int J Antimicrob Agents 2003a; 21: 39-48.

[212] Urbina JA, Lira R, Visbal G, Bartroli J. *In vitro* antiproliferative effects and mechanism of action of the new triazole derivative UR-9825 against the protozoan parasite *Trypanosoma (Schizotrypanum) cruzi.* Antimicrob Agents Chemother 2000; 44(9): 2498-502.

[213] Urbina JA, Payares G, Sanoja C, Lira R, Romanha AJ. *In vitro* and *in vivo* activities of ravuconazole on *Trypanosoma cruzi*, the causative agent of Chagas disease. Int J Antimicrob Agents 2003; 21(1): 27-38.

[214] Apt W, Aguilera X, Arribada A, Pérez C, Miranda C, Sánchez G, *et al*. Treatment of chronic Chagas' disease with itraconazole and allopurinol. Am J Trop Med Hyg 1998; 59: 133-8.

[215] Guedes PM, Urbina JA, de Lana M, Afonso LC, Veloso VM, Tafuri WL, *et al*. Activity of the new derivative Albuconazole against *Trypanosoma cruzi* in dog hosts. Antimicrob Agents Chemoter 2004; 48(11): 4286-92.

[216] Buckner FS, Wilson AJ, White TC, Van Vooris WC. Induction of resistance to azole drugs in *Trypanosoma cruzi*. Antimicrob Agents Chemother 1998; 42(12): 3245-50.

[217] Buckner F, Yokoyama K, Lockman J, Aikenhead K, Ohkanda J, Sadilek M, *et al.* A class of sterol 14-demethylase inhibitors as anti- *Trypanosoma cruzi* agents. Proc Natl Acad Sci USA 2003; 100(25): 15149-53.

[218] Lepesheva GI, Hargrove TY, Kleshchenko Y, Nes WD, Villalta F, Waterman MR. CYP51: A major drug target in the cytochrome P450 superfamily. Lipids 2008; 43(12): 1117-25.

[219] Benaim G, Sanders JM, Garcia-Marchán Y, Colina C, Lira R, Caldera AR, *et al.* Amiodarone has intrinsic anti-

Trypanosoma cruzi activity and acts synergistically with posaconazole. J Med Chem 2006; 49(3): 892-9.
[220] Paniz-Mondolfi AE, Pérez-Alvarez AM, Lanza G, Márquez E, Concepción JL. Amiodarone and itraconazole: A rational therapeutic approach for the treatment of chronic Chagas' disease. Chemotherapy 2009; 55(4): 228-33.
[221] Buckner FS. Sterol 14-demethylase inhibitors for *Trypanosoma cruzi* infections. Adv Exp Med Biol 2008; 625: 61-80.
[222] Lepesheva GI. Design or screening of drugs for the treatment of Chagas disease: what shows the most promise? Expert Opin Drug Discov 2013; 8(12): 1479-89.
[223] Keenan M, Chaplin JH. A new era for Chagas disease drug discovery? Prog Med Chem 2015; 54: 185-230.
[224] Hoekstra WJ, Hargrove TY, Wawrzak Z, da Gama Jaen Batista D, da Silva CF, Nefertiti AS, *et al.* Clinical Candidate VT-1161's Antiparasitic Effect *In vitro*, activity in a murine model of Chagas Disease, and structural characterization in complex with the target enzyme CYP51 from *Trypanosoma cruzi*. Antimicrob Agents Chemother 2015; 60(2): 1058-66.
[225] Andriani G, Amata E, Beatty J, Clements Z, Coffey BJ, Courtemanche G, *et al*. Antitrypanosomal lead discovery: Identification of a ligand-efficient inhibitor of *Trypanosoma cruzi* CYP51 and parasite growth. J Med Chem 2013; 56(6): 2556-67.
[226] Suryadevara PK, Olepu S, Lockman JW, Ohkanda J, Karimi M, Verlinde CL, *et al*. Structurally simple inhibitors of lanosterol 14alphademethylase are efficacious in a rodent model of acute Chagas disease. J Med Chem 2009; 52(12): 3703-15.
[227] Suryadevara PK, Racherla KK, Olepu S, Norcross NR, Tatipaka HB, Arif JA, *et al*. Dialkylimidazole inhibitors of *Trypanosoma cruzi* sterol 14α-demethylase as anti-Chagas disease agents. Bioorg Med Chem Lett 2013; 23(23): 6492-9.
[228] Hucke O, Gelb MH, Verlinde CL, Buckner FS. The protein farnesyltransferase inhibitor tipifarnib as a new lead for the development of drugs against Chagas disease. J Med Chem 2005; 48(17): 5415-8.
[229] Kraus JM, Tatipaka HB, McGuffin SA, Chennamaneni NK, Karimi M, Arif J, *et al*. Second generation analogues of the cancer drug clinical candidate tipifarnib for anti-Chagas disease drug discovery. J Med Chem 2010; 53(10): 3887-98.
[230] Buckner FS, Bahia MT, Suryadevara PK, White KL, Shackleford DM, Chennamaneni NK, *et al*. Pharmacological characterization, structural studies, and *in vivo* activities of anti-Chagas disease lead compounds derived from tipifarnib. Antimicrob Agents Chemother 2012; 56(9): 4914-21.
[231] Doyle PS, Chen CK, Johnston JB, Hopkins SD, Leung SS, Jacobson MP, *et al.* A non azole CYP51 inhibitor cures Chagas' disease in a mouse model of acute infection. Antimicrob Agents Chemother 2010; 54(6): 2480-8.
[232] Konkle ME, Hargrove TY, Kleshchenko YY, von Kries JP, Ridenour W, Uddin MJ*, et al*. Indomethacin amides as a novel molecular scaffold for targeting *Trypanosoma cruzi* sterol 14alpha-demethylase. J Med Chem 2009; 52(9): 2846-53.
[233] Choi JY, Calvet CM, Gunatilleke SS, Ruiz C, Cameron MD, McKerrow JH, *et al*. Rational development of 4-aminopyridyl-based inhibitors targeting *Trypanosoma cruzi* CYP51 as anti-chagas agents. J Med Chem 2013; 56(19): 7651-68.
[234] Choi JY, Calvet CM, Vieira DF, Gunatilleke SS, Cameron MD, McKerrow JH*, et al*. R-Configuration of 4-aminopyridyl-based inhibitors of CYP51 confers superior efficacy against *Trypanosoma cruzi.* ACS Med Chem Lett 2014; 5(4): 434-39.
[235] Calvet CM, Vieira DF, Choi JY, Kellar D, Cameron MD, Siqueira-Neto JL, *et al*. 4-Aminopyridyl-based CYP51 inhibitors as anti-*Trypanosoma cruzi* drug leads with improved pharmacokinetic profile and *in vivo* potency. J Med Chem 2014; 57(16): 6989-7005.
[236] Vieira DF, Choi JY, Calvet CM, Siqueira-Neto JL, Johnston JB, Kellar D, *et al*. Binding mode and potency of N-indolyloxopyridinyl-4-aminopropanyl-based inhibitors targeting *Trypanosoma cruzi* CYP51. J Med Chem. 2014; 57(23): 10162-175.
[237] Choy JW, Bryant C, Calvet CM, Doyle PS, Gunatilleke SS, Leung SS, *et al*. Chemical-biological characterization of a cruzain inhibitor reveals a second target and a mammalian off-target. Beilstein J Org Chem 2013; 9: 15-25.
[238] Lepesheva GI, Hargrove TY, Rachakonda G, Wawrzak Z, Pomel S, Cojean S *et al*. VFV as a new effective CYP51 structure-derived drug candidate for Chagas Disease and visceral Leishmaniasis. J Infect Dis 2015; 212(9): 1439-48.
[239] Dobish MC, Villalta F, Waterman MR, Lepesheva GI, Johnston JN. Organocatalytic, enantioselective synthesis of VNI: A robust therapeutic development platform for Chagas, a neglected tropical disease. Org Lett 2012; 14(24): 6322-25.
[240] Villalta F, Dobish MC, N de PN, Kleshchenko YY, Hargrove TY, Johnson CA, *et al*. VNI cures acute and chronic experimental Chagas disease. J Infect Dis 2013; 208(3): 504-11.
[241] Soeiro Mde N, de Souza EM, da Silva CF. *In vitro* and *in vivo* studies of the antiparasitic activity of sterol 14α-demeth ylase (CYP51) inhibitor VNI against drug-resistant strains of *Trypanosoma cruzi*. Antimicrob Agents Chemother 2013; 57(9): 4151-63.
[242] Cherkesova TS, Hargrove TY, Vanrell MC, Ges I, Usanov SA, Romano PS, *et al*. Sequence variation in CYP51A from the Y strain of *Trypanosoma cruzi* alters its sensitivity to inhibition. FEBS Lett 2014; 588(21): 3878-85.
[243] de Macedo-Silva ST, de Souza W, Fernandes Rodrigues JC. Sterol biosynthesis pathway as an alternative for the anti-protozoan parasite chemotherapy. Curr Med Chem 2015; 22(18): 2186-98.
[244] Jouvert BM, Buckner FS, Matsuda SP. Trypanosome and animal lanosterol synthases use different catalytic motifs. Org Lett 2001; 14: 1957-60.
[245] Buckner FS, Griffin JH, Wilson AJ, Van Voorhis WC. Potent anti-*Trypanosoma cruzi* activities of oxidosqualene cyclase inhibitors. Antimicrob Agents Chemother 2001; 45(4): 1210-15.
[246] Oliaro-Bosso S, Ceruti M, Balliano G, Milla P, Rocco F, Viola F. Analogs of squalene and oxidosqualene inhibit oxidosqualene cyclase of *Trypanosoma cruzi* expressed in Saccharomyces cerevisiae. Lipids 2005; 40(12): 1257-62.
[247] Prestwich, G.D., Buckner, F.S., Hinshaw, J.C. Methods related to metabolism of parasites and mycobacteria. WO0076316A1 **(2000)**.
[248] Balliano G, Dehmlow H, Oliaro-Bosso S, Scaldaferri M, Taramino S, Viola F, *et al*. Oxidosqualene cyclase from *Saccharomyces cerevisiae*, *Trypanosoma cruzi, Pneumocystis carinii* and *Arabidopsis thaliana* expressed in yeast: A model for the development of novel antiparasitic agents. Bioorg Med Chem Lett 2009; 19(3): 718-23.
[249] Abe I, Prestwich GD. In Cane DE Ed, Comprehensive natural products chemistry. New York, Elsevier 1999; 2(10): 267-98.
[250] Urbina JA. Lipid biosynthesis pathways as chemotherapeutic targets in kinetoplastid parasites. Parasitology 1997; 114: 91-9.
[251] Gerpe A, Odreman-Nuñez I, Draper P, Boiani L, Urbina JA, González M, *et al*. Heteroallyl-containing 5-nitrofuranes as new anti-*Trypanosoma cruzi* agents with a dual mechanism of action. Bioorg Med Chem 2008; 16(1): 569-77.
[252] Gerpe A, Alvarez G, Benítez D, Boiani L, Quiroga M, Hernández P, *et al*. 5-Nitrofuranes and 5-nitrothiophenes with anti-*Trypanosoma cruzi* activity and ability to accumulate squalene. Bioorg Med Chem 2009; 17(21): 7500-09.
[253] Urbina JA, Concepcion JL, Rangel S, Visbal G, Lira R. Squalene synthase as a chemotherapeutic target in *Trypanosoma cruzi* and *Leishmania mexicana*. Mol Biochem Parasitol 2002; 125(1-2): 35-45.
[254] Urbina JA, Concepcion JL, Caldera A, Payares G, Sanoja C, Otomo T, *et al*. *In vitro* and *in vivo* activities of E5700 and ER-119884, two novel orally active squalene synthase inhibitors, against *Trypanosoma cruzi.* Antimicrob Agents Chemother 2004; 48(7): 2379-87.
[255] Szajnman SH, Yan W, Bayley BN, Docampo R, Elhalem E, Rodriguez JB. Design and synthesis of aryloxyethyl thiocyanate derivatives as potent inhibitors of *Trypanosoma cruzi* proliferation. J Med Chem 2000; 43(9): 1826-40.

[256] Urbina JA, Concepcion JL, Montalvetti A, Rodriguez JB, Docampo R. Mechanism of action of 4- phenoxyphenoxyethyl thiocyanate (WC-9) against *Trypanosoma cruzi*, the causative agent of Chagas'disease. Antimibrob Agents Chemoter 2003; 47(6): 2047-50

[257] Elhalem E, Bailey BN, Docampo R, Ujvary I, Szajnman SH, Rodriguez JB. Design, synthesis, and biological evaluation of aryloxyethyl thiocyanate 50 derivatives against *Trypanosoma cruzi*. J Med Chem 2002; 45(18): 3984-99.

[258] Braga MV, Urbina JA, de Souza W. Effects of squalene synthase inhibitors on the growth and ultrastructure of *Trypanosoma cruzi.* Int J Antimicrob Agents 2004; 24(1): 72-8.

[259] Lorente OS, Gomez R, Jimenez C, Cammerer S, Yardley V, de Luca-Fradley K, *et al*. Biphenylquinuclidines as inhibitors of squalene synthase and growth of parasitic protozoa. Bioorg Med Chem 2005; 13(10): 3519-29.

[260] Lorente OS, Jimenez CJ, Gros L, Yardley V, de Luca-Fradley K, Croft SL*, et al*. Preparation of transition-state analogues of sterol 24-methyl transferase as potential anti-parasitics. Bioorg Med Chem 2005; 13(18): 5435-43.

[261] Urbina JA, Lazardi K, Marchan E, Visbal G, Aguirre T, Piras MM, *et al*. Mevinolin (lovastatin) potentiates the antiproliferative effects of ketoconazole and terbinafine against *Trypanosoma cruzi: In vitro* and *in vivo* studies. Antimicrob Agents Chemoter 1993; 37(3): 580-91.

[262] Yardley V, Crof SL. *In vitro* and *in vivo* activity of amphotericin B-lipid formulations against experimental *Trypanosoma cruzi* infections. Am J Trop Med Hyg 1999; 61(2): 193-97.

[263] Hamilton, A.D., Van Vooris, W.C., Yokoyama, K., Buckner, F.S., Ohkanda, J., Gelb, M. Compounds and methods for the inhibition of *Trypanosoma cruzi.* WO03006012A1 **(2003)**.

[264] Lockman J., Hamilton, A.D., Gelb, M., Buckner, F.S., Ohkanda, J., Yokoyama, K., Van Vooris, W.C. Compounds and methods for the inhibition of *Trypanosoma cruzi.* CA2453396A **(2003)**.

[265] Hamilton, A.D., Van Vooris, W.C., Yokoyama, K., Buckner, F.S., Ohkanda, J., Gelb, M., Lockman J. Compounds and methods for the inhibition of *Trypanosoma cruzi.* BR0211098A **(2004)**.

[266] Ferella M, Li ZH, Andersson B, Docampo R. Farnesyl diphosphate synthase localizes to the cytoplasm of *Trypanosoma cruzi* and *T. brucei.* Exp Parasitol 2008; 119(2): 308-12.

[267] Montalvetti A, Bailey BN, Martin MB, Severin GW, Oldfield E, Docampo R. Bisphosphonates are potent inhibitors of *Trypanosoma cruzi* farnesyl pyrophosphate synthase. J Biol Chem 2001; 276(36): 33930-37.

[268] Martin MB, Grimley JS, Lewis JC, Heath HT 3rd, Bailey BN, Kendrick H, *et al*. Bisphosphonates inhibit the growth of *Trypanosoma brucei*, *Trypanosoma cruzi, Leishmania donovani, Toxoplasma gondii*, and *Plasmodium falciparum*: A potential route to chemotherapy. J Med Chem 2001; 44(6): 909-16.

[269] Garzoni LR, Caldera A, Meirelles M de N, de Castro SL, Docampo R, Meints GA, *et al*. Selective *in vitro* effects of the farnesyl pyrophosphate synthase inhibitor risedronate on *Trypanosoma cruzi*. Int J Antimicrob Agents 2004; 23(3): 273-85.

[270] Garzoni LR, Waghabi MC, Baptista MM, de Castro SL, Meirelles Mde N, Britto CC, *et al*. Antiparasitic activity of risedronate in a murine model of acute Chagas' disease. Int J Antimicrob Agents 2004; 23(3): 286-90.

[271] Szajnman SH, Montalvetti A, Wang Y, Docampo R, Rodriguez JB. Bisphosphonates derived from fatty acids are potent inhibitors of *Trypanosoma cruzi* farnesyl pyrophosphate synthase. Bioorg Med Chem Lett 2003; 13(19): 3231-35.

[272] Szajnman SH, Ravaschino EL, Docampo R, Rodriguez JB. Synthesis and biological evaluation of 1- amino-1,1-bisphosphonates derived from fatty acids against *Trypanosoma cruzi* targeting farnesyl pyrophosphate synthase. Bioorg Med Chem Lett 2005; 15(21): 4685-90.

[273] Docampo R, Moreno SN. Bisphosphonates as chemotherapeutic agents against Trypanosomatid and apicomplexan parasites. Curr Drug Targets Infect Disord 2001; 1(1): 51-61.

[274] Gabelli SB, McLellan JS, Montalvetti A, Oldfield E, Docampo R, Amzel LM. Structure and mechanism of the farnesyl diphosphate synthase from *Trypanosoma cruzi*: Implications for drug design. Proteins 2006; 62(1): 80-8.

[275] Sigman L, Sanchez VM, Turjansky AG. Characterization of the farnesyl pyrophosphate synthase of *Trypanosoma cruzi* by homology modeling and molecular dynamics. J Mol Graph Model 2006; 25 (3): 345-52.

[276] Recher M, Barboza AP, Li ZH, Galizzi M, Ferrer-Casal M, Szajnman SH, *et al*. Design, synthesis and biological evaluation of sulfur-containing 1,1-bisphosphonic acids as antiparasitic agents. Eur J Med Chem 2013; 60: 431-40.

[277] Gelb MH, Van Voohris WC, Buckner FS. Protein farnesyl and N-myristoyl transferases: Piggy-back medicinal chemistry targets for the development of anti-Trypanosomatid and antimalarial therapeutics. Mol Biochem Parasitol 2003; 126(2): 155-63.

[278] Karp JE. Farnesyl transferase inhibition in hematologic malignancies. J Natl Comp Cancer Netw 2005; 3(1):37-40.

[279] Esteva MI, Kettler K, Maidana C, Fichera L, Ruiz AM, Bontempi EJ, *et al*. Benzophenone-based farnesyl transferase inhibitors with high activity against *Trypanosoma cruzi.* J Med Chem 2005; 48(23): 7186-91.

[280] Kraus JM, Verlinde CL, Karimi M, Lepesheva GI, Gelb MH, Buckner FS. Rational modification of a candidate cancer drug for use against Chagas disease. J Med Chem 2009; 52(6): 1639-47.

[281] Kraus JM, Tatipaka HB, McGuffin SA, Chennamaneni NK, Karimi M, Arif J, *et al*. Second generation analogues of the cancer drug clinical candidate tipifarnib for anti-Chagas disease drug discovery. J Med Chem 2010; 53(10): 3887-98.

[282] Field, M.C., Ali, B.R.S. Manumycin derivatives for treating parasitic disorders. WO00105384A3 **(2001)**.

[283] Windsor, W.T., Weber, P.C., Stricland, C., Syto, R., Girijavallabhan, V.M., Kaminski, J.J., Guo, Z. Treatment of *Trypanosoma brucei* with farnesyl protein transferase inhibitors. US2003134846A1 **(2003)**.

[284] Sandes JM, Borges AR, Junior CG, Silva FP, Carvalho GA, Rocha GB, *et al*. 3-Hydroxy-2-methylene-3-(4 nitrophenyl-propanenitrile): A new highly active compound against epimastigote and trypomastigote form of *Trypanosoma cruzi.* Bioorg Chem 2010; 38(5): 190-95.

[285] Yokoyama K, Gillespie JR, Van Voorhis WC, Buckner FS, Gelb MH. Protein geranylgeranyltransferase-I of *Trypanosoma cruzi.* Mol Biochem Parasitol 2008; 157(1): 32-43.

[286] Krauth-Siegel RL, Inhoff O. Parasite-specific trypanothione reductase as a drug target molecule. Parasitol Res 2003; 90(2): 77-85.

[287] Krauth-Siegel RL, Bauer H, Schirmar RH. Dithiol proteins as guardians of the intracellular redox milieu in parasites: Old and new drug targets in trypanosomes and malaria-causing plasmodia. Angew Chem Int Ed Engl 2005; 44(5): 690-715.

[288] Straniero V, Pallavicini M, Chiodini G, Ruggeri P, Fumagalli L, Bolchi C, *et al*. Farnesyltransferase inhibitors: CAAX mimetics based on different biaryl scaffolds. Bioorg Med Chem Lett 2014 1; 24(13): 2924-27.

[289] Augustins K, Amssoms K, Yamani A, Rajan PK, Haemers A: Trypanothione as a target in the design of anti-trypanosomal and antiLeishmanial agents. Curr Pharm Des 2001; 7(12): 1117-41.

[290] Faundez M, Pino L, Letelier P, Ortiz C, López R, Seguel C, *et al*. Buthionine sulfoximine increases the toxicity of nifurtimox and benznidazole to *Trypanosoma cruzi*. Antimicrob Agents Chemother 2005; 49: 126-30.

[291] Stoppani AO. The chemotherapy of Chagas disease. Medicina (Bs As) 1999; 59(2):147-65.

[292] Henderson GB, Ulrich P, Fairlamb AH, Rosenberg I, Pereira M, Sela M, *et al*. "Subversive" substrates for the enzyme trypanothione disulfide reductase: Alternative approach to chemotherapy of Chagas disease. Proc Natl Acad Sci USA 1988; 85(15): 5374-78.

[293] Flohé L. The trypanothione system and the opportunities it offers to create drugs for the neglected kinetoplast diseases. Biotechnol Adv 2012; 30(1): 294-301.

[294] Bernardes LS, Zani CL, Carvalho I. Trypanosomatidae diseases: from the current therapy to the efficacious role of trypanothione reductase in drug discovery. Curr Med Chem 2013; 20(21): 2673-96.
[295] Manta B, Comini M, Medeiros A, Hugo M, Trujillo M, Radi R: A unique bis-glutathionyl derivative in trypanosomatids. Biochim Biophys Acta 2013; 1830(5): 3199-216.
[296] Maya JD, Salas CO, Aguilera-Venegas B, Diaz MV, López-Muñoz R. Key proteins in the polyamine-trypanothione pathway as drug targets against *Trypanosoma cruzi.* Curr Med Chem 2014; 21(15): 1757-71.
[297] Salmon-Chemin L, Buisine E, Yardley V, Debreu MA, Landry V, Sergheraert C, *et al*. 2- and 3-substituted 1, 4-naphthoquinone derivatives as ubversive substrates of trypanothione reductase and lipoamide dehydrogenase from *Trypanosoma cruzi*: synthesis and correlation between redox cycling activities and *in vitro* cytotoxicity. J Med Chem 2001; 44(4): 548-65.
[298] Paulino M, Iribarne F, Dubin M, Aguilera-Morales S, Tapia O, Stoppani AO. The chemotherapy of Chagas' disease: An overview. Mini Rev Med Chem 2005; 5(5): 499-519.
[299] Blumenstiel K, Schoeneck R, Yardley V, Croft SL, Krauth-Siegel RL. Nitrofuran drugs as commonsubversive substrates of *Trypanosoma cruzi* lipoamide dehydrogenase and trypanothione reductase. Biochem Pharmacol 1999; 58(11): 1791-99.
[300] Cenas N, Bironaite D, Dickancaite E, Anusevicius Z, Sarlauskas J, Blanchard JS. Chinifur, a selective inhibitor and "subversive substrate" for *Trypanosoma congolense* trypanothione reductase. Biochem Biophys Res Commun 14; 204(1):224-29.
[301] Paulino M, Iribarne F, Dubin M, Aguilera-Morales S, Tapia O, Stoppani AO. The chemotherapy of Chagas' disease: an overview. Mini Rev Med Chem 2005; 5(5): 499-519.
[302] Davies C, Marino Cardozo R, Sánchez Negrette O, Mora MC, Chung MC, Basombrío MA. Hydroxymethylnitrofurazone is active in a murine model of Chagas Disease. Antimicrob Agents Chemother 2010; 54(9): 3584-9.
[303] De Moura KCG, Emery FS, Neves-Pinto C. Synthesis and trypanocidal activity of naphthoquinones isolated from *Tabebuia* and heterocyclic derivatives: A review from an interdisciplinary study. J Braz Chem Soc 2001; 12: 325-38.
[304] Cuellar MA, Salas C, Cortes MJ, Morello A, Diego Maya J, Preite MD. Synthesis and *in vitro* trypanocide activity of several polycyclic drimane-quinone derivatives. Bioorg Med Chem 2003; 11(12): 2489-97.
[305] Santos AF, Ferraz PA, de Abreu FC, Chiari E, Goulart MO, Sant'Ana AE. Molluscicidal and trypanocidal activities of lapachol derivatives. Planta Med 2001; 67(1): 92-3.
[306] Buchholz K, Comini MA, Wissenbach D, Schirmer RH, Krauth-Siegel RL, Gromer S. Cytotoxic interactions of methylene blue with Trypanosomatid-specific disulfide reductases and their dithiol products. Mol Biochem Parasitol 2008; 160(1): 65-9.
[307] Stump B, Kaiser M, Brun R, Krauth-Siegel RL, Diederich F. Betraying the parasite's redox system: diaryl sulfide-based inhibitors of trypanothione reductase: Subversive substrates and anti-trypanosomal properties. Chem Med Chem 2007; 2(12): 1708-12.
[308] Gallwitz H, Bonse S, Martinez-Cruz A, Schichting I, Schumacher K, Krauth-Siegel RL. Ajoene is an inhibitor and subversive substrate of human glutathione reductase and *Trypanosoma cruzi* trypanothione reductase: Crystallographic, kinetic, and spectroscopic studies. J Med Chem 1999; 42(3): 364-72.
[309] Farrell NP, Williamson J, McLaren DJ. Trypanocidal and antitumour activity of platinum-metal and platinum-metal-drug dual-function complexes. Biochem Pharmacol 1984; 33(7): 961-71.
[310] Bonse S, Richards JM, Ross SA, Lowe G, Krauth-Siegel RL. (2, 2': 6', 2"-Terpyridine) platinum (II) complexes are irreversible inhibitors of *Trypanosoma cruzi* trypanothione reductase but not of human glutathione reductase. J Med Chem 2000; 43(25): 4812-21.
[311] Sanchez-Delgado RA, Lazardi K, Rincon L, Urbina JA. Towards a novel metal-based chemotherapy against tropical diseases. Enhancement of the efficacy of clotrimazole against *Trypanosoma cruzi* by complexation to ruthenium in RuCl2 (clotrimazole) 2. J Med Chem 1993; 36(14): 2041-3.
[312] Sanchez-Delgado RA, Anzellotti A. Metal complexes as chemotherapeutic agents against tropical diseases: trypanosomiasis, malaria and Leishmaniasis. Mini Rev Med Chem 2004; 4(1): 23-30.
[313] Lowe, G. Platinum (ii) compounds. WO0050431A1 **(2000)**.
[314] Lee B, Bauer H, Melchers J, Ruppert T, Rattray L, Yardley V, *et al*. Irreversible inactivation of trypanothione reductase by unsaturated Mannich bases: a divinyl ketone as key intermediate. J Med Chem 2005; 48(23): 7400-10.
[315] Otelo L, Vieites M, Boiani L, Denicola A, Rigol C, Opazo L, *et al*. Novel trypanosomal agents based on palladiumnitrofurylthiosemicarbazone complexes: DNA and redox metabolism as potential therapeutic targets. J Med Chem 2006; 49(11): 3322-31.
[316] Gutierrez-Correa J, Fairlamb AH, Stoppani AO. *Trypanosoma cruzi* trypanothione reductase is inactivated by peroxidase-generated phenothiazine cationic radicals. Free Radic Res 2001; 34(4): 363-78.
[317] Rivarola HW, Paglini Oliva P. *Trypanosoma cruzi* trypanothione reductase inhibitors: Phenothiazines and related compounds modify experimental Chagas' disease evolution. Curr Drug Targets Cardiovasc Haematol Disord 2002; 2(1): 43-52.
[318] Rivarola HW, Bustamante JM, Lo Presti S, Fernández AR, Enders JE, Gea S, *et al*. *Trypanosoma cruzi*: Chemotherapeutic effects of clomipramine in mice infected with an isolate obtained from an endemic area. Exp Parasitol 2005; 111(2):80-86.
[319] Fauro R, Lo Presti S, Bazan C, Baez A, Strauss M, Triquell F, *et al*. Use of clomipramine as chemotherapy of the chronic phase of Chagas disease. Parasitology 2013; 140(7): 917-27.
[320] O'Sullivan MC, Durham TB, Valdes HE, Dauer KL3, Karney NJ3, Forrestel AC, *et al*. Dibenzosuberyl substituted polyamines and analogs of clomipramine as effective inhibitors of trypanothione reductase; molecular docking, and assessment of trypanocidal activities. Bioorg Med Chem 2015; 23(5): 996-1001.
[321] Jacoby EM, Schlichting I, Lantwin CB, Kabsch W, Krauth-Siegel RL. Crystal structure of the *Trypanosoma cruzi* trypanothione reductase-mepacrine complex. Proteins 1996; 24(1): 73-80.
[322] Persch E, Bryson S, Todoroff NK, Eberle C, Thelemann J, Dirdjaja N, *et al*. Binding to large enzyme pockets: Small-molecule inhibitors of trypanothione reductase. Chem Med Chem 2014; 9(8): 1880-91.
[323] Kumar S, Ali MR, Bawa S. Mini review on tricyclic compounds as an inhibitor of trypanothione reductase. J Pharm Bioallied Sci. 2014; 6(4): 222-8.
[324] Girault S, Davioud-Charvet E, Salmon L, Berecibar A, Debreu MA, Sergheraert C. Structure-activity relationships in 2-aminodiphenylsulfides against trypanothione reductase from *Trypanosoma cruzi.* Bioorg Med Chem Lett 1998; 8(10): 1175-80.
[325] Girault S, Davioud-Charvet TE, Maes L, Dubremetz JF, Debreu MA, Landry V, *et al*. Potent and specific inhibitors of trypanothione reductase from *Trypanosoma cruzi*: Bis (2-aminodiphenylsulfides) for fluorescent labeling studies. Bioorg Med Chem 2001; 9(4): 837-46.
[326] Bonnet B, Soullez D, Davioud-Charvet E, Landry V, Horvath D, Sergheraert C. New spermine and spermidine derivatives as potent inhibitors of *Trypanosoma cruzi* trypanothione reductase. Bioorg Med Chem 1997; 5(7): 1249-56.
[327] Li Z, Fennie MW, Ganem B, Hancock MT, Kobaslija M, Rattendi D*, et al*. Polyamines with N-(3-phenylpropyl) substituents are effective competitive inhibitors of trypanothione reductase and trypanocidal agents. Bioorg Med Chem Lett 2001; 11(2): 251-4.
[328] Flohe L, Hecht HJ, Steinert T. Glutathione and trypanothione in parasitic hydroperoxide metabolism. Free Radic Biol Med 1999; 27(9-10): 966-84.

[329] Hamilton CJ, Saravanamuthu A, Fairlamb AH, Eggleston IM. Benzofuranyl 3,5-bis-polyamine derivatives as time-dependent inhibitors of trypanothione reductase. Bioorg Med Chem 2003; 11(17): 3683-93.

[330] Fournet A, de Arias RA, Ferreira ME, Nakayama H, Torres de Ortiz S, Schinini A*, et al*. Efficacy of the bisbenzylisoquinoline alkaloids in acute and chronic *Trypanosoma cruzi* murine model. Int J Antimicrob Agents 2000; 13(3): 189-95.

[331] Khan MO, Austin SE, Chan C, Yin H, Marks D, Vaghjiani SN, *et al*. Use of an additional hydrophobic binding site, the Z site, in the rational drug design of a new class of stronger trypanothione reductase inhibitor, quaternary alkylammonium phenothiazines. J Med Chem 2000; 43(16): 3148-56.

[332] Parveen S, Khan MO, Austin SE. Anti-Trypanosomal, anti-Leishmanial, and antimalarial activities of quaternary arylalkylammonium 2-amino-4-chlorophenyl phenyl sulfides, a new class of trypanothione reductase inhibitor, and of N-acyl derivatives of 2-amino-4-chlorophenyl phenyl sulfide. J Med Chem 2005; 48(25): 8087-97.

[333] Barbosa EG, Pasqualoto KF, Ferreira MM. The receptor-dependent LQTA-QSAR: Application to a set of trypanothione reductase inhibitors. J Comput Aided Mol Des 2012; 26(9): 1055-65.

[334] Lo Presti MS, Bazán PC, Strauss M, Báez AL, Rivarola HW, Paglini-Oliva PA.Trypanothione reductase inhibitors: Overview of the action of thioridazine in different stages of Chagas disease. Acta Trop 2015; 145:79-87.

[335] Aguirre G, Cabrera E, Cerecetto H, Di Maio R, González M, Seoane G*, et al*. Design, synthesis and biological evaluation of new potent 5- nitrofuryl derivatives as anti-*Trypanosoma cruzi* agents. Studies of trypanothione binding site of trypanothione reductase as target for rational design. Eur J Med Chem 2004; 39(5): 421-31.

[336] Aguirre G, Boiani M, Cabrera E, Cerecetto H, Di Maio R, González M, *et al*. New potent 5-nitrofuryl derivatives as inhibitors of *Trypanosoma cruzi* growth. 3D-QSAR (CoMFA) studies. Eur J Med Chem 2006; 41(4): 457-66.

[337] Meiering S, Inhoff O, Mies J, Vincek A, Garcia G, Kramer B, *et al*. Inhibitors of *Trypanosoma cruzi* trypanothione reductase revealed by virtual screening and parallel synthesis. J Med Chem 2005; 48(15): 4793-802.

[338] Rodrigues RF, Castro-Pinto D, Echevarria A, dos Reis CM, Del Cistia CN, Sant'Anna CM, *et al*. Investigation of trypanothione reductase inhibitory activity by 1,3,4-thiadiazolium-2-aminide derivatives and molecular docking studies. Bioorg Med Chem 2012; 20(5): 1760-6.

[339] Majumder S, Kierszenbaum F. N,N'-thiophene-substituted polyamine analogs inhibit mammalian host cell invasion and intracellular multiplication of *Trypanosoma cruzi*. Mol Biochem Parasitol 1993; 60(2): 231-9.

[340] Cerecetto H, González M. Anti-*T. cruzi* agents: Our experience in the evaluation of more than five hundred compounds. Mini Rev Med Chem 2008; 8(13): 1355-83.

[341] Galarreta BC, Sifuentes R, Carrillo AK, Sanchez L, del Amado MR, Maruenda H. The use of natural product scaffolds as leads in the search for trypanothione reductase inhibitors. Bioorg Med Chem 2008; 16(14): 6689-95.

[342] Cota BB, Rosa LH, Fagundes EM, Martins-Filho OA, Correa-Oliveira R, Romanha AJ, *et al*. A potent trypanocidal component from the fungus *Lentinus strigosus* inhibits trypanothione reductase and modulates PBMC proliferation. Mem Inst Oswaldo Cruz 2008; 103(3): 263-70.

[343] Cota BB, Rosa LH, Caligiorne RB, Rabello AL, Almeida Alves TM, Rosa CA*, et al*. Altenusin, a biphenyl isolated from the endophytic fungus Alternaria sp., inhibits trypanothione reductase from *Trypanosoma cruzi.* FEMS Microbiol Lett 2008; 285(2):177-82.

[344] Czechowicz JA, Wilhelm AK, Spalding MD, Larson AM, Engel LK, Alberg DG. The synthesis and inhibitory activity of dethiotrypanothione and analogues against trypanothione reductase. J Org Chem 2007; 72(10): 3689-93.

[345] de Paula da Silva CH, Bernardes LS, da Silva VB, Zani CL, Carvalho I. Novel aryl β-aminocarbonyl derivatives as inhibitors of *Trypanosoma cruzi* trypanothione reductase: binding mode revised by docking and GRIND2-based 3D-QSAR procedures. J Biomol Struct Dyn 2012; 29(6): 702-16.

[346] Duyzend MH, Clark CT, Simmons SL, Johnson WB, Larson AM, Leconte AM*, et al*. Synthesis and evaluation of substrate analogue inhibitors of trypanothione reductase. J Enzyme Inhib Med Chem 2012; 27(6): 784-94.

[347] Olin-Sandoval V, Moreno Sanchez R, Saavedra E. Targeting trypanothione metabolism in trypanosomatid human parasites. Curr Drug targets 2010; 11(12): 1614-30.

[348] Wilkinson SR, Obado SO, Mauricio IL, Kelly JM. *Trypanosoma cruzi* expresses a plant-like ascorbate dependent hemoperoxidase localized to the endoplasmic reticulum. Proc Natl Acad Sci USA 2002; 99(21): 13453- 8.

[349] Leroux AE, Krauth-Siegel RL.Thiol redox biology of trypanosomatids and potential targets for chemotherapy. Mol Biochem Parasitol 2015 pii: S0166-6851(15)30039-6.

[350] Eberle C, Lauber BS, Fankhauser D, Kaiser M, Brun R, Krauth-Siegel RL, Diederich F. Improved inhibitors of trypanothione reductase by combination of motifs: Synthesis, inhibitory potency, binding mode, and antiprotozoal activities. Chem Med Chem 2011; 6(2): 292-301.

[351] Olin-Sandoval V, González-Chávez Z, Berzunza-Cruz M, Martínez I, Jasso-Chávez R, Becker I, *et al*. Drug target validation of the trypanothione pathway enzymes through metabolic modelling. FEBS J 2012; 279(10): 1811-33.

[352] Taylor MC, Lewis MD, Fortes Francisco A, Wilkinson SR, Kelly JM. The *Trypanosoma cruzi* vitamin C dependent peroxidase confers protection against oxidative stress but is not a determinant of virulence. PLoS Negl Trop Dis 2015; 9(4): e0003707.

[353] Marquez VE, Arias DG, Piattoti CV, Rovello C, Iglesias AA, Guerrero AS. Cloning, expression, and characterization of a dithiol glutaredoxin from *Trypanosoma cruzi*. Antioxid Redox Signal 2010; 12(6): 787-92.

[354] Ravaschino EL, Docampo R, Rodriguez JB. Design, synthesis, and biological evaluation of phosphinopeptides against *Trypanosoma cruzi* targeting trypanothione biosynthesis. J Med Chem 2006; 49(1): 426-35.

[355] Oza SL, Chen S, Wyllie S, Coward JK, Fairlamb AH. ATP-dependent ligases in trypanothione biosynthesis-kinetics of catalysis and inhibition by phosphinic acid pseudopeptides FEBS J 2008; 275(21): 5408-21.

[356] Aravena CM, Olea AC, Cerecetto H, González M, Maya JD, Rodríguez-Becerra J. Potent 5-nitrofuran derivatives inhibitors of *Trypanosoma cruzi* growth: Electrochemical, spectroscopic and biological studies. Spectrochim Acta A Mol Biomol Spectrosc 2011; 79(2): 312-9.

[357] Castro H, Romao S, Carvalho S, Teixeira F, Sousa C, Tomás AM. Mitochondrial redox metabolism in trypanosomatids is independent of tryparedoxin activity. PLoS One 2010; 5(9): e12607.

[358] Piñeyro MD, Parodi-Talice A, Portela M, Arias DG, Guerrero SA, Robello C. Molecular characterization and interactome analysis of *Trypanosoma cruzi* tryparedoxin 1. J Proteomics 2011; 74(9): 1683-92.

[359] Arias DG, Marquez VE, Chiribao ML, Gadelha FR, Robello C, Iglesias AA, *et al*. Redox metabolism in *Trypanosoma cruzi*: functional characterization of tryparedoxins revisited. Free Radic Biol Med 2013; 63: 65-77.

[360] González-Chávez Z, Olin-Sandoval V, Rodíguez-Zavala JS, Moreno-Sánchez R, Saavedra E. Metabolic control analysis of the *Trypanosoma cruzi* peroxide detoxification pathway identifies tryparedoxin as a suitable drug target. Biochim Biophys Acta 2015; 1850(2): 263-73.

[361] Arias DG, Piñeyro MD, Iglesias AA, Guerrero SA, Robello C. Molecular characterization and interactome analysis of *Trypanosoma cruzi* tryparedoxin II. J Proteomics 2015; 120: 95-104.

[362] Wyllie S, Fairlamb AH. Methylglyoxal metabolism in trypanosomes and leishmania. Semin Cell Dev Biol 2011; 22(3): 271-7.

[363] Engel JC, de Cazzulo FBM, Stoppani AO, Cannata JJ, Cazzulo JJ. Aerobic glucose fermentation by *Trypanosoma cruzi* axenic culture amastigote-like forms during growth and dif-

ferentiation to epimastigotes. Mol Biochem Parasitol 1987; 26 (1-2): 1-10.
[364] Greig N, Wyllie S, Vickers TJ, Fairlamb AH. Trypanothione-dependent glyoxalase I in *Trypanosoma cruzi.* Biochem J 2006; 400(2): 217-23.
[365] Greig N, Wyllie S, Patterson S, Fairlamb AH. A comparative study of methylglyoxal metabolism in Trypanosomatids. FEBS J 2009; 276(2): 376-86.
[366] Flohe, L., Koenig, K., Menge, U. Glutathionylspermidine synthetase and processes for recovery and use thereof. US6291217 **(2001)**.
[367] Sousa Silva M, Ferreira AE, Gomes R, Tomás AM, Ponces Freire A, Cordeiro C. The glyoxalase pathway in protozoan parasites. Int J Med Microbiol 2012; 302(4-5): 225-9.
[368] Souza DH, Garatt RC, Araujo AP*, Guimarães BG, Jesus WD, Michels PA, et al*. *Trypanosoma cruzi* glycosomal glyceraldehyde-3-phosphate dehydrogenase: Structure, catalytic mechanism and targeted inhibitor design. FEBS Lett 1998; 424(3): 131-5.
[369] Rodríguez E, Lander N, Ramirez JL. Molecular and biochemical characterisation of *Trypanosoma cruzi* phosphofructokinase. Mem Inst Oswaldo Cruz 2009; 104(5): 745-8.
[370] Brimacombe KR, Walsh MJ, Liu L, Vásquez-Valdivieso MG, Morgan HP, McNae I*, et al*. Identification of ML251, a potent inhibitor of *T. brucei* and *T. cruzi* phosphofructokinase. ACS Med Chem Lett 2013; 5(1): 12-7.
[371] Aronov A, Suresh S, Buckner FS, Van Voorhis WC, Verlinde CL, Opperdoes FR, *et al*. Structure-based design of submicromolar, biologically active inhibitors of Trypanosomatid glyceraldehyde-3-phosphate dehydrogenase. Proc Natl Acad Sci USA 1999; 96(8): 4273-8.
[372] Bressi JC, Verlinde CL, Aronov AM, Shaw ML, Shin SS, Nguyen LN, *et al*. Adenosine analogues as selective inhibitors of glyceraldehyde- 3-phosphate dehydrogenase of Trypanosomatidae via structure-based drug design. J Med Chem 2001; 44(13): 2080-93.
[373] Maluf FV, Andricopulo AD, Oliva G, Guido RV. A pharmacophore-based virtual screening approach for the discovery of *Trypanosoma cruzi* GAPDH inhibitors. Future Med Chem 2013; 5(17): 2019-35
[374] Pariona-Llanos R, Pavani RS, Reis M, Noël V, Silber AM, Armelin HA, *et al*. Glyceraldehyde 3-phosphate dehydrogenase-telomere association correlates with redox status in *Trypanosoma cruzi.* PLoS One 2015; 10(3): e0120896.
[375] de Marchi AA, Castillo MS, Nascimento PG, Archanjo FC, del Ponte G, Oliva G, *et al*. New 3-Piperonylcoumarins as inhibitors of glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) from *Trypanosoma cruzi*. Bioorg Med Chem 2004; 12(18): 4823-33.
[376] Belluti F, Uliassi E, Veronesi G, Bergamini C, Kaiser M, Brun R, *et al*. Toward the development of dual-targeted glyceraldehyde-3-phosphate dehydrogenase/trypanothione reductase inhibitors against *Trypanosoma brucei* and *Trypanosoma cruzi*. Chem Med Chem 2014; 9(2): 371-82
[377] da Silva R, Saraiva J, de Albuquerque S, Curti C, Donate PM, Bianco TN, *et al*. Trypanocidal structure-activity relationship for cis- and trans-methylpluviatolide. Phytochemistry 2008; 69(9): 1890-94.
[378] Ladame S, Fauré R, Denier C, Lakhdar-Ghazal F, Willson M. Selective inhibition of *Trypanosoma cruzi* GAPDH by "bisubstrate" analogues. Org Biomol Chem 2005; 3(11): 2070-2.
[379] Freitas RF, Prokopczyk IM, Zottis A. Discovery of novel *Trypanosoma cruzi* glyceraldehyde-3- phosphate dehydrogenase inhibitors. Bioorg Med Chem 2009; 17(6): 2476-82
[380] Pereira JM, Severino RP, Vieira PC, Fernandes JB, da Silva MF, Zottis A*, et al*. Anacardic acid derivatives as inhibitors of glyceraldehyde-3-phosphate dehydrogenase from *Trypanosoma cruzi* phosphate dehydrogenase inhibitors. Bioorg Med Chem 2008; 16(19): 8889-95.
[381] Silva JJ, Guedes PM, Zottis A, Balliano TL, Nascimento Silva FO, França Lopes LG, *et al*. Novel ruthenium complexes as potential drugs for Chagas's disease: Enzyme inhibition and *in vitro*/*in vivo* trypanocidal activity. Br J Pharmacol 2010; 160(2): 260-9.
[382] Hudock MP, Sanz-Rodriguez CE, Song Y, Chan JM, Zhang Y, Odeh S, *et al*. Inhibition of *Trypanosoma cruzi* hexokinase by bisphosphonates. J Med Chem 2006; 49(1): 215-23.
[383] Cáceres AJ, Quiñones W, Gualdrón M, Cordeiro A, Avilán L, Michels PA, *et al*. Molecular and biochemical characterization of novel glucokinases from *Trypanosoma cruzi* and *Leishmania* spp. Mol Biochem Parasitol 2007; 156 (2): 235-45.
[384] Cordeiro AT, Cáceres AJ, Vertommen D, Concepción JL, Michels PA, Versées W. The crystal structure of *Trypanosoma cruzi* glucokinase reveals features determining oligomerization and anomer specificity of hexose-phosphorylating enzymes. J Mol Biol 2007; 372(5): 1215-26.
[385] Sanz-Rodríguez CE, Concepción JL, Pekerar S, Oldfield E, Urbina JA. Bisphosphonates as inhibitors of *Trypanosoma cruzi* hexokinase: Kinetic and metabolic studies. J Biol Chem 2007; 282(17): 12377-87.
[386] Chelesky J, Freites RF, Wiggers HJ, Rocha JR, de Araujo AP, Montanari CA. Expression, purification and kinetic characterization of His-tagged glyceraldehyde-3-phosphate dehydrogenase from *Trypanosoma cruzi*. Protein Expr Purif 2011; 76(2): 190-6.
[387] Gupta S, Cordeiro AT, Michels PA. Glucose-6-phosphate dehydrogenase is the target for the trypanocidal action of. Mol Biochem Parasitol 2011; 176 (2): 112-5.
[388] Alvarez G, Aguirre-Lopez B. Varela J, Cabrera M, Merlino A, López GV, *et al*. Massive screening yields novel and selective *Trypanosoma cruzi* triosephosphate isomerase dimer-interface-irreversible inhibitors with anti-trypanosomal activity. Eur J Med Chem 2010; 45(12): 5767-72.
[389] Gao XG, Maldonado E, Pérez-Montfort R, Garza-Ramos G, de Gómez-Puyou MT, Gómez-Puyou A, *et al*. Crystal structure of triosephosphate isomeras from *Trypanosoma cruzi* in hexane. Proc Natl Acad Sci USA 1999; 96(18): 10062-7.
[390] Aguirre Y, Cabrera N, Aguirre B, Pérez-Montfort R, Hernandez-Santoyo A, Reyes-Vivas H, Different contribution of conserved amino acids to the global properties Proteins 2014; 82(2): 323-35.
[391] Téllez-Valencia A, Avila-Ríos S, Pérez-Montfort R, Rodríguez-Romero A, Tuena de Gómez-Puyou M, López-Calahorra F*, et al*. Highly specific inactivation of triosephosphate isomerase from *Trypanosoma cruzi*. Biochem Biophys Res Commun 2002; 295(4): 958-63.
[392] Espinoza-Fonseca LM, Trujillo-Ferrara JG. Exploring the possible binding sites at the interface of triosephosphate isomerase dimer as a potential target for anti-tripanosomal drug design. Bioorg Med Chem Lett 2004; 14(12): 3151-4.
[393] Hassan N, Gárate MP, Sandoval T, Espinoza L, Piñeiro Á, Ruso JM. On the self-assembly of a highly selective benzothiazole-based TIM inhibitor in aqueous solution. Langmuir 2010; 26(22): 16681-9.
[394] Alvarez G, Martínez J, Aguirre-López B, Cabrera N, Pérez-Díaz L, de Gómez-Puyou MT, *et al*. New chemotypes as *Trypanosoma cruzi* triosephosphate isomerase inhibitors: A deeper insight into the mechanism of inhibition. J Enzyme Inhib Med Chem 2014; 29(2): 198-204.
[395] Minini L, Álvarez G, González M, Cerecetto H, Merlino A. Molecular docking and molecular dynamics simulation studies of *Trypanosoma cruzi* triosephosphate isomerase inhibitors. Insights into the inhibition mechanism and selectivity. J Mol Graph Model 2015; 58: 40-9.
[396] Alvarez G, Martínez J, Varela J, Birriel E, Cruces E, Gabay M, *et al*. Development of bis-thiazoles as inhibitors of triosephosphate isomerase from *Trypanosoma cruzi.* Identification of new non-mutagenic agents that are active *in vivo*. Eur J Med Chem 2015; 100: 246-56.
[397] Couto M, Sánchez C, Dávila B, Machín V, Varela J, Álvarez G, *et al*. 3-H-[1,2]Dithiole as a new Anti-*Trypanosoma cruzi* chemotype: Biological and mechanism of action studies. Molecules 2015, 20: 14595-610.
[398] Leroux AE, Maugeri DA, Cazzulo JJ, Nowicki C. Functional characterization of NADP-dependent isocitrate dehydro-

genase isozymes from *Trypanosoma cruzi*. Mol Biochem Parasitol 2011; 177(1): 61-4.

[399] Avilán L, Gualdrón-López M, Quiñones W, González-González L, Hannaert V, Michels PA, *et al*. Enolase: A key player in the metabolism and a probable virulence factor of trypanosomatid parasites-perspectives for its use as a therapeutic target. Enzyme Res 2011; 2011: 932549.

[400] Trapani S, Linss J, Goldenberg S, Fischer H, Craievich AF, Oliva G. Crystal structure of the dimeric phosphoenolpyruvate carboxykinase (PEPCK) from *Trypanosoma cruzi* at 2 A resolution. J Mol Biol 2001; 313(5): 1059-72

[401] Maugeri DA, Cannata JJ, Cazzulo JJ. Glucose metabolism in *Trypanosoma cruzi.* Essays Biochem 2011; 5: 15-30.

[402] Maugeri DA, Cazzulo JJ. The pentose phosphate pathway in *Trypanosoma cruzi*. FEMS Microbiol Lett 2004; 234(1): 117-23.

[403] Igoillo-Esteve MI, Cazzulo JJ. The glucose-6-phosphate dehydrogenase from *Trypanosoma cruzi*: Its role in the defense of the parasite against oxidative stress. Mol Biochem Parasitol 2006; 149(2): 170-81.

[404] Igoillo-Esteve M, Maugeri D, Stern AL, Beluardi P, Cazzulo JJ. The pentose phosphate pathway in *Trypanosoma cruzi:* A potential target for the chemotherapy of Chagas disease. Acad Bras Cienc 2007; 79(4): 649-63.

[405] Stern AL, Burgos E, Salmon L, Cazzulo JJ. Ribose 5-phosphate isomerase type B from *Trypanosoma cruzi*: Kinetic properties and site-directed mutagenesis reveal information about the reaction mechanism. Biochem J 2007; 401(1): 279-85.

[406] Stern AL, Naworyta A, Cazzulo JJ, Mowbray SL. Structures of type B ribose 5-phosphate isomerase from *Trypanosoma cruzi* shed light on the determinants of sugar specificity in the structural family. FEBS J 2011; 278(5): 793-808.

[407] Mielniczki-Pereira AA, Chiavegatto CM, López JA, Colli W, Alves MJ, Gadelha FR. *Trypanosoma cruzi* strains, Tulahuen 2 and Y, besides the difference in resistance to oxidative stress, display differential glucose-6-phosphate and 6-phosphogluconate dehydrogenases activities. Acta Trop 2007; 101(1): 54-60.

[408] Filipin MV, Caetano LC, Brazão V, Santello FH, Toldo MP, Prado JC Jr. DHEA and testosterone therapies in *Trypanosoma cruzi*-infected rats are associated with thymic changes. Res Vet Sci 2010; 89(1): 98-103.

[409] Cordeiro AT, Thiemann OH. 16-Bromoepiandrosterone, an activator of the mammalian immune system, inhibits glucose 6-phosphate dehydrogenase from *Trypanosoma cruzi* and is toxic to these parasites grown in culture. Bioorg Med Chem 2010; 18(13): 4762-8.

[410] Mercaldi GF, Ranzani AT, Cordeiro AT. Discovery of new uncompetitive inhibitors of glucose-6-phosphate dehydrogenase. J Biomol Screen 2014; 19(10): 1362-71.

[411] Marino C, Mariño K, Miletti L, Manso Alves MJ, Colli W, de Lederkremer RM. 1-Thio-beta-D-galactofuranosides: Synthesis and evaluation as beta-D-galactofuranosidase inhibitors. Glycobiology 1998; 8(9): 901-4.

[412] Miletti LC, Mariño K, Marino C, Colli W, Alves MJ, de Lederkremer RM. Evidence for exo beta-D-galactofuranosidase in *Trypanosoma cruzi*. Mol Biochem Parasitol 2003; 127(1): 85-8.

[413] Oppenheimer M, Valenciano AL, Sobrado P. Biosynthesis of galactofuranose in kinetoplastids: Novel therapeutic targets for treating leishmaniasis and Chagas' disease. Enzyme Res 2011; 2011: 415976.

[414] Marino C, Baldoni L. Synthesis of D-galactofuranose-containing molecules: Design of galactofuranosyl acceptors. Chembiochem 2014; 15(2): 188-204.

[415] Oppenheimer M, Valenciano AL, Kizjakina K, Qi J, Sobrado P. Chemical mechanism of UDP-galactopyranose mutase from *Trypanosoma cruzi*: A potential drug target against Chagas' disease. PLoS One 2012; 7(3): e32918.

[416] Dhatwalia R, Singh H, Oppenheimer M, Sobrado P, Tanner JJ. Crystal structures of *Trypanosoma cruzi* UDP-galactopyranose mutase implicate flexibility of the histidine loop in enzyme activation. Biochemistry 2012; 51(24): 4968-79.

[417] Boechi L, de Oliveira CA, Da Fonseca I, Kizjakina K, Sobrado P, Tanner JJ, *et al*. Substrate-dependent dynamics of UDP-galactopyranose mutase: Implications for drug design. Protein Sci 2013; 22(11): 1490-501.

[418] Pereira CA, Alonso GD, Paveto MC, Iribarren A, Cabanas ML, Torres HN, *et al*. *Trypanosoma cruzi* arginine kinase characterization and cloning. A novel energetic pathway in protozoan parasites. J Biol Chem 2000; 275(2): 1495-501.

[419] Pereira CA, Alonso GD, Ivaldi S, Silber A, Alves MJ, Bouvier LA, *et al.* Arginine metabolism in *Trypanosoma cruzi* is coupled to parasite stage and replication. FEBS Lett 2002; 526(1-3): 111-4.

[420] Miranda MR, Canepa GE, Bouvier LA, Pereira CA. *Trypanosoma cruzi*: Oxidative stress induces arginine kinase expression. Exp Parasitol 2006; 114(4): 341-4.

[421] Fernandez P, Haouz A, Pereira CA, Aguilar C, Alzari PM. The crystal structure of *Trypanosoma cruzi* arginine kinase. Proteins 2007; 69(1): 209-212.

[422] Miranda MR, Bouvier LA, Canepa GE, Pereira CA. Subcellular localization of *Trypanosoma cruzi* arginine kinase. Parasitology 2009; 27: 1-7.

[423] Paveto C, Guida MC, Esteva MI, Martino V, Coussio J, Flawiá MM, *et al*. Anti-*Trypanosoma cruzi* activity of green tea (*Camellia sinensis*) catechins. Antimicrob Agents Chemother 2004; 48(1): 69-74.

[424] Silber AM, Colli W, Ulrich H, Alves MJ, Pereira CA. Amino acid metabolic routes in *Trypanosoma cruzi*: Possible therapeutic targets against Chagas' disease. Curr Drug Targets Infect Disord 2005; 5(1): 53-64.

[425] Ivanenkov YA, Chufarova NV. Small-molecule arginase inhibitors. Pharm Pat Anal 2014; 3(1): 65-85.

[426] Chamond N, Grégoire C, Coatnoan N, Rougeot C, Freitas-Junior LH, da Silveira JF, *et al*. Biochemical characterization of proline racemases from the human protozoan parasite *Trypanosoma cruzi* and definition of putative protein signatures. J Biol Chem 2003; 278(18): 15484-94.

[427] Chamond N, Goytia M, Coatnoan N, Barale JC, Cosson A, Degrave WM, *et al*. *Trypanosoma cruzi* proline racemases are involved in parasite differentiation and infectivity. Mol Microbiol 2005; 58(1): 46-60.

[428] Buschiazzo A, Goytia M, Schaeffer F, Degrave W, Shepard W, Grégoire C, *et al*. Crystal structure, catalytic mechanism, and mitogenic properties of *Trypanosoma cruzi* proline racemase. Proc Natl Acad Sci USA 2006; 103(6): 1705-10.

[429] Coatnoan N, Berneman A, Chamond N, Minoprio P. Proline racemases: insights into *Trypanosoma cruzi* peptides containing D-proline. Mem Inst Oswaldo Cruz 2009; 104(4): 295-300.

[430] Minoprio, P., Chamond, N., Degrave, W., Berneman, A. Cloning, sequencing and expression of a gene encoding an eukaryotic amino acid racemase, and diagnostic, therapeutic, and vaccination applications of parasite and viral mitogens. US7262015 **(2007),** US7556939 **(2009)**.

[431] Minoprio, P., Alzari, P., Buscchiazzo, A., Gregoire, C., Bernenam, A., Degrave, W.M. Crystallographic structure of *Tc*PRACA and uses therefor. US7585656 **(2009)**.

[432] Coutinho L, Ferreira MA, Cosson A, Batista MM, Batista Dda G, Minoprio P, *et al*. Inhibition of *Trypanosoma cruzi* proline racemase affects host-parasite interactions and the outcome of *in vitro* infection. Mem Inst Oswaldo Cruz 2009; 104 (8): 1055-62.

[433] Minoprio, P., Arala Chaves, M., Fernandez de Almeida, S.I., Coutinho, A., San Martin, B.R., Rougeot, C., Degrave, W., Cosson, A. Cloning, sequencing and expression of a gene encoding an eukaryotic amino acid racemase, and diagnostic, therapeutic, and vaccination applications of parasite and viral mitogens. US7732563, US7851603 **(2010)**.

[434] Berneman, A., Blondel, A., Minoprio, P. Inhibitors of the proline racemase of *Trypanosoma cruzi.* EP2272510A1, EP2451450A1/B1, WO2011004323A1/A9 **(2011)**.

[435] Mantilla BS, Paes LS, Pral EM, Martil DE, Thiemann OH, Fernández-Silva P, *et al*. Role of Δ1-pyrroline-5-carboxylate

dehydrogenase supports mitochondrial metabolism and host-cell Invasion of *Trypanosoma cruzi*. J Biol Chem 2015; 290(12): 7767-90.

[436] Magdaleno A, Ahn IY, Paes LS, Silber AM. Actions of a proline analogue, L-thiazolidine-4-carboxylic acid (T4C), on *Trypanosoma cruzi*. PLoS One 2009; 4(2): e4534.

[437] Naula C, Parsons M, Mottram JC. Protein kinases as drug targets in Trypanosomes and Leishmania. Biochim Biophys Acta 2005; 1754 (1-2): 151-9.

[438] Bao Y, Weiss LM, Hashimoto M, Nara T, Huang H. Protein kinase A regulatory subunit interacts with P type ATPases in *Trypanosoma cruzi*. Am J Trop Med Hyg 2009; 80(6): 941-3.

[439] Belaunzarán ML, Lammel EM, Giménez G, Wainszelbaum MJ, de Isola EL. Involvement of protein kinase C isoenzymes in *Trypanosoma cruzi* metacyclogenesis induced by oleic acid. Parasitol Res 2009; 105(1): 47-55.

[440] Braga MV, de Souza W. Effects of protein kinase and phosphatidylinositol-3 kinase inhibitors on growth and ultrastructure of *Trypanosoma cruzi.* FEMS Microbiol Lett 2006; 256(2): 209-16.

[441] Saraiva VB, Wengert M, Gomes-Quintana E, Heise N, Caruso-Neves C. Na (+)-ATPase and protein kinase C are targets to 1-O-hexadecylphosphocoline (miltefosine) in *Trypanosoma cruzi*. Arch Biochem Biophys 2009; 481(1): 65-71.

[442] Deinhardt K, Chao MV. Trk receptors. Handb Exp Pharmacol 2014; 220:103-19.

[443] Takeuchi, J., Ikura, M., Higashino, M., Iwahashi, M., Hashimura, K. Trk-inhibiting compounds. WO2013161919 **(2013)**, JP20150111865 **(2015)**.

[444] Lountos GT, Tropea JE, Waugh DS. Structure of the *Trypanosoma cruzi* protein tyrosine phosphatase PTP1, a potential therapeutic target for Chagas' disease. Mol Biochem Parasitol 2013; 187(1):1-8.

[445] Woodring JL, Patel G, Erath J, Behera R, Lee PJ, Leed SE, *et al*. Evaluation of aromatic 6-substituted thienopyrimidines as scaffolds against parasites that cause Trypanosomiasis, Leishmaniasis and Malaria. Medchemcomm 2015; 6(2): 339-46.

[446] Ariyanayagam MR, Fairlamb AH. Diamine auxotrophy may be a universal feature of *Trypanosoma cruzi* epimastigotes. Mol Biochem Parasitol 1997; 84(1): 111-21.

[447] Majumder S, Wirth JJ, Bitonti AJ, McCann PP, Kierszenbaum F. Biochemical evidence for the presence of arginine decarboxylase activity in *Trypanosoma cruzi*. J Parasitol 1992; 78(2): 371-4.

[448] Carrillo C, González NS, Algranati ID. *Trypanosoma cruzi* as a model system to study the expression of exogenous genes coding for polyamine biosynthetic enzymes. Biochim Biophys Acta 2007; 1770(12): 1605-11.

[449] Menezes D, Valentim C, Oliveira MF, Vannier-Santos MA. Putrescine analogue cytotoxicity against *Trypanosoma cruzi*. Parasitol Res 2006; 98(2): 99-105.

[450] Audette, M., Charest-Gaudrealt, R. Polyamine transport inhibitors. US6949679 **(2005)**.

[451] Goebel T, Ulmer D, Projahn H, Kloeckner J, Heller E, Glaser M, *et al*. In search of novel agents for therapy of tropical diseases and human immunodeficiency virus. J Med Chem 2008; 51(2): 238-50.

[452] Heby O, Persson L, Rentala M. Targeting the polyamine biosynthetic enzymes: A promising approach to therapy of African sleeping sickness, Chagas' disease, and Leishmaniasis. Amino Acids 2007; 33(2): 359- 66.

[453] Pereira CA, Carrillo C, Miranda MR, Bouvier LA, Canepa GE. *Trypanosoma cruzi*: transport of essential metabolites acquired from the host. Medicina (Bs As) 2008; 68(5): 398-404.

[454] Birkholtz LM, Williams M, Niemand J, Louw AI, Persson L, Heby O. Polyamine homoeostasis as a drug target in pathogenic protozoa: peculiarities and possibilities. Biochem J 2011; 438(2): 229-44.

[455] Díaz MV, Miranda MR, Campos-Estrada C, Reigada C, Maya JD, Pereira CA, *et al*. Pentamidine exerts *in vitro* and *in vivo* anti-*Trypanosoma cruzi* activity and inhibits the polyamine transport in *Trypanosoma cruzi*. Acta Trop 2014; 134: 1-9.

[456] Ullman B, Carter D. Molecular and biochemical studies on the hypoxanthine-guanine phosphoribosyltransferases of the pathogenic haemoflagellates. Int J Parasitol 1997; 27(2): 203-13.

[457] Nakajima-Shimada J, Hirota Y, Aoki T. Inhibition of *Trypanosoma cruzi* growth in mammalian cells by purine and pyrimidine analogs. Antimicrob Agents Chemother 1996; 40(11): 2455-8.

[458] Avila JL, Avila A, Munoz E. Effect of allopurinol on different strains of *Trypanosoma cruzi*. Am J Trop Med Hyg 1981; 30(4): 769-74.

[459] Eakin AE, Guerra A, Focia PJ, Torres-Martinez, Craig SP 3rd. Hypoxanthine phosphoribosyltransferase from *Trypanosoma cruzi* as a target for structure-based inhibitor design: crystallization and inhibition studies with purine analogs. Antimicrob Agents Chemother 1997; 41(8): 1686-92.

[460] Freymann DM, Wenck MA, Engel JC, Feng J, Focia PJ, Eakin AE, *et al*. Efficient identification of inhibitors targeting the closed active site conformation of the HPRT from *Trypanosoma cruzi*. Chem Biol 2000; 7(12): 957-68.

[461] Wenck MA, Medrano FJ, Eakin AE, Craig SP. Steady-state kinetics of the hypoxanthine phosphoribosyltransferase from *Trypanosoma cruzi*. Biochim Biophys Acta 2004; 1700 (1): 11-8.

[462] Reche P, Arrebola R, Santi DV, Pacanowska GD, Perez RLM. Expression and characterization of the *Trypanosoma cruzi* dihydrofolate reductase domain. Mol Biochem Parasitol 1996; 76(1-2): 175-85.

[463] Gilbert IH. Inhibitors of dihydrofolate reductase in Leishmania and Trypanosomes. Biochim Biophys Acta 2002; 1587(2-3): 249-57.

[464] Schormann N, Senkovich O, Walker K, Wright DL, Anderson AC, Rosowsky A, *et al*. Structure-based approach to pharmacophore identification, *in silico* screening, and three-dimensional quantitative structure-activity relationship studies for inhibitors of *Trypanosoma cruzi* dihydrofolate reductase function. Proteins 2008; 73(4): 889-901.

[465] Senkovich O, Schormann N, Chattopadhyay D. Structures of dihydrofolate reductase-thymidylate synthase of *Trypanosoma cruzi* in the folate-free state and in complex with two antifolate drugs, trimetrexate and methotrexate. Acta Crystallogr D Biol Crystallogr 2009; 65(7): 704-16.

[466] Chitnumsub P, Yuvaniyama J, Chahomchuen J, Valaivan T, Yuthavon Y. Crystallization and preliminary crystallographic studies of dihydrofolate reductase-thymidylate synthase from *Trypanosoma cruzi*, the Chagas disease pathogen. Acta Crystalllogr Sect F Struct Biol Cryst Commun 2009; 65(11): 1175-8.

[467] Schormann N, Velu SE, Murugesan S. Synthesis and characterization of potent inhibitors of *Trypanosoma cruzi* dihydrofolate reductase. Bioorg Med Chem 2010; 18(11): 4056-66.

[468] Chowdhury SF, Guerrero RH, Brun R, Ruiz-Perez L, Pacanowska DG, Gilbert IH. Synthesis and testing of 5-benzyl-2, 4-diaminopyrimidines as potential inhibitors of Leishmanial and Trypanosomal dihydrofolate reductase. J Enzyme Inhib Med Chem 2002; 17(5): 293-302.

[469] Pez D, Leal I, Zuccotto F. 2,4-Diaminopyrimidines as inhibitors of Leishmanial and Trypanosomal dihydrofolate reductase. Bioorg Med Chem 2003; 11(22): 4693-4711.

[470] Khabnadidesh S, Pez D, Musso A. Design, synthesis and evaluation of 2,4-diaminoquinazolines as inhibitors of Trypanosomal and Leishmanial dihydrofolate reductase. Bioorg Med Chem 2005; 13(7): 2637-49.

[471] Mendoza-Martínez C, Correa-Basurto J, Nieto-Meneses R, *Márquez-Navarro A, Aguilar-Suárez R, Montero-Cortes MD, *et al*. Design, synthesis and biological evaluation of quinazoline derivatives as anti-trypanosomatid and anti-plasmodial agents. Eur J Med Chem. 2015; 96:296-307.

[472] Sencovich O, Bhatia V, Garg N, Chattopadhyay D. Lipophilic antifolate trimetrexate is a potent inhibitor of *Trypanosoma cruzi.* Prospect for chemotherapy of Chagas disease. Antimicrob Agents Chemother 2005; 49(8):3234-8.

[473] Nare B, Luba J, Hardy LW, Beverley SM. New approaches to Leishmania chemotherapy: pteridine reductase 1 (PTR1) as a target and modulator of antifolate sensitivity. Parasitology 1997; 114 (Suppl): 101-10.

[474] Schormann N, Pal B, Chattopadhyay D. Expression, purification, crystallization and preliminary crystallographic analysis of recombinant pteridine reductase of *Trypanosoma cruzi*. Acta Crystallogr D Biol Crystallogr 2001; 57(11): 1671-3.

[475] Senkovich O, Pal B, Schormann N, Chattopadhyay D. *Trypanosoma cruzi* genome encodes a pteridine reductase 2 protein. Mol Biochem Parasitol 2003; 127(1): 89-92.

[476] Schormann N, Pal B, Senkovich O, Carson M, Howard A, Smith C, *et al*. Crystal structure of *Trypanosoma cruzi* pteridine reductase 2 in complex with a substrate and an inhibitor. J Struct Biol 2005; 152(1): 64-75.

[477] Lowe, G. Dihydrofolate reductase inhibitors. WO0153276A1 **(2001)**.

[478] Lawton P. Purine analogs as antiparasitic agents. Expert Opin Ther Pat 2005; 15(8): 987-94.

[479] Bottaro, J.C., Schmitt, R.J., Petrie, M.A., Penwell, P.E. Nucleoside pyrophosphate and triphosphate analogs and related compounds as therapeutic agents. WO0114401A1 **(2001)**.

[480] Cavazzuti A, Paglietti G, Hunter WN, Gamarro F, Piras S, Loriga M, *et al*. Discovery of potent pteridine reductase inhibitors to guide antiparasite drug development. Proc Natl Acad Sci USA 2008; 105(5): 1448-53.

[481] Heere, J., Augustyns, K., Van Der Veken, P., Joosens, J., Muthusamy, V., Maes, L.L., Paulus, J. Disubstituted triazine dimers for treatment and/or prevention of infectious diseases. WO2013068551A1 **(2013)**, EP2776410A1 **(2014)**.

[482] Nara T, Kamei Y, Tsubouchi A. Inhibitory action of marine algae extracts on the *Trypanosoma cruzi* dihydroorotate dehydrogenase activity and on the protozoan growth in mammalian cells. Parasitol Int 2005; 54 (1): 59-64.

[483] Inaoka DK, Tashamira E, Osanai A. Expression, purification and crystallization of *Trypanosoma cruzi* dihydroorotate dehydrogenase complexed with orotate. Acta Cryst Sec F Struct Biol Cryst Commun 2005; 61(10): 875-8.

[484] Pinheiro MP, Iulek J, Nonato CM. Crystal structure of *Trypanosoma cruzi* dihydroorotate dehydrogenase from Y strain. Biochem Biophys Res Commun 2008; 369(3): 812-7.

[485] Inaoka DK, Sakamoto K, Shimizu H, Shiba T, Kurisu G, Nara T, *et al*. Structures of *Trypanosoma cruzi* dihydroorotate dehydrogenase complexed with substrates and products: atomic resolution insights into mechanisms of dihydroorotate oxidation and fumarate reduction. Biochemistry 2008; 47(41): 10881-91.

[486] Cheleski J, Rocha JR, Pinheiro MP, Wiggers HJ, da Silva AB, Nonato MC, *et al*. Novel insights for dihydroorotate dehydrogenase class 1A inhibitors discovery. Eur J Med Chem 2010; 45(12): 5899-909.

[487] Berg M, van der Veken P, Goeminne A, Haemers A, Augustyns K. Inhibitors of the purine salvage pathway: A valuable approach for antiprotozoal chemotherapy? Curr Med Chem 2010; 17(23): 2456-81.

[488] Gonzales-Perdomo M, de Castro SL, Meirelles MN, Goldenberg S. *Trypanosoma cruzi* proliferation and differentiation are blocked by topoisomerase II inhibitors. Antimicrob Agents Chemother 1990; 34(9): 1707-14.

[489] Morales J, Mogi T, Mineki S, Takashima E, Mineki R, Hirawake H, *et al*. Novel mitochondrial complex II isolated from*Trypanosoma cruzi* is composed of 12 peptides including a heterodimeric Ip subunit. J Biol Chem 2009; 284(11): 7255-63.

[490] Balaña-Fouce R, Alvarez-Velilla R, Fernández-Prada C, García-Estrada C, Reguera RM. Trypanosomatids topoisomerase re-visited. New structural findings and role in drug discovery. Int J Parasitol Drugs Drug Resist 2014; 4(3):326-37.

[491] Calderano SG, de Melo Godoy PD, da Cunha JP, Elias MC. Trypanosome prereplication machinery: A potential new target for an old problem. Enzyme Res 2011; 2011: 518258.

[492] Jonckers TH, van Miert S, Cimanga K, Bailly C, Colson P, De Pauw-Gillet MC, *et al*. Synthesis, cytotoxicity, and antiplasmodial and antitrypanosomal activity of new neocryptolepine derivatives. Med Chem 2002; 45(16): 3497-508.

[493] Bilbao-Ramos P, Galiana-Roselló C, Dea-Ayuela MA, González-Alvarez M, Vega C, Rolón M, *et al*. Nuclease activity and ultrastructural effects of new sulfonamides with antileishmanial and trypanocidal activities. Parasitol Int 2012; 61(4): 604-13.

[494] Galiana-Roselló C, Bilbao-Ramos P, Dea-Ayuela MA, Rolón M, Vega C, Bolás-Fernández F, *et al*. *In vitro* and *in vivo* antileishmanial and trypanocidal studies of new N-benzene- and N-naphthalenesulfonamide derivatives. J Med Chem 2013; 56(22): 8984-98.

[495] Abdul-Rahman, S. Compounds with antibacterial and antiparasitic properties. US6967205 **(2005)**.

[496] Estevez Y, Juiliano M, Burguete A, Cabanillas B, Zimic M, Málaga E, *et al*. Trypanocidal properties, structure-activity relationship and computational studies of quinoxaline 1,4-di-N-oxide derivatives. Exp Parasitol 2011; 127 (4):745-51.

[497] Stephens CE, Brun R, Salem MM, Werbovetz KA, Tanious F, Wilson WD, *et al*. The activity of diguanidino and 'reversed' diamidino 2, 5- diarylfurans versus *Trypanosoma cruzi* and *Leishmania donovani*. Bioorg Med Chem Lett 2003; 13 (12): 2065-9.

[498] Werbovetz K. Diamidines as anti-Trypanosomal, antiLeishmanial and antimalarial agents. Curr Opin Investig Drugs 2006; 7(2): 147-57.

[499] Boykin, D., TIidwell, R.R., Wilson, W.D., Perfect, J.R., Stephens, C.E. Synthesis and antimicrobial activityof novel dicationic 'reversed amidines'. WO02057224A2 **(2002)** and WO02057224A3 **(2002)**.

[500] da Silva CF, Batista MM, Batista D da G, de Souza EM, da Silva PB, de Oliveira GM, *et al*. *In vitro* and *in vivo* studies of the trypanocidal activity of a diarylthiophene diamidine against *Trypanosoma cruzi*. Antimicrob Agents Chemother 2008; 52(9): 3307-14.

[501] Silva CF, Batista MM, Mota RA, de Souza EM, Stephens CE, Som P, *et al*. Activity of "reversed" diamidines against *Trypanosoma cruzi* "*in vitro*". Biochem Pharmacol 2007; 73(12): 1939-46.

[502] Soeiro MN, Werbovetz K, Boykin DW, Wilson WD, Wang MZ, Hemphill A. Novel amidines and analogues as promising agents against intracellular parasites: A systematic review. Parasitology 2013; 140(8): 929-51.

[503] Timm BL, Da Silva PB, Batista MM, Farahat AA, Kumar A, Boykin DW, *et al*. *In vitro* investigation of the efficacy of novel diamidines against *Trypanosoma cruzi*. Parasitology 2014; 141(10): 1272-6.

[504] de Castro SL, Batista DG, Batista MM, Batista W, Daliry A, de Souza EM, *et al*. Experimental chemotherapy for Chagas disease: A morphological, biochemical, and proteomic overview of potential *Trypanosoma cruzi*. targets of amidines derivatives and naphthoquinones. Mol Biol Int 2011; 2011: 306928.

[505] Werbovetz, K., Bhatta Charya, G., Sackett, D., Salem, M.M. Antileishmanial dinitroaniline sulfonamides with activity against parasite tubulin. WO03090678A2/A3 **(2003)**.

[506] Chohan ZH, Hernandes MZ, Sensato FR, Moreira DR, Pereira VR, Neves JK. Sulfonamide-metal complexes endowed with potent anti-*Trypanosoma cruzi* activity. J Enzyme Inhib Med Chem 2014; 29(2): 230-6.

[507] Benítez J, Guggeri L, Tomaz I, Arrambide G, Navarro M, Pessoa JC, *et al*. Design of vanadium mixed-ligand complexes as potential anti protozoa agents. J Inorg Biochem 2009; 103(4): 609-16.

[508] Scalese G, Benítez J, Rostán S, Correia I2, Bradford L1, Vieites M, *et al.* Expanding the family of heteroleptic oxidovanadium (IV) compounds with salicylaldehyde semicarbazones and polypyridyl ligands showing anti-*Trypanosoma cruzi* activity. J Inorg 2015;147:116-25.

[509] Demoro B, Rossi M, Caruso F, Liebowitz D, Olea-Azar C, Kemmerling U, *et al*. Potential mechanism of the antitrypanosomal activity of organoruthenium complexes with bioactive thiosemicarbazones. Biol Trace Elem Res 2013; 153(1-3): 371-81.

[510] Sarniguet C, Toloza J, Cipriani M, Lapier M, Vieites M, Toledano-Magaña Y, *et al*. Water-soluble ruthenium complexes bearing activity against protozoan parasites. Biol Trace Elem Res 2014; 159(1-3):379-92.
[511] Glass LS, Nguyen B, Goodwin KD, Dardonville C, Wilson WD, Long EC, *et al*. Crystal structure of a trypanocidal 4,4'-bis (imidazolinylamino)diphenylamine bound to DNA. Biochemistry 2009 30; 48(25): 5943-52.
[512] Stolic I, Miskovic K, Magdaleno A, Silber AM, Piantanida I, Bajić M, *et al*. Effect of 3,4-ethylenedioxy-extension of thiophene core on the DNA/RNA binding properties and biological activity of bisbenzimidazole amidines. Bioorg Med Chem 2009; 17(6): 2544-54.
[513] Ochola DO, Prichard RK, Lubega GW. Classical ligands bind tubulin of trypanosomes and inhibit their growth *in vitro*. J Parasitol 2002; 88(3): 600-4.
[514] Morgan RE, Werbovetz KA. Selective lead compounds against kinetoplastid tubulin. Adv Exp Med Biol 2008; 625: 33-47.
[515] Cai S, Li QS, Borchardt RT, Kuczera K, Schowen RL. The antiviral drug ribavirin is a selective inhibitor of S-adenosyl-L-homocysteine hydrolase from *Trypanosoma cruzi*. Bioorg Med Chem 2007; 15(23): 7281-7.
[516] Cai S, Li QS, Fang J, Borchardt RT, Kuczera K, Middaugh CR, *et al*. The rationale for targeting the NAD/NADH cofactor binding site of parasitic S-adenosyl-L-homocysteine hydrolase for the design of anti-parasitic drugs. Nucleosides Nucleotides Nucleic Acids 2009; 28(5): 485-503.
[517] Li QS, Cai S, Fang J, Borchardt RT, Kuczera K, Middaugh CR, *et al*. Evaluation of NAD (H) analogues as selective inhibitors for *Trypanosoma cruzi* S-adenosylhomocysteine hydrolase. Nucleosides Nucleotides Nucleic Acids 2009; 28(5): 473-84.
[518] Cai S, Fang J, Li QS, Borchardt RT, Kuczera K, Middaugh CR, *et al*. Comparative kinetics of cofactor association and dissociation for the human and trypanosomal S-adenosylhomocysteine hydrolases. 3. Role of lysyl and tyrosyl residues of the C-terminal extension. Biochemistry 2010; 49(38): 8434-41.
[519] Bukrinsky, M. I., Cerami, A., Ulrich, P., Berger, B. Compounds and methods of use to treat infectious diseases US6906076 **(2005)**.
[520] Docampo R, de Souza W, Miranda K, Rohloff P, Moreno SN. Acidocalcisomes-conserved from bacteria to man. Nat Rev Microbiol 2005; 3(3): 251-61.
[521] Vercesi AE, Grijalba MT, Docampo R. Inhibition of Ca2+ release from *Trypanosoma brucei* acidocalcisomes by 3,5-dibutyl-4-hydroxytoluene: role of the Na+/H+ exchanger. Biochem J 1997; 328 (Pt 2): 479-82.
[522] Lang, H.J., Lanzer, M., Wiesner, J., Sanchez, C., Wunsch, S.E.P. Use of inhibitors of the sodium-hydrogen exchanger for the production of a pharmaceutical for the treatment of disorders which are caused by protozoa. US6114393 **(2000)**.
[523] Menna-Barreto RF, Henriquez-Pons A, Pinto AV, Morgado-Diaz JA, Soares MJ, De Castro SL. Effect of a beta-lapachone-derived naphthoimidazole on*Trypanosoma cruzi*: identification of target organelles. J Antimicrob Chemother 2005; 56(6): 1034-41.
[524] Montalvetti A, Rohloff P, Docampo RA. A functional aquaporin co-localizes with the vacuolar proton pyrophosphatase to acidocalcisomes and the contractile vacuole complex of *Trypanosoma cruzi*. J Biol Chem 2004; 279(37): 38673-82.
[525] Beitz E. Aquaporins from pathogenic protozoan parasites: structure, function and potential for chemotherapy. Biol Cell 2005; 97(6): 373-83.
[526] Rohloff P, Docampo R. A contractile vacuole complex is involved in osmoregulation in *Trypanosoma cruzi.* Exp Parasitol 2008; 118(1): 17-24.
[527] Li ZH, Alvarez VE, De Gaudenzi JG, Sant'Anna C, Frasch AC, Cazzulo JJ, *et al*. Hyperosmotic stress induces aquaporin-dependent cell shrinkage, polyphosphate synthesis, amino acid accumulation, and global gene expression changes in *Trypanosoma cruzi*. J Biol Chem 2011; 286(51): 43959-71.
[528] Landfear SM. Drugs and transporters in kinetoplastid protozoa. Adv Exp Med Biol 2008; 625: 22-32.
[529] Schoijet AC, Miranda K, Girard-Dias W, de Souza W, Flawiá MM, Torres HN, *et al.* A *Trypanosoma cruzi* phosphatidylinositol 3-kinase (*Tc*Vps34) is involved in osmoregulation and receptor-mediated endocytosis. J Biol Chem 2008; 283(46): 31541-50.
[530] Gimenez AM, Gesumaría MC, Schoijet AC, Alonso GD3, Flawiá MM3, Racagni GE, *et al*. Phosphatidylinositol kinase activities in *Trypanosoma cruzi* epimastigotes. Mol Biochem Parasitol 2015; 203(1-2): 14-24.
[531] Fang J, Ruiz FA, Docampo M, Luo S, Rodrigues JC, Motta LS, *et al*. Overexpression of a Zn2+-sensitive soluble exopolyphosphatase from *Trypanosoma cruzi* depletes polyphosphate and affects osmoregulation. J Biol Chem 2007; 282(44): 32501-10.
[532] Moreira OC, Rios PF, Esteves FF, Meyer-Fernandes JR, Barrabin H. CrATP as a new inhibitor of ecto- ATPases of Trypanosomatids. Parasitology 2009; 136(1): 35-44.
[533] Einicker-Lamas M, Nascimento MT, Masuda CA, Oliveira MM, Caruso-Neves C. *Trypanosoma cruzi* epimastigotes: Regulation of myo-inositol transport by effectors of protein kinases A and C. Exp Parasitol 2007; 117(2): 171-7.
[534] Boutaleb-Charki S, Marín C, Maldonado CR, Rosales MJ, Urbano J, Guitierrez-Sánchez R, *et al*. Copper (II) complexes of [1,2,4]triazolo [1,5-a]pyrimidine derivatives as potential anti-parasitic agents. Drug Metab Lett 2009; 3(1): 35-44.
[535] Inbar E, Canepa GE, Carrillo C, Glaser F, Suter Grotemeyer M, Rentsch D, *et al*. Lysine transporters in human trypanosomatid pathogens. Nucleosides Nucleotides Nucleic Acids 2009; 28(5): 473-84.
[536] Kunz S, Oberholzer M, Seebeck T. A FYVE-containing unusual cyclic nucleotide phosphodiesterase from *Trypanosoma cruzi*. FEBS J 2005; 272: 6412-22.
[537] Schoijet AC, Miranda K, Medeiros LC, de Souza W, Flawiá MM, Torres HN, *et al*. Defining the role of a FYVE domain in the localization and activity of a cAMP phosphodiesterase implicated in osmoregulation in *Trypanosoma cruzi*. Mol Microbiol 2011; 79(1): 50-62.
[538] Makin L, Gluenz E. cAMP signalling in trypanosomatids: role in pathogenesis and as a drug target. Trends Parasitol 2015; 31(8): 373-9.
[539] King Keller S, Li M, Smith A, Zheng S, Kaur G, Yang X, *et al*. Chemical validation of phosphodiesterase C as a chemotherapeutic target in *Trypanosoma cruzi*, the etiological agent of Chagas' disease. Antimicrob Agents Chemother 2010; 54(9): 3738-45.
[540] Tagoe DN, Kalejaiye TD, de Koning HP. The ever unfolding story of cAMP signaling in trypanosomatids: vive la difference! Front Pharmacol 2015; 6: 185.
[541] Michels PA, Bringaud F, Herman M, Hannaert V Metabolic functions of glycosomes in trypanosomatids. Biochim Biophys Acta 2006; 1763(12): 1463-77.
[542] Wilkinson SR, Prathalingam SR, Taylor MC, Horn D, Kelly JM. Vitamin C biosynthesis in trypanosomes: A role for the glycosome. Proc Natl Acad Sci USA 2005; 102(33): 11645-50.
[543] Barros-Alvarez X, Gualdrón-López M, Acosta H, Cáceres AJ, Graminha MA, Michels PA, *et al*. Glycosomal targets for anti-trypanosomatid drug discovery. Curr Med Chem 2014; 21(15): 1679-706.
[544] Barros-Álvarez X, Cáceres AJ, Ruiz MT, Michels PA, Concepción JL, Quiñones W. The glycosomal-membrane associated phosphoglycerate kinase isoenzyme A plays a role in sustaining the glucose flux in *Trypanosoma cruzi* epimastigotes. Mol Biochem Parasitol 2015; 200(1.2): 5-8.
[545] Lisvane Silva P, Mantilla BS, Barisón MJ, Wrenger C, Silber AM. The uniqueness of the *Trypanosoma cruzi* mitochondrion: Opportunities to identify new drug target for the treatment of Chagas disease.Curr Pharm Des 2011; 17(20): 2074-99.

[546] Menna-Barreto RF, De Castro SL. The double-edged sword in pathogenic trypanosomatids: the pivotal role of mitochondria in oxidative stress and bioenergetics. Biomed Res Int 2014; 2014:614014.

[547] Menna-Barreto RF, Gonçalves RD, Costa EM, Silva RS, Pinto AV, Oliveira MF, *et al*. The effects on *Trypanosoma cruzi* of novel synthetic naphthoquinones are mediated by mitochondrial dysfunction. Free Radic Biol Med 2009; 47(5): 644-53.

[548] Matsuo AL, Silva LS, Torrecilhas AC, Pascoalino BS, Ramos TC, Rodrigues EG, *et al*. *In vitro* and *in vivo* trypanocidal effects of the cyclopalladated compound 7a, a drug candidate for treatment of Chagas' disease. Antimicrob Agents Chemother 2010; 54(8): 3318-25.

[549] Boiani L, Aguirre G, González M, Cerecetto H, Chidichimo A, Cazzulo JJ, *et al*. Furoxan-, alkylnitrate-derivatives and related compounds as anti-Trypanosomatid agents: Mechanism of action. Bioorg Med Chem 2008; 16(17): 7900-7.

[550] Batista D da G, Batista MM, de Oliveira GM. Arylimidamide DB766, a potential chemotherapeutic candidate for Chagas' disease treatment. Antimicrob Agents Chemother 2010; 54(7): 2940-52.

[551] Daliry A, Pires MQ, Silva CF. The trypanocidal activity of amidine compounds does not correlate with their binding affinity to *T. cruzi* kinetoplast DNA. Antimicrob Agents Chemother 2011; 55(10): 4765-73.

[552] da Silva CF, Batista Dda G, Oliveira GM, de Souza EM, Hammer ER, da Silva PB, *et al. In vitro* and *in vivo* investigation of the efficacy of arylimidamide DB1831 and its mesylated salt form DB1965 against *Trypanosoma cruzi* infection. PLoS One 2012; 7(1): e30356.

[553] Benitez D, Cabrera M, Hernández P, Boiani L, Lavaggi ML, Di Maio R, *et al*. 3-Trifluoromethylquinoxaline N, N'-dioxides as anti-trypanosomatid agents. Identification of optimal anti-*T. cruzi* agents and mechanism of action studies. J Med Chem 2011; 54(10): 3624-36.

[554] Pardo Andreu GL, Inada NM, Pellón RF, Docampo ML, Fascio ML, D'Accorso NB, *et al*. *In vitro* effect of a new cinnamic acid derivative against the epimastigote form of *Trypanosoma cruzi*. Arzneimittelforschung 2009; 59(4): 207-11.

[555] Zuma AA, Cavalcanti DP, Zogovich M, Machado AC, Mendes IC, Thiry M, *et al*. Unveiling the effects of berenil, a DNA-binding drug, on *Trypanosoma cruzi*: Implications for kDNA ultrastructure and replication. Parasitol Res 2015; 114(2): 419-30.

[556] Jimenez V, Paredes R, Sosa MA, Galanti N. Natural programmed cell death in *T. cruzi* epimastigotes maintained in axenic cultures. J Cell Biochem 2008; 105(3): 688-98.

[557] Menna-Barreto RFS, De Castro SL. Between armour and weapons: Cell death mechanisms in trypanosomatid parasites. In: Tobias Ntuli. (Org). Cell Death - Autophagy, Necrosis and Apoptosis, (2015) 1 ed., Rijeka: Intech, p. 1-10.

[558] Lozano E, Barrera P, Tonn C, Nieto M, Sartor T, Sosa MA. The effect of the diterpene 5-epi-icetexone on the cell cycle of *Trypanosoma cruzi*. Parasitol Int 2012; 61(2): 275-9.

[559] Tfouni E, Truzzi DR, Tavares A, Gomes AJ, Figueiredo LE, Franco DW. Biological activity of ruthenium nitrosyl complexes. Nitric Oxide 2012; 26(1): 38-53.

[560] Lazarin-Bidóia D, Desoti VC, Ueda-Nakamura T, Dias Filho BP, Nakamura CV, Silva SO. Further evidence of the trypanocidal action of eupomatenoid-5: Confirmation of involvement of reactive oxygen species and mitochondria owing to a reduction in trypanothione reductase activity. Free Radic Biol Med 2013; 60:17-28.

[561] Piacenza L, Peluffo G, Alvarez MN, Martínez A, Radi R. *Trypanosoma cruzi* antioxidant enzymes as virulence factors in Chagas disease. Antioxid Redox Signal 2013; 19(7): 723-34.

[562] Jimenez V, Kemmerling U, Paredes R, Maya JD, Sosa MA, Galanti N. Natural sesquiterpene lactones induce programmed cell death in *Trypanosoma cruzi*: A new therapeutic target? Phytomedicine 2014; 21(11): 1411-8.

[563] Benítez D, Casanova G, Cabrera G, Galanti N, Cerecetto H, González M. Initial studies on mechanism of action and cell death of active N-oxide-containing heterocycles in *Trypanosoma cruzi* epimastigotes *in vitro*. Parasitology 2014; 141(5): 682-96.

[564] Sandes JM, Fontes A, Regis-da-Silva CG, de Castro MC4, Lima-Junior CG5, Silva FP*, et al*. *Trypanosoma cruzi* cell death induced by the Morita-Baylis-Hillman adduct 3-Hydroxy-2-methylene-3-(4-nitrophenylpropanenitrile). PLoS One 2014; 9(4): e93936.

[565] Damasceno FS, Barisón MJ, Pral EM, Paes LS, Silber AM. Memantine, an antagonist of the NMDA glutamate receptor, affects cell proliferation, differentiation and the intracellular cycle and induces apoptosis in *Trypanosoma cruzi.* PLoS Negl Trop Dis 2014; 8(2): e2717.

[566] Vanrell MC, Cueto JA, Barclay JJ, Carrillo C, Colombo MI, Gottlieb RA, *et al*. Polyamine depletion inhibits the autophagic response modulating *Trypanosoma cruzi* infectivity. Autophagy 2013; 9(7): 1080-93.

[567] Magalhaes Moreira DR, Lima Leite AC, Srivastava RM, Hernandes MZ, Rabello MM, *et al*. Structural Design, Synthesis and structure-activity relationships of thiazolidinones with enhanced anti-*Trypanosoma cruzi* activity. Chem Med Chem 2014, 9: 177-88

[568] Volpato H, Desoti VC, Valdez RH. Mitochondrial dysfunction induced by N-butyl-1-(4-Dimethylamino)phenyl-1,2,3,4-tetrahydro-β-carboline-3-carboxamide is required for cell death of *Trypanosoma cruzi*. PLoS One 2015; 10(6): e0130652.

[569] Lazarin-Bidóia D, Desoti VC, Martins SC. Dibenzylideneacetones are potent trypanocidal compounds that affect the *Trypanosoma cruzi* redox system. Antimicrob Agents Chemother 2015;60(2): 890-903.

[570] Schenkman S, Chaves LB, de Carvalho PLC, Eichinger D. A proteolytic fragment of *Trypanosoma cruzi* trans-sialidase lacking the carboxyl-terminal domain is active, monomeric, and generates antibodies that inhibit enzymatic activity. J Biol Chem 1994; 269(11): 7970-5.

[571] Frasch AC. Functional diversity in the trans-sialidase and mucin families in *Trypanosoma cruzi*. Parasitol Today 2000; 16(7): 282-6.

[572] Buschiazzo A, Amaya MF, Cremona ML, Frasch AC, Alzari PM. The crystal structure and mode of action of trans-sialidase, a key enzyme in *Trypanosoma cruzi* pathogenesis. Mol Cell 2002; 10(4):757-68.

[573] Streicher H, Busse H. Building a successful structural motif into sialylmimetics cyclohexenephosphonate monoesters as pseudo-sialosides with promising inhibitory properties. Bioorg Med Chem 2006; 14 (4):1047-57.

[574] Horenstein, B.A., Parr, I.B. Neuraminidase inhibitors. WO9906369A1 **(1999)**.

[575] Luo, M. White, C.L. Inhibitors of bacterial sialidase. US6114386 **(2000)**.

[576] Carvalho I, Andrade P, Campo VL. 'Click chemistry' synthesis of a library of 1,2,3-triazole-substituted galactose derivatives and their evaluation against *Trypanosoma cruzi* and its cell surface trans-sialidase. Bioorg Med Chem 2010; 18(7): 2412-27.

[577] Pierdominici-Sottile, Roitberg AE. Proton Transfer Facilitated by Ligand Binding. An energetic analysis of the catalytic mechanism of *Trypanosoma cruzi* trans-sialidase. Biochemistry 2011; 50(5): 836-42.

[578] Carvalho ST, Sola-Penna M, Oliveira IA. A new class of mechanism-based inhibitors for *Trypanosoma cruzi* trans-sialidase and their influence on parasite virulence. Glycobiology 2010; 20(8): 1034-45.

[579] Meinke S, Schroven A, Thiem J. Sialic acid C-glycosides with aromatic residues: investigating enzyme binding and inhibition of *Trypanosoma cruzi* trans-sialidase. Org Biomol Chem 2011; 9(12): 4487-97.

[580] Campo VL, Sesti-Costa R, Carneiro ZA, Silva JS, Schenkman S, Carvalho I. Design, synthesis and the effect of 1,2,3-triazole sialylmimetic neoglycoconjugates on *Trypanosoma cruzi* and its cell surface trans-sialidase. Bioorg Med Chem 2011; 20(1): 145-56.

[581] Meinke S, Thiem J. Trypanosomal trans-sialidases: Valuable synthetic tools and targets for medicinal chemistry. Top Curr Chem 2015; 367: 231-50.
[582] Kai H, Hinou H, Naruchi K, Matsushita T, Nishimura S. Macrocyclic mechanism-based inhibitor for neuraminidases. Chemistry 2013; 19(4): 1364-72.
[583] Miller BR 3rd, Roitberg AE. *Trypanosoma cruzi* trans-sialidase as a drug target against Chagas disease (American trypanosomiasis). Future Med Chem 2013; 5(15): 1889-900.
[584] Martins-Teixeira MB, Campo VL, Biondo M. α-Selective glycosylation affords mucin-related GalNAc amino acids and diketopiperazines active on. Bioorg Med Chem 2013; 21(7):1978-87.
[585] Santa-Rita SM, Barboza SH, Meirelles MN, de Castro SL. Effect of the alkyl lysophospholipids on the proliferation and differentiation of *Trypanosoma cruzi*. Acta Trop 2000; 75 (2): 219-28.
[586] Lira R, Contreras LM, Rita RM, Urbina JA. Mechanism of action of anti-proliferative lysophospholipid analogues against the protozoan parasite *Trypanosoma cruzi*: Potentiation of *in vitro* activity by the sterol biosynthesis inhibitor ketoconazole. J Antimicrob C hemother 2001; 47(5): 537-46.
[587] de Castro SL, Santa-Rita RM, Urbina JA, Croft SL. Antiprotozoal lysophospholipid analogues: a comparison of their activity against Trypanosomatid parasites and tumor cells. Mini Rev Med Chem 2004; 4(2): 141-51.
[588] Santa Rita RM, Lira R, Barbosa HS, Urbina JA, de Castro SL. Anti-proliferative synergy of lysophospholipid analogues and ketoconazole against *Trypanosoma cruzi* (Kinetoplastida: Trypanosomatidae): cellular and ultrastructural analysis. J Antimicrob Chemother 2005; 55(5): 780-4.
[589] Santa Rita RM, Barbosa HS, de Castro SL. Ultrastructural analysis of edelfosine-treated trypomastigotes and amastigotes of *Trypanosoma cruzi*. Parasitol Res 2006; 100(1): 189-90.
[590] Menna-Barreto RF, Salomão K, Dantas AP. Different cell death pathways induced by drugs in *Trypanosoma cruzi*: An ultrastructural study. Micron 2009; 40(2): 157-68.
[591] Jones SM, Urch JE, Brun R, Harwood JL, Berry C, Gilbert IH. Analogues of thiolactomycin as potential anti-malarial and anti-trypanosomal agents. Bioorg Med Chem 2004; 12(4): 683-92.
[592] Fügi MA, Kaiser M, Tanner M, Schneiter R, Mäser P, Guan XL. Match-making for posaconazole through systems thinking. Trends Parasitol 2015; 31(2):46-51.
[593] Cardoso MS, Junqueira C, Trigueiro RC. Identification and functional analysis of *Trypanosoma cruzi* genes that encode proteins of the glycosylphosphatidylinositol biosynthetic pathway. PLoS Negl Trop Dis 2013; 7(8): e2369.
[594] Goldston AM, Sharma AI, Paul KS, Engman DM. Acylation in trypanosomatids: an essential process and potential drug target. Trends Parasitol 2014; 30(7): 350-60.
[595] Salomão K, de Souza EM, Carvalho SA, da Silva EF, Fraga CA, Barbosa HS, *et al. In vitro* and *in vivo* activities of 1,3,4-thiadiazole-2-arylhydrazone derivatives of megazol against *Trypanosoma cruzi*. Antimicrob Agents Chemother 2010; 54(5): 2023-31.
[596] Keenan M, Abbott MJ, Alexander PW, Armstrong T, Best WM, Berven B, *et al*. Analogues of fenarimol are potent inhibitors of *Trypanosoma cruzi* and are efficacious in a murine model of Chagas disease. J Med Chem 2012; 55(9): 4189-204.
[597] Keenan M, Alexander PW, Diao H Best WM, Khong A, Kerfoot M, *et al*. Design, structure-activity relationship and *in vivo* efficacy of piperazine analogues of fenarimol as inhibitors of *Trypanosoma cruzi*. Bioorg Med Chem 2013; 21(7): 1756-63.
[598] Jacobs RT, Plattner JJ, Keenan M. Boron-based drugs as antiprotozoals. Curr Opin Infect Dis 2011; 24(6): 586-92.
[599] Moraes CB, Giardini M A, Kim H. Nitroheterocyclic compounds are more efficacious than CYP51 inhibitors against *Trypanosoma cruzi*: implications for Chagas disease drug discovery and development. Sci Rep 2014; 4: 4703-14.
[600] Paez Prosper, JA., Campillo Martin NE., Guerra Alvarez A., Gonzalez Hormaiztegui., M.M. Cerecetto Meyer. H., Family of antichagasics derived from imidazo[4,5-C][1,2,6]thiadiazine 2,2-dioxide. EP2392577A1/A4, US20120035160 A1 **(2012),** WO2010086481A1, US8815846 B2 **(2014)**.
[601] Hernández P, Rojas R, Gilman RH, Sauvain M, Lima LM, Barreiro EJ, *et al*. Hybrid furoxanyl N-acylhydrazone derivatives as hits for the development of neglected diseases drug candidates. Eur J Med Chem 2013; 59: 64-74.
[602] Paula FR, Jorge SD, de Almeida LV, Pasqualoto KF, Tavares LC. Molecular modeling studies and *in vitro* bioactivity evaluation of a set of novel 5-nitro-heterocyclic derivatives as anti-*T. cruzi* agents. Bioorg Med Chem 2009; 17(7): 2673-9.
[603] García Mellado O, Cortes, E. Derivatives of 5-R1-2[8N-R2)-furfuryliden: Thiophenylliden semicarbazones and thiosemicarbazones, method for obtaining and using the same for the preparation of a drug for the Chagas disease. MX2007013128A **(2007, 2009)**.
[604] Papadopoulou MV, Bloomer WD, Rosenzweig HS, Wilkinson SR, Kaiser M. Novel nitro (triazole/imidazole)-based heteroarylamides/sulfonamides as potential antitrypanosomal agents. Eur J Med Chem 2014; 87:79-88.
[605] Papadopoulou MV, Bloomer WD, Rosenzweig HS, O'Shea IP, Wilkinson SR, Kaiser M.3-Nitrotriazole-based piperazides as potent antitrypanosomal agents. Eur J Med Chem 2015; 103: 325-34.
[606] Grael CF, Albuquerque S, Lopes JL. Chemical constituents of *Lychnophora pohlii* and trypanocidal activity of crude plant extracts and of isolated compounds. Fitoterapia 2005;76(1): 73-82.
[607] Mosqueira, V.C.F., Lana, M., Guimaraes, D.A.S., Branquinho, RT. Pharmaceutical compositions containing sesquiterpene lactones belonging to the class of furan heliangolides for the treatment of parasitic infections and tumours. WO2013059898A1 **(2013)**.
[608] Branquinho RT, Mosqueira VC, de Oliveira-Silva JC, Simões-Silva MR, Saúde-Guimarães DA, de Lana M. Sesquiterpene lactone in nanostructured parenteral dosage form is efficacious in experimental Chagas disease. Antimicrob Agents Chemother 2014; 58(4): 2067-75.
[609] Melis V, Magbagbeolu M, Rickard JE, Horsley D, Davidson K, Harrington KA*, et al*. Effects of oxidized and reduced forms of methylthioninium in two transgenic mouse tauopathy models. Behav Pharmacol 2015; 26(4): 353-68.
[610] Storey, J.M.D., Marshall, C., Sinclair, J.P., Baddeley, T.C., Wischik, C.M. Methods of synthesis and/or purification methods of diaminophenothiazinium compounds. AU2013200264A1 **(2013)**.
[611] Khraiwesh MH, Lee CM, Brandy Y, Akinboye ES, Berhe S, Gittens G, *et al*. Antitrypanosomal activities and cytotoxicity of some novel imido-substituted 1,4-naphthoquinone derivatives. Arch Pharm Res 2012; 35(1): 27-33.
[612] Bakare, O., Lee, C.M., Brandy, Y., Khraiwesh, M.H. Method for inhibiting *Trypanosoma cruzi.* US2015073177A1 **(2015)**.
[613] Vazquez K, Espinosa-Bustos C, Soto-Delgado J. New aryloxy-quinone derivatives as potential anti-Chagasic agents: Synthesis, trypanosomicidal activity, electrochemical properties, pharmacophore elucidation and 3D-QSAR analysis. RSC Adv 2015; 5: 65153-66.
[614] Tapia RA, Salas CO, Vázquez K, Espinosa-Bustos C, Soto-Delgado J, Varela J, *et al*. Synthesis and biological characterization of new aryloxyindole-4,9-diones as potent trypanosomicidal agents. Bioorg Med Chem Lett 2014; 24(16): 3919-22.
[615] Olmo F, Rotger C, Ramírez-Macías I, Martínez L, Marín C, Carreras L, *et al*. Synthesis and biological evaluation of N,N'-squaramides with high *in vivo* efficacy and low toxicity: toward a low-cost drug against Chagas disease. J Med Chem 2014; 57(3): 987-99.
[616] Rotger P.M.D.C., Costa T.A.N., Sánchez M.M., Marín S.C. Antiparasitic activity from squaramides. WO2014184416A1 **(2014)**.

[617] Hoorelbeke B, Kim Y, Oki T, Igarashi Y, Balzarini J. Role of cations in the interaction of pradimicins with HIV-1 envelope gp120. Curr Top Med Chem 2013; 13(16): 1907-15.
[618] Balsarini, J., González, P., Rui Pérez, L.M., Castillo Acosta, V., Igarashi, Y, New pradimicin derivatives for the treatment of diseases caused by kinetoplastids.WO2015051422A1 **(2015)**.
[619] Souza-Neta LC, Menezes D, Lima MS, Cerqueira MD, Cruz FG, Martins D, *et al*. Modes of action of arjunolic acid and derivatives on *Trypanosoma cruzi* cells. Curr Top Med Chem 2014; 14(8): 1022-32.
[620] Olmo F, Guardia JJ, Marin C*, et al*. Prospects of an alternative treatment against *Trypanosoma cruzi* based on abietic acid derivatives show promising results in Balb/c mouse model.Eur J Med Chem 2015; 89: 683-90.
[621] Ancizu S, Moreno E, Torres E, Burguete A, Pérez-Silanes S, Benítez D, *et al*. Heterocyclic-2-carboxylic acid (3-cyano-1,4-di-N-oxidequinoxalin-2-yl)amide derivatives as hits for the development of neglected disease drugs. Molecules 2009;14(6): 2256-72.
[622] Gerpe A, Boiani L, Hernández P, Sortino M, Zacchino S, González M, *et al*. Naftifine-analogues as anti-*Trypanosoma cruzi* agents. Eur J Med Chem 2010; 45(6): 2154-64.
[623] Rodrigues JH, Ueda-Nakamura T, Corrêa AG, Sangi DP, Nakamura CV. A quinoxaline derivative as a potent chemotherapeutic agent, alone or in combination with benznidazole, against *Trypanosoma cruzi*. PLoS One 2014; 9(1): e85706.
[624] Cogo J, Kaplum V, Sangi DP, Ueda-Nakamura T, Corrêa AG, Nakamura CV. Synthesis and biological evaluation of novel 2,3-disubstituted quinoxaline derivatives as antileishmanial and antitrypanosomal agents. Eur J Med Chem 2015; 90: 107-23.
[625] Monge A., Perez, S., Aldana, I., Moreno, E., Torres, E.; Cerecetto, M.H. Gonzalez H.M., Paniagua S.J.F. Quinoxaline derivatives which are selective against trypanosoma cruzi and do not cause mutagenic effects. WO2012096556A1 **(2012)**.
[626] Aldana I., Meyer Cerecetto H., Hormaiztegui Gonzalez M. M., Monge A, Moreno E., Solis Paniagua J. F., Perez S, Torres E. Quinoxaline derivatives which are selective against trypanosoma cruzi and do not cause mutagenic effects. WO 2012096556 A1 **(2012)**.
[627] Bellera CL, Balcazar DE, Vanrell MC*, Casassa AF3, Palestro PH1, Gavernet L et al*. Computer-guided drug repurposing: Identification of trypanocidal activity of clofazimine, benidipine and saquinavir. Eur J Med Chem 2015; 93: 338-48.
[628] Bellera CL, Sbaraglini ML, Balcazar DE, Fraccaroli L, Vanrell MC, Casassa AF, *et al*. High-throughput drug repositioning for the discovery of new treatments for Chagas disease. Mini Rev Med Chem 2015; 15(3): 182-93.
[629] Palace-Berl F, Pasqualoto KF, Jorge SD, Zingales B, Zorzi RR, Silva MN *et al*. Designing and exploring active N'-[(5-nitrofuran-2-yl) methylene] substituted hydrazides against three *Trypanosoma cruzi* strains more prevalent in Chagas disease patients. Eur J Med Chem 2015; 96: 330-9.
[630] Rios Vasquez L.M., Ocampo Cardona, R., Duque S.M., Robledo, S.M., Velez Berna, I.D., Cedeflo Medina D.L., Jones M.A. Quaternary n-(halomethyl) ammonium salts as therapeutic agents. US0194640A1 **(2014)**.
[631] Long TE, Lu X, Galizzi M, Docampo R, Gut J, Rosenthal PJ. Phosphonium lipocations as antiparasitic agents. Bioorg Med Chem Lett 2012; 22(8): 2976-9.
[632] Long T.E. Small molecule naphthoquinone- and phthalimide-based lipocations as anti-parasitic agents. WO2013036766A1 **(2013)**.
[633] Reid CM, Ebikeme C, Barrett MP, Patzewitz EM, Müller S, Robins DJ, *et al*. Synthesis and anti-protozoal activity of C2-substituted polyazamacrocycles. Bioorg Med Chem Lett 2008; 18(7): 2455-8.
[634] Sánchez-Moreno M, Marín C, Navarro P, Lamarque L, García-España E, Miranda C, *et al*. *In vitro* and *in vivo* trypanosomicidal activity of pyrazole-containing macrocyclic and macrobicyclic polyamines: Their action on acute and chronic phases of Chagas disease. J Med Chem 2012; 55(9): 4231-43.
[635] Olmo F, Marín C, Clares MP, Blasco S, Blasco S, Albelda MT, Soriano C, *et al*. Scorpiand-like azamacrocycles prevent the chronic establishment of *Trypanosoma cruzi* in a murine model. Eur J Med Chem 2013; 70: 189-98.
[636] Garcia-Espana, M.E., Clares G.M.P., Blasco L.S., Soriano C.C., Gonzalez G.J.,Verdejo V.B., Inclan N.M., Sanchez M.M., Marin S.C., Olmo, A.F. Compuestos macrocíclicos de tipo escorpiando y su uso como antiparasitarios. WO2013087965A1 **(2013)**.
[637] Peña S, Scarone L, Serra G. Macrocycles as potential therapeutic agents in neglected diseases. Future Med Chem 2015; 7(3): 355-82.
[638] Cardoso MFC, Salomão K, Bombaça AC. Synthesis and anti-*Trypanosoma cruzi* activity of new 3-phenylthio-nor-b-lapachone derivatives. Bioorg Med Chem 23 (2015): 4763-8.
[639] Sakhuja R, Panda SS, Khanna L, Khurana S, Jain SC. Design and synthesis of spiro[indole-thiazolidine]spiro[indole-pyrans] as antimicrobial agents. Bioorg Med Chem Lett 2011; 21(18): 5465-9.
[640] Sundar JK, Rajesh SM, Sivamani J, Perumal S, Natarajan S. Synthesis and crystal structures of 5'-phenylspiro[indoline-3, 2'-pyrrolidin]-2-one derivatives. Chem Cent J 2011; 5(1): 45.
[641] Ang, S.H., Krastel,P., Leong, S.Y. Tan, L.J., Wong, W.L.J., Yeung, B.K.S., Zou, B. Spiro-indole derivatives for the treatment of parasitic diseases. NZ588217A **(2012)**.
[642] Moreira DR, Costa SP, Hernandes MZ, Rabello MM, de Oliveira Filho GB, *et al*. Structural investigation of anti-*Trypanosoma cruzi* 2-iminothiazolidin-4-ones allows the identification of agents with efficacy in infected mice. J Med Chem 2012; 55(24): 10918-36.
[643] de B Moreira TL, Barbosa AF, Veiga-Santos P, Henriques C, Henriques-Pons A, Galdino SL, *et al*. Effect of thiazolidine LPSF SF29 on the growth and morphology of *Trypanosoma cruzi.* Int J Antimicrob Agents 2013; 41(2): 183-7.
[644] Carvalho Tecia, MU., De Lima, M., Alves, D.C., De Souza, W., Lins, S.G., Henriques, C., Hernandes, M.Z. De Barros Moreira, T.L., Alves Pereira, V.R., Da Rocha Pitta, I. Imidazolidine and thiazolidine molecules with anti-t. cruzi activity. WO2012119212A1 **(2012)**.
[645] Honorato SB, Porcal W, Merlino A*, et al*. Understanding the solid forms of 5-Ephenylethenylbenzofuroxan with different *in vivo* anti-*T. cruzi*. activity. Rev Virtual Quim 2003; 5(6): 1179-90.
[646] Masataka, I., Jian-Feng, G., Chika, A., Ismail, N., Saad M., Mei, Y., Isamu, I., Reto, B. Medicinal composition containing benzo[a]phenoxanthin compound as the active ingredient for preventing or treating protozoal disease. CN102026989A/B, EP2269996A1/A4, EP2269996B1, US8288374, US20110021450, WO2009113569A1 **(2011)**.
[647] Álvarez G, Varela J, Cruces E , Fernández M, Gabay M, Leal SM, *et al*. Identification of a new amide-containing thiazole as a drug candidate for treatment of Chagas' disease. Antimicrob Agents Chemother 2015; 59(3): 1398-1404.
[648] Timm BL, da Silva PB, Batista MM, da Silva FH, da Silva CF, Tidwell RR *et al*. *In vitro* and *in vivo* biological effects of novel arylimidamide derivatives against *Trypanosoma cruzi.* Antimicrob Agents Chemother 2014; 58(7): 3720-6.
[649] Moreno-Rodríguez A, Salazar-Schettino PM, Bautista JL, Hernández-Luis F, Torrens H, Guevara-Gómez Y, *et al*. *In vitro* antiparasitic activity of new thiosemicarbazones in strains of *Trypanosoma cruzi.* Eur J Med Chem 2014; 87: 23-9.
[650] Kaiser M, Maes L, Tadoori LP, Spangenberg T, Ioset JR. Repurposing of the open access malaria box for kinetoplastid diseases identifies novel active scaffolds against Trypanosomatids. J Biomol Screen 2015; 20(5): 634-45.
[651] Parrilha GL, Dias RP, Rocha WR. 2-Acetylpyridine- and 2-benzoylpyridine-derived thiosemicarbazones and their antimony(III) complexes exhibit high anti-trypanosomal activity. Polyhedron 2012; 31: 614-21.
[652] Parrilha GL, Ferraz KS, Lessa JA, de Oliveira KN3, Rodrigues BL1, Ramos JP, *et al*. Metal complexes with 2-acetylpyridine-N(4)-orthochlorophenylthiosemicar-bazone:

Cytotoxicity and effect on the enzymatic activity of thioredoxin reductase and glutathione reductase. Eur J Med Chem 2014; 84: 537-44

[653] Ramírez-Macías I, Marín C, Chahboun R, Messouri I, Olmo F, Rosales MJ, *et al*. *In vitro* and *in vivo* studies of the trypanocidal activity of four terpenoid derivatives against *Trypanosoma cruzi*. Am J Trop Med Hyg 2012; 87(3): 481-8.

[654] Raviolo MA, Solana ME, Novoa MM, Gualdesi MS, Alba-Soto CD, Briñón MC. Synthesis, physicochemical properties of allopurinol derivatives and their biological activity against *Trypanosoma cruzi*. Eur J Med Chem 2013; 69: 455-64.

[655] Aguilera-Venegas B, Olea-Azar C, Arán VJ, Speisky H. Indazoles: A new top seed structure in the search of efficient drugs against *Trypanosoma cruzi.* Future Med Chem 2013; 5(15): 1843-59.

[656] Boiani L, Gerpe A, Arán VJ, Torres de Ortiz S, Serna E, Vera de Bilbao N, *et al*. *In vitro* and *in vivo* antitrypanosomatid activity of 5-nitroindazoles. Eur J Med Chem 2009; 44(3): 1034-40.

[657] Muro B, Reviriego F, Navarro P, Marín C, Ramírez-Macías I, Rosales MJ, *et al*. New perspectives on the synthesis and antichagasic activity of 3-alkoxy-1-alkyl-5-nitroindazoles. Euro J Med Chem 2014; 74: 124-34.

[658] Díaz-Urrutia CA, Olea-Azar CA, Zapata GA, Lapier M, Mura F, Aguilera-Venegas B, *et al*. Biological and chemical study of fused tri- and tetracyclic indazoles and analogues with important antiparasitic activity. Spectrochim Acta A Mol Biomol Spectrosc 2012; 95: 670-8.

[659] Soares RO, Echevarria A, Bellieny MS, Pinho RT, de Leo RM, Seguins WS, *et al*. Evaluation of thiosemicarbazones and semicarbazones as potential agents anti-*Trypanosoma cruzi*. Exp Parasitol 2011; 129(4): 381-7.

[660] Torres E, Moreno-Viguri E, Galiano S, Devarapally G, Crawford PW, Azqueta A, *et al*. Novel quinoxaline 1,4-di-N-oxide derivatives as new potential antichagasic agents. Eur J Med Chem 2013; 66: 324-34.

[661] Varela J, Alves Lessa J, Lavaggi ML, Beraldo H, Cerecetto H, González M. Coordination of 3-aminoquinoxaline-2-carbonitrile 1,4-dioxides to antimony(III) as a strategy for anti-*Trypanosoma cruzi* activity improvement. Med Chem Res 2012; 21: 4120-8.

[662] Villalobos-Rocha JC, Sánchez-Torres L, Nogueda-Torres B, Segura-Cabrera A, García-Pérez CA, Bocanegra-García V, *et al*. Anti-*Trypanosoma cruzi* and anti-leishmanial activity by quinoxaline-7-carboxylate 1,4-di-N-oxide derivatives. Parasitol Res. 2014; 113 (6): 2027-35.

[663] Bot C, Hall BS, Bashir N, Taylor MC, Helsby NA, Wilkinson SR. Trypanocidal activity of aziridinyl nitrobenzamide prodrugs. Antimicrob Agents Chemother 2010; 54(10): 4246-52.

[664] Wilkinson SR, Bot C, Kelly JM, Hall BS.Trypanocidal activity of nitroaromatic prodrugs: current treatments and future perspectives. Curr Top Med Chem 2011; 11(16): 2072-84.

[665] Fernández M, Varela J, Correia I, Birriel E, Castiglioni J, Moreno V, *et al*. A new series of heteroleptic oxidovanadium (IV) compounds with phenanthroline-derived co-ligands: Selective *Trypanosoma cruzi* growth inhibitors. Dalton Trans, 2013, 42, 11900-11.

[666] Fernández M, Becco L, Correia I, *et al*. Oxidovanadium (IV) and dioxidovanadium (V) complexes of tridentatesalicylaldehydesemicarbazones: Searching for prospectiveantitrypanosomal agents. J Inorg Biochem 2013; 127: 150-60.

[667] Merlino A, Otero L, Gambino D, Laura Coitiño E. In search of patterns over physicochemical properties and pharmacological activities for a set of [MCl_2(thiosemicarbazone)] complexes (M=Pt/Pd): Support for multiple mechanisms of antichagasic action excluding DNA-bonding *in vivo*? Eur J Med Chem 2011; 46(7): 2639-51.

[668] Aguilera-Venegas B, Olea-Azar C, Norambuena E, Arán VJ, Mendizábal F, Lapier M, *et al*. ESR, electrochemical, molecular modeling and biological evaluation of 4-substituted and 1,4-disubstituted 7-nitroquinoxalin-2-ones as potential anti-*Trypanosoma cruzi* agents. Spectrochim Acta A Mol Biomol Spectrosc 2011; 78(3): 1004-12.

[669] Pérez-Cruz F, Serra S, Delogu G, Lapier M, Maya JD, Olea-Azar C, *et al*. Antitrypanosomal and antioxidant properties of 4-hydroxycoumarins derivatives. Bioorg Med Chem Lett 2012; 22(17): 5569-73.

[670] Guíñez RF, Matos MJ, Vazquez-Rodriguez S, Santana L, Uriarte E, Olea-Azar C, *et al*. Synthesis and evaluation of antioxidant and trypanocidal properties of a selected series of coumarin derivatives. Future Med Chem 2013; 5(16): 1911-22.

[671] Vazquez-Rodriguez S, Figueroa Guíñez R, João Matos M. Synthesis and trypanocidal properties of new coumarin-chalcone derivatives 2015; 5(4): 173-7.

[672] Kumar M, Makhal B, Gupta VK, Sharma A. *In silico* investigation of medicinal spectrum of imidazo-azines from the perspective of multitarget screening against malaria, tuberculosis and Chagas disease. J Mol Graph Model 2014; 50: 1-9.

[673] Neitz RJ, Chen S, Supek F, Yeh V, Kellar D, Gut J *et al*. Chagas disease affects 8 million lead identification to clinical candidate selection: drugs for Chagas disease. J Biomol Screen 2015; 20(1): 101-11.

[674] Ferrer-Casal M, Li C, Galizzi M, Stortz CA, Szajnman SH, Docampo R, *et al*. New insights into molecular recognition of 1,1-bisphosphonic acids by farnesyl diphosphate synthase. Bioorg Med Chem 2014; 22(1): 398-405.

[675] Caballero AB, Rodríguez-Diéguez A, Salas JM. Lanthanide complexes containing 5-methyl-1,2,4-triazolo [1,5-a] pyrimidin-7(4H)-one and their therapeutic potential to fight leishmaniasis and Chagas disease. J Inorg Biochem 2014; 138: 39-46.

[676] Caballero AB, Rodríguez-Diéguez A, Quirós M, Salas JM, Huertas Ó, Ramírez-Macías I, *et al*. Triazolopyrimidine compounds containing first-row transition metals and their activity against the neglected infectious Chagas disease and leishmaniasis. Eur J Med Chem 2014; 85: 526-34.

[677] Wong-Baeza C, Nogueda-Torres B, Serna M, Meza-Toledo S, Baeza I, Wong C. Trypanocidal effect of the benzyl ester of N-propyl oxamate: a bi-potential prodrug for the treatment of experimental Chagas disease. BMC Pharmacol Toxicol 2015; 16(1): 10-23.

[678] Olmo F, Urbanová K, Rosales MJ, Martín-Escolano R, Sánchez-Moreno M, Marín C. An *in vitro* iron superoxide dismutase inhibitor decreases the parasitemia levels of *Trypanosoma cruzi* in BALB/c mouse model during acute phase. Int J Parasitol Drugs Drug Resist 2015; 20; 5(3): 110-6.

[679] Kudryashova EV, Leferink NG, Slot IG, van Berkel WJ. Galactonolactone oxidoreductase from *Trypanosoma cruzi* employs a FAD cofactor for the synthesis of vitamin C. Biochim Biophys Acta 2011; 1814(5): 545-52.

[680] Arias DG, Cabeza MS, Erben ED, Carranza PG, Lujan HD, Téllez Iñón MT, *et al*. Functional characterization of methionine sulfoxide reductase A from *Trypanosoma* spp. Free Radic Biol Med 2011; 50(1): 37-46.

[681] Aguilera E, Varela J, Birriel E, Serna E, Torres S, Yaluff G, *et al.* Potent and selective inhibitors of *Trypanosoma cruzi* triosephosphate isomerase with concomitant inhibition of cruzipain: Inhibition of parasite growth through multitarget activity. Chem Med Chem 2016; 11(12): 1328-38.

[682] Nowicki C, Cazzulo JJ. Aromatic amino acid catabolism in trypanosomatids. Comp Biochem Physiol A Mol Integr Physiol 2008; 151(3): 381-90.

[683] Rivera G, Bocanegra-García V, Ordaz-Pichardo C, Nogueda-Torres B, Monge A. New therapeutic targets for drug design against *Trypanosoma cruzi*, advances and perspectives. Curr Med Chem 2009; 16(25): 3286-93.

[684] Salavati R, Moshiri H, Kala S, Shateri Najafabadi H. Inhibitors of RNA editing as potential chemotherapeutics against trypanosomatid pathogens. Int J Parasitol Drugs Drug Resist 2011; 2: 36-46.

[685] Soares MB, Silva CV, Bastos TM, Guimarães ET, Figueira CP, Smirlis D, *et al*. Anti-*Trypanosoma cruzi* activity of nicotinamide. Acta Trop 2012; 122(2): 224-9.

[686] Sacconnay L, Smirlis D, Queiroz EF, Wolfender JL, Soares MB, Carrupt PA, *et al*. Structural insights of SIR2rp3 pro-

teins as promising biotargets to fight against Chagas disease and leishmaniasis. Mol Biosyst 2013; 9(9): 2223-30.

[687] Bayona JC, Nakayasu ES, Laverrière M, Aguilar C, Sobreira TJ, Choi H, *et al*. SUMOylation pathway in *Trypanosoma cruzi*: Functional characterization and proteomic analysis of target proteins. Mol Cell Proteomics 2011; 10(12): M110.007369.

[688] Benaim B, Garcia CR. Targeting calcium homeostasis as the therapy of Chagas' disease and leishmaniasis - a review. Trop Biomed 2011; 28(3): 471-81.

[689] Benaim G, Paniz Mondolfi AE. The emerging role of amiodarone and dronedarone in Chagas disease. Nat Rev Cardiol 2012; 9(10): 605-9.

[690] Kappes B, Tews I, Binter A, Macheroux P. PLP-dependent enzymes as potential drug targets for protozoan diseases. Biochim Biophys Acta 2011; 1814(11): 1567-76.

[691] Rodrígues-Poveda CA, Pérez-Moreno G, Vidal AE, Urbina JA, González-Pacanowska D, Ruiz-Pérez LM. Kinetic analyses and inhibition studies reveal novel features in peptide deformylase 1 from *Trypanosoma cruzi*. Mol Biochem Parasitol 2012; 182(1-2): 83-7.

[692] Kawano DF, Carvalho I. Targeting *Trypanosoma cruzi* platelet-activating factor receptors: scope for the development of novel drugs to treat Chagas Disease. Mini Rev Med Chem 2013; 13(7): 997-1004.

[693] Pan P, Vermelho AB, Scozzafava A, Parkkila S, Capasso C, Supuran CT. Anion inhibition studies of the α-carbonic anhydrase from the protozoan pathogen *Trypanosoma cruzi*, the causative agent of Chagas disease. Bioorg Med Chem 2013; 21(15): 4472-6.

[694] André J, Bach M, Xie J, Rigolet P. A new binding site involving the C-terminal domain to design specific inhibitors of PepX. Protein Pept Lett 2013; 20(1): 45-53.

[695] Khare S, Roach SL, Barnes SW, Hoepfner D, Walker JR, Chatterjee AK, *et al*. Utilizing chemical genomics to identify cytochrome b as a novel drug target for Chagas disease. PLoS Pathog 2015; 11(7): e1005058.

[696] Amano Y, Namatame I, Tateishi Y, Honboh K, Tanabe E, Niimi T, Sakashita H. Structural insights into the novel inhibition mechanism of *Trypanosoma cruzi* spermidine synthase. Acta Crystallogr D Biol Crystallogr 2015; 71(Pt 9): 1879-89.

[697] Peña I, Pilar Manzano M, Cantizani J, Kessler A, Alonso-Padilla J, Bardera A, *et al*. New compound sets identified from high throughput phenotypic screening against three kinetoplastid parasites: An open resource. Sci Rep 2015; 5: 8771-3

[698] Torrico, F. In: Proceedings of the 62nd annual meeting of the american society of tropical medicine and hygiene; 2013; November 13-17; Washington, DC.

[699] Molina I, Gómez I Prat J, Treviño B, Sulleiro E, Serre N, *et al*. Randomized trial of posaconazole and benznidazole for chronic Chagas' disease. N Engl J Med 2014; 370(20):1899-908.

[700] Urbina JA. Recent clinical trials for the etiological treatment of chronic chagas disease: Advances, challenges and perspectives. J Eukaryot Microbiol 2015; 62(1): 149-56.

[701] Riley J, Brand S, Voice M, Caballero I, Calvo D, Read KD. Development of a fluorescence-based *Trypanosoma cruzi* CYP51 inhibition assay for effective compound triaging in drug discovery programmes for Chagas disease. PLoS Negl Trop Dis 2015; 9(9): e0004014.

[702] Khare S, Liu X, Stinson M, Rivera I, Groessl T, Tuntland T, *et al*. Anti-trypanosomal treatment with benznidazole is superior to posaconazole regimens in mouse models of Chagas disease. Antimicrob Agents Chemother 2015: 59(10): 6385-94.

[703] Engel JC, Ang KKH, Chen S, Aekin MR, McKerrow JH, Doyle PS. Image-based high-throughput drug screening targeting the intracellular stage of *Trypanosoma cruzi*, the agent of Chagas' disease. Antimicrobial Agents and Chemother 2010; 54(8): 3326-34.

[704] Rhee SW, Bradford WW, Malerich JP, Tanga MJ.Carbon-14 labeling of K777•HCl, a therapeutic agent for Chagas disease. J Labelled Comp Radiopharm 2013; 56(9-10): 461-3.

[705] Sosa-Estani S, Segura EL. Integrated control of Chagas disease for its elimination as public health problem - A Review Mem Inst Oswaldo Cruz, Rio de Janeiro 2015 110(3): 289-98.

[706] Fonseca-Berzal C, Palmeiro-Roldán R, Escario JA, Torrado S2, Arán VJ4, Torrado-Santiago S, *et al*. Novel solid dispersions of benznidazole: Preparation, dissolution profile and biological evaluation as alternative antichagasic drug delivery system. Exp Parasitol 2015; 149: 84-91.

[707] García MC, Manzo RH, Jimenez-Kairuz AF. Extemporaneous benznidazole oral suspension prepared from commercially available tablets for treatment of Chagas disease in paediatric patients.Trop Med Int Health 2015; 20(7): 864-70.

[708] Sadok Menna-Barreto RF, Belloze KT, Perales J, Silva-Jr FP. Proteomic and bioinformatic analysis of *Trypanosoma cruzi* chemotherapy and potential drug targets: New pieces for an old puzzle. Curr Drug Targets 2014; 15(3): 255-71.

[709] Smirlis D, Soares MB. Selection of molecular targets for drug development against trypanosomatids. Subcell Biochem 2014; 74: 43-76.

[710] Bahia MT, Diniz Lde F, Mosqueira VC.Therapeutical approaches under investigation for treatment of Chagas disease. Expert Opin Investig Drugs 2014; 23(9): 1225-37.

[711] Don R, Ioset JR. Screening strategies to identify new chemical diversity for drug development to treat kinetoplastid infections. Parasitology 2014; 141(1):140-6.

[712] Carmo AA, Rocha MO, Silva JL, Ianni BM, Fernandes F, Sabino EC *et al*. Amiodarone and *Trypanosoma cruzi* parasitaemia in patients with Chagas disease. Int J Cardiol 2015; 189: 182-4

[713] Duschak VG, Couto AS. An insight on targets and patented drugs for chemotherapy of Chagas disease. Recent Pat Anti-infect Drug Discov 2007; 2(1): 19-51.

[714] Apt W. Current and developing therapeutic agents in the treatment of Chagas disease. Drug Des Devel Ther 2010; 4: 243-53.

[715] Assíria Fontes Martins T, de Figueiredo Diniz L, Mazzeti AL, da Silva do Nascimento ÁF, Caldas S, Caldas IS *et al*. Benznidazole/itraconazole combination treatment enhances anti-*Trypanosoma cruzi* activity in experimental Chagas disease. PLoS One 2015; 10(6): e0128707

[716] Grosso NL, Alarcon ML, Bua J, Laucella SA, Riarte A, Fichera LE. Combined treatment with benznidazole and allopurinol in mice infected with a virulent *Trypanosoma cruzi* isolate from Nicaragua. Parasitology 2013; 140(10): 1225-33.

[717] Perez-Mazliah DE, Alvarez MG, Cooley G, Lococo BE, Bertocchi G, Petti M, *et al*. Sequential combined treatment with allopurinol and benznidazole in the chronic phase of *Trypanosoma cruzi* infection: A pilot study. J Antimicrob Chemother 2013; 68(2): 424-37.