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Therapeutic Targets for the Development of Anti-*Trypanosoma cruzi* Drugs: A Brief Review

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Bocanegra-García V

Abstract: Chagas disease is a neglected disease caused by *Trypanosoma cruzi* (*T. cruzi*) that remains a serious public health problem in Latin America since there are approximately 7 million infected people, making it a matter of worldwide concern. Advances in new therapeutic strategies to combat Chagas disease have been scarce over the last decades. Efforts have been made to explore *T. cruzi* enzymes as potential therapeutic targets. Inhibitors that act on enzymes such as triose phosphate isomerase (TIM*Tc*), glyceraldehyde 3-phosphate dehydrogenase (GAPDH*Tc*), trypanothione reductase (TR), cruzipain, squalene synthase (SQS*Tc*), farnesyl pyrophosphate synthase (FPPS*Tc*) and sterol 14 α-demethylase (CYP51*Tc*) of *T. cruzi* have been studied to develop selective inhibitors that interrupt the lifecycle of *T. cruzi*. These selected drug targets are part of indispensable *T. cruzi* survival systems such as the glycolysis pathway, cellular detoxification, the host adhesion complex and sterol synthesis pathways, which exhibit relevant features useful in the design of selective inhibitors that may be useful for treating Chagas disease. This review discusses recent progress in the exploration of these enzymes as therapeutic targets and their relevant structural features to develop new drugs against American trypanosomiasis

Keywords: Cell detoxificaha, cell metabolism enzyme, Chagas disease, neglected diseases, therapeutic target, *Trypanosoma cruzi*.

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INTRODUCTION

American trypanosomiasis, also known as Chagas disease, is an endemic burden in the American continent. It is caused by the flagellated protozoa *Trypanosoma cruzi* (*T. cruzi*). The World Health Organization (WHO) estimates that about 7 million people are already infected worldwide, mainly in Latin American countries. The mortality rate is about 12000 per year, and it is considered one of the most important parasitic diseases in Latin America [1, 2].

The most common route of transmission of *T. cruzi* to individuals is through an infected triatomine vector commonly known as "kissing bug", "chipo" or "barberio". When it feeds on humans, infected forms are discharged with the feces. Additional transmission may be by transfusion of contaminated blood, ingestion of contaminated food, infected organ transplantation, and from an infected mother to the fetus. The main triatomine Chagas disease vector species are *Triatoma infestans*, *T. dimidiata*, *Panstrongylus megistus*, and *Rhodnius prolixus* [3].

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The biological cycle of *T. cruzi* is heteroxenous and involves vertebrate and non-vertebrate species. Infection begins by the biting of a hematophagous triatomine (through the proboscis) and the discharge of feces contaminated with *T. cruzi* (infective trypomastigotes phase), allowing the entrance of *T. cruzi* through the biting site. Once the parasite enters the body, it invades human host cells and transforms into an amastigote by binary fission. Then, amastigotes transform into trypomastigotes which lyse host cells and spread through the lymph and blood to other host cells, mainly muscle and ganglion cells. If a triatomine then feeds with human infected blood, it will ingest some of these trypomastigotes that will enter into the bug intestine medium to go into the epimastigote phase and then to the posterior gut, where it stages a second transformation now into infective trypomastigotes; these are then released in the feces reinitiating the biological cycle [4, 5].

Current therapy against Chagas disease is based on two drugs: a) nifurtimox, a nitrofuran derivative that causes deoxyribonucleic acid (DNA) damage and inhibits protein synthesis through the release of nitrile metabolites and reactive oxygen species (ROS), and b) benznidazole (Bnz), a nitroimidazole (Nfx) derivative that also forms ROS and oxidizes nucleotides during DNA replication, causing double-stranded DNA breaks [6, 7]. Both drugs are only effective during the acute phase of the disease [8] with a 50-80% cure

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rate. In the chronic stage, cure rates are reduced to 20-60% [9]. The low efficacy of trypanocidal therapy brings new opportunities to explore known and new therapeutic targets for drug design and development against Chagas disease.

Interrupting the lifecycle of *T. cruzi* by inhibiting different enzymes may guide the search for new trypanocidal drugs. This paper provides an overview of progress in the discovery of new chemical inhibitors of *T. cruzi* enzymes that are involved in cellular metabolism, in the cellular detoxification complex and host adhesion, and in sterol metabolism. Therefore, this review is mainly focused on promising targets such as triose phosphate isomerase (TIM*Tc*), glyceraldehyde 3-phosphate dehydrogenase (GAPDH*Tc*), trypanothione reductase (TR), cruzipain, squalene synthase (SQS*Tc*), farnesyl pyrophosphate synthase (FPPS*Tc*), and sterol 14 α-demethylase (CYP51*Tc*).

T. CRUZI THERAPEUTIC TARGETS

Thiol dependent metabolism, the sterol production pathway, vesicular production such as acidocalcisome and glycosomes, are some of the main metabolic differences that *T. cruzi* has from mammalian cells [10]. Thanks to advances in proteomic studies and basic biology of *T. cruzi*, functions and key characteristics of enzymes that *T. cruzi* requires in these differential metabolic pathways have been determined [11], so new active molecules have been designed to inhibit these *T.* cruzi enzymes [11]. Rational drug design strategies have been a widely used approach, and along with compound screening, have allowed the obtention of molecules with a higher affinity for the drug target, enhancing the inhibitory concentrations of lead compounds. On the other hand, new discoveries in the metabolic routes and information derived from *T. cruzi* genome sequencing [12] will further suggest new drug targets for Chagas disease treatment alternatives [10].

T. CRUZI CELL METABOLISM ENZYMES

Triosephosphate Isomerase (TIM*Tc*)

Because glycolysis takes place inside essential compartments of *T. cruzi* called glycosomes and not in the cytosol as in mammalian cells, inhibition of involved enzymes of this pathway represent promising therapeutic targets for new developments of anti *T. cruzi* drugs [13]. Such is the enzyme TIM*Tc*, which is composed of two monomers of 250 amino acid residues each. It catalyzes dihydroxyacetone phosphate interconvertion to glyceraldehyde 3-phosphate**.** It has several differences from human TIM in its amino acid sequence in the catalytic site [14]. TIM*Tc* is active only in its dimeric form and it has been proposed as a therapeutic target. Particularly, in the oligomers interphase there is a hydrophobic pocket formed by eight amino acids, of which only three are identical to the human TIM, so these amino acid differences can be used to design molecules that selectively inhibit the parasitic enzyme [15].

Olivares *et al.* evaluated the compound dithiodianiline Fig. (**1**), a molecule that presents specific inhibition of TIM*Tc* in nanomolar concentrations range (IC_{50} = 258 nM). In 4-8 μM concentrations, dithiodianiline induces significant inhibition of growth of epimastigotes of Ninoa strains of *T. cruzi*, and in 10 and 15 μM concentrations kills all parasites in cell culture. Dithiodianiline is shown to be specific in TIM*Tc* inhibition; however, higher concentrations are required to inhibit the enzyme in cell culture assays, perhaps because dithiodianiline may bind to proteins of the intracellular space. The compound has cytotoxic activity, but it is still considered a pharmacophore for development of inhibitors of this enzyme [15].

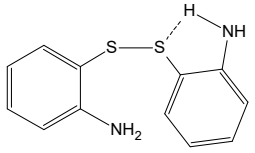

Fig. (1). Dithiodianiline.

Gayosso *et al.* in 2009, reported selective inactivation of TIM*Tc* by brevifolin carboxilate derivatives. Through molecular docking studies, these authors demonstrated the interaction of methylbrevifolin carboxylate Fig. (**2**), with the dimeric form of TIM*Tc*; then with *in vitro* tests they demonstrated selective inhibition of the parasite's enzyme, with a median inactivation concentration (I_{50}) of 6.5 μM, indicating that the compound did not have any effect (I_{50} = 1 mM) on human TIM enzyme, proposing brevifolin carboxylate derivatives as lead molecules for synthesis of more potent compounds against *T. cruzi* [16].

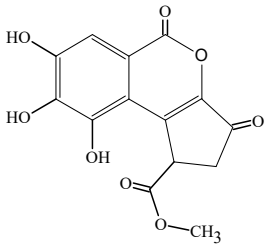

Fig. (2). Methylbrevifolin carboxylate derivative.

Álvarez *et al.* from a screening of 230 different chemotypes, obtained 26 compounds from 8 different chemotypes that were active against TIM*Tc* in inhibition tests; of these, three compound types showed selective inhibition of TIM*Tc*: 1, 2, 6-thiadiazines with an IC_{50} = 13-20 μM, Fig. (**3**), phenazines with an IC_{50} = 26 μM, Fig. (**3**), and thiadiazole with an IC_{50} = 3.5 μM, Fig. (**3**) [17]. Then with *in vitro* assays with the Tulahuen strain, phenazine 118 derivative showed to be more potent (ID_{50} = 2.9 μM), than Bnz (ID_{50} = 7.4 μM), and the phenazine 118 derivative did not inhibit human TIM, therefore, it was considered an ideal lead compound (chemotype) [18]. In studies to determine phenazine derivatives and thiadiazine inhibition action mechanisms of TIM*Tc*, they found that these compounds were non-competitive inhibitors that induce enzyme monomer dissociation and thus inactivation of the enzyme.

Several research reports include thiadiazol derivatives as a new class of selective inhibitors of TIM*Tc*, such as 1,2,4-thiadiazol-5 toluil (*4H*)-2 one, Fig. (**4**), which showed inhibition of TIM*Tc* with an IC_{50} = 3.5 μM, and it was about 10 times more selective against TIM*Tc* than against human TIM; however, in *in vitro* assays on epimastigotes of Tulahuen strain, this thiadiazole derivative was unable to pass through the parasite membrane, thus it had a poor inhibitory effect. When the compound was included in liposomes and

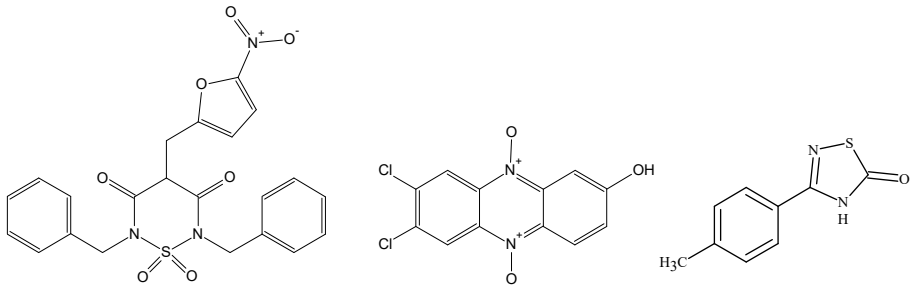

Fig. (3). Thiadiazines, phenazines and thiadiazole derivates.

tested again, it showed similar inhibition values to the ones obtained in the enzymatic assays (IC_{50} = 8 μM). This demonstrated that this thiadiazol-based derivative can inhibit the growth of *T. cruzi* in micromolar concentrations [19].

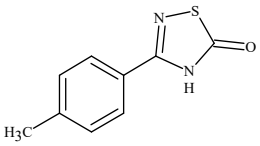

Fig. (4). Thiadiazole derivative with inhibitory activity of TIM.

Álvarez *et al*. also designed and developed a new series of bis-thiazoles, which demonstrated to be promising lead compounds against *T. cruzi*. The derivative (5), 3,3'-Allyl-2,2'-bis[3-(2-furyl)-2-propenyl idenehydrazono]-tetrahydro-4,4'-bisthiazole Fig. (**5**), showed the best selectivity profile when it was tested *in vitro* against amastigotes of Tulahuen strains (EC_{50} = 1.2 μM *vs* Nfx = 0.4 μM, and Bnz = 3.3 μM). However the best TIM*Tc* inhibitor was the derivative (14), 3-Allyl-2-[3-(2-furyl)-2-propenylidenehydrazono]-3'-phenyl-2'-(3-phenyl-2-propenyl idenehydrazono]-tetrahydro-4, 4'-bisthiazole (EC_{50} = 16.6 μM), Fig. (**5**). It is noteworthy that although these compounds showed excellent results when tested, the anti-trypanocidal activity of compound 5 did not relate to the ability to inhibit TIM. Additionally compound 14 showed an inhibitory activity for human TIM, which discarded these compounds as anti-*T cruzi* viable candidate drugs [20].

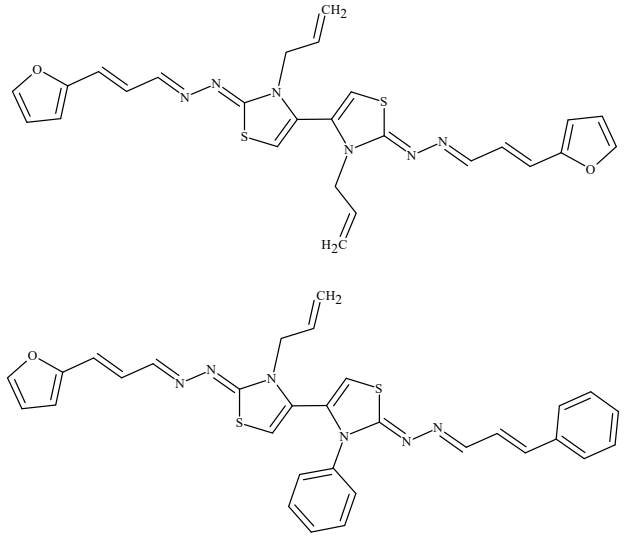

Fig. (5). Bis-thiazole derivates.

Diverse molecular coupling studies of the catalytic site of TIM*Tc* have clearly defined the true scope of TIM*Tc* as a therapeutic target. It has also been possible to elucidate the mechanism of action of some inhibitors of this enzyme; however, there are no molecules that meet the desired characteristics of low cytotoxicity and solubility. On the other hand, dithiodianiline, one of the most promising molecules for this therapeutic target, has remained a prototype inhibitor, therefore, optimizing this molecule is one of the most promising possibilities

Glyceraldehyde-3-Phosphate Dehydrogenase

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH*Tc*) is an enzyme that catalyzes *D*-glyceraldehyde-3-phosphate phosphorylation into 1, 3-diphosphoglycerate using NAD^+ and inorganic phosphate in the glycolysis pathway. GAPDH*Tc* is a homotetramer with a molecular mass of 156 kDa and shares about 50% similarity with human GAPDH. Each monomer is composed of two domains, one being an *N*-terminal portion where NAD^+ binds and the other a C-terminal ending with the protein active site [21].

The NAD^+ binding site in GAPDH is one of the main structural differences with GAPDH*Tc*, and designs of inhibitory compounds has been based on this difference. Molecules such as coumarin have been reported as inhibitors of GAPDH*Tc* activity. Chalepin isolation Fig. (**6**), a natural compound occurring in *Pilocarpus spicatus*, showed a potent activity of GAPDH*Tc* with IC_{50} = 64 μM [22]. Through molecular modeling approaches Menezes *et al.* designed chalepin derivatives, obtaining 13 coumarins with a probable inhibitory effect on GAPDH*Tc*, and the most effective compound showed an IC_{50} = 55.5 μM. Since this compound shows strong receptor-ligand interactions, it is considered an important discovery of GAPDH*Tc* inhibitors [23].

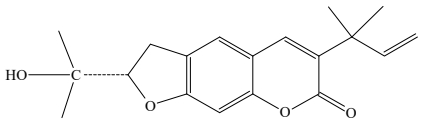

Fig. (6). *Pilocarpus spicatus* metabolite, Chalepin.

In another report on inhibiting glycolysis pathway enzymes, Gallo *et al*. in 2008 reported the inhibitory activity of hexanic, methanolic and hydroalcholic extracts of *Siphoneugena densiflora* and *Vitex polygama* on GAPDH*Tc*, which showed trypanocide effects in *in vitro* assays on *T. cruzi* and *T. brucei*. Extracts from polar solvents of *S. densiflora* showed best results (60% of inhibition on *T. cruzi*

strain Y), of which the compounds flavonol quercetin and gallic acid Fig. (**7**) were the most active with an IC_{50} = 24 and 25 μM respectively; however, in *in vitro* assays both compounds showed poor inhibition of the parasites (34-41% of lysis in 1 mM concentrations), concluding that glycosylated derivatives with a high volume reduce contact in the active site, producing poor a inhibition effect against the whole parasite [24].

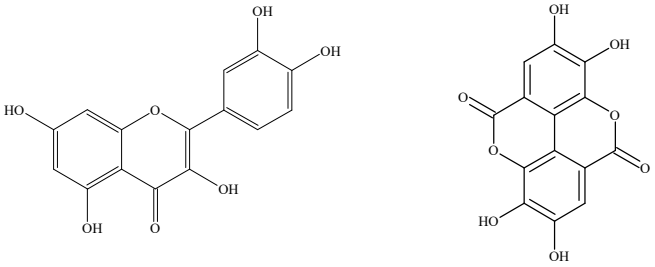

Fig. (7). Flavonol quercetin and gallic acid.

Because the mechanism of action of Bnz and Nfx depends on free radical release, compound design with such properties has recovered special interest to find new trypanocidal compounds [25]. In this context, Silva *et al.* in 2010, reported the synthesis of ruthenium derivatives Fig. (**8**), which showed in *in vitro* assays inhibition of GAPDH*Tc* (Y strain). The most active compounds were *cis*-$[Ru(NO)(bpy)_2imN](PF_6)_3$, *cis*-$[Ru(NO)(bpy)_21\text{-}miN](PF_6)_3$ and *cis*-$[Ru(NO)(bpy)_2SO_3]$ PF_6 with an inhibition percentage of 97% and an IC_{50} from 89 to 153 μM. In other *in vitro* assays on trypomastigotes, only one ruthenium derivative was found as effective as Bnz (IC_{50} = 52 μM *vs* IC_{50} = 53 μM). In *in vivo* assays with murine models, compounds caused reduced parasitemia (60-80% survival in 20 days), compared with the Bnz treated group (with no survival at 20 day period). Authors suggested that the inhibitory activity of those compounds could be through an *S*-nitrosylation mechanism making it a good lead compound to synthesize bioactive molecules [26].

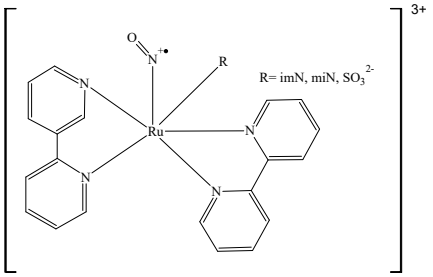

Fig. (8). Derivatives based on ruthenium.

Soares *et al.* in 2013 reported results including design, synthesis and biological activity evaluation of ribonucleoside derivatives as inhibitors of GAPDH*Tc*. Derivative 1, 4-dihydro-4-oxoquinoline ribonucleoside, Neq135, Fig. (**9**), was considered the most active inhibitor with 16 μM, and on *in vivo* assays with mice, similar concentrations as those of Bnz were effective (18 μML^{-1} *vs* 19 μML^{-1}). Those compounds had no cytotoxic effects on fibroblasts, being Neq135 a prototype with good characteristics such as selectivity index and inhibitory profile to serve as a lead compound in the design of inhibitory molecules of GAPDH of *T. cruzi* [27].

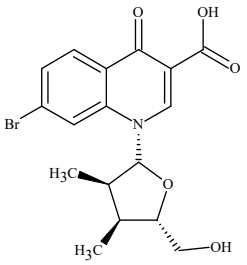

Fig. (9). Ribonucleoside Neq135.

In other approaches, using a new innovative approach called "multitarget ligands" (MTL), hybrid quinone-coumarin compounds synthesis was obtained, Fig. (**10**), with an inhibitory effect on GAPDH and TR in *T. cruzi* and *T. brucei* [28]. These compounds showed potent activity against *T. brucei* (EC_{50} = 0.05 μM); however, in *T. cruzi*, the compounds 2-{4-[6-(3-dimethylaminopropoxy)-2-oxo-2*H*-chromen-3-yl]phenoxy} naphtalene-1,4-dione and 2-{4-[6-(2-dimethylaminoethoxy)-2-oxo-2*H*-chromen-3-yl]phenoxy} naphtalene-1,4-dione showed similar values of EC_{50} (1.24 and 1.47 μM, respectively), in contrast with the reference drug Bnz (1.70 μM), and on *in vitro* assays the dimethylaminoethoxy derivative did not shown interference with the human glutathione reductase, so it is a good candidate against both African and American trypanosomiasis.

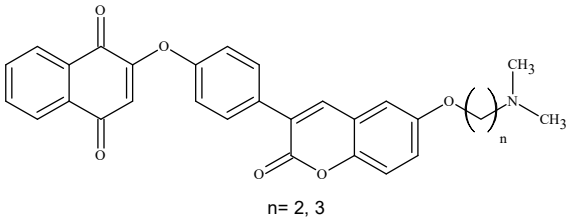

Fig. (10). Quinone-coumarin derivates

Due to the excellent results of inhibition of the previously reported compounds, GAPDH*Tc* is positioned as one of the most promising therapeutic targets in the search for anti- *T. cruzi* drugs; however, even with the advances in design of bioactive molecules, the lack of reference inhibitors for GAPDH*Tc* causes bias when confronting the results of assays with the results of control drugs such as Bnz and Nfx, which do not act as inhibitors of GAPDH*Tc.* It is important to establish reference points for inhibitory activity to evaluate if molecules possess low IC_{50} or only small inhibition values. New GAPDH*Tc* inhibitors, such as Neq135 and quinone coumarin hybrids, are molecules with biological potential as prototypes of new molecules with better characteristics, although it is necessary to understand the mechanism of action that make them effective against GAPDH*Tc*.

CELL DETOXIFICATION AND HOST ADHESION COMPLEX

Trypanothione Reductase (TR)

TR is a FAD-cysteine-oxidoreductase reported in 1985 by Fairlamb *et al.* and only recently it has been possible to fully known the structure and function of this enzyme, which has 492 amino acid residues and a molecular weight of 53.2

kDa [29]. The glutathione reductase system in humans has a counterpart in trypanosomatids with trypanothione reductase that plays an important role in oxygen reactive species detoxification and thiol-redox balance [30]. TR has three binding domains to FAD and NADPH, and the disulphide binding site that is a pocket composed of hydrophobic amino acid residues. The amino acid residues involved in oxide-reduction catalysis are located in the lower part of the pocket and close to the isoalloxacine ring of FAD, close to cysteine 53 and 58 [31]. TR has been recognized a one of the most promising enzymes to be used as a drug target in the search for inhibitory molecules of *T. cruzi* [32].

In the search for compounds to enhance oxidative stress, Porcal *et al*. informed of the inhibitory activity of *N*-oxide benzofuroxane derivatives, Fig. (**11**), which had been associated with free radicals production. Three thiosemicarbazones was tested *in vitro* on epimastigotes in Tulahuen strains showing IC_{50} values of 6.8-8.7 μM**,** quite similar to the reference drugs Nfx and Bnz (7.4 μM). In cell lines assays these compounds had poor solubility and poor activity, and presented unspecific cytotoxicity in J774 cells lines. Authors concluded that the mechanism of action of this compounds series was not related with TR inhibition [33].

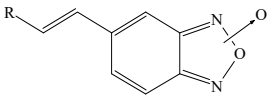

Fig. (11). *N*-oxide benzofuroxane derivatives.

Mesoionic heterocycles have distinguishing bipolar charges, thus they present additional biological properties because they have the ability to interact with molecules such as DNA, and are also being capable of passing through cell membranes. Such compounds have been reported as active against trypanosomatids [34]. In recent work Rodrigues *et al.* in 2012, reported 4 new mesoionic derivatives of thiadizolium aminide as potential inhibitors of TR. The compound 4-phenyl-5-(4-nitrostyryl)-1, 3, 4-thiadiazolium-2-phenilamine chloride, Fig. (**12**) showed inhibition in up to 69% of TR. All of the rest of evaluated compounds were not as effective, but mesoionic molecules are promising in the development of new prototypes with better inhibitory effect profiles [35].

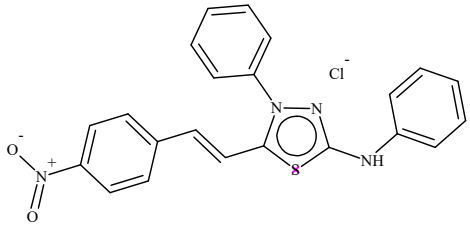

Fig. (12). Mesoionic derivate with activity against TR.

In *in vivo* assays with Albino Swiss mice, tricyclic compounds such as clomipramine, a TR inhibitor of *T. cruzi*, (K_i = 6.5μM), Fig. (**13**), has been evaluated. It was observed that at 5 mg/kg, the parasite load was reduced in the different stages of infection, resulting in less tissue lesion, especially in the heart, and less mortality. According to these results, clomipramine is a good candidate for Chagas disease treatment in its chronic stage because it reduces cardiac tissue damage and enhances survival of infected test individuals [36].

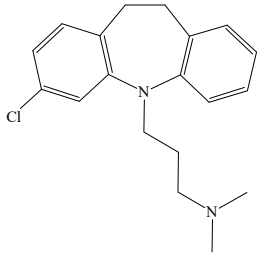

Fig. (13). Clomipramine, inhibitor drug of TR.

Recently, another tricyclic compound was reported with anti-TR activity, thioridazine, Fig. (**14**), which was tested in the different stages of Chagas disease in *in vivo* models [37]. The authors reported the results on the Tulahuen strains of *T. cruzi* and in SGO-Z12 isolate, using these to infect Swiss mice with the trypomastigotes and then treating them with two different treatment schemes, one simulating the treatment used in acute phase, with a dose of thioridazine (80 mg/kg/day) for three days, beginning the treatment 1 hour after infection; and in a second model representing the chronic phase with a dose of 80 mg/kg/day for twelve days beginning the treatment 180 days after infection. The thioridazine effect in infected mice with Tulahuen strains was successful, and the compound was able to inhibit parasitemia in the acute phase with an 80% survival rate up to one year after infection. In the chronic stage model, thioridazine stopped disease progression, necrosis, and fibrosis. These results suggested that this compound is able to impede the progression of the cardiomyopathy produced by *T. cruzi* infection.

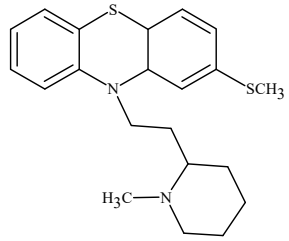

Fig. (14). Tricyclic drug, thioridazine.

In another recent publication, O'Sullivan *et al*. in 2015 reported the biological evaluation of new tricyclic derivatives as TR inhibitors. Two main compound types were prepared, one series with a dibenzosuberyl (DBS) ring in its structure, being classified as clomipramine analogues, and another series of DBS-polyamine conjugates. The most active compounds to inhibit TR *in vitro* were the N^4-(dibenzosuberyl) spermidine with K_i = 4 μM, Fig. (**15**) and N^4, N^8-Bis (dibenzosuberyl) spermine with K_i = 0.2 μM, Fig. (**15**), (reference clomipramine with K_i = 8.4 μM), but in *in vivo* assays, those compounds did not have any effect in increasing mean survival of *T. brucei* infected mice. This lack of correlation between *in vitro* and *in vivo* assays suggests that additional characteristics such as molecules passing through the parasite membrane are very important to the trypanocidal effect. Analysis *in silico* of the binding site of clomipramine and N^4, N^8-Bis (dibenzosuberyl) spermine

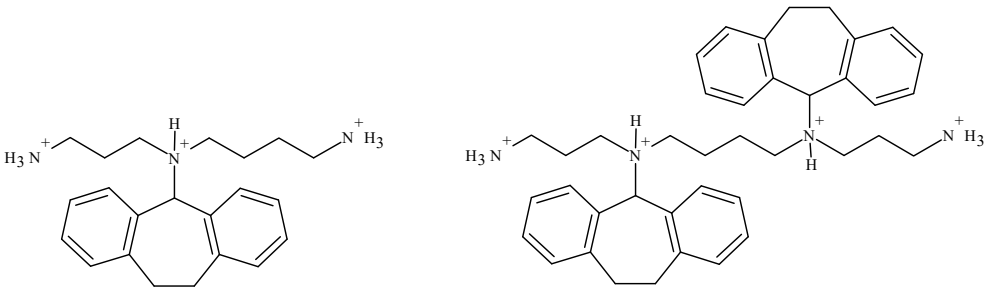

Fig. (15). Clomipramine and polyamine derivatives.

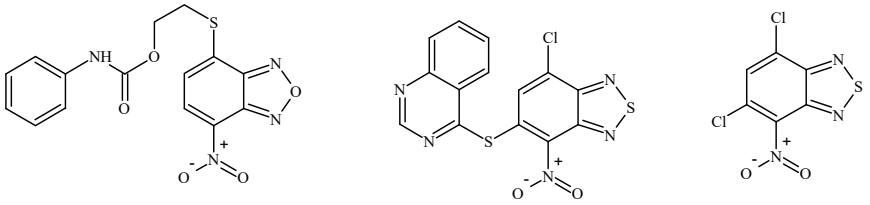

Fig. (16). Lead compounds with antiparasitic activity.

suggested that incorporation of halogen radicals in the aryl groups could increase the binding affinity of this new TR inhibitor [38].

Using combined *in vitro* and *in silico* approaches, Beig *et al.* selected structures with potential inhibition activity on TR. In an enzymatic assay they determined that 21 compounds had an inhibitory effect on *T. cruzi* TR (CL Brener strain) with an IC_{50} < 1.1 μM, with these being more potent in their inhibition effect than other well know inhibitors such as chlorhexidine (IC_{50} = <26 μM). Using such compounds as lead compounds in an *in silico* assay, they looked for related structures, finding 61 compounds with potential inhibition activity on TR. Out of 82 molecules that were tested *in vitro* using *T. brucei* trypomastigotes**,** three were the most active, Fig. (**16**), finding that EC_{50} values of 5-50 μM caused inhibition of parasite proliferation; however, this was higher than reference compounds (chlorhexidine with EC_{50} = 200 nM). To find out if these compounds specifically inhibited TR, they were assayed against transfected parasites with *Tb*TR; however, no significant difference was observed between wild-type parasites and cells that also expressed the TR-copy**.** Therefore, it was not possible to demonstrate that these compounds have a direct inhibitory effect on TR. Despite this, the combined *in vitro–in silico* approach represents a new strategy in the search for new molecules with activity against *T. cruzi* [39].

Diverse studies have reported inhibition of TR in *in vitro* models; however, there is little research that evaluates this activity in animal models. In this context, tricyclic derivatives such as clomipramine and thioridazine have been evaluated *in vivo* with promising results and they have also served as controls in TR inhibition, contrasting their inhibitory effect with other compounds. Moreover, one of the inconveniences when evaluating molecules with possible anti-TR activity is the membrane system of the parasite, which impedes the entrance of bioactive molecules blocking them from target sites like TR. Therefore, the use of carriers that can pass through the membrane of the parasite are very useful in therapeutic targets where inhibition values are effective but intracellular presence of the inhibitor is scarce.

Cruzipain

Cruzipain is the main cysteine-protease in *T. cruzi*, and it is a glycoprotein with an approximate molecular weight of 41 kDa. It is localized in the lysosomes, but it can also be found in several isoforms in the plasma membrane [40]. Cruzipain has been considered a therapeutic target for Chagas diseases [41] because of its role in tissue invasion and immune response evasion. Also, this protein is expressed in all stages of the biological cycle of the parasite [42].

Several cruzipain inhibitors has been reported and one of the best prototypes is the protease inhibitor K777, Fig. (**17**). In preclinical assays it has been shown to have an inhibitory effect on cruzipain (IC_{50} = 0.004 μM) and an MIC of 0.5-5.0 μM; however, its efficacy varies depending on the tested strains, according to its infectivity and pathogenicity [43]. It is worth mentioning that in mice models infected with *T. cruzi*, compound K777 has eliminated the infection in 20- to 30-day periods, and in *in vitro* assays showed a synergic effect with Bnz, enhancing the parasite infected muscle cells survival period with a co-treatment of 5 μM, with a survival rate of 47 days in comparison with Bnz and K777 alone with 27 days of survival [44].

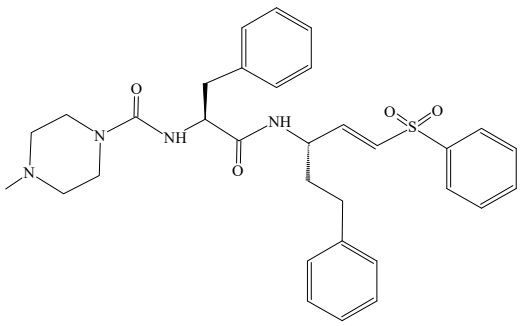

Fig. (17). K777, Currently in clinical trials.

Based on a vinyl sulfone K777 prototype, along with a new compound screening method known as "substrate activity screening" (SAS), several potent cruzipain inhibitors were designed [45]. To do this, optimized candidate substrates were converted to potential inhibitors by adding a cysteine protesase mechanism based-pharmacophore. A tetrafluorophenoxymethyl ketone derivative, Fig. (**18**) was particularly interesting because it showed biological activity at 10 μM on *in vitro* assays, eradicating completely the trypomastigotes from macrophages J744 in 40 days post-infection. Because of these excellent results in *in vitro* assays, a aryloxyketone derivative was tested in mouse plasma, in order to prove its stability and bioavailability for future animal assays. Such assays indicated that this compound was 100% stable under these conditions.

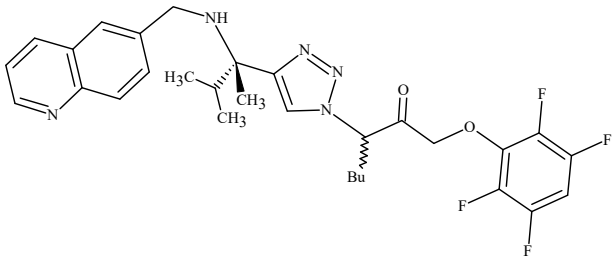

Fig. (18). Tetrafluorophenoxymethyl ketone, inhibitor of cruzipain.

In 2009, Bryant *et al.* reported 11 vinyl sulfone K777 analogues (IC_{50} = 0.004 μM). In *in vitro* assays on *T. brucei*, the compounds 7a (IC_{50} = 0.05 μM) and 8a (IC_{50} = 0.1 μM) resulted in almost the same effectivity as its predecessor. In contrast with results from the cytotoxic assays of K777 (3% at 10 μM on Jurkat cell line), compounds 7a and 8a, Fig. (**19**) did not have the desired results (3% > 100 μM). However, in crystallographic studies the vinyl sulfone 8a derivative showing effective binding in the active sites of cruzipain (sub sites S1 and S3), information of vital importance that will be very helpful in the design of new cruzipain inhibitors [46].

Several small molecules containing an electrophilic warhead acting as cruzipain inhibitors have been reported. Luci *et al*. used reversible inhibitors of cruzipain (ML217: IC_{50} = 2 μM, ML090 and ML092: IC_{50} = 2 nM and 1.2 μM, respectively), Fig. (**20**), to find new effective cruzipain inhibitors. They evaluated 40 benzimidazole analogs, Fig. (**21**) as potential cruzipain inhibitors and the 4 most effective compounds showed an IC_{50} = 1.3-1.8 μM, and *in vivo* assays with mice models demonstrated a parasitemia reduction at day 19 and no toxicity (10 mg/kg/day), but some had low solubility which could represent low absorption and bad oral bioavailability [47].

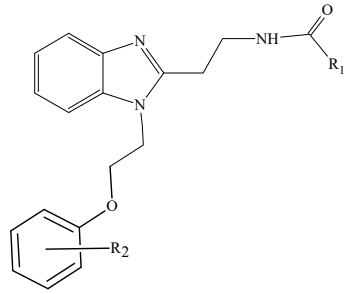

Fig. (21). Benzimidazole analogs.

Recently, Choy *et al.* reported synthesis of vinylsulfone K777 analogs, and out of 13 evaluated compounds, derivative *N*-[(2*S*)-1-[[(*E*, 3*S*)-1-(bencenesulfonyl)-5-phenylpent-1-

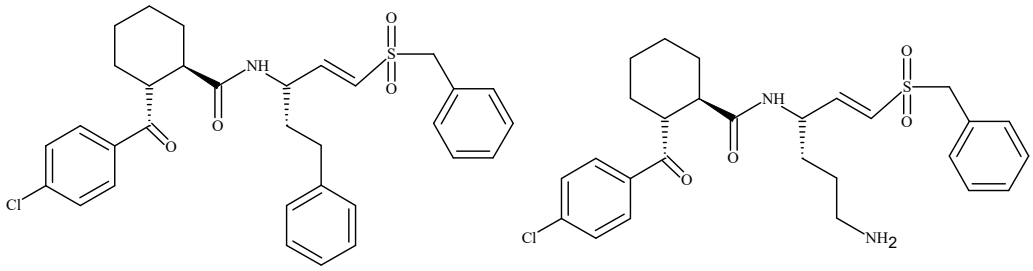

Fig. (19). Compounds 7a and 8a, analogues of K777.

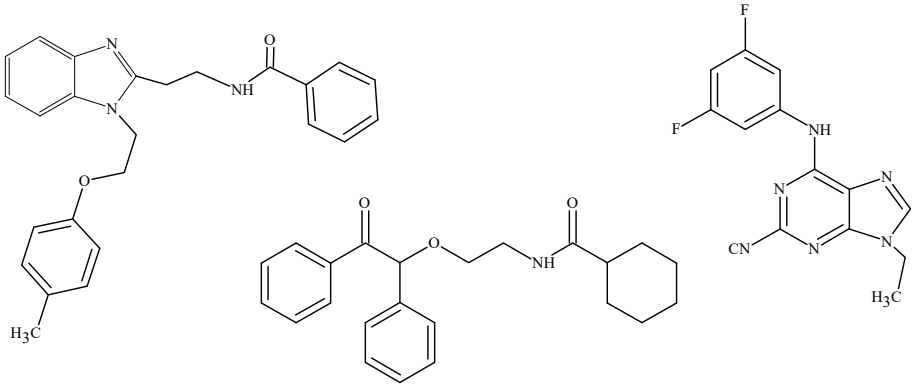

Fig. (20). Compounds ML217, ML090 and ML092.

en-3-yl] amino]-3-(4-methylphenyl)-1-oxopropan-2-yl] pyridin-4-carboxamide (4), Fig. (**22**), had effective minimum concentrations *in vitro* (0.6 μM); about 10 times lower than the lead K777 (8 μM). However, this excellent activity could also be because of a compound interaction on a second therapeutic target, so UV-visible compound 4 affinity to CYP51*Tc* was determined, and it showed similar results to other know CYP51*Tc* (K_D = 5 nM *vs* nonazole K_D = 4.8 nM) inhibitors. The authors suggested that trypanocidal activity of such a compound may be derived from CYP51 inhibition on *T. cruzi*; however, the compound also inhibited mammalian CYP isoforms [48].

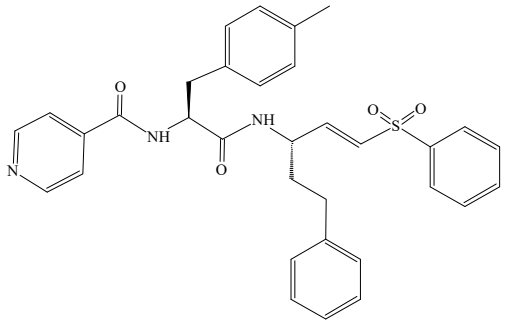

Fig. (22). Truncated analogue of K777.

A biological evaluation of aryl-thiosemicarbazone derivatives on *T. cruzi* showed promising results, and out of 12 tested compounds, derivative 2-amino-1-(4-nitro) acetophenone thiosemicarbazone, Fig. (**23**), had an IC_{50} = 6.3 μM in *in vitro* assays with *T. cruzi* epimastigotes being much lower that with the reference drug Bnz (IC_{50} = 3.6 μM). However, this compound only caused a 20% inhibition of cruzipain activity. All thiosemicarbazones derivatives had an inhibitory effect on cruzipain (5-65% at 10 μM) but this could not be correlated with trypanocidal activity, so authors suggest that arylthiosemicarbazones may be also inhibiting TR activity [49].

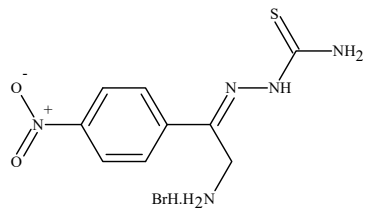

Fig. (23). Acetophenone thiosemicarbazone derivate.

In another study by Braga *et al.* in 2014, the synthesis and biological activity of 18 new bis-(arylmethylidene)-cycloalkanone, Fig. (**24**) derivatives was reported. All compounds were evaluated on *T. cruzi* amastigotes and 6 compounds showed trypanocidal activity on intracellular amastigotes with an IC_{50} = 7-250 μM; however, these values were above Bnz values (IC_{50} = 3.8 μM). As some of the cruzipain inhibitors have electrophilic groups (vinyl ketones), several cycloalkanones were evaluated as cruzipain inhibitors, and out of 8 evaluated compounds, none showed significant activity (16-66% cruzipain inhibition at 100 μM). The authors suggested that these derivatives may have a low structural complementarity with cruzipain [50].

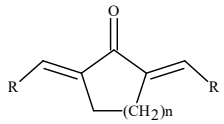

Fig. (24). bis-(arylmethylidene)-cycloalkanone derivatives.

Oliveira-Cardozo *et al.* in 2014, reported the synthesis and pharmacological evaluation of 25 new derivatives of 2-pyridyl thiazoles Fig. (**25**) as potential cruzipain inhibitors [51]. In a first *in vitro* assay on Y strain epimastigotes, 19 derivatives showed activity in the range of IC_{50} = 2.1-5.6 μM, being more potent than Bnz (IC_{50} = 6.6 μM). In enzymatic assays, derivatives (2-(1-(Pyridin-2-yl) ethylene) hydrazinyl)-4-(4-methoxyphenyl)-1,3-thiazole and (2-(1-(Pyridin-2-yl) ethylene) hydrazinyl)-4-(naphthalenyl)-1,3-thiazole had values of IC_{50} = 0.04 μM and 0.01 μM, respectively. They demonstrated that cruzipain is strongly inhibited by thiazole derivatives; in contrast, it has been reported that thiazole derivatives have a trypanocidal effect affecting ergosterol biosynthesis [52].

One of the greatest advances in obtaining molecules with biological activity against *T. cruzi* has been in this particular therapeutic target. The protease inhibitor K777 is being tested in preclinical studies and is by far one of the most promising molecules for fighting Chagas disease. Analogues of this vinyl sulfone have also shown excellent results in *in vitro* evaluations and have overcome other pharmacological barriers such as bioabsorption and bioavailability. Thiosemicarbazones, cycloalkanones and other molecules have been evaluated as selective cruzipain inhibitors; however, the effect of non-specific inhibition and low solubility are some major drawbacks to overcome.

STEROL METABOLISM

Squalene Synthase

Squalene synthase (SQS*Tc*) is an important enzyme in the sterol production pathway, and it has been validated as a therapeutic drug target in *T. cruzi* and *Leishmania mexicana* [53]. It has a monomeric structure of 404 amino acid residues and it is associated to the endoplasmic reticulum, where it catalyzes farnesyl pyrophosphate (FPP) molecule conden-

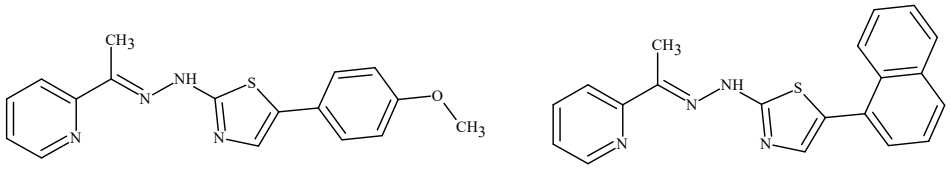

Fig. (25). Pyridyl thiazole derivatives.

sation to yield squalene as a final product [54].

Elhalem *et al*. reported phenoxy and aryloxy ethylthiocyante derivatives with a potent *in vitro* activity on *T. cruzi* amastigotes; however, the molecular action mechanisms were not clearly elucidated [55]. Urbina *et al*. reported the action mechanism and inhibition activity on SQS*Tc* of compound WC-9 Fig. (**26**), which in an enzymatic assay had an IC_{50} = 88-129 nM and an MIC = 1 μM, inhibiting endogenous sterol production. This suggested that these compounds could be part of a new inhibitor class of SQS*Tc* [56].

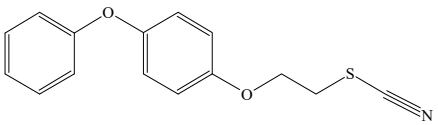

Fig. (26). WC-9, inhibitor compound of SQS*Tc*.

Active inhibitors of SQS*Tc* through the enteral route were reported with the quinuclidines E5700 and ER-119884 Fig. (**27**) being the compounds that inhibited SQS at 5.4 and 5.2 nM, respectively. In *in vitro* evaluation on epimastigotes, quinuclidine derivatives had an IC_{50} = 8 and 11 mM and an MIC of 30 and 100 nM, respectively. Also it was demonstrated that these compounds had no harmful effect on host cells. In *in vivo* murine models infected with trypomastigotes, it was observed that E5700 was able to offer complete protection against infection and parasitemia was halted at 50 mg/kg in a 30-day period; ER-119884 provided only partial protection [57].

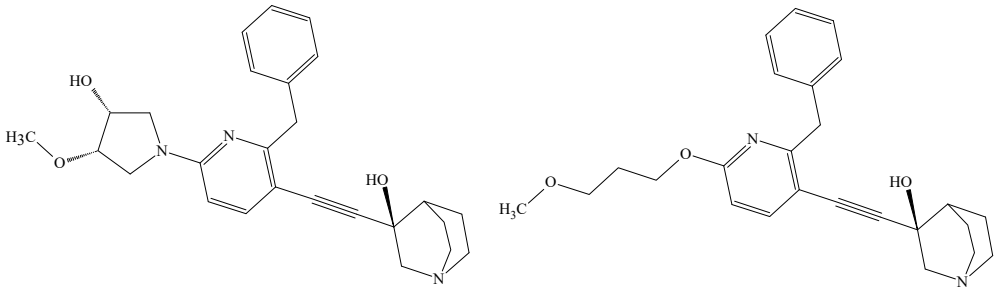

Fig. (27). Quinuclidine derivates E5700 and ER-119884.

Cardona *et al*. reported the biological evaluation of quinuclidine derivatives. On enzymatic inhibition assays against SQS*Tc*, 4 compounds evaluated had an IC_{50} between 0.5-1.5 μM, which allowed assessment of the effectiveness of quinuclidine derivatives as SQS*Tc* inhibitors, since the controls E5700 and ER119884 have a lower IC_{50} (0.0008 y 0.003 μM, respectively).

On *in vitro* assay on amastigotes, the best compound Fig. (**28**) had an IC_{50} = 3.1 μM, value also above the control compounds with IC_{50} = 0.008 and 0.01 μM, respectively. Furthermore, all the compounds tested had an inhibitory effect on human SQS, again with a more pronounced effect observed in the control compounds E5700 and ER-119884 (IC_{50} = 0.001 and 0.006 μM), demonstrating that such inhibitors are not selective against the SQS of the parasite. However, all this data suggest some hints in the design of new more efficient and selective inhibitors of *T. cruzi* SQS [58].

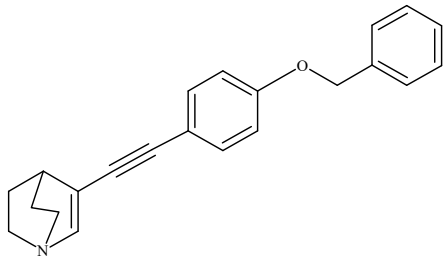

Fig. (28). Quinuclidine analogues.

Recently, Rodriguez-Poveda *et al.* described 2-alkylaminoethyl-1,1-bisphosphonic acids derivatives Fig. (**29**), as potent inhibitors of SQS*Tc*, and the most effective compounds were [(*n*-pentylamino) etil] 1,1-bisphosphonic acid (IC_{50} = 5 nM), [(*n*-hexylamino) ethyl] 1,1-bisphosphonic acid (IC_{50} = 21 nM); and [(*n*-heptylamino) ethyl] 1,1-bisphosphonic acid (IC_{50} = 12 nM). During *in vitro* assay, these same derivatives showed the best results in the inhibition of intracellular amastigotes with an IC_{50} = 0.5-0.08 μM. In a previous report, these compounds had been identified as FPPS*Tc* inhibitors [59] but recent results suggest that the main target of these compounds may be SQS instead of FPPS as was initially thought [54].

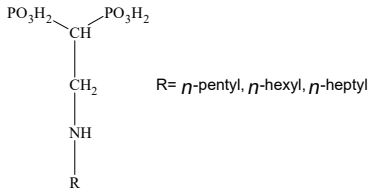

Fig. (29). 2-alkylaminoethyl-1, 1-bisphosphonic acids derivatives.

Elicio *et al.* worked with a derivative series from compound WC-9 and a nitro derivative, 4-(3-nitrophenoxy) phenoxyethyl thiocyanate Fig. (**30**) that in *in vitro* assay had an ED_{50} = 5.2 μM on *T. cruzi* amastigotes, being as potent as its predecessor WC-9 (ED_{50} = 5 μM). On the other hand, the WC-9 (3-phenoxy phenoxyethyl thiocyanate) regioisomer Fig. (**30**), showed an inhibitory activity similar to that of its lead (ED_{50} = 11.2 μM), which indicates that the substitution pattern in the *para*-aryl groups of WC-9 may not be essential for biological activity. It is important to note that analogs of WC-9 also had inhibitory effect on *Toxoplasma gondii*; analogs have a reasonable drug-like character that overcomes the approach to establish a structure/activity relationship [60].

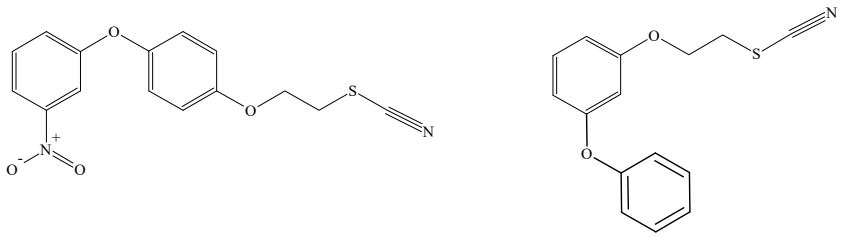

Fig. (30). 4-(3-nitrophenoxy) phenoxyethyl thiocyanate and regioisomer of WC-9.

The anti-tuberculosis drug in phase II clinical trial, SQ110 Fig. (**31**), has also been evaluated as a trypanocidal agent [61]. In an *in vitro* assay with Y strain of *T. cruzi* trypomastigote infected cells, SQ110 showed better results than reference drugs (IC_{50} = 50 nM *vs* violet crystal IC_{50} = 12 μM) and Bnz (IC_{50} = 400 μM). In assays on epimastigotes (IC_{50} = 4.6 μM) this compound caused dramatic changes in the Golgi apparatus and mitochondrial damage. In assays of SQ110 on CL Brener strain, it was more active (IC_{50} = 520 nM) than Bnz (IC_{50} = 1.2 μM). It is important to note that in previous reports, SQ110 was an inhibitor of bacterial enzymes such as dehydrosqualene synthase and homolog synthases [62, 63], but in *T. cruzi* assays SQ110 had an IC_{50} = 100 μM, which was above the expected results. However, through electron microscopy, it was determined that SQ110 induces damage in the mitochondrial membrane through H^+ release into cell compartments, which may explain the excellent results on epimastigotes.

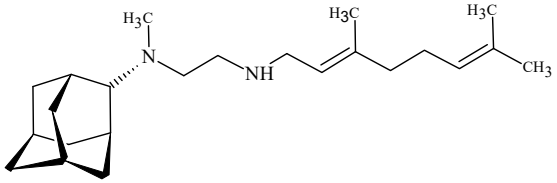

Fig. (31). Anti-tuberculosis drug, SQ110.

With the results of quinuclidine and analog inhibition, SQS also ranks as one of the promising therapeutic targets for the development of treatments for Chagas disease although it is necessary to guide efforts toward finding new more selective inhibitors than WC-9. On the other hand, the action of SQ110, an antituberculosis drug in clinical evaluation, has been assessed against SQSTc, showing effective results in *in vitro* assays. In this context, elucidating the mechanism of action of SQ110 would be a major step towards the search for analogous compounds with better features.

Farnesyl Pyrophosphate Synthase

Farnesyl pyrophosphate synthase (FPPS*Tc*) is a cytoplasmic enzyme composed of 364 amino acid residues with a molecular weight of 41.2 kDa [64]. It is involved in mevalonate and farnesyl pyrophosphate (FPP) pathway synthesis [65]. In trypanosomatids FPPS is inhibited by bisphosphonates Fig. (**32**) with an IC_{50} = 65 μM and up to 300 μM. These compounds are currently used in post-menopause osteoporosis therapy, Paget disease, and some bone metastasis [66]. The specific action mechanism depends on compound buildup in the acidocalcisome, because they cannot be metabolized and thus produce apoptosis. It has been postulated that the acidocalcisome of the parasite, being rich in calcium and pyrophosphate, has an equivalent role of the bone mineral in humans. This could explain the high affinity of these inhibitors to this enzyme [67].

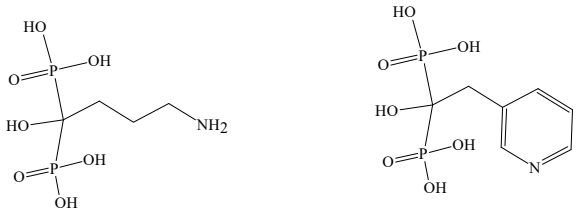

Fig. (32). TcFPPS inhibitors, alendronate and risendronate.

Because bis-phosphonates are promising anti-*T cruzi* agents, in 2008 Szajnman *et al*. reported the evaluation of bis-phosphonic acid derivatives, and out of 14 derivatives evaluated as possible inhibitors of FPPS*Tc*, the ones with propylamino ethyl, *n*-heptylamino ethyl and cyclohexylamino ethyl Fig. (**33**) substitutes showed better results in enzyme assays against FPPS*Tc* with IC_{50} values of 0.03, 0.05 and 0.01 μM, respectively [59]. On *in vitro* assays using *T. cruzi* amastigotes, eight derivatives (among them the [(*n*-heptylamino) ethyl] 1,1-bisphosphonic) acid showed an IC_{50} below that of the reference compound WC-9 (0.5-10 μM against 12 μM of the control). With all this data, bisphosphonic acid derivatives are promising molecules as lead compounds in the search for selective inhibitors of FPPS*Tc*.

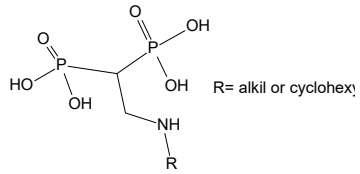

Fig. (33). Bis-phosphonic acid derivatives.

Despise the data of several reports that bis-phosphonate derivatives have inhibitory action in *T. cruzi*, one problem is their poor oral bioavailability [68] that could be overcome with the addition of metals in coordination with bisphosphonates. Taking that into account, Demoro *et al.* reported the inhibitory activity against FPPS*Tc* of new derivatives of risendronate metal complexes. Compounds such as $[Mn^{II}(Ris)_2]\cdot 4H_2O$, and $[Ni^{II}(Ris)_2(H_2O)_2]\cdot H_2O$ Fig. (**34**), showed excellent results with IC_{50} values of 0.0029 and 0.0027 μM, respectively, and in *in vitro* assays, analogues (Ris, (1- hydroxy-1-phosphono-2-pyridin-3-yl-ethyl) phosphonate), along with the previously mentioned compounds

showed good inhibitory concentrations in amastigote assays (IC_{50} = 14-55 μM). It is important to note that in the protein interaction test, all of them had strong interactions with albumin, which may represent an adequate media for transport *in vivo* to tissues [69].

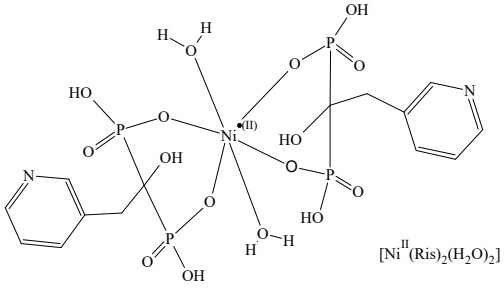

Fig. (34). Metal complexes with bioactive bisphosphonates as ligand.

In cytotoxicity assays, bis-phosphonates did not cause damage to eukaryotic cells and presented an additional characteristic that few compounds have: these compounds are inexpensive and easily synthesized, so they are a promising option for anti-*T cruzi* drug development. Aripirala *et al*. indicated that 2-alkyl, aminoethylbisphosphonate derivatives Fig. (**33**) are inhibitory compounds of FPPS*Tc*, and by isothermal titration calorimetry, showed to inhibit the enzyme in ranges of IC_{50} = 38 nM to 1.84 μM, so these derivatives are also considered potential antiparasitic agents that can inhibit the enzymatic target in nanomolar concentrations [70].

Ferrer-Casal *et al.* reported the synthesis and biological evaluation of new derivatives of bis-phosphonic acid Fig. (**35**), but despite this previous reports indicated that bisphosphonates had excellent results against *T. cruzi*. The 4 compounds evaluated by these authors had not inhibitory effect against FPPS*Tc* (IC_{50} = >10 μM *vs* risendronate IC_{50} = 0.02 μM). Also, these compounds had a poor effect in *in vitro* evaluations on *T. cruzi* amastigotes (ED_{50} = >20 μM *vs* Bnz ED_{50} = 1.4 μM). The lack of biological activity of this compounds series may be due to the fact that they may adopt limited spatial conformations that can interfere with the molecular interaction with FPPS [71].

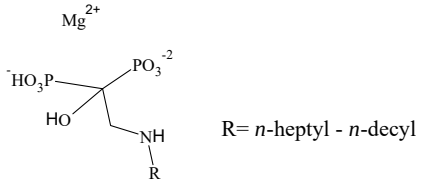

Fig. (35). Derivatives of bis-phosphonic acid.

Bis-phosphonic acid analogs, such as risedronate and alendronate represent an important class of FPPS inhibitors, insomuch that for this target most evaluated molecules are bisphosphonate analogs. A challenge in this area is to develop new molecules with improved bioavailability. It is noteworthy that although the previously described compounds have excellent inhibition values in *in vitro* assays, there are no published reports of bisphosphonate new derivatives evaluation *in vivo*.

STEROL 14A-DEMETHYLASE

Lipids are essential components of eukaryotic membranes because they help regulate their fluidity and permeability. Because of this, sterol 14α-demethylase (CYP51*Tc*) becomes a key enzyme in the search for anti-*T. cruzi* drugs. CYP51 is implicated in the conversion of 24-methylenedehydrolanosterol into ergosterol, and it is a highly susceptible inhibitor of sterol biosynthesis, as special attention is given to it as a therapeutic target to fight Chagas disease [72].

The first CYP51*Tc* inhibitor was reported in 1980 by Docampo *et al.* and in this work they reported the alterations in *T. cruzi* produced by classic antifungals such as miconazole and econazole. Currently, analogs of 24-methylenedehydrolanosteol have been reported, such as MCP (14α-methylenecyclopropyl-Δ7-24, 25-dihydrolanosterol) Fig. (**36**) which had inhibitory effects against CYP51 in several trypanosomatid species. In the I105 (wild) and I105F (mutated) strain of *T. cruzi*, values were 10 μM and 30 μM with 95 and 90% of inhibition, respectively. The inhibitory effect of MCP was correlated in amastigotes because it produces a reduction in their proliferation. With all of this, MCP is an excellent prototype for the development of new structures able to interact and selectively block sterol synthesis in *T. cruzi* [73].

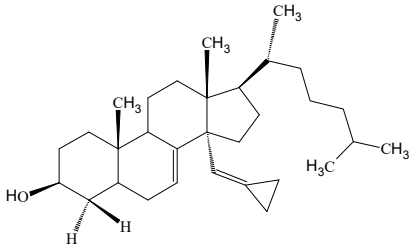

Fig. (36). MCP, inhibitor of CYP51.

By high-throughput screening (HTS) approaches it has been possible to detect compounds with activity against *T. cruzi*. With this approach Andriani *et al*. reported the inhibitory activity on CYP51*Tc* out of a screening of 21 chemotypes of imidazole. The derivatives NEU 321: 1-(3-(4-Chloro-3,5-dimethylphenoxy)benzyl)-1*H*-imidazole and NEU 704: 1-(3-((4-Chlorobenzyl)oxy)benzyl)-1*H*-imidazole Fig. (**37**) showed inhibition of *T. cruzi* growth in *in vitro* assays (NEU 321: EC_{50} = 0.08 μM and NEU 704: EC_{50} = 0.04 μM), being more efficient than the reference drug Bnz (1.9 μM). In the enzyme assay of CYP51*Tc*, the derivative NEU 321 had a K_d that was similar to the reference drug posaconazole (0.06), and was more selective on host cells with a ligand efficiency (LE) NEU 321 = 0.33 y NEU 704 > 0.30 against posaconazole LE = 0.14 [74].

New derivatives of imidazolyl-phenylethanol and aminopyridines have been reported as anti-CYP51*Tc* by Friggeri *et al*. in 2013. Such compounds were designed as analogs of other CYP51*Tc* inhibitors. Out of 8 initial compounds, in the biological evaluation of Tulahuen strain C2C4 amastigotes, the derivatives 2-(1*H*-imidazolyl)-1-phenylethyl-3-(biphenyl) benzoate (IC_{50} = 0.014 μM), 2-(1*H*-imidazolyl)-1-(4-chlorophenyl) ethyl-3-(propan-2-yl phenylamino) benzoate (IC_{50} = 0.005 μM), 2-(1*H*-imidazolyl)-1-(4-fluorophenyl) ethyl-3-(propan-2-yl phenylamino) benzoate (IC_{50} = 0.005 μM), and 2-(1*H*-imidazolyl)-1-(4-chlorophenyl) ethyl-3-(2,6-dichloropyridin-4-

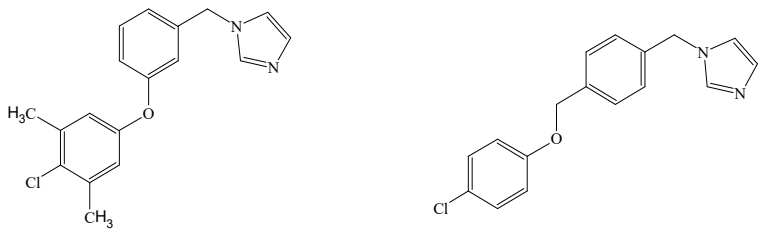

Fig. (37). NEU321 and NEU704 derivatives.

yl amino) benzoate (IC_{50} = 0.036 μM) were more potent that control Bnz (IC_{50} = 1.6 μM). Later in *in vitro* assays to test the inhibitory activity of enantiomers of these compounds, they find that (*S*) enantiomers were more active than (*R*) enantiomers. Interestingly, derivatives such as 2-(1*H*-imidazolyl)-1-(4-chlorophenyl) ethyl-3-(propan-2-yl phenylamino) benzoate Fig. (**38**), were up to 1000-fold more potent than Bnz. Unfortunately, when evaluated on L6 cell lines, all compounds showed toxicity. These derivatives represent a good starting point for the development of molecules with better characteristics than their predecessors [75].

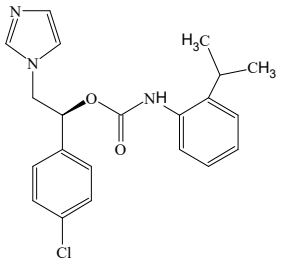

Fig. (38). Imidazolyl-phenylethanol derivatives.

In other reports, Suryadevara *et al*. mention that antifungal posaconazole is effective against *T. cruzi*, but its high production cost could probably restrict its extended use against Chagas disease. They developed a new class of CYP51*Tc* inhibitor taking the posaconazole structure as a lead and other analogues with similar activity (EC_{50} = >1 nM) were synthetized compounds with excellent yields. The inhibitors 4-((5-(((4-chloro-3-methylphenyl) amino) methyl)-1*H*-imidazol-1-yl) methyl)-[1,1'-biphenyl]-3-amine, 4-{5-[(4-Chloro-phenylamino)-methyhl]-imidazol-1-ylmethyl}-biphenyl-3-amine, and 4-((5-(((4-fluorophenyl)amino)methyl)-1*H*-imidazol-1-yl)methyl)-[1,1'-biphenyl]-3-amine Fig. (**39**) had EC_{50} values of 0.6-2.1 nM. These analogues were well tolerated in animal models with the acute stage of the disease, and they presented physical chemical activities (a simple structure, a low molecular weight and low-cost direct synthesis) that make them good candidates as oral drugs against Chagas disease [76].

In the search for new and effective anti-*T. cruzi* drugs for sterol 14α- demethylase, posaconazole, a triazole derivative, demonstrated in *in vitro* and *in vivo* trials that it could counteract parasitemia. One advantage of these type inhibitors is that they have high solubility and selectivity for the therapeutic target; however, the high cost that the synthesis of these compounds entails has made research groups synthesize low-cost analogs from which they can obtain high yields in synthesis. For that matter, dialkyl imidazole inhibitors have been shown to eliminate amastigotes of *T. cruzi* at nanomolar concentrations. Suryadevara *et al*. have also reported that such molecules are well tolerated in animals; therefore, future research could be aimed at the development of this inhibitor as an oral drug for treatment of Chagas disease.

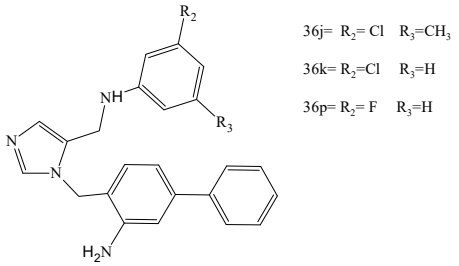

Fig. (39). Dialkylimidazole inhibitors.

CONCLUSION

In Table **1** we provide the general scheme of the recent advances reviewed in this work in the search for molecules with an inhibitory effect on enzymes of *T. cruzi*, some of which in the near future could be good candidates as drugs against Chagas disease. Crucial enzymes for *T. cruzi* have been studied with the aim of developing bioactive molecules. The use of rational inhibitor design and several synthesis strategies have provided important advances in the research of inhibition, action mechanisms, functional role and organization of essential enzymes of the parasite. The combined use of antitrypanosomial drugs, suggests that some aspects to consider for the development of new formulates that are based on efficacy and synergism may move forward all stages of pharmacological evaluation. Besides of this, techniques such as crystallography, have been situated very useful tools to elucidate ways of binding inhibitors and proteins, and, in the other hand, liposomes have also been recognized as a useful technology for compound evaluation since high hydrophobicity impedes passage through parasite membranes.

However, results from diverse research in different trypanosomatid species are not fully equivalent because of the inherent differences in study designs, enzymes quantities, and their specific characteristics and functions [77]. Herein, we have reviewed the recent advances in antiparasitic therapy of *T. cruzi*, taking in account reports as well as established drug targets with a wide knowledge of their inhibition and effect on *T. cruzi*. Important reported enzymes, such as phosphodiesterase C (PDE-C*Tc*) [78], and kynureninase (KI*Tc*) [79], have recently been proposed as relatively new drug targets because of their recent discovered characteristics, but their action mechanisms has not been fully elucidated.

Table 1a. Description of recent advances in antiparasitic therapy against Chagas disease (Enzymes implicated in the cellular metabolism).

Enzyme	Author and Year	Assessed Molecule	Enzymatic Inhibition Range in *T. cruzi*	Antiparasitic Activity in *T. cruzi*	Study Stage
TIM*Tc*	Olivares *et al.* 2007	Dithiodianiline	IC_{50} = 250 nM	IC_{50} = 4 μM epimastigotes ninoa (*in vitro*)	*In vitro* assays
	Gayosso *et al.* 2009	Brevifolin carboxilate derivatives	I_{50} = 6.5 μM	----[a]	*In vitro* assays
	Alvarez *et al.* 2010	Thiadiazole, Thiadiazines, and phenazines	IC_{50} = 3.5-26 μM	ID_{50} = 3 μM epimastigotes Tulahuen (*in vitro*)	*In vitro* assays
	Alvarez *et al.* 2013	Thiadiazole	IC_{50} = 3.5 μM	IC_{50} = 8 μM epimastigotes Tulahuen (*in vitro*)	*In vitro* assays
	Alvarez *et al.* 2015	Bis-thiazoles	EC_{50} =16 μM	EC_{50} = 1.2 μM amastigotes Tulahuen (*in vitro*)	*In vitro* assays
GAPDH*Tc*	Vieira *et al.* 2001	Chalepin	IC_{50} = 64 μM	----[a]	*In vitro* assays
	Menezes *et al.* 2003	Chalepin derivatives	IC_{50} = 55.5 μM	----[a]	*In vitro* assays
	Gallo *et al.* 2008	Quercetin and gallic acid	IC_{50} = 24-25 μM	34-41% of lysis at 1.7 mM (*in vitro*)	*In vitro* assays
	Silva *et al.* 2010	Ruthenium derivatives	IC_{50} = 89-153 μM	IC_{50} = 52 μM (*in vitro*), 60-80% of survival at 20 days (*in vivo*)	*In vivo*, preclinical assays
	Soares *et al.* 2013	Ribonucleoside Neq 135	K_i = 16 mM	18 μM/ L^{-1} (*in vitro*)	*In vivo*, preclinical assays
	Belluti *et al.* 2014	Hybrid quinone-coumarin	IC_{50} = 5.4 mM (GAPDH*Tb*)[b]	EC_{50} = 1.2-1.4 μM Tulahuen (*in vitro*)	*In vitro* assays

Table 1b. Description of recent advances in antiparasitic therapy against Chagas disease (continuation 2) (Enzymes from the cellular detoxification complex and host adhesion).

Enzyme	Author and Year	Assessed molecule	Enzymatic Inhibition Range in *T. cruzi*	Antiparasitic Activity in *T. cruzi*	Study Stage
TR	Porcal *et al.* 2008	*N*-oxide benzofuroxane	9-50 % of inhibition	IC_{50} = 6.8-8.7 μM epimastigotes Tulahuen (*in vitro*)	*In vitro* assays
	Rodrígues *et al.* 2012	Thiadizolium aminide derivatives	69 % of inhibition	----[a]	*In vitro* assays
	Fauro *et al.* 2013	Clomipramine	K_i = 6.5 μM	5 mg/kg^{-1} reduction of parasite load trypomastigotes Tulahuen (*in vivo*)	*In vivo* assays and used as TR inhibition control
	Lo presti *et al.* 2015	Thioridazine	----[a]	80 mg/Kg/day reduction of parasitemia in 80 % *in vivo* trypomastigotes Tulahuen	*In vivo* assays
	O'Sullivan *et al.* 2015	Clomipramine and polyamine derivatives	K_i = 0.2-4 μM	IC_{50} = 4.5 μM (*in vitro T. brucei*),	*In vivo* assays
	Beig *et al.* 2015	Anti-TR compounds	IC_{50} = 1.1 μM	50 mg/Kg/day (*in vivo T. brucei*) EC_{50} = 50-5 μM (*in vitro T. brucei*)	*In vitro* assays
Cruzipain	McKerrow, 2009	K777	IC_{50} = 0.004 μM	----[a]	phase: clinical assays
	Doyle *et al.* 2007	K777	----[a]	5 μM cells survival to 47 days (*in vitro*)	phase: clinical assays
	Brak *et al.* 2008	Aryloxyketone derivatives	----[a]	10 μM eradication of parasites at 40 days post infection (*in vitro*)	*In vitro* assays
	Bryant *et al.* 2009	K777 analogues	IC_{50} = 0.05 μM	----[a]	*In vitro* assays
	Luci *et al.* 2010	Benzimidazole analogues	IC_{50} = 1.3-1.8 μM	10 mg/Kg/day reduction of parasitemia at 19 days	investigation *in vitro* ADME

	Choy *et al.* 2013	K777 analogues	----[a]	MTC[c]= 0.6 μM	*In vitro* assays
	Blau *et al.* 2013	Aryl-thiosemicarbazone derivatives	5-65% of inhibition at 10 μM	IC_{50}= 6.3 μM epimastigotes (*in vitro*)	*In vitro* assays
	Braga *et al.* 2014	Cycloalkanones	66% of inhibition at 100 μM	IC_{50}= 7-250 μM amastigotes (*in vitro*)	*In vitro* assays
	Oliveira *et al.* 2014	Pyridyl thiazole derivatives	IC_{50}= 0.01-0.04 μM	IC_{50}= 2.1-5.6μM epimastigotes Y (*in vitro*)	*In vitro* assays

Table 1c. Description of recent advances in antiparasitic therapy against Chagas disease (continuation 3) (Enzymes from sterol metabolism).

Enzyme	Author and Year	Assessed Molecule	Enzymatic Inhibition Range in *T. cruzi*	Antiparasitic Activity in *T. cruzi*	Study Stage
SQS*Tc*	Urbina *et al.* 2003	WC-9	IC_{50}= 88-129 nM	----[a]	*In vivo* assays and used as SQS inhibition control
	Urbina *et al.* 2004	Quinuclidines	IC_{50}=5.2-5.4 nM	IC_{50}= 8-11 nM epimastigotes (*in vitro*), 50 mg/kg x 30 days arrest parasitemia *in vivo*	*In vivo* assays and orally active SQS inhibitor
	Cardona *et al.* 2007	Quinuclidine analogues	IC_{50}=0.5-1.5 μM	IC_{50}= 3 μM amastigotes (*in vitro*)	*In vitro* assays
	Rodrígues *et al.* 2012	Bis-phosphonic acid derivatives	IC_{50}= 5-21 nM	IC_{50}= 0.5-0.8 nM amastigotes (*in vitro*) ED_{50}= 5.2 μM amastigotes (*in vitro*)	*In vitro* assays
	Elicio *et al.* 2013	WC-9 analogues	----[a]	IC_{50}= 50 nM trypomastigotes Y IC_{50}= 4.6 μM epimastigotes Y	*In vitro* assays
	Veiga *et al.* 2015	SQ110	IC_{50}= 100 μM	IC_{50}= 520 nM amastigotes brener (*in vitro*)	*In vitro* assays
FPPS*Tc*	Szajnman *et al.* 2008	Bis-phosphonic acid derivatives	IC_{50}= 0.01-0.05 μM	IC_{50}= 0.5-10 μM amastigotes (*in vitro*)	*In vitro* assays
	Huang *et al.* 2010	Bis-phosphonates	IC_{50}= 65-300 μM	----[a]	*In vivo* assays and used as FPPS inhibition control
	Demoro *et al.* 2010	Derivatives of risendronate metal complexes	IC_{50}=0.0027 mM	IC_{50}= 14-55 μM amastigotes (*in vitro*)	*In vitro* assays
	Aripirala *et al.* 2012	Aminoethylbisphosphonate derivatives	IC_{50}= 38 nM-1.84 μM	----[a]	*In vitro* assays
	Ferrer *et al.* 2014	Bis-phosphonic acid derivatives	IC_{50}= >10 mM	ED_{50}= >20 μM amastigotes (*in vitro*)	*In vitro* assays
CYP51*Tc*	Hargrove *et al*; 2012	Lanosterol analogues (MCP)	95 % of inhibition at 10 mM	----[a]	*In vitro* assays
	Andriani *et al.* 2013	Chemotypes of imidazole	K_d= 0.06	EC_{50}= 0.04-0.08 μM trypomastigotes (*in vitro*)	*In vitro* assays
	Friggeri *et al.* 2013	Imidazolyl-phenylethanol and amino-pyridines	----[a]	IC_{50}= 5-36 nM amastigotes (*in vitro*)	*In vitro* assays
	Suryadevara *et al.* 2013	Dialkylimidazole derivatives	----[a]	EC_{50}= 0.6-2.1 nM amastigotes (*in vitro*)	*In vivo* assays

a: Not tested, data no show
b: Glyceraldehyde-3-phosphate dehydrogenase of *T. brucei*
c: Minimum effective concentration that clears J774 host cells of parasites at day 40 of the experiment

Despite all the recent advances on therapeutic targets, it has not been possible to obtain compounds that surpass all the clinical phases in animal models, so, most of are considered prototypes. To date, we have not obtained new specific compounds with activity on the chronic or acute stage of Chagas disease [80]. TR, cruzipain and enzymes of the sterols pathway are currently the most deeply studied therapeutic targets, and it is from these that a large amount of information can be used to generate inhibitory molecules because these enzymes do not exist in humans. While inhibition assays on FPPS and SQS of *T cruzi* with bis-phosphonates [67, 70, 71], and quinuclidines [57] are promising, *in silico* analysis could further determinate the inhibitor effects of these molecules in mammals. The search for more efficient drugs against Chagas disease will depend on the amount of information and their rational use (for instance, in therapeutic targets). Therefore, more research on the rational design of inhibitory compounds of therapeutic targets is essential.

LIST OF ABBREVIATIONS

Bnz	=	Benznidazole
CYP51	=	Sterol 14 α-demethylase
DNA	=	Deoxyribonucleic acid
DBS	=	Dibenzosuberyl
FAD	=	flavin adenine dinucleotide
FPPS	=	Farnesyl pyrophosphate synthase
GAPDH	=	Glyceraldehyde 3-phosphate dehydrogenase
HTS	=	High-throughput screening
KI	=	kynureninase
LE	=	Ligand efficiency
MTL	=	Multi-target ligands
NAD^+	=	Nicotinamide adenine dinucleotide
NADPH	=	Reduced nicotinamide adenine dinucleotide phosphate
Nfx	=	Nifurtimox
PDE-C	=	Phosphodiesterase C
ROS	=	Reactive oxygen species
SAS	=	Substrate activity screening
SQS	=	Squalene synthase
TIM	=	Triose phosphate isomerase
T. brucei (*Tb*)	=	*Trypanosoma brucei*
T. cruzi (*Tc*)	=	*Trypanosoma cruzi*
TR	=	Trypanothione reductase
WHO	=	World Health Organization

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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