

An Insight on Targets and Patented Drugs for Chemotherapy of Chagas Disease

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Abstract: Chagas disease or American Trypanosomiasis, a parasitic infection typically spread by triatomine bugs, affects millions of people throughout Latin America. Current chemotherapy based on the nitroaromatic compounds, benznidazole and nifurtimox provides unsatisfactory results and suffers from considerable side effects and low efficacy. Therefore, there is an urgent need for new drugs to treat this neglected disease.

Over the last two decades, new advances and understanding in the biology and the biochemistry of *Trypanosoma cruzi* has allowed the identification of multiple targets for Chagas disease chemotherapy. This review summarizes antichagasic agents obtained based on i) target metabolic biochemical pathways or parasite specific enzymes, ii) natural products and its derivatives, iii) design and synthesis of lead compounds. Related patents filed and issued from 2000 to early 2006 are also discussed. Most of them claimed inhibitors on specific parasite targets such as cysteine proteinase, sterol biosynthesis, protein farnesyltransferase, etc. Particularly, those related to cysteine proteinase inhibitors were the most represented. Natural products also displayed many anti-*T. cruzi* lead compounds. In addition, a few patents claiming natural or synthetic compounds with antichagasic activity, disclosed no specific target. However, only a small proportion of all these patents displayed specific data of biological trypanocidal activity.

Keywords. Chagas disease, *Trypanosoma cruzi*, drug targets, natural and synthetic inhibitor compounds.

INTRODUCTION

Tropical parasitic diseases are produced by different eukaryotic protozoa. Among them, trypanosomes are known to be responsible for sickness presenting quite different clinical manifestations, geographical distribution, life cycle and insect vectors [1]. Chagas disease, also known as American Trypanosomiasis is one of the most serious protozoan diseases which occurs throughout Latin America, particularly in South America. Its etiological agent is *Trypanosoma cruzi* (*T. cruzi*), a flagellate protozoan, which is transmitted to humans and other mammals mostly by hematophagous insects of the Reduviidae family, Triatominae subfamily. *T. cruzi* has a complex life cycle, with proliferative stages in the vector (epimastigotes) and the vertebrate (intracellular amastigotes), as well as non-proliferative infectious stages (trypomastigotes) in both hosts. The World Health Organization has estimated that some 16-18 million people are infected throughout the American continent (including some 100.000 in the United States), and that more than 100 million are at risk [2]. Despite Chagas disease transmission has been eliminated in several countries by control of the Triatomine vector using insecticide spraying and serological screening of blood donors [3, 4], the disease continues to be endemic in large areas of Latin America.

The disease is characterized by three clinical forms named acute, indeterminate and chronic. In the acute phase, a local inflammatory lesion appears at the site where metacyclic trypomastigotes enter, and the parasite spreads throughout the host organism. The indeterminate phase comprises a period that may last 10-20 years between the acute and chronic phases and is generally asymptomatic. On the contrary, the chronic phase is characterized by the presence of myocarditis and/or pathological disturbances in the peripheral nervous and gastrointestinal systems. Thirty to forty per cent of chronic infected individuals develop cardiac abnormalities and as many as 10 % develop digestive tract disease [5]. Recently, night blindness has been investigated as new clinical symptom in patients with chronic Chagas disease and retinal dysfunction has been associated to anti-*Trypanosoma cruzi* antibodies that cross-react with rhodopsin [6]. Two mechanisms were proposed for pathogenesis in the chronic phase: inflammatory reactivity due to the persistence of the parasite inside the host tissues and induction of autoimmune responses targeted in infected tissues. Both events would indicate that the elimination of *T. cruzi* from infected patients would lead to arrest the evolution of the disease [7].

Diagnosis of Chagas disease has been performed by the traditional direct detection of the parasite in blood during the acute phase or by serodiagnosis. DNA amplification using the polymerase chain reaction (PCR) as well as single or mixtures of recombinant antigens used for serodiagnosis, are currently available tools to evidence the presence of the parasite [8,9]. In addition, the use of chimerical synthetic peptides containing antigenic sequences of immunodominant regions of *T. cruzi* as coating antigens seems to be useful for

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the immunodiagnosis of this disease [10]. To date, there are no prophylactic drugs to prevent infection with *T. cruzi*. Moreover, current chemotherapy of Chagas disease based on the nitroaromatic compounds benznidazole (Radanil, Roche), and nifurtimox (Lampit, Bayer, discontinued to the public) is questionable as these compounds are effective only for recent (acute, congenital or experimental) infections and their utility during the chronic phase of Chagas disease is of limited efficacy [11,12]. In addition, both drugs have severe side-effects including anorexia, vomiting, allergic dermatopathy and peripheral polyneuropathy.

Taking into account that nifurtimox and benznidazole are far from the requirements to consider them ideal as trypanocidal drugs (very safe, very effective, very stable and inexpensive) in addition to the fact that in the last decade trials with allopurinol showed poor results [13], the search for new compounds with anti-*T. cruzi* activity, with low toxicities and increased efficacies during the indeterminate and chronic phases, continues. The identification of new antichagasic agents may be based not only on rational drug design and natural products screening [14], but also taking advantage of compounds already in use against other human diseases, which have already passed several of the clinical trials necessary for the development of any new drug. Thus, there is an urgent need to identify specific enzymes and metabolic pathways in the parasite useful as potential targets for drug development. However, in spite of the urgency of the matter, pharmaceutical industry has restricted investment in research and development in this disease [15]. Recently, health innovation networks to help developing countries address neglected diseases have been created [16]. On the other hand, as a result of the parasite genome sequencing project, available since 2005 [17] the possibility of identifying new specific pathways and novel drug targets in the near future is open.

Over the last two decades, new advances and understanding in the biology and the biochemistry of *T. cruzi* has allowed the identification of multiple targets for Chagas disease chemotherapy. The main promising targets for antiparasitic agents involve proteinases (particularly cysteine proteases), sterols and isoprenoids biosynthetic pathways and thiol-dependent redox metabolism. In addition, polyamine metabolism and transport pathways, enzymes of the glycolytic and pentose phosphate biosynthetic pathways, lipidic (alkyl-lysophospholipids, glycosphingolipids) and purine salvage pathways, have also been intensively studied. Moreover, some organelles functions including DNA modulation in nucleus and kinetoplast involving topoisomerases as well as the exchanger Na^+/H^+ mechanism from acidocalcisomes are also considered promising targets for antiparasitic drugs. Among them, particularly those that target the validated biochemical pathways of the parasite including cysteine proteinase inhibitors (CPIs) and inhibitors able to block ergosterol biosynthesis are currently in the pipeline. In summary, the aim of this review is to present a whole view including patents and recent advances on antichagasic agents obtained from different sources. Data were divided into three major sections: Part I will include targets and most patents referring to specific drug targets. Part II will refer to natural compounds and their derivatives as chemotherapeutic agents. Part III will include designed

and synthesized parasiticidal drugs. Metabolic pathways or specific enzymes used as targets and patents will also be discussed.

The search strategy for patent literature claiming for trypanocidal activity against *T. cruzi* was performed through the Delphion Research intellectual property network including international and US patent search database (2000-early 2006).

DEVELOPMENT OF NOVEL DRUGS AGAINST CHAGAS DISEASE

Part I. Drug Targets and Lead Compounds

***T. cruzi* metabolism-Targets** Trypanosomes diverged very early from the common eukaryotic lineage, probably due to independent evolution of Kinetoplastida, one of the oldest lineages of protozoa [18]. Thus, they have several unusual biochemical pathways which differ in numerous aspects from that of mammalian cells. This fact may provide selective targets for drug development, particularly, rational design of metabolic pathway inhibitors or for specific enzymes chosen as drug targets. However, it is worth mentioning that a simple difference between host and parasite is not sufficient to consider a compound as a drug target. Target validation is an essential step in any rational approach to chemotherapy. The usual method to verify that an enzyme is essential for an organism is based on the use of a highly specific inhibitor, but such compounds are not always available, so genetic approaches such as knock-out mutants or the inducible depletion of the specific mRNA by RNA interference, are now widely in use [19,20]. Moreover, although an enzyme has proven to be essential, it may not constitute necessarily a drug target. In fact, a high protein abundance of the target enzyme will difficult the maintenance of the high drug concentration required for the binding of a reversible inhibitor within the cell. On the other hand, a rapid *de novo* synthesis of the essential enzyme would overcome the effect of an irreversible inhibitor.

1- Proteinases

Proteinases play multiple roles in disease pathogenesis. They have been involved in host invasion, in the migration of the parasite through tissue barriers, in the degradation of haemoglobin and other blood proteins, in immune evasion as well as in activation of the inflammation process. The multiple roles suggested for several of the *T. cruzi* proteolytic enzymes make them attractive potential targets for the development of new drugs against Chagas disease [21]. *Trypanosoma cruzi* contains cysteine, serine, threonine and metallo proteinases but no report about aspartyl proteinases has been presented to date.

1A-Cysteine Proteinases Cysteine proteinases regulate host-parasite interaction being involved in modulation of a variety of pathobiological effects including nutrient uptake, immune evasion and host tissues degradation. The specific inhibition of these enzymes by immunoprophylaxis or chemotherapy may potentially impair the survival mechanisms of the parasite. Therefore, cysteine proteinases are promising targets for vaccines or chemotherapy.

Cruzipain (Cz), also known as cruzain or GP57/51 [22-24], the most abundant member of the papain C1 family of

cysteine proteinases (CPs) of the parasite, is expressed as a complex mixture of isoforms by the major developmental stages of the parasite and present microheterogeneities [25]. Although the bulk of the enzyme is lysosomal, it is also present in an epimastigote-specific pre-lysosomal organelle called 'reservosome'. In addition, some plasma membrane-bound isoforms [26] and cruzipain forms released into the medium [27] have been reported. The *T. cruzi* enzyme consists of a catalytic domain with high homology to cathepsins S and L and a particular C-terminal domain (C-T) which is absent in all other CPs of the C1 families described so far [28]. The enzyme is an immunodominant antigen in human chronic Chagas disease and seems to be important in the host-parasite relationship, it was associated with virulence [29], the interaction between plasma membrane-bound isoforms with alpha-macroglobulins was reported [30] and the humoral immune response to cruzipain appeared to be related with the severity of chronic Chagas disease [31]. Since membrane bound isoforms of cruzipain were detected, and sialylation is a surface reaction in *T. cruzi*, it was interesting to identify the presence of sialic acid in the C-terminal domain of cruzipain. In addition, *N*-acetyl-D-glucosamine in O-glycosidic linkages has also been determined. These findings might contribute to elucidate the migratory route followed by cruzipain [32]. Recently, we have reported the presence of sulfated structures in this glycoprotein [33]. Thus, studies to show if sulfate-bearing glycoproteins in Trypanosomatids are antigenic for humoral and cellular immune responses are being performed in our laboratory¹.

The addition of fluoromethyl ketone as cysteine proteinase inhibitor to infected mammalian cells showed that the enzyme (Cz) is essential for replication of the intracellular parasite and the differential susceptibility of parasite versus host cysteine proteases to these inhibitors suggested that *T. cruzi* major cysteine protease might represent a potential lead target for new chemotherapy of Chagas disease [34]. However, the possibility that some other, minor and highly specific, CPs may be involved in the inhibition of the parasite life cycle, should not be discarded. Recently, we have reported a novel CP present during *T. cruzi* metacyclogenesis [27]. In addition, the presence of a group of atypical cruzipain molecules which do not bind to ConA-Sepharose columns (NACrI), that represent a minor subclass with a different oligosaccharide pattern and different preference of chromogenic substrates, was also studied [35].

The advances in the study of the structure and specificity of cruzipain, including the obtention of the crystal structure bound to various inhibitors [36,37], favoured the development of new and more specific inhibitors. However, not only the presence of minor CPs but also atypical responses to inhibitors of other classes of proteinases should not be discarded. In fact, it was described that the serine proteinase oligopeptidase B was strongly inhibited by the CP inhibitor Z-Phe-Arg-fluoromethylketone [38] in a similar way to the

atypical serine peptidase oligopeptidase B from *Trypanosoma brucei* [39].

CPs inhibitors (CPIs) Studies were performed with synthetic peptidyl and non peptidyl inhibitors. Among peptidic compounds, the following groups of irreversible or reversible inhibitors can be mentioned (Table 1):

I₁-Irreversible Peptidic Inhibitors

a- Peptidyl diazomethane inhibitors. The interaction between Cz and biotin-labelled peptidyl diazomethane inhibitors showed a strong reaction when the inhibitor included a spacer arm containing part of the sequence of the known proteic inhibitor cystatin at difference with the mammalian counterparts, probably due to differences in the topologies of the binding site [40].

b- Peptidyl ketone based inhibitors. The design and synthesis of a variety of peptidyl fluoromethylketones, potent irreversible inhibitors of Cz, revealed that dipeptidyl alpha', beta'-epoxy ketones resulted more effective inhibitors of Cz than E-64c. In addition, D-Phe- and D-Tyr containing epoxysuccinate derivatives from the peptidyl-epoxysuccinate E-64, selective irreversible inhibitor of CP obtained by substituting the L-Leu residue of this compound, showed to be potent irreversible inhibitors of Cz but they were little effective against *T. cruzi* in cell cultures [41].

c- Peptidyl sulphone inhibitors. The potential toxicity associated to the use of the known irreversible inhibitors led to the screening of compounds including vinyl sulphones. The first report of trypanocidal activity involved to these compounds. It was demonstrated by *in vivo* assays that vinyl sulphone derivatized dipeptides were able to effectively rescue the mice from an acute lethal inoculation of *T. cruzi* resulting in the complete cure of the disease [42]. In particular, the vinyl sulphones morpholinourea-FhF-vinyl sulphone phenyl (MFhFVSPH) and morpholinourea-FhF-fluoromethylketone arrested growth of the epimastigotes and caused parasite death, probably due to accumulation of the enzyme in the Golgi [43]. The fact that these compounds inhibit Cz allowed to identify it as a promising therapeutic target in the treatment of Chagas disease [44]. Besides, a second generation of new potent N-alkoxyvinylsulfonamide inhibitors of Cz has been developed. One of them, named inhibitor 13 resulted to be highly effective against *T. cruzi* trypomastigotes in a tissue culture assay [45]. In addition, the novel dipeptidyl allyl sulphones were determined to be more potent than the dipeptidyl vinyl sulfones [46].

I₂-Reversible Peptidic Inhibitors

d-Bis-arylacylhydrazides, aryl ureas. The structure activity relationship (SAR) based design has evolved focusing on reversible compounds, most of which rely particularly on covalent attachment to the enzyme thiol group, to minimize the potential toxicity associated to the use of irreversible inhibitors. Some reversible inhibitors, have been designed based on the known structure of the active site of Cz, and synthesized including a family of bis-arylacylhydrazides [47] and some aryl ureas [48] as new class of CPIs.

e- Ketone based inhibitors. Among potent ketone based peptides, some of them reversible against Cz by formation of

¹Arnaiz MR, Acosta DM, Esteva MI, Torres S, Laucella SA, Couto AS, Duschak V.G. Importance of sulfated oligosaccharides in the antigenicity of cruzipain, the major cysteine proteinase of *Trypanosoma cruzi*. Kinetoplastid Diseases, Dakar, Senegal, Africa (2006).

Table 1. Cysteine Proteinase Inhibitors (CPIs). Representative Compounds

Type of inhibitor ^a		
I-Peptide-based		
I₁-Irreversible		
a- Peptidyl diazomethane	b- Peptidyl ketone based	c- Peptidyl sulphone
[40]	[41]	[42-46]
I₂- Reversible		
d- Bis-arylacylhydrazides.	e- Ketone based (cyclic structures)	
[47, 48]	[37, 49-59]	
f- Azepanone based	g- Nitrile based	
[60-62]	[63-67]	
II- Non peptidic based CPIs		
Thiosemicarbazone		
	[68-78]	

^a numbers under chemical structures correspond to the references in the text.

hemithioacetal complexes with CPs, inhibiting the enzyme in the nM range, have been developed by using solid-phase parallel synthesis [49]. Crystal structures of these reversible ketone-based inhibitors of Cz were studied [37]. Choe and co-workers synthesized a novel series of alpha-ketoamide-,

alpha-ketoacid-, alpha-ketoester-, and aldehyde-based inhibitors of Cz. Some of them displayed picomolar potency in *in vitro* assays and three inhibitors representing different alpha-keto-based inhibitor scaffolds demonstrated anti-trypansomal activity in cell culture [50].

A search for patents showed that scientist from Glaxo Smith Kline Corp designed a series of cyclic ketone compounds as protease inhibitors which form a hemithioacetal with the cys 25 residue and retain reliable oral bioavailability and improved pharmacokinetics. However, these compounds have not been tested against Cz [51]. In order to address the epimerization problem in the ketone based inhibitors, Medivir UK Ltd, Genzyme Corp have reported the synthesis of a series of substituted amides and 2 acylamide-bicyclic ketone derivatives as inhibitors of CPs and its potential use in infectious diseases including Chagas disease. In the first patent dealing with substituted amides [52], a tetrahydropyran-3-one derivative was used as cathepsin S inhibitor but no biological data were presented. Similarly, Incenta have also designed a series of peptide mimics 2-acylamino bicyclic ketone derivatives including tetrahydrofuran-3-one derivatives which claimed to be more potent inhibitors of Cz than those of the previous series mentioned. In addition, Amura Therapeutics Ltd. also patented inhibitors of Cz and other cysteine proteases [53-56]. Similarly, Amura disclosed a series of pyrrole compounds, with activity on Cz and also cathepsins K, S and L, useful for the *in vivo* therapeutic treatment of diseases in which participation of a cysteine protease is implicated [57] and other peptide based CPIs, claimed by Corvas International Inc as useful antiparasitic agents, were tested as effective against Cz (IC₅₀ values lower than 50 nM), but no specific biological data are available [58]. Recently, Amura Therapeutics Ltd. have patented some amide molecules that inhibit Cz more effectively than they inhibit mammalian CPs, such as bovine cathepsin S, human cathepsins L and K [59].

f- Azepanone based inhibitors. In their search for cathepsin K inhibitors, Smith Kline Beecham Corp published several patents describing the synthesis and use of peptidomimetics based on azepine or thiazepane [60,61]. These compounds were tested as cathepsin K inhibitors and claimed to be useful against different parasitic diseases including Trypanosomiasis. However, only two patents reported biological data [60-62] and only the latter [62] disclosed the inhibition by 4-aminoazepan-3 one derivatives of seven parasitic proteases including Cz in the analysis. 43 out of about 222 compounds tested, showed Ki values lower than 5 nM against Cz. The most potent CPIs against Cz were the 1-(pyridin 2-ylsulfonyl) azepan-3 one derivatives [62].

g- Nitrile based inhibitors. Novartis has patented a series of novel peptidic heteroaryl nitrile derivatives as therapeutic agents [63] for the treatment of osteoporosis and several parasitic diseases, one of them with Ki value of about 50 nM for human cathepsin K. The patent assessed that the compound would be useful in the prevention and treatment of several parasitic diseases including Chagas disease. The Combio Company [64] has recently disclosed a series of novel alpha-amino-carbonitrile-derived inhibitors of human dipeptidyl peptidase and cathepsin B, H and L, claiming that can be used for Chagas disease. However, data with regard to their efficacy against parasitic diseases have not been reported. Boehringer Ingelheim Pharmaceuticals, Inc. disclosed 404 novel nitrile compounds claiming they were useful as reversible inhibitors for treatment of diseases mediated by CPs, particularly cathepsin K and S and a variety of pathological conditions exacerbated by these

proteases but no detailed experimental results were shown [65,66]. Ten compounds were assayed against Cz with Ki values ranging from 0.09 to 20 μM [67]. Although the specific claim of these patents, biological data for these nitrile based inhibitors (including some of them non-peptidic) regarding their efficacy on parasitic diseases are also absent.

II-Non peptidic inhibitors. Structure activity relationships (SAR) for non peptidic inhibitors of Cz based on different scaffolds were reported, including the following:

a-Thiosemicarbazone: among non-peptidic inhibitors those based on the thiosemicarbazone lead were reported as active Cz inhibitor at the nM range; many of them, of small size and low cost showed trypanocidal activity against intracellular amastigotes *in vivo* making them attractive candidates for drug development [68-71].

However, the emergence of parasite populations resistant to some of these inhibitors was reported. A phenotypically stable cell line of *T. cruzi* (R-Dm28) displayed increased resistance to the irreversible cysteine proteinase inhibitor Z-(SBz)Cys-Phe-CHN 2, which preferentially inactivates cathepsin L-like enzymes suggesting that this fact would represent a possible limitation of CPs as targets for chemotherapy [72,73]. On the other hand, the ChemBridge database was used for virtual screening to identify novel druglike non-peptidic inhibitors of parasitic cysteine proteases. Several non-peptidic inhibitor compounds were able to avoid protease hydrolysis in living systems, retaining *in vivo* activity as well as selectivity [74]. Recently, it was reported that the treatment of dogs with K177, inhibitor of Cz, abrogated myocardial damage by *T. cruzi*, as documented by histopathological lesion scores and serum troponin I levels [75]. The design of lead optimization libraries of thiosemicarbazone inhibitors was performed. The screening of some of these compounds on different CPs and on their respective parasites showed that they were able to kill several species of protozoan parasites through the inhibition of CPs as well as other novel targets [76]. Among the active CPIs tested, several inhibited proliferation of cultures of *T. brucei* potently but only a modest activity was observed in inhibition of *T. cruzi* growth [71]. Finally, a novel series of thiosemicarbazone and aminoacyl thiazolidones derivatives were also synthesized. Some of them were able to inhibit *T. cruzi* growth in non-cytotoxic concentrations to mammalian cells [77]. Recently, Regents of the University of California presented a patent related to thiosemicarbazone and semicarbazone inhibitors of CPs and methods of using such compounds to prevent and treat protozoan infections such as trypanosomiasis, malaria and leishmaniasis [78].

Minor CPs. The presence of cathepsin B-like CPs in *T. cruzi* was demonstrated but it is not still known neither how many different enzymes of this type are present nor their possible functions. Among them, a 30 kDa cathepsin B-like enzyme has been described [72,79]. Recently, the presence of a novel CP, TcCPmet, secreted by metacyclic trypomastigotes was reported. This novel CP presented a different elution pattern on ConA-Sepharose than Cz and was not recognized by anti-cruzipain serum. In addition, TcCPmet was able to hydrolyse the same chromogenic peptides as Cz at optimal alkaline pH values, although with a different order

of effectiveness. The results obtained strongly suggest a different nature between TcCPmet and Cz [27]. Although there is no data still available, these minor CPs may constitute new targets for the development of novel inhibitors.

In the near future, an effective chemotherapy of the American Trypanosomiasis based on CPs seems to be possible, regarding the results obtained so far with this type of drugs on animal models.

1B-Serine peptidases (SPs). Oligopeptidase B is a member of the prolyl oligopeptidase family involved in Ca²⁺ signaling during mammalian cell invasion [38,80]. A secreted prolyl endopeptidase (Tc80), with collagenolytic activity, was also purified and partially characterized from *T. cruzi* [81]. The inhibition pattern and its ability to hydrolyze peptide bonds at the carboxyl side of Pro residues suggested that the enzyme is a prolyl endopeptidase also belonging to the S9ASP family, but distinct from the oligopeptidase B. Selective inhibitors of the enzyme have been synthesized [82,83], with Ki values in a low nM range, and shown to be able to block the entry of the parasite into the host cells [84]. This SP looks, therefore, as a new very promising target for the development of new drugs against Chagas disease. Other putative serine proteinases have also been described [21].

Serine Proteinase Inhibitors. Synthetic prolylprolyl-isoxazoles and prolylprolylisoxazolines, potent inhibitors of human and trypanosomal prolyl oligopeptidase (POP), were shown to inhibit *T. cruzi* and *T. brucei* *in vitro* systems with ED50 in the lower μM range [85]. Novel inhibitors were assayed with rPOP Tc80, and the most efficient ones presented values of inhibition coefficient Ki lower than 1.52 nM. Infective parasites treated with these specific POP Tc80 inhibitors attached to the surface of mammalian host cells, but were incapable of infecting them [86].

1C-Metalloproteinases. Enzymes with homology to the gp63 of *Leishmania spp.* are also present in *T. cruzi* [21, 87]. Studies related with metalloproteinases inhibitors have not been reported in *T. cruzi* yet.

1D-Threonine Proteinases (Proteasome). In protozoan parasites, the proteasome is involved in cell differentiation and replication, and could therefore be a promising therapeutic target [88]. In *T. cruzi*, the presence of proteasome with properties similar to those of other eukaryotes was reported [89] and its inhibition by lactacystin blocks some differentiation steps in the life cycle of the parasite. However, clasto-lactacystin, an inactive analogue of lactacystin, and cell-permeant peptide aldehyde inhibitors of *T. cruzi* CPs did not show effect. The use of proteasome inhibitors determined the accumulation of ubiquitinated proteins and showed that cytoskeletal proteins associated with the flagellum are targets of the ubiquitin-proteasome pathway [90]. Although several parasite proteasome subunits have been cloned and sequenced showing homology to the corresponding subunits from other eukaryotic proteasomes [21], at difference with other kinetoplastida no studies about proteasome specific inhibitors are available for *T. cruzi* yet.

Nereus Pharmaceuticals, Inc. [91] presented a patent claiming the use of analogue compounds of salinosporamide A, a bacterial marine natural product, as proteasome inhibitor for the treatment of neoplasm, inflammation and

microbial infection. This heterocyclic compound was able to inhibit proteasome activity with an IC50 value of 11.8 nM. However, despite the well known potential of proteasome inhibitors against trypanosomes *in vitro* [21,92], no biological data of antitrypanosomal activity was disclosed.

2-ERGOSTEROL BIOSYNTHESIS PATHWAY

The sterols are essential structural components of cellular membranes serving as precursors of steroid hormones and vitamin D in mammals and modulators of growth and development in unicellular organisms [93,94]. Trypanosomatids contain sterols in plasma, inner mitochondrial and glycosomal membranes [95]. Depletion of sterol end products causes trypanosomal cell death as a result of membrane disruption, especially in the exponentially dividing stages of the parasite [96,97]. The finding that the main sterol in *T. cruzi* metabolism is ergosterol instead of cholesterol unlike human hosts triggered an intensive search for the identification and potential effect of inhibitors of ergosterol biosynthesis (EBIs) [98]. The singularity of this pathway in kinetoplastid parasites, the strict requirement of *T. cruzi* for specific endogenous sterols for cell viability and growth, similarly to fungi and yeast, and the susceptibility to sterol biosynthesis inhibitors (EBI) *in vitro* [99-102] and *in vivo* [99,101-104] have shown sterol biosynthesis pathway as a promising target for drug therapy against *T. cruzi* [105].

Among potential drug target enzymes of sterol biosynthesis for treatment of Chagas disease can be mentioned the following enzymes from this metabolic pathway (Fig. 1):

2A-Sterol C14 -demethylase. Sterol C14 -demethylases are essential enzymes in sterol biosynthesis in eukaryotes and drug targets in antifungal therapy. These enzymes catalyze oxidative removal of the C14 -methyl group from postsqualene sterol precursors (Fig. 1). They are found in Trypanosomatids. It was reported that even with only 22-33 % aminoacid identity across the biological kingdoms the orthologous enzymes from bacteria to mammals preserve strict catalytic regio- and stereospecificity and have a very limited range of substrates [106]. The sterol C14 -demethylase from *T. cruzi* (TcCYP51) was found to be catalytically closely related to animal/fungi-like CYP51 and prefers C4-dimethylsterols. By contrast, the ortholog from *T. brucei*, similarly to plant CYP51 requires C4-monomethylated sterol substrates. The substrate preferences of these enzymes imply differences in the postsqualene portion of sterol biosynthesis in different trypanosomes. The phyla specific residue can be used to predict preferred substrates of new CYP51 sequences and subsequently for the development of new artificial substrate analogues, which might serve as highly specific inhibitors [107]. Recent results describe the effects of sterol biosynthesis inhibitors (simvastatin, zaragasic acid, terbinafine, ketoconazole, and others) on the regulation of different sterol biosynthesis genes and their protein products, demonstrating that *T. cruzi* can specifically regulate sterol C14-demethylase gene expression [108].

I-Azole inhibitors. The azole drugs (ketoconazole, itraconazole, Table 2), target the lanosterol C14- -demethylase enzyme in the ergosterol biosynthesis pathway causing the accumulation of 14 -methylsterols and decrea-

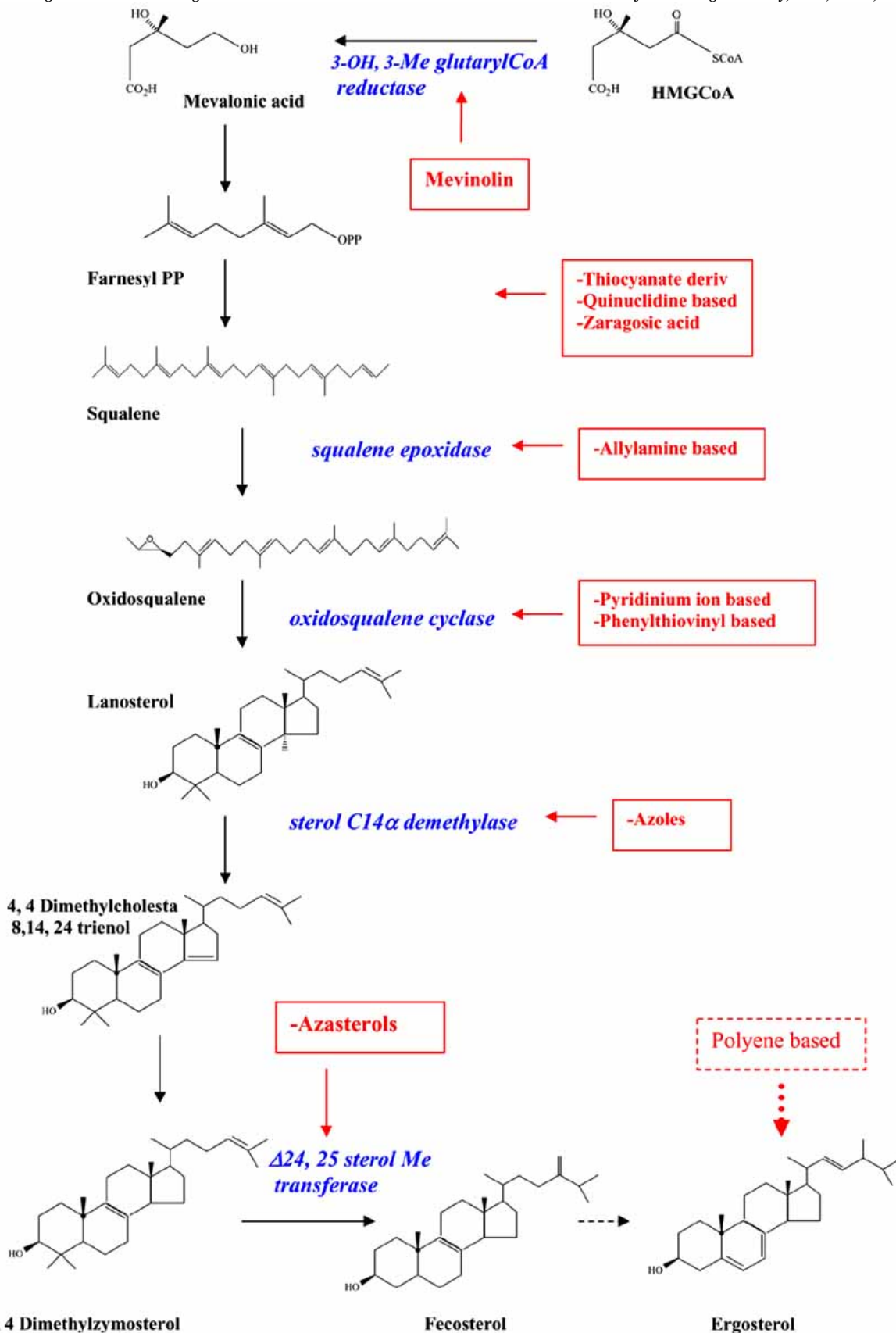
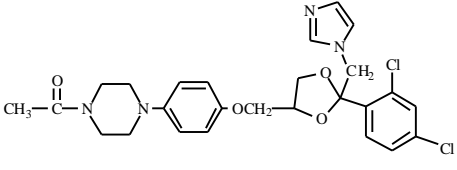
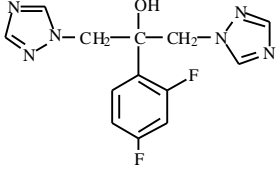
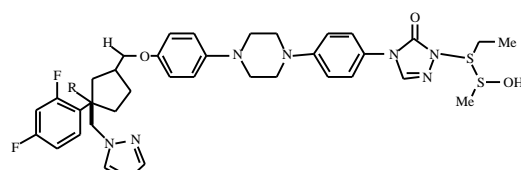
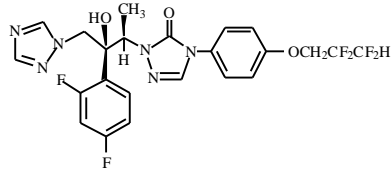
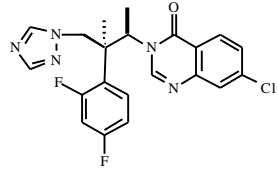
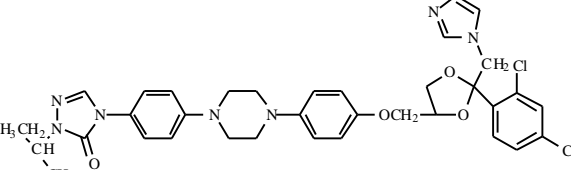
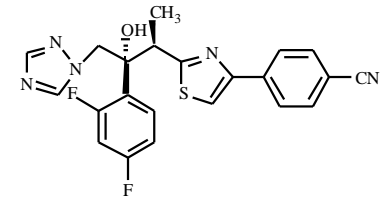
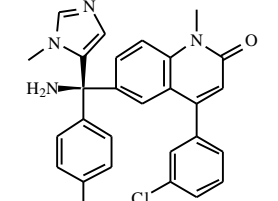
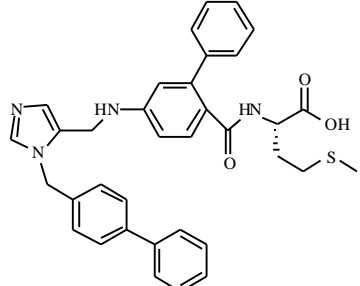
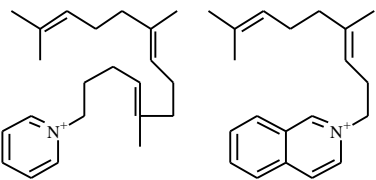
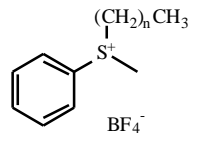
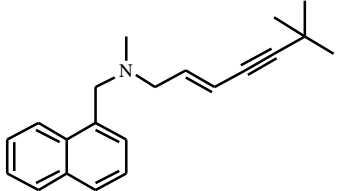
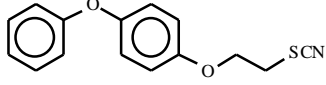
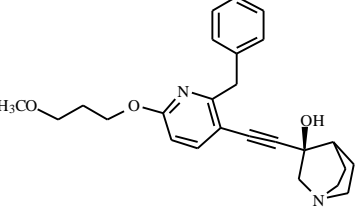
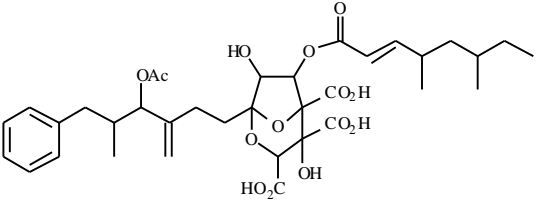
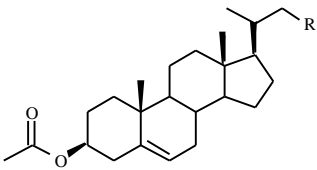
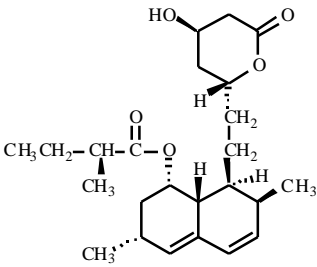
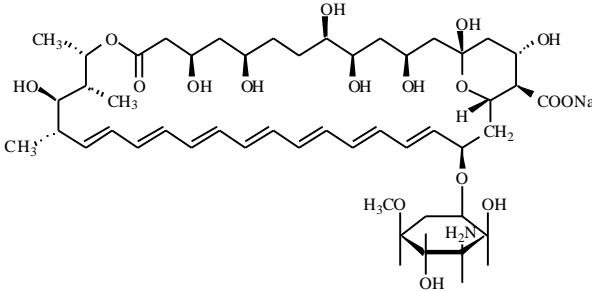


Fig. (1). Enzymes of the Ergosterol biosynthetic pathway as drug targets. The scheme shows the chemical structures and names of the major intermediates of the ergosterol biosynthesis. Enzymes are shown in blue italics and drug classes that act on them are shown in red squares.

Table 2. Inhibitors of Ergosterol Metabolism

1-Azole inhibitors		
-sterol C14- demethylase [106-115, 133-135]		
<p>Ketoconazole</p> 	<p>Fluconazole</p> 	<p>Posaconazole</p> 
<p>TAK-187</p> 	<p>Albaconazole</p> 	<p>Itraconazole</p> 
<p>Ravuconazole</p> 	<p>Tipifarnib</p> 	<p>Peptidomimetic Imidazol compounds</p> 
2-Non-Azole Inhibitors		
-oxidosqualene cyclase [116-120]		- Squalene epoxidase [121, 122]
<p>Pyridinium ion based</p> 	<p>-phenylthio based</p> 	<p>-allylamine based</p> 
- squalene synthase [123-129]		
<p>-thiocyanate derivatives</p> 	<p>- quinuclidine based</p> 	<p>zaragasic acid</p> 

(Table 2) Contd....

24-SMT [130]	HMGCoA reductase [131]	No enzyme target assigned [132]
- azasterol	- mevinoxin	-polyene based
		

sing production of ergosterol. It was reported that the triazole derivatives, inhibitors of fungal P-450-dependant C14-sterol demethylase, posaconazole, (SCH56592, Schering-Plough Research Institute), D0870 (Astra-Zeneca Pharmaceuticals), and TAK-187 (Takeda Chemical Company) are capable of inducing parasitological cure in murine models of both acute and chronic Chagas disease with no toxic side effects to the hosts [99,103,104,109].

Compounds such as itraconazole and fluconazole markedly reduced or prevented chronic phase symptoms [110]. D0870, D (+) isomer of fluconazole, displayed a striking inhibitory activity *in vivo*, both in acute and chronic models, leading to unprecedented percentages of parasitological cure [103]. Albaconazole (UR-9825; Uriach & Company, Barcelona, Spain) resulted one of the most potent EBIs tested against this organism [100,111]. Among triazole derivatives of probed antifungal activity, ketoconazole, failed to eradicate *T. cruzi* from experimentally infected animals or human patients [98,101] whereas ravuconazole resulted one of the most advanced candidates for clinical trials for a new, rationally developed trypanocidal activity *in vivo* and *in vitro* [109]. However, the use of such compounds as chemotherapeutic agents was questioned due to the cross resistance between ketoconazole, miconazole and itraconazole revealed in *in vitro* experiments, in addition to the induction of resistance of *T. cruzi* to some azoles [112]. Interestingly, a series of peptidomimetic disubstituted imidazoles resulted highly effective against *T. cruzi*. The compounds were administered orally to mice with acute *T. cruzi* infection and caused significant decrease in parasitemia leading to 100% survival [113]. Moreover, Tipifarnib (R115777), an inhibitor of human protein farnesyltransferase (PFT), is shown to be a highly potent inhibitor of *T. cruzi* growth (ED₅₀: 4 nM). Surprisingly, this is due to the inhibition of CYP51, the cytochrome P450 sterol C14-demethylase [114]. Recently, the antiarrhythmic compound amiodarone, frequently prescribed for the symptomatic treatment of Chagas disease patients, was reported to have direct activity against *T. cruzi*, both *in vitro* and *in vivo*, and that it acts synergistically with posaconazole [115]. These results open up the possibility of novel combination therapy approaches for the treatment of Chagas disease using currently approved drugs.

2B-Oxidosqualene cyclase or lanosterol synthase (OSC) is a key enzyme in sterol biosynthesis, which converts 2, 3-oxidosqualene to the tetracyclic product, lanosterol (Fig. 1). The synthesis of lanosterol is an essential step in the production of mature sterols. In yeast and higher eukaryotes (including humans), OSC directly 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. The fact that OSCs from Trypanosomes and animals use different catalytic motifs could lead to the development of specific inhibitors for this enzyme [116].

II-Non azole inhibitors. Among this type of inhibitors (Table 2), the following can be mentioned:

-Pyridinium ion based inhibitors. N-Alkyl- and N-prenylpyridinium ions showed to be potent and specific inhibitors on *C. albicans* oxidosqualene-lanosterol cyclase and to exhibit antifungal activity [117]. Besides, it was reported that these compounds have potent activities against *T. cruzi* and inhibit sterol biosynthesis in these organisms. The antitrypanosomal activities from specific non-azole inhibitors including the lead compound N-(4E, 8E)-5, 9, 13-trimethyl-4, 8, 12-tetradecatrien-1-ylpyridinium and a series of compounds designed to inhibit OSC, were tested against mammalian stages and 12 of them resulted highly active in the nM range against trypanosomes [118].

-Phenylthiovinyl derivatives. By using a recombinant *T. cruzi* OSC expressed in yeast, 19 inhibitors: aza, methylidene, vinyl sulfide, and conjugated vinyl sulfide derivatives of oxidosqualene and squalene, were tested. Many inhibitors of control OSC showed comparable IC₅₀ for *T. cruzi* OSC, but some phenylthiovinyl derivatives showed to be 10-100 times more effective on the *T. cruzi* enzyme than on the control enzymes [119].

Buckner *et al.* presented a patent claiming that OSC inhibitors could be used to treat fungal, bacterial and parasite infections including trypanosomatids based on the drug induced blockade of sterol biosynthesis (University of Utah Research Foundation) [120]. Five promising compounds were described with *in vitro* growth inhibitory effects against *T. cruzi* and *L. mexicana* with IC₅₀ values in the nM range

and antiparasitic activity confirmed in a murine model of Chagas disease.

2C-squalene epoxidase. This enzyme catalyzes the conversion of squalene to (3S) 2, 3-oxidosqualene (Fig. 1). It was described in vertebrates as a nonmetallic, flavoprotein monooxygenase and is also considered as potential target for the design of therapeutic agents to be used against different pathogen organisms [121].

-Allylamine based inhibitors. It is known that among antifungal drugs, the allylamine terbinafine (Table 2) inhibits squalene epoxidase in the sterol biosynthesis pathway and was shown to be synergistic with ketoconazole against cultures of *T. cruzi* [122].

2D-squalene synthase (SQS) catalyzes a head-to-head reductive dimerization of two molecules of farnesyl pyrophosphate (FPP) in a two-step reaction to form squalene (Fig. 1), the first step in sterol biosynthesis. This enzyme is currently under intense study as a possible target for cholesterol-lowering agents and has been recently shown as a promising target for antiparasitic chemotherapy [123,124].

-Thiocyanate derivatives. The 4-phenoxyphenoxyethyl thiocyanate (Table 2) resulted to be an effective and potent agent against epimastigote proliferation and produced the accumulation of low molecular weight metabolites from mevalonate to squalene [125]. Searching for new chemotherapeutic and chemoprophylactic agents, some aryloxyethyl thiocyanate derivatives, structurally related to 4-phenoxyphenoxyethyl thiocyanate were designed, synthesized, and evaluated. Some of these drugs proved to be effective growth inhibitors of *T. cruzi* with values comparable with those presented by ketoconazole, others proved to be potent inhibitors of epimastigotes multiplication, and one of them was reported to be an effective antichagasic agent with perspectives as a lead drug for further *in vivo* studies [126]. The growth inhibition of *T. cruzi* epimastigotes induced by 4-phenoxyphenoxyethyl thiocyanate (WC-9) was associated with a reduction in the content of the parasite's endogenous sterols due to a specific blockade of their *de novo* synthesis at the level of squalene synthase [127].

-Quinuclidine based inhibitors. Among the synthesized quinuclidine inhibitors (Table 2), 3-(biphenyl-4-yl)-3-hydroxyquinuclidine (BPQ-OH) showed to be a powerful non-competitive inhibitor of *T. cruzi* SQS, with a K_i value in the nM range. This compound was able to eradicate intracellular *T. cruzi* amastigotes from culture Vero cells with no side effects on host cells [96,123]. In addition, the compounds E5700 and ER-119884 were found to be potent noncompetitive or mixed-type inhibitors of *T. cruzi* SQS with K_i values in the low nanomolar to subnanomolar range. *In vivo* studies indicated that E5700 by oral administration is capable of providing complete protection against acute Chagas disease [124]. *In vitro* and *in vivo* activities of these two novel quinuclidine SQS inhibitors are currently under development by Eisai Company Ltd. (Ibaraki, Japan) as cholesterol- and triglyceride-lowering agents in humans [128]. Recently, some biphenylquinuclidine derivatives have been evaluated as inhibitors of SQS in order to explore their potential in the treatment of the parasitic diseases such as leishmaniasis and Chagas disease. The compounds were

screened against a recombinant leishmanial SQS, against *L. mexicana* promastigotes, and *T. cruzi* intracellular amastigotes. Compounds that inhibited the enzyme also reduced the levels of steroids and caused growth inhibition of *L. mexicana* promastigotes [129].

2E-Delta 24(25)-methyltransferase (24-SMT). This enzyme is essential for the biosynthesis of ergosterol, but not required for the biosynthesis of cholesterol (Fig. 1). A series of potential transition state analogues of 24-SMT were designed, synthesized and evaluated against recombinant *L. major* 24-SMT and the parasites *L. donovani* and *T. cruzi* *in vitro*. Some of the compounds (Table 2) showed inhibition of the recombinant *L. major* 24-SMT and inhibited parasite growth. Others, although they did not show enzyme inhibition, presented anti-parasitic activity against *T. cruzi* [130].

2F-3-hydroxy-3-methyl-glutaryl-coenzymeA (HMGCoA) reductase: The antiproliferative effects of mevinolin (Table 2), an inhibitor of HMGCoA, were tested on *T. cruzi* both *in vitro* and *in vivo* (Fig. 1). In addition, its ability to potentiate the action of specific EBIs, such as ketoconazole and terbinafine was evaluated. A synergic action against the proliferative stages of *T. cruzi* of combined EBIs suggested that mevinolin combined with azoles, such as ketoconazole, could be used in the treatment of human Chagas disease [131].

Finally, among other antifungal drugs, different Amphotericin B-lipid formulations which associate with ergosterol to disrupt the integrity of the cell membrane were also tested in *in vitro* and *in vivo* assays against experimental *T. cruzi* infections showing potent anti-*T. cruzi* activities [132].

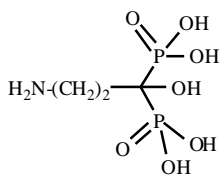
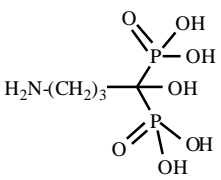
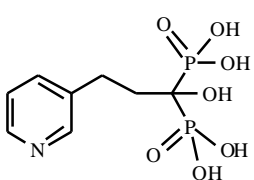
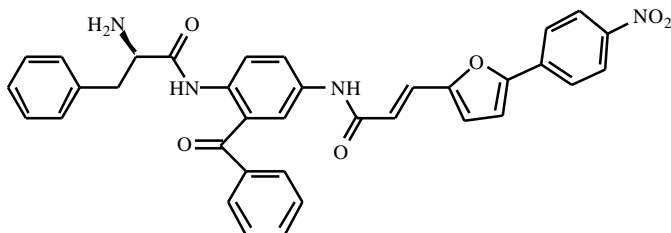
Despite the enzymes of this biosynthetic pathway are showing growing indications, this is not reflected in the number of disclosed patents. Only the synthesis of 5-amino-1-benzyl-imidazole derivatives, inhibitors of the C-14 -demethylase, with antibacterial, antifungal and antitrypanosomal activity was presented by scientists of Yale University. The compounds were tested on intracellular amastigotes, they were non toxic to the cells and showed a remarkable IC_{50} from μM to pM values. The authors analyzed *in vivo* assays in mice and suggested that a phenylbenzylimidazole moiety is responsible for the inhibition of the enzyme and consequent antiparasitic activity. However, no data of enzymatic inhibition is presented [133-135].

3-BIOSYNTHESIS OF POLYISOPRENOIDS

3A- Farnesylpyrophosphate synthase (FPPS). In pathogenic protozoa, farnesylpyrophosphate synthase (FPPS) is the enzyme responsible for the formation of farnesylpyrophosphate that marks the branching point in the synthesis of a variety of sterols and other essential isoprenoids. In *T. cruzi* the gene TcFPPS that codifies for this enzyme was cloned, sequenced and expressed.

-Farnesylpyrophosphate synthase inhibitors. The above mentioned pathways can be blocked by biphosphonates (Table 3), metabolically inert inorganic PP analogues that inhibit FPPS [136]. The recombinant enzyme was inhibited by the nitrogen-containing bisphosphonates risedronate and pamidronate causing the latter a decrease of parasitemia in infected mice and inhibiting the *in vitro*

Table 3. Isoprenoid Metabolism Inhibitors

-farnesyl PP synthase [136-144]		
Biphosphonates		
Pamidronate 	Alendronate 	Risedronate 
-farnesyl transferase [145-150]		
Benzophenone based 		

intracellular replication of amastigotes [98]. By contrast, the non-nitrogen-containing bisphosphonate etidronate did not affect parasite growth [136]. Risedronate inhibited the proliferation of epimastigotes and sterol biosynthesis at a pre-squalene level as shown by sterols analysis in treated parasites, associating these results with the inhibition of FPPS, turning out as a promising lead compound for the development of new drugs against *T. cruzi* [137-139].

The treatment of human bone resorption disorders currently involves bisphosphonate-containing drugs which due to their potential innocuousness are good candidates to control tropical diseases. Some fatty acids-derived bisphosphonate compounds resulted potent inhibitors of the proliferation of *T. cruzi* intracellular amastigotes at low μM level, but none of them was effective against epimastigotes [140,141]. The drug accumulation in parasite acidocalcisomes seems to be responsible for the selective action displayed by bisphosphonate compounds against *T. cruzi* [142]. FPPS condenses the diphosphates of C5 alcohols to form C10 and C15 diphosphates (geranyl and farnesyl). The analysis of the structures of the *T. cruzi* FPPS alone and in complexes with substrates and inhibitors revealed that after binding the enzyme undergoes conformational changes facilitating the enzyme to bind a bisphosphonate inhibitor. Structural studies as well as molecular dynamics may lead to the design of new, more potent anti-trypanosomal bisphosphonates [143,144].

3B- Protein Farnesyltransferase (PFT). This enzyme catalyzes the transfer of a farnesyl residue from farnesylpyrophosphate to the thiol of a cysteine side chain of proteins which carry at the C-terminus the so called CAAX-sequence. The attachment of polyisoprenoids to specific proteins, protein prenylation, is involved in signal transduction and anchorage of protein to cell membranes.

Prenylation was demonstrated in Trypanosomatids, [145,146] and PFT of both *T. cruzi* and *T. brucei* were cloned, finding differences with its mammalian counterpart. These facts validated the use of PFT as trypanocidal chemotherapeutic target [146].

-Farnesyl transferase inhibitors. PFT inhibitors are known as potent antitumoral drugs in experimental animals, some of them assayed in treatment of human cancer [147]. The development of PFT inhibitors is clearly directed towards the so-called non-thiol farnesyltransferase inhibitors (Table 3) because of adverse drug effects connected to free thiols. Recently, several farnesyltransferase non-thiol inhibitors based on the benzophenone scaffold have been assayed *in vitro* and *in vivo* with *T. cruzi*. The common structural feature of all inhibitors resulted to be an amino function which can be protonated. *R*-phenylalanine and *N*-propylpiperazinyl derivatives showed the best *in vitro* activity with IC50 values in the nM range. These inhibitors showed no cytotoxicity to cells. When tested *in vivo*, the survival rates of infected animals were 60 to 80 % at day 115 post infection [148].

The use of PFT inhibitors, such as the natural antibiotic manumycin A and other synthetic cyclic hexenone compounds, to treat parasitic diseases was patented by Mark Field from the Imperial College of Sciences, Technology and Medicine (UK) [149] but no description of the synthesis procedure was included. Schering Corp recently disclosed 21 PFT inhibitors based on piperazine or piperidine scaffold for the treatment of *T. brucei* infection [150]. The compounds were claimed to inhibit PFT in a μM range and *in vivo* inhibition of the parasite ranged between 0.2-10 μM . However, no patents specifically related with PFT inhibitors acting on *T. cruzi* have been reported yet.

4-THIOL-DEPENDENT REDOX METABOLISM

Biosynthesis of Trypanothione: Trypanosomatids present a unique thiol-dependant redox metabolism, which is based on trypanothione (a low molecular weight thiol-polyamineconjugate, N1, N8-bis(glutathionyl)spermidine T(SH)₂), exclusively found in parasitic protozoa of the order Kinetoplastida and specific enzymes including a trypanothione reductase (TR) in replacement of the ubiquitous glutathione reductase (GR) [151]. The sensitivity of trypanosomatids towards oxidative stress and the absence of trypanothione in the mammalian host validate the enzymes of the trypanothione metabolism as drug-target molecules.

Trypanothione reductase (TR) is a key enzyme of the parasite antioxidant defense, and is essential for all trypanosomatids studied so far. It is an NADPH-dependent flavoprotein that maintains trypanothione in its reduced form and able to be oxidized by trypanothione oxidase, leading to reduction of free radicals levels and contributing to the maintenance of an intracellular reducing environment. X ray crystallography studies solved the three-dimensional structure of the purified TR in free form, in complex with substrates and in the presence of inhibitors [152]. The differences on the substrate specificity found between TR and the mammalian counterpart determined that TR had been widely used as a target for rational drug design against trypanosomiasis [153].

Trypanothione reductase inhibitors. A great proportion of trypanocidal agents are involved in the trypanothione metabolism [154]. Among them, a lot of them are inhibitors of *T. cruzi* TR (Table 4).

I-Irreversible inhibitors

a-Subversive substrates or sabotage inhibitors are molecules that convert an antioxidative disulfide reductase into a prooxidative enzyme. Typical subversive substrates are reduced in single-electron steps to the respective radicals which then react with molecular oxygen to yield superoxide anion radicals, enhancing the effect of oxidative stress. Among the compounds capable to act as subversive substrates of TR and other flavoenzymes are nitrofurans and naphthoquinones [155,156]. These compounds can be reduced by a variety of cellular reductases triggering the production of oxygen radicals, followed by the consumption of thiol species. When the acting reductase is TR, the subversive process may take place avoiding the regeneration of T (SH)₂ [156]. On the basis of the redox properties, nitrofurans compounds, resulted moderate inhibitors of TR and GR and some of them, namely nifuroxime and nifuroxazide were no substrates for GR and proved to be better inhibitors of *T. cruzi* in culture as compared to nifurtimox. Among some promising nitrocompounds reported, Chinifur, a bactericidal nitrofurans derivative, is an inhibitor and subversive substrate of TR, but it interacts weakly with some structurally related antioxidant enzymes [157]. However, a series of nitroderivatives (nitrofurazones and nitrothienyl analogues), were not found to be significantly better inhibitors of *T. cruzi* *in vitro* growth [158].

Naphthoquinones group is composed of very reactive molecules capable to undergo redox cycling, present in all aerobic cells, which display multiple applications in medi-

cine. Some of them, menadione, plumbagin, and lapachol showed notable trypanocidal activities but interacted with TR as well as human GR [156]. With respect to parasite infections, some naphthoquinone derivatives, both synthetic and obtained from natural sources, have been assayed as trypanocidal agents [159-161]. With the aim of obtaining trypanocidal compounds with specificity for *T. cruzi* TR, a series of menadione, plumbagin, and juglone derivatives have been synthesized. The most potent derivatives contained two 1, 4-naphthoquinone moieties linked by a polyamine spacer. It was reported that the inhibition of TR alone is not sufficient for a significant trypanocidal activity but the combination of both inhibition of T(SH)₂ reduction and redox cycling would render the parasite more susceptible to the harmful effects of free radical species [156]. The trypanocidal activity of new synthesized naphthoimidazoles from beta-lapachone with an aromatic moiety linked to the imidazole ring using phenylic and heterocyclic aldehydes was assayed finding no correlation between biological activity and the structure of the phenylic series [162]. In addition, several oxyranes structurally related to -lapachone, nor- -lapachone, -lapachone, and 4-methoxy-1,2-naphthoquinone showed similar trypanocidal activity to -lapachone although less cytotoxicity than the corresponding naphthoquinones [163].

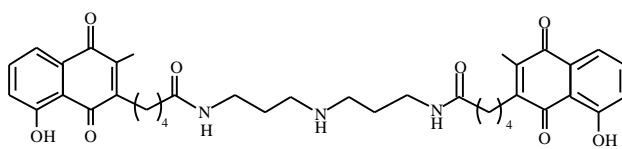
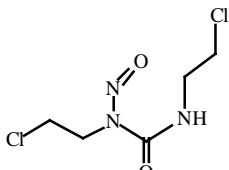
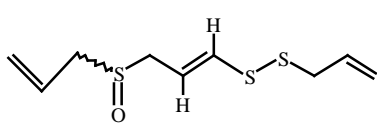
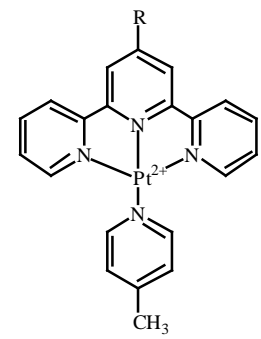
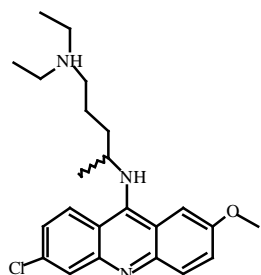
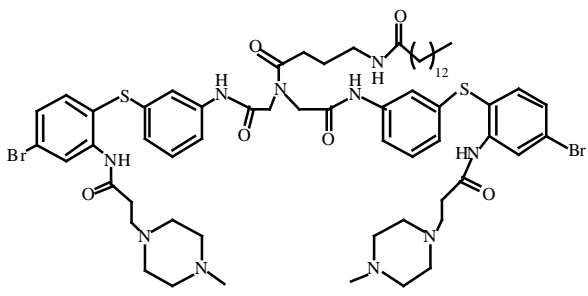
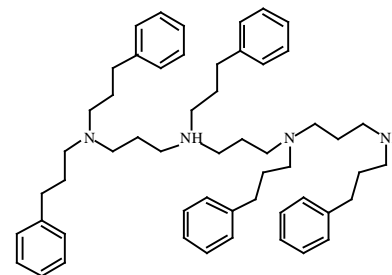
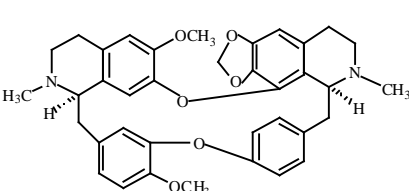
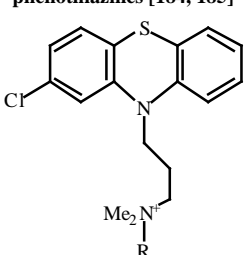
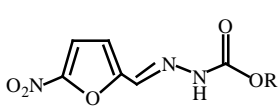
Finally, four new naphthofuranquinones were evaluated for trypanocidal activity in assays with *T. cruzi* trypomastigotes. The IC₅₀ values for these compounds were between 157 and 640 μM, while those for crystal violet were about 540 μM. The trypanocidal activity of the new naphthofuranquinones bearing redox properties reinforces a rational approach in the chemotherapy of Chagas disease [164].

b-Nitrosooureas The fact that substrate accumulation cannot overcome inhibition constitutes an advantage for using covalent inhibitors. Among them, the drug carmustine is an irreversible inhibitor of TR, however, it also inactivates human GR [165].

c-Ajoene ((E,Z)-4,5,9-Trithiadodeca-1,6,11-triene-9-oxide), the spontaneous degradation product of allicin, a major sulfur garlic-derived natural compound, is known for its antifungal, antiviral, antitrypanosomal, and antimalarial activity and is a covalent inhibitor and subversive substrate of both human GR and *T. cruzi* TR. A crystal structure of GR inhibited by (E)-ajoene revealed a mixed disulfide between the active site Cys58 and a specific moiety of ajoene. The interactions between the flavoenzymes and ajoene are expected to increase the oxidative stress of the respective cell. The antiparasitic and cytostatic actions of ajoene may at least in part be due to the multiple effects on key enzymes of the antioxidant thiol metabolism [166].

d-Organo-metallic complexes. Platinum II organo-metallic complexes extensively used in cancer therapy are also irreversible ligands of *T. cruzi* TR but not of human GR. They display trypanocidal activity both *in vivo* and *in vitro* assays [167,168]. It was also reported that complexation of known antiparasitic drugs such as ketoconazol with ruthenium II or III and rhodium II enhances the activity of the parental drugs overcoming primary and secondary drug resistance [169]. The evaluation of synthesized copper (II) and gold (I) clotrimazole and ketoconazole complexes

Table 4. Trypanothione Reductase Inhibitors. Representative Compounds

I-Irreversible Inhibitors		
a- Subversive substrates or sabotage inhibitors [155-164] 	b-Nitrosoureas [165] 	
c- Ajoene [166] 	d- Organo-metallic complexes [170-173] 	
II-Reversible Inhibitors		
a- Tricyclic compounds [174-177] 	b- Aminodiphenylsulfides [178, 179] 	c-Polyamine derivatives. [180-182] 
d- Bisbenzylisoquinoline alkaloids [183] 	e-Quaternary arylalkylammonium phenothiazines [184, 185] 	f- Nitrofuryl derivatives [186, 187] 

against *T. cruzi* growth exhibited significantly higher inhibitory activity than their respective parental compounds [170].

A patent from Isis Innovation Ltd claimed that some (2, 2', 6', 2''terpyridine) platinum II complexes resulted useful as antitumoral and antiprotozoal agents [171]. About 40 complexes were synthesized and characterized including

pyridine-2-thiolate-(4-chloro-2, 2', 6', 2''terpyridine) platinum (II), which inhibit the reduced form of the TR, are active against tumoral cell lines and on *T. cruzi* and other trypanosomatids.

Unsaturated Mannich bases irreversibly inactivated TR from *T. cruzi* and structural studies revealed a divinyl ketone

as the active compound responsible for the enzyme inactivation. It was proposed that the interaction of these compounds with both trypanothione and TR could account for their potent trypanocidal effect reported against *T. brucei* [172]. Sixteen novel palladium (II) complexes with bioactive nitrofurans-containing thiosemicarbazones as ligands were synthesized. Most complexes showed higher *in vitro* growth inhibition activity against *T. cruzi* than nifurtimox. The complexes showed strong DNA binding; however the main trypanocidal mechanism of action seems to be due to the production of oxidative stress as a result of their bioreduction and extensive redox cycling. Moreover, the complexes were found to be irreversible inhibitors of TR [173].

II-Reversible Inhibitors

a-Tricyclic compounds. The structure of tricyclic neuroleptic compounds showed to be a promising class of TR inhibitors [174]. Phenothiazines and related compounds are tricyclic drugs with different biological activities. These drugs also exert trypanocidal effects upon epimastigote and trypomastigote forms: anticalmodulin action (clomipramine); disruption of mitochondria (trifluoperazine and thioridazine); serious cell membrane disorganization (prometazine). Moreover, clomipramine and thioridazine were also effective in treatment of mice with experimental Chagas disease [175]. Clomipramine, a tricyclic antidepressant drug with anti-TR and anti-calmodulin effects, was used for treating mice infected with trypomastigotes. 70 % of the mice survived for more than 2 years demonstrating that clomipramine could be a promising trypanocidal agent for the treatment of Chagas disease [176].

Mepacrine, the acridine derivative that prevents the transmission of Chagas disease by blood transfusion, similar to phenothiazines, is a reversible competitive inhibitor of TR but not of GR. The coupling of mepacrine to the active site of *T. cruzi* TR allowed the obtention of a crystallographic TR-inhibitor complex [177].

b-Aminodiphenylsulfides. Some compounds of the series of 2-amino diphenylsulfides, with lower neuroleptic activity than phenothiazines, were potent inhibitors of TR [178]. To avoid the disadvantages of the neuroleptic activity of phenothiazines, some compounds of the series of 2-amino diphenylsulfides, were synthesized resulting potent inhibitors of TR and showing that the active site of TR easily accommodates extremely bulky ligands [178,179].

c-Polyamine derivatives. Several potent spermidine and spermine-based inhibitor compounds have been synthesized. In many cases, the spermine derivatives were significantly more effective than the corresponding spermidines [180]. Screening of a library of spermidine-peptide conjugates revealed that N 1, N 1, N 4, N 8, N 12-penta (3-phenylpropyl) spermine was the most effective competitive inhibitor of *T. cruzi* TR. The compounds of this series were strong trypanocides but a clear correlation between enzyme inhibition and antiparasitic activity was not observed. Several polyamine derivatives were prepared and found to be potent competitive inhibitors of *T. cruzi* TR. The most effective inhibitor studied was compound 12 with a K_i value of 0.151 μM [181]. The antihypertensive agent Kukoamine A, a natural spermine derivative from the root bark of

Lycium chinense, is a mixed-type inhibitor of TR. Kukoamine showed no significant inhibition of human GR providing thus a novel selective drug lead [182].

d-Bisbenzylisoquinoline alkaloids were also studied in their capacity of inhibiting TR of *T. cruzi*. Among them, daphnoline and cepharanthine were shown to be TR inhibitors. Daphnoline led to a significant decrease in parasitemia as well as an increase in parasitological cure rate in comparison with Benznidazol-treated acute infected mice and in 70 % of the treated chronic mice no parasite was detected [183].

e-Quaternary arylalkylammonium phenothiazines. Substituted benzyl [3-(2-chloro-phenothiazine-10-yl)propyl] dimethylammonium salts were synthesized to introduce a permanent positive charge into inhibitor molecules. These compounds were linear competitive inhibitors against trypanothionedisulfide. The strongest inhibitor of this series rendered a K_i value of 0.12 μM , approximately 2 orders of magnitude more inhibitory than the parent chlorpromazine [184]. Quaternization of the nitrogen atom of 2-amino-4-chlorophenyl phenyl sulfide analogues of chlorpromazine improved *T. cruzi* TR inhibition approximately 40-fold with a linear competitive K_i value in the μM range. The quarterized analogues of the 2-chlorophenyl phenyl sulfides had strong antitrypanosomal and antileishmanial activity *in vitro* [185].

f-Nitrofuryl derivatives: New 5-nitrofuryl derivatives were synthesized and tested as anti-*T. cruzi* agents finding that more than 75 % of the prepared derivatives showed higher activity than nifurtimox [186]. The design of 5-nitrofuryl derivative compounds combining in the same molecule the recognized 5-nitrofuryl group, an oxidative stress promoter, and lateral chains that could interact with biomolecules such as TR showed to be very active against the epimastigote forms of the parasite in comparison with the reference drug nifurtimox [187].

Two structurally new types of inhibitors of TR but not of GR were studied: the antimicrobial chlorhexidine {1, 1'-hexamethylenebis [5-(4-chlorophenyl) biguanide]}, a linear competitive inhibitor and a piperidine derivative acting as mixed inhibitor. Although these compounds did not exert an improved inhibitory potency compared to chlorhexidine, the change from competitive to mixed-type inhibition resulted advantageous, since substrate accumulation does not overcome inhibition [188].

Other enzymes of the trypanothione metabolism, such as trypanothione synthetase without counterparts in the mammalian host, could be also mentioned as potential drug targets [152]. Particularly, TcAPX, a plant-like ascorbate-dependent hemoperoxidase was reported in *T. cruzi*. This enzyme belongs to the oxidative defense system of the parasite and is involved in the reduction of the parasite-specific thiol trypanothione by ascorbate in a process that involves non-enzymatic interaction. The absence of this redox pathway in the human host may be therapeutically exploitable [189].

Glutathione cycle A gene codifying for a novel *T. cruzi* protein containing the glutaredoxin (Grx) pattern CXXC was cloned. TcGrx, the recombinant protein, showed homology

to glutathione-S-transferases (GSTs) and was recognized by a serum anti-recombinant TcGrx in parasite lysates. It was confirmed that it is a thiol containing NADPH dependent reductase and binding assays suggested that it might use another thiol different from GCS as substrate². TcGrx could be a putative target for the design of specific inhibitors with antiparasitic properties.

-Phosphinopeptides structurally related to glutathione. In addition, a series of phosphinopeptides structurally related to glutathione was designed, synthesized, and evaluated as *T. cruzi*-antiproliferative agents. Two of them resulted potent growth inhibitors against amastigote forms [190].

5-GLYCOLYSIS

It is known that *T. cruzi* amastigotes possibly derive its energy entirely from glycolysis, that is the reason why the inhibition of glycolytic enzymes of trypanosomes may be considered attractive targets for the development of anti-*T. cruzi* drugs [191].

5A-Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The structural differences found between the glycosomal GAPDH (gGAPDH) in comparison with that of the mammalian counterpart led to the development of specific inhibitors [192]. Adenosine was found to be a very poor inhibitor, however the addition of substituents to the 2' position of ribose and the N6-position of adenosine led to a series of disubstituted nucleosides, finding that the adenosine derivative [N6-(1-naphthalenemethyl)-2'-(3-chlorobenzamido)adenosine] inhibited the proliferation of amastigotes without effect on the corresponding GAPDH human enzyme. A tight binding competitive inhibitor of an enzyme in the glycolytic pathway has been suggested to block the energy production in trypanosomatids [193,194]. Besides, flavonoids from the fruits of *Neoraputia magnifica* were isolated, and among these compounds 3', 4', 5', 5, 7- penta-methoxy-flavone resulted to be the most active over flavones and pyrano chalcones displaying inhibitory effect against the GAPDH of the parasite [195]. Studies performed on *T. cruzi* and *T. brucei* gGAPDHs showed that despite the high homology between the two trypanomatid enzymes (> 95%), some specific interactions identified could be useful to design selective irreversible inhibitors against *T. cruzi* gGAPDH [196].

-3-Piperonylcoumarins were designed as inhibitors of gGAPDH from *T. cruzi* based on the structures of previously identified natural products. The molecules could be clustered in different groups according to the chemical substitutions regarding the biological activity, finding that the most active synthesized derivatives contained heterocyclic rings at position 6. Molecular modeling studies by docking suggested a different binding mode for these derivatives, when compared to natural chalepin [197].

5B-Hexoquinase. It is well known that hexokinase is the first enzyme involved in glycolysis in most organisms. In *T.*

cruzi, unlike the human enzyme, it presents an unusual inhibition by inorganic diphosphate (PPi).

-Bisphosphonates. It was recently reported that bisphosphonates, non-hydrolyzable analogues of PPi, are potent inhibitors of *T. cruzi* hexokinase (TcHK). The most active compound against *T. cruzi* hexokinase was found to have a 2.2 μ M IC₅₀ versus intracellular amastigote forms showing selective activity against the parasite [198].

6-PENTHOSE PHOSPHATE PATHWAY

Recent results regarding the pentose phosphate pathway (PPP) have been reported in *T. cruzi*. All the enzymes of the PPP are present in the four major developmental stages of the parasite [199]. The third enzyme of this pathway, 6-phosphogluconate dehydrogenase (6PGDH), from *T. cruzi*, was cloned and sequenced. The kinetic parameters from a recombinant form of *T. cruzi* 6PGDH showed to be identical to the values reported for 6PGDHs from mammals, however Km for NADP was significantly lower than the value reported for the human enzyme, and closer to that for the *T. brucei* enzyme, suggesting that inhibitors of the *T. brucei* 6PGDH might also be successful for the chemotherapy of Chagas disease. It was recently reported that the enzyme shows a similar behaviour to the redox regulated G6PDHs from chloroplasts and cyanobacteria in addition to a considerable G6PDH increase in metacyclic trypomastigotes under oxidative stress conditions, suggesting that the enzyme may play a prominent role in the defense mechanisms of the parasite against oxidative stress becoming an important target for chemotherapy [200].

7- ARGININE KINASE

Vertebrates, including human, use creatine kinase for the storage of ATP in the form of phosphocreatine, capable to maintain ATP homeostasis during muscle contraction. A few years ago, it was reported that *T. cruzi* and *T. brucei*, possess an alternative pathway which uses arginine kinase as the catalyst for arginine phosphorylation to produce the analogous phosphagen, phosphoarginine. Phosphagens, phosphoarginine and phosphocreatine, play a critical role as energy reserve because the high-energy phosphate is ready to be transferred to adenosine diphosphate ADP when the production of ATP is required. In addition, the molecular and biochemical characterization of arginine kinases in trypanosomes have been reported [201]. This pathway is also widespread through the invertebrate phylum, including a great variety of phosphagens other than arginine, but not creatine.

Creatine kinase and arginine kinase are homologous proteins belonging to the family of guanidino kinases, conserved proteins with phosphotransferase activity. There is a close relationship between the energy requirements within the cell and the activity of guanidino kinases. Particularly, in *T. cruzi*, it has been suggested that the action of arginine kinase acquires relevance during the vertebrate stage of the parasite life cycle, due to variations in the insect feeding condition. Thus, during bursts of cellular activity or under starvation stress conditions, phosphoarginine results a rapid source of energy allowing the parasite to adaptate either to environmental changes or stress conditions [202].

²García GA, Garavaglia PA, Esteva MI, Duschak VG, Ruiz AM. Identification, characterization and purification of a putative thiol containing NADPH dependent reductase from *Trypanosoma cruzi* Reunión de Protozoología y Enfermedades Parasitarias, Rosario, Santa Fe, Argentina (2004).

Some reports showed that arginine kinase inhibition resulted in parasite growth inhibition in culture. Arginine kinase was also inhibited by the arginine analogs agmatine, canavanine, nitroarginine and homoarginine. Among them, canavanine turned out to be a potent inhibitor of arginine kinase. The trypanocidal action of green tea catechins against two different developmental stages of *T. cruzi* was demonstrated. In addition, recombinant *T. cruzi* arginine kinase was inhibited by the polyphenols catechin, gallate or gallo-catechin gallate [203]. However, patents related with catechins compounds in the last years relate these compounds with anticancer activity.

It is worth mentioning that aminoacid metabolic routes as possible therapeutic targets against Chagas disease have been recently reviewed by Silber *et al.*, 2005 [204].

8-PROTEIN KINASES

Protein kinases (PK) represent promising drug targets for a number of human and animal diseases including Trypanosomiasis and Leishmaniasis. Genome sequences of the three human-infective Trypanosomatid protozoa, *L. major*, *T. brucei* and *T. cruzi* have been completed, thus defining the eukaryotic protein kinases or kinome for each parasite representing one third of the human complement. Kinome analysis will allow exploiting differences between parasite and mammalian protein kinases to develop novel anti-parasitic chemotherapeutic agents [205].

Protein kinase inhibitors. To evaluate PKs as drug target, three PK inhibitors: staurosporine (serine/threonine kinase inhibitor), genistein (tyrosine kinase inhibitor), and wortmannin (phosphatidylinositol 3' (PI3) kinase inhibitor) were tested on the growth and ultrastructure of *T. cruzi* epimastigotes and the effect of these drugs on intracellular amastigotes were evaluated. Wortmannin inhibited parasite growth at the lowest concentrations. However, staurosporine was the most effective after 24 h treatment and genistein caused the stronger inhibition during the whole treatment (60-70 % inhibition) whereas wortmannin showed the lower IC₅₀ in the mM range. In addition these PK inhibitors showed strong ultrastructural effects on the epimastigotes, they did not interfere neither with the division of intracellular amastigotes nor with their differentiation to trypomastigotes. However, as trypanosomes have kinomes that contain a large set of protein kinases and phosphatases, PKs should not be disregarded as an important target for chemotherapy of Chagas disease [206].

9-POLYAMINE METABOLISM AND TRANSPORT PATHWAYS

In parasitic protozoa, polyamine metabolism and transport pathways comprise valuable targets for chemotherapy. Polyamines are involved in multiple functions inside the cell: in chromatin condensation, in stabilization of tRNA's structure, in DNA conformational transitions, in neurotransmission modulation and post-translational modification of proteins [207]. In *T. cruzi*, the polyamine spermidine forms a part of trypanothione, essential member of the dithiol redox metabolism, contributing to the maintenance of an intracellular reducing environment. Polyamines are essential requirements for parasite cell growth and differentiation and

polyamine metabolism has attracted considerable attention as a chemotherapeutic target in parasite infections.

Although ornithine decarboxylase (ODC) is a key enzyme of the polyamine biosynthesis pathway usually inhibited by the rationally designed drug difluormethylornithine, it has not been detected in any stage of *T. cruzi*'s life cycle and *T. cruzi* is not affected by difluormethylornithine. However, *T. cruzi* was found to be susceptible to a compound related to ODC, difluoromethylarginine (DFMA), which is supposed to inhibit arginine decarboxylase (ADC) but ADC activity in *T. cruzi* was only found in the trypomastigote form although at almost undetectable levels [208]. On the other hand, taking into account that *T. cruzi* does not synthesize putrescine, but uptakes it from the extracellular milieu, the putrescine analogue 1,4 -diamino-2-butanone (DAB) inhibited *T. cruzi* epimastigotes' *in vitro* proliferation and produced remarkable signs of oxidative stress such as mitochondrial destruction and cell architecture disorganization. In addition, thiobarbituric-acid-reactive substances were measured to assess lipid peroxidation. A dose-dependent response was found indicating that putrescine uptake by this diamine auxotrophic parasite may be important for epimastigote axenic growth and cellular organization [209]. A patent related with polyamine transport inhibitors from Laval University, Canada includes design, synthesis and therapeutic use of a variety of novel inhibitors of polyamine transport to prevent polyamines salvage in tumoral cells [210]. However, the importance of polyamines in cell survival as well as the complete knowledge of the synthetic pathways in *T. cruzi* still needs further investigation.

10-PURINE SALVAGE PATHWAY AND NUCLEOTIDE SYNTHESIS

Whereas in mammals nucleotides are synthesized both *de novo* and salvaged from recycled purine bases, most parasites are obligate purine auxotrophs, it means that they must salvage purines from their host and they have developed systems to transport, internalize and metabolize the required substrates: the components of nucleic acids and ATP. Accordingly, *T. cruzi* depends on the scavenging of exogenous purines for nucleotide synthesis. Among trypanosomatid enzymes involved in the scavenging of purines from the host can be mentioned:

10A-Purine(hypoxanthine/guanine)-phosphoribosyl-transferase (HGPRT). The HGPRT catalyzes the transfer of a phosphoribosyl moiety on the nucleobase hypoxanthine or guanine converting purine bases to ribonucleotides and is responsible for the initiation in the parasite of the metabolism of certain cytotoxic purine base analogues, such as allopurinol. Thus, either inhibitors or substrates of HGPRT are good targets for effective and selective chemotherapeutic agents. The *hgprt* genes from *T. cruzi* and other pathogenic trypanosomatids have been cloned, sequenced and overexpressed in *Escherichia coli*, and the recombinant proteins have all been purified and characterized [211]. It was reported that the purine (3'-azido-3'-deoxyinosine, 3'-deoxyadenosine) and pyrimidine (3'-azido-3'-deoxythymidine) analogues inhibited the proliferation of amastigotes in culture cell lines [212]. Allopurinol (4-hydroxypyrazol-(3,4d)-pyrimidine) has been used in humans for the treatment of gout and it is transformed in vertebrates in

oxypurinol, a potent inhibitor of xanthine oxidase (XO). In Trypanosomatids, deficient in XO, the compound acts as a purine analogue and is incorporated via HGRPT into DNA disrupting the synthesis of RNA and proteins. Allopurinol was shown to be active in murine models of acute Chagas disease with differences in susceptibilities among *T. cruzi* strains [213] however, there are some conflicting reports related to its efficiency in humans. The drug did not show *in vivo* activity due to low incorporation in vertebrate stages of *T. cruzi* and probably to inadequate pharmacokinetic properties. Purine analogues were assayed for their interaction with the HGPRs from *T. cruzi* and its human counterpart and some of them showed affinity for the trypanosomal enzyme [214]. A structure-based docking method identified several potential inhibitors of the trypanosomal HRPT. Among them, three compounds (2,4,7-trinitro-9-fluorenylidene-malononitrile, 3-(2-fluoro-phenyl)-5-(phenoxy)-1,2,4-triazolo(4,3-C)-quinazoline and 3,5-diphenyl-4'-methyl-2-nitrobiphenyl) showed trypanostatic activity in cell culture (against intracellular amastigotes) and one [6-(2,2-dichloroacetamido)chrysene] was a potent inhibitor of the enzyme [215]. Wenck and co-workers (2004) stated the difficulty in designing a mechanism-based inhibitor of the trypanosomal HPRT that would only inhibit the human cognate enzyme based on kinetic parameter analysis [216].

10B-Dihydrofolate reductase (DHFR). Dihydrofolate reductase and thymidylate synthase are two widespread enzymes involved in DNA nucleotide synthesis. Both constitute a bifunctional protein present in different species of protozoa which has been successfully used as a drug target in chemotherapy of cancer, malaria and infectious diseases.

The gene coding for the DHFR domain from *T. cruzi* was cloned and expressed [217]. Several derivatives of methotrexate, inhibitor of the human enzyme, were designed and synthesized using a structure-based approach, and some of them showed higher selectivity for the parasite enzyme than for the human counterpart. Another group of compounds were designed, synthesized and screened as inhibitors of DHFR of trypanosomatids, showing weak activity in *in vitro* assays with intracellular amastigotes of *T. cruzi* [218]. The inhibitors described are:

a-2, 4 -Diaminopyrimidines. It was shown that 5-benzyl-2, 4-diaminopyrimidines are selective inhibitors of the trypanosomal as well as leishmanial enzymes. Various compounds with alkyl/aryl substitution on the 6-position of the pyrimidine ring were prepared and evaluated against both the recombinant enzymes and the intact organisms finding that the presence of a substituent did not enhance the inhibitor activity neither against the enzyme nor intact parasites in comparison with unsubstituted compounds [219]. On the other hand, the synthesis of 4'-substituted and 3', 4'-disubstituted 5-benzyl-2, 4-diaminopyrimidines was performed and these compounds were then assayed against the recombinant parasite and human DHFRs. Some of the compounds showed good activity against *T. cruzi* in *in vitro* assays. A molecular modelling showed that those compounds which bound within the enzyme pocket of trypanosomatid enzymes presented the highest selectivity [220].

b-2,4-diaminoquinazolines. A series of 2, 4-diaminoquinazolines were designed, synthesized and evaluated as inhibitors of dihydrofolate reductase of different Trypanosomatids. Some of these compounds showed potent activity against *T. cruzi* [221].

c-Antifolate drugs. Recently, the lipophilic trimetrexate (TMQ), a FDA-approved drug for the treatment of *Pneumocystis carinii* infection in AIDS patients, showed to be a potent inhibitor of *T. cruzi* DHFR activity and was also highly effective in killing *T. cruzi* trypomastigotes and amastigotes. Unluckily, TMQ also showed to be a good inhibitor of human enzyme [222].

10C-Pteridine reductase (PTR). Many important cellular functions require reduced pteridines. Trypanosomatids unlike their mammalian host are pteridine auxotrophs and salvage the precursor pteridines from the host and reduce them to the respective biologically active tetrahydro forms using parasite enzymes which may serve as drug targets. The enzyme pteridine reductase 1 (PTR1), only found in trypanosomatids and plant pathogens, was first related with reduction of unconjugated pteridins. However, it also catalyzes the reduction of folate to dihydrofolate and tetrahydrofolate mediating in the salvage of oxidized pteridines showing a lower sensitivity to methotrexate than DHFR, interfering in the effectiveness of antifolate drugs targeting DHFR [223]. In addition, pteridine reductase 2 (PTR2), which can only reduce dihydropterin and dihydrofolate substrates but not oxidized pteridines was identified and expressed in *T. cruzi* [224,225]. A docking study was recently performed on a set of pteridine analogues at the active site of PTR2 and better results than that of methotrexate, were obtained for the assayed compounds [225]. Recently, the crystal structure of an inhibitor (methotrexate) and a substrate (dihydrofolate)-complex of this enzyme was performed [226].

Isis Innovation Ltd, 2001 described triazine derivatives as useful novel DHFR inhibitors claiming that these compounds were useful for parasitic infections including Chagas disease [227]. Most of the recently disclosed patents on purine analogues are related to antiviral and/or anticancer activity [228], only a few claim their effects on parasitic diseases. Among ATP analogues, Bottaro *et al.*, claimed that nucleoside pyrophosphate and triphosphate analogues, were useful against infectious diseases caused by some protozoans including Chagas disease. However no experimental evidences were given [229].

10D-Dihydroorotate dehydrogenase (DHOD). In *T. cruzi*, the fourth enzyme of the pathway catalyzing production of orotate from dihydroorotate, markedly differs from the human enzyme. Searching for potent inhibitors against *T. cruzi* DHOD activity, a number of methanol extracts prepared from green, brown, and red algae were tested. *T. cruzi* DHOD activity was inhibited by the extracts from two brown algae, *Fucus evanescens* and *Pelvetia babingtonii*. In addition these extracts were effective against the protozoan infection and proliferation in mammalian cells [230] and a recombinant enzyme form, Tc DHOD, was recently crystallized complexed to orotate [231], opening the possibility for future inhibitors design.

11-ORGANELLES AS TARGETS

11-1-Nucleus, kinetoplast and DNA modulation. DNA topoisomerases are essential enzymes for nucleic acid biosynthesis and cell survival which modify the topology of DNA. In kinetoplastids, topoisomerases are involved in the metabolism of both nuclear and mitochondrial (kinetoplast) DNA. DNA topoisomerases from parasites have been the focus of molecular and cellular biology studies and have been also considered as target for antiparasitic chemotherapy, particularly, topoisomerase II, required for kinetoplast replication. Several inhibitors of bacterial DNA topoisomerase II showed to be effective against *T. cruzi*, producing damage to kinetoplast and/or the nucleus of epimastigotes and inhibiting both proliferation and differentiation processes, suggesting that both organelles could be the targets of the drugs [232].

-Quinolone derivatives. *T. cruzi* is particularly sensitive to quinolone derivatives probably through DNA topoisomerase II inhibition. Quinolone derivatives were patented by New Pharma Research Sweden AB as useful agents in treatment of bacterial and parasitic diseases including those caused by trypanosomatids but no specific data for the claimed compound were reported [233]. Currently, these compounds are patented as antibacterial compounds and especially suitable for treatment of coccidiosis. On the other hand, Campthotecin, an antitumor drug and a well-characterized inhibitor of eukaryotic DNA topoisomerase I, caused disruption of nuclear and mitochondrial DNA in *T. cruzi* [234].

-Dicationic guanidine and reverse amidine derivatives. Among DNA modulating agents, described as promising agents for the treatment of African trypanosomiasis, twenty dicationic molecules containing either diguanidino or reversed amidine cationic groups were tested *in vitro* versus *T. cruzi*. The most active compounds belong to the reversed amidine series and six exhibited IC50 values of less than 1 μM [235,236]. Scientist from University of North Carolina at Chapel Hill synthesized dicationic reversed amidines such as novel 2, 5-bisalkyl (or aryl) imino aminophenyl furans and thiophenes, compounds with strong DNA binding affinities and a patent claimed them useful for mycobacterial, fungal and protozoal infections including *Trypanosoma cruzi* [237].

The inhibition of trypanosome growth was caused by the specific interaction of typical ligands (benzimidazoles, colchicine and vinblastine) with trypanosome tubulin. Then, in kinetoplastids, tubulin has been proposed as a potential target [238].

-Dinitroaniline sulfonamide derivatives These antimetabolic compounds with activity against tubulin were disclosed by scientist from Ohio State University as useful for the treatment of diseases caused by parasitic protozoa, particularly leishmaniasis. Despite their good *in vitro* activity, these compounds failed to cure parasite infected mice. The putative toxicity of compounds with nitroaromatic groups remains to be addressed [239].

Among patents related with compounds and methods of use to treat infectious diseases, scientist from Bradley Cytokine Pharmasciences, Inc, describe some of them, used to target specific nuclear localization, signal blocking

importation of specific proteins or molecular complex into the nucleus of a cell claiming their use for treatment or prevention of infectious diseases, such as parasitic and viral diseases [240].

11-2-Acidocalcisomes and Exchanger Na^+/H^+ Mechanism

The storage of calcium in specialized acidic organelles, termed acidocalcisomes constitutes another unusual feature of *T. cruzi*, in comparison with mammalian cells. These structures are involved in polyphosphate and calcium storage as well as in adaptation to environmental oxidative stress [241]. 3,5 Dibutylhydroxitolueno blocks Ca^{2+} release via the acidocalcisomal exchanger Na^+/H^+ . Then, the acidocalcisomal exchanger Na^+/H^+ is a mechanism involved in Ca^{2+} and pH homeostasis exclusive of this organism to be potentially used as drug target.

-Guanidine derivative compounds. Hoechst Marion Russel Deutschland GmbH claimed the use of Na^+/H^+ exchange inhibitors for the treatment of protozoal infections including Chagas disease. However, these compounds were described but neither synthesis nor characterization was shown [242].

-lapachone-derived naphthoimidazoles. Among 45 semi-synthetic derivatives of naphthoquinones isolated from *Tabebuia spp.*, naphthoimidazole N1 resulted one of the most active compounds against *T. cruzi* trypomastigotes. The effect of N1 against the proliferative forms of *T. cruzi* suggested that in epimastigotes, reservosomes, mitochondrion, and nucleus contain N1 targets. In trypomastigotes, in which reservosomes are absent, the organelles affected by the compound were also the mitochondrion and nucleus, as well as acidocalcisomes, in which the decrease in electron density could be due to the use of polyphosphate as an alternative energy supply [243].

11-3 Membrane components, contractile vacuole complex and osmoregulation. Searching for novel drug targets, among parasite membrane components, transport proteins for nutrients and metabolites of the parasite-host interface are getting into focus. Genes coding for aquaporin water and solute channels have been identified in the protozoan genomes. Six protozoan aquaporins have been cloned and functionally characterized. Amino acid compositions of the individual pore entries were compared and permeability properties attributed to specific protein features. Furthermore, possible physiological roles in osmotic protection and metabolism were assigned to aquaporins. The presence of *Tc* AQP, corresponding to an aquaporin gene from *T. cruzi*, was reported in acidocalcisomes and contractile vacuole complex of the parasite [244]. The potential of protozoan aquaporins for use as a target or entry pathway for chemotherapeutic compounds was recently reviewed by Beitz and co-workers [245].

11-4 Glycosome and vitamin C synthesis. It was demonstrated that both *T. brucei* and *T. cruzi* have the capacity to synthesize vitamin C and the reaction occurs in a unique single-membrane organelle of the parasite, the glycosome. Taking into account that the capacity to synthesize vitamin C (ascorbate) is widespread in eukaryotes but is absent from humans, this aspect constitutes another potential chemotherapeutic drug target [246].

12- SIALIC ACIDS TRANSFERENCE

Trypanosomes are unable to synthesize sialic acids but can scavenge them from its mammalian hosts by using a unique neuraminidase with trans-sialidase activity able to transfer sialic acid molecules from host glycoconjugates to mucin-like acceptors present in the parasite surface membrane. In addition, the action of this particular developmentally regulated trans-sialidase (TS) seems to be essential for *T. cruzi* survival and cell invasion in the host [247,248]. Then, TS inhibitors are also considered potential trypanocidal therapeutic agents. The X-ray structure of TcTS and TcTS in complex with substrates and sialidase inhibitors has been published. A significant number of amino acid residues are conserved within the active site of TcTS that are common to all known sialidases, reflecting a strong evolutionary link to other microorganisms. However, critical amino acid residue differences between mammalian sialidases and the parasite trans-sialidase provide a basis for an explanation of the particular glycotransfer enzymatic activity of TcTS [249].

A recent report describes some target synthetic sialyl-mimetics-cyclohexenophosphonate monoester compounds displaying promising inhibitory properties when tested with parasitic or bacterial sialidases [250]. Among patented compounds, novel N-substituted piperidines were disclosed by Horenstein and Parr from the University of Florida, claiming that these compounds with neuraminidase inhibitory activity could be used for the treatment of bacterial, viral and parasitic infections including diseases caused by trypanosomes [251]. In addition, scientist from University of Alabama disclosed inhibitor and methods of treating and preventing bacterial or trypanosomal infection using a bacterial sialidase inhibitor [252]. However, in both cases no data on their activity against the parasite enzymes were presented.

13-BIOSYNTHESIS OF LIPIDS

13-A-Alkyl-lysophospholipids (ALPs). Another group of promising compounds active against proliferation and differentiation of *T. cruzi*, *in vitro* and *in vivo* are alkyl-lysophospholipids (ALPs). These synthetic analogues of lysophospholipids designed as potential immunomodulators have been developed as antitumoral and antileukaemic agents [253]. Although the mechanism related with antiparasitic activity is still not known, the anti-*T. cruzi* activity of ALPs has been related with a selective blockade of phosphatidyl-choline (PC) biosynthesis in the parasite involving the transmethylation pathway, in contrast with the situation in the vertebrate host, where the CDP-choline pathway is predominant. These ALPs present good oral activity and low toxicity [254].

In addition, lysophospholipid analogues (LPAs) originally developed as anti-cancer agents, have also shown significant activity against *Leishmania spp.* and *T. cruzi*, both *in vitro* and *in vivo*. Miltefosine was registered in 2002 for the oral treatment of visceral leishmaniasis. LPAs interfere with lipid synthesis in *T. cruzi* and cancer cells, but the activity is about >20-fold higher against the parasite [255]. It was reported that LPAs present antiproliferative synergy with ketoconazole against both epimastigotes and

intracellular amastigotes of *T. cruzi*. Whereas edelfosine or ketoconazole alone induced morphological alterations in the plasma membrane and reservosomes of the parasites, combined also led to severe mitochondrial damage, formation of autophagic structures and multinucleation, possibly by interference with lipid metabolism [256]. Recently, the LPA edelfosine was also tested on trypanomastigotes. LPAs induced alterations in the plasma membrane of the three developmental stages of the parasite and in the mitochondria in epimastigotes suggesting that these organelles are potential targets of these analogues [257].

A series of analogues of the naturally occurring antibiotic thiolactomycin (TLM) has been evaluated against *P. falciparum* proliferation taking into account that TLM is an inhibitor of Type II fatty acid synthase but not of Type I fatty acid synthase in mammals. A number of the analogues showed inhibition equal to or greater than TLM and some of them showed activity when assayed against the parasitic protozoa, *T. cruzi* and *T. brucei* [258].

13-B- Glycosphingolipids (GSLs). Lipid metabolism has also been attracting a lot of attention with respect to basic biology and applications for chemotherapeutic purposes. Although glycosphingolipids (GSLs) are ubiquitous in eukaryotic cells, very little is known about their role in parasites. The presence of an active Glucosylceramide synthase (GCS) in the intraerythrocytic stages of *P. falciparum* has been demonstrated [259]. Taking into account that glucosylceramide is a pivotal precursor of numerous GSLs, the special features presented by this enzyme compared with the mammalian counterpart signal GCS as a potential target [260]. In *T. cruzi*, different GCS inhibitors were tested as antiproliferative agents in culture and bloodstream forms. PPMP produced 79 to 95.5 % of parasite lysis in *in vitro* assays. *In vivo* assays in infected mice are under development with PPMP as well as with some citostatic drugs involved in the alteration of this GSL pathway³.

PART II. DRUGS DERIVED FROM NATURAL SOURCES

The use of natural products for the treatment of protozoal infections is well known and has been early documented. Several recent works report the investigation of trypanocidal activity of a wide variety of crude natural extracts or compounds isolated, particularly of vegetal origin as well as semi-synthetic analogues. Among them different groups can be found in the literature:

1- Anti-microtubule agents. Microtubules play fundamental roles in eukaryotic cells. The antimicrotubule drug taxol, obtained from the bark of *Taxus brevifolia* as well as its synthetic derivatives, employed in cancer chemotherapy, also interferes with the proliferation of *Crithidia fasciculata* and *T. cruzi*, leading to morphological alterations, interruption of nuclear division and cytokinesis, and inhibitory effect on endocytosis of proteins by epimastigotes [261,262]. On the other hand, the antimicrotubule agents vinblastine and vincristine, alkaloids obtained from *Vinca rosea* showed

³Duschak, VG; Landoni, M; Garabaglia, P.; Esteva, MI; Couto, A.S. Glucosylceramide synthase as target for new antiparasitic drugs. Kinetoplastid Diseases, Dakar, Senegal, Africa (2006).

selective and reversible effects inhibiting both nuclear division and cytokinesis thus interfering with epimastigotes proliferation [263].

2- Alkaloids. A variety of alkaloids have been tested against epimastigotes of *T. cruzi*. The activity of apomorphine [264] as well as the activity of β -carboline alkaloids on nifurtimox resistant parasites [265] was associated to the inhibition of respiratory chain. Besides, some glycoalkaloids including β -chaconine and β -solanargine as well as some aglycones (demissidine, solanidine, etc) were tested against epimastigotes, bloodstream and metacyclic trypomastigotes, showing higher activity than ketoconazole [266]. Five new bisbenzylisoquinoline derivatives were isolated from the stem bark of *Guatteria boliviana*, among them, funiferine, antioquine and guatteboline were active against trypomastigotes [267]. In addition, trypanocidal effects of the natural alkaloid piperine were evaluated and twelve synthetic derivatives were tested against epimastigote and amastigote forms of *T. cruzi*, pointing out piperidine as a suitable template for the development of new drugs with trypanocidal activity [268]. Recently, five out of 64 diterpenoid alkaloids tested, were active on *T. cruzi* epimastigotes: atisinium chloride and 13-oxocardiopetamine were potent *T. cruzi* epimastigote growth inhibitors with activity levels similar to that of benznidazole. *in vitro* Assays showed that these compounds reduced metacyclic forms capacity of invasion to mammalian cell, their intracellular replications and their transformation into trypomastigotes, with no toxicity to the host cell suggesting that these alkaloids are structural leads of clinically active compounds against *T. cruzi* [269].

3- Stilbenoids. Isonothalaenic acid, a natural dihydrostilbenoid and some synthetic series of related heterocyclic compounds were tested on cultures of epimastigote and trypomastigote forms of *T. cruzi*, finding that some of these compounds showed activity similar to benznidazole against epimastigotes, and others were more active against trypomastigotes than the reference drug gentian violet [270].

4- Gangliosides. Ganglioside treatment of acute infected mice determined long-term survival and clearance of parasites from the bloodstream and organs, producing additional complete prevention of clinical manifestations of the infection, and progression into the chronic stages of the disease, for at least 18 months post-infection. It was suggested that the effect of gangliosides could be due to inhibition of phospholipase A2 enzymes, which are involved in membrane destabilization interfering parasite penetration into the host cells. However, the fact that these compounds had no toxic effect on the parasite turned non probable this hypothesis, considering that the *in vivo* effect could be due to modulation of the host immune system [271].

5- Snake venom. Venom from three different snake species was tested *in vitro* against *T. cruzi*. Epimastigotes proliferation was inhibited by Venom from *Cerastes cerastes* and *Naja haje* at levels similar to benznidazole. Venom from *C. cerastes* was also active against trypomastigotes [272].

6- Juvenile hormone and analogues. The juvenile hormone-III and the analogues methoprene and fenoxycarb inhibited macromolecular biosynthesis and growth of epimastigotes [273]. Some analogues showed lytic activity

on blood trypomastigotes and reduced the parasitemia and mortality levels in infected mice in a moderate degree [274]. Sulphur-containing derivatives structurally related to fenoxycarb showed to be potent growth inhibitors against the intracellular form of the parasite [275]. On the other hand, whereas *in vitro* experiments showed that methoprene cause cellular death of *T. cruzi*, this compound failed to clear bloodstream trypomastigotes in *in vivo* experiments but a decrease of parasitemia levels of infected mice was observed, suggesting that this compound might serve as an effective agent to sterilize blood for transfusions [276].

7- Flavonoids and propolis. Antiplasmodial, leishmanicidal and antitrypanosomal activities of eight natural biflavonoids were estimated *in vitro* on the respective parasites. Among them, ginkgetin and isoginkgetin showed the best antitrypanosomal activity with low IC50 values in the μ M range [277]. In addition, the strong antimicrobial activity of propolis, the natural resin produced by honey bees is associated mainly with flavonoids and also with derivatives of hydroxycinnamic acid. In *T. cruzi*, the effect of different types of propolis was evaluated, finding *in vitro* activity against epimastigotes, trypomastigotes and intracellular amastigotes but no effect was observed on the course of acute infection [278]. In the last years, four derivatives of hydroxycinnamic acid isolated from a Brazilian propolis were assayed against trypomastigotes showing lower activity than crystal violet [279]. In addition, two ethanolic Bulgarian propolis extracts with a high content of flavonoids presented strong inhibitory activity against *T. cruzi* proliferative epimastigotes, but were more susceptible than trypomastigotes [280]. Multivariate analysis was applied to evaluate the efficiency of different extracts of a Brazilian propolis from *Apis mellifera* finding different degrees of trypanocidal activity [281]. Recently, it was reported that treatment of *T. cruzi*-infected mice with ethanolic extracts of Bulgarian propolis interferes with the basic properties of immune cells promoting changes in the immune response [282].

8- Natural naphthoquinones. Among natural naphthoquinones present in plants, bioactive compounds known are lapachol, which was isolated from the heartwood of *Tabebuia spp.* and β -lapachone, both obtained as contaminants in the process of lapachol isolation. Lapachol derivatives were assayed against infective trypomastigote blood forms of *T. cruzi* and the triacetoxo derivative of reduced lapachol showed relevant trypanocidal activity [159]. β -Lapachone showed trypanocidal activity against epimastigotes, which was associated to generation of free radicals and inhibition of nucleic acids and protein synthesis [160]. Other quinone compounds isolated from natural products were assayed against *T. cruzi* and showed trypanocidal activity including trihydroxylated anthrax-quinone purpurin, obtained from the roots of *Rubia tinctorum* (Rubiaceae) [283]; the 1,4-naphthoquinone 2,3,3-trimethyl-2-3-dihydro-naphtho[2,3-b]furan-4,9-quinone isolated from *Calceolaria sessilis*, [284]; and the polyprenylated benzoquinone 7-epiclusianone, isolated from *Rheedia gardneriana* (Clusiaceae). The latter was active *in vitro* against trypomastigote, but showed no effect on experimentally infected mice [285].

9- Cyclosporin analogues. Cyclophilin and FK506-binding protein families, known as immunophilins, include the major binding proteins of certain immunosuppressive drugs: cyclophilins for the cyclic peptide cyclosporin A and FK506-binding proteins for the macrolactones FK506 and rapamycin. Taking into account the antiparasitic activities of cyclosporins, macrolactones and non-immunosuppressive derivatives of these compounds, immunophilins may mediate drug action and/or may themselves represent potential antiparasitic drug targets [286]. In *T. cruzi*, cyclosporin A (CsA) nonimmunosuppressive analogues were evaluated against the parasite and on a parasite cyclophilin named TcCyP19. Among them, two out of eight CsA analogues, showed the best anti-parasitic effects on epimastigote proliferation, trypomastigote lysis and inhibition of trypomastigote infection *in vitro* assays in comparison to CsA control suggesting that this cyclophilin might be involved in the trypanocidal effects [287].

10- Crude plant extracts and its components. *in vitro* Screenings of plant extracts testing the antiprotozoal activity from different plant families have been performed. Preliminary studies on Bolivian medicinal plants evidenced that some of the extracts showed activity against epimastigotes of different strains of *T. cruzi* [288]. Similarly, extracts from several plants used in Guatemala for the treatment of protozoal infections showed high activity against trypomastigotes. Among them, *Neurolaena lobata* showed *in vitro* and *in vivo* trypanocidal activity [289]. When 79 total extracts obtained from Asteraceae, Araceae, Moraceae, Solanaceae, Rhamnaceae, Zingiberaceae, Leguminosae and Sapotaceae were tested on different parasite models, only nine of them showed trypanocidal activity [290]. The evaluation of trypanocidal activity against trypomastigotes of crude plant extracts of different species of Rutaceae showed that eight out of 32 were significantly active, being the most active the one obtained from the stems of *Pilocarpus spicatus* [291]. Besides, crude ethanolic extracts and several fractions obtained by solvent partition of 13 plants from Brazilian Rain Forest were tested for trypanocidal activity with promising *in vitro* activity against different forms of the parasite. Particularly, activity was observed in both dichloromethane and hexane fractions of *Polygala sabulosa* and *P. paniculata* [292]. In addition, extracts obtained from *C. podantha* and *M. arenosa* showed high percentages of growth inhibition of epimastigote forms from *T. cruzi* [293]. Moreover, among selected plants, *Casearia sylvestris* var. *lingua* was the most active against both *T. cruzi* and *L. donovani* and extracts of *Annona crassiflora*, *Duguetia furfuracea*, and *Casearia sylvestris* var. *lingua* were active with IC50 values between 0.3-10 µg/ml against amastigotes of *T. cruzi* [294].

On the other hand, a variety of organic crude extracts obtained from 65 Mexican medicinal plants was screened for trypanocidal activity, the methanolic extract of seeds of *Persea americana* (avocado), six 1,2,4-trihydroxy-heptadecane derivatives and two 1,2,4-trihydroxy-nonadecane derivatives, isolated from the active fractions showed a moderate activity against epimastigotes and trypomastigotes [295]. Finally, organic and aqueous extracts from 12 Argentine medicinal plants were tested for their *in vitro* trypanocidal activity on epimastigote forms from *T. cruzi*.

Among the selected species, the organic extracts of *Ambrosia scabra*, *Ambrosia tenuifolia*, *Baccharis spicata*, *Eupatorium buniifolium*, *Lippia integrifolia*, *Mulinum spinosum* and *Satureja parvifolia*, and the aqueous extracts of *E. buniifolium*, *L. integrifolia*, *M. spinosum* and *S. parvifolia* showed trypanocidal activity with a percentage of growth inhibition higher than 70 % at a concentration of 100 µg/ml [296].

Some plant extract components were isolated and also tested for trypanocidal activity. Among them can be mentioned acetogenins from the seeds of *Annona glauca* (glaucanisin, annonacin A, squamocin and annonacin) which showed activity against trypomastigotes [297], or those extracted from the stem barks of *Rollinia emarginata* showing *in vitro* leishmanicidal and trypanocidal properties [298]; cryptofolione derivatives isolated from *Cryptocarya alba* fruit, were active against trypomastigotes, but with moderate cytotoxicity for both amastigotes and macrophages, indicating little selectivity for *T. cruzi* [299]; among antibiotic macrolides, megalomicin, produced by *Micromonospora megalomicea*, showed potent activity against epimastigotes and intracellular amastigotes at lower concentrations than those that interfere with the mammalian organelle [300] while some polyene macrolides produced by genetically modified *Streptomyces* appeared to be especially potent and selective trypanocidal compounds [301] and among lignans, methylpluviatolide extracted from the leaves of *Zanthoxylum naranjillo* (Rutaceae) which was tested both *in vitro* and *in vivo* assays against different strain of *T. cruzi* resulted highly effective [302] or eupomatenoid-5, a neolignan dihydrobenzofuranic compound isolated from leaves of *Piper regnellii* var. *Pallescens* which showed antiprotozoal activity against the epimastigote proliferative stages and intracellular amastigote forms of *T. cruzi* produced ultrastructural alterations [303]. Recently, several flavonoid glycosides from a Turkish plant were tested in mouse models showing a moderate activity against *T. cruzi*, and only chrysin dimethylether and 3-hydroxydaidzein had IC50s lower than 5.0 µg/ml. In addition, it was reported that 7, 8 dihydroxyflavone and quercetin appear to ameliorate parasitic infections in mouse models resulting potent and effective antiprotozoal agents [304].

Terpenes. A variety of terpene compounds were isolated from different plants as follows:

a- Diterpenes. Some kaurane diterpenes, isolated from the aerial parts of *Wedelia paludosa* (Asteraceae), showed activity in *in vitro* assays against trypomastigotes [305]; among diterpenoids isolated from *Azorella compacta*, the products azorellanol and mulin-11,13-dien-20-oic acid were active against amastigotes and the cytotoxicity to mammalian cells was lower than that of nifurtimox [306]. Besides, two new norditerpen aldehydes and five known diterpenes from the fruits of *Vitex trifolia* also showed *in vitro* trypanocidal activity with minimum lethal concentrations against epimastigotes in the µM range [307]. In addition, komaroviquinone, a potent trypanocidal diterpene, was reduced by *T. cruzi* old yellow enzyme (TcOYE) to its semiquinone radical. The reductase activity in trypanosome lysates was completely immunoabsorbed by anti-TcOYE antibody. It was suggested that the fact that TcOYE is

expressed throughout the *T. cruzi* life cycle, turns komarovicquinone in an interesting candidate for developing new antichagasic drugs [308]. On the other hand, the oleoresin from *Pinus oocarpa* was fractioned yielding two diterpenes, pimaric acid and dehydroabietic acid among other compounds, which were tested *in vitro* against epimastigotes of *T. cruzi* resulting pimaric acid as well as the sesquiterpene longifolene and the oleoresin the most active compounds, being as active as the reference compound nifurtimox [309]. Recently, a novel icetexane diterpene, 5-epi-icetexone (ICTX) from *Salvia gilliessi* has resulted active against epimastigotes from different *T. cruzi* strains [310].

b- Triterpenes. Crude extracts and fractions of *Bertholletia excelsa* stem barks were tested for trypanocidal activity. *in vitro* Assays performed with the acetonic and methanolic extracts showed significant activity against trypomastigote forms since in the concentration of 500 µg/ml, the parasites were reduced in 100 % and 90.3 % respectively, whereas a triterpene betulinic acid pure isolated from an hexane extract presented 75.4 % [311]. In addition, some bioactive constituents were obtained from an ethanolic extract of *Dracocephalum subcapitatum* including five flavonoids, calycopterin, xanthomicrol, isokaempferide, luteolin and apigenin, together with five terpenoids, oleonic acid, ursolic acid, geranial, neral and limonene-10-al. Among them, citral and limonene-10-al were the most effective components against epimastigotes of *T. cruzi* [312]. Recently, triterpene acids were isolated from methylene chloride extracts of the *Miconia sellowiana* and *M. ligustroides* species and their activities against the trypomastigote blood forms of *T. cruzi* were evaluated. The *in vitro* assays showed that ursolic acid and oleanolic acid were the most active showing IC₅₀ values in the µM range. *In vivo* assays, showed that ursolic acid and its salt derivative produced the most significant reduction in parasitemia (75.7 % and 70.4 %, respectively) increasing the survival time for all the treated mice [313].

c- Sesquiterpenes. The sesquiterpene lactone dehydroleucodine affects the growth of cultured epimastigotes of *T. cruzi*, resulting lethal for the parasites at the higher concentrations tested [314]. By contrast, the sesquiterpene lactones: helenalin and some structurally related derivatives showed anti-trypanosomal activity towards both *T. cruzi* and *T. brucei*. Helenalin was the most active compound in the series with IC₅₀ values in the µM range [315]. Recently, new assays on trypanocidal effect of sesquiterpene lactones including helenalin and mexicanin on cultured epimastigotes has been analyzed concluding that both are deleterious for *T. cruzi* epimastigotes and that their mechanism of action is different from that of the related lactone, dehydroleucodine [316]. Besides, the ethyl acetate extract from leaves plus inflorescences of *Lychnophora salicifolia* showed significant trypanocidal activity against trypomastigote forms of *T. cruzi*, which was due to the flavonoid quercetin-7, 3', 4'-trimethyl ether and the sesquiterpenoid lychnopholic acid [317]. In addition, new sesquiterpene hydroperoxides with trypanocidal activity from *Pogostemon cablin* were also described by Kiuchi and co-workers [318]. Moreover, chemical constituents of *L. pohlii*, crude extracts from leaves

plus inflorescences of *L. pohlii* and the active sesquiterpene lactones lychnopholide, centratherin, goyazensolides, caffeic acid as well as the isolated flavonoids luteolin and vicenin-2 were analyzed for trypanocidal activity [319].

11- Macrophytes and Algae. Some Turkish freshwater macrophytes and marine macroalgae were assayed for their *in vitro* antiprotozoal activity. Whereas all crude extracts displayed appreciable trypanocidal activity on different trypanosomatids and plasmodia none of the extracts was active against *T. cruzi* [320].

Patents related to natural compounds. Among chemotherapeutic drugs derived from natural products, 6 patents claimed the use of natural extracts or synthetic natural products derivatives against Chagas disease (Table 5).

-Quinoline derivatives. A series of 3, 3-dimethyl-8-oxoisoquinoline derivatives from natural naphthylisoquinoline alkaloids, originally isolated from African plants, were synthesized. These compounds claimed as useful antiviral and antitumoral agents as well as for treatment of neurodegenerative diseases showed *in vitro* activity on *T. cruzi* infected L6-cells with values ranging from 10 to 50 µM. One of them displayed selectivity towards the parasite but with cytotoxicity on the cells at concentrations higher than 440 µM [321]. Similar claims were also described for 1-phenyl-2-aminomethyl naphthalene derivatives. Among them, a sulfonate derivative showed selectivity and an IC₅₀ value in the µM range [322]. Moreover, antidesmone, an isoquinoline alkaloid isolated from different species of Euphorbiaceae and Ancistrocladaceae A and B, two new bioactive naphthylisoquinolines, and related naphthoic acids which were isolated from *Ancistrocladus ealaensis*, exhibited *in vitro* activity against *L. donovani* and *T. cruzi* [323]. Some of these tetrahydroisoquinoline derivatives were disclosed as useful for treating tropical diseases, especially leishmaniasis, trypanosomiasis or Chagas disease showing *in vitro* activity against *T. cruzi* with high IC₅₀ values ranging from 0.02 to 30 µM [324].

-Derivatives from plant extracts. The use of canthin 6-one and its derivatives, extracted from *Zanthoxylum chiloperone*, were disclosed for treatment of *T. cruzi* infection. This natural compound was assayed in chronic and acute mouse models resulting in both cases more effective than benznidazole. Mice treated with this drug resulted parasite free and protected from death [325]. The lignans obtained from leaves of *Zanthoxylum naranjillo* such as cubebin, or methylpluviatolide showed trypanocidal activity [302]. A patent was presented by Fundação Pesquiza do Estado de São Paulo claiming that these lignans and semisynthetic dibenzylbutyrolactonic derivatives were useful for the treatment and prophylaxis of Chagas disease. Assays performed with cubebin with blood trypomastigotes showed IC₅₀ values ranging from about 2 to 270 µM. Some of them displayed *in vitro* total inhibitory activity as well as were claimed to show chemoprophylactic ability into healthy mice [326]. Recently, five cubebin derivative compounds have been evaluated on *in vitro* assays against free amastigotes finding that hinokinin was the most active compound (IC₅₀ = 0.7 µM) [327].

Table 5. Patent Protected Drug Targets, Natural and Synthetic Trypanocidal Compounds

	Target	Inhibitor	Patent Number
Part I	CPs	CP inhibitors	<p>2001: WO0195911 [51] 2002: WO0240462 [52]; WO02057246 [53]; WO02057248 [54]; WO02057249A1 [54]; WO02057270A1 [54]; CA2436462AA [54]; WO00217924 [62] WO02100849 [65]; 2003: EP1362052A1 [55]; NO20033220 [55]; WO0248097A1 [58]; WO0248097B1/C2 [58]; WO03053331 [60]; WO03103574 [61]; WO03104257 [61]; WO03097593 [61]; WO03097664 [67] 2004: CN1486320A [56]; MX3006224A [56]; ZA0305259A [56]; NZ0526913A [56]; WO04007501A1 [57]; WO04020441A1 [63] WO04110988A1 [64] 2005: US20056958358 [59]; US20056897240 [78] 2006: US20066982263 [66]</p>
	Ergosterol Biosynthesis	OSC inhibitors C14 demethylase	<p>2000: WO0076316A1 [120] 2003: WO03006012A1 [133]</p>
	Synthesis of Poliisoprenoids	PFT inh. (in <i>T. brucei</i>)	<p>2001: WO00105384A3 [149] 2003: US2003134846A1 [150]</p>
	Redox metabolism	TR inh	2000: WO0050431A1 [171]
	DNA nucleotide synthesis	DHFR inh	<p>2001: WO0153276A1 [227]; WO0114401A1 [229]</p>
	Acidocalcisome nucleus	Exch. Na ⁺ /H ⁺ inh DNA binder antimitotic drugs topoisomerase II	<p>2000: US20006114393 [242] 2002: WO02057224 [237] 2003: WO03090678 [239] 2005: US20056967205 [233]; US20056906076 [240]</p>
	Sialic acid transference	Neuraminidase / sialidase inh.	<p>1999: WO9906369A1 [251] 2000: US20006114386 [252]</p>
Part II	nd	Natural compound and its derivatives	<p>2003: WO03000272A1 [324]; WO 03080600A1 [326] 2004: WO04067514A1 [321]; WO 04065349 [322]; WO04050092A1/B1 [325]</p>
Part III	nd	Synthetic compounds	<p>2000: BR 09805381A [346] WO0032201A2 [347]; 2004: WO04062590 [345]; WO04080390 [348]</p>

* 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

PART III. DESIGN AND SYNTHESIS OF NEW ANTI *T. CRUZI* DRUGS

The literature describes rationally developed drugs, some of them active against different parasitic forms as potential new candidates for the treatment of the Chagas disease [328]. Recent advances include:

-Benzotropolone derivatives. This new synthetic series of compounds bearing an endocyclic hydrazine moiety were evaluated as potential anti-protozoal agents showing limited or no *in vitro* activity against *L. donovani*, *P. falciparum* and *T. brucei rhodesiense*. However, several of these compounds were active against *T. cruzi* in the μM range, comparable to that of benznidazole [329].

-Nitroimidazole derivatives. New 1, 3, 4-thiadiazole-2-arylhydrazone derivatives of nitroimidazole or phenyl series were synthesized. The evaluation of the activity against bloodstream trypomastigote forms of *T. cruzi* allowed the identification of brazilzone A, a new potent trypanocidal compound which present an IC₅₀/24 h=5.3 μM [330].

-Aromatic diamidines. Furamidine (DB75) and its phenyl-substituted analogue (DB569) were tested. DB569 displayed higher trypanocidal activity compared to furamidine and also had higher ability to induce apoptosis-like death in treated parasites [331,332].

-Melamine-based nitroheterocycles. Various nitro heterocycles compounds were tested on different trypanosomatids. Some of them showed significant activity *in vitro* against *T. cruzi* [333].

-Quinone compounds A set of 25 quinone compounds with anti-trypanosomal activity were studied by using the density functional theory (DFT) method. Two of them were predicted as active against *T. cruzi* [334].

-Thiosemicarbazone derivatives. A series of new derivatives were designed combining in the same molecule the thiosemicarbazone function recently described as a potent C_z-inhibitor moiety and the recognized 5-nitrofuryl group, an oxidative stress promoter. Some of the derivatives were found to be very active against epimastigotes, being 1.5-1.7-fold more active than nifurtimox [335].

-Quinoxaline derivatives. *in vitro* Assays of some synthetic compounds presented similar inhibitor growth activity than nifurtimox. Among them, 13, a quinoxaline N, N'-dioxide derivative, and the reduced derivatives 19 and 20 were the most cytotoxic compounds against the protozoan [336].

-Benzofuroxan derivatives. A series of new benzo [1, 2-c] 1, 2, 5-oxadiazole N-oxide derivatives as antitrypanosomal compounds were generated. *in vitro* Activity of these compounds was tested against *T. cruzi*. The most effective derivatives showed IC₅₀ of the same order as that of the reference drug [337,338].

-2-propen-1-amine derivatives. The *cis* and *trans* isomers of the unsubstituted and bromo-2-propen-1-amine derivatives were evaluated *in vitro* and *in vivo* assays on *T. cruzi*. It was suggested that these derivatives should inhibit the enzyme squalene synthase of the parasite ergosterol biosynthesis pathway [339].

-N-oxide derivatives. 3-Cyano-2-(4-iodophenyl)-2H-indazole N1-oxide among a series of synthesized N-oxide derivatives exhibited interesting antichagasic and leishmanocidal activity in some of the parasitic strains evaluated [340]. In addition, three series of benzimidazole N-oxide derivatives were developed and were examined for their activity against trypanosomatid parasites in *in vitro* and *in vivo* assays (*T. cruzi* and *Leishmania spp.*). Among them, the series of 2H-benzimidazole 1, 3-dioxides displayed remarkable *in vitro* activities against both parasites resulting selective toward both trypanosomatid parasites [341].

-Goniothalamine analogues. Sixteen 5, 6-dihydro-2H-pyran-2-ones were evaluated in an *in vitro* assay against trypomastigotes forms of *T. cruzi*. The relevant structural

features for the trypanocidal activity of these goniothalamine analogues against *T. cruzi* were established by a structure-activity relationship study (SAR), finding that the non-natural form of goniothalamine was threefold more potent than the natural styryl lactone. Some analogues were identified as potent compounds against *T. cruzi* with IC₅₀ values in the mM range and significant low toxicities [342].

Finally, in the search for new antitrypanosomal compounds, computational approaches were described. A novel non-stochastic quadratic fingerprints-based approach was satisfactorily applied for virtual evaluation. The antitrypanosomal activity of a series of 10 already synthesized compounds was *in silico* predicted as well as *in vitro* and *in vivo* explored against *T. cruzi*. The model was able to predict correctly the behaviour of these compounds in 90 % of the cases [343]. A new ligand-based approach applying non-stochastic linear fingerprints to the identification of potential antichagasic compounds was recently introduced. A few compounds with trypanocidal activity against epimastigotic forms of *T. cruzi* were predicted with a confidence of 95 % [344].

Patents related with screened synthetic compounds

Among them, the synthesis and *in vitro* activity of different bicyclic carbohydrates as antiprotozoal bioactive for the treatment of parasitic diseases, such as leishmaniasis and trypanosomiasis was disclosed by Kemin Pharma Europe [345]. Besides, scientist from the Universidade Estadual de Campinas in Brazil described the synthesis and *in vitro* activity against the different stage forms of *T. cruzi*, among other parasites from a series of 4-bromophenyl metanone and 2-propen-1-amine derivatives, claiming that among them a furanil derivate showed a considerable IC₅₀ value (9.5 μM) [346]. In addition, Hollis-Eden Pharmaceuticals, Inc., claimed the use of 17-ketosteroid compounds and derivatives, metabolites and precursors in the treatment of Malaria and African and American trypanosomiasis or to ameliorate or reduce one or more symptoms associated with a *Plasmodium* or *Trypanosoma* infection [347]. Later, Merck and Co, Inc presented a series of novel synthesized imidazopyridine compounds and N-oxide derivatives and claimed to be useful in the treatment and prevention of protozoan diseases including Trypanosomiasis Americana among other parasitic diseases but no relevant biological data were included [348] (Table 5).

CURRENT & FUTURE DEVELOPMENTS

The drugs available for the treatment of the Trypanosomiasis Americana are not satisfactory; they present toxic side effects and are expensive. Moreover, there is currently no drug effective once the disease has progressed to the chronic stage. In addition, these drugs are not dispensed in pediatric version, which complicates the treatment of children. The disease affects 16-18 million people in the Americas, particularly in South America, only in Argentina about two million people are infected and it is estimated that from 5000 to 10000 people die every year. Therefore, there is an urgent need to solve this problem. However, it is well-known that pharmaceutical industry has restricted investment in research and development of diseases affecting primarily poor populations in low-income developing countries [15].

The identification of new antichagasic agents may be based not only on rational drug design and synthetic or natural products screening [14], but also taking advantage of compounds already in use against other human diseases which have already passed several of the clinical trials necessary for the development of any new drug. The current state of knowledge of parasite biochemistry has favoured the development of new chemotherapeutic approaches based on newly validated biochemical targets. Multiple metabolic pathways and specific enzymes useful for the development of targeted trypanocidal drugs have been investigated. In addition, as a result of the parasite genome sequencing project, available since 2005 [17] the possibility of identifying new specific pathways and novel drug targets in the near future is open.

As it has been shown, the biology of the parasite has been intensively studied and a large number of compounds have emerged, however, despite all the new information available, a true applicable drug has not been identified so far. Thus, a huge effort of the global research community is needed, gathered to sustainable financial resources, in order to translate the basic scientific knowledge into a number of selected drug candidates in the pipeline. In fact, only some cysteine proteinase inhibitors and ergosterol biosynthesis inhibitors are currently in the pipeline.

A search through the patent literature during the last five years involving parasitocidal activity against *T. cruzi* was performed including target-based drugs, natural products and its derivatives and new synthetic compounds as well as old ones rediscovered as novel drugs against Chagas disease. Most patents found are related with specific target-based drugs, and some of them that claim compounds useful for the treatment of human diseases such as various cancers, bone diseases or antiviral activity also report possible trypanocidal activity. Among them, can be mentioned those related with cysteine protease inhibitors, purine analogues, organometallic complexes. Others disclose compounds with specific protozoal, parasitic or trypanocidal activity as the main claim, including Chagas disease [227,237,242]. Only some of them are related with targets in pipeline (CPIs, sterol biosynthesis inhibitors). It is worth mentioning that despite the abrupt increase of knowledge about the parasite biochemistry, this is not reflected in the number of disclosed patents, furthermore only a few number of the analyzed patents showed specific data of biological anti-*T. cruzi* activity.

Whereas most patents found are related with parasitic targets disclosing interesting *in vitro* activity against *T. cruzi*, only a few important *in vivo* results were reported. In particular, those inhibitors based on the drug induced blockade of specific enzymes involved in sterol biosynthesis specially, C-14 demethylase [133] and OSC can be mentioned [120]. In the last years, patents related with CPIs were the most represented among those claiming potential chemotherapeutic agents against *T. cruzi*, involving azapanone based inhibitors [60] or thiosemicarbazone and semicarbazone inhibitors [78].

Among patents related with natural compounds, two of them showed interesting results. Some cubebin derivatives showed total *in vitro* inhibitory activity [326]. In addition, cantin-6-one derivatives showed not only an important

inhibitory activity but also showed interesting *in vivo* results [325] suggesting that screening of natural products as well as libraries of synthetic compounds against *T. cruzi* should allow the discovery of new trypanocidal lead compounds. Moreover, structural studies as well as molecular dynamics leading to the development of more potent antichagasic drugs based on specific molecule analogues should help to find *Trypanosoma cruzi* specific chemotherapeutic agents. On the other hand, multiple libraries of synthetic compounds are under evaluation and some of them were patented as possible antitrypanosomal agents. However, no clinical successful results have been shown yet. Hence, as Dardonville suggested, a pragmatic approach for the rapid development of new anti-*T. cruzi* chemotherapy would be based on the clinical assessment of drug combination with existing trypanocides [349]. In fact, synergistic effects between an antiarrhythmic compound commonly prescribed for the symptomatic treatment of the disease with azole drugs have been recently reported [115] opening the possibility of novel approaches including combination of current approved drugs for the treatment of the disease.

Under the light of the results obtained so far, despite the multiple efforts done, currently there are no drugs in clinical trial for Chagas disease. Unluckily, Chagas disease still remains a challenge for effective chemotherapy.

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