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50 Years After Nifurtimox: Current and Future Chemotherapy For Chagas Disease

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50 Years After Nifurtimox: Current and Future Chemotherapy For Chagas Disease

ABSTRACT

American trypanosomiasis, commonly called Chagas disease, is one of the most neglected illnesses in the world and remains one of the most prevalent chronic infectious diseases of Latin America with thousands of new cases every year. The only treatments available have been introduced five decades ago. They have serious, undesirable side effects and disputed benefits in the chronic stage of the disease – a characteristic and debilitating cardiomyopathy and/or megavisceras. Several laboratories have therefore focused their efforts in finding better drugs. Although recent years have brought new clinical trials, these are few and lack diversity in terms of drug mechanism of action, thus resulting in a weak drug discovery pipeline. This fragility has been recently exposed by the failure of two candidates; posaconazole and E1224, to sterily cure patients in phase 2 clinical trials. Such setbacks highlight the need for continuous, novel and high quality drug discovery and development efforts to discover better and safer treatments.

In this article we will review past and current findings on drug discovery for *Trypanosoma cruzi* made by academic research groups, industry and other research organizations over the last half century. We will also analyze the current research landscape that is now better placed than ever to deliver alternative treatments for Chagas disease in the near future.

KEYWORDS

Trypanosoma cruzi, Chagas disease, benznidazole, nifurtimox, drug discovery, chemotherapy.

1. INTRODUCTION

Chagas disease is named after the Brazilian physician Carlos Chagas who first described the disease in 1909 [1]. Chagas disease is caused by the parasite *Trypanosoma cruzi* and is considered to be the parasitic infirmity with the biggest social and economic burden in Latin America [2, 3]. There is an estimate of 7-8 million people currently infected with *T. cruzi*, while there are approximately 25 million people at risk of acquiring the disease [4]. Every year, Chagas disease claims 10,000 deaths in endemic countries [5]. The parasite has a complex life cycle, alternating between the mammalian host and the hematophagous triatomine insect vector. The infection begins when the infected bug feeds on the host, which can be a wild or domestic mammal or a human. Infective metacyclic trypomastigote forms of *T. cruzi* are found in the feces of the bug which are released during the blood meal, and gain access through the lesion to infect dendritic cells [6]. Once inside a cell, the parasite breaks free of its entering organelle, the endosome/lysosome, and differentiates into a replicative amastigote form. The amastigote divides several times and matures into bloodstream trypomastigotes that rupture the host cell and are released into the bloodstream or lymph, free to infect a wide range of cells or be ingested by the transmitting vector, thus closing the cycle. In the triatomine gut, the parasite transforms once again into a replicative stage called the epimastigote and after clonal divisions it migrates to the final portion of the intestine and differentiates again into infectious metacyclic trypomastigote.

Although vectorial transmission has been greatly reduced due to vector control campaigns carried out by the World Health Organization (WHO), the Pan-American Health Organization (PAHO) and national health ministries of participating countries, there are still about 41,000 [5] new cases each year due to vectorial transmission. Many thousands of cases can be also attributed to secondary infection routes like transfusion of contaminated whole blood and derivate products, transplant of organs from chronically infected patients, congenital and oral transmissions. Oral transmission is a growing concern, with 138 outbreaks responsible for the appearance of 776 new cases in the period of 2000 – 2010. Oral infection is usually acquired through ingestion of food, sugar cane and other juices, water or soup contaminated with infected triatomines or their feces [7]. Human migration in recent years have increased the incidence of new cases in non-endemic countries, making Chagas a health and medical problem in North America, Europe, Japan and Australia, requiring governments to implement screenings for blood and organs donations, as well as implement infrastructures to treat infected patients [8].

Due to the huge vertebrate reservoir and the variety of triatomine insects, the eradication of this zoonotic parasite is practically impossible [9].

The symptomatology of Chagas disease may vary according to the route of infection: while vectorial transmission is usually asymptomatic or presents nonspecific symptoms, oral infection may increase the chance of acute cardiomyopathy because of the higher parasite loads associated. About

20 to 30% of acute cases develop serious chagasic cardiomyopathy with evolving symptoms and the risk of sudden death [10]. Fifteen to 20% develop digestive tract manifestations. The remaining infected individuals are considered to have the indeterminate form of the disease and the majority may not have symptoms or signs of the disease for all their life [10].

To treat new acute cases, intermediate phase patients or reactivations from chronic patients, the only drugs available were introduced more than four decades ago, with no alternatives. Benznidazole and nifurtimox (Figure 1A & B) are effective in treating acute infections, but efficacy is thought to decrease with the disease progression, with little to no effect in the chronic phase. Additionally, they must be administered for long periods of time and display numerous side effects. Some of the most serious side effects require monitoring and ultimately, treatment interruption.

For the above reasons, new drugs to fight this disease are a dire need. New formulations of old drugs, old drugs with new applications as well as innovative drugs are feeding the pipeline for the treatment of Chagas disease [11].

In the following sections we will discuss the therapies available today and their limitations followed by the advances in the drug discovery and the candidates currently in preclinical and clinical studies to treat Chagas disease.

2. CURRENT THERAPIES

2.1 BENZNIDAZOLE

Benznidazole (figure 1A) is a nitroimidazole (*N*-benzyl-2-(2-nitro-1*H*-imidazol-1-yl)acetamide) discovered in 1972 at Roche Laboratories, was and originally marketed as RochaganTM or RodanilTM. Despite its age, it is still the front-line treatment for the disease, although it is not approved by FDA [12]. Benznidazole is considered to be effective in reducing symptom severity and to shorten the clinical course and the duration of detectable parasitemia. Clinical cures are thought to be achieved in 60 to 85% of the acute cases and in more than 90% of congenitally infected infants, if treated in their first year of life [13]. Efficacy of benznidazole in chronic Chagas disease is still debatable, with reports varying from 15 - 35% of cure rates [14, 15]. The benefits of the drug in preventing cardiac and/or megacolon and megaesophagus manifestations are not yet clear [16]. To address this uncertainty, a large, multicenter, double-blind, randomized, placebo-controlled clinical trial called BENEFIT (The Benznidazole Evaluation For Interrupting Trypanosomiasis, ClinicalTrials.gov, ID: NCT00123916) with 3,000 patients in several endemic countries is underway and will evaluate the efficacy of a daily dose during 40 to 80 days of treatment in reducing mortality and morbidity in patients with chronic

Chagas cardiomyopathy [17]. Problems with precise dosing in young children and the adverse effects observed has led to the development of a new pediatric formulation of benznidazole. This lower dose, easily dispersible tablet that should improve dosing accuracy, safety, and adherence to treatment is currently in clinical trials (Population Pharmacokinetics Study of Benznidazole in Children With Chagas'Disease - Pop PK Chagas, ClinicalTrials.gov, ID: NCT01549236) [18].

The mechanism of action of benznidazole is thought to require the reduction of its nitro group by parasite nitroreductases, and in the process originate free radical intermediates and electrophilic metabolites that react with proteins, lipids and DNA that disrupt normal cell function and metabolism. It is also thought that *T. cruzi* NADH-fumarate reductase inhibition, phagocytosis improvement and death by INF- γ are additional mechanisms involved in parasite killing by benznidazole [19]. On the other hand, reduction by human liver NADPH, cytochrome P-450 reductase, P450, xanthine oxidase and aldehyde oxidase are thought to be responsible by the adverse side effects in patients [20].

Benznidazole is very toxic, but remains one of the few drugs with nitroaromatic groups still in use today [21] from where it derives its major toxicity. This toxicity is the main reason why benznidazole is far from an optimal drug and why new drugs are urgently needed. The most common side effect is dermatitis from hypersensitivity to the drug, the later appearing in 20 to 25% of the patients, usually after 10 days on the treatment, and for this reason, onwards weekly monitoring is recommended [22]. Digestive intolerance, peripheral neuropathy, depression of bone marrow, toxic hepatitis and lymphomas are other occurring side effects. Treatment interruption is most frequently due to dermatitis and digestive intolerance, although studies reveal that low-fat and hypoallergenic diet and daily dose administrations can reduce their incidence [22]. In addition, benznidazole should not be administered to pregnant women nor patients with severe renal or hepatic dysfunction, because of drug metabolism by these organs [23].

Strains resistant to benznidazole have been reported and are a major and increasing concern. An example is the Colombian strain, with benznidazole only being able to cure up to 16% of the mice infected with different clones [24]. *In vitro* results using real-time PCR suggest nitroreductases (NTRs) as the main mechanism of resistance *in vitro*, probably due to loss of a NTR gene copy [25]. A recent study warns of the relative ease in which benznidazole can develop resistance *in vitro* by a couple of different mechanisms such as chromosome loss and different point mutations in the NTR gene, all arising from a single population [26].

2.2 NIFURTIMOX

Nifurtimox (N-(3-methyl-1,1-dioxo-1,4-thiazinan-4-yl)-1-(5-nitrofuran-2-yl)methanimine) a 5-nitrofuran derivative (figure 1B), constitutes the second and only alternative to benznidazole for the treatment of Chagas disease. Its use is also not approved by the FDA either. Also known as Bayer 2502, the drug, marketed as LampitTM, was originally discovered in that pharmaceutical company in 1965, exactly 50 years ago and provided, for the first time, a treatment for chagasic patients. It is also used in combination therapy with eflornithine to treat second stage African trypanosomiasis caused by the parasite strain *Trypanosoma brucei gambiense* [27, 28].

Nifurtimox efficacy is similar to benznidazole, but it has a much higher frequency of adverse effects. There is a frequency of adverse effects in 98% of patients, with only 56% of them completing the 60-day course treatment and 29% not tolerating it for more than 30 days. Digestive symptoms are predominant and neurological alterations the most persistent. An estimated 7% of patients had severe adverse effects like angioedema, myocarditis and grade-3 anaphylactic reactions [29].

Recently, a study highlighted the possible biochemical mechanisms that may be associated with some of nifurtimox adverse side effects and as well as from other nitro-aromatic derived drugs [30].

Similar to benznidazole, nifurtimox also acts through a mechanism of intracellular nitro reduction with the generation of the nitro radical, followed by redox cycling. In contrast, there is a greater role for oxygen reactive species, like superoxide ion and hydrogen peroxide which are toxic to *T. cruzi*. This parasite is sensitive to oxidative stress due to weak detoxification mechanisms due to the absence of catalase or peroxidase activity and reduced superoxide dismutase activity [19, 31]. RNA interference studies on *Trypanosoma brucei*, responsible for Human African Trypanosomiasis, show that besides NTR, other proteins linked to ubiquinone synthesis are also involved in nifurtimox mechanism of action in that species, and it is likely that the same mechanism is also present in *T. cruzi* [32].

Resistance to nifurtimox is readily obtainable *in vitro* and it seems parasite nitroreductases play a major role in its resistance, mounting up evidence that cross-resistance with benznidazole can occur as has been reported [33, 34], increasing the pressure to find alternative drugs to treat patients refractive to the only available therapies.

The renewed interest in nitro-heterocycles has spurred research into finding alternative nitro-heterocycles, these include heteroallyl-containing 5-nitrofuranes, 5-nitrofuryl containing thiosemicarbazones and 2- or 3-nitro-1H-imidazole-based amides and sulfonamides. Despite some of these compounds being 10-50 times more potent than nifurtimox, they too seem to have the same issues of cross-resistance [35-37]. However, some recently synthesized novel nitrofuran amides, which are up to a 1000-fold more potent than nifurtimox, with excellent selectivity, have a trypanocidal activity that seems to be independent of nitroreductase activity [38].

3. DRUG DISCOVERY AND DEVELOPMENT FOR CHAGAS DISEASE

3.1 DRUG DISCOVERY STRATEGIES

Various approaches can be adopted when considering developing drugs for neglected diseases Nwaka and Hudson³⁹: (i) “*De novo* synthesis” is the classical way that focuses on the identification of new chemical entities through target discovery and compound screening. Although this is a very important strategy in the discovery of novel drugs for neglected diseases, it is a long-term approach and usually has constraints like high risk, high attrition rate of candidate compounds and needs high human and financial resources. Because of that and the perspectives of low market return and profits, the majority of companies do not make neglected tropical diseases a priority [40]. Populations affected by neglected diseases, and Chagas disease in particular, are very poor and don’t have the means to pay for expensive medication [41]. (ii) “Piggy-back” discovery is the process that takes advantage of the development of drugs for other diseases that may share some mechanistic identity in terms of molecular target, providing strong chemical start points to be followed and developed in the next phases. An example is the use of kinase inhibitors research data from cancer treatment to provide shortcuts for the development of a kinase inhibitor versus a parasitic target (iii). Label extension or drug repurposing is the approach that has some of the most immediate results, in that it uses already approved drugs for some pathologies, and repurposes them to be used in neglected diseases, saving considerable time and costs for approval processes after efficacy confirmation. Most of the toxicological data and sometimes, clinical tests are already available. An example of successful application of this strategy is the case of praziquantel for schistosomiasis and ivermectin for filariasis/onchocerciasis [39, 42]. More recently, Auranofin, an approved drug for rheumatoid arthritis, has been identified as an amebicide [43] and a clinical trial is being launched in Bangladesh.

Independently of the strategy used, an essential feature of the drug discovery process that plays a guiding role is the target product profile (TPP). TPPs are a set of criteria to be followed through the development process and describe the needs and characteristic that the new candidate has to meet in order to constitute an improvement over the current available therapies. Drugs for Neglected Diseases initiative (DNDi), a non-profit drug research and development organization founded with the

objective to develop therapies for neglected diseases, has recently updated a TPP for Chagas disease [44].

3.2 STARTING POINTS: SCREENING FOR HITS

Hits is the name given to the compounds that are first identified in a screening as interesting molecules able to yield a positive read or specific phenotype, usually in a similar way to a positive control or reference drug. Two different approaches can be taken to identify these starting points for drug discovery: a molecular target or target-based approach, and a phenotypic approach, also known as untargeted drug discovery. Target-based drug discovery relies on the previous discovery and characterization of a given molecular target and subsequent target validation by chemical or genetic means. Each has advantages and disadvantages [45]. Ideally, a target should be validated by more than one method. Chemical and biochemical validation is the proof that a molecular target, usually a protein, is able to be inhibited by a small molecule and that the use of such molecule in the parasite and/or *in vitro* and *in vivo* models of the disease leads to deficient parasite growth or ability to establish a normal infection. Genetic validation implies the reduction or elimination of the molecular target at the cellular level and the consequent observation of the interference in the parasite fitness/survival. The only reliable way to genetically validate a protein target in *T. cruzi* is by gene-knockout. A selection marker - a coding sequence of a gene that confers resistance to a given antibiotic used to select parasites - is cloned between two homologous regions of the locus to eliminate, in a way that when the DNA is electroporated into the parasite, homologous recombination occurs with the substitution of the endogenous gene by the exogenous selection marker. Because of the low recombinogenic potential of a parasite and slow growth kinetics, these transgenic techniques are extremely hard and time consuming to perform, with high failure rates and many weeks just to select stable transfected cells [46]. There are only two genes, which code to oligopeptidase B and N-myristoyltransferase that have been properly genetically validated and only the later seems to be interesting to explore as a drug target [47, 48]. Unfortunately and unlike the related species *Trypanosoma brucei*, *T. cruzi* does not have functional RNAi machinery. Apparently some of its components have been lost or mutated during evolution [49, 50]. Most recently, and in the wake of a revolution in genome editing technology, CRISPR-Cas9 technology has been successfully applied to *T. cruzi* with major breakthroughs like expression knocking down of an enzyme gene family consisting of 65 members [51].

After proper drug target validation, biochemical assays can be set up to screen for inhibitors. These assays usually make use of proteins, frequently of recombinant origin. In the past, many of the labs working with *T. cruzi* have screened only a small quantity of compounds, from either synthetic or natural origin, because of the limited access to large compound collections. As a consequence, the small, scattered and independent scale of the efforts greatly reduces the chances of discovering interesting compounds.

The scenario changed dramatically with the development of high throughput technology based on assay miniaturization and automation of protocols, from procedure to analysis, thus opening the door to large scale screening campaigns for this parasite. Among the improvements are time saving, since many compounds are tested in simultaneous, assay cost reduction, and data reproducibility. As an example, one high-throughput screen of 200 000 compounds against cruzipain, yielded 921 hit compounds that were subsequently screened by computational docking analysis and revealed 5 chemical scaffolds of common hits. These scaffolds are good starting points for further optimization and evidentiate the advantages of combining biological and bioinformatics analysis for prioritization of molecules after an high-throughput screening campaign has been performed [52]. In another example of target-based drug discovery, CYP51 from *Mycobacterium tuberculosis* was screened against a library of 20 000 organic compounds and resulted in two very active compounds [53], of which one (ChemDiv C155-0123) later showed selective inhibitory activity against the *T. cruzi* orthologous enzyme [54].

The big limitations of molecular target approaches are the possibility of poor disease linkage, low or impossible druggability of the target, risk of off-target effects that may translate into significant toxicity and the chance of overlapping research by different groups since there are so few targets characterized [55].

To circumvent such limitations, phenotypic base approaches have been developed. Instead of a single molecular target, whole cells are tested directly with the compounds and selection is made based on the observance of the required phenotype. This allows the selection of only those compounds that are active against the parasite, despite their mechanism of action. Also, it readily selects those compounds with the minimal pharmacodynamic and pharmacokinetic properties needed: proper intracellular distribution and accumulation, physiological binding and inhibition to target, etc., that are very difficult to predict with target-based strategies. However, this method requires that the target must be elucidated in the discovery process, a task not always easy but achievable [56]. The most recent trend in whole-cell assays has employed the use of high-content screening analyzers – automated microscopes that can image many conditions (compounds) in clear bottom culture microplates. When the technology appeared one assay was developed that made use of mammalian cells expressing GFP

and parasites stained with DAPI, and both manual and automated data analysis was performed [57]. With critics of genetically modified parasites/host cells and the further development of technology an improved assay was developed that used whole unmodified cells and parasites. This assay was validated with a small library of FDA-approved drugs [58]. The development of analysis software further automates the campaigns and allows the additional mining of important data, for example, the toxicity for host cells [59]. The first multi-thousand screening campaign described in the literature has been recently published [60].

Balancing the benefits and disadvantages of both strategies in hit identification for parasitic diseases such as Chagas, experts lean towards phenotypic approaches as the most promising methodologies [39] in which hits are selected for their ability to kill or not the parasite, coupled with cytotoxicity evaluation. In fact, when we take a look at the recent first-in-class new drugs with innovative molecular mechanism of action, we see that many of these drugs were discovered by phenotypic screening (28 vs. 17) [61]. The development of such high-throughput, high-quality, cheap and reliable assays like the described above is considered one of the biggest contributions to the advance of the Chagas disease drug discovery effort.

3.3 FROM HIT TO LEAD AND BEYOND

Once hits have been obtained, the most promising are further confirmed with the same assay in a dose response-curve to confirm activity and interpolate the EC_{50} value, a measure of the potency of the compound that is the concentration of a compound where 50% of its maximal effect is observed. Most guidelines indicate an EC_{50} lower than 10 μ M as a good starting value, although recommendations can vary if other criteria are met, like a high selectivity index, for instance. The confirmed hits can also be subject to complementary activity assays. These can be of a different configuration, employ a different readout, or even access activity against other strains. A recent paper shows that a set of compounds in clinical trials have significantly different activity profiles depending on the strain they are tested on [62]. As has been discussed above, it is a requisite of the TPP for Chagas disease that a future drug is active against a large set of different DTUs. Another key unanswered question is whether a compound must clear the infection totally, as the reference compound, benznidazole, does. Does total clearance of infected cells, or parasites in animals or humans correlate with multistrain activity or, more importantly, with the clinical course of disease in the subsequent 20 years? Compounds that still meet an agreed upon pharmacological and biological properties are called lead compounds. Leads are at the end of the screening campaign, but are the starting points of yet another phase in the drug discovery

and development called lead optimization. In this phase, compounds enter a cycle of further testing, commonly with *in vivo* testing of activity and toxicity, and are in parallel modified and optimized with medicinal chemistry to try to improve potency, selectivity, reduce toxicity and enhance pharmacokinetic parameters [40]. The medicinal chemistry necessary for lead optimization is very costly and constitutes a bottleneck for many drug discovery efforts. Few in academia have the resources or access to the synthetic chemistry capacity necessary to produce the tens to hundreds of compounds usually required for lead optimization. Organizations such as DNDi and recently launched consortia-based projects like the FP7 (Seventh Framework Program supported by the European Commission) KINDReD (Kinetoplastid Drug Development), NMTrypI (New Medicines for Trypanosomatidic Infections), PDE4NPD (Phosphodiesterase Inhibitors for Neglected Parasitic Disease) and A-PARADISE (Anti-Parasitic Drug Discovery in Epigenetics) have attempted to address this issue by coordination or outsourcing.

The optimized lead compound is one which can be called a pre-clinical candidate and enter the pre-clinical phase.

3.4 ANIMAL MODELS

Animal models are used to extract the maximum possible information on drug efficacy and toxicity before testing the drug candidates in humans. Since the translation of data is of the utmost importance, several animal models have been studied to reproduce the physiopathology of Chagas disease. Models like mouse, rat, rabbit, dogs and non-human primates have been tried, but none of them completely mimics what happens in the human host [63]. The rat has been used in the past, but early observations concluded that it is somewhat resistant to *T. cruzi* infection, developing a mild and slow pathology [64]. Rabbits proved to be capable of developing some of the chronic alterations such as focal myocarditis with a fibrous nature, but did not show more severe forms of chronic myocarditis or severe histological lesions in digestive track and skeletal muscles found in typical infections [65]. The Syrian hamster has also been proposed as an animal model for chronic Chagas cardiomyopathy. It was not able to display all the characteristics findings of human cases [66]. Dogs, on the other hand, develop most of the clinical aspects of the disease found in humans, in particular the indeterminate form characterized by a latent infection, without disease symptoms and with normal electrocardiograms; just a fraction of the animals develop chronic phase symptoms [67, 68]. However, this is a disadvantageous characteristic of the dog model when the larger amount of time and number of animals needed to obtain enough chronically infected dogs is considered in terms of the discovery

process. Monkeys are phylogenetically the closest related species to be used to study Chagas disease. Similarly to other pathologies like Leishmaniasis and HIV, Chagas disease findings in these animals are easily extrapolated to the humans [68, 69].

For the above reasons, the mouse has remained the preferred animal model. Mice are easy to handle, house and are cheaper. Additionally, mouse models resemble many immunological, pathological and physiological aspects of human Chagas disease. One of the commonly used strains in chemotherapy is Swiss mice, an outbred strain very sensitive to diverse *T. cruzi* genotypes [70]. Regarding inbred strains, Balb/C has also been extensively used and is considered one of the most susceptible to parasite infection in general [71]. C3H are a mildly resistant mouse strain commonly used to obtain chronic-like infection in these animals [72]. C57BL/6 are considered to be among the most resistant strains, although susceptibility can vary widely depending on the strain of trypanosome used [73]. This genetic background is frequently used to obtain chronically infected mice in attempts to reproduce the pathophysiology of the human disease.

The obtention of a valid model for chronic Chagas disease remains one of the biggest challenges in research. Current chronically infected mouse models develop an anti-inflammatory infiltrate and fibrosis in the heart, hallmarks of the disease in humans, but development of a model closely resembling human chronic Chagas cardiomyopathy with extensive fibrosis, segmental myocardial abnormalities and macroscopic ventricle dilatation after a period of absence of signs is still to report [66]. According to current protocols, four strategies have been employed to try to mimic chronic Chagas disease in mice: (a) a combination of susceptible mice strain, pathogenic *T. cruzi* DTU, age of animals and inoculation route that guarantees the survival of the animals to the acute phase; (b) infection of mice with a lethal dose of *T. cruzi* followed by the treatment with a reference drug that assures animal survival, but not parasite clearance; (c) infection of resistant strains of mice with sub lethal inoculum of low pathogenic DTU; (d) infection of animals immunized by attenuated strains with a pathogenic DTU [74].

There are also dozens of different *T. cruzi* strains that have been used in animal models of the disease. Each research group works with a limited set of biological specimens that may reflect the history of the lab. An illustrative example of this variability is the case of A/J and C3H/HePAS mice infected with the same clone of Sylvio X10/4. Distinct histopathological findings are reported, suggesting a host genetic role in the manifestations and progress of the disease [75]. This variability hinders the extrapolation of results to other animal models and ultimately, to humans.

Recent guidelines for *in vivo* testing of compounds in Chagas disease drug discovery have been elaborated. One protocol suggests three independent and consecutive *in vivo* evaluations of drug candidates: (1) testing for the effect of the compound on parasitemia reduction using Swiss female

mice infected with Y strain, three doses of compound with the highest one set at the maximum tolerated dose, orally or intraperitoneally, and after five days of infection for a duration of five consecutive days; (2) analysis of parasitological cure during the acute phase using Swiss female mice infected with Y strain, with the dose established in the previous stage; (3) cure the acute phase of parasitemia caused by Colombian strain, which is benznidazole resistant [76]. Parasitemia is analyzed at 5, 8 and 10 days post infection (dpi) for (1) and (2) and at 20, 25 and 30 dpi for (3). Mortality is evaluated for all the three phases at 30 days and PCR, after immunosuppression with cyclophosphamide, to detect “latent” parasites. This technique was employed because it was proved to be more sensitive and time-efficient than haemoculture. All the tests are done against a positive control of 100 mg benznidazole per kilogram of weight per day [76].

The effectiveness in the chronic and indeterminate stage comes further on the development process, and the lack of it does not invalidate the drug since, if the TPP is followed, it should be already an advance over existing therapies.

3.5 BIOMARKERS

Another obstacle in the drug discovery for Chagas has been the lack of reliable biomarkers of cure. Traditionally, the definitive test of cure relies on conventional serology methods that have the limitation that it can take many years for the seroconversion to take place. Also, the majority of currently used methods employ crude antigen preparations from parasite life-cycle stages not present in the mammalian host. Polymerase chain reaction is the standard method of cure in the current clinical trials and although useful, there is no proof of efficacy and it is only an indication of sterile cure for a given therapy [77]. Newer tests using recombinant proteins or peptides may be an improvement, but results are often inconsistent [78]. A recent a promising discovery in the field has been the identification of unusual fragments of human apolipoprotein A1 (APOA1) that are specifically present in chagasic patients and seem to disappear after treatment with nifurtimox [79, 80]. In mouse models, different methodologies to access parasitological cures were used after treatment with benznidazole and found out that even mice considered cured by hematological criteria still showed positive PCR tissues, either indicating a residual infection or residential parasite nucleic acid [81].

4. TARGET CANDIDATES IN THE PIPELINE

4.1.ERGOSTEROL BIOSYNTHESIS INHIBITORS

Inhibitors of sterol 14 α -demethylase (CYP51) constitute a major fraction of all the drugs in the Chagas disease pipeline [11]. This enzyme is involved in the *de novo* synthesis of sterols in *T. cruzi*. Sterols are membrane lipids present in eukaryotes and have essential functions such as control of membrane fluidity and permeability, signal transduction and modulation of membrane-bound enzyme activity [82]. While in mammals the major sterol is cholesterol, in plants, fungi and protozoa the major sterol present is ergosterol. The difference consists of a second double bond at the B ring and a fully saturated side chain with a methyl group at C24 in cholesterol [83]. CYP51 catalyzes a critical step of this biosynthetic pathway, removing the C14 methyl group from the sterol intermediate eburicol and originating 14 α -demethyl-14dihydroeburicol [84].

Ergosterol biosynthesis inhibitors are among the most common drugs used to treat fungal infections, and after the validation of this pathway in *T. cruzi*, compounds that were originally developed as antifungals were tested against the parasite. While some of the early generations imidazoles (e.g. miconazole, ketoconazole) and triazole (e.g. itraconazole, fluconazole) sterol biosynthesis inhibitors have some attenuating effect on the infection, they failed in achieving parasitological cures [85]. However, as newer azoles to treat fungi infections are still an ongoing interest for pharmaceutical companies, latest generations drugs have also been tested for anti-*T. cruzi* activity.

One of the most promising molecules of the past decade was posaconazole (figure 2A). This triazole originally marketed as Noxafil by Schering-Plough pharmaceutical and active against *Candida spp.* and *Aspergillus spp.* is one example of the previously described drug repurposing strategy. Early assays demonstrated its potent and specific *in vitro* activity against *T. cruzi*, especially against the amastigote stage. Moreover, the effect on murine acute and chronic models was curative, rather than suppressive, as some earlier tested antifungal compounds demonstrated [86]. Later, posaconazole also proved to be an efficient trypanocidal against benznidazole and nifurtimox resistant strains, even in immunosuppressed mouse models, where the parasite would have a favorable environment to multiply [87]. A comparative study between posaconazole and benznidazole in a mouse model of Chagas disease showed both drugs led to 100% survival rates, suppression of parasitemia and negative *T. cruzi* antibodies. Only posaconazole-treated mice had completely negative haemocultures 54 dpi, whilst 50% of the benznidazole-treated had positive results. Also, plasma enzymatic assessment of cardiac lesion was indistinguishable from uninfected control for posaconazole, but significantly higher for benznidazole [88]. These promising results led to two clinical trials: one phase 2 trial sponsored by Hospital Universitari Vall d'Hebron Research Institute (ClinicalTrials.gov, ID: NCT00349271, CHAGASOL) that evaluated posaconazole and benznidazole for the treatment of Chagas disease

chronic infection [89] and a phase 2 trial by Schering-Plough (now merged with Merck & Co.) for the treatment of asymptomatic Chagas disease, comparing a posaconazole with a placebo regimen and a combination of posaconazole with benznidazole (ClinicalTrials.gov, ID: NCT01377480, STOP CHAGAS) [90]. When evaluating posaconazole in patients, it is noteworthy that a woman with chronic Chagas disease and systemic lupus erythematosus requiring immunosuppression, was treated with posaconazole eliminating *T. cruzi* completely, whereas benznidazole treatment failed in this patient [91].

Unfortunately, the first clinical trial of posaconazole in humans did not replicate the results reported for the first patient. The treatment, consisting of two doses delivered orally for 60 days, had initial marked antitrypanosomal activity in chronic Chagas disease affected patients, but follow-up at the end of treatment suggested reactivation of infection, as documented by PCR. All but one patient treated with benznidazole showed negative PCR. The second clinical trial has finished in January 2015 and the final results should be published soon thereafter.

The activity of posaconazole is attributed in part to its pharmacokinetic characteristics, with a large volume of distribution and long terminal half-life, coupled with the fact that this lipophilic drug accumulates in cell membranes. This is expected to give high local concentrations of the drug to interact with the membrane-bound CYP51 target [92]. A drawback is the difficult synthesis of posaconazole and the associated costs of about €8,000 per treatment, a value that clearly is incompatible with the economical impoverished majority of the population affected [91].

Another ergosterol biosynthesis inhibitor in the recent pipeline was E1224 (figure 2B) from Eisai Co. E1224 is the monolysine salt of ravuconazole, thus a pro-drug of an antifungal with a short half-life. In this trial, which began in Bolivia in July 2011 as a partnership of DNDi and Eisai, adults with chronic “intermediate” Chagas disease were given placebo, E1224 or benznidazole (ClinicalTrials.gov, ID: NCT01489228). A series of examinations were then carried out in the following months in order to evaluate parasitological cures [93]. According to DNDi, the drug failed to maintain sustained efficacy 1 year after the end of treatment. The advantage of E1224, was that the structure of this compound was simpler and thus synthesis should be less expensive.

A third ergosterol biosynthesis inhibitor in clinical trials is Tak187 (figure 2C), a triazole synthesized in the 1990's and the property of Japanese Takeda Chemical Industries. There was 100% survival in the acute model of Chagas disease in mice treated with Tak-187 as well as a very high parasitological cure (80%). In the chronic model, not all mice survived, but those that did there was 100% parasitological cures even with benznidazole and nifurtimox resistant *T. cruzi* strains [94]. Subsequent studies confirmed that the drug could produce reductions of parasitemia similar to benznidazole, but at 10 times less dosage. Furthermore, it was superior to benznidazole in reducing

inflammatory infiltrates and tissue damage in the heart and skeletal muscle of infected mice. The superior efficacy was attributable to higher intrinsic activity and long terminal half-life [95]. Tak-187 has completed phase I trials [11].

The fungicide fenarimol, another inhibitor of CYP51, has been found to affect *T. cruzi* growth. After synthesis of analogues, the most promising compounds were tested *in vivo* in a Swiss mouse model with three dosing regimens. One analogue was effective in the 20 days regimen, reducing parasitemia to negligible levels that only reactivated after three cycles of immunosuppression [96].

While remaining controversial as to long term clinical effects, the failure of repurposed antifungals in Chagas disease clinical trials has focused attention to drug leads targeting the *T. cruzi* CYP51 (TcCYP51) itself [97, 98]. TcCYP51 is one of the most studied enzymes of *T. cruzi* as represented by crystallographic data for 18 structures of the protein with 16 different ligands in the protein databank (www.rcsb.org). Three structural features make this protein particularly interesting for a rational drug design approach: (i) high structure rigidity, particularly in its substrate binding cavity; (ii) a substrate access channel in both ligand-free and bound structures that remains open and well defined; (iii) a substrate binding cavity that extends deeper inside the molecule than in other CYP structures [99]. Figure 3 depicts the key structural regions of TcCYP51: the active site residues within the BC-region (residues 100-120, PDB ID: 3KSW sequence numbering) that close the active site and isolate the substrate from solvent; the substrate tunnel through which substrate and ligands enter the active site; and the deeper substrate regions occupied by smaller ligands such as VNF and LFT (figure 4A & B) [98, 100, 101]. Some novel compounds designed to target TcCYP51 possess a nitrogen atom as a warhead, included in an azole or pyridine heterocycle that are able to form a coordination bond with the CYP51 catalytic heme iron and are represented in figure 4A-C. These compounds are simple and easy to synthesize and demonstrated strong inhibitory potential of intracellular amastigote growth of *T. cruzi* [98, 102-104]. VNI, in particular, was able to cure infected mice, has oral bioavailability and low toxicity, making it an excellent drug candidate [102].

Other enzymes of the ergosterol biosynthesis pathway may be targeted as potential drug targets for Chagas disease, including squalene synthase. This enzyme is responsible for the first step of ergosterol biosynthesis and was suggested as target in the parasites *Leishmania mexicana* and *T. cruzi* [105]. The effective and potent squalene synthase inhibitor 4-phenoxyphenoxyethyl thiocyanate effective against epimastigote proliferation producing an accumulation of mevalonate pathway intermediates is an example of compound targeting this enzyme [106, 107]. E5700, a drug from the Eisai Co. which is in development as human cholesterol lowering agent, is efficacious against *T. cruzi* [108]. Amiodarone (figure 2E) also inhibits ergosterol biosynthesis and is currently in clinical trials (as well as dronedarone) against the chronic phase of the disease [11]. This antiarrhythmic drug is used in

the treatment of cardiac failure in chronic chagasic patients and has been found to act on a synergistic manner with azoles in disrupting *T. cruzi* biology. Amiodarone interferes with the calcium hemostasis but also inhibits ergosterol biosynthesis, while posaconazole or itraconazole also affects calcium hemostasis, suggesting a viable and advantageous drug combination [109-111]. Allylamine terbinafine, a squalene epoxidase inhibitor, and mevinolin inhibit 3-hydroxy-3-methylglutaryl-coenzyme A reductase, are antiproliferative against *T. cruzi* and both have been shown to be synergistic with ketoconazole against cultures of the parasite [112], suggesting they could be used in the treatment of human Chagas disease [113].

4.2. CRUZIPAIN INHIBITORS

K-777, a vinyl sulfone cysteine protease inhibitor was originally synthesized at Khepri Pharmaceuticals as an anti-inflammatory lead (figure 2D). It is an irreversible inhibitor of cruzipain, also known as cruzain or gp51/57. Cruzipain is a cathepsin L-like cysteine protease responsible for the majority of proteolytic activity in all the stages of *T. cruzi*. It may be essential for metabolism, metacyclogenesis, immune evasion, and invasion of host cells [114-117]. It has been suggested not only as a drug target but also as a vaccine target.

Early experiments with mouse models of Chagas disease showed that cysteine protease inhibitors were able to rescue mouse from lethal infection, displaying repetitive negative haemocultures and so indicating parasitological cure [118]. K-777 was able to rescue mice from an acute and lethal *T. cruzi* infection even with a non-functional immune system, as seen in immunocompromised patients (e.g. HIV/AIDS) or immunosuppressed individuals (e.g. transplantation patients) [119]. K-777 also abrogated myocardial damage in beagle dogs treated orally for seven days [120].

Several other classes of inhibitors of cruzipain have been reported as potential drug leads, including selenosemicarbazones [121], amidines bearing benzofuroxan or benzimidazole [122], and others scaffolds [123, 124]. Effective nitrile inhibitors of cruzipain have also been identified and serve to chemically validate this target [125].

A variety of approaches has been considered to rationally design inhibitors for cruzipain. Most of the compounds synthesized were originally designed to target the catalytic cysteine (Cys) of the enzyme in order to obtain irreversible inhibitors. The protein databank reports 24 crystallographic structures of cruzipain in complexes with inhibitors. They show that cruzipain is composed of one polypeptide chain folded into two domains: one mainly α -helix and the other with an extended antiparallel β -sheet. The catalytic triad is composed by Cysteine25, Histidine162 and Asparagine182 and together with the extended substrate-binding site, they are found in the cleft between the two domains [126]. Within the substrate binding site, different regions (S1', S1, S2 and S3), each devoted

to the interaction with and binding of a residues of the peptidic substrate, have been recognized (figure 5) [127]. Region S2 in particular residue Glutamine208 at its bottom is the key determinant for substrate specificity. These residues adopt a substrate-directed conformation in case that the S2 site is occupied by a basic or uncharged hydrogen bonding residues (such as Arginine and Tyrosine, respectively) whereas it assumes a solvent-directed conformation when an hydrophobic residue (such as Phenylalanine) is present [128]. Examples scaffolds designed to inhibit cruzipain are acylhydrazones, thiosemicarbazones and methoxyphenyl ketone derivatives. The discovery of acylhydrazone compounds as antiparasitic Cys protease inhibitors originated from an high throughput screening against brucipain, the major Cys protease of *T. brucei* [129]. Optimized scaffolds of this class of compounds have since been synthesized and also showed to inhibit cruzipain (figure 4D) [130, 131]. Interestingly, acylhydrazones share some similarity with chalcones, in which the unsaturated arylketone subunit can act as a Michael acceptor (figure 6). Chalcones possess anti *T. cruzi* activity, but few studies are associated with cruzipain inhibitory activity [132, 133].

After the initial discovery of a peptide vinyl sulfone as an irreversible (Michael acceptor) cruzipain inhibitor able to cure parasitic infections in animal models, but with low oral bioavailability owing to its peptidic nature, an optimized compound derived from a methoxyphenyl ketone scaffold and with desirable physicochemical properties has been reported (figure 4E). Its mechanism of action, supported by the crystal structure of the complex, is depicted in figure 7 [134].

Thiosemicarbazones are another class of covalent inhibitors originated from a screening of compounds able to inhibit cruzipain. Thiosemicarbazones inhibit Cys proteases through the formation of a reversible tetrahedral adduct by attack of the Cys thiolate to the carbon of the thiocarbonyl group (figure 4F) [135]. However, several members of this class of compounds, inactive on the enzyme, were shown to be active on *T. cruzi* parasites [136-138], suggesting that cruzipain could not be the main target for at least some of these compounds. This class of compounds was further modified according to the strategy represented in figure 9 [139] and led to compounds that could act in a way that differs from a simple cruzipain inhibition. Compound G from figure 4 inhibits cruzipain whereas its derivatives, as exemplified by compound in figure 4H, did not, but exhibited strong antiparasitic activity.

4.3. PURINE SALVAGE INHIBITORS

Allopurinol is an analogue of hypoxanthine that is used to treat gout, a condition characterized by deposits of uric acid in bone joints. The mechanism of action for this drug involves the inhibition of xanthine oxidase, an enzyme responsible for the consecutive conversion of hypoxanthine to xanthine and xanthine to uric acid. *T. cruzi* is not able to perform *de novo* synthesis of purines and needs to

acquire them from the host. Since the microorganism does not possess xanthine oxidase, allopurinol is incorrectly sensed as a purine substrate and is directly incorporated in the parasite DNA by hypoxanthine-guanine phosphoribosyltransferase, disrupting DNA-related processes. Previous assays showed the potential of the drug in arresting infection in cultured tissues [140], and a later study with other purine and pyrimidine analogues confirmed this activity [141]. In an animal model, allopurinol was able to reduce parasite blood levels, but with mild cardiac inflammatory infiltrates at the heart. Altogether, the results demonstrated the drug modified the evolution of the infection and prevented the acute phase from evolving into chronic cardiac disease [142]. A comparative study between itraconazole and allopurinol in preventing chronic Chagas disease in Chile showed similar results in preventing cardiomyopathy, but itraconazole was preferred due to the fewer adverse effects [143]. A combination of allopurinol with clomipramine to treat Chagas disease in an acute mouse model was found to be no better than the use of clomipramine alone [144]. Although there has been an interest in the label extension of this drug, early clinical evidence have discouraged the development of allopurinol as a drug to treat Chagas [145]. There is an interest in exploiting this pathway for Chagas chemotherapy, but to our knowledge no other compounds have been recently tested *in vivo* [146]. Several 4'-substituted and 3',4'-disubstituted 5-benzyl-2,4-diaminopyrimidines are selective inhibitors of *T. cruzi* dihydrofolate reductase and showed good *in vitro* activity against the parasite [147].

4.4. INHIBITORS OF PYROPHOSPHATE METABOLISM

Another pathway that has gained attention is the one responsible for pyrophosphate metabolism. This process does not take place in the cytosol but rather at acidocalcisomes, parasite-specific organelles that are also involved in calcium hemostasis, response to cell stress, osmoregulation, and energy transduction [148]. It has been demonstrated that bisphosphonates, drugs currently used to treat osteoporosis, accumulate in the acidocalcisomes and can inhibit a key enzyme of pyrophosphate metabolism – farnesyl pyrophosphate synthase [149].

The first report of this activity in animal models demonstrated that risedronate could reduce parasitemia with reductions in mortality, but no complete parasitological cures were achieved [150]. In another study, a significant reduction in mortality was observed when CD-1 mice were treated with risedronate, but myocardial pathology and ventricular dilatation was unchanged in comparison with control. On the other hand, Tulahuen strain infected C57BL/6 mice had no improvement in mortality [151].

More recently, metal complexes of the bioactive, bisphosphonates alendronate [152] pamidronate [152] and risendronate [153] were synthesized and showed activity against amastigotes, with no toxicity for the mammalian host cells tested. These complexes are thought to protect phosphonate

groups from ionization at physiological pH, increasing bioavailability to target the parasitic farnesyl diphosphate synthase [152]. Newly synthesized bisphosphonates also proved to be potent inhibitors of *T. cruzi* farnesyl diphosphate synthase [154].

4.5. TRYPANOTHIONE BIOSYNTHESIS INHIBITORS

Instead of the glutathione and glutathione reductase, trypanosomatids produce trypanothione and trypanothione reductase for thiol-dependent redox metabolism. This is essential in detoxification of free radicals and maintenance of the intracellular reducing environment. As this parasite-specific system does not exist in the humans, it is considered a potential target [155]. Recently, a study validated the trypanothione pathway as drug target with a metabolic modeling approach, suggesting that all the constituent enzymes and transporters present are essential for proper pathway function, but not all of them have therapeutic potential [156]. Many inhibitors have been found for this enzyme, including polyamine derivatives, crystal violet, acridine-based tricyclics, phenothiazine, benzoazepine, isoalloxazine, pyridoquinoline and many more have been synthesized [157-160]. *In vivo* testing of a trypanothione reductase inhibitor, thioridazine, promoted heart protection but failed to completely eradicate the parasite [161].

4.6. LIPID BIOSYNTHESIS INHIBITORS

Alkyllysophospholipids and lysophospholipid analogues, such as miltefosine and edelfosine have been shown to be active against proliferation and differentiation of *T. cruzi*, *in vitro* and *in vivo* with good oral activity and low toxicity [162-167].

Surprisingly little is known about the role of glycosphingolipids in trypanosomatids, however, various glycosphingolipids inhibitors have shown antiproliferative activity lysing 79 to 95.5% of parasite in an *in vitro* assay and showed cytostatic activity in mouse model [168].

Although *T. cruzi* glycosphosphatidylinositol GPI anchors share the same conserved core as other eukaryotes, many often contain a fourth mannose on which resides a galactofuranosyl (Gal_f) linked to the O-3 and an obligatory 2-aminoethylphosphonate (2AEP), also known as ciliate, linked to O-6 of the glucosamine of all *T. cruzi* GPIs and GPI-anchored mucins [169-171].

Enzymes common to the biosynthesis of the *T. cruzi* GPI have been shown to have sensitivities to various inhibitors in different organisms [172, 173]. A recent work identified the orthologous sequences of all genes involved in the biosynthesis of the *T. cruzi* GPI and three sequences showed they acted to complement yeast conditional mutants of genes of this pathway. Unsuccessful attempts to generate *T. cruzi* knockouts for three of these genes further suggested that the GPI is an essential component of the organism [174].

Although the role of 2AEP in *T. cruzi* has not yet been identified, it has been shown to be a virulence factor in human pathogenic organisms such as *Bacteroides fragilis* [175]. The absence of the 2AEP biosynthetic enzymes (phosphoenolpyruvate mutase, phosphoenolpyruvate decarboxylase & 2-aminoethylphosphonate transaminase) in humans, make the pathway an excellent candidate for drug targeting. Recent investigations by Coron and Smith (unpublished) have genetically validated the AEP biosynthetic pathway and identified compounds with potent *in vitro* activity against the recombinant enzymes of the 2AEP pathway as well as epimastigotes.

CONCLUSION

Since the discovery of the etiological agent of Chagas disease more than 100 years ago, a cure has been pursued. It took 50 years to discover a specific drug to treat this neglected disease. However, apart from the discovery and development of benznidazole some years later, no improvements in chemotherapy have been made compared to these highly toxic compounds despite a half century of intense research. Nevertheless, there are reasons to be optimistic. The global nature of the disease and the information about its pathology, has brought new researchers to the field. Novel basic and applied research is constantly feeding our knowledge of the disease. New partnerships, including with pharmaceutical companies, are accelerating efforts traditionally made solely by academia. Clinical trials for some candidates have been recently completed, some are under way and a few more are planned to begin shortly. But we should not ignore the still long way and challenges to find a better treatment for Chagas: resources and funding are scarce, and there is a critical need to define beneficial intellectual property agreements and improve data sharing.

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Figures

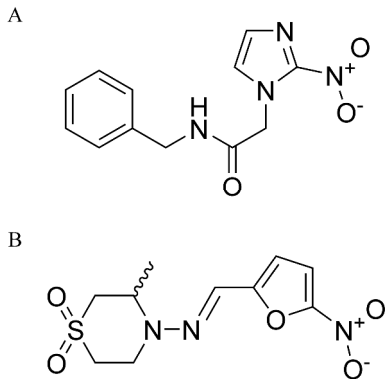


Figure 1. Current drugs for Chagas disease: (A) benznidazole and (B) nifurtimox.

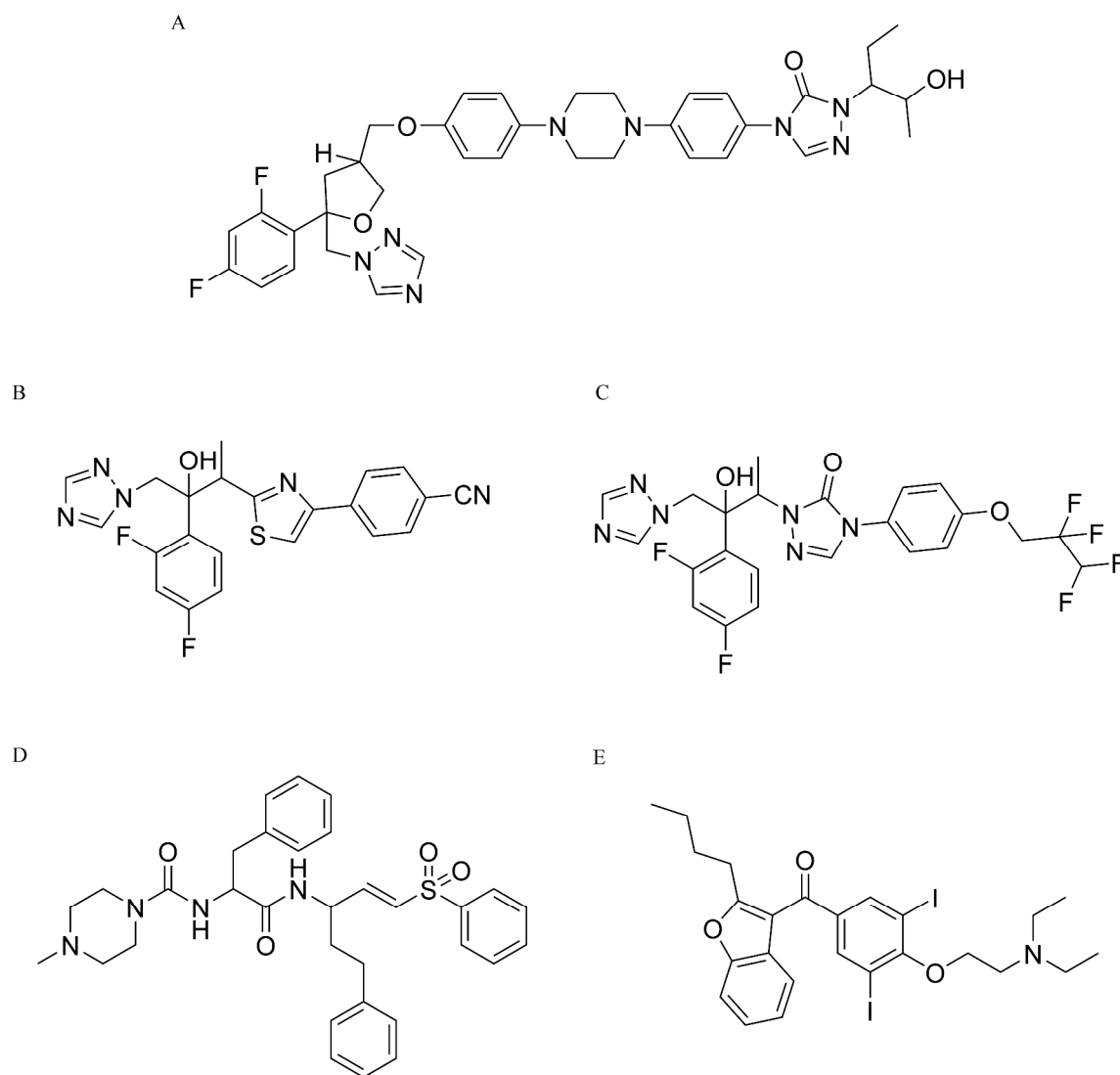


Figure 2. Drugs on the pipeline for Chagas disease: (A) posaconazole, (B) E1224, (C) Tak187, (D) K-777, (E) amiodarone.

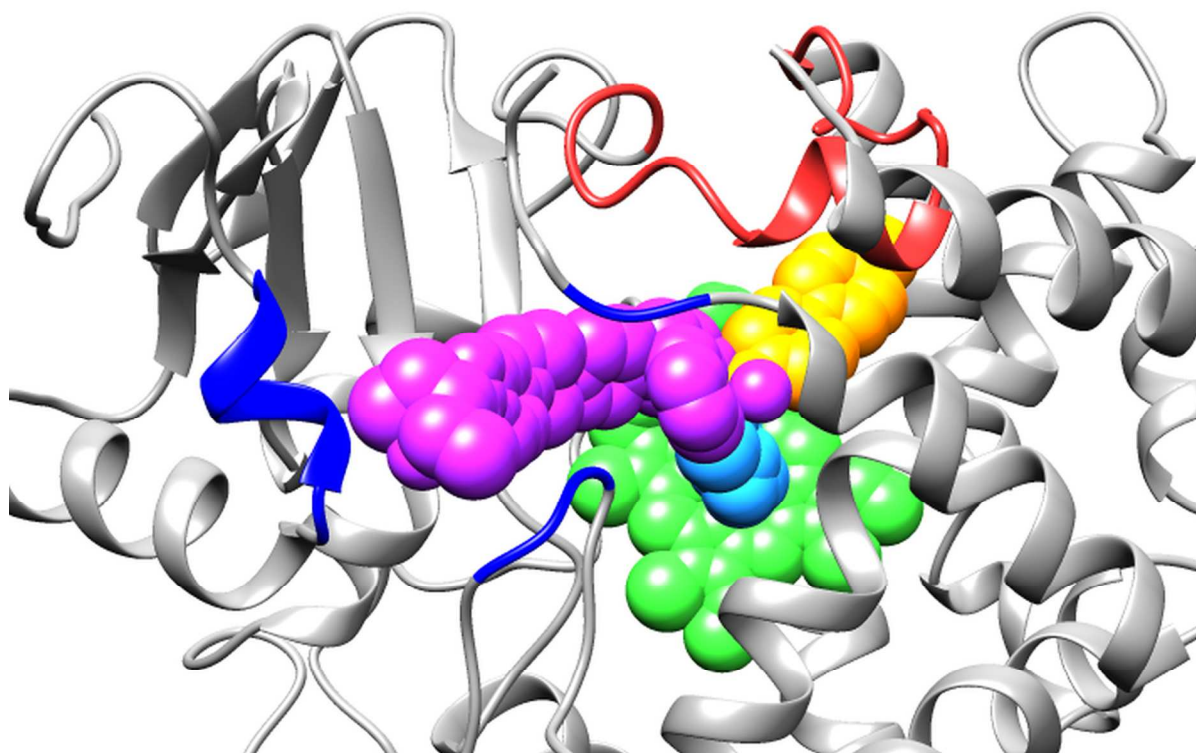
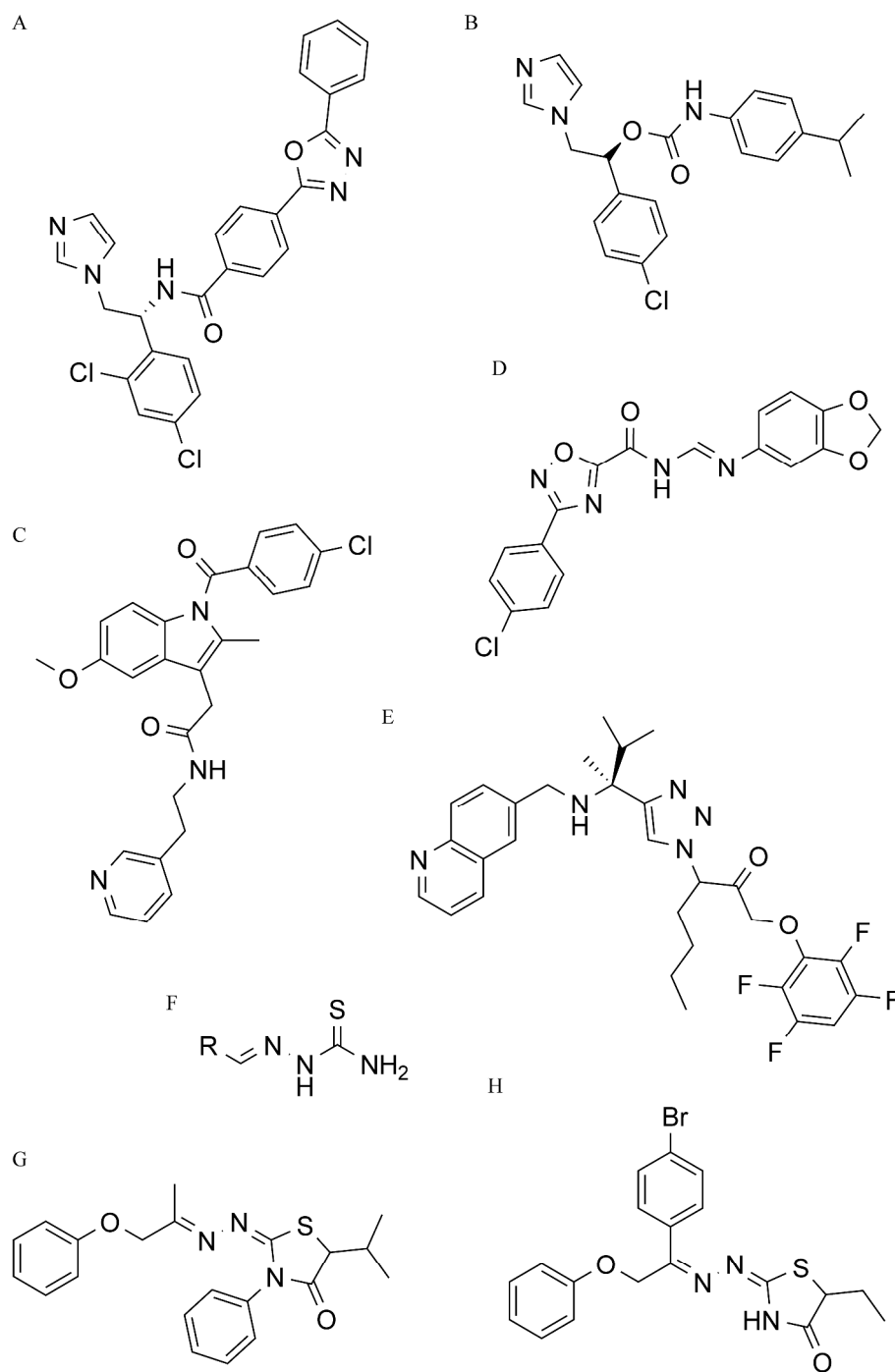


Figure 3: TcCYP51 crystallographic structure (PDB ID: 3KSW). The protein is represented as a gray ribbon, the BC-region that close the active site and includes residues important of the catalytic activity is colored in red, residues surrounding the entrance of the substrate channel are colored in blue. Inside the enzyme, the heme group is colored in green with atoms represented as sphere; the binding cavity is occupied by ligands (POZ (PDB ID: 3K1O) and VNF (PDB ID: 3KSW)), whose atoms are represented by sphere and colored as follow: purples the moieties that occupy the substrate channel; orange the moieties that occupy the deeper binding region; cyan the moieties involved in the interaction with the heme group.



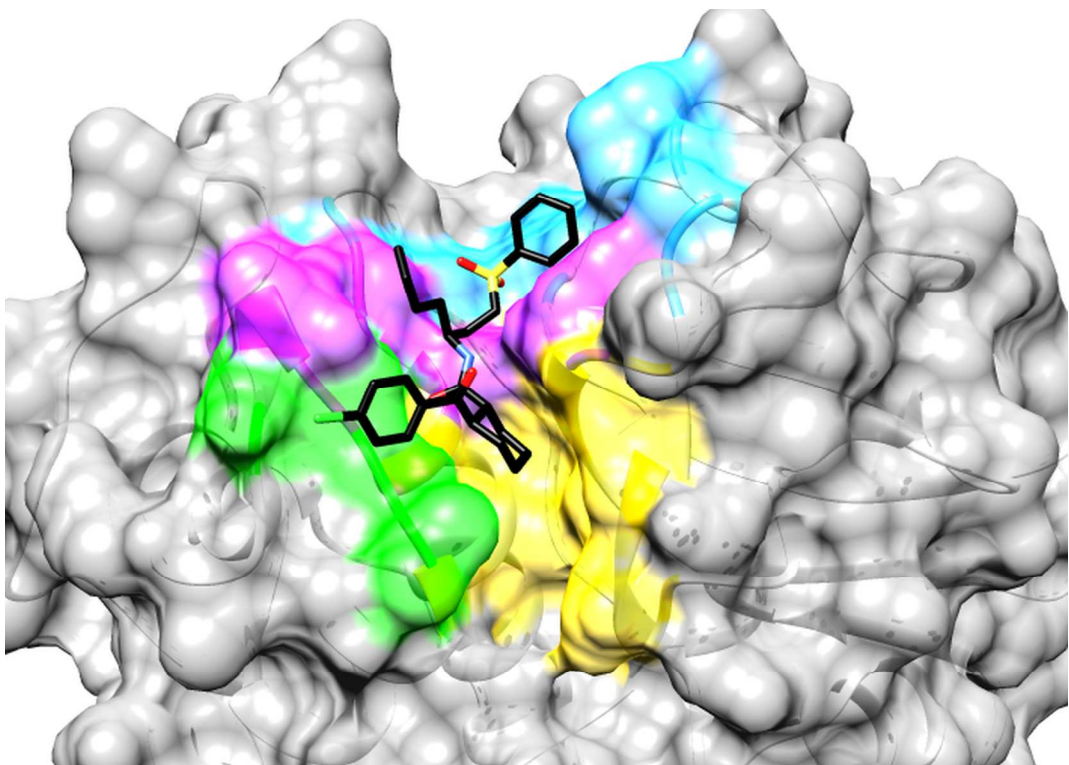


Figure 5. Crystallographic structure of cruzipain in complex with a non-peptidic vinylsulfone derivative (PDB-ID: 3HD3). The protein is represented as a surface colored in gray, specific sites are colored as follow: S1' in cyan, S1 in purple, S2 in yellow, S3 in green. The ligand in represented in stick, colored by-atom as follow: C in black, O in red, N in blue, S in yellow.

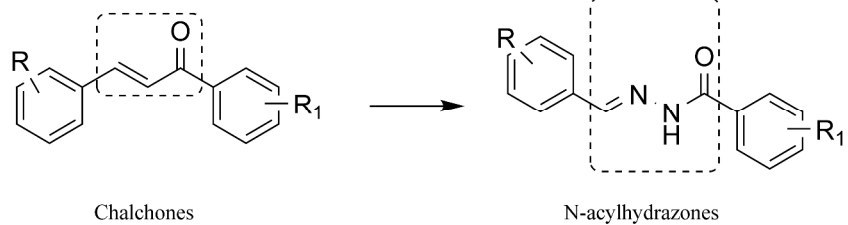


Figure 6: Similarity between chalcones and N-acylhydrazones.

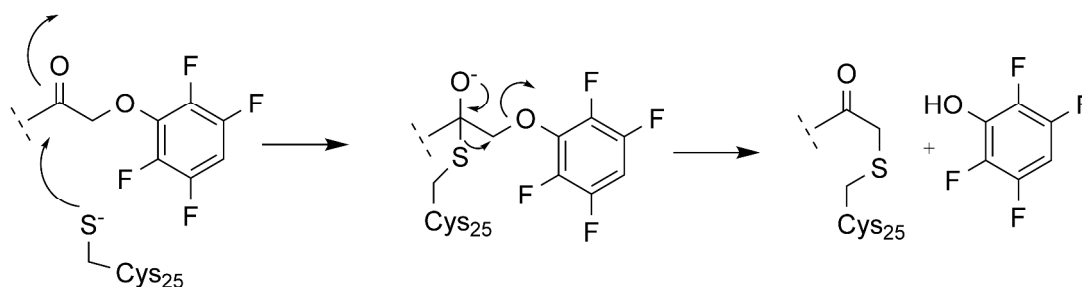


Figure 7: Mechanism of inactivation of cruzipain by a tetrafluorophenoxymethyl ketone derivative.

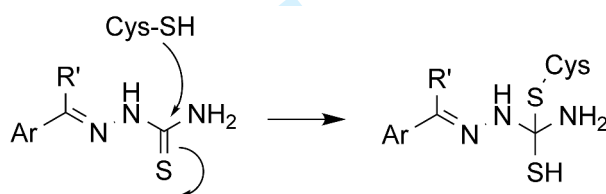


Figure 8: Mechanism of inactivation of Cys proteases by thiosemicarbazones.

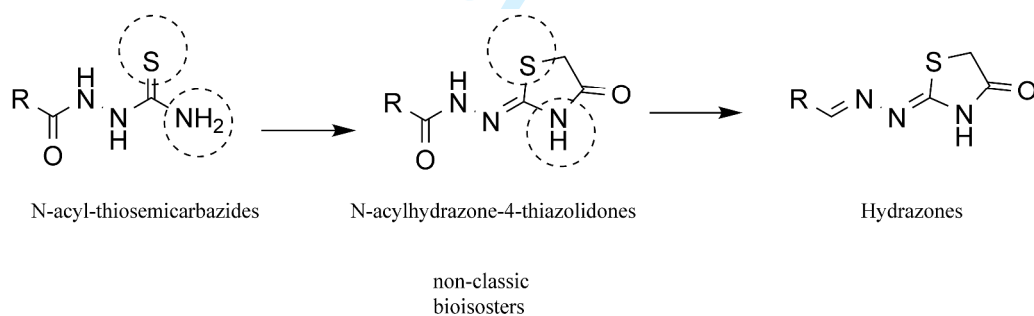


Figure 9: Design of hydrazones as anti *T. cruzi* parasites.

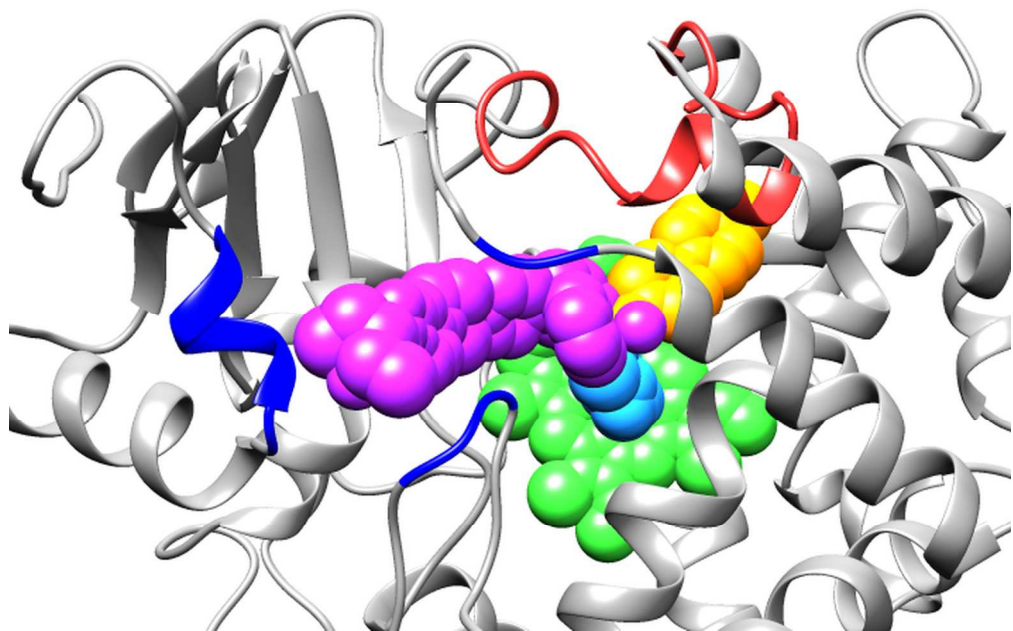


Figure 3: TcCYP51 crystallographic structure (PDB ID: 3KSW). The protein is represented as a gray ribbon, the BC-region that close the active site and includes residues important of the catalytic activity is colored in red, residues surrounding the entrance of the substrate channel are colored in blue. Inside the enzyme, the heme group is colored in green with atoms represented as sphere; the binding cavity is occupied by ligands (POZ (PDB ID: 3K1O) and VNF (PDB ID: 3KSW)), whose atoms are represented by sphere and colored as follow: purples the moieties that occupy the substrate channel; orange the moieties that occupy the deeper binding region; cyan the moieties involved in the interaction with the heme group.

157x99mm (300 x 300 DPI)

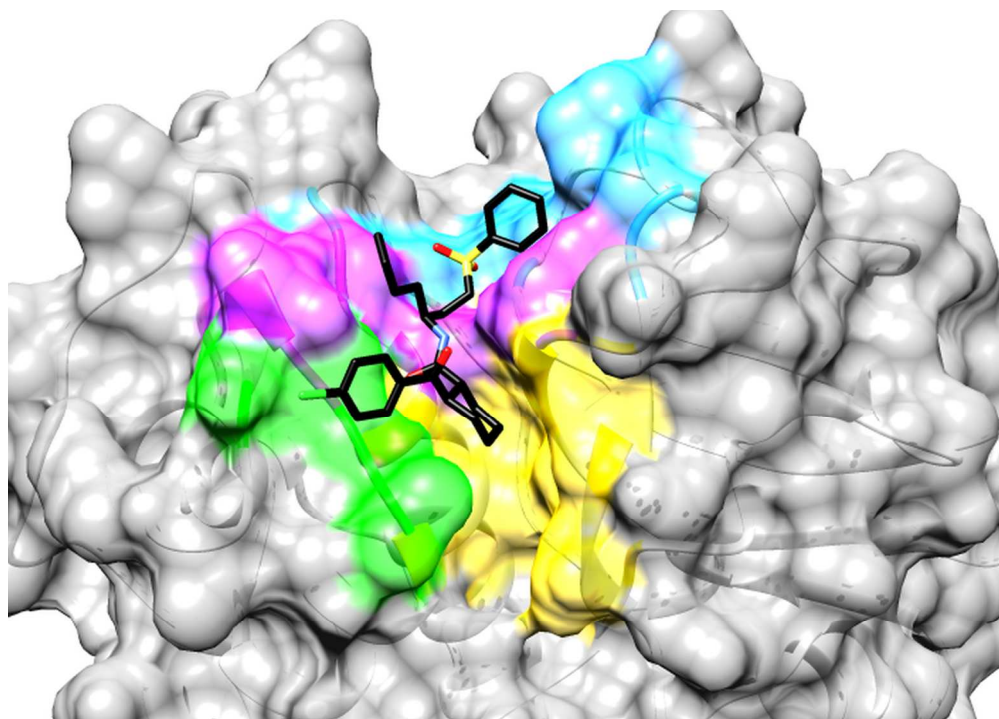


Figure 5. Crystallographic structure of cruzipain in complex with a non-peptidic vinylsulfone derivative (PDB-ID: 3HD3). The protein is represented as a surface colored in gray, specific sites are colored as follow: S1' in cyan, S1 in purple, S2 in yellow, S3 in green. The ligand in represented in stick, colored by-atom as follow: C in black, O in red, N in blue, S in yellow.

140x99mm (300 x 300 DPI)