3. Recent advances in the experimental chemotherapy for Chagas disease

Wanderley de Souza¹,²,³, Emile Barrias³, Phercyles Veiga-Santos¹,² and Tecia Maria Ulisses de Carvalho¹,²

¹Laboratório de Ultraestrutura Celular Hertha Meyer, CCS, Instituto de Biofísica Carlos Chagas Filho
Universidade Federal do Rio de Janeiro, Bloco G, Ilha do Fundão, Rio de Janeiro, CEP 21941-902
Brazil; ²Instituto Nacional de Ciência e Tecnologia em Biologia Estrutural e Bioimagens
Universidade Federal do Rio de Janeiro, Cidade Universitária, Ilha do Fundão, Rio de Janeiro, Brazil; ³Instituto Nacional de Metrologia, Normalização e Qualidade
Industrial–INMETRO, Duque de Caxias, Rio de Janeiro, Brazil

Abstract. Trypanosoma cruzi, the causative agent of Chagas’ disease, which affects a large number of individuals in Central and South America, is transmitted to vertebrate hosts by blood-sucking insects. This protozoan is an obligate intracellular parasite. The infective forms of the parasite are the metacyclic trypomastigotes released with the feces of the insect. After invading the mammalian host, they penetrate cells, multiply as amastigotes, and transform again into trypomastigotes, which in turn are released into the bloodstream. In this review, we describe basic aspects of the biology of Trypanosoma cruzi and its interaction with host cells. Special emphasis is given to structures, organelles, and metabolic pathways that may constitute potential targets for the development of new antiparasitic drugs.
Introduction

*T. cruzi* and its life cycle

Protozoa of the *Trypanosomatidae* family are agents of parasitic diseases that have a high incidence of occurrence and a negative economic impact in developing countries. Among them, leishmaniasis, sleeping sickness, and Chagas disease - caused by several species of *Leishmania*, *Trypanosoma brucei*, and *T. cruzi*, respectively, are the most relevant. One characteristic feature of the trypanosomatids is the ability to change their general shape during the life cycle. In those species switching from the vertebrate to the invertebrate host, these changes may be dramatic, involving the appearance of developmental stages that do not divide and stages which are highly infective. This is achieved through a process generally described as protozoan differentiation or transformation (De Souza et al., 2002). Among the trypanosomatids, *T. cruzi* presents one of the most complex life cycles, involving several developmental stages found in the vertebrate and the invertebrate hosts, as well as in the bloodstream and within vertebrate host cells. Figure 1 shows a general view of the life cycle. The cycle starts with insects from the Reduvidae family sucking the blood of vertebrates infected with the parasite and containing trypomastigote forms circulating in the bloodstream. Once ingested together with the blood, most of the trypomastigotes are lysed following interaction with bacteria existent in the insect stomach (Castro et al., 2007). The surviving trypomastigotes transform a few days later either in spherical forms, known as spheromastigotes, or in epimastigote forms. Organisms in the latter form migrate to the intestine, where they intensely divide and attach through a specific interaction process involving recognition of surface-exposed GPI-linked macromolecules by components of the intestinal cell surface and the perimicrovillar membranes (Alves et al., 2007; Nogueira et al., 2007). At the most posterior regions of the intestine and at the rectum, many epimastigotes detach from the intestinal surface and transform into trypomastigote forms that are subsequently released together with the faeces or with the urine (Garcia et al., 1991). These forms are also designated as metacyclic trypomastigotes and are highly infective for several mammalian species, including humans. Usually the infection of mammals takes place through direct inoculation of these forms through the ocular mucosa or skin lesions from insect bites. Other important mechanisms of transmission are by blood transfusion, transplacental transmission, and organ transplant, though these are much less common methods of transmission at present due to programmes of vector control and careful analysis of blood donors. More recently, it has been shown that these forms are also infective through an oral route (Nobuko, 2009).
Figure 1. Life cycle of *Trypanosoma cruzi*, showing the various forms of the protozoan in the invertebrate (triatomine) and vertebrate (mammalian) hosts. Figure reproduced from the Center of Disease Control (CDC) homepage.

Once in the vertebrate host, the metacyclic trypomastigotes invade cells found in the inoculation site (fibroblasts, macrophages, and epithelial cells, for example) through several endocytic mechanisms in a process involving at least three well-defined stages: (i) adhesion and recognition, (ii) signaling, and (iii) invasion. Following invasion, the parasite is found within an endocytic vacuole known as the parasitophorous vacuole (PV). A few hours after cell infection, the long and thin trypomastigote forms gradually become rounded and have a short flagellum, corresponding to the amastigote form. The membrane lining the PV is lysed gradually, and the trypomastigote-amastigote transition form enters into direct contact with the host cell cytoplasm. After several successive divisions, a large number of amastigotes are formed within the host cell. Then, they start a process of transformation into highly motile trypomastigote forms that are subsequently released into the intercellular space following host cell rupture. The trypomastigotes are then able to penetrate into new host cells.
Clinical aspects and epidemiology

Following natural inoculation of *T. cruzi* into humans during a triatomine blood meal, an acute phase of the disease may take place after some days or weeks, depending on the infection route. Most of the time this phase is asymptomatic, but in some cases a periophthalmic unilateral oedema, regional lymphoadenopathy, hepatosplenomegaly, and fever may be observed. The acute phase is usually more severe in children, of whom up to 10% may succumb to acute myocarditis. Patients surviving this phase enter into a chronic phase that can be either asymptomatic or symptomatic. In symptomatic cases, heart problems (arrhythmia, palpitation, syncope, dyspnea) as well as digestive alterations (loss of peristalsis, regurgitation, dysphagia, and progressive dilatation of the oesophagus and colon) may be observed (Rassi et al., 2010).

It is estimated that approximately 8 to 10 million individuals are infected in the Americas (Rassi et al., 2012) and nearly 80 million are at risk of infection (www.WHO.org). The number of infected people decreases every year due to better control of the transmission routes, especially from improvements in housing, as well as vigilance over blood transfusion quality. However, in recent years there has been an increase in the number of oral infections due to the ingestion of fruit juices in the Amazon region (açai and guayaba).

Complete control of transmission is almost impossible, mainly due to the wide distribution of the parasite in many Reduvidae insects, as well as in many domestic and peridomestic animals. Chagas disease is found throughout the Americas, from Argentina to some parts of the United States. Most of the cases of Chagas disease are concentrated in Argentina, Bolivia, and Brazil (Figure 2).

Drugs currently used for Chagas disease treatment

Two nitro-heterocyclic compounds have been used for Chagas disease-specific treatment since the 1970s: nifurtimox (NFX) (4-{(5-nitrofurfurylidene)amino}-3-methylthiomorpholine-1,1-dioxide) (Lampit® Bayer) (Figure 3A) and benznidazole (BZN), an N-benzyl-2-(2-nitro-1H-imidazol-1-yl) acetamide, commercially known as Rochagan® and Radanil®, and initially produced by Roche (Figure 3B). Today this drug is commercially available by the Brazilian Pharmaceutical Laboratory of the Pernambuco state (LAFEPE). These compounds are very active when used during the acute phase of the disease, though with different efficacies depending on the geographical area of use, which probably reflects varying
susceptibility among the different *T. cruzi* strains. BZN was shown also to be effective if used during early chronic infections (reviewed in Coura and Castro, 2002; Urbina, 2009; Coura, 2009). These drugs present low antiparasitic effect during the chronic phase - the prevalent form of the disease - which is most likely due to their short terminal half-lives and limited tissue penetration (Urbina and Docampo, 2003). During the chronic phase of the disease, the parasite is mainly found inside host cells in a tissue, so a high concentration of the drug most likely fails to reach the parasites. Both drugs cause side effects in the patient, sometimes leading to the interruption of the treatment. The drugs must be used daily for 30 to 60 days.

The mechanism of action of NF is based on its reduction by nitroreductases, leading to nitroanion radical production that, in the presence
of oxygen, generates reactive oxygen species (ROS). *T. cruzi* was shown to be partially deficient in free radical detoxification mechanisms (Docampo and Moreno, 1986). NF and BZN were shown to be activated by nitroreductases from rat heart and pancreas, which may explain host cytotoxicity and the presence of side effects from using the drug (Urbina, 2010). NF is administered orally in 3 or 4 doses for 90 days as follows: between 10-15 mg/kg/day for children ≤10 years old, 12.5-15 mg/kg/day for those 11-16 years, and 8-10 mg/kg/day for those 17 years or older.

The mechanism of action of BZN seems to be the covalent modification of macromolecules by the molecules derived from the nitroreduction reaction (Docampo, 1990), although free radicals are produced during nitroradical autoxidation. BZN is commercially available in tablets of 100 mg and 12.5 mg. LAFEPE BZN of 100 mg was included in the World Health Organization’s (WHO) Essential Medicines List in 1988. The pediatric version of 12.5 mg was developed in association with the Drugs for Neglected Diseases Initiative (DNDi; http://www.lafepe.pe.gov.br/lafepe/). BZN dosage regimens are 10 mg/kg/day for children younger than 12 years and 5–7 mg/kg/day for those 12 years or older. The drug is administered orally in two divided doses for 60 days.

Since BZN is only used in the acute and early chronic phases of infection, a randomized double-blind controlled clinical trial investigating the role of BZN in patients with chronic Chagas heart disease was launched. This project evaluated BZN treatment of chronic patients in several countries from South America, including Brazil (Viotti et al., 2006). This study showed that treatment with BZN reduces the number of patients demonstrating progression to severe cardiomyopathy, suggesting an alteration of the evolution of the disease. PCR is being used as a tool to establish the cure criteria of patients, towards the establishment of a consensus real-time qPCR to monitor *T. cruzi* parasitemia in patients with chronic Chagas disease cardiomyopathy (a substudy from the BENEFIT trial) (Moreira et al., 2013).

**Potential chemotherapeutic targets**

For many years, antiparasitic drugs were selected based on tests carried out with all available compounds. Although such an approach is still used, more recent efforts have focused on identifying new parasitic targets, availing of better knowledge of the parasite’s biology, its main components, and specific metabolic pathways. In this review, we will focus mainly on some structures and organelles found in trypanosomatids that are potential drug targets due to their characteristic features. However, some metabolic pathways, as well as key molecules, will be also mentioned.
Structures and organelles

The structural organization of *T. cruzi*, as revealed using several microscopical techniques, has been reviewed recently (De Souza, 2008; De Souza, 2009). Here, we will concentrate only on those structures and organelles that, in our view, constitute potential drug targets.

The kinetoplast-mitochondrion complex

Morphological studies have shown that all trypanosomatids contain a dark, electron-dense and slightly bent inclusion known as the kinetoplast. It is situated close to the nucleus and localized in a portion of the unique and highly ramified mitochondrion, which is in close association with the basal body from where the flagellum originates. The kinetoplast appears as a dense structure and is made of a special type of DNA, known as the kinetoplast DNA (kDNA) (Figure 4, A-C). Filamentous structures connect the kinetoplast to the basal body (Figure 5) (Souto-Padron et al., 1984; Ogbadoiry et al., 2003). A protein designated as p166 was identified and shown to be located in between the kDNA disk and the flagellar basal body (Zhao et al., 2008).

Histone-H1-like proteins are known to be involved in the condensation of *T. cruzi* kDNA (reviewed in Cavalcanti et al. 2008). Two types of circular DNA are present in the kinetoplast: minicircles and maxicircles. There are several thousand minicircles, which range in size from 0.5 - 2.5 kb (depending on the trypanosomatid species), and a few dozen maxicircles, which range from 20 - 40 kb (reviewed in Shapiro and Englund, 1995). The minicircles encode guide RNAs that modify the maxicircle transcripts by extensive uridylate insertion or deletion, a process known as RNA editing.

![Figure 4](image-url) **Figure 4.** Kinetoplast organization of trypomastigote (A), amastigote (B) and epimastigote (C) forms of *Trypanosoma cruzi*. F: flagellum; K: kinetoplast; N: nucleus. The white arrows point to the area of adhesion of the flagellum to the cell body. White arrowheads point to profiles of the endoplasmic reticulum. Bars =1 μm. (After De Souza et al., 2009 – figures A and B; after Rocha et al., 2006 – figure C).
Figure 5. Kinetoplast of *Trypanosoma cruzi*’s epimastigotes, as seen in a replica of quick-frozen, freeze-fractured, deep-etched and rotary-replicated cells. The network of DNA molecules which make the kinetoplast (K) is shown, as well as filamentous structures (arrow) connecting the basal bodies (BB) to each other and to the mitochondrial membrane. The axonemal microtubules that comprise the flagellum (F) are also seen. Bar =100 nm. (After Souto-Padron *et al.*, 1984).

The maxicircles are structurally and functionally analogous to the mitochondrial DNA that encodes rRNAs and subunits of the respiratory complexes in higher eukaryotes. In situ analysis of the kDNA structure revealed that the network is formed not by circles, but by irregular polygonal structures (Figure 6). Similar images of the whole kDNA network have been obtained using atomic force microscopy (Cavalcanti *et al.*, 2011). In light of the special characteristics of the kDNA, the observations that it is essential for life of the parasite, and the fact that it is not found in mammalian cells, this structure has been considered an important target for the development of new antiparasitic drugs.

Acridine and acridine derivatives have been tested as possible drug candidates since the 1980s (De Castro, 1993; reviewed by Coura and De Castro, 2002). One of these compounds is a bisacridine (*bis*(9-amino-6-chloro-2-methoxyacridin)), which showed co-localization with kDNA of epimastigotes (Girault *et al.*, 2000). Another class of acridine derivatives that demonstrates activity against *T. cruzi* includes 9-thioalkylacridines (Bsiri *et al.*, 1996). 9-Amino and 9-thioacridines have been reported to inhibit the enzyme trypanothione (Bonse *et al.*, 2000). Recently, with the use of DNA-binding drugs, it was reported that a compound named berenil caused remarkable kDNA disorganization in epimastigote forms (Zuma *et al.*, 2011).
Metal-based drugs based on a vanadyl complex, including the bidentate polypyridyl DNA intercalator, are capable of inhibiting *T. cruzi* growth. Using gel electrophoresis and atomic force microscopy, it was suggested that the complex interacts with DNA (Benitez et al., 2009). Members of the same group have used another compound based on a vanadyl complex which demonstrated activity against amastigotes in concentrations significantly lower than that which is toxic for mammalian host cells (Benitez et al., 2011; Benitez et al., 2013). Other metal compounds that have demonstrated activity against trypanosome DNA are mixed-chelate copper (II) complexes such as Casiopeina®. Becco and colleagues (2012) showed that the interaction of casiopeinas trigger DNA cleavage by a free radical mechanism.

Another way to interfere with DNA is with the use of drugs that target enzymes involved in the kDNA replication, transcription and segregation known as topoisomerases (Zuma et al., 2011). The first topoisomerase-targeting experiments were based on the use of the antibiotic novamycin, which has been previously shown to inhibit the bacterial enzyme topoisomerase II (DNA gyrase), thus interfering with amastigote DNA synthesis.

**Figure 6.** Organization of the kinetoplast DNA fibers in maxicircles and minicircles. The kinetoplast DNA network was dispersed in water, collected in a grid, and shadowed at a low angle with platinum. Bar =0.3 μm. (courtesy of David Pérez-Morga).
(Kerschmann et al., 1989). Posteriorly, using novamycin and another class of topoisomerase inhibitors (ofloxacin and the commercial compound Tarivid), Gonzales-Perdomo and colleagues (1990) showed that epimastigote-metacyclic trypomastigote or amastigote-trypomastigote transformation was inhibited by these compounds in a dose-dependent manner. Using these compounds, different physiological effects were observed, which could be attributed to factors such as permeability to cell membranes, resistance due to alterations in drug-binding sites of distinct enzymes, and pharmacokinetic properties. The knowledge about the mechanism of action of topoisomerase inhibitors can stimulate the development of selective drugs against pathogenic trypanosomatids.

The glycosome

All trypanosomatids contain spherical structures with a homogeneous matrix that are surrounded by a unit membrane and distributed throughout the cell. These are a special type of peroxisome, designated as glycosomes, due to the concentration of glycolytic pathway enzymes in their matrices (reviewed in Opperdoes, 1987; Opperdoes and Borst, 1977). In other cells, glycolysis takes place in the cytosol. Like other peroxisomes, glycosomes contain proteins involved in metabolic pathways, such as peroxide metabolism, $\beta$-oxidation of fatty acids, carbon dioxide fixation, purine salvage, de novo pyrimidine biosynthesis, fatty acid elongation, sterol biosynthesis, isoprenoid biosynthesis, and ether phospholipid synthesis (Opperdoes and Cotton 1982; reviewed in Opperdoes, 1987). The glycosome does not possess a genome; therefore, all of the proteins found in it are encoded by nuclear genes, translated on free ribosomes, and post-translationally imported into the organelle. The biogenesis of the glycosome seems to be similar to that of peroxisomes and involves the participation of proteins known as peroxins (PEX). These proteins participate in protein import into glycosomes, a process that involves the presence of peroxisome targeting sequence (PTS). By genetic manipulation, it was shown that decreasing the expression of PEX leads to growth arrest and death of the parasites. These proteins also show a low degree of sequence conservation (reviewed by De Souza et al., 2002). These characteristics make peroxins interesting potential drug targets.

The acidocalcisome and contractile vacuole

For many years, morphological analysis of trypanosomatids has shown the presence of spherical structures distributed throughout the cell body and
initially designated as “polyphosphate granules” or “volutin granules”. Electron microscopy observations revealed that they appear as vacuolar structures containing electron-dense deposits. Biochemical and physiological studies have shown that these are acidic organelles capable of transporting protons and calcium, thus prompting the designation as “acidocalcisomes” (reviewed in Docampo et al., 2005) (Figure 7). Electron microscopy microanalysis has shown that acidocalcisomes contain calcium, phosphorous, sodium, potassium, zinc, and even iron (Miranda et al., 2004).

Acidocalcisomes have many functions, including: (a) the storage of calcium, magnesium, sodium, potassium, zinc, iron, and phosphorous compounds, especially inorganic pyrophosphate and polyphosphate, as determined by biochemical analysis and X-ray microanalysis, (b) pH homeostasis and (c) osmoregulation, a function that involves interaction of the acidocalcisome with the contractile vacuole (reviewed in Docampo et al., 2005). This later structure consists of several tubules connected to a central vacuole located close to the flagellar pocket (Linder and Staehelin, 1977; Girard-Dias et al., 2012). Aquaporin, a protein involved in water transport, was identified in T. cruzi epimastigotes and localized to both the acidocalcisomes and the contractile vacuole (Montalvetti et al., 2004). These structures seem to be involved in the process of osmoregulation. The fusion of acidocalcisomes to the contractile vacuole was shown to take place in a process mediated by cyclic AMP (reviewed in Rohloff and Docampo, 2008).

**Figure 7.** Acidocalcisome morphology observed by transmission electron microscopy. Bar =200 nm. (After De Souza et al., 2009).
The cytoskeleton

Transmission electron microscopy of thin sections of trypanosomatids showed the presence of sub-pellicular microtubules distributed throughout the cell body, except in the flagellar pocket region. The microtubules are separated by a constant distance from each other (about 44 nm) and from the plasma membrane (about 12 nm) in all portions of the cell body. In favorable sections, it is possible to see filamentous structures connecting the microtubules to the plasma membrane and to other microtubules. Profiles of the endoplasmic reticulum (ER) can be seen in between and below the sub-pellicular microtubules (Pimenta and De Souza 1985; Souto-Padron et al., 1984). It is important to point out that microtubules are dynamic structures, and in a process involving the binding and hydrolysis of GTP, these structures grow and shorten due to a reversible process of polymerization and depolymerization, i.e., reversible non-covalent addition and loss of tubulin dimers at their ends (Vaart et al., 2009).

The microtubule cytoskeleton is one of the major targets in cancer chemotherapy. Several compounds interact with tubulin, altering microtubule dynamics and leading to cell death. Microtubule stabilization or destabilization alters the cellular unpolymerized to polymerized tubulin ratio, thereby inducing cell death. Natural antimicrotubule compounds have been used in the clinic for many years and can be classified in three different groups: (i) alkaloids, obtained from Colchicum autumnale, including colchicine and colcemid; (ii) alkaloids obtained from Vinca rosea, including vinblastine and vincristine, and (iii) taxoids, such as Taxol, isolated from the bark of Taxol brevifolia. These compounds present different mechanisms of action, though all target the microtubules. The first two groups bind to the end of growing microtubules and prevent dimer addition, leading to the accumulation of mammalian cells at metaphase with abnormal mitotic spindles (Lobert and Correia, 2000). The taxoid compounds stabilize microtubules and prevent the disassembly process, which leads to a blockage of mitosis at the metaphase/anaphase phase, multinucleation, and ultimately cell death (reviewed by Jordan and Wilson, 1999). These drugs are potent antitumoral drugs; however, their clinical use is limited by hematological and neurological toxicities, as well as by tumor resistance, leading to tumor resurgence and death (Calligaris et al., 2010).

There have been several attempts to use antimicrotubule compounds to kill trypanosomatids (Chan and Fong 1990, 1994; Chan et al., 1991, 1993). These attempts altered the cell cycle, leading to the appearance of aberrant cells with multiple flagella, which was most likely due to interference with spindle microtubules (Chan et al., 1993; Grellier et al., 1999; Zaidenberg
et al., 2006). It is important to point out that *T. cruzi* is relatively resistant to classical drugs such as colchicine. Conversely, vinca alkaloids, Taxol, and especially the dinitroanilines, such as trifluralin and oryzalin, showed significant effects against the protozoan by affecting microtubule assembly and stability, inhibiting the processes of proliferation and metacyclogenesis, and decreasing the number of *T. cruzi* intracellular amastigotes (Baum et al., 1981; Bogitsh et al., 1999; Grellier et al., 1999; Traub-Cseko et al., 2001; Dantas et al., 2003; Rodrigues and De Souza, 2008).

Microfilaments have never been observed in the cytoplasm of *T. cruzi*. However, it has been shown (Correa et al., 2007; 2008) that cytochalasin B treatment leads to morphological alterations in cytoskeletal elements associated with the cytostome-cytopharynx complex, which is responsible for transferrin uptake. *T. cruzi* has, in addition to an actin gene, an expanded myosin family and a CapZ F-actin capping complex. Comparative genomic analysis identified a potential role for an actin-myosin system, and it has been suggested that an actin-myosin system might function at the cytostome. Actin and actin-binding proteins were recently characterized in *T. cruzi* (De Melo et al., 2008). *T. cruzi* actin (Tc Actin) was observed in several patch-like cytoplasmic structures, distributed throughout the *T. cruzi* developmental stages. However, while the structure of Tc Actin is similar to that of higher eukaryotic actin, homology modeling has revealed fundamental differences, predominantly in the loops responsible for oligomerization and interactions with actin-binding proteins. As a consequence, actin filaments have never been detected in *T. cruzi."

**The flagellum**

All trypanosomatids have one flagellar complex, with a canonical basal body at the base of the flagellum. The flagellum consists of the typical array of nine pairs of peripheral microtubule doublets and one central pair (Figure 8). In addition, it contains an intriguing structure made up of a complex array of filaments linked to the axoneme that, due to its location, is called the paraxial or paraflagellar rod (PFR). Two regions, designated as proximal (consisting of two plates) and distal (consisting of several plates), were identified in the PFR. The plates are formed by an association of 25nm- and 7 nm-thick filaments that are oriented at an angle of 50° in relation to the major axis of the axoneme (Farina et al., 1986). Biochemical analyses have shown that the PFR is composed of a large number of proteins, most of which have not been characterized. However, two major proteins have been characterized in some detail. These proteins, known as PFR1 and PFR2, have molecular weights of 73 and 79 kDa, respectively. They are highly antigenic and thus
Figure 8. Flagellum of trypanosomatids as seen in longitudinal (A, D) and transversal (B, C) views of thin sections and in quick-frozen, freeze-fractured, deep-etched, and rotary-replicated samples (e). The axonemal (A) microtubules, as well as filaments that make the paraflagellar rod (PFR) structure, can be seen. Bridges connecting the axoneme to the PFR (arrows) and the plates that form the PFR are seen. Bar =50 (A–C) and 100 nm (D, E). (After Farina et al., 1986).

are potential targets for vaccine and diagnostic kit development. Available evidence indicates that the PFR is an essential structure for parasite survival (reviewed in Bastin and Gull, 1999).

The endocytic pathway

Trypanosomatids are highly polarized cells, and their endocytic activity is restricted to the flagellar pocket and cytostome regions (reviewed in De Souza et al., 2009). Studies of *T. cruzi* have shown that this protozoan exhibits certain peculiarities in its endocytic pathway that distinguish it from other cells. First, endocytosis only occurs at high levels in the epimastigote form. Endocytosis is low or absent in metacyclic and bloodstream...
trypomastigotes and in intracellular amastigotes. Second, epimastigotes have two sites in which macromolecule uptake takes place: the flagellar pocket and a highly specialized structure known as the cytostome. Third, the cargo of the endocytic vesicles is delivered to unusual structures called reservosomes, which are located at the posterior end of the cell. The cytostome is a plasma membrane invagination that has a diameter of up to 0.3 μm and is coupled to a few special microtubules that penetrate the cell almost to the nucleus. Following binding to the cytostome and flagellar pocket, macromolecules are rapidly internalized and appear in small endocytic vesicles, which bud from regions of these structures. Coated vesicles, suggestive of a clathrin coat, were first reported to bud from the Golgi complex. Transferrin uptake was shown to be dependent on membrane cholesterol as well as cytoskeletal elements that are associated with the cytostome (Corrêa et al., 2007, 2008). In silico analysis revealed the presence of clathrin, adaptin, and clathrin self-assembly genes. Moreover, clathrin expression in T. cruzi has been demonstrated with western blots using polyclonal antibodies raised against bovine clathrin heavy chain. T. cruzi clathrin (Tc-Clathrin) has been localized to the Golgi complex and flagellar pocket region. Curiously, agents that disturb receptor-mediated endocytosis do not impair transferrin uptake in epimastigotes (Correa et al., 2007; 2008).

The major protease of T. cruzi, cruzipain, belongs to the cysteine protease family. It is very active in epimastigotes and is concentrated in reservosomes. The enzyme is a glycoprotein that is synthesized in the ER-Golgi system as a proenzyme and is then targeted to the endocytic pathway. The pro-peptide sequence is necessary and sufficient to drive cruzipain to reservosomes (Souto-Padron et al., 1990).

It has been shown that typical reservosomes disappear during the in vitro transformation of epimastigotes into metacyclic trypomastigotes. Morphometrical analysis showed that the reservosomes may account for 6% of the total cell volume in epimastigotes. During the transformation into trypomastigotes, the lipid-like structures disappear first, followed by the contents of the reservosome matrix. (Soares et al., 1989)

Reservosomes are thought to be the final destination for macromolecules captured from the extracellular medium and are also a major site of accumulation of parasite proteases. This organelle most likely has lysosomal functions, especially because classical lysosomes have never been identified in T. cruzi. Nevertheless, aryl sulfatase activity, which is characteristic of lysosomes, has been detected inside small vesicles distributed all over the cell body of epimastigotes and trypomastigotes (Adade et al., 2007). The digestive function of these compartments has not been addressed.
**Metabolic pathways**

We will briefly describe some recent studies carried out on sterol biosynthesis and phospholipid biosynthesis.

**Ergosterol biosynthesis inhibitors**

Sterols are essential components of eukaryotic cells that maintain the cell viability and serve as precursors for biologically active molecules that regulate growth and development processes. Mammalian cells utilize cholesterol, which can be synthesized by endogenous pathways or can be obtained from exogenous sources through an endocytic process. Sterols with structures similar to cholesterol are found in other eukaryotic cells, such as sitosterol in plants and ergosterol in fungi and trypanosomatids (Janmey and Kinnunen, 2006). Figure 9 shows the various steps of the sterol biosynthesis pathway.

**Figure 9.** The biosynthesis of ergosterol and cholesterol, showing the main steps, the enzymes involved, and the known inhibitors. (After De Souza and Rodrigues, 2009).
In the 1980s, several azoles, ergosterol inhibitors first used against fungal infections, were assayed against *T. cruzi*. The compounds ketoconazole, itraconazole, or terbinafine hydrochloride showed suppressive, but surprisingly not curative, effects in *T. cruzi* infections in humans or experimental animals, and in most cases failed to stop progression of the disease (Docampo et al., 1981; Beach et al., 1986; Urbina et al., 1988, 2009). More recently, new triazole derivatives with potent and selective action against fungal and protozoa infections were developed and assayed with promising results against Chagas disease. The selective activities of the compounds D-0870 (AstraZeneca), posaconazole (SCH-56592; Merck & Co.), ravuconazole (Eisai), TAK-187 (Takeda), and albaconazole (Uriach) were obtained due to their exclusive target - the enzyme cytochrome P450-dependent lanosterol 14 \( \alpha \)-demethylase (CYP51) - an essential enzyme in sterol biosynthesis (Urbina, 2010). In recent studies, these drugs induced a parasitological cure in murine models of acute and chronic Chagas disease and were active against different *T. cruzi* strains previously shown to be resistant to nitrofuran and nitroimidazole drugs (Urbina and Docampo, 2003). Among these, the compound posaconazole is a promising drug for Chagas disease therapy due to its in vitro and in vivo antiparasitic activities, pharmacokinetic properties (long terminal half-life and large volumes of distribution), and its excellent safety profile in humans (Urbina et al., 1998; Moton et al., 2009; Buckner and Navabi, 2010). Posaconazole has been shown to eradicate amastigotes from cultured cardiomyocytes while allowing full reassembly of the host cell’s cytoskeleton and contractile apparatus (Silva et al., 2006). Very recently, the drug was shown to cure an immunosuppressed patient with concomitant chronic Chagas disease and systemic lupus erythematosus who had previously failed to achieve parasitological cure after BZN treatment (Pinazo et al., 2010). Posaconazole is already registered for the treatment of invasive fungal infections and is currently undergoing phase II clinical trials to evaluate its efficacy and safety for the specific treatment of chronic human Chagas disease (Urbina, 2010). However, there are some problems involved with the approval of this drug for clinical use, such as the fact that posaconazole is a complex compound which is difficult to synthesize, and therefore is too costly for the global cure of Chagas disease (Lepesheva et al., 2011). In addition, it has been shown that the antiarrhythmic drug amiodarone, frequently used for the symptomatic treatment of chronic Chagas disease in patients with compromised cardiac function, also selectively targets *T. cruzi* by inhibiting de novo ergosterol biosynthesis at the level of lanosterol synthesis (Benaim et al., 2006; Adesse et al., 2011). Surprisingly, amiodarone acts synergistically with posaconazole against *T. cruzi*, showing promising results in both in vitro and in vivo
assays. Both drugs, used synergistically, could be effective in arresting parasite proliferation in the mammalian host at doses lower than those required when each is used alone, reducing the potential cost of therapy or the toxic effects involved (Benaim et al., 2006; Veiga-Santos et al., 2012). Inside host cells, amastigotes exhibit myelin-like structures, membrane shedding, and plasma membrane disruption (Figure 10) when labelled with LC3B, therefore appearing to suffer autophagic death.

Another azole, the ravuconazole, also is a promising drug for Chagas disease treatment and is currently in phase II clinical trials as an antifungal agent. This compound is considered by the DNDi as an option for clinical development for Chagas disease therapy (Lepesheva et al., 2011).

Although azole derivatives are the most potent CYP51 inhibitors identified so far, other non-azole compounds can also inhibit the *T. cruzi* sterol 14α-demethylase and are promising alternative therapies. One such

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**Figure 10.** Transmission electron microscopy images of intracellular amastigotes of *Trypanosoma cruzi* treated with posaconazole and amiodarone for 96 h. (A) Untreated intracellular amastigote displays a normal kinetoplast (k), nucleus (n), mitochondrion (m) and flagellum (f). (B and C) Parasites treated with 0.25 nM posaconazole (PCZ) plus 1 μM amiodarone (AMD) shows formation of vesicles (black arrowhead) and vacuoles (white arrowhead) in the cytoplasm. (D and E) Parasite treated with 0.20 nM PCZ plus 1.5 μM AMD exhibits myelin-like structures (black arrow), membrane shedding (white arrow), and disruption of the amastigote plasma membrane. (F) Parasites treated with the same concentrations of PCZ and AMD demonstrate vacuoles that resemble modified reservosomes, with lipid inclusions (large white arrow). (After Veiga-Santos et al., 2012).
example is a pyridine derivative ChemDiv-3124-01167, assayed by Doyle and colleagues (2010). This compound showed excellent antiparasitic effects against *T. cruzi*, leading to curative activity against Chagas disease in a murine model.

**Phospholipid biosynthesis inhibitors**

Unlike mammalian cells that use the Kennedy pathway, trypanosomatids synthesize phosphatidylcholine using the Greenberg pathway, and these two different methods are very important in the surveillance of the parasite (Van Meer et al., 2008). The drug therapy based on alkyl phospholipids (APs) for the treatment of Chagas disease could offer an important means to selectively target the parasite, thereby avoiding any cytotoxic effects on the host cells. APs were first considered for their potential as immunomodulators and phospholipid antimetabolites, and have shown cytotoxic effects on malignant cells both in vitro and in vivo (Arthur and Bittman, 1998). The antitumor activity of APs is thought to be mediated by the inhibition of cell proliferation and invasion, and the differentiation - as well as induction and inactivation - of cytotoxic macrophages, which involves the inhibition of phosphatidylcholine (PC) synthesis (Giantonio et al., 2009), the inhibition of signal transduction enzymes (e.g., phosphatidylinositol-phospholipase C) (Berdel, 1991), and protein kinase C (Vogler, 1996), besides intracellular calcium level alteration. More recently, APs have been extensively tested in vitro and in vivo against *Leishmania* and *T. cruzi*, and these studies have reported selective antiparasitic activity (Bergman et al., 1994; Croft et al., 2003; Urbina, 2006). Some APs, such as ilmofosine, miltefosine, and edelfosine, are active against the three developmental stages of *T. cruzi*, causing plasma membrane alterations, decrease in electron-dense lipid inclusions, and cytoplasmic vacuolization (Santa-Rita et al., 2000). In *T. cruzi* amastigotes (the host cell proliferative stage), IC₅₀ values between 0.2 and 5.0 µM were reported (Santa-Rita et al., 2006). Recently, our group examined the effect of three ring-substituted alkyl phosphocholines bearing a cyclopentadecyl, an adamantyl, and a 1, 2, 3-triazolyl moiety - compounds TC19, TCAN26, and TC70, respectively. Two of them (TC70 and TCAN26), were highly effective against epimastigotes, amastigotes, and trypomastigotes of *T. cruzi* without significant host cell toxicity. The antiproliferative effects of TC70 and TCAN26 were more accentuated in intracellular amastigotes than in trypomastigotes, and were more efficient than those of miltefosine. The presence of a ring in the lipid portion most likely renders the respective compounds more active and less toxic than miltefosine (Calogeropoulou et al., 2008; Papanastasiou et al., 2010; Godinho et al., 2013). This result supports
the view that phospholipids of the membranes are important as a potential route for the development of new therapeutic agents to treat Chagas disease.

**Trypanothione reductase (TR) biosynthesis**

Trypanothione is a molecule involved in protozoan protection against oxidative stress, and is now considered as a virulence factor in Chagas disease (Piacenza et al., 2012). Thus, the use of molecules that interfere with trypanothione biosynthesis may lead to parasite death. Since this enzyme is not found in the mammalian host, it is an attractive target for structure-based drug design (Krauth-Siegel and Inhoff, 2003). Nowadays, inhibitors of TR are designed based on a rational design approach (Maccari et al., 2011), which has identified a tricyclic antidepressant framework as a selective TR lead-inhibitor (Benson et al., 1992). McKie and colleagues (2001) tested some peptide derivatives as specific TR inhibitors, demonstrating some effect on *T. cruzi*.

**Cysteine proteinases**

Cruzipain, or cruzin, is the major *T. cruzi* cysteine proteinase that is present in all stages of the parasite life cycle. This endoproteinase is the most abundant member among the cysteine-, serine-, threonine-, and metallo-proteinases, and it is expressed as a complex mixture of isoforms. Cruzipain has been demonstrated as essential for host cell infection and parasitic replication, and also plays multiple roles in disease pathogenesis. Besides, there are no human enzymes homologous to *T. cruzi*’s cysteine proteinase (Steverding et al., 2006; Caffrey and Steverding, 2009). Based on these facts, this enzyme has emerged as one of the best targets for the development of vaccines and drugs with anti-*T. cruzi* activity. It is one of the few *T. cruzi* molecules with the crystal structure currently available in the Protein Data Bank (Scharfstein et al., 1986). Since the mid-1990s, some groups have invested their efforts in the search for a candidate able to inhibit cruzipain. Chung and colleagues (1997) demonstrated that derivatives of an antimalarial drug known as primaquine are capable of affecting all stages of *T. cruzi*. Subsequently, Engel et al. (1998) demonstrated that a vinyl sulfone cysteine protease inhibitor (K777) blocked *T. cruzi* infection both in vitro and in a murine model. Subsequent tests showed that K777, orally administrated for 14 days, was able to drastically reduce heart damage in experimentally infected dogs (Barr et al., 2005). Studies with infected mice (acute phase) demonstrated that this drug can cure infection following treatment for 20–30
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days (Engel et al., 1998; Doyle et al., 2007). Studies in human hepatocytes showed no toxicity at concentrations up to 100 µM, whereas healing studies in mice used a range of 0.5–5 µM (Doyle et al., 2007). After more than a decade of research with K777 against Chagas’ disease, McKerrow and colleagues received permission in 2012 from the U.S. Food and Drug Administration (FDA) to launch a phase I safety trial of the compound (Leslie, 2011). K777 is currently considered a hope for this neglected disease.

The first efficient compounds against cysteine proteases are peptidic inhibitors, which present low toxicity. However, attempts have been made in the development of nonpeptidic inhibitors to improve their oral bioavailability and thus their efficacy. Attempts to identify nonpeptidic enzyme inhibitors have been made by the substrate activity screening (SAS) method, which consists of the identification of non-peptidic substrate fragments, substrate optimization, and conversion of optimal substrates to inhibitors. Based on this technique, rational compounds have been identified as acyl andaryl-oxyethyl ketone cruzain inhibitors that eradicate intracellular amastigotes with no effects to host cells (Brak et al., 2008). Another nonpeptidic compound identified was quinoxaline-N-acylhydrazone; however, this compound only inhibited proliferation of the noninfective form of *T. cruzi* (Romeiro et al., 2009). New cysteine protease inhibitors will be developed since the nuclear resonance magnetic structure of cruzipain has also been generated. This will facilitate analysis of molecular docking between inhibitors and the protein structure, allowing for the experimental testing of new competitive inhibitors.

Another class of compound known as thiosemicarbazones (TSCs) has been described as inhibitors of *T. cruzi* cysteine proteases by Du and co-workers (2002). Based on this, new 5-nitrofurane derivatives containing thiosemicarbazone moieties have been designed and synthesized, and show significant anti-*T. cruzi* activity (Aguirre et al., 2004; Rigol et al., 2005). Caputto and colleagues (2011) also demonstrated that TSCs derived from 1-indanones with different patterns of substitution in the aromatic ring inhibit *T. cruzi* growth without host cell toxicity.

Metal complexes of gold, platinum, iridium, palladium, rhodium, and osmium have been reported to have activity against a variety of trypanosomatids, but the molecular targets of these compounds have not been identified (Meshnick et al., 1984; Croft et al., 1992; Mesa-Vale, 1997; Mesa-Vale et al., 1998). Fricker and colleagues (1998) analyzed the effect of metal complexes against the cysteine proteases (cruzipain) from *T. cruzi* and observed that the most potent parasitic growth inhibitors were the two rhenium complexes (2(1H)-pyridinethionato-jS2)[2,6-bis[( mercapto-jS)methyl] pyridine-jN1]oxorhenium(V)(15) and chloro[2,20-(thio-jS)bis[ethanethiolato-
oxorhenium(V). Besides these, another two oxorhenium(V) compounds ((p-methoxyphenylthiolato-S)[2,6-bis[(mercapto-S)methyl]pyridine-N1]) oxorhenium (V) and (methanethiolato) [2,20-(thio-S)bis[ethanethiolato-S]] oxorhenium (V) and the palladium compound also inhibited T. cruzi intracellular growth.

**Host cell interaction**

In the case of intracellular parasites such as T. cruzi, it is very important to know in detail the mechanisms used by the parasite to penetrate into host cells. If penetration is blocked, there is no amplification of the infection that only takes place once the amastigote forms proliferate in the cytoplasm of the host cells. Trypomastigotes that remain in the intercellular space or in the bloodstream will be ultimately lysed. As mentioned previously, the process of interaction of infective forms (amastigotes and trypomastigotes) with the host cell involves a first adhesion step, with the participation of surface exposed molecules present on both interacting cells, a process of cell-to-cell recognition, which is subsequently followed by the initiation of an endocytic process involving several signaling pathways. We cannot exclude the possibility that molecules secreted by the parasite also play some role on this process, as clearly shown by members of the Apicomplexa phylum (Dubremetz et al., 1998). Adhesion and internalization are clearly different processes that can be separated. Adhesion was shown to only take place when the cells are allowed to interact at 4°C (reviewed in De Souza et al., 2010). Treatment with inhibitors of actin polymerization, such as cytochalasins, also allows a better observation of the adhesion step. Adhesion is a process that depends on receptors restricted to membrane domains. The adherence of the parasite to a host cell does not mean that invasion will take place.

Several molecules exposed on both cell surfaces, like glycoproteins, glycolipids, and lectin-like proteins, have been considered to be involved in this initial recognition (De Souza et al., 2010). **Figure 11** indicates the proteins that have been implicated in the interaction process. One of the most studied molecules, present in the plasma membrane of trypomastigote forms (but also in amastigotes, although in small amounts), is a protein with neuraminidase and trans-sialidase activity. This enzyme removes sialic acid residues (located at the position α-1-3) of glycoproteins, glycolipids, and oligosaccharides present in the environment and transfers them to acceptor molecules (named mucins) present in the plasma membrane of trypomastigote forms (reviewed in De Souza et al., 2010). Sialic acid has been shown to act as a modulating molecule in the adhesion process between T. cruzi and the host cell.
Figure 11. Schematic model summarizing the molecules involved on the parasite-host cell interaction process and exposed on the surface of a hypothetical host cell and in trypomastigotes of *Trypanosoma cruzi*. (After De Souza et al., 2010).

Since penetration of *T. cruzi* into host cells is a key step in the process of evolution of parasitism, drugs that inhibit it would also be potential candidates for the chemotherapy of Chagas disease. *In vitro* experiments have shown that drugs such as dynamin, which inhibits GTPases involved in membrane fission in endocytosis, inhibited cellular infection by *T. cruzi* by 98% (Barrias et al., 2010).

References


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