Challenges in the chemotherapy of Chagas disease: Looking for possibilities related to the differences and similarities between the parasite and host

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Abstract

Almost 110 years after the first studies by Dr. Carlos Chagas describing an infectious disease that was named for him, Chagas disease remains a neglected illness and a death sentence for infected people in poor countries. This short review highlights the enormous need for new studies aimed at the development of novel and more specific drugs to treat chagasic patients. The primary tool for facing this challenge is deep knowledge about the similarities and differences between the parasite and its human host.

Key words: Trypanosoma cruzi; Trans-sialidase; Trypanothione reductase; CYP51; Cruzipain; Tubulin

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Core tip: Chagas disease remains the most neglected parasitic illness the world. Here, we note that detailed knowledge of both the differences and similarities between the Trypanosoma cruzi parasite and the human host’s biochemical targets may be the key to developing novel effective drugs to treat patients who are suffering with this severe and debilitating sickness.
INTRODUCTION

American trypanosomiasis (or Chagas disease) is a parasitic illness that results from infection by the hemoflagellate protozoan Trypanosoma cruzi (T. cruzi). The discovery of this parasite was made in 1908 by Chagas[1], and it was followed by his complete description of the disease's pathology[2], as well as diagnostic methods[3]. This sequence of events has established Carlos Chagas as the only scientist in the entire history of medicine to elucidate all aspects of an infectious disease completely, from the etiological agent to the vector, transmission, and its hosts and clinical manifestations[4]. Carlos Chagas' discoveries were characterized by their unusual deductive reasoning steps: The initial identification of the etiological agent in primates from Minas Gerais State (and not the disease itself), followed by the identification of vectors and their association with the occurrence of recurring infestations caused by triatomines in the region [predominantly blood-feeding insects such as Triatoma infestans (T. infestans)]. The confirmation of the disease, which is usually the first step in the elucidation of an infectious disease process, was subsequently performed by observing the protozoa in the blood of individuals who were living under the precarious health conditions of the people in the region; they presented the lethargic symptoms of some well-known parasitic infections[5]. This unusual sequence of stages to understanding Chagas disease is the result of a process of social stratification in which the poorest individuals, who lived in wattle-and-daub houses in rural areas, were more vulnerable to T. cruzi exposure. Almost one hundred years after an important speech by Carlos Chagas for his inaugural lecture as the Professor of the Tropical Medicine Course[6] in which Chagas said that tropical diseases should not be analyzed through a simplistic approach, but as a biologically, culturally and economically complex phenomenon, Chagas disease remains neglected in all its aspects. At present, there is no effective drug that can cure chagasic patients.

After the publication of the first studies on Chagas disease in the early twentieth century, there was a series of disparaging campaigns against the relevance of Carlos Chagas’ work that were led by members of the Brazilian National Academy of Medicine[7]; it undermined the interest of Brazilian researchers in the disease during the years that followed. However, studies on Chagas disease continued to be performed around the world, and we highlight the contributions of the Argentine researcher Cecilio Romaña as one of the most important. Romaña made a great contribution to the classification of vectors as well as knowledge of the transmission and diagnosis of Chagas disease. Romaña described the first pathognomonic symptom associated with Chagas disease as follows: A one-sided bialalpebral inflammatory edema called unilateral conjunctivitis, which became known as the “mark of Romaña” in accordance with the recommendations of Evandro Chagas (Medical Doctor and son of Carlos Chagas), in recognition of Cecilio Romaña's contributions to our knowledge of the disease[8].

The transmission of Chagas disease occurs primarily through the bite of an infected triatamine bug on an individual. Triatomines are insects that usually belong to the genera Triatoma, Rhodnius or Panstrongylus, which are commonly known as “barbeiros” in Brazil and “kissing bugs” in the United States, due to their preference for biting the faces of sleeping people. These insect genera include more than 140 species, of which 61 are endemic to Brazil[9]. The insect’s bite itself does not cause the transmission of viable forms of T. cruzi. However, the triatomine's physiology is characterized by a short digestive apparatus, leading these insects to defecate upon blood suction, releasing the infective trypomastigote forms of the parasite, which are present in high quantities in their feces. The itching caused by the bite causes the individual to move the infective forms to the wound, where the parasite enters the bloodstream[10], causing infection. Despite the fact that the primary form of contamination is due to vector bites, there are other clinically relevant transmission pathways, with blood transfusion and organ transplantation among them[11]. Vertical transmission can occur via the placenta or breastfeeding[12] or less commonly by oral contamination due to the consumption of fresh infected food[13]. After infection, the disease in the human host has two phases: Acute and chronic. The acute phase occurs during the first months after infection, and it is characterized by a high parasitic load in the host's bloodstream. It may be asymptomatic, or it may present moderate symptoms that are of low diagnostic value. These characteristics hinder drug intervention at this stage. Although the acute phase is asymptomatic, sometimes it leads to the enlargement of the liver and lymph nodes, rashes, a loss of appetite, a swelling at the bite site (chagoma), and, occasionally, the Romaña’s mark[14]. After the acute phase, infected individuals spend long periods without symptoms, after which some patients evolve to the chronic phase. This stage of the infection is characterized by the appearance of severe degenerative disorders in the host's vital organs including megacolon, megaesophagus and cardiomegaly[15].

SOCIO-ECONOMIC IMPACT OF CHAGAS DISEASE

Damage to vital organs such as the heart contributes
Table 1  Estimated number of Disability-Adjusted Life Year (× 1000) by cause and by region (excluding the Europe)¹ 2004

| Neglected disease | World² | Region (OMS criteria) |
|-------------------|--------|-----------------------|
|                   |        | Africa | Americas | East of Mediterranean | Southeast Asia | Pacific West |
| Sleeping sickness | 1673   | 1609   | 0       | 62                   | 0              | 0           |
| Chagas disease    | 430    | 0      | 426     | 0                    | 0              | 0           |
| Schistosomiasis   | 1707   | 1502   | 46      | 145                  | 0              | 13          |
| Leishmaniasis     | 1974   | 328    | 45      | 281                  | 1264           | 51          |
| Filariasis        | 5941   | 2263   | 10      | 75                   | 3525           | 65          |
| Onchocerciasis    | 389    | 375    | 1       | 11                   | 0              | 0           |
| Leprosy           | 194    | 25     | 16      | 22                   | 118            | 13          |
| Dengue            | 670    | 9      | 73      | 28                   | 391            | 169         |
| Trichomoniasis    | 1334   | 601    | 15      | 208                  | 88             | 419         |
| Ascaridiasis³     | 1851   | 915    | 60      | 162                  | 404            | 308         |
| Trichuriasis      | 1012   | 236    | 73      | 61                   | 372            | 269         |
| Ancylostomiasis   | 1092   | 377    | 20      | 43                   | 286            | 364         |

¹Source: The global burden of disease: 2004 update. Geneva, World Health Organization¹⁶; ²Europe was omitted, so the sum of the regions will not be equal to the total value; ³Soil-transmitted helminthiasis.

greatly to the reduced economic capacity of a population of individuals and influences their economic and social conditions. This approach is currently used by the World Health Organization, through the use of a modern indicator for measuring the economic impact of diseases over certain regions using a number called the Disability-Adjusted Life Year (DALY). This number corresponds to the number of productive years lost to death or disability resulting from an illness in a given population¹⁶. This indicator has the advantage of accounting for two complementary factors as follows: Mortality, as measured by the number of years lost due to premature death [Years of Life Lost (YLL)]; and a new parameter for years lived with disability and economic output [Years Lived with Disability (YLD)]. The YLD indicator also indicates the burden to social security systems as a result of early retirement²⁷. The YLL values are calculated by multiplying the number of deaths for the life expectancy of a particular group of individuals; the YLD can be calculated as the product of the number of cases, the duration of the disease (a parameter that is particularly relevant for chronic diseases) and a constant for each disease (disability weight (DW)) that varies depending on the severity of the disability caused, ranging from zero (healthy) to one (dead). The resulting formula is shown below:

\[
\text{DALY} = \text{YLL} + \text{YLD} \\
\text{YLL} = N \times L \\
\text{YLD} = I \times \text{DW} \times L'
\]

Where \(N\) = number of deaths, \(L\) = life expectancy, \(I\) = number of individuals affected by the disease, and \(\text{DW}\) = disability weight; \(L'\) = duration of the disease. Table 1 shows the impacts of various neglected diseases on the economies of certain regions.

A closer look at Latin America shows that the geographical regions that are affected by higher rates of \(T.\ cruzi\) infection are also those in which the population is traditionally poorer. In countries such as Panama, Costa Rica, Bolivia and Venezuela and the hinterlands of northeastern Brazil and Northern Argentina¹⁹, there is an estimated loss of 752,000 d of work per year due to the early deaths of individuals with Chagas disease. In addition, United States $1.2 billion is lost each year from Latin American countries, with at least United States $5.6 million lost from Brazil²⁰. Taking into account that this financial loss is absorbed mostly by a specific group of people, it makes the discussion of Chagas disease even more complex since it is no longer a consequence of poverty but an agent that maintains poverty. These findings are due to decreasing productive capacities with a consequent reduction in the capital movement of a particular group of people in these geographical areas²¹.

The governmental programs aimed at both insect control and the quality of the blood used in transfusions in the countries where Chagas disease is endemic (primarily in Central and Latin America) led to an important decrease in the notifications of new cases. However, in non-endemic countries such as the United States and some countries in Europe, there was a significant increase in the number of infected individuals²²²³. First, this increase is associated with the increased migratory flux of people that has occurred in recent decades. Additionally, there is an expectation that global warming could also contribute to the advance of vector-transmitted tropical diseases, including American trypanosomiasis²³. There are several species of triatomine bugs that are capable of vectoring Trypanosoma cruzi in United States²⁴. In a recent study conducted in the metropolitan area of Tucson, Arizona (United States), investigators found that 41.5% of 164 triatomine bugs collected tested positive for \(T.\ cruzi\)²⁴. These data are alarming, and they show that the population of the southern part of the United States is exposed and at risk of infection by \(T.\ cruzi\).

THE AVAILABLE TREATMENTS

FOR CHAGAS DISEASE: OLD AND INEFFECTIVE DRUGS

The prevalence of Chagas disease in certain regions over
many years is primarily due to a lack of interest among pharmaceutical companies in developing drugs to treat Chagas disease. Despite the existence of a high demand, the potential consumers have no money to pay for medicines. In other words, there is demand, but there is no market. In this scenario, only two almost 100-year-old drugs are used to treat Chagas disease, namely the heterocyclic derivatives benznidazole (1) and nifurtimox (2), as shown in Figure 1. However, neither of these drugs is effective during the chronic phase of the disease, and both of them cause numerous toxic side effects.

Nifurtimox (2) is a 5-nitrofuran that is commercially available under the name Lampit®, and it was initially described as a promising alternative for the treatment of Chagas disease. This drug was developed by Bayer® under the name Bay-2502. It was shown to be effective for treating sleeping sickness (or African trypanosomiasis), which is caused by the trypanosomatid Trypanosoma brucei. In 1969, this compound yielded a total of 11 publications in the Bulletin of the Chilean Parasitology, in which the clinical outcomes of patients with the disease were described, as well as the biological properties of the drug in vivo. Despite the fact that nifurtimox (2) is still on the list of essential medicines, its distribution has been discontinued due to its low efficacy during the chronic phase of the disease, as well as its severe adverse effects, such as gastrointestinal problems, central nervous system disturbances and peripheral neuropathy. Its mechanism of action (Figure 2) involves the participation of the type I and II nitroreductases that are present in the parasite.

Type I nitroreductases cause electron transfer, converting nitrofuran (2) into the corresponding nitroanion radical through the conversion of molecular oxygen to a superoxide anion radical. These reactive oxygen species (ROS) are substrates of superoxide dismutase, which disproportionates superoxide into molecular oxygen and hydrogen peroxide (other ROS). Hydrogen peroxide is converted into water through the oxidation of the trypanothione in its reduced form, which is T(SH)₂. This process is reversed by the action of the trypanothione reductase (TR) enzyme. H₂O₂ can also oxidize ferrous ions from microsomal systems using the classical Haber-Weiss reaction to form hydroxyl radicals, which is harmful to the parasite.

Through the action of type I nitroreductases, the transfer of two electrons takes place (provided by NADH), and nitrofuran (2) is reduced directly to the nitroso derivative that is sequentially reduced to N-furan-2-yl-hydroxylamine. The hydroxylamine intermediate undergoes ring opening after losing water through the generation of an unsaturated nitrile, which is reduced again to the corresponding saturated derivative. The unsaturated nitrile is toxic to both the parasite and to the host cells. This high toxicity is due to the presence of a Michael-type acceptor that can bind bionucleophiles from both the host and parasite irreversibly. Although it has relevant activity against the intracellular form of the parasite, nifurtimox (2) is no longer marketed in Brazil due to the emergence of many resistant T. cruzi strains and the significant genotoxic effects caused by metabolites derived from the opening of its nitro-furan rings.

Benznidazole (1), a 2-nitroimidazole, is the drug of choice for treating patients who are infected with T. cruzi, and it was introduced to the market by Roche under the name Rochagan®. The rights to the drug were given to the Brazilian government in 2003, allowing the Pharmaceutical Laboratory of the State of Pernambuco to prepare and market benznidazole. Despite having a nitro-heterocyclic fragment in its structure, the mechanism of anti-chagasic action of benznidazole (1) differs from the proposed mechanism for nifurtimox (2) because its 2-nitroimidazole subunit has a lower electrochemical potential for the reduction when compared to the 5-nitrofuran moiety. Thus, the concentration of superoxide anion radicals is sufficiently low for the parasite to perform the detoxification on its own. The selective toxicity shown by benznidazole (1) is due to the transfer of an electron to its nitro-aryl motif, which disproportionates, then generating nitroimidazole and a nitrosimidazol that binds irreversibly to trypanothione, which is an essential cofactor for the viability of parasite cells. The addition reaction may happen to the nitrous group, but the work of Trochine et al. showed that adducts are also formed by an aromatic electrophilic substitution at position 4 of the imidazole ring. Another possible mechanism of action for benznidazole is proposed by Patterson et al. in which the drug is converted to an N-aryl-hydroxylamine in the same way as it occurs with nifurtimox (2). Then, a number of non-enzymatic reactions take place, culminating in the formation of a metabolite containing a guanidine subunit and a glyoxal molecule, which has cytotoxic properties; these properties could explain the trypanocidal activity shown by benznidazole (1). Figure 3 shows the two possible mechanisms of benznidazole activation.

However, nifurtimox (2) and benznidazole (1) are active only during the acute phase of the disease, which is usually asymptomatic and has a short duration. During the chronic phase, the long-term administration of these two nitroderivatives leads to the development of severe side effects in patients, making treatment with these compounds non-viable.

Based on the information provided above, there is a clear need for new studies on the development of novel and more specific drugs with low toxicities to the host. An important point to highlight here is the lack
of interest of big pharmaceutical corporations in the development of new and more effective therapeutic alternatives for the treatment of Chagas patients. This lack of interest is one of the most important factors that contributes to the fact that this disease remains a death sentence for infected people in poor countries. Chagas disease and other parasitic illnesses have left a huge mark of destruction and economic loss on humanity.

**POTENTIAL TARGETS FOR TRYPANOCIDAL DRUGS: T. CRUZI**

**VIRULENCE FACTORS AS A TARGET**

The search for novel compounds that are selectively harmful to the parasite without compromising the host's health is the primary paradigm in the search for new antiparasitic drugs. The approach to antiparasitic chemotherapy is usually based on two broad classes of targets, namely those targets that are specific to the parasite (such as trypanothione and cruzipain) and those shared between the parasite and the host (such as tubulin and sterol 14α-demethylase). When a common target is shared between the parasite and host cells, there should be some selectivity by the bioactive substance for the receptors/enzymes of the parasite, to the detriment of those related to the host cells, with the aim of exerting greater effects on the parasite and fewer effects on the host.

Some of the most common targets for Chagas
disease are shown below (Figure 4). These targets were chosen as some of the most relevant ones on the basis of research from the Scopus database, in which the term "T. cruzi" was searched together with the target's name. The numbers of published papers available on the Scopus database (https://www.scopus.com/) were compared, resulting in the graph shown in Figure 4.

This result shows that some targets, such as trans-sialidase and trypanothione reductase, are well-known targets with a high average number of papers published in the last two decades. Cruzipain appears to have had a significant increase in the number of papers, possibly due to the large number of deposits that contain crystalline structures with better resolution in databases such as the Protein Data Bank, which enhances the search for new substances that are capable of acting as enzyme inhibitors. Tubulin and CYP51 have a smaller number of published papers, despite their importance. These two targets, which also have distinct isoforms in human cells, have shown an increased growth trend in publications over the past five years. This trend could provide a possible increase in their relevance to antichagasic chemotherapy over the next decade. In this particular case, the selectivity needs to be considered because the modification of these substance dynamics in human cells can cause severe side effects in the host. Therefore, this review aims to present a literature review and critical analysis of the importance of each one of these targets in the development of substances with possible antichagasic activity.

**CRUZIPAIN**

Cruzipain, which is also called GP 57/51 (recombinant cruzain), is a cysteine protease from the papain family; its primary feature is an atypical C-terminal segment that is highly glycosylated. Cruzipain is encoded by a polymorphic gene (i.e., its expression is regulated differently at different developmental stages of the parasite), which suggests the existence of specific functions for the enzyme in each form of the parasite.
In trypomastigotes, cruzipain is located in lysosomes, whereas in the amastigote form, it is present primarily at the cell surface. However, in the epimastigote form, cruzipain is compartmentalized in reservosomes, which are related to penetration processes in the host cell, intracellular nutrition and the escape mechanism from the cell for the trypomastigote form. Because they are cysteine proteases, the first cruzipain inhibitors were thought to be peptoids that were capable of binding irreversibly to the enzyme, e.g., vinyl sulfone (3). Next, computational tools were used to design non-peptide derivatives, such as (4) [44], which was a more potent inhibitor of the enzyme (Figure 5, entry A) [45].

**TRYPANOTHIONE REDUCTASE**

TR, an enzyme that is present in many trypanosomatids (e.g., Trypanosoma, Leishmania and Crithidia sp.), is responsible for the catalysis reaction shown in Figure 6. This enzyme maintains the redox balance of trypanothione and is responsible for maintaining an intracellular reducing environment by decreasing the concentration of ROS and other free radicals, which are consumed by the reduced form of trypanothione [46].

The inhibition of this enzyme leads to the accumulation of ROS in the parasite cell, causing a potentially lethal oxidative stress in *T. cruzi*. The development of TR inhibitors began in 1992 with the work of Benson et al [47], who isolated the TR and performed *in vitro* assays of enzymatic inhibition with some selected synthetic compounds. These studies identified clomipramine (5) as the first prototype for TR selective inhibition *in vitro* based on the redox potential of the glutathione system that is present in the vertebrate host [47]. In the work of Benson et al [47], compound 5 has an inhibition constant of Ki = 6.53 ± 0.59 μmol/L for *T. cruzi* TR and no inhibition of human glutathione reductase at the maximum concentration of 1 mmol/L. In the early 90s, most rationally planned TR inhibitors were structurally related to the substrate, which usually led to irreversible inhibitions and little selectivity. The work of Zhang et al [48] made an important contribution to the development of TR inhibitors because it was the very first one that was planned through computational studies of non-peptidic inhibitors that were rationally designed with structural information from the target (Figure 7). Zhang et al [48] used the known crystallographic structure of trypanothione reductase from *Crithidia fasciculata* to create a homology model for the corresponding enzyme in *T. cruzi*. *C. fasciculata* is a kinetoplastid that is able to parasitize mosquitoes but is harmless to humans. TR from *C. fasciculata* shares 69% of its identity with the trypanosome TR, especially in terms of the active sites of the two enzymes [49]. In the same year, Jacoby...
et al[50] elucidated the structure of the enzyme from the crystallography of TR that was co-crystallized with mepacrine (6). Since then, several TR inhibitors have been discovered by using the models of Zhang and Jacoby, especially the aminoacridine (7), a higher homolog of (6), as described by Bonse et al[51], and a pyridazine (8) and a carbazole (9) described by Horvath[52]. More recently, the inhibitory activity of more potent derivatives such as thioridazine (10) and aminoquinoline (11) was described by Lo Presti et al[53] and Sola et al[54], respectively.

A series of twenty-one small peptides or peptide conjugates were assessed by McKie et al[55]. Among all the evaluated peptidic derivatives, two of them, namely N-benzyloxy carbonyl-Ala-Arg-Arg-4-methoxy-β-naphthylamide (12) and Bz-Leu-Arg-Arg-β-naphthylamide (13, Figure 8), showed good inhibitory activity against TR with Ki values of 2.4 μmol/L and 13.8 μmol/L, respectively. Additionally, the former derivative showed good selectivity for the parasitic enzyme (TR) compared to the host enzyme (human glutathione reductase).

**TRANS-SIALIDASE**

Another conspicuous target on *T. cruzi* is *trans*-sialidase (TcTS), an enzyme that was more often expressed on the trypomastigote forms of the parasite. *Trans*-sialidase is associated with the infective process, and it is a key component of the parasite's biology and its ability to evade both the innate and adaptive immune systems of the host[56]. The cell recognition processes can occur in mammals, through sugars that are present in the cell glycocalyx. One of the sugars with fundamental
importance in mammalian cell recognition is sialic acid, which is not produced by *T. cruzi*. The parasite has developed an enzyme that is capable of transferring sialic acid from the host cell to its own. Therefore, the parasite is no longer recognizable as a foreign agent and can then infect host cells without triggering the immune response. In trypomastigotes, trans-sialidase is anchored as a non-integral membrane protein to glycosyl-phosphatidyl inositol. This enzyme has the ability to sialylate mucin, a very abundant glycoprotein in the cell membrane of the parasite, through the transfer of sialic acid from the host membrane to a \(\beta\)-galactopyranose that is present at the glycosylated hydrophilic site of the parasite's mucin (Figure 9). Thus, by using a specific carbohydrate in the host membrane, the parasite can engage in a non-phagocytic invasion process without activating an immune response.

To understand the trans-sialidase kinetic properties, Damager et al. performed studies on the enzyme catalytic properties using *in vitro* studies in which sialyllactose was used as a sialic acid donor, and \(N\)-acetyllactosamine was used as an acceptor. The kinetic isotopic effect studies led to the proposal of a so-called "ping-pong mechanism", in which the sialic acid donor binds first, followed by the acceptor, suggesting two near-lactose-binding sites leading to the chemical mechanism proposed in Figure 10.

The classical inhibitors of trans-sialidase are all weak and non-specific, with an inhibition constant (Ki) being at the millimolar order, and some of them are shown in

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*Figure 8* Structure of peptidic inhibitors of *T. cruzi* trypanothione reductase.

*Figure 9* Transfer of sialic acid from a host glycoprotein to a \(\beta\)-galactopyranose from the parasite. This transfer occurs at the position 3 of the terminal sugar of the oligosaccharide which it is attached. Adapted from GIORGI and LEDERKREMER.

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Figure 11. 2-Deoxy-2,3-didehydro-\( N \)-acetylneuraminic acid (DANA, 14) was used by Paris et al.\(^{[63]}\) to compare the inhibition of \( T. \) cruzi trans-sialidase and \( T. \) rangeli sialidase (TrSA, an enzyme that hydrolyzes sialic acid but lacks transglycosidase ability). Despite being a potent inhibitor of TrSA (\( K\text{i} = 1.5 \mu\text{mol/L} \)), DANA was reported to inhibit TcTS at a very high concentration, at \( K\text{i} = 12.3 \text{ mmol/L} \).\(^{[63]}\) Watts et al.\(^{[64]}\) used 2,3-difluorosialic acid (15) to show the covalent binding of sialic acid with Tyr342 by mass spectra analysis. The TcTS complexed with (15) was readily subjected to peptide digestion. The LC-MS analysis of the hydrolysis product shows an \( m/z \) fragment of 1392, which corresponds to the peptide DENSAYSSVL+3OHSial. The ESI tandem MS daughter ion spectrum of this fragment shows a pattern in which only the fragments with tyrosine included the 3-hydroxy sialil label, indicating that sialic acid would probably bind covalently to that area.\(^{[64]}\) Lactitol (16) also acts as an inhibitor, competing with the parasite's sugars (e.g., lactose) for the sialic acid, inhibiting TcTS activity toward conventional substrates. However, this inhibition demands a high concentration of (16), which shows that these lactose analogs are not suitable inhibitors for \textit{in vivo} studies.\(^{[65]}\) With the aim of synthesizing analogs that can inhibit the transfer of sialic acid into different organisms, Streicher and Buse planned a series of pseudo-sialosides with a cyclohexene and a phosphonate ester moiety, and they tested it against some trans-sialidases, including TcTS.\(^{[66]}\) However, the most active compound (17) showed an IC\(_{50} = 4.7 \text{ mmol/L} \), or the same magnitude as the previous inhibitors (14-16). With a different approach, Neres et al.\(^{[67]}\) designed a series of benzoic acid derivative analogs to pyridoxal phosphate, a well-known TcTS inhibitor with a \( K\text{i} = 7.3 \text{ mmol/L} \). This strategy was useful in the inhibition of the influenza virus neuraminidase, and it acted by replacing sialic acid with more simple structures such as benzene and pyridine.

Figure 11. 2-Deoxy-2,3-didehydro-\( D \)-\( N \)-acetylneuraminic acid (DANA, 14) was used by Paris et al.\(^{[63]}\) to compare the inhibition of \( T. \) cruzi trans-sialidase and Trypanosoma \textit{rangeli} sialidase (TrSA, an enzyme that hydrolyzes sialic acid but lacks transglycosidase ability). Despite being a potent inhibitor of TrSA (\( K\text{i} = 1.5 \mu\text{mol/L} \)), DANA was reported to inhibit TcTS at a very high concentration, at \( K\text{i} = 12.3 \text{ mmol/L} \). Watts et al.\(^{[64]}\) used 2,3-difluorosialic acid (15) to show the covalent binding of sialic acid with Tyr342 by mass spectra analysis. The TcTS complexed with (15) was readily subjected to peptide digestion. The LC-MS analysis of the hydrolysis product shows an \( m/z \) fragment of 1392, which corresponds to the peptide DENSAYSSVL+3OHSial. The ESI tandem MS daughter ion spectrum of this fragment shows a pattern in which only the fragments with tyrosine included the 3-hydroxy sialil label, indicating that sialic acid would probably bind covalently to that area.\(^{[64]}\) Lactitol (16) also acts as an inhibitor, competing with the parasite's sugars (e.g., lactose) for the sialic acid, inhibiting TcTS activity toward conventional substrates. However, this inhibition demands a high concentration of (16), which shows that these lactose analogs are not suitable inhibitors for \textit{in vivo} studies.\(^{[65]}\) With the aim of synthesizing analogs that can inhibit the transfer of sialic acid into different organisms, Streicher and Buse planned a series of pseudo-sialosides with a cyclohexene and a phosphonate ester moiety, and they tested it against some trans-sialidases, including TcTS.\(^{[66]}\) However, the most active compound (17) showed an IC\(_{50} = 4.7 \text{ mmol/L} \), or the same magnitude as the previous inhibitors (14-16). With a different approach, Neres et al.\(^{[67]}\) designed a series of benzoic acid derivative analogs to pyridoxal phosphate, a well-known TcTS inhibitor with a \( K\text{i} = 7.3 \text{ mmol/L} \). This strategy was useful in the inhibition of the influenza virus neuraminidase, and it acted by replacing sialic acid with more simple structures such as benzene and pyridine.
The compound (18) was the most potent among the synthesized compounds, with a $K_i = 300 \mu mol/L$.

Because the previous inhibitors described here were not very active against TcTS, a search for more potent scaffolds was led by Arioka et al.\(^{[68]}\) to explore novel chemical scaffolds by assessing the TcTS inhibition properties of non-sialyl derivatives. The capacity of sulfonamide chalcones to inhibit $\alpha$-glucosidase was previously described, and since both enzymes have a sugar subtraction, Kim et al.\(^{[69]}\) planned and synthesized a series of chalcones based on the bioisosteric relationship between the carboxylic acid subunit of sialic acid with the sulfonamide moiety of the compounds (19–21, Figure 12). This synthesis resulted in the identification of the first TcTS inhibitors with an IC\(_{50}\) of lower than ten micromolar.

The need to find new chemical scaffolds of TcTS inhibitors led Arioka et al.\(^{[69]}\) to design a massive in vitro screening from a natural product library containing 2283 compounds, to search for more potent derivatives. The first trial selected the hit compounds that showed TCT inhibition above 40% at a 1 $\mu mol/L$ concentration, resulting in 103 compounds. Then, the second screening selected those compounds with IC\(_{50}\) < 100 $\mu mol/L$, picking out a group of 50 compounds. The promiscuous inhibitors were excluded by a data analysis of the IC\(_{50}\) determination in the presence of 0.1% Triton X-100, and the resulting 16 selected compounds were critically evaluated using the Lipinski rules, culminating in the selection of two lead compounds called myricetin (22) and 6-chloro-9,10-dihydro-4,5,7-trihydroxy-9,10-dioxo-2-anthracenecarboxylic acid (23). These compounds represented novel chemical scaffolds in the scope of TcTS inhibition, and they were submitted to structure/activity relationship (SAR) studies with the aim of optimizing these leads. Despite their great contributions to understanding the SAR of these scaffolds, none of the new derivatives were more potent than the natural prototypes shown in Figure 13. After a paper was published by Arioka et al.\(^{[69]}\), many groups synthesized TcTS inhibitors (all sialyl mimetics), but none of them were more potent than compound (23).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Figures/figure12.png}
\caption{Chemical structures of the sulfonamide-chalcone derivatives designed by Kim et al.\(^{[69]}\) (19, IC\(_{50}\) = 0.9 $\mu$mol/L; 20, IC\(_{50}\) = 2.5 $\mu$mol/L; 21, IC\(_{50}\) = 0.6 $\mu$mol/L).}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Figures/figure13.png}
\caption{Chemical structures of myricetin (22, IC\(_{50}\) = 17 $\mu$mol/L) and the anthraquinone (23, IC\(_{50}\) = 0.58 $\mu$mol/L), TcTS inhibitors found by Arioka et al.\(^{[69]}\) from a natural products library.}
\end{figure}

**STEROL 14$\alpha$-DEMETHYLASE (CYP51)**

In addition to those mentioned above, many other targets have been investigated for their antichagasic activities. One of the studied targets acts in regulating parasite cell membrane steroids, which play a fundamental role in cell division (since they are the primary components of the cell membrane) and cell maintenance (since its presence on the membrane is fundamental for maintaining selective permeability). As a class of molecules that are essential to the maintenance of cell viability, steroids are lipophilic biomolecules that act on the cell membrane, modulating its fluidity, integrity and permeability. The biosynthesis of steroids differs significantly between the Kingdoms, and squalene oxide is a key intermediate in all eukaryotes. From this point, there is a divergence in the biosynthetic pathways that leads to different steroids; among animals, the major steroid is cholesterol (24). In fungi and protozoa, the primary steroid is ergosterol (25); and in plants, it is sitosterol (26), as shown above.

The differences shown in Figure 14 may be an advantage in the design of biologically active compounds that target enzymes involved in the biosynthetic pathways since this condition allows for the search for new derivatives possessing toxicity that is selective for parasites. The clinical use of the azole derivatives that modulate steroid biosynthesis is well established and clinically useful for fungal infection chemotherapy. In infections caused by trypanosomatids, the use of an azole derivative previously known as an antifungal called ketoconazole (28) was studied by McCabe et al.\(^{[71]}\), and the results led to a series of studies about the enzyme
sterol 14α-demethylase (or CYP51), a key enzyme in the regulation of sterol biosynthesis in eukaryotes\(^7\) (Figure 15).

The endogenous steroids in \(T. \text{ cruzi}\) have a direct role in the cell viability and activity regulation of cell membrane enzymes\(^7\)\(^3\). Although ergosterol is a final product of biosynthetic pathways that are common to both trypanosomatids and fungi, there are specificities regarding the synthesis of this lipid, principally during the demethylation step mediated by the CYP51 of each species. Despite the low similarity between the different isoforms of CYP51\(^7\)\(^4\), all the enzymes have both high regio- and stereoselectivity to the reactions they catalyze, which reduces the number of possible substrates. There are three known substrates in all the sterol-14α-demethylases families; for example, lanosterol (29), eburicol (31) and obtusifoliol (32)\(^7\)\(^5\). However, this phenomenon diminishes the possibility that an azole is capable of inhibiting CYP51 from both protozoa and fungi since each isoenzyme possesses different affinities for ligands (Figure 16).

The inhibitors of \(T. \text{ cruzi}\) CYP51 are the only class of drug candidates that have reached clinical trials for Chagas disease chemotherapy\(^7\)\(^6\). One example is the imidazole derivative VNI (33), which was found to be active during the chronic phase of the disease in \(\text{in vivo}\) experiments\(^7\)\(^7\). The use of trypanocidal CYP51 inhibitors occurred before this enzyme was identified as a potential target. Antifungal agents such as itraconazole (34), fluconazole (35) and ketoconazole (28) were assessed \(\text{in vitro}\) and \(\text{in vivo}\) in Chagas models in the 80s, and they led to reductions in the parasite load in infected animals\(^7\)\(^1\),\(^7\)\(^8\). The motivation for the first work involving ketoconazole (28) activity in a murine model of \(T. \text{ cruzi}\) infection was derived from previous reports of its activity against \(\text{Plasmodium falciparum}\)\(^7\)\(^9\) and \(\text{Leishmania tropica}\)\(^8\)\(^0\). The azole compounds act on \(T. \text{ cruzi}\) CYP51 through interactions with the nitrogen heterocycles and the iron atom present in the central HEME (Figure 17) enzyme. This enzyme is responsible for the demethylation of eburicol, preventing the formation of a zymosterol intermediate (30) from lanosterol (29), thereby preventing the formation of ergosterol (25)\(^7\)\(^2\),\(^8\)\(^1\). Thus, the consequence of inhibiting the final stages of ergosterol
biosynthesis is the accumulation of toxic biosynthetic precursors in the *T. cruzi* cell membrane, compromising its integrity, which is similar to what happens in yeast \[82,83\] (Figure 18). This finding suggests that CYP51 inhibition as a promising approach to the development of new antichagasic molecules.

Recently, Franklim *et al* \[84\] described the synthesis of a novel series of triazole derivatives that were prepared from the natural amide piperine (36), and they were designed as CYP51 inhibitors of *T. cruzi* based on the bioisosteric relationship between the amide from (36) and the bioisomeric relationship between the amide from (36) and the 1,2,4-triazole-3-thione from the antifungal drug prothioconazole (37). Derivative (38), as shown in Figure 19, showed the best trypanocidal profile.

Despite their potential as trypanocidal agents, the new CYP51 inhibitors should be developed very carefully since these compounds can inhibit other enzymes that are involved in the hepatic microsomal system, leading to severe side effects such as hepatotoxicity and alterations in basal steroidogenesis. Long-term exposure to CYP51 inhibitors can cause deleterious effects on both the cellular biosynthesis of steroids and the phase I metabolism of drugs and xenobiotics, leading to a lack of clearance of toxic substances\[85\]. One of the most relevant side effects

![Figure 15 Ergosterol (25) biosynthesis in yeast (Saccharomyces cerevisiae), from squalene oxide.](image-url)
of the administration of CYP51 inhibitors is the non-specific binding of these inhibitors to another important enzyme that is present in the hepatic microsomal system, which is known as CYP19 (aromatase). CYP19 is an enzyme that is located in the endoplasmic reticulum and is responsible for the demethylation of different steroids at position 10, e.g., during the conversion of androstenedione (39) into estrone (40) or testosterone (41) to estradiol (42), as shown in Figure 20. The inhibition of CYP19 leads to the accumulation of (39) and (41) that impairs the balance between the steroid hormones, which is crucial for the development and maintenance of the reproductive system as well as for the differentiation of the sexual phenotype.

TUBULIN

In addition to CYP51, tubulin is another promising target in the development of compounds that can modulate the cell cycle of *T. cruzi*. Tubulin is a class of globular protein whose isoforms comprise microtubules, which are cytoskeletal filaments that are responsible for maintaining the fundamental functions of eukaryotic cells. These functions include the segregation of chromosomes during cell division, the transport of intracellular components and the maintenance of the cell shape, cell motility and distribution of plasma membrane components.

Microtubule formation occurs through the polymerization of two tubulin isoforms called α and β. Both subunits form a heterodimer of α and β-tubulin, which polymerizes, forming a filamentous cylindrical structure in the "head-to-tail" direction where the α subunit of a dimer binds to the β unit of the other. This polymerization leads to an initial polymer protofilament, which is grouped with other similar protofilaments to form the microtubule itself, as shown in Figure 21.

Once the microtubule is formed, it becomes a dynamic structure in which the continuous processes of polymerization and depolymerization take place in equilibrium. This feature makes it possible for the microtubule to change its size and adapt to different situations, such as those that occur during the cell cycle. The α-terminal portion ([region (-)]) is less dynamic, whereas the β-terminal ([region (+)]) is more dynamic and can lengthen/shorten more quickly. This characteristic confers polarity to microtubules, which gives the different (+) and (-) regions different properties and causes them to be oriented in different directions. This characteristic is given by the fact that each tubulin subunit (both α and β) has a binding site for guanosine triphosphate (GTP), which binds more strongly to α than to β-tubulin. In this way, the GTP bound to β-tubulin is more easily hydrolyzed to guanosine-diphosphate (GDP) after polymerization. The kinetics of polymerization in this case are more favorable than the kinetics of GTP hydrolysis, allowing the growth of the microtubule. In that case, the increase or decrease of the microtubule length in the region (+) closely depends on the nucleotide linked to β-tubulin; a microtubule with a GTP molecule tends to polymerize, while those associated with GDP will try to...
Since tubulin is a key component of cell proliferation, it is an important target in the development of cancer chemotherapy, and the tubulin inhibitors are some of the most effective anti-cancer drugs\textsuperscript{[94]}. Similarly, tubulin plays the same role in cell division in parasites such as \textit{T. cruzi} that possess cell proliferation kinetics comparable to those found in cancer cells. The cell division processes in parasites are strictly dependent on the polymerization/depolymerization equilibrium of tubulin\textsuperscript{[95]}, and they act on the parasite motility process as well, which is essential for the maintenance of the host infection.

Although there is a strong shared identity between the sequences of tubulin amino acids from different species, the search for new therapeutic agents against parasitic diseases remains a crucial area of research. 

Figure 17 Structures of azole derivatives active in \textit{T. cruzi} infection model: Ketoconazole (28), VNI (33), itraconazole (34) and fluconazole (35). Crystallographic structure of \textit{T. cruzi} CYP51 with fluconazole (35) linked to the catalytic site of the enzyme [available at Protein Data Bank under the code 2wx2 (left)]. Bidimensional scheme of fluconazole (35) binding interaction with the catalytic site of the enzyme, generated by the program LigPlot\textsuperscript{+} from the same code (right).
organisms, e.g., mammalian cells and yeast may have shared identities from 70% to 90% in their tubulin isoforms, there are several reports of tubulin inhibitor drugs being used in anti-parasitic chemotherapy in the literature. The antifungal benzimidazolic drug Benomyl® (43) has high selectivity for yeast tubulin; Kilmartin et al.[96] showed that (43) is 300 times more potent at inhibiting S. cerevisiae tubulin than bovine brain tubulin. Thus, despite the high structural similarity between tubulins from diverse species, the small differences are probably responsible for the selective recognition of these compounds in different organisms, making tubulin an important target for Chagas disease chemotherapy.

Figure 18  Schematic representation of the mechanism of actions of azolic compounds upon ergosterol (25) synthesis and subsequent alteration of composition and organization of cell membrane.

Figure 19  Structures of piperine (36), prothioconazole (37) and cycloexyltriazole (38), and its IC₅₀ values against epi- and amastigotes of T. cruzi and the toxic profile against murine macrophages.
The search for selective tubulin inhibitors is a current challenge in the development of new antichagasic drugs. The work of Werbovetz et al. identified some sulfonamide-dinitroaniline derivatives that were structurally analogous to oryzalin (49), an herbicide that acts by depolymerizing microtubules from plants and thereby prevents its anisotropic growth. In this study, compound GB-II-5 (47) was found to have greater potency against kinetoplastids than mammalian cells, as shown in Figure 24.

The sulfonamide derivatives (46-48) bind in a tubulin region called “the colchicine site” (99), which is a region between the α and β tubulin subunits. This site is where colchicine (49) interacts with tubulin as a well-known tubulin inhibitor. Colchicine (49) is a natural product that is extracted from Colchicum sp. (e.g., Colchicum autumnale or meadow saffron), which is used to treat gout (100). When colchicine (49) binds to the region between two tubulin heterodimer subunits, it induces the depolymerization of microtubules by altering the conformation adopted by the β subunit after its binding. Once bound to (49), the dimer assumes a curved conformation that generates steric hindrance upon the formation of protofilaments that will generate the microtubules (101).

Another binding site in the tubulin structure is located at the interface of two heterodimers, more specifically, on the β subunit (102). The vinca (Catharanthus roseus) alkaloïds, e.g., vinblastine (50) and vincristine (51), bind to this site. Despite the fact that C. roseus had been used in popular medicine for a long time in various locations such as India, China and Hawaii, it attracted the interest of a group of Canadian scientists in the 1950s who wanted to study its popular use in a diabetes treatment from Jamaica (103). Although their efforts to pursue antidiabetic substances did not succeed, strong cytostatic activity was identified in the crude extract of C. roseus, which led to the isolation of two alkaloids (50-51). These bis-indole monoterpenoid alkaloids are produced in very small quantities in the leaves of C. roseus through the reaction of two other alkaloids called catharanthine (52) and vindoline (53) (103), as shown in Figure 26. The mechanism of action of these alkaloids involves the suppression of their polymerization in the positive region (104) and the promotion of depolymerization in the negative region of the microtubules. This characteristic
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![Chemical structures of compounds](image)

| Compound     | L. donovani (amastigotes) | T. b. brucei (variant 221) | T. b. brucei (macrophages) | J774 | PC3 |
|--------------|--------------------------|---------------------------|--------------------------|------|-----|
| Orizalin (46) | 72 ± 10                  | 11 ± 0                    | 6.6 ± 1.0                | 41 ± 5 | 57 ± 4 |
| GB-Ⅱ-5 (47)  | 5.0 ± 0.6                | 0.41 ± 0.02               | 0.73 ± 0.09              | 29 ± 1 | 35 ± 1 |
| GB-Ⅱ-46 (48) | 20 ± 2                   | 2.6 ± 0.3                 | 1.9 ± 0.7                | 9.4 ± 2.0 | 23 ± 4 |

Figure 24 Structures and IC₅₀ values (micromolar) of orizalin (46) and the sulfonamide-dinitroanilin derivatives (47 and 48) against kinetoplastide and mamalian cells.

![Chemical structure of colchicine](image)

Figure 25 Chemical structure of colchicine (49).

allows these alkaloid derivatives to be able to change their microtubule dynamics during the formation of the mitotic spindle in the cell division process⁹⁷. In addition to the change in the polymerization/depolymerization dynamics, Vinca alkaloids also promote the fragmentation of the existing microtubules through the detachment of some regions near the (-) region of the biopolymer¹⁰⁸.

The third-most common approach to the modulation of the microtubule dynamics does not involve increases in the depolymerization process, but instead involves its blockage. Thus, through the stabilization of microtubules, these organelles lose their dynamics, which is necessary for the maintenance of their functionality. This phenomenon occurs when paclitaxel (54, also known as Taxol®) binds to a specific site of the tubulin β subunit¹⁰⁹. Paclitaxel (54) is a natural compound that was initially identified as a secondary metabolite of the Pacific yew (Taxus brevifolia). Due to the small amount of taxol available in the plant, together with the difficulty of sustainably managing T. brevifolia cultures, a semisynthetic method was employed to manufacture paclitaxel (54) based on the isolation of 10-deacetylbaclactin III (55) from the leaves of Taxus baccata. Ojima et al.¹¹⁰ developed a method in which compound (55) is coupled with the lactam at C-13 (56), as shown in Figure 27. Subsequently, a great number of biotechnological approaches involving cell culture and gene expression in bacteria allowed for the preparation of appreciable amounts of (54) in a less costly way. However, Taxol® remains a very expensive drug¹¹¹.

In the β-terminal subunit of tubulin [region (+)], the nature of the anchored nucleotide determines whether there will be polymerization or depolymerization. The presence of GTP provides for polymerization, and the presence of GDP promotes microtubule depolymerization instead. These processes take place because GDP hydrolysis alters the conformation of the β-tubulin, which causes a cascade of events that changes the structure of protofilaments, making them more curved and causing them to protrude out of the microtubules (structure previously shown in Figure 22, item 3). The presence of paclitaxel (54) anchored in the region adjacent to the GDP-binding site (the so-called “taxol site”) stabilizes the polymer structure, preventing the depolymerization needed to maintain the microtubule dynamic equilibrium. The microtubule stabilization compromises different processes that depend on the microtubules, such as mitosis, disabling cell duplication¹¹². These three tubulin
binding sites are the primary paradigms in the research and development of bioactive compounds, with the aim of modulating cellular phenomena through involvement with microtubules, and they are shown in Figure 28. Each of these sites has a number of known ligands, the structures of which are depicted in Figure 29.

The first work reporting the capacity of taxol (54) to act against \( T. \) cruzi was published by Baum et al\(^{[112]} \) in 1981, in which the authors used transmission and scanning electron microscopy experiments to find several parasites containing multiple flagella and multiple intracellular organelles such as the nucleus and kinetoplastids. However, cell division by binary fission does not occur, which corroborates the hypothesis of (54), showing that it acts on a specific structure during cytokinesis\(^{[112]} \). After that, other authors studied the effects of different compounds such as the natural amide piperine (36), as reported by Freire-de-Lima et al\(^{[113]} \). Natural piperine (36) acts in the blockage of cytokinesis of \( T. \) cruzi epimastigotes, and it leads to cellular ultra-structural alterations similar to those observed in taxol-treated parasites\(^{[113]} \).

Some of the well-known tubulin inhibitors can interact with other sites on the tubulin heterodimer. Curcumin (67, Figure 30), for example, is a natural diarylheptanoid with a recognized involvement in cell cycle modulation. It acts by binding to tubulin in HeLa and MCF-7 cells, reducing the GTPase activity and partially inhibiting the activity of colchicine (49) in these cells\(^{[114]} \). Banerjee et al\(^{[115]} \) also reported that curcumin acts by suppressing the dynamic instability of microtubules in MCF-7 cells, maintaining the microtubules in a metastable state, similar to paclitaxel (54). However, Banerjee's work suggested that curcumin did not interact with any of the three most popular binding sites of tubulin (taxol, vinca alkaloids and colchicine sites), which led Chakraborti et al\(^{[116]} \) to perform experiments that allowed them to elucidate the binding site of curcumin (67) to tubulin. Using Fluorescence Resonance Energy Transfer, Chakraborti et al\(^{[116]} \) determined that curcumin (67) interacts between two \( \alpha, \beta \)-tubulins, which are heterodimers that are 32 Å away from the colchicine binding site. This interaction features a new binding site that is potentially useful for planning new antitubulin agents. Aiming to justify the trypanocidal properties of curcumin (67) and other natural diarylheptanoids, Sueth-Santiago et al\(^{[117]} \) build a theoretical model of \( T. \) cruzi tubulin. Molecular docking studies have shown a good correlation between the binding scores and the trypanocidal activities of the four natural diarylheptanoids. The results obtained from the cell cycle studies corroborated this hypothesis, showing alterations on parasites cell cycle in the same way of the positive control with accumulation of parasite cells in the G2 phase\(^{[117]} \).

**CONCLUSION**

Despite the fact that Chagas disease was described more than 100 years ago, it remains a death sentence to millions of people who are in the chronic phase of the illness. Due to the lack of interest of the pharmaceutical industry in developing new drugs to treat neglected illnesses such as Chagas disease, there is no effective treatment available at present. However, given that, we have found a huge and growing amount of information that has been published about the parasite and its complex relationship with the host, the discovery of an effective drug comes closer each day. In this sense, detailed knowledge of both the differences and similarities...
between T. cruzi and its human host's biochemical targets may be the key for curing this severe and debilitating sickness.

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