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Current drug therapy and pharmaceutical challenges for Chagas disease

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ABSTRACT

One of the most significant health problems in the American continent in terms of human health, and socioeconomic impact is Chagas disease, caused by the protozoan parasite *Trypanosoma cruzi*. Infection was originally transmitted by reduviid insects, congenitally from mother to fetus, and by oral ingestion in sylvatic/rural environments, but blood transfusions, organ transplants, laboratory accidents, and sharing of contaminated syringes also contribute to modern day transmission. Likewise, Chagas disease used to be endemic from Northern Mexico to Argentina, but migrations have earned it global. The parasite has a complex life cycle, infecting different species, and invading a variety of cells – including muscle and nerve cells of the heart and gastrointestinal tract – in the mammalian host. Human infection outcome is a potentially fatal cardiomyopathy, and gastrointestinal tract lesions. In absence of a vaccine, vector control and treatment of patients are the only tools to control the disease. Unfortunately, the only drugs now available for Chagas' disease, Nifurtimox and Benznidazole, are relatively toxic for adult patients, and require prolonged administration. Benznidazole is the first choice for Chagas disease treatment due to its lower side effects than Nifurtimox. However, different strategies are being sought to overcome Benznidazole's toxicity including shorter or intermittent administration schedules—either alone or in combination with other drugs. In addition, a long list of compounds has shown trypanocidal activity, ranging from natural products to specially designed molecules, re-purposing drugs commercialized to treat other maladies, and homeopathy. In the present review, we will briefly summarize the upturns of current treatment of Chagas disease, discuss the increment on research and scientific publications about this topic, and give an overview of the state-of-the-art research aiming to produce an alternative medication to treat *T. cruzi* infection.

Contents

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1. Introduction

Neglected Tropical Diseases are a group of 17 parasitic infections that affect people living with low income mainly in developing countries, causing large physical, economic and health problems in patients and their communities. According to the World Health Organization (WHO), these infections include dengue, rabies, trachoma, human African trypanosomiasis (sleeping sickness), leishmaniasis, leprosy, Chagas disease, Buruli ulcer, echinococcosis, lymphatic filariasis, onchocerciasis, schistosomiasis, dracunculiasis (Guinea worm disease), foodborne trematodiases, taeniasis/cysticercosis, soil-transmitted helminth infection, and yaws (WHO, 2013). American trypanosomiasis, also called Chagas disease after the Brazilian physician Carlos Chagas who described the infection in 1909, is a vector-borne infection caused by the protozoan parasite *Trypanosoma cruzi* (Chagas, 1909). It was originally found throughout South and Central America, but owing to migrations it has been recorded in every continent. The endemicity of the disease is a complex phenomenon that roots in history, when sedentary and grain accumulation developed in ancient human populations facilitating their interaction with vectors (Brenière et al., 1998; Aufderheide et al., 2004; Coura, 2007) and in the modern economic and social scenery of Latin America (Dias et al., 2014; WHO, 2002). Chagas disease represents one of the most significant health problems in the American continent in terms of human health (i.e., number of people infected with and dying from it), socioeconomic impact, and geographic distribution. Even though the incidence of new infections decreased in Brazil and other countries due to urbanization and improved living conditions, an estimated number of 8 million people remains infected (WHO, 2013).

In its natural life cycle, *T. cruzi* is transmitted by reduviid insects to vertebrates while they sleep. While feeding, infected reduviids (Order Hemiptera, Family Reduviidae, Sub-Family Triatominae) defecate on the sleeping vertebrate and parasites present in the feces (metacyclic trypomastigotes) enter the host through skin abrasions provoked by scratching the bite. Due to this complex life cycle that can be sustained in both sylvatic and urban environments, *T. cruzi* oral transmission has been reported in rural areas in close association with the sylvatic cycle. One of the main sylvatic reservoirs is *Didelphis* spp., marsupials that have anal glands where all *T. cruzi* stages can be found (epi, trypo and amastigote forms). With these glands' secretions, oppossums can contaminate with parasites fruits and utensils used to prepare juice in sylvatic/rural environments in the Amazon region (Shikanai-Yasuda et al., 1991; Roque et al., 2008; Coura, 2015; Barbosa et al., 2015). Crushing infected bugs among the fruits and ingestion of raw or undercooked meat has also been reported as a source of oral outbreaks (Pereira et al., 2010; Cardoso et al., 2006). Oral transmission is usually manifested as acute Chagas, and it has been demonstrated that metacyclic trypomastigotes invade the gastric mucosal epithelium (Camandaroba et al., 2002; Pinto et al., 2008; Pinto Dias, 2006; Yoshida, 2009). A comparison between oral and gastric infections in mice showed that oral infections induced higher levels of parasitemia, mortality, liver lessions, and pro-inflammatory cytokines (INF-γ and TNF-α) than gastric infections (Barreto-de-Albuquerque et al., 2015). Similarly, intragastric infections showed slower development of parasitemia, with lower peaks, as well as lower mortality than intraperitoneally infected mice (Castellanos-Domínguez et al., 2015), demonstrating that the initial site of parasite entrance strongly affects the host immune response and disease outcome.

Human infection results in a myriad of clinical symptoms arising from the initial deposition of infective trypomastigotes, occasionally originating swelling or "chagoma" at the site of infection (WHO, 2002). The development of Chagas' disease varies considerably and there are marked differences between individuals and geographic localities. This suggests that genetic differences on both the parasite and the host (Trischmann and Bloom, 1982; Anon., 1992; Silva et al., 1992; Williams-Blangero et al., 2012) are important for disease outcome, which is characterized by three phases. In the early, acute phase of infection trypomastigotes circulate in blood (parasitemia), and infect cells where they transform into the asexually-multiplying amastigotes. When the cell containing amastigotes is broken, parasites are released to the blood and infect other cells in a cycle lasting a few weeks. During this period there are unspecific symptoms (fever, allergic reactions, and more rarely acute heart failure or meningoencephalitis). Acute Chagas disease can be life threatening if acute myocarditis develops, but it can also be a non-specific febrile illness that in some cases resolves spontaneously without diagnosis or therapy (WHO, 2002). The acute phase might be fatal in children, but most patients survive to enter a prolonged, asymptomatic indeterminate phase where parasites reach and establish in their target organs, forming amastigote nests (Estani et al., 1998). Chronic Chagas disease progresses at a relatively slow pace and 70% of chronic patients have no further evidence of disease. Only 30% develop chronic Chagasic cardiomyopathy or mega-organs—esophagus, liver or intestines- decades later (WHO, 2002). Chronic Chagasic cardiomyopathy (CCC) is characterized by heart hypertrophy and dilatation, which cause severe arrhythmias and progressive systolic dysfunction (Pearson et al., 2003). Severe inflammation of the myocardium (myocarditis) was found to be positively associated with parasite persistence and interstitial fibrosis (Benvenuti et al., 2008). Destroyed myocardial cells are also found, with lymphocyte, plasma cell, and macrophage infiltration often forming "microabscesses" that later heal by fibrosis. Mega-organ disease is associated with destruction of the myenteric plexus in the gastrointestinal tract (Pearson et al., 2003). Inflammatory infiltrate cells and their cytokine and chemokine expression in CCC heart lesions are well characterized. Moreover, given the fact that only 20–40% of patients develop CCC (Rassi et al., 2009; Bern, 2015; Organization WH, 2015), and the importance of inflammatory mechanisms in its development, it is expected to find genetic polymorphisms and susceptibility markers to CCC in genome-wide association studies (Cunha-Neto and Chevillard, 2014).

A consequence of research about Chagas disease has been the description and study of its causative agent, *T. cruzi* (Domain: Eukarya, Phylum: Euglenozoa, Class: Kinetoplastea, Order: Tripanosomatida, Family: Tripanosomatidae, Genus: *Trypanosoma*,

Species: *cruzi*). Classification of unicellular eukaryotes is under constant review, but the most recent studies state that Phylum Euglenozoa comprises single-cell, flagellated organisms with different nutrition forms, including parasitism. Among these, Class Kinetoplastea groups obligatory parasites characterized by the "kinetoplast", a mitochondrion-like organelle with extensive DNA content (kDNA). Family Tripanosomatidae groups many organisms of human medical importance baring only one flagellum, such as *Trypanosoma brucei, T. cruzi* and *Leishmania* spp. (Lukes, 2009). In particular, *T. cruzi* not only has unique enzymes that are absent in other organisms, such as *trans*-sialidase and cruzi pain, but also unique metabolic pathways (RNA *trans*-splicing), or proteins shared with others but with distinctive characteristics.

Since *T. cruzi* reproduces asexually in both its vertebrate and invertebrate hosts it is postulated to have a clonal population structure, with high diversity reflected by its isoenzymes (Morel et al., 1980) and their codifying genes (Tibayrenc et al., 1986; Tibayrenc and Breniere, 1988; Tibayrenc et al., 1993). However, some authors suggested that genetic hybridization between parasites may also take place (Sturm and Campbell, 2010). It is assumed that parasite strains or isolates obtained from mammals or vectors are multiclonal entities, and their filogenetic relationships have impact on the parasites' diversity and biological characteristics (Oliveira et al., 1998). Current classification of *T. cruzi* variability is based on Discrete Typing Units (DTUs) that describes "a set of stocks that are genetically more similar to each other than to any other stock, and are identifiable by common genetic, molecular, or immunological markers, constituting relevant units for molecular epidemiology and experimental evolution" (Tibayrenc, 2003). There are six main DTUs, each one having distinct biological properties such as infectivity, tissue tropism, and drug susceptibility (Zingales et al., 2009). These characteristics must be taken into account when performing experiments on new drugs, for example, because different DTUs show variations in expression and activity of some metabolic enzymes (Zingales et al., 2014).

Besides humans, *T. cruzi* infects a wide range of domestic and wild mammals including dogs, cats, bats, rats and armadillos. Therefore, control of Chagas disease is difficult because there are many potential parasite reservoirs in different vertebrate species and environmental settings (PAHO, 2008). In addition, some triatomine populations have developed insecticide resistance (Vassena and Picollo, 2003; Cardozo et al., 2010). Other transmission paths of *T. cruzi* infection are blood transfusions, organ donation, congenitally from mother to fetus, and laboratory accidents. Although numerous studies have been conducted aiming to produce a vaccine against Chagas' disease, success has been impeded by two main difficulties: finding protective antigens, and generating attenuated parasites that will not trigger pathology in the long term (Sánchez-Valdéz et al., 2014; Vázquez-Chagoyán et al., 2011). Therefore, in absence of vaccines, control measures of Chagas disease are limited to case detection and treatment, vector control by insecticide applications within domestic buildings, screening of blood banks and organ donors, detection of infected pregnant women and congenital cases (WHO, 2002).

Based on these facts, we will discuss the treatment of human Chagas disease with the drugs currently in use, as well as the potential new drugs under trial and innovative strategies for treating Chagas disease.

2. Treatment

Since Neglected Tropical Diseases occur mainly in developing countries, some of the drugs needed to treat them are not authorized to use in industrialized countries, and therefore have not been approved by a regulatory drug agency. The guarantee of product quality is evaluated at the time of its registration in every country. National regulatory drug agencies of Neglected Tropical Diseases in many endemic countries have limited ability to assess whether drug producers comply with guidelines established by WHO in its Good Manufacturing Practices. The risk associated with these products is further complicated because some drugs are outdated, and guidance on the product's specifications is not available in international pharmacopoeia monographs.

In the particular case of *T. cruzi* infection, Benznidazole (BZL) and Nifurtimox (NFX), launched in the early 1970s, are the only drugs approved for human treatment. Both compounds share some characteristics: better tolerance by children, more effectiveness during the acute phase of *T. cruzi* infection, higher toxicity in adults, and different susceptibility among *T. cruzi* DTUs. There is documented evidence that some DTUs are naturally resistant to nitroheterocyclic compounds (Filardi and Brener, 1987; Andrade et al., 1985; Toledo et al., 2004; Martins et al., 1998), but drug-induced resistant parasites can also be obtained in response to the selective pressure of adding drugs to the culture medium (Buckner et al., 1998; Wilkinson et al., 2008; Campos et al., 2014). In addition, P-glycoprotein (Pgp) efflux and Pgp ATPase activity were described in *T. cruzi*, and Pgp inhibitors cyclosporine A and verapamil were able to revert BZL resistance in treated parasites, thus implicating Pgp efflux pumps in drug resistance (Campos et al., 2013). However, overexpression of *Pgp* genes did not seem to play a role in *T. cruzi* drug resistance, rather than qualitative differences in Pgp function (Murta et al., 2001). The controversy about the relationship between drug resistance and parasite virulence is also a matter to be settled; some authors claim that resistant strains are more virulent (Andrade et al., 1985) while others did not find this association (Filardi and Brener, 1987).

According to WHO, there is enough evidence supporting that parasitic persistence is the main cause of progression towards cardiomyopathy, and therefore treatment with BZL or NFX during the chronic phase can soften its development. For that reason, there has been a change of paradigm in the guidelines of WHO that now recommends treatment to all patients. However, treatment must be handled with care in advanced chronic patients since the existing pathology may not be reversed, but those with gastrointestinal manifestations could have lower risk of developing cardiomyopathy after treatment (WHO, 2002; Viotti et al., 2014).

2.1. Benznidazole

Benznidazole (BZL) or *N*-benzyl-2-(2-nitro-1*H*-imidazol-1-yl) acetamide, is a 2-nitroimidazole derivative used as the first line treatment of Chagas disease (Fig. 1A). According to Maximiano et al. (2010), based on its low solubility in water BZL can be assigned to class 4 in the Biopharmaceutical Classification System (Leonardi et al., 2009). It is recommended that BZL treatment should be given orally for 60 days on a daily basis at 5–7 mg/kg for adults, and 10 mg/kg for children (WHO, 2002). The low solubility of BZL, combined to a high-dose treatment for a long period of time trigger adverse reactions (WHO, 2013; Lima et al., 2011) that include hypersensitivity -ranging from light-sensitive rashes to exfoliative dermatitis, bone marrow suppression (thrombocytopenia, neutroenia, agranulocytosis) and peripheral neuropathy (Weiss Louis et al., 2011). The low solubility of BZL also affects its bioavailability, decreasing its effectiveness during the chronic phase of infection—when parasites are localized mainly in skeletal and heart-muscle cells. Pinazo et al. (2013) tested whether adverse reactions were related to BZL concentration in serum, and therefore analyzed 54 patients diagnosed with *T. cruzi* infection that were given BZL regardless the phase of disease. BZL concentration in serum was not statistically different in either patients that stopped treatment due to adverse effects or those who finished

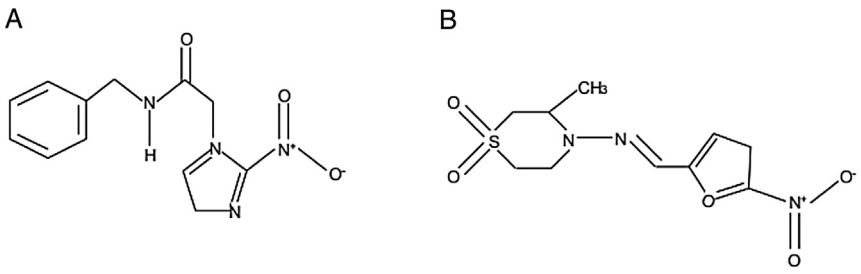

Fig. 1. Structures of benznidazole (A) and nifurtimox (B), drugs currently used against Chagas disease.

it without complications. Even though the authors did not find any association between BZL concentration in serum and adverse effects, they established that BZL given at 5 mg/kg/day resulted in mean serum concentrations of 3–6 μg/mL-assumed to be the highest trypanocidal concentration, thus indicating that 5 mg/kg/day is appropriated to obtain therapeutic drug concentrations in serum (Pinazo et al., 2013). In agreement with these results, empirical data from patients treated with 5 mg/kg/day of BZL during the chronic phase have shown lower clinical progression to cardiomyopathy than untreated patients (Viotti et al., 1994; Viotti et al., 2011).

2.2. Nifurtimox

Nifurtimox, or (*RS*)-3-methyl-*N*-[(1E)-(5-nitro-2-furyl) methylene] thiomorpholin-4-amine 1,1-dioxide (Fig. 1B) is produced by Bayer, and has been the mainstay of therapy for Chagas' disease in the United States. For more than a decade Bayer has been providing the WHO with 1 million tablets a year free of charge, along with financial assistance for its distribution mainly in Honduras and El Salvador (AGB, 2015). Doses recommended by WHO are 8–10 mg/kg daily in three divided doses for adults, and 15–20 mg/kg daily in four divided doses for children, during 60–90 days (WHO, 2002). Gastrointestinal maladies (nausea, vomiting, abdominal pain) are predominant adverse effects observed with nifurtimox, but 30% of patients can also experience central nervous system perturbations such as polyneuritis, confusion or focal or generalized seizures, and even psychosis that resume when treatment is stopped (Weiss Louis et al., 2011). Some patients may also develop skin rashes, and individuals with glucose-6-phosphate dehydrogenase deficiency can experience drug-induced hemolytic anemia. An increase in chromosomal aberrations has been seen in children given nifurtimox (Gorla et al., 1989). NFX has shown higher toxicity and adverse effects than BZL, including increased oxidative stress in rat pancreas (de Mecca et al., 2007) and heart (Mecca et al., 2008). In the context of heart damage caused by the parasite during a chronic infection, treatment with NFX may represent a higher risk of heart failure than BZL treatment. For these reasons, NFX is not the treatment of choice for Chagas disease in most endemic countries.

It is accepted that toxicity of nitro-compounds arises from their metabolic conversion after enzymatic reduction of the nitro group, which results in molecules considered free radicals (Castro and Diaz, 1988). This statement stands true for both BZL and NFX, although there are some differences in the nature of their reactive metabolites. Several enzymes are proposed to be involved in BZL and NFX metabolism, including trypanothione reductase (Henderson et al., 1988) and nitroreductases (Wilkinson et al., 2008; Kubata et al., 2002). The general mechanism proposed cyclic reduction of the nitro group that yields a nitro-radical, which in turn undergoes auto-oxidation and forms superoxide, regenerating the parent compound. Wilkinson et al. (2008) proposed that type I nitroreductase (NTR) is the main enzyme involved in the bioactivation of nitroheterocyclic drugs in *T. cruzi* and *T. brucei*, and that impairment of its activity confers resistance to BZL and NFX. In further experiments, Hall and Wilkinson (2012) demonstrated that reduction of BZL generates a hydroxylamine derivative that ultimately converts to glyoxal, a highly cytotoxic and mutagenic compound (Hall and Wilkinson, 2012). However, in a recent work, Trochine et al. (2014) failed to detect glyoxal in a metabolomics study carried out on *T. cruzi* treated with BZL. Instead, they found that BZL binds to low molecular weight thiols, and to protein thiols thus inactivating enzymes whose active site involves cysteine residues, such as the tryparedoxin peroxidase-tryparedoxin system. They also found BZL covalently bound to pyroglutamic acid and valine residues. Hence the authors proposed a new mechanism of action BZL, but its exact pathway and enzymes involved will be discovered in future experiments (Trochine et al., 2014). On the other side, reduction of NFX by NTR brakes the furan ring yielding an unsaturated open chain nitrile that is as cytotoxic as the parent compound, evidencing that NFX typanocidal activity does not necessarily involve oxidative stress (Hall et al., 2011).

3. Public health information and specific research on chagas disease

Disease profiles show variations among developing and fully developed countries. These profiles are also reflected in the information about health needs of different countries. Taking the number of scientific publications as a general measurement of health information, it is possible to observe that the vast majority of global knowledge on health topics -including clinical trials- is produced by developed countries in response to their own local needs. This situation also raises concern about information on research infrastructure in lower income populations. However, the number of research articles remains an imperfect approach of accurate knowledge about biomedical development. Since some diseases are harder to understand, prevent, diagnose, and treat than others, many publications contain a high amount of data aiming to tackle these difficulties, often with limited success. In the present section we will describe how scientific publications about Chagas disease have shifted during the past 100 years, in terms of number of publications, topics, and countries that make contributions to the subject.

It has been mentioned before that Chagas disease was first described in 1909, and some articles of that time have been indexed by Latin-American search engines such as LILACS and Scielo.

The first references in search engines such as Scopus and Pubmed were registered in 1933 (Villela and Villela Von, 1933) and 1938 (Wood, 1938), when presence of non-infectious forms of *T. cruzi* were described in USA. Nevertheless, the increasing interest of scientific research on Chagas disease has been boosted by the combination of different factors. One of them, and perhaps the main, is a consequence of the economic support derived from policies implemented following the "Special Program for Research and Training in Tropical Diseases" (TDR) by WHO (Rowe, 1977). Other factors include the general improvement in global com-

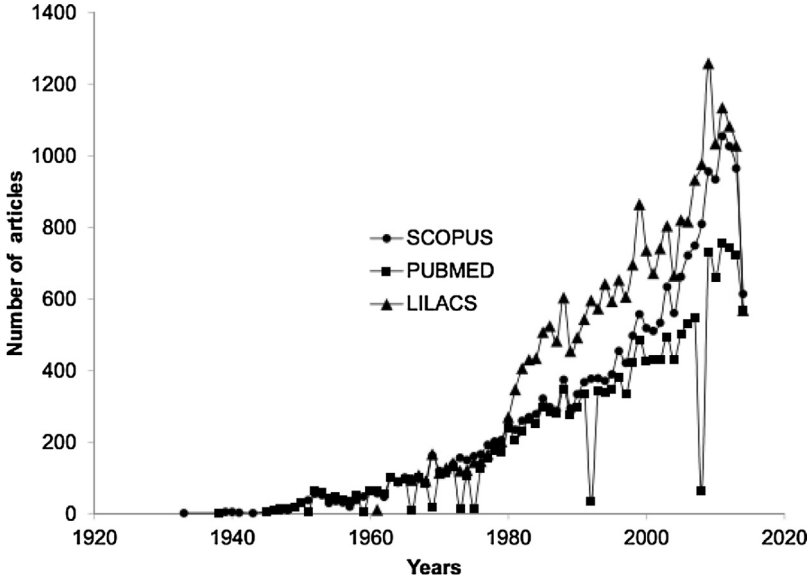

Fig. 2. Number of publications by year/database.

munications that triggered systematic organization of specialized meetings, the existence of resources exclusively dedicated to certain topics, along with discussion places for researchers and public exposure of research papers.

The increasing scientific interest on Chagas disease can be clearly seen in Fig. 2, which shows the rising number of published articles using the keywords "Chagas"; "Chagas disease"; "*Trypanosoma cruzi*"; "Chagas cardiomyopathy" and "Drug Therapy" in different databases.

While WHO and PAHO policies triggered an increase in publication numbers, there was a weak articulation between knowledge production and specific intervention opportunities on the disease. More than 70% of the total amount of papers published on the subject refers to basic/experimental research, and the most explored topics are related to the insect vector, including descriptions of its biochemical functions, its feeding, reproductive habits, geographical distribution, and insecticide resistance. Less than 10% of the total amount of publications is related to clinical trials. Leaving aside research about the biology of *T. cruzi* and the different cellular and animal models employed for its study, case reports are by far the largest type of study on Chagas disease (Fig. 3).

Private-funded laboratories lack interest on research about Chagas disease, while equipment and technological restrictions in public laboratories jeopardize the development of new drugs and treatment strategies, leading to the actual situation where currently available drugs are the same as in 1970. Considering that research about this topic are mainly produced by the academy, we embraced a "classic" scientific production evaluation criterion (quantity and quality of publications) to assess the development of interest on Chagas disease. In that regard, the number of publications related to other diseases such Alzheimer, hypertension, HIV, tuberculosis is at least triple than the number of publications on Chagas disease (Fig. 4).

Academically, the number of publications should be analyzed using a criterion that allows assessing their quality as well. The impact factor can be used as a (rather subjective) measure of quality. Since it is obtained by calculating the average of the times an article published in a certain journal is referenced, it is therefore assumed that the higher the impact factor is, the higher the visibility of the journal, and the greater the number of researchers who will intend to publish in that journal. Resulting from that competition, there is a correlation between the quality and impact of the publications in a journal with its impact factor. There are two indicators for scientific journals (journal metrics): Scimago Journal & Country Rank (SJR), and Scopus Source Normalized Impact per Paper (SNIP). The journal metrics show that articles about Chagas disease are more frequently published in Latin-American journals with low impact factor (Fig. 5).

It is not surprising that the highest number of research articles are published in -and proceed from- American countries (Fig. 6), since Chagas is endemic in 21 countries of the region. Moreover, since 1991 there are explicit PAHO initiatives committed to eliminate or at least reduce neglected diseases, implementing policies to promote active research and scientific development supported by the Ministries of Health of Argentina, Bolivia, Brazil, Chile, Paraguay and Uruguay.

Increased interest on research about Chagas disease from US and European countries arose from higher migrations rates in recent decades, causing a new health problem in non-endemic countries. Therefore the affected countries acknowledged the new situation, taking measures to prevent congenital transmission, carrying out controls at blood banks, and last but not least, developing new lines of research for treatment of this old disease.

4. Challenges for new therapies

Taking a deeper insight on the information presented above, one of the fields studied with equal interest in higher and lower income countries is the improvement of existing therapies, and the development of new ones to treat American trypanosomiasis. Given the complexity of Chagas disease, the characteristics of the parasite, and the evidence gathered in clinical practice, it is assumed that any new drug would be administered for at least 30 days, and in order to achieve patient compliance in low income settings it needs to be given orally (Buckner, 2011). In this section we will briefly summarize the most recent upturns in treatment with BZL, and the new drug candidates that may struggle to reach the market in the future.

4.1. Improvements to current treatment

According to new WHO recommendations (2002) of giving treatment to all Chagasic patients regardless the phase of the disease they are suffering, there have been many initiatives to

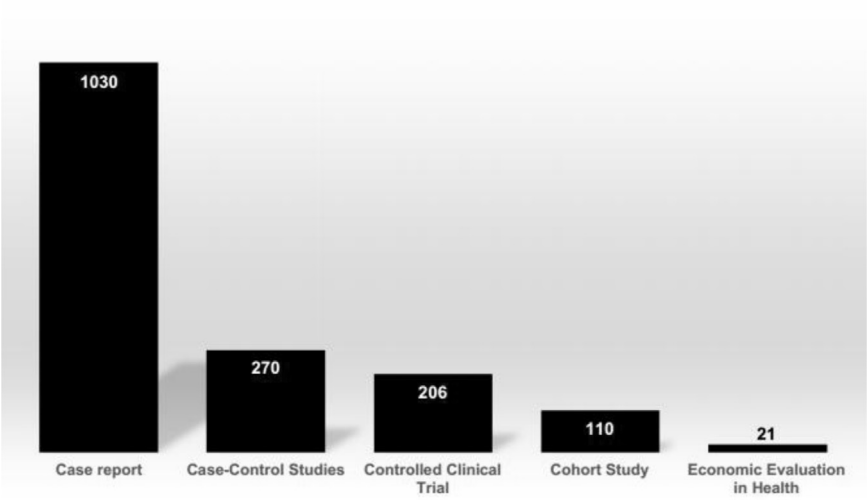

Fig. 3. Main types of studies on human Chagas disease.

evaluate BZL treatment in the chronically infected population. One of these initiatives was the trial "Benznidazole Evaluation for Interrupting Trypanosomiasis (BENEFIT)", designed to clarify the role of treatment with BZL in patients with CCC, and its effect on disease progression in endemic countries (Argentina, Bolivia, Brazil, Colombia, and El Salvador) (Morillo et al., 2015). It was a multicenter, international, randomized, double-blind, placebo-controlled trial in which 2854 patients received treatment during 40–80 days with either BZL (5 mg/kg/day, later adjusted by body weight to 300 mg/day) or placebo, and were followed up for at least 5 years. Patients were evaluated twice intra-treatment, at the end of treatment, at 6 months, and then annually until the end of the study for side-effects, liver-function, ECG, and parasitic burden in blood. Parasite load was determined by conventional PCR in 50% of the population, and serum samples were stored for future studies. Adverse effects, which include cutaneous rash, gastrointestinal symptoms and nervous system disorders, lead to significantly higher interruption of treatment in BZL (23.9%) than in placebo groups (9.5%). In conclusion, the study demonstrated that treatment with BZL significantly diminished parasite load circulating in blood, but this reduction did not correlate with ameliorating cardiac deterioration.

An alternative often proposed is the combination of BZL with other compounds to increase effectiveness. Allopurinol and allopurinol riboside have been under investigation for a long time as possible treatments of American trypanosomiasis (Gallerano et al., 1990), and it has been demonstrated that allopurinol and itraconazole, alone or in combination have produced beneficial responses in patients with chronic disease (Apt et al., 1998; Apt et al., 2003). Recently, Perez-Mazliah et al. (2013) have shown that a combination of BZL and allopurinol is well tolerated, and effective in reducing infection parameters (parasite burden and changes in B- and T-cell response) in chronically infected humans.

Nowadays, BZL is only available as 100 mg tablets, and they must be fractioned for administration in suspension to newborns and children, leading to inadequate doses and possible adverse reactions. Since a large part of the population affected by Chagas disease are children, one line of research is focused on the discovery of new pediatric formulations. Tarragona et al. (2013) developed BZL sugary gels and chewable tablets for pediatric administration. These were innovative formulations that masked the bad taste of the drug and increased its dissolution profile compared to the commercially available tablet. Moreover, Manarin et al. (2013) formulated novel BZL-water-polyethylene glycol 400 solutions at pH 2.5, which were useful vehicles that highly improved BZL solubility. The advantage

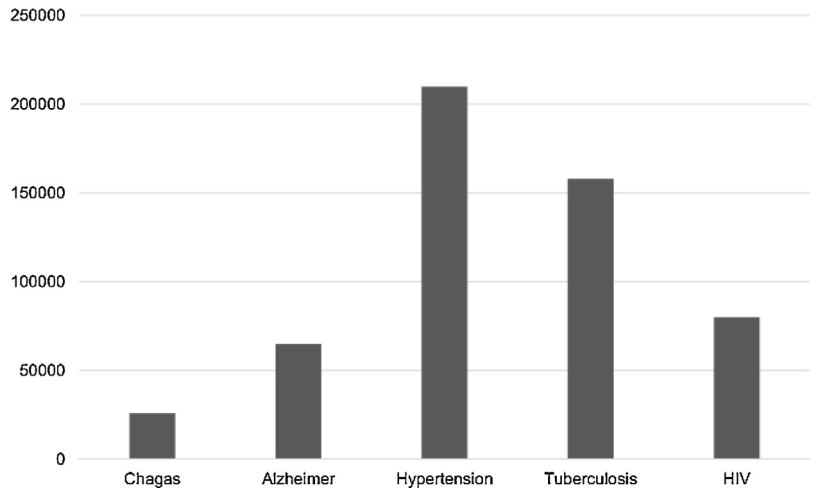

Fig. 4. Number of publications related to Chagas disease and other diseases until present day.

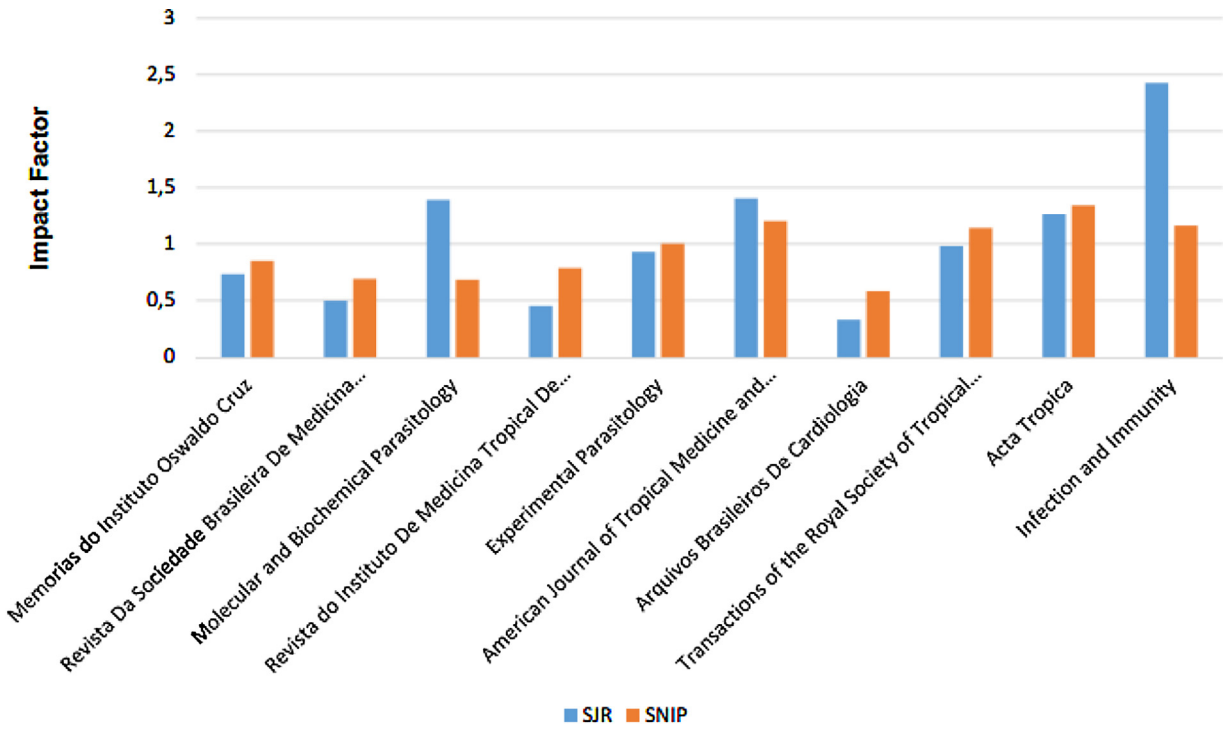

Fig. 5. Impact factor of the top 10 journals arranged by number of publications about Chagas, according Scopus (left to right).

of liquid formulations is to avoid manual disruption of BZL tablets needed to prepare suspensions suitable for small children.

Regarding the children population, recent advances were reported by Alonso-Vega et al. (2013) on Bolivia's Congenital Chagas Disease Program. All children diagnosed with congenital Chagas were treated with BZL at 10 mg/kg/day for 30 days, in two daily doses, excepting the first week when it was 7 mg/kg/day in two doses. Children were monitored to control compliance with treatment, side effects and dose-adjustment according to weight variations. There were no reports of side effects in more than 90% treated children, and those who presented adverse effects were treated accordingly. Side effects were not a cause of withdrawal, rather than migration to other places in Bolivia. Treatment outcome was evaluated by serological tests (HAI and ELISA) 6 months after treatment; if the result was positive the test was repeated 6 months later, and if that was still positive it was considered treatment failure, and the child received a second round of treatment. Serological negativization (treatment success) was found in 98% of children, demonstrating that BZL is effective as an anti-Chagasic medication in the children population of several Bolivian departments (Alonso-Vega et al., 2013).

Results about the effectiveness of an alternative drug to BZL were published by Molina et al. (2014) that conducted CHAGAZOL, a randomized, open-labeled clinical trial carried out in Catalunya, Spain. The aim was to evaluate the efficacy of posaconazole vs. BZL in chronic Chagasic patients in a 1:1:1 ratio, who randomly recived oral treatments twice daily for 60 days (BZL group: 150 mg, posaconazole (low-dose): 100 mg, posaconazole (high-dose): 400 mg). They evaluated treatment outcome only by real time PCR (qPCR) determination of *T. cruzi* DNA circulating in blood at 8, 16, 24, and 40 weeks after the end of the treatment. They found treatment failure (measured as positive qPCR) in 90% of patients treated with high dose posaconazole, in 80% of low dose posaconazole, and in 5.9% of BZL-treated patients. Positive results occurred at an earlier time in posaconazole-treated patients than in those receiving BZL. Side effects were not reported in either of the

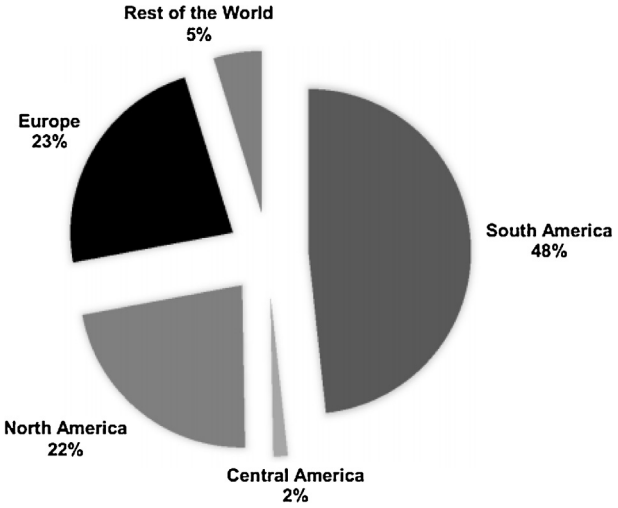

Fig. 6. Distribution of publications about Chagas disease by regions.

posaconazole groups, but 5 patients withdraw from BZL treatment due to adverse side effects. Even though the authors did not evaluate changes in the serologic profile after treatment, they postulate that posaconazole was not as effective as BZL in chronic Chagasic patients (Molina et al., 2014). However, as reviewed by Urbina (2015); this failure of posaconazole treatment in human patients compared to better results in mice models is related to a suboptimal dosage and administration schedules. Posaconazole concentration measured in human plasma is 10–20% of that measured in mice, which could be improved by new formulations of the drug with better plasma exposure (currently under development). Likewise, increasing time of administration may improve parasitological cure rates (Wood, 1938).

In 2012, Alvarez et al. (2012) suggested that BZL dosing may be reduced and still be trypanocidal in human subjects. Since

side effects are the main cause of BZL-treatment withdrawal, the authors analyzed a cohort of 81 patients that were treated for only 10 days and found that 20% meet the criteria of cure. These data suggested that BZL dosing regimen could be adjusted to avoid side effects and achieve cure (Alvarez et al., 2012). Different authors have found evidence that supports Alvarez and cols. data, and demonstrated that reducing BZL dosing to diminish adverse effects while maintaining its trypanocidal effect is possible in mice models. In addition, co-administration of BZL with azoles can improve BZL effectiveness and contribute to reduce its dosing. Evaluation of the interaction between BZL and itraconazole (ITC) by Moreira da Silva et al. (2012) demonstrated that co-administration of both drugs in mice diminished maximum BZL plasma concentration, improving its half-life and volume of distribution. Since BZL and ITC are metabolized by the same cytochrome P450 enzymes, the prolonged half-life of BZL could be due to the preference of ITC for the metabolizing enzymes, but this hypothesis needs confirmation in animals with larger volumes of blood than mice (Moreira da Silva et al., 2012). In a recent work, the interaction between BZL and ITC was shown again in an experiment of combined therapy at suboptimal doses of both compounds. In a mice model of acute *T. cruzi* infection, treatment with suboptimal doses (50 and 75 mg/kg/day) or optimal doses (100 mg/kg/day) of BZL/ITC in combination therapy had equivalent or superior efficacies than these compounds given at their optimal doses separately (Assíria Fontes Martins et al., 2015). This is a promising scenario for a combined therapy, since the good pharmacokinetic properties of ITC can be used to reduce the dosage and time of administration of BZL, concomitantly lowering the emergence of side-effects. Similarly, a synergistic effect using a combined treatment of BZL and the azole posaconazole was observed by other authors. Cencig et al. (2012) demonstrated that BZL and NFX, alone or in combination with posaconazole or amphotericin B (Ambisome) administered during shorter periods of time were able to cure *T. cruzi* infection in mice. The best combination was BZL + posaconazole in both susceptible and partially resistant strains; whereas, BZL + Ambisomedid not cure the infection (Cencig et al., 2012). The synergistic effect of BZL-posaconazole at suboptimal doses or shorter treatment schedules during the acute phase of *T. cruzi* infection, even with the BZL-resistant strain VL-10, has also been shown (Diniz Lde et al., 2013). The authors used 1/2 (50 mg/kg/day) and 1/4 (25 mg/kg/day) of the optimal doses of BZL and posaconazole for the combined therapies. Similarly, a sequential treatment at optimal dosage starting with posaconazole followed by BZL significantly reduced parasite burden. The authors suggest that BZL would rapidly reduce parasite biomass for subsequent action of posaconazole, facilitated by its large volume of distribution and long half-life. These results indicate that it is possible to reduce dosage and/or treatment duration to diminish BZL toxicity and the azole cost. This is a promising scenario for life-threatening acute Chagas cases, such as reactivation after an immunosuppression. However, these combinations remain to be tested in chronic phase models of the infection to assess their efficacy in reducing the progression of CCC.

Likewise, using a short, intermittent dosage schedule, Bustamante et al. (2014) also found promising results in mice infected with susceptible and resistant *T. cruzi* strains treated with BZL, NFX, allopurinol, posaconazole, an oxaborale, and a nitrotriazole, alone and in combination (Bustamante et al., 2014). Mice infected with susceptible strains were cured under regimens of intermintent administration of BZL or NFX over a reduced period of time (13 doses given at 5 days intervals). A short-term, intermittent regime of a combination of BZL + posaconazole rendered the same result as a 40 consecutive days of BZL or NFX alone. In contrast, the oxaborale was effective only in a 40-day administration schedule. Therefore the authors suggested that BZL may have a maximum-dose mechanism of action, which displays an extended post-antibiotic effect even after the compound has been completely removed (Craig, 1998). This finding is opposite to the previous assumption that BZL should be maintained in a concentration above the minimum inhibitory concentration, considering its half-life of 1–2 h in mice and 12 in humans. In addition, Planer et al. (2014) found some new combinations of amlodipine (a calcium-channel blocker) and posaconazole, as well as the combination of clemastine (anti-histamine) and posaconazole, to be effective in reducing parasitemia in mice (Planer et al., 2014).

4.2. Molecular targets

Since *T. cruzi* diverged from other eukaryotes a long time ago, there are many metabolic pathways and enzymes unique to this parasite that represent excellent molecular targets for drug development. However, despite the specificity of new compounds targeting parasite molecules, the effect of these drugs on mammalian metabolism must be carefully addressed before they reach the market. A few of the most studied compounds targeting specific *T. cruzi* enzymes will be reviewed in this section.

4.2.1. Nitroreductase type I

This enzyme is involved in activation of nitroheterocycles, as mentioned earlier (Wilkinson et al., 2008), and therefore represents a molecular target for BZL/NFX-susceptible strains. New compounds that inhibit NTR have shown anti-*T. cruzi* activity, including aziridinyl nitrobenzamide derivatives (Bot et al., 2010; Wilkinson et al., 2011), and 3-nitrotriazoles, the latter against amastigotes and infected mice (Papadopoulou et al., 2013; Papadopoulou et al., 2015). Moreover, nitroheterocyclic compounds dependent on NTR activation demonstrated to be more effective than ergosterol biosynthesis inhibitors when tested on the same clones. Experiments conducted on amastigotes of the same clones and strains representative of each DTU showed that nitroheterocyclic compounds were rapidly active towards a broader range of clones and strains; whereas, ergosterol synthesis inhibitors showed variable activity and were unable to eradicate intracellular infection after 7 days of continuous compound exposure (Moraes et al., 2014). In turn, these findings could explain results obtained in mice treated with itraconazole (Toledo et al., 2004), and in patients treated with posaconazole (Molina et al., 2014).

4.2.2. Ergosterol synthesis inhibitors

The existence of *T. cruzi* populations naturally resistant to benznidazole and nifurtimox (Murta et al., 1998; Camandaroba et al., 2003; Mejía-Jaramillo et al., 2012) led to search for compounds with a different mechanism of action, such as ergosterol biosynthesis inhibitors. Ergosterol is a precursor in the synthesis of cholesterol, and therefore essential for membrane structure in animal cells. One step in its synthesis reaction is catalized by a member of the cytochrome P450 family, the sterol C14-demethylase (CYP51), which in *T. cruzi* is highly specific and different from organisms closely related, like *T. brucei* (Lepesheva et al., 2006). This finding led to test *T. cruzi* susceptibility to several azole compounds that inhibit C14-demethylase synthesis, which were already approved to treat fungal infections (Lepesheva et al., 2007). Azole molecules with trypanocidal activity include the compound TAK-187 that showed promising results *in vitro* (Corrales et al., 2005) but whose production was discontinued, and some commercially available antifungals such as fluconazole, itraconazole, ravuconazole, and posaconazole. Fluconazole resistance was *in vitro*-induced in *T. cruzi*, shown to be stable in absence of the drug, and even maintained in animal models thus hampering its further use as an alternative treatment (Buckner et al., 1998). In addition, strains naturally resistant to itraconazole (ITC) were reported. ITC-resistance was apparently linked to phylogenetic relationships among popu-

lations, since all the studied ITC-resistant populations belonged to DTU I and susceptible ones were DTU II, showing an even clearer pattern than BZL-resistance (Toledo et al., 2004; Toledo et al., 2003). However, promising results were obtained in Chile, where after a 20-year follow up chronic patients treated with ITC showed normal ECG but remained serologically positive (Apt et al., 2013). It is worth noting that DTU II, ITC-susceptible populations described by Toledo in 2003 were isolated from Chile (Toledo et al., 2003). Ravuconazole showed potent trypanocidal activity *in vitro*, but its activity in a mice model was limited (Urbina et al., 2003). Tested in dogs, ravuconazole showed suppressive but not curative activity against *T. cruzi*, probably due to its unfavorable pharmacokinetic properties (a half-life of 8.8 h). However, its half-life in humans is 4–8 days, thus ravuconazole might still be a good candidate for a combined therapy (Diniz et al., 2010). Posaconazole has also been tested as trypanocidal agent, showing promising results as described earlier (Molina et al., 2014). Other CYP51 inhibitors were also tested against *T. cruzi*. Buckner et al. (2012) reported that the oncologic drug tipifarnib, as well as some of its analogs, were effective against amastigotes *in vitro*, but less effective in mice infection. Their results suggest that chemical modification of the lead molecule may increase its trypanocidal activity (Buckner et al., 2012). Soeiro et al. (2013) described the activity of VNI/VNF -a carboxamide-containing β-phenyl imidazole designed to fill the majority of the CYP51-binding cavity- *in vivo* against parasite strains with different drug susceptibility. Even though sterile cure was not achieved in the drug-resistant Colombiana strain, ultrastructural alterations were observed, suggesting that further modifications in the molecule and different dosage schemes may improve its efficacy (Soeiro et al., 2013). SQ19, a compound with activity against *Mycobacterium tuberculosis* has also shown trypanocidal properties and a complex mechanism of action that includes NADPH-dependent reductase inhibition, collapsing the mitocondrial membrane potential, releasing H^+ from intracellular acidic compartments, and partial CYP51 inhibition, in addition to synergy with posaconazole in amastigotes (Veiga-Santos et al., 2015).

4.2.3. Topoisomerase inhibitors

DNA topoisomerases are a family of enzymes that control DNA supercoiling and entanglement in all living organisms from bacteria to multicellular eukaryotes. Their importance arises from the double helical structure of DNA, which also determines topoisomerase subfamilies: ATP-independent, type I subfamily passes one strand of the DNA through a break in the opposing strand; and ATP-dependent, type II subfamily passes a duplex strand from the same or a different molecule through a double-brake gap in the DNA (Champoux, 2001; Pommier, 2013). Type II topoisomerase was found in the nucleus of *T. cruzi*, downregulated in trypomastigotes, and upregulated in the replicative epimastigotes and amastigotes (Fragoso et al., 1998). Consistently, topoisomerase inhibitors like tarivin, oxoflacin, novobiocin and nalixidic acid were shown to be effective against epimastigotes and amastigotes (Gonzales-Perdomo et al., 1990). Type I topoisomerase was found to be localized in both nucleus and kinetoplast, and parasites were susceptible to the inhibitor camptothecin (Bodley and Shapiro, 1995). Due to their use as antitumoral agents, there has been renewed interest in testing new topoisomerase inhibitors on *T. cruzi*. Camptothecin caused ultrastructural alterations in the kinetoplast and heterochromatin unpacking in the nucleous; enoxacin and mitoxantrone, topoisomerase II inhibitors, were also effective to inhibit *T. cruzi* proliferation (Zuma et al., 2015). However, camptothecin derivatives topotecan and irinotecan were not as effective as the parent compound in imparing *T. cruzi* growth (Lacombe et al., 2014). Recent work has revealed that camptothecin treatment triggers *T. cruzi* apoptosis, but some parasites do not progress to late apoptosis signaling and remain in a “senescence-like” state (Zuma et al., 2014). Taken together, these results show that topoisomerase inhibitors are compounds that will still be studied as promising trypanocidal compounds.

4.2.4. Cruzipain

The main cysteine proteinase of *T. cruzi*, encoded by tandemly arranged genes located on 2–4 chromosomes in different isolates, constitutes a family of proteins with 30–70% sequence identity with their homologues in *T. brucei* (Campetella et al., 1992). Being the main *T. cruzi* lysosomal proteinase, cruzipain is involved in parasite nutrition, but also in penetration of trypomastigotes into the host cell, in the defense of parasites against the mammalian immune system, and in the differentiation processes from one stage to another. Its catalytic domain has homology with cathepsines B and L, but the specificity of its C-terminal domain renders cruzipain as one of the most specific families of *T. cruzi* proteins, and therefore it has been extensively studied as a drug target candidate (Cazzulo, 2002). Even though most cruzipain inhibitors were also able to inhibit mammalian cathepsins, mammalian cells are not adversely affected at concentrations which effectively kill the parasite. This selective effect may be due to the redundancy of proteolytic activities in higher eukaryotic cells compared to parasitic protozoa. In *T. cruzi*, cruzipain inhibitor-resistant parasites were not cross-resistant to nifurtimox and benznidazole, indicating different mechanisms of action. Among the compounds tested against this enzyme, the most promising has been K777, a vinyl sulfone derivative described by McKerrow et al. (2009). Compound K777 was active against a wide range of susceptible and resistant strains representative of the main circulating DTUs. It was able to cure *T. cruzi* acute and non-acute infection in mice, showing synergistic activity with BZL, low hepatotoxicity, and ameliorated cardiac damage in treated dogs (McKerrow et al., 2009). While K777 is now under pre-clinical trials (Zingales et al., 2014), some of its analogs have been documented to display higher trypanocidal potency, unexpectedly due to inhibition of C14-α-demetilase (*Tc*CYP51) rather than inhibition of cruzipain (Choy et al., 2013).

4.2.5. Trans-*sialidase (TS)*

This enzyme has been extensively studied since the first reports of its activity in the early 1990s (Schenkman et al., 1991). It has been demonstrated that TS is composed by 3 domains: the N-terminal, *trans*-sialidase catalylic domain; the globular lectin-like domain involved in binding to nerve growth factor receptor, and the C-terminal, antigenic part, which ends in a glycophosphatidyl inositol membrane anchor that provides the hydrolyzing point to cleavage the molecule for shedding into the extracellular milieu (Rubin and Schenkman, 2012). Briefly, TS is related to viral and bacterial hydrolases, and catalizes transfer of sialic acid from sialoglycoconjugates in the external milieu of mammalian cells to glycoconjugates (mucins) in the cell membrane of *T. cruzi* (Buschiazzo et al., 2002). Sialation in *T. cruzi* membranes plays important roles in *T. cruzi* biology, but probably the most remarkable are evading the early complement-mediated immune response, and host-cell invasion (Schenkman and Eichinger, 1993; Rubin-de-Celis et al., 2006). For these important roles and its absence in mammalian organisms, TS has been proposed as a possible drug target. The binding of sialic acid to TS triggers conformational changes that create a sugar-acceptor binding site for β-galactose, and a Tyr-residue switch that allows the covalent bond with sialic acid during the intermediary state of the reaction (Watts et al., 2003). Agustí et al. (2004) tested lactose derivatives modified in the glucose constituent, demonstrating that lactitol inhibited *trans*-sialidase reaction *in vitro* by being a preferencial sialyl residue acceptor during the transfer reaction, and effectively interfered with parasite infection in cultured cells (Agustí et al., 2004). Due to the short half-life of lactitol in blood (Mucci

et al., 2006), covalent conjugation of lactitol analogues (lactose, lactobionolactone, and benzyl-β-D-galactopyranosyl-(1 → 6)-2-amino-2-deoxy-α-D-glucopyranoside) with polyethylene glycol (PEG) have proven to inhibit TS (Giorgi et al., 2010). Eight-arm PEG with a star-shape increased the molecular weight and half-life of benzyl-β-D-galactopyranosyl-(1 → 6)-2-amino-2-deoxy-α-D-glucopyranoside (Giorgi et al., 2012). Buchini et al. (2008) described compounds that specifically bind to the catalytic site of the enzyme (BFNs: 9-benzoyl-3-fluoro-*N*-acetylneuraminic acid, and its 3-fluorosialyl fluorides-bearing substituents at C9). BFNs formed long-lived intermediates that inactivated mammalian TS 150 times more slowly than *T. cruzi* TS (Buchini et al., 2008). Similarly, sulfonamide chalcones and quinolones were specific inhibitors of *T. cruzi* TS, not showing inhibition of human TS even at high concentrations (Kim et al., 2009). There is a mouse monoclonal antibody (mAb 13G9) that recognizes *T. cruzi* TS with high specificity and subnanomolar affinity, but its high molecular weight must be chemically modified to improve its bioavailability (Buschiazzo et al., 2012). More recently, a virtual screening of a database with more than 4 million compounds detected 2 molecules (ZINC13359679 and ZINC02576132) that are promising candidates as TS inhibitors (Miller Iii and Roitberg, 2013). However, data on the activity of all these synthetic inhibitors *in vivo* is yet to be published.

4.3. Discovering new compounds

There are several lines of research that may lead to the discovery of new compounds with anti-*T. cruzi* activity while having low toxicity towards mammalian hosts. In fact, many promising drug candidates have been documented, but the process of validation is slow and rigorous and very few of these candidates will be approved in the end. Some of them will be briefly described in this section.

An interesting approach was performed by Wang and Sun (2013) that reported metal–drug complexes as novel alternatives for Chagas disease treatment. Their work was based on the concept of 'metal–drug synergism' described by Sanchez-Delgado et al. (1993), where the coordination of an organic drug to a metal center stabilizes the drug and enhances its activity. The metal compounds may have dual or even multiple mechanisms of action, combining the pharmacological properties of both the ligand and the metal, and also triggering additive effects. Toxicity of the metal ion is reduced by complexation of the ion to drug ligands, which diminishes its interaction with biomolecules. Santos et al. (2012) exhaustively studied Pd(II), Pt(II), Ru(II) and Ru(III) coordination compounds of bioactive 5-nitrofuryl and 5-nitroacroleine thiosemicarbazones. Many of the Pd and Pt compounds showed increased activity against trypanosomes in comparison to free ligands. A severe disadvantage is that these metal complexes lead to deleterious effects on mammalian cells, and a strategy to overcome it is to use commercially available drugs that have low toxicity. In that regard, recent work by Martínez et al. (2012) showed that ruthenium-clotrimazole did not induce toxicity in normal mammalian cells while keeping its trypanocidal activity (Martínez et al., 2012). Similarly, Iniguez et al. (2013) demonstrated that ruthenium-ketoconazole complexes are non-toxic to murine macrophages, or to human fibroblasts and osteoblasts (Iniguez et al., 2013).

The development of bioactive metal complexes with commercial drugs is a promising new approach in the search for a treatment of Chagas disease. In this sense, Batista et al. (2011) obtained $MnCl_2{\cdot}4H_2O$, $CoCl_2{\cdot}4H_2O$ and $CuCl_2$ (phen) (phen = 1, 10-phenanthroline) complexes with the quinolones norfloxacin and sparfloxacin. The trypanocidal effect *in vitro* against amastigotes and bloodstream trypomastigotes was evaluated, showing that quinolones were poorly effective against *T. cruzi*. However, $[CoC_{l2}(NOR)(H_2O)_2]$ and $[CoCl_2(SPAR)(H_2O)_2]$ were active against amastigotes, and [CuCl2(phen)(NOR)] and $[CuCl_2(phen)(SPAR)]$ displayed a higher activity against both bloodstream typomastigotes and amastigotes (Batista et al., 2011).

Several bisphosphonates that are used for the treatment of bone diseases are active against *T. cruzi*. The main target of these compounds is the parasitic farnesyl diphosphate synthase enzyme which is involved in the biosynthesis of polyisoprenoids and sterols. This enzyme is competitively inhibited by commercial bisphosphonate drugs, like risedronate. However, a significant clinical disadvantage of bisphosphonates is their poor oral bioavailability. This problem could potentially be attenuated by coordination to a metal ion. In this sense, Demoro et al. (2010) have developed metal complexes with bioactive bisphosphonates as ligands. Results demonstrated that the coordination of risedronate to different metal ions improved its antiproliferative effect against amastigotes (Demoro et al., 2010).

Thiosemicarbazones and their metal complexes represent a class of compounds with a wide range of pharmacological applications, including anti-*T. cruzi* activity. An interesting approach was performed by Batista et al. (2010) that obtained Mn (II) complexes with N4-methyl-4-nitrobenzaldehyde thiosemicarbazone ($H4NO_2Fo4$ M), N4-methyl-4-nitroacetophenone thiosemicarbazone ($H4NO_2Ac4$ M) and N4-methyl-4-nitrobenzophenone thiosemicarbazone ($H4NO_2$ Bz4 M). The molecules $H4NO_2Fo4$ M, $H4NO_2Ac4$ M, and their Mn (II) complexes displayed poor effect on bloodstream trypomastigotes, as well as $H4NO_2Bz4$ M, but $Mn(H4NO_2Bz4\,M)_2Cl_2$ was significantly active against trypomastigotes, encouraging further *in vitro* and *in vivo* studies (Batista et al., 2010). However, the toxicity of these compounds is an important factor that must be taken into account at the moment of designing experiments in animal models.

Plant-derived products are an immense source of lead compounds that could be potentially active against protozoa (Croft et al., 2005; Salem and Werbovetz, 2006); and despite being extensively studied, there are still many compounds to be discovered. Essential oils from aromatic plants have shown many biological activities against various microorganisms (Boyraz and Özcan, 2006; Tagboto and Townson, 2001; Tepe and Sokmen, 2007) including *T. cruzi* (Santoro et al., 2007) and *Leishmania* spp. (De Medeiros et al., 2011; Oliveira et al., 2009). Just as a brief example, extracts from worldwide known plants like rosemary (Abe et al., 2002) and green tea (Paveto et al., 2004) have shown trypanocidal activity. Borges et al. (2012) investigated the trypanocidal activity of essential oils from Brazilian medicinal plants that inhibited parasite growth in a dose-dependent way, and were well tolerated by mammalian cells. Similarly, Bou et al. (2014) described the antileishmanial and trypanocidal activities of casearins isolated from *Casearia sylvestris* leaves, a plant geographically distributed throughout Latin America (Lorenzi and de Abreu Matos, 2002). Among the latest promising candidates from vegetal extracts are dehydroleucodine (DhL) and helenalin, sesquiterpene lactones found in many plant families. It has been demonstrated that DhL and helenalin induce programmed cell death in *T. cruzi* epimastigotes and trypomastigotes, which in turn may help to modulate the host's immune response by lowering the inflammation triggered by non-programmed cell death of parasites. In addition, the combination of DhL with BZL or NFX increases its trypanocidal activity (Jimenez et al., 2014). Actinomycetes are another rich source of bioactive compounds. It has recently been described that the macrolide actinoallolide A is active against *T. cruzi* epimastigotes at a lower dose than BZL, and further experiments are being carried out to elucidate its mechanism of action and *in vivo* activity (Inahashi et al., 2015). (Annang et al., 2015) reported a high-throughput platform to screen 5976 microbial extracts from MEDINA Natural Products library, and found that actinomycin D, bafilomycin B1, chromomycin A3, echinomycin,

hygrolidin, and nonactin were active against *T. cruzi in vitro* at subnanomolar concentrations, which support further studies of these promising compounds. Similarly, (Neitz et al., 2015) performed a high-throughput screening of 160,000 compounds from Novartis library and found promising results in xanthine and xanthine derivatives that warrant further *in vivo* studies.

Since both commercial trypanocidal drugs have a nitroaromatic moiety—NFX is a nitrofuran and BZL is a nitroimidazole, the chemical modification of nitroaromatic molecules is another approach to search for new anti-*T. cruzi* compounds. For example, some authors have designed compounds combining a furoxan—a widely studied nitric oxide donor with cytotoxic and cytostatic activity (Cerecetto and Porcal, 2005), and N-acylhydrazone—a molecule with potential to interact with different biological targets (Hernández et al., 2013; Massarico Serafim et al., 2014). In previous work, quinoxaline-*N*-acylhydrazones proved to have trypanocidal activity against epimastigotes by inhibition of cruzipain, the main *T. cruzi* cysteine proteinase (Romeiro et al., 2009). *N*-acylhydrazone compounds are stable in both simulated biological matrixes and plasma, have low mutagenic potential, and target cruzipain (Romeiro et al., 2009; Hernández et al., 2013). In addition, some of these compounds have shown lower cytotoxicity than the reference drug BZL *in vitro* (Massarico Serafim et al., 2014).

Repositioning, or finding new therapeutic uses for already known drugs is also a valid approach taken to discover new active compounds with anti-*T. cruzi* activity. Phenothiazines are tricyclic drugs employed as antidepressant, anxiolytic and antipsychotic in psychiatric treatments, but have also been found to have trypanocidal activity by inhibiting trypanothione reductase, equivalent to mammalian glutathione reductase (Paglini-Oliva and Rivarola, 2003; Lo Presti et al., 2015). One example is clomipramine, a drug prescribed to treat obsessive-compulsive disorder. Clomipramine has been used to treat *T. cruzi* infected mice during acute phase at 5 mg/kg/day and 40 mg/kg/day intraperitoneally, showing a decrease in parasitemia, structural heart damage and electrocardiographic alterations in comparison with untreated mice (Rivarola et al., 2001; Rivarola et al., 2005). For chronic phase treatment, a dose of 5 mg/kg/day induced a significatively lower amount of *T. cruzi* DNA in heart and skeletal muscle, milder inflammatory infiltrates, and reduction in heart fibrosis, as well as a decrease in anti-*T. cruzi* antibody titers and longer survival (Bazán et al., 2008; Fauro et al., 2013). Similar results were found with thioridazine treatment (Lo Presti et al., 2015; Lo Presti et al., 2004). These results point towards a decreased, retarded chagasic cardiomyopathy after treatment with clomipramine, but further studies will be needed to test its effectiveness in oral doses and in different DTUs, as well as possible side effects of equivalent doses in humans. Bellera and co-workers (Bellera et al., 2015) used a computer-aided drug screening, combined with biochemical, cellular and preclinical tests to study clofazimine (used to treat leprosy), benidipine (for hypertension and angina pectoris treatment) and saquinavir (an antiviral) as potential trypanocidal agents. Only clofazimine and binidipine were tested on mice models, because saquinavir maximum steady state concentration was below the concentration needed to kill parasites. Clofazimine and binidipine were not as effective as BZL at the doses tested in the mice model, but showed promising results to be tested in further studies.

Using the concept of latentiation by which a pro-drug is metabolically converted to an active compound, Chung et al. (2003) described the trypanocidal activity of hydroxymethylnitrofurazone (NFOH). Even though NFOH is a precursor of nitrofurazone (NF) in the chemical synthesis from the nitrofuran ring, it can also be obtained from NF in a single step. Nitrofurazone (NF) has proven to be effective against Gram positive and Gram negative bacteria, and to have trypanocidal activity (Henderson et al., 1988) but is also a known carcinogen that affects the reproductive system in both male and female mice (Heindel et al., 1997), causing single strand DNA brakes and oxidative damage (Takegawa et al., 2000; Hiraku et al., 2004). For these reasons, FDA regulations only allow commercialization of NF as topical medications formulated for dermatologic applications (FDA, 1998). However, the remarkable properties of NFOH as a trypanocidal agent guaranteed further academic investigation. In that sense, NFOH was shown to be 4 times less mutagenic in Ames test than its parental compound (Guido et al., 2001), demonstrated higher trypanocidal activity than BZL against amastigotes and typomastigotes *in vitro*, and a LD50 higher than 2000 mg/kg in rats (Melo, 2006). Its proposed mechanism of action involves the classical nitroaromatic activation, interference with mRNA transcription (Barbosa et al., 2007), and inhibition of cruzipain (Trossini et al., 2010). Tested *in vivo*, NFOH was as effective as BZL treatment during the acute phase of infection in mice (Davies et al., 2010). In addition to its trypanocidal activity, NFOH showed higher solubility in water, and lower adverse effects than BZL or the parental compound NF. Research about its pharmacological showed that 50% of NFOH was converted to NF in human plasma, with a volume of distribution 20 times higher than that of NF. In rats, levels of NF converted from NFOH were 4 times lower than those of NF directly administered (Serafim et al., 2013). Toxicity studies conducted in HepG2 cells and in liver of healthy, non-infected mice showed that NFOH was less hepatotoxic than BZL (Davies et al., 2014). The *N*-hydroxymethylation at the primary amide of NF leading to NFOH – equivalent to that occurring in phase I and II during liver metabolism of xenobiotics – resulted in higher water solubility, lower liver toxicity and other favorable pharmacological properties of NFOH (Serafim et al., 2013; Davies et al., 2014; Nogueira Filho et al., 2013) that ensure further studies of this promising molecule.

Olmo et al. (2015) synthesized some abietic acid derivatives (abietane diterpenoids) that show anti *T. cruzi* activity. Abietane diterpenoids have shown antiprotozoal activity against *T. brucei* and *Plamsmodium* spp, low toxicity against Vero cells, and good efficacy against *T. cruzi* both *in vitro* and in mice models of acute infection. In addition to their good trypanocidal activity and low toxicity, being easily synthesized from non-expensive substrates makes these abietane diterpenoids good candidates to develop new trypanocidal agents.

Another interesting advance was performed by Cogo et al. (2015). They synthesized 2,3-disubstituted quinoxalinederivates showing effectiveness against *T. cruzi*. Their activity was directly related to the methylsulfoxyl, methylsulfonyl, and amine groups as well as the presence of chorine or bromine in the molecules. The authors assume that these molecules are promising candidates because of their privileged scaffold, but their effectivity *in vivo* remains to be evaluated.

Finally, homeopathic treatment has also been considered as an alternative in *T. cruzi* mice models of infection. A suspension of 10^7 blood trypomastigotes/mL from mice on the 7th day of infection, diluted $1{:}10^7$ in 7% ethanol has been chosen for administration by gavage to mice infected with 1400 *T. cruzi*, Y strain trypomastigotes (Aleixo et al., 2012; Sandri et al., 2015). This dilution given to mice at 4 days post-infection delayed the appearance of parasitemia and increased the survival of treated (27 days) vs. untreated (15 days) mice; however, mortality was still high in the treated (92%) compared to the untreated group (100%) (Aleixo et al., 2012). The age of treated mice was an important factor in susceptibility to homeopathic treatment given at the 5th day post-infection for 20 days. Mice 4-week old were less susceptible than 8-week old, the latter showing lower inflammatory infiltrates, higher apoptosis, lower TGF-β, and lower intracellular parasitism in spleen and liver cells (Sandri et al., 2015). These results confirm the finely tuned immune response and exquisite relationship between *T. cruzi* and its mam-

malian hosts, pointing towards many more years of interesting research.

5. Concluding remarks

Chagas disease currently poses one of the greatest therapeutic challenges in tropical medicine, because *T. cruzi* has a complex life cycle in sylvatic, rural and urban environments, and susceptibility factors in both hosts and parasites have different influence on disease outcome. This complexity has also hampered the development of a vaccine against *T. cruzi* even using methodologies as different as designing specific protective antigens, or by generating attenuated parasites. An additional problem remains in the progressive loss of interest and visibility of Chagas disease as it disappears from endemic areas after implementation of vector control policies from Public Health Organizations in endemic countries. Since climate change is re-shaping our landscapes, remaining foci of vectors and insecticide-resistant triatomines will constitute permanent reservoirs of *T. cruzi*.

Prolonged treatment course, side-effects and naturally resistant parasite populations hinder treatment with BZL or NFX. Recent research demonstrated that shorter treatment schedules with BZL are as effective as the classical 2-month protocol (Viotti et al., 2014; Alvarez et al., 2012; Bustamante et al., 2014), and that there are synergistic effects when BZL is administered with commercial ergosterol synthesis inhibitors like posaconazole or ITC. Both have good pharmacokinetic properties—such as the affinity of ITC for CYP450 enzymes that improves the half-life of BZL by substrate competition (Moreira da Silva et al., 2012), or the large volume of distribution of pozaconazole. Posaconazole does not seem to be effective as a monotherapy against chronic *T. cruzi* infection, as suggested by the persistence of parasites circulating in blood during the CHAGAZOL study (Molina et al., 2014). In contrast, treatment with ITC improved ECG outcome in chronic patients, but these results belong to a limited cohort in Chile (Apt et al., 2003). Being azole molecules with the same molecular target, ITC and posaconazole show the same synergistic behavior when administered in combination with BZL or in short-alternate schedules during the acute phase in mice models (Assíria Fontes Martins et al., 2015; Cencig et al., 2012; Diniz Lde et al., 2013; Bustamante et al., 2014). The possibility of using these combined schemes of treatment aims to reduce the dose and time of exposure of BZL thus lowering its side-effects while diminishing the economic cost of either posaconazole or ITC administration. This is especially important in life-threatening acute cases such as in immunosuppressed patients, in oral outbreacks, or in congenital cases. Moreover, the combination of BZL + posaconazole was effective even against the BZL-resistant strain VL-10, which is a promising result for treatment of infections with naturally resistant parasites (Diniz Lde et al., 2013).

Therefore, in the near future it is reasonable to expect that Chagas disease treatment will involve combinations of BZL and other approved compounds such as CYP51 inhibitors (posaconazole and/or ITC), in short and intermittent administration schedules to minimize BZL overdosing and its side effects. New formulations with increased water solubility, higher volume of distribution or plasma concentration are also expected for posaconazole, ITC, and BZL. In the long term, some molecules from the list of promising compounds will finally hit the market as new trypanocidal drugs with high efficacy and no secondary effects, particularly to treat chronic cases of Chagas disease. This list includes natural products, molecules specifically designed to inhibit a particular enzyme, chemically-modified existing molecules to increase their trypanocidal activity, and drugs that were approved to treat other maladies. From these, the cruzipain inhibitors that have passed phase II trials are the closest to be approved as new medications for Chagas disease (Zingales et al., 2014; McKerrow et al., 2009), while metal–drug complexes will take longer to hit the market due to the metal toxicity towards mammalian cells.

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