

# Product Development (Preclinical And Clinical) Research Of New Therapeutic Tools For The Specific Treatment Of Chagas Disease

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Although the relevance of specific anti-parasitic treatment for the management of Chagas disease in its chronic stage has been the subject of many controversies, recent results on the pathogenesis of the disease have led to an increasing consensus over the notion that that elimination of *T. cruzi* from infected patients may be a prerequisite to arrest the evolution of the disease and to avert its irreversible long-term consequences. Unfortunately, current chemotherapeutic approaches, based in nifurtimox and benznidazole, have very low efficacy in the prevalent established chronic stage of the disease and suffer from significant side effects, which can lead to treatment discontinuation. Several rational chemotherapeutic approaches for the specific treatment of Chagas are being developed, as a result of our increasing knowledge of the biochemistry and physiology of *T. cruzi*, which could potentially have significant advantages over currently available drugs in terms of both efficacy and safety for the human host. Among the most advanced agents in development are new triazole derivatives, specific ergosterol biosynthesis inhibitors that act at the level of C14 sterol demethylase, which are poised to undergo clinical development in the short term (within 5 years), but there are other compounds that could enter clinical development in the next decade, among them cysteine protease (cruzipain) inhibitors and N-alkyl-bisphosphonates, inhibitors of farnesyl-pyrophosphate synthase. Several other promising approaches (among them trypanothione reductase inhibitors, hypoxanthine-guanine phosphoribosyl transferase inhibitors and novel ergosterol biosynthesis inhibitors that act at the level of squalene synthase and oxidosqualene cyclase) are also undergoing pre-clinical development, but their potential clinical development is only expected in the medium to long term (10-15 years).

## **Introduction. The relevance of specific treatment of Chagas disease**

According to the World Health Organization estimates Chagas disease remains by far the largest parasitic disease burden of the America Continent, despite significant advances in the control of the vectorial and transfusional transmission of its causative agent, *Trypanosoma cruzi* [86]. No vaccines are available to prevent this infection and the perspectives of their eventual development remain unclear [10,69].

Although the role of the parasite in the pathology of acute phase of Chagas disease and the importance of antiparasitic treatment in that stage is widely accepted [10,16], the participation of *T. cruzi* in the etiology of chronic Chagas disease has been the subject of many debates [21,30,40,69,71]. Several studies have strongly implicated autoimmune phenomena as a primary factor leading to the pathological phenomena associated with the chronic phase of the disease, including classic chagasic cardiomyopathy [26,35]. This hypothesis is based on the apparent absence of parasites in the characteristic inflammatory lesions of the heart and gastrointestinal tract and the presence of anti-self immune responses in chronic Chagas disease patients. The latter are postulated to result from "molecular mimicry" between parasite antigens and host cellular components or from

bystander activation resulting from release of self-antigens mediated by parasite-mediated cytolysis [26,35]. According to such hypothesis, after the autoimmune response establishes in the host, parasite persistence should not play a pivotal role in the pathogenesis of the disease and even a successful antiparasitic treatment might not lead to an improvement of the clinical outcome of the patients. Such conceptualization, in fact, stalled for many years the development of new specific chemotherapeutic approaches for this disease because they were considered irrelevant [20,76,80].

However, the autoimmune hypothesis of the pathology of Chagas disease has been strongly challenged by more recent studies, reviewed in [46,69,70,72,80], which have concluded that the persistence of parasites, coupled with an unbalanced immune response in some individuals that could include autoimmune reactions, is a necessary and sufficient condition to generate the sustained inflammatory response in infected tissues that underlies the characteristic lesions of the chronic stage of the disease [69,80]. These findings indicate that elimination of *T. cruzi* from infected patients may be a prerequisite to arrest the evolution of the disease and to avert its irreversible long-term consequences. Thus, the current prevalent opinion is that this condition should be treated as a parasitic, not as an autoimmune, disease [45,65,69,80].

### **Current specific chemotherapy of Chagas disease and its limitations**

Although this topic is treated in other presentations to this meeting some key points should be stressed, in the light of the light of concepts touched upon in the previous section. The drugs currently used for the treatment of Chagas disease are nitroheterocyclic compounds, a nitrofurantoin, nifurtimox (Lampit® Bayer) and benznidazole, a nitroimidazole derivative (Rochagan®, Radanil®, Roche), whose anti-*T. cruzi* activities were discovered empirically over three decades ago. Nifurtimox acts via the reduction of the nitro group to unstable nitroanion radicals, which in turn react to produce highly toxic reduced oxygen metabolites (i.e. superoxide anion, hydrogen peroxide). *T. cruzi* has been shown to be deficient in detoxification mechanisms for oxygen metabolites, particularly hydrogen peroxide, and is thus more sensitive to oxidative stress than are vertebrate cells [24]. Benznidazole seems to act via a different mechanism (reductive stress), which involves covalent modification of macromolecules by nitroreduction intermediates [24]. These findings have led to the important conclusion that the antiparasitic activities of these compounds are inextricably linked to their toxicity towards the human host.

Both nifurtimox and benznidazole have significant activity in the acute (up to 80% of parasitological cures in treated patients, defined as a negative result for all parasitological and serological tests (16)) and early chronic phases (up to 60% cures) [22,23,65,66], but some dissenting results have also been published [63,64]. However, their efficacy of these drugs varies according to the geographical area, probably due to differences in drug susceptibility among different *T. cruzi* strains [2,16] and there are common side effects, including anorexia, vomiting, peripheral polyneuropathy and allergic dermatopathy, which can in some cases lead to treatment discontinuation [16]. But the major limitation of these compounds is their very low antiparasitic activity in the chronic form of the disease, as  $\geq 80\%$  of treated patients is not parasitologically cured [16]. These conclusions, based on the persistence of positive anti-*T. cruzi* serology and clinical evolution of these patients, have been confirmed using PCR-based methods [3,9,11,41]. The reasons for the marked difference in the antiparasitic efficacy of nitroheterocyclic compounds between the acute and chronic stages of the disease are not clear, but they could be related to unfavorable pharmacokinetic properties of the drugs in the chronic stages [73,74]. Nevertheless, it has been shown that in some studies chronic patients subjected to antiparasitic treatment with benznidazole, although not parasitologically cured, have a significant reduction in the occurrence of electrocardiographic changes and a lower frequency of deterioration of their clinical condition [6,85]. This has been rationalized in terms of the parasite persistence

hypothesis, on the basis of a drug-induced reduction of parasite loads in infected tissues, which should reduce the severity of the associated inflammatory processes and subsequent organ lesions [6,69,85]. Thus, although there is now a growing consensus that antiparasitic treatment should be given to all seropositive individuals to reduce or eliminate their parasite loads, many physicians still have strong reservations concerning the use of nifurtimox or benznidazole in chronic patients, because of the unfavorable risk/benefit profile of these drugs.

## **New chemotherapeutic tools in development for the specific treatment of Chagas disease**

### **1. Ergosterol biosynthesis inhibitors**

Research in the last two decades has consistently demonstrated that *T. cruzi*, like most fungi and yeasts, requires specific sterols for cell viability and proliferation in all stages of its life cycle and the ergosterol biosynthesis pathway has been chemically validated at many different steps, *in vitro*, reviewed in [73,80]. However, several studies have shown that commercially available ergosterol biosynthesis inhibitors (EBI), which are highly successful in the treatment of fungal diseases (such as ketoconazole, itraconazole or terbinafine) are not powerful enough to eliminate *T. cruzi* from chronically infected animals or humans, or to stop the progression of the disease [73,80]. However, in the past decade new triazole derivatives (Fig. 1), which are potent and selective inhibitors of fungal and protozoan cytochrome P-450-dependent C14 $\alpha$  sterol demethylase (CYP51), such as D0870 (Zeneca Pharmaceuticals) and posaconazole (SCH 56592, Schering-Plough Research Institute), see Fig. 1, were found to be capable of inducing radical parasitological cure in murine models of acute and chronic Chagas disease [73,80,82]. These were the first compounds reported to display curative activity both forms of the disease. Furthermore, such compounds were able to eradicate nitrofurantoin- and nitroimidazole-resistant *T. cruzi* strains from infected mice, even if the hosts were immunosuppressed [73,80]. It has been argued that the remarkable *in vivo* antiparasitic activities of these triazole derivatives result from a combination of their potent and selective intrinsic anti-*T. cruzi* activity (the minimal growth inhibitory concentrations against the intracellular amastigote form is in the nanomolar to sub-nanomolar range) with special pharmacokinetic properties (long terminal half-life and large volumes of distribution) [73,76,80]. More recent studies with posaconazole, a structural analogue of itraconazole, have shown that this compound can eradicate the intracellular amastigote forms from cultured cardiomyocytes and at the same time allow the full reassembly of the host cells cytoskeleton and contractile apparatus [62]. Furthermore, it has also been shown that the anti-*T. cruzi* activity of this compound in a murine model of acute Chagas disease is much less dependent on interferon- $\gamma$  than that of benznidazole [31]. Posaconazole was recently registered in the USA, European Union and Australia for the prophylaxis and treatment of invasive fungal infections andazole-resistant candidiasis and is a prime candidate for clinical trials in Chagas disease patients. Other triazoles (Fig. 1) such as TAK-187 (Takeda Chemical Company) [19,84], UR-9825 (Uriach & Company) [36,81] and ravuconazole (BMS 207,147; Bristol-Myers Squibb) [83], have also been shown to have trypanocidal activity, both *in vitro* and *in vivo*. TAK-187 is a long lasting triazole derivative with broad-spectrum antifungal activity, which has very potent anti-*T. cruzi* activity *in vitro* and is capable of curing both acute and chronic infections in murine hosts even when the infecting strain is nitrofurantoin- and nitro-imidazole resistant [84]; more recent work has shown that this compound is superior to benznidazole in preventing cardiac damage in a murine model Chagas disease [18]. UR-9825 is another potent fungal and protozoan CYP51 inhibitor with remarkable *in vitro* anti-*T. cruzi* activity [81]; although its very short half life in the mouse (<0.5 h) precluded *in vivo* studies in this animal model, work in a canine model have demonstrated that the compound has curative activity in established infections of the virulent Y strain of *T. cruzi* with very low toxicity, although drug resistance was encountered with the Berenice-78 strain [36]. Finally, ravuconazole has also been shown to be very active against *T. cruzi* *in vitro*, but its *in vivo* activity in mice was limited, probably due to inadequate pharmacokinetic properties in this animal model [83]; however, these results do not

necessarily rule out the potential utility of this compound in the treatment of human *T. cruzi* infections, as its minimal inhibitory concentration against intracellular amastigotes (1 nM) is 1,000- to 5,000 lower than the levels attainable in human plasma with multiple oral dosing and its terminal half-life in man is  $\geq 120$  hrs [1,49]. Most of these compounds have now completed their pre-clinical development as anti-*T. cruzi* agents and all of them has already undergone pharmacokinetic and safety studies in humans, aimed at their development as systemic antifungal agents. Thus, pending on legal and economic agreements with the pharmaceutical companies that originally developed these compounds as anti-fungal agents, the new antifungal triazoles are poised for clinical development for the treatment of human Chagas disease in the near future (within 5 years).

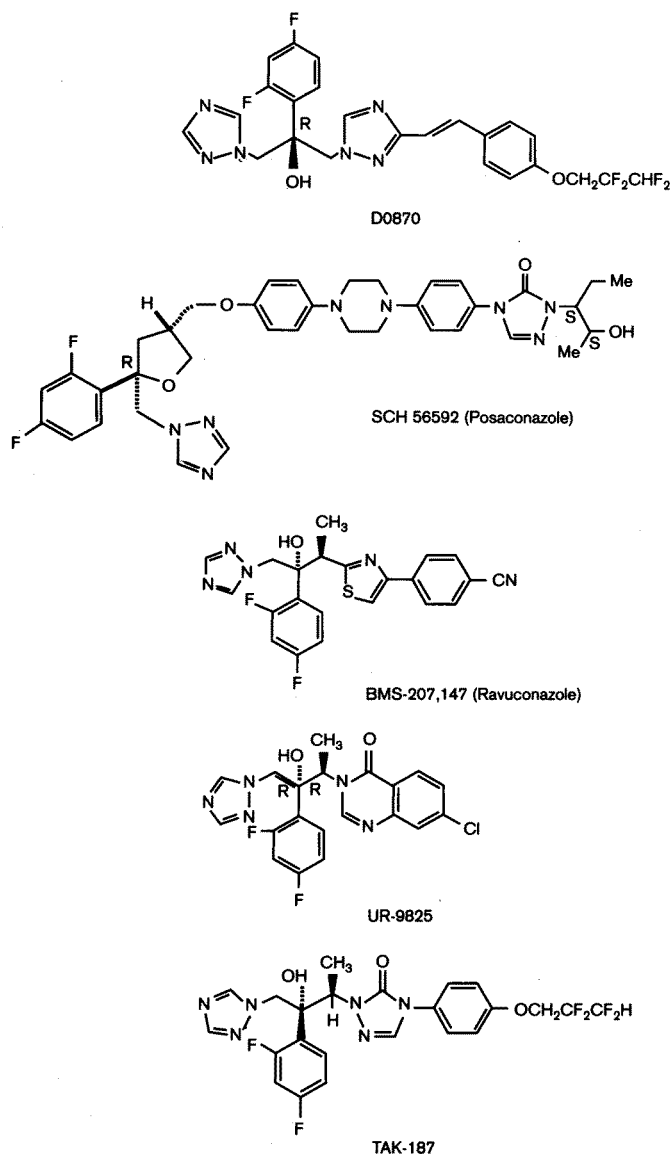
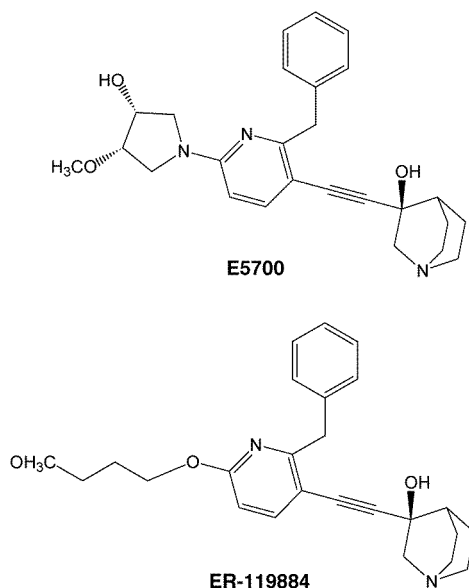


Figure 1

Another promising *T. cruzi* agents among EBI are squalene synthase (SQS) inhibitors. SQS is the first committed step in sterol biosynthesis and had been the subject of intense study by both academic and industrial group as it as an attractive target for cholesterol lowering agents, with potential significant advantages over currently available statins [48,68]. This enzyme has been recently chemically validated as a chemotherapeutic target in *T. cruzi* and *Leishmania mexicana* [79]; further studies have

shown that E5700 and ER-119884, two novel quinuclidine SQS inhibitors currently in development as cholesterol and triglyceride lowering agents in humans by Eisai Company (Fig. 2) have very potent anti-*T. cruzi* activity and one of them (E5700) was able to provide full protection against death and completely arrested development of parasitemia in a murine model of acute disease when given orally [78]; this is the first report of an orally-active SQS inhibitor as an ant-infective agent. Although these compounds and other aryl-quinuclidines are also potent inhibitors of mammalian SQS [38,78,79,88], their selective antiparasitic activity is probably explained by the capacity of the host's cells to compensate for the blockade of de novo cholesterol synthesis by up-regulating the expression LDL receptors and taking this sterol from the growth LDL receptors and taking this sterol from the growth medium or serum [35]; in contrast, there is no way for the parasite to compensate in this manner for the quinuclidine-induced blockade of ergosterol biosynthesis, as there are no appreciable amounts of ergosterol in the host cells or growth media. However, the requirement of some key organs (such as testis) of an elevated *endogenous* cholesterol supply could pose a significant limitation for the prolonged use of currently available SQS inhibitors and parasite-specific inhibitors will probably have to be developed. Recent work has demonstrated progress towards this goal, as the gene coding *T. cruzi* SQS has been cloned and expressed in *E. coli*, allowing the production of a soluble, fully active, recombinant enzyme, which has been used to identify parasite-specific SQS inhibitors [51,61]. Despite these advances, the clinical development of SQS inhibitors for the treatment of human Chagas disease can only be expected in the medium term (within 10 years).



**Figure 2**

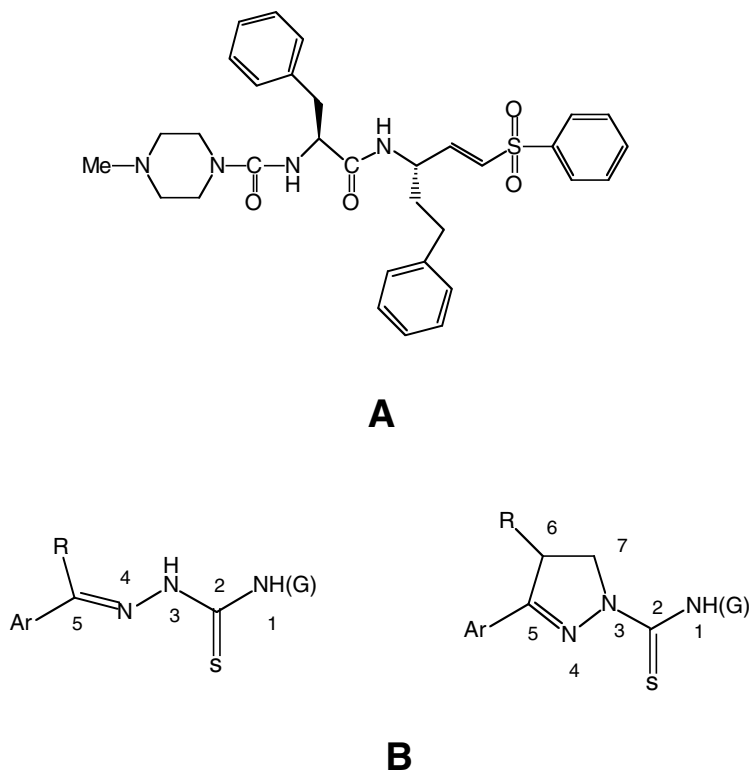
Another recent development in this area is the validation of oxidosqualene cyclase (OSC, lanosterol synthase) as a novel chemotherapeutic target in *T. cruzi* and related parasites [12,13,19]. Buckner et al. [12] have demonstrated the potent and selective in vitro antiparasitic activities of OSC inhibitors, but no evidence of in vivo activity has been published yet, although a recent patent by the same authors claims the use of oxidosqualene cyclase inhibitors as chemotherapeutic agents for the specific treatment of parasitic infections, including Chagas disease (U.S Patent WO0076316, see ref. [75]). Giving this limited advance in preclinical development, the clinical development of OSC inhibitors for

the treatment of human Chagas disease can only be expected in the medium to long term (10 to 15 years). An interesting and very recent development in this area was the demonstration that amiodarone, the antiarrhythmic drug most frequently used in chronic Chagas disease patients with cardiac compromise [52,58], has intrinsic anti-*T. cruzi* activity in vitro and in vivo and combinations of this drug with posaconazole produce synergistic effects [8]. It was found that amiodarone has dual mechanism of action against this parasite, disruption of Ca<sup>2+</sup> homeostasis and blockade of de novo ergosterol biosynthesis at the level of OSC, which explained the synergistic effects observed with posaconazole. The results indicate that Chagas disease patients under treatment with amiodarone may have the added benefit of a reduction of their parasite burden and enhancement of the effects of antiparasitic treatment [8].

## **New chemotherapeutic tools in development for the specific treatment of Chagas disease.**

### **2. Cysteine protease (cruzipain) inhibitors**

*T. cruzi* contains a cathepsin L-like cysteine protease termed as cruzipain, also known as cruzain or gp51/57, which is responsible for the major proteolytic activity of all stages of the parasite life cycle [15,17]. Selective inhibitors of this protease block the proliferation of both extracellular epimastigotes and intracellular amastigotes and arrest metacyclogenesis (transformation of epimastigotes to metacyclic trypomastigotes) in vitro, indicating that the enzyme performs essential functions for parasite survival and growth [15,17]. The genes encoding cruzipain have been cloned and expressed, the crystal and molecular structures of the recombinant enzyme have been determined and structure-activity relationships of inhibitors established. Rationally designed selective cruzipain inhibitors, such as N-methyl-piperazine-urea-F-hF-vinyl-sulfone-phenyl, also known as CRA-3316 or K-777 (Fig. 3A), are able to markedly reduce the parasitemia levels and prolong survival in murine models of acute and chronic Chagas disease, with minimal toxicity [29]. However, there is no published rigorous evidence of parasitological cure induced by this type of compounds in any of the animal models used. A recent study in a canine model of acute Chagas disease indicated that, although treatment K-777 was unable to cure the infected animals, it significantly reduced parasite-induced cardiac damage [7]. In 2002, Celera Genomics, announced that the Institute for One World Health (IOWH) and the National Institutes of Health had initiated development of K-777, as a potential new treatment for Chagas disease, but in 2005 IOWH announced that it was terminating this project, citing hepatotoxicity and serious problems with the manufacture of this compound (<http://www.oneworldhealth.org/diseases/chagas.php>). Recently, new lead scaffolds for inhibitors of cruzipain, with potent and selective activity against *T. cruzi* in vitro, have been identified [14,28].



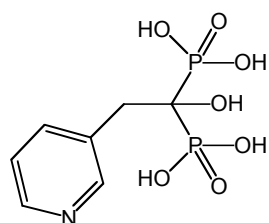
**Figure 3**

For example, structure-activity relationships for non-peptidic inhibitors of cruzipain, based on the thiosemicarbazone scaffold [28], were described and explained on the basis of the known structure and mechanism of the enzyme (Fig. 3B). Many of the compounds that were active in the low nanomolar range against pure cruzipain had trypanocidal activity against intracellular amastigotes cultured in mammalian cells in vitro [28]; the non-peptide nature of this series of compounds, their small size and extremely low cost make them promising leads for drug development. Taken together, these results indicate that cruzipain is an attractive anti-*T. cruzi* target and accordingly seven patents dealing with cruzipain inhibitors as potential anti-*T. cruzi* agents were identified from a survey of the patent literature for the period 1999-2002 [75]. However, given their current stage of pre-clinical development, according to the published scientific and patent literature, the clinical development of cruzipain inhibitors for the treatment of human Chagas disease can only be expected in the medium term (within 10 years).

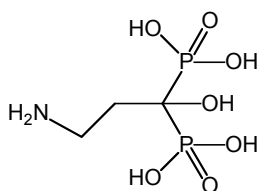
### **New chemotherapeutic tools in development for the specific treatment of Chagas disease. 3. Inhibitors of pyrophosphate metabolism**

Trypanosomatid and Apicomplexan parasites contain specialized organelles, termed acidocalcisomes that are involved in polyphosphate and cation storage [25,26].  $\text{Ca}^{2+}$  uptake and release from the acidocalcisomal matrix is regulated by a series of mechanisms, including a  $\text{Ca}^{2+}$  ATPase, a  $\text{Na}^+/\text{H}^+$  exchanger and  $\text{H}^+$ -pumping ATPases and pyrophosphatases, [25,26]. Short chain polyphosphates (mainly pyrophosphate and triphosphate) are involved in the response of these microorganisms to environmental stress, osmoregulation and energy transduction [25,26]. Bisphosphonates (metabolically inert inorganic pyrophosphate analogs, Fig. 4), which are currently used for the treatment of bone resorption disorders in humans [56], are selectively accumulated in the parasite and

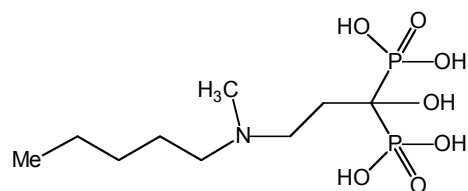
can inhibit enzymes involved in inorganic and organic pyrophosphate reactions such as farnesyl-pyrophosphate synthase (FPPS) [47,50], squalene synthase [77] or proton-pumping pyrophosphatases. Specifically, N-alkyl-bisphosphonates, specific FPPS inhibitors, have potent and selective activity against *T. cruzi*, *in vitro* and *in vivo* [27,33,34]; however, although radical parasitological cure in murine model of cutaneous leishmaniasis using pamidronate have been reported [57] no cures were reported in a murine model of acute Chagas disease using risedronate, a more potent FPPS inhibitor [34], probably due to the disseminated nature of the infection and the short treatment periods used. Thus, although bisphosphonates are promising lead compounds for anti-Trypanosomatid and anti-Apicomplexan chemotherapy and are currently approved for human use for the treatment of osteoporosis, their potential use as antiparasitic agents may require new pharmacological formulations (including pro-drugs) with pharmacokinetic properties appropriate for this new application. Based on those facts, the clinical development of bisphosphonates for the treatment of human Chagas disease can only be expected in the medium term (within 10 years).



**RISEDRONATE**



**PAMIDRONATE**



**IBANDRONATE**

**Figure 4**

## New chemotherapeutic tools in development for the specific treatment of Chagas disease.

### 4. Inhibitors of trypanothione synthesis and metabolism

Several research groups, using complementary approaches have identified the enzymes involved in the synthesis and redox metabolism of trypanothione ( $N^1, N^8$ -bis(glutathionyl)-spermidine) as potential chemotherapeutic targets [60]. This biochemical pathway is unique to Kinetoplastid protozoa, where it replaces glutathione and glutathione reductase in these cells intracellular thiol-redox system (Fig. 5), making it a promising target for antiparasitic chemotherapy [59,60]. The genes of all the enzymes of this pathway have been cloned and expressed and the 3D structures of these proteins have been determined using X-ray crystallography (Fig.5). Also, several of the enzymes in this pathway, including trypanothione reductase (TR) and trypanothione synthase, have been genetically validated [60]. The design and testing of specific inhibitors is currently underway and several families of compounds identified as specific TR inhibitors and trypanocidal agents in vitro [37,43,55,59], but very few studies demonstrating selective in vivo activity have appeared. Thioridazine, a known inhibitor of TR in vitro [37] is able to reduce the parasitemia, increase survival and prevent cardiac damage in murine models of acute Chagas disease [44,54], but no parasitological cures were obtained and the selectivity of the drug action against the parasite has not been demonstrated. Giving this limited advance in preclinical studies, the clinical development of TR inhibitors for the treatment of human Chagas disease can only be expected in the medium to long term (10 to 15 years).

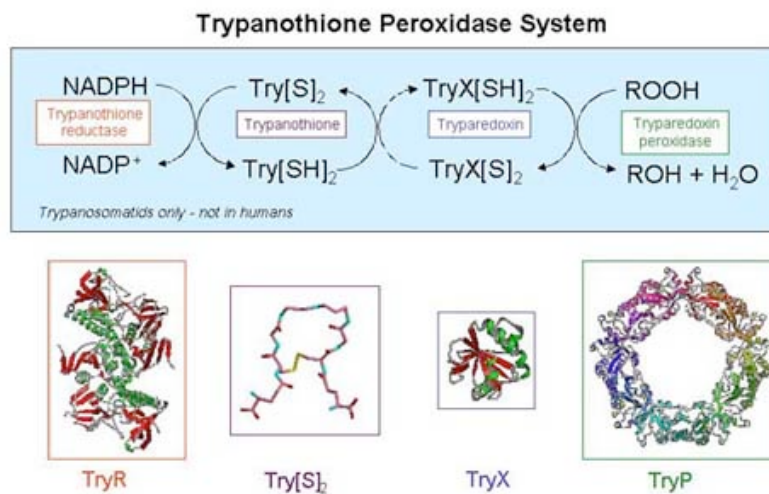


Figure 5

## New chemotherapeutic tools in development for the specific treatment of Chagas disease.

### 5. Inhibitors of purine salvage

Trypanosomatid parasites are absolutely deficient in the *de novo* biosynthesis of purines. Instead, they scavenge these essential compounds from their growth medium or mammalian hosts. A key enzyme of this pathway is hypoxanthine-guanine phosphoribosyl transferase (HGPRT), a validated biochemical target in these organisms [67]. Allopurinol (4-hidroxy-pyrazol-(3,4d)-pyrimidine) has been used for many decades in humans for the treatment of gout, as it is transformed in vertebrates to oxypurinol, a potent inhibitor of xanthine oxidase. In trypanosomatids, which are deficient in xanthine oxidase,

allopurinol acts as a purine analog and is incorporated, through HGPRT, into the parasite's DNA thus disrupting the synthesis of RNA and proteins [67]. Allopurinol was shown to be active in murine models of acute Chagas disease, but marked differences in susceptibilities to the drug among different *T. cruzi* strains were also reported [67]. There have been conflicting reports of the therapeutic efficacy of allopurinol in humans. An early report from Brazil indicated its ineffectiveness in acute Chagas disease patients [42], a finding confirmed by a multicentric study in chronic patients launched in 1992 in Argentina, Brazil and Bolivia, which was stopped as it was unable to control parasitemia in treated patients [53,87]. In contrast, Apt et al. [4] found that allopurinol, at 8.5 mg.Kg<sup>-1</sup>.day<sup>-1</sup> for 60 days, induced disappearance of positive xenodiagnosis tests in a high percentage of chronic patients in Chile and was able to reverse (in 49% of the cases) or prevent (75% of the cases) the development of electrocardiographic abnormalities after a 9 year follow-up [5]. However, it must be stressed that no parasitological cures could be demonstrated, according to serological criteria [16]. A possible explanation for the difference in results obtained in the Chilean study when compared to those of previous work in Brazil, Argentina and Bolivia could be the higher intrinsic allopurinol susceptibility of the *T. cruzi* strains present in that region, also observed with itraconazole [4,5]. Freymann et al. [32] found, using the crystal structure of *T. cruzi* HGPRT in a conformation that is similar to the transition state and a flexible docking program, 22 compounds from the Available Chemicals Directory, 16 of which had potent inhibitory activity on HGPRT in vitro and eight of these were capable of blocking the proliferation of intracellular *T. cruzi* amastigotes in cultured vertebrate cells, but no in vivo activities were reported. Giving this limited advance in preclinical development, the clinical development of new HGPRT inhibitors for the treatment of human Chagas disease can only be expected in the medium to long term (10 to 15 years).

## Conclusions

Several rational chemotherapeutic approaches for the specific treatment of Chagas are being developed as a result of our increasing knowledge of the biochemistry and physiology of *T. cruzi*, which could potentially have significant advantages over currently available drugs in terms of both efficacy and safety for the human host. Among the most advanced agents in development are new triazole derivatives, specific ergosterol biosynthesis inhibitors that act at the level of C14 $\alpha$  sterol demethylase (CYP51), which have completed in vitro and in vivo preclinical studies as anti-*T. cruzi* agents and have been registered or are completing clinical trials as systemic antifungals. These compounds are poised to undergo clinical development for Chagas disease in the short term (within 5 years), if legal and economic agreements with the pharmaceutical companies that originally developed them can be reached. Other compounds that could enter clinical development in the next decade include cysteine protease (cruzipain) inhibitors and N-alkyl-bisphosphonates, inhibitors of farnesyl-pyrophosphate synthase. There are several other promising approaches (among them trypanothione reductase inhibitors, hypoxanthine-guanine phosphoribosyl transferase inhibitors and novel ergosterol biosynthesis inhibitors that act at the level of squalene synthase and oxidosqualene cyclase) that are also undergoing pre-clinical development, but their potential clinical development is only expected in the medium to long term (10-15 years).

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