Novel Therapeutic Strategies for Chagas` Disease

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1. Introduction

Chagas' disease, also called American trypanosomiasis, is one of the most neglected parasitic diseases in the world. An estimated 10 million people are infected worldwide, mostly in Latin America where Chagas disease is endemic. More than 25 million people are at risk of the disease. It is estimated that in 2008 Chagas disease killed more than 10,000 people. Its infectious agent is the protozoan parasite *Trypanosoma cruzi* with symptoms progressing from mild swelling to intestinal disease and ultimately heart failure.

Currently, 2 antiparasitic drugs are recommended for the treatment of chagasic patients: nifurtimox and benznidazole. However, the effectiveness of both varies according to (i) the phase of the disease (acute and early latent infection), (ii) different parasite isolates, (iii) period of treatment and dosage and (iv) age of patient. Also, their well-known toxicity and limited effect make the search for new drugs imperative. Many trypanocidal compounds have been screened in the past few decades and some promising targets have been reported since the introduction of nifurtimox and benznidazole (1960-1970).

2. Nitro-heterocyclic derivatives as trypanocidal agents in the treatment against Chagas` disease

The history of chemotherapy for Chagas' disease can be divided into three phases: The first phase begins with the discovery of the disease in 1909 by the researcher Carlos

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Chagas and ends with the publication of "Manual of Tropical and Infectious Diseases" in 1935, at which point no major advances in the discovery of trypanocidal drugs had been made. In the second phase, between 1936 and 1960, numerous substances were empirically tested for the treatment of the disease, generating controversial results. The third phase, which started in 1961, was characterized by studies that clearly demonstrated the effectiveness of some compounds, e.g. nitrofurazone (1) (Figure 1), in experimental models of infection with T. cruzi in mice. The clinical trials showed that nitrofurazone (1) might be effective in the therapy for the disease but most patients were unable to tolerate the side effects at the doses and the time required for healing. However, the discovery of nitrofurazone (1) began a new era in the therapy of Chagas' disease (Coura & de Castro, 2002).

2.1 Nifurtimox and benznidazole

In the beginning of the 1970s, the discovery of the nitroheterocyclic derivatives nifurtimox (Lampit®, Bayer) (2) and benznidazole (Rochagan®, Roche) (3) brought new perspectives for the treatment of Chagas disease, due to their efficacy in the acute phase and tolerance (Bock et al., 1969; Richle, 1973).
Recently, the patent of benznidazole (3) was transferred to LAFEPE (the Pharmaceutical Laboratory of Pernambuco State, Brazil). Since the1980s, nifurtimox (2) sales were discontinued in Brazil and then in other Latin American countries.

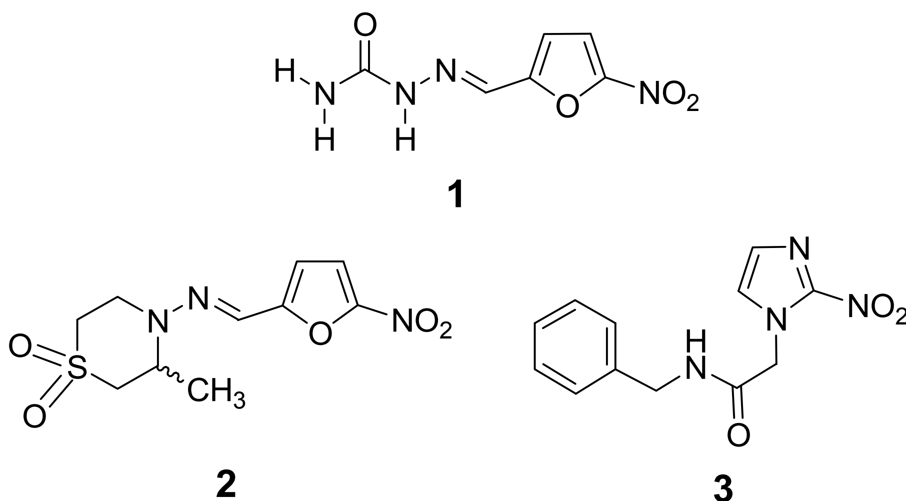


Fig. 1. Representation of chemical structures of nitrofurazone (**1**), nifurtimox (**2**) and benznidazole (**3**).

The adverse reactions associated with nifurtimox include digestive tract disturbances, such as lack of appetite, nausea, vomiting and weight loss and those induced by treatment with benznidazole (**3**) are dermo- and polyneuropathies. The main limitations of these drugs are the long-term administration and the severe side effects (Coura, 1996; Cansado, 1997). Additionally, another limitation lies in the fact that most cases of Chagas Disease are

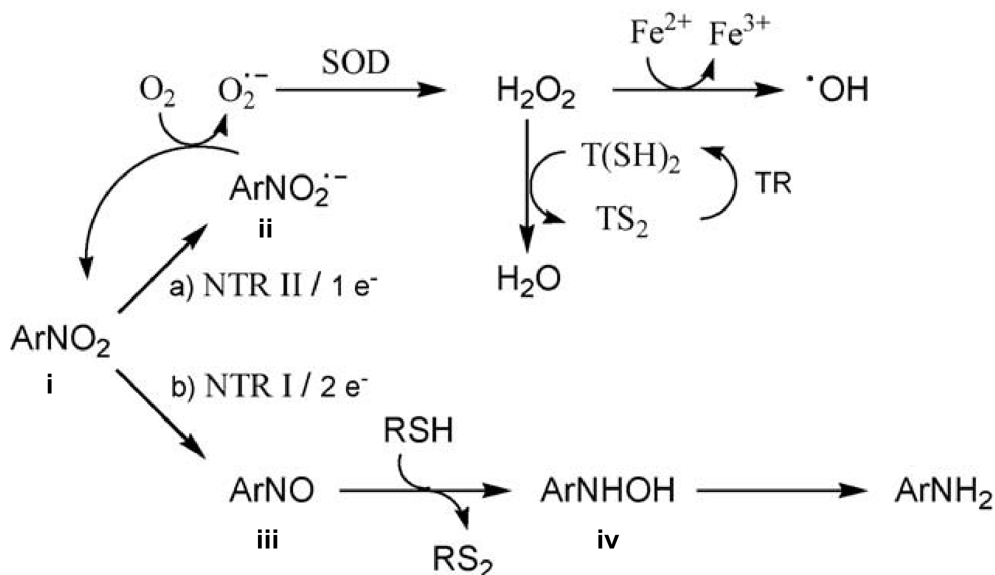
diagnosed in the chronic phase (de Castro, 1993), where these compounds have a reduced efficacy profile.

The mode of action of nifurtimox (**2**) is not yet fully elucidated despite being studied by many research groups,. The hypothesis that nifurtimox (**2**) acts through an oxidative stress has been based on the differences between the detoxification mechanisms of trypanosomatids and humans. The trypanosomatids have low activity of superoxide dismutase, absence of catalase and glutathione peroxidase (except for *Crithidia fasciculata*) (Turrens, 2004, Wilkinson & Kelly, 2003), and further explore a detoxification system based on the redox cycle of trypanothione (Fairlamb *et al.* 1985; Krauth-Siegel *et al.*, 2007), a system similar to that of glutathione. For these reasons the defence system of the parasite against reactive oxygen species (ROS) has been deficient, when compared to the host (Krauth-Siegel *et al.*, 2003; Turrens, 2004).

Scheme 1 shows the two possible routes proposed for the reductive process and bioactivation of nitro-heterocyclic derivatives with a general structure (**i**). Currently the most likely mode of action is one in which nifurtimox (**2**) exerts its biological activity through bioreduction of the nitro group (Maya *et al.*, 2007; Docampo, 1990). This process begins with the reduction of the nitro group to the nitro-anion radical (**ii**) in a reaction catalyzed by NADPH / NADH-nitroredutase (Maya *et al.*, 2007). Under aerobic conditions, the nitro-anion radical (**ii**) reacts with oxygen regenerating (**i**) and forming the superoxide anion, which is transformed into hydrogen peroxide by reacting with superoxide dismutase (SOD). The accumulation of H_2O_2 in the presence of iron can generate the hydroxyl radical via the Haber-Weiss reaction (Haber & Weiss, 1932; Koppenol, 2002). The accumulation of these ROS would lead to a possible oxidative stress in the parasite, which an impaired detoxification mechanism (Docampo & Moreno 1984; Cadenas, 1989). The hypothesis that the trypanocidal activity of nifurtimox (**2**) depends on the aerobic reductive cycle is based on various evidences, such as: (a) treatment of *T. cruzi* Tulahuen strains with **2** resulted in increased consumption of O_2, H_2O_2 production and the release of a superoxide ion (Nuñez-Vergara *et al.*, 1997; Maya *et al.*, 2007; Giulivi *et al.*, 1998). Additionally, after the use of different concentrations of **2**, it was also detected the presence of nitro-anion radical (**ii**) able to promote the anti-proliferative effect against the parasite, in the same range of serum concentrations achieved in adults after a single dose of 15 mg/kg (Docampo & Stoppani, 1979). The presence of nitro-anion radical (**ii**) was also observed by electron-spin resonance spectroscopy (ESR) (Nuñez-Vergara *et al.*, 1997) and (b), there was a reduction of essential thiols (mainly glutathione and trypanothione) in the parasite after treatment with nifurtimox (**2**) (Maya *et al.*, 1997).

Although the formation of ROS by nifurtimox (**2**) could be involved in its anti-*T. cruzi* activity, this does not seem to be the main mode of action. A study carried out by Wilkinson *et al.* (2000) provided evidence that a cell line over-expressing peroxidases was also likely to be vulnerable to oxygen metabolites generated by nifurtimox (**2**) that the typical lineage, suggesting that the trypanocidal activity of nifurtimox (**2**) is not predominantly mediated by ROS damage. One study with *C. fasciculate*, showed that organisms with low levels of the enzyme trypanosomatid catalase maintain their sensitivity to nifurtimox (**2**). These results contradict the hypothesis that the action of nifurtimox (**2**) involves the accumulation of hydrogen peroxide due to the absence of catalase (Gutteridge *et al.*, 1982). Moreover, recently, five peroxidases were identified in *T. cruzi*: two tryparedoxin peroxidases (Wilkinson *et al.*, 2000; Piacenza *et al.*, 2008; Trujillo *et al.*, 2004), two glutathione-dependent

peroxidases (Wilkinson *et al.* 2002a; Wilkinson *et al.*, 2002b), and one ascorbate-dependent heme peroxidase (Wilkinson *et al.*, 2002c) showing, in contrast to previous studies that the parasite has a complex and effective system to deal with oxidative stress.



Scheme 1. Two possible routes for the metabolic reduction of the nitro group in nitro-heteroaromatic derivatives (**i**). The aerobic route (a) and the anaerobic route (b). NTR = nitroreductase; SOD = superoxide dismutase; TSH = trypanothione; TR = trypanothione reductase. Adapted from Chauviere *et al.*, 2003.

Another likely possibility for the mode of action of nifurtimox (2) considers that the metabolic reduction of the nitro group is a process mediated by two electrons that result in the formation of the nitroso intermediate (**iii**) and then the hydroxylamine derivative (**iv**) (Kedderis & Miwa, 1988; Morello, 1988; Viodé *et al.*, 1999) (Scheme 1). Recently, Wilkinson et al. (2008) identified a nitroreductase (NTR) responsible for activation of nifurtimox (**2**) and benznidazole (**3**) in *T. cruzi* and *T. brucei* strains. This NTR of subtype I works through a sequence of two-electron reductions of species (**i**) by using NADH as a cofactor. Decreased activity of this NTR confers resistance to nitro heteroaromatic drugs, demonstrating its role in the metabolic activation of these compounds in trypanosomatids through the formation of nitroso species (**iii**) and hydroxylamine (**iv**), rather than the nitro-anion (**ii**). Additionally, it is known that nitroso compounds are electrophilic and promote the reduction of thiol levels, which can also be related to the mechanism of action of some anti-*T. cruzi* agents (Kedderis & Miwa, 1988; Viodé et al., 1999).
The oxidative stress was discarded as the main mode of anti-*T. cruzi* action of benznidazole (**3**), since at the concentrations where the drug has trypanocidal activity there was no production of superoxide ion and H_2O_2. Only at concentrations much higher than those needed to affect the parasite, *i.e.* 10 mM, was the formation of the corresponding nitro-anion radical detected (Moreno *et al.*, 1982). Some authors believe that benznidazole (**3**) is reduced

by the anaerobic route (b) and that its mode of action on *T. cruzi* may involve a direct effect on the biosynthesis of macromolecules by covalent or other interactions between its nitroreduction intermediates (Polak & Richle, 1978; Goijman *et al.*, 1985; Docampo & Moreno, 1986, Diaz de Toranzo *et al.*, 1988). Diaz de Toranzo and colleagues (1988) described the binding of benznidazole (**3**) to DNA, lipids and proteins from the epimastigote forms of *T. cruzi*. Also nifurtimox (**2**) and benznidazole (**3**) inhibited the synthesis of *T. cruzi* nucleic acids and proteins (Goijman & Stoppani, 1985; Goijman *et al.*, 1985; Gonzalez & Cazzulo, 1989).
Nifurtimox (**2**) and benznidazole (**3**) also act on the replication, transcription and translation processes in *T. cruzi* strains, inhibiting the biosynthesis of DNA, RNA and proteins respectively (Goijman & Stoppani, 1985; Goijman *et al.*, 1985).

2.2 Megazol

Few synthetic compounds have the same remarkable curative effects for the treatment of Chagas disease as megazol [2-amine-5-(1-methyl-5-nitro-2-imidazol-2-yl) 1,3,4 thiadiazole] (**4**) (Berkelhammer & Asato, 1968), a 1,3,4-thiadiazole nitroimidazole derivative that in experiments with mice infected with Colombian and ϒ *T. cruzi* strains showed higher rates of cure compared to the standard treatment using nitrofurazone (**1**), nifurtimox (**2**) and benznidazole (**3**) (Table 1) (Filardi & Brener, 1982).

T. cruzi strain	Compounds	Dose (mg.kg^{-1})	Number of Doses	No. cured/ No. treated	% cured
Y	Megazol (4)	25	20	9/18	50.0
		50	20	19/19	100.0
		100	20	17/17	100.0
		50♦	20	17/20	85.0
		500^{T}	1	8/9	88.8
	Nitrofurazone (**1**)	100	20	5/18	27.7
	Benznidazole (3)	100	20	5/17	29.4
	Nifurtimox (2)	25	20	1/17	5.8
		500^{T}	1	0/10	0.0
Colombiana	**Megazol (4)**	50	20	13/15	85.6
		100	20	14/14	100.0
	Nifurtimox (2)	50	20	0/15	0.0

♦Treatment began 5 days after inoculation, when the parasitemia was apparent. / ^{T}single dose given in the day after inoculation.

Table 1. Percentage of cured mice inoculated with *T. cruzi* strains treated with nitrofurazone (**1**), nifurtimox (**2**), benznidazole (**3**) and megazol (**4**) after oral administration.

Chauvière and co-workers (2003), who synthesized a series of megazol analogs with several structural modifications, studied the structure-activity related to their antiprotozoal profile. These new derivatives were tested against *T. cruzi*, *Trypanosoma brucei* (*T. brucei*) and *Leishmania infantum* (*L. infantum*), and their activities were compared with those exhibited by specific standard drugs, *e.g.* sodium stibogluconate (**5**) for Leishmania, suramin (**6**) for *T. brucei* and nifurtimox (**1**) for *T. cruzi* (Figure 2).

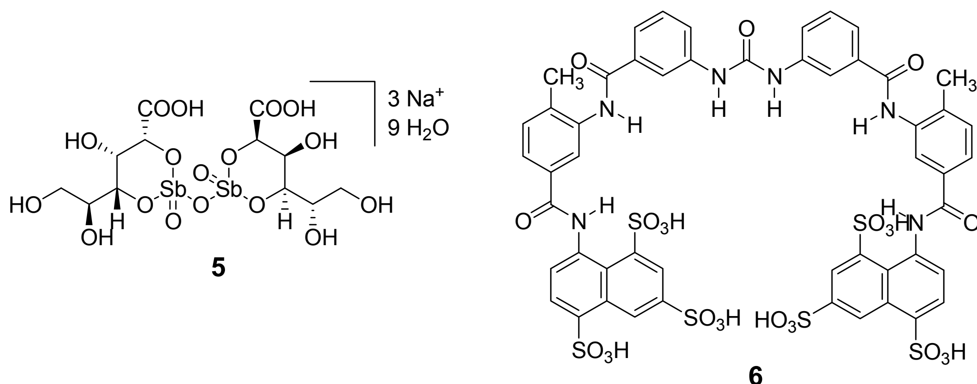


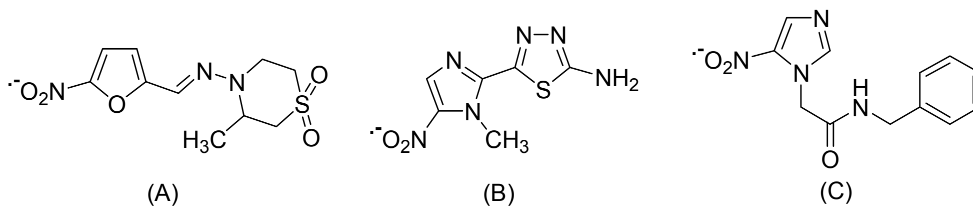
Fig. 2. Chemical structures of sodium stibogluconate (5) and suramin (6).

All derivatives tested by Chauvière and colleagues (2003) produced a decrease in trypanocidal activity; however the molecular architecture of the prototype megazol (**4**) is the one that presents the best bioactivity profile against *T. brucei*, *T. cruzi*, *L. infantum* and *L. donovani*, without cytotoxicity to macrophage cells.

Little is known about the metabolism of megazol, and despite its nitro-heterocyclic nature, some authors disagree on the metabolic reduction of the nitro group by nitroreductases as being critical for its trypanocidal activity. The biochemical studies performed by Tsuhako *et al.* (1989), with megazol (**6**) in the presence of NAD(P)H/cellular fractions of *T. cruzi* (*Y* strain) or in the presence of NAD(P)H/rat liver microsomes (Figure 3) were not able to show the presence of it corresponding nitro-anion radical (Figure 3, B).

The ineffectiveness of the rat liver microsomes in reducing megazol (**4**) was confirmed by experiments showing the slow disappearance of the band corresponding to the nitro chromophore measured by absorption spectroscopy of visible light (Figure 4). Although *T. cruzi* nitro-reductases are not well characterized (Marr and Docampo, 1986, Kuwahara *et al.*, 1984, Henderson *et al.*, 1988), the results (Figure 3) show that NADPH: cytochrome P450 reductase (Em = -0.328 mV) (McLane *et al.*, 1983) is also inefficient in promoting the reduction of the nitro group present in megazol (**4**) to the corresponding nitro-anion radical (Figure 3, B). Under these experimental conditions, the nitro-anion radical of megazol (**4**) was not detected, while the corresponding radicals of nifurtimox (**2**) and benznidazole (**3**) (Figure 3) were clearly detected. However, in the presence of NADPH and ferredoxin: $NADP^+$ oxirredutase (E_m = -0.442 mV) (Batie & Kamin, 1981), the megazol nitro-anion radical was easily detected by electron-spin resonance spectroscopy (ESR) directly under anaerobic conditions (Figure 5A), and was well marked by computer simulation (Figure 5C) (Rao *et al.*, 1987). Corroborating these data, Tsuhako *et al.*, (1989) demonstrated that the metabolic

reduction of megazol (**4**) generates the nitro-anion radical (B), but requires enzymes with low reductive potential, suggesting that the trypanocidal activity of megazol (**4**) is not related to the metabolic bio-reductive process, differing from the mode of action of nifurtimox (**2**) and benznidazole (**3**).



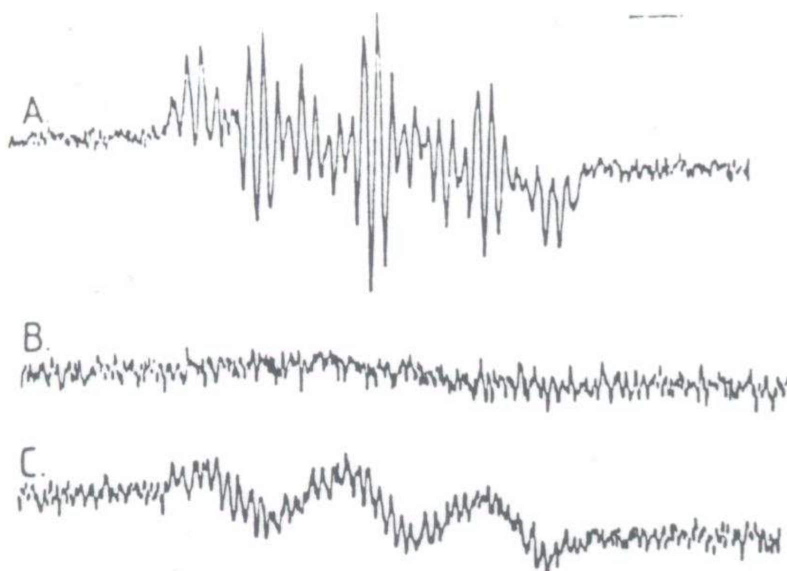


Fig. 3. ESR spectrum obtained during anaerobic incubation of rat liver microsomes with different nitro-heterocyclic compounds. The reaction mixtures in phosphate buffer (20 mM), pH 7.4, containing rat liver microsomes (2 mg protein /mL), NADPH (5 mM), DTPA (1 mM), KCl (150), DMSO (10% v/v) and: (A) nifurtimox (5 mM) (B) megazol (10 mM), and (C) benznidazole (10 mM). Adapted from Tsuhako *et al.*, 1989.

Viodé and colleagues (1999) studied the interaction of megazol (**4**) with three different enzymes with distinct redox potential, and compared the results with those of nifurtimox (**2**) and metronidazole (**7**) (Figure 6). The results indicated that the reduction of megazol (**4**) and nifurtimox (**2**) by the enzyme cytochrome b_2 (E_m = -0.01 V) occurred at similar rates, however with the enzyme cytochrome P-450 reductase (E_m = -0.328 mV), the reaction of the nitrofuran derivative was faster by one order of magnitude (Table 2). The reduction of nifurtimox (**2**) with the ADR enzyme (adrenodoxin reductase) was also faster when compared to megazol (**4**). The Km values for these enzymes (Tshako *et al.*, 1989) as well as

other reductases (Table 2) indicate that the catalytic efficiency is correlated with the reduction potential of the corresponding nitro-heterocyclic derivatives.

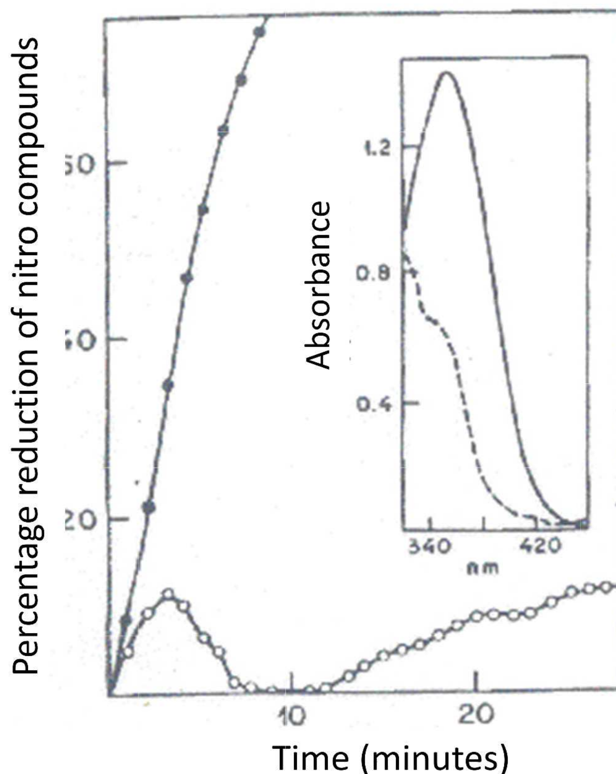


Fig. 4. Reduction of the nitro-heterocyclic compounds under anaerobic conditions by rat liver chromosomes. (•) nifurtimox (0.2 mM), and (o) megazol (0.2 mM). The graph shows the spectrum of visible light absorption of megazol (0.2 mM) in buffer (—) before and after (- - -) the addition of sodium dithionate (control).

The activities of ADR and cytochrome P-450 reductase represent the rate of NADPH oxidation at 25°C. The activity of L-lactate cytochrome *c* reductase was determined by measuring O_2 consumption. The k_{cat} values of the other enzymes were obtained from the coupled cytochrome *c* assays. ND = not determined.

The ESR experiments showed expressive enzymatic production of the nitro-anion radical of megazol (**4**), under anaerobic conditions, in comparison with nifurtimox (**2**), used as control (Figure 7).

The corresponding spectra are consistent with those published for the nitrofurane derivative (**2**) and those obtained for megazol (**4**) after reduction by ferredoxin: $NADP^+$ oxidoreductase (Tsuhako et al., 1989). The signal of ESR spectra obtained from the reduction of megazol (**4**), even at a lower intensity, under the same conditions, lasted longer than that of nifurtimox

(**2**). The half-lives determined under the same conditions were 10 and 3 minutes, respectively.

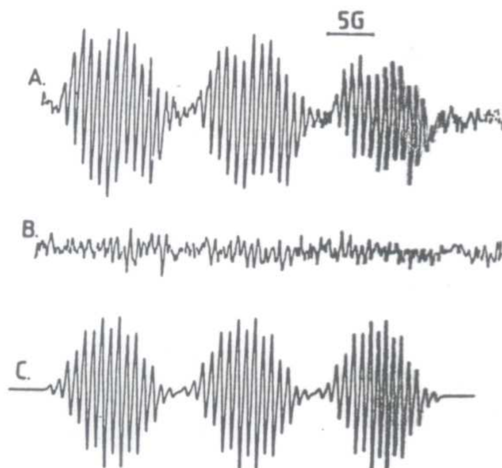


Fig. 5. ESR data obtained during the reduction of megazol. Megazol (1 mM) was incubated with ferredoxin: $NADP^+$ oxidoreductase and NADPH in phosphate buffer containing DTPA and DMSO under: (A) anaerobic conditions and (B) air ; (C) computer simulation of (A).

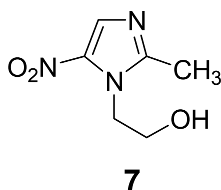


Fig. 6. Chemical structure of the antiprotozoal agent metronidazole (**7**).

Nitro compound E^7_1 (mV) (Tshako *et al.*, 1989)	Nifurtimox - 260	Megazol - 438	Metronidazole - 485
Enzyme	K_{cat}/K_m [M^{-1} . sec^{-1}]		
L-lactase cit. c reductase	$2.8X10^3$	$4,7X10^3$	ND
ADR	$5.0X10^4$	$3,0X10^3$	$3,0X10^2$
Cit. P-450 redutase	$2.0X10^4$	$2,0X10^3$	ND
T. cruzi LipDH	$7.6X10^2$	$2,3X10^3$	ND
Pig heart LipDH	$3.0X10^2$	$4,3X10^3$	ND
T. cruzi TR	$1.5X10^3$	$1,8X10^3$	ND
Human GR	≤14	≤5	ND

Table 2. Enzymatic reduction of nitro-heterocyclic compounds by different reductases. Adapted from Viodé *et al.*, 1999.

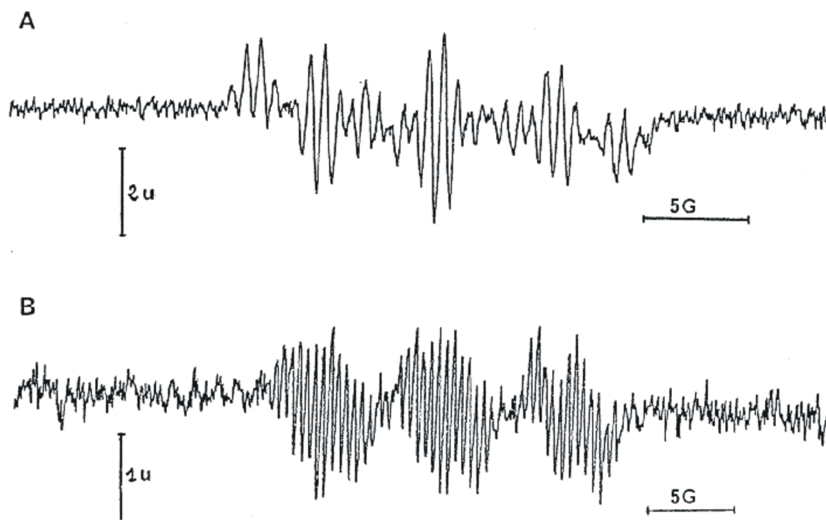


Fig. 7. The ESR spectrum of the nitro-anion radical obtained during the reduction of megazol (B) and nifurtimox (A) under anaerobic incubation at 37°C with microsomes, NADPH in phosphate buffer.

The generation of the nitro-anion radical by cytochrome P-450 reductase and rat liver microsomes also confirms that megazol (**4**) is significantly reduced, since the corresponding nitro-anion radical can be accumulated under anaerobic conditions.

The values of k_{cat}/K_m indicate that megazol (**4**) has a slower enzymatic reduction than the other two nitro-heterocyclic compounds (Table 2), whose high trypanocidal activities are not related to the first step of the reductive process.

Viodé and co-workers (1999) conclude that there is no doubt that megazol (**4**) can interfere with the oxygen metabolism of the parasite by inducing oxidative stress, but its greater potency compared to nifurtimox could be related to factors not yet known at this time.

De Castro and Meirelles (1990) studied the megazol mode of action by incorporating macromolecular precursors, *e.g.* leucine, uridine, and thymidine. Megazol (**4**) showed a potent effect on selective inhibition of protein biosynthesis, where benznidazole (**3**) and nifurtimox (**2**), even at high concentrations, caused no change in the incorporation pattern.

Maya *et al.* (2003) tested the hypothesis that nitroreduction metabolites of **4**, *e.g.* nitroso species (**iii**) (Scheme 1), act as electrophilic scavenging bionucleophilic species from the parasite. Consequently the effect of **4** on the amount of free thiols, *i.e.* $T(SH)_2$ and GSH, was investigated.

Megazol (4) caused a progressive decrease in the amount of thiol in the parasite in two hours, with a half life of 88 minutes for $T(SH)_2$ and greater than 200 minutes for GSH (Figure 8). It is an efficient scavenger of thiols, in contrast to the profile shown by nifurtimox (2).

However, the mechanism involved in the trypanocidal activity of **4** is strictly dependent on the bioformation of reactive oxygen species (Declerck *et al.*, 1986; Declerck *et al.*, 1987), produced by the reduction of its nitro group, which interacts with DNA producing mutagenicity (Ferreira & Ferreira, 1986; Nesslany *et al.*, 2004; Poli *et al.*, 2002). This undesirable profile limits the use of megazol (**4**) as a drug for Chagas' disease.

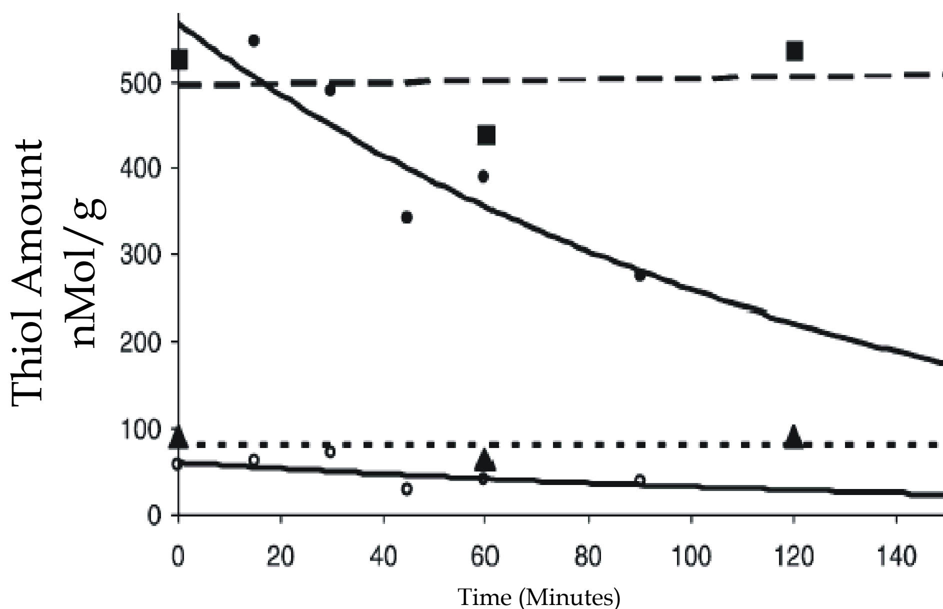


Fig. 8. Effect of megazol (**4**) on the amount of thiols in epimastigote forms of *T. cruzi*. The half-life of trypanothione is 88 min. Control -T $(SH)_2$ (■), T $(SH)_2$-megazol (•), GSH-control (▲), GSH-megazol (o).

2.3 Discovery of brazilizones A and N

Considering this background, Carvalho *et al.* (2004, 2008a) synthesized novel 1,3,4-thiadiazole derivatives structurally designed by exploiting the molecular hybridization of megazol (**4**) with the guanylhydrazone derivative (**8**), which proved to be active against trypomastigotes forms of *T. cruzi* with IC_{50}/24h = 17 μM (Messeder *et al.*, 1995). The molecular design explored the introduction of the pharmacophoric *N*-arylhydrazone subunit (A) from **8** (Figure 9) to the nitroimidazole derivative (**4**), in order to act as a radical scavenger (Mahy *et al.*, 1993; Prusis *et al.*, 2004) that would abolish the oxidative stress that induces the formation of toxic species resulting from the formation of reactive nitro-derivatives.

Among a series of substituted arylhydrazone derivatives tested as trypanocidal agents, the catechol derivative called brazilizona A (**9**) (Figure 9) showed remarkable activity (IC_{50} = 5.3 μM), being two fold more active than megazol (**4**) (IC_{50} = 9.9 μM) against blood trypomastigote forms of *T. cruzi* (Carvalho *et al.*, 2004). Parasites treated with **9** displayed an expansion of the flagellar membrane structure, dilation of the nuclear envelope, the formation of autophagosome-like structures, and cellular disorganization (Salomão *et al.*, 2010). The profile confirmed that the hydrazine framework introduced into this novel molecular pattern was successful to optimize the trypanocidal action of megazol (**4**), since the nitro imidazole group is kept. The corresponding phenyl analogue (**10**) (Figure 9) is twelve fold less active than **9** as a trypanocide agent, confirming the pharmacophoric behaviour of the nitro heterocyclic ring (Carvalho *et al.*, 2004).

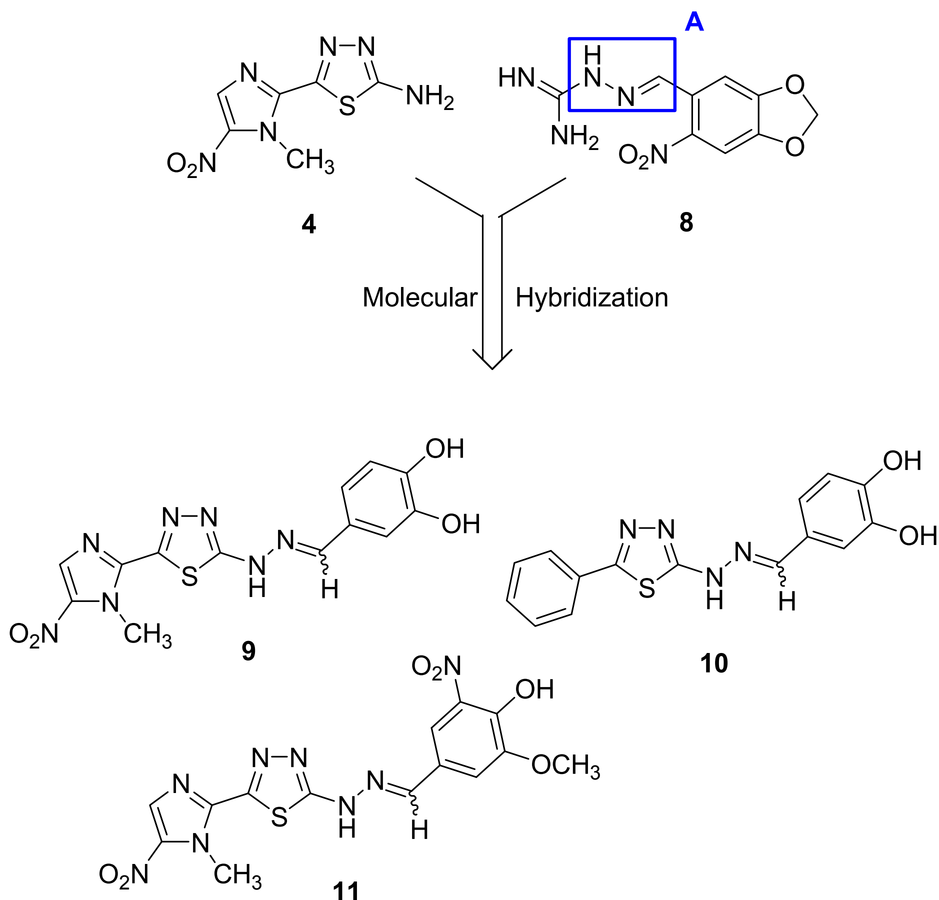


Fig. 9. Structural planning of the novel 1,3,4-thiadiazole-*N*-arylhydrazone derivatives Brazilizone A (**9**), (**10**) and Brazilizone N (**11**).

The construction of QSAR-3D models for these derivatives exploiting the CoMFA program allowed as to identify the brazilizone N (**11**), nitro vanillin analogue of **9** that also presented good trypanocide activity, *i.e.* IC_{50} = 19.0 μM) (Carvalho *et al.*, 2008).
Theoretical calculations have shown that brazilizone A (**9**) and brazilizone N (**11**) have good potential for absorption *in vivo* (Carvalho *et al.*, 2008). For an in vivo screening, compound **9** was administered to infected mice in a single dose of 200 mg/kg at the onset of parasitemia, and the body weights, levels of parasitemia, and mortality rates of the mice were monitored up to 40 days post-infection. There was no significant decrease in parasitemia or mortality in the two different regimens of treatment in vivo (Salomão *et al.*, 2010). The treatment of infected mice with **9** (200 mg/kg) did not interfere with the course of infection, with the values of body weight, parasitemia, and mortality being similar to those of the infected control group and 50% cumulative mortality occurring at 16 days post-infection (Salomão *et al.*, 2010). This behaviour is indicative of the necessity to optimize the pharmacokinetic

properties of brazilizone A (**9**) so that the use of this potent trypanocide agent as an anti-Chagasic drug can become viable.

3. Essential oils: State-of-the-art and Chagas' disease

Essential oils are very complex mixtures composed of natural volatile organic compounds characterized by a strong aroma. They are produced as secondary metabolites in plants, where the biosynthesis can be carried out in a constitutive way, independently of the action of pathogens (phytoanticipins) or induced as a defensive response of the plant against infection by bacteria, fungi and nematodes (phytoalexins). Indeed, they can be synthesized by different organs, i.e. buds, flowers, leaves, stems, twigs, seeds, fruits, roots, wood or bark, and are stored in secretory cells, cavities, canals, epidermic cells or glandular trichomes (Bakkali et al., 2008; Jalali-Heravi & Parastar, 2011). In general, we can classify the effective components of essential oils into two main groups, volatile and nonvolatile fractions. The former group contains monoterpenes, sesquiterpenes and their oxygenated derivatives, aliphatic aldehydes, alcohols and esters. The volatile compounds are the main fraction of the oil. The non-volatile fraction is mainly composed of hydrocarbons, fatty acids, sterols, carotenoids, waxes, coumarins, psoralens and flavonoids (Jalali-Heravi and Parastar, 2011). In fact, the chemical composition of essential oils may vary according to many factors such as (i) the difference between plant species and varieties, (ii) different climates, (iii) seasonal and geographical conditions and (iv) harvest periods. In addition, different parts of the same plant can provide varying compositions of essential oils. So, in order to obtain essential oils of constant composition, they have to be extracted under the same conditions from the same organ of the plant which has been growing on the same soil, under the same climate and has been picked in the same season.

Plant essential oils are extracted from various aromatic plants generally localized in temperate to warm countries like Mediterranean and tropical countries. They are usually obtained by steam or hydro-distillation and analyzed by gas-chromatography mass spectrometry. In fact, there are other methods for extracting essential oils. These may include use of liquid carbon dioxide or microwaves, as well as low or high pressure distillation with boiling water or hot steam. Depending on the type of extraction the chemical profile of the essential oils could also differ not only in the number of molecules but also in the stereochemical type of molecules extracted (Masotti et al. 2003; Angioni et al., 2006).

In nature, essential oils play an important role in protecting plants against numerous organisms of bacterial, fungal and viral origin. Sometimes these oils can have a double function; on one hand they can attract some insects to ensure pollination and seed dispersal, and on the other hand they can repel predators. These valuable natural products can be used in many fields based on their properties already observed in nature, i.e. for their antimicrobial and repellent activities. The ethnopharmacological approach, which is used as a strategy to investigate essential oils, combines information acquired from users of medicinal plants (traditional communities and experts), with chemical and pharmacological studies. Brazil has a rich cultural and biological diversity and is also home to 55,000 species of higher plants as well as almost 7% of the global animal diversity (Garcia et al., 2010). Despite the large Brazilian biodiversity and the extensive work of researchers in the search for new chemotherapeutic agents against Chagas' disease, only one study is related to the

essential oil of a native species, *Croton cajucara*. Other studies are being carried out with exotic species, commonly used as spices and medicinal plants.

Today there are approximately 3,000 known essential oils, 10% of which are commercially used for the perfume, cosmetic, food and pharmaceutical industries. From a pharmaceutical point of view, many herbs and related essential oils have revealed medicinal properties including anticancer, antibacterial, antiviral, antitoxigenic and antiparasitic activities (Bakkali et al., 2008; Alviano & Alviano, 2009). Indeed, the complex composition of essential oils makes them both a promising and challenging source of drug candidates. Essential oils may contain up to 100 individual components at completely different concentrations, many of them with one or more biological activitie. Structures of natural products feature wide chemical diversity, biochemistry specificity and other molecular properties that make them favourable as lead structures in drug discovery. Therefore, besides the rational drug design and novel synthetic efforts, natural products, such as essential oils, are still undergoing investigation for novel chemical structures that may interact with known and unknown microbial targets.

With the discovery of Chagas' disease by Carlos Chagas in 1909, a large number of drugs with trypanocidal activity have been evaluated by several research groups, but all without therapeutic success. So since the late 1960s nifurtimox (4 [(5-nitrofurfurilideno) amino]-3-metiltiomorfolino-1,1-dioxide), a nitrofuran derivative, and benznidazole (Nbenzil-2-nitroimidazole-1-acetamide), a nitroimidazole derivative have been used to treat Chagas' disease. The healing potential of nifurtimox and benznidazole varies depending on the stage of the disease, treatment period and dosage, and patient age. Generally, satisfactory results are achieved when the patient is treated in the acute phase, in recent chronic infection, in congenital infection and in laboratory accidents. The main limitation of these compounds is the low efficacy in the treatment of patients in the chronic phase (Coura & Castro, 2002; Urbina, 2002). The reasons for the difference in the effectiveness of treatment between the acute and chronic stages of the disease are not known. The major disadvantage in the treatment of Chagas' disease is the development of resistance to the chemotherapeutic drugs currently being used. Due to their high toxicity, these drugs are administered at low doses, and thus resistance may appear (Coura & Castro, 2002). In addition, the high cost of treatment and the consequent abandonment of it, along with the permanence of infected people in endemic areas have contributed to parasite resistance which causes a major impact on controlling this disease.

The treatment with nifurtimox can lead to side effects such as psychological disorders, anorexia, somnolence and gastrointestinal symptoms (nausea, vomiting and diarrhea). The recommended doses for benznidazole in the treatment of Chagas' disease may cause hypersensitivity and dermatitis with skin rashes and generate neuropathologies such as paresthesia and peripheral nerve polyneuritis (Maya et al., 2007). However, more serious reactions to benznidazole include bone marrow depression, which can lead to thrombocytopenia purpurea, and agranulocytosis. While thrombocytopenia purpurea can cause hemorrhagic manifestations, agranulocytosis may lead the patient to septicaemia. Because of these characteristic side effects, nifurtimox and benznidazole should not be prescribed to pregnant women and elderly patients or patients with any disease associated with severe Chagas' disease such as heart and respiratory diseases, and in cases of renal or hepatic impairment, systemic infection or malignancies (Coura & Castro, 2002). Since the inefficacy of current chemotherapies has grown, mainstream medicine is increasingly receptive to the use of antimicrobials and other drugs derived from plants (Cowan, 1999).

However, the full acceptance of phytopharmaceuticals and the integration of phytotherapy into the concept of classical medicine will arise only if they meet the same criteria of quality as synthetic pharmaceuticals and if they are submitted to the major pharmacological and toxicological assays for standardization.
The long association between the coexistence of parasites, humans and herbal remedies, has made plants an undeniable source for drug candidates particularly for infectious diseases. Most research efforts into the effects of plants on parasite infections has been undertaken using aqueous or alcoholic extractions; however purified plant essential oils could also be efficacious in treating or preventing parasitic diseases (Anthony et al., 2005). So far, however, most of the studies concerning the biological effects of essential oils have been focused on the bactericidal effects. Only a few studies have addressed the effect of essential oils and their components against trypanosomes including *Trypanosoma cruzi*.

3.1 Essential oils: Characteristics as active agents

Properties such as low density and rapid diffusion across cell membranes (owing to their lipid solubility) can enhance the targeting of active components within an oil to intracellular parasites (Alviano & Alviano, 2009). Table 3 lists some of the essential oils of plant species commonly used in food and folk medicine and whose functional group of their main component presents activity against *T. cruzi*.

Botanical name	Common name	Part used	Main or active component / Main functional group	Reference
Allium sativa	garlic	garlic cloves	ajoene / disulfide bond	Urbina et al., 1993
Chenopodium ambrosioides	mexican tea	leaves	(−)-(1S,4S)-p-mentha- 2,8-dien-1-hydroperoxide / peroxide	Kiuchi et al., 2002
Laurus nobilis	bay	leaves	(1R,4S)-1-hydroperoxy-p-menth-2-en-8-ol / peroxide	Uchiyama et al., 2002
Cymbopogon citratus	lemongrass	leaves	citral / aldehyde	Santoro et al., 2007a Cardoso & Soares, 2010
Achillea millefolium	yarrow	leaves and flowers	chamazulene / C-C double bonds	Santoro et al., 2007b
Syzygium aromaticum	clove	flowers	eugenol / phenol	
Ocimum basilicum	basil	leaves	linalool / alcohol	
Origanum vulgare	oregano	leaves	3-cyclohe n-1-ol / alcohol	Santoro et al., 2007c
Thymus vulgaris	thyme	leaves	thymol / phenol	
Croton cajucara	white sacaca	leaves	linalool / alcohol	Rodrigues, 2010

Table 3. Plant essential oils and main constituents with trypanocidal activity against *Trypanosoma cruzi*.

3.2 Essential oils: Mode of action against *Trypanosoma cruzi*

Most studies of new agents with anti-protozoal activity are based on the direct action of these drugs on parasites and their immunomodulatory effects, particularly on macrophage responses. In fact, promising results have been obtained by some groups in the search of essential oils and related compounds with activity against pathogenic microorganisms, including *T. cruzi*.

Some plant essential oils have immunomodulatory effects that are useful for treating infectious diseases, particularly in cases where the oil has no direct adverse effect on the host. Linalool-rich essential oils extracted from many plants are reported as antimicrobials for bacteria, fungi and protozoal species (Rosa et al., 2003; Alviano et al., 2005; de Almeida et al., 2007). In fact, the effects of linalool-rich essential oil from *Croton cajucara* (white sacaca) on *Leishmania amazonensis* have been successfully investigated. The median lethal doses and absolute lethal doses of the essential oil and purified linalool (Figure 10) from *C. cajucara* for promastigotes and amastigotes are very low (Rosa et al., 2003). The same essential oil is active against *T. cruzi* infection, promoting parasite damages such as mitochondrial swelling and important alterations in the organization of the nuclear and kinetoplast chromatin. Along these lines, the essential oil from *C. cajucara* could be a useful source of novel drugs. *T. cruzi* is also susceptible to the action of the *Cymbopogom citratus* essential oil (Santoro et al., 2007a; Cardoso & Soares, 2010). The essential oil is effective in killing *T. cruzi* with a low 50% inhibitory concentration (IC_{50}) at 15 µg/mL for bloodstream trypomastigotes. Furthermore, this essential oil also inhibits epimastigote growth at low concentrations, inducing ultrastructural alterations on parasite morphology. The alterations caused by *C. citratus* can be observed in Figure 11. In addition, the treatment of *T. cruzi* with citral (Figure 10), the main constituent of *C. citratus* essential oil, results in epimastigote growth inhibition and decrease of viable trypomastigotes at 42 and 14.2 µg/mL, respectively, showing its high microbicidal activity (Santoro et al., 2007a). This monoterpene also exerts inhibitory effects on *T. cruzi* metacyclogenesis (Cardoso & Soares, 2010). Although no effect is observed at concentrations lower than 20 µg/mL, metacyclogenesis is almost totally abolished at 40 µg/mL after 24, 48 or 72 h of incubation in TAU3AAG (differentiation) medium. Higher concentrations (60, 80 or 100 µg/mL) induce 100% cell lysis.

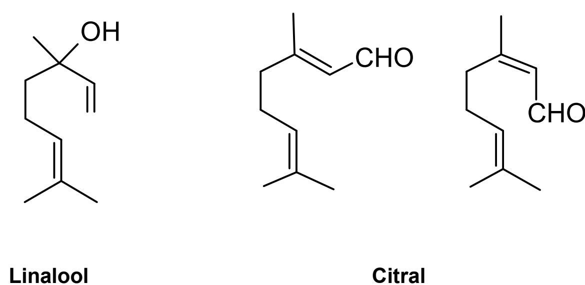


Fig. 10. Linalool and citral structures.

The essential oils of *C. cajucara* and *C. citratus* have no toxicity for macrophages, the main cells involved in the early steps of infection. In fact, the number of internalized parasites (amastigote forms of *T. cruzi*) drastically decreases when infected macrophages are treated with the essential oil of *C. citratus*. Interestingly, the *C. cajucara* can not only reduce the number

of internalized parasites such as *L. amazonensis* and *T. cruzi* amastigotes in infected macrophages, but it can also promote macrophage activation with a consequent production of nitric oxide. However, how these oils cause macrophage activation is still poorly understood.

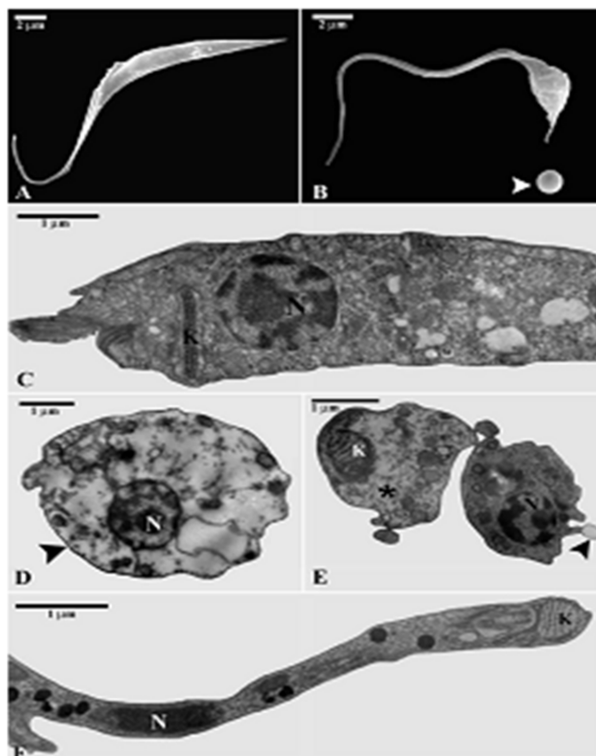


Fig. 11. Effect of lemongrass essential oil on epimastigotes and bloodstream trypomastigotes, as observed by scanning (SEM) or transmission (TEM) electron microscopy, after treatment with IC_{50}/24 h. (A) Untreated, control parasite observed by SEM. (B) SEM of lemongrass-treated epimastigote after incubation with 126.5 µg/mL essential oil. Note the rounding of the parasite body and a small vesicle (arrowhead) that appears to have been detached from the parasite plasma membrane. (C) Untreated epimastigote showing normal organelles by TEM. (D) Treated epimastigote, showing cytoplasmic extraction. The plasma membrane and the subpellicular microtubules remain unaltered (arrowhead). (E) Bloodstream trypomastigotes treated with IC_{50} (15.5 µg/mL) of lemongrass essential oil as observed by TEM, showing intense cytoplasmic extraction (asterisk) and formation of a membrane bleb (arrowhead). (F) Control, untreated bloodstream trypomastigote showing typical organelles. **K**, kinetoplast; **N**, nucleus (Santoro et al., 2007). Image reproduced with permission from Memórias do Instituto Oswaldo Cruz editor in chief.

The trypanocidal activity of the essential oils of *Origanum vulgare* (oregano) and *Thymus vulgaris* (thyme) demonstrated that epimastigotes treated with different concentrations of these essential oils resulted in growth inhibition and dose-dependent inhibitory

concentration. Trypomastigotes proved to be more susceptible to the action of the essential oils and especially to the action of thymol (Figure 12), the active phenolic component of the thymus essential oil with 50% inhibitory concentration, approximately 60 µg/mL. Different pathways may account for these variations in sensitivity to the oils evaluated, given that epimastigotes and trypomastigotes are adapted to survive in different environmental conditions (Santoro et al., 2007c).
Trypomastigotes treated with *Syzygium aromaticum* (clove) essential oil demonstrated swelling of the parasite body, cytoplasmic extraction and nuclear alterations, whereas the plasma membrane remained unchanged. Similar observations were made in parasites treated with essential oils *Ocimum basilicum* (basil) and *Achillea millefolium* (yarrow) (Santoro et al., 2007b). The phenolic compound eugenol (Figure 12) is the major component of *S. aromaticum* essential oil, but it is also an important chemical constituent of essential oils from many aromatic plants, such as *Dicipelium cariophyllatum*, *Pimenta dioica*, *Croton zehntneri* var. eugenoliferum, and *C. zehntneri* (De Vincenzi et al., 2000). However, incubation of *T. cruzi* with eugenol alone results in a less potent activity with 50% inhibitory concentration values equal to 246 µg/mL for epimastigotes and 76µg/mL for trypomastigotes (Santoro et al., 2007b). The contrast of results obtained with essential oils of *S. aromaticum* and eugenol in the experiments carried out with *T. cruzi* could be explained by synergistic effects of different compounds of the plant essential oil used in this work. The synergistic phenomenon is well known in several other systems (Zee-Cheng, 1992; Alviano & Alviano, 2009). Additionally, comparing the effectiveness of the phenolic compounds, eugenol and thymol, the increased availability of the phenolic group in the latter may be the reason for its higher activity, reinforcing the importance of this group for its mode of action (Ultee et al., 2002).

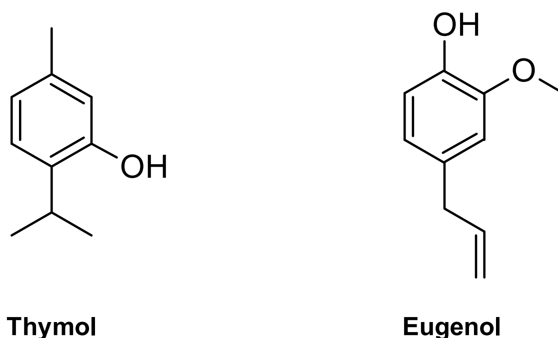
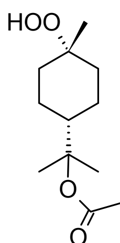


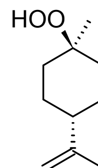
Fig. 12. Thymol and eugenol structures

Many essential oils are known to exert antimicrobial activity but the mode of action is often not entirely understood. The large number of constituents in essential oils makes it possible to have more than one cellular target. Perhaps this could explain why some essential oils present higher activity than their isolated compounds. However, some characteristics of essential oils can help us to understand their efficacy. First, the lipophilic constituents from essential oils may be involved in antimicrobial mechanisms by permeating cell membranes and disrupting the structure of the different layers of membrane polysaccharides, fatty acids

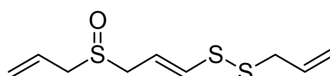
and phospholipids, which permeabilize the membranes (Bakkali et al., 2008). The membrane permeabilization leads to the leakage of macromolecules and lysis. Second, essential oil metabolic targets could lead to serious cellular damages. Alterations such as mitochondrial damage, with membranous arrangements within this organelle and in the flagellar pocket, and the formation of autophagic structures observed in some parasites are associated with depletion of ergosterol and the alteration of the physical properties of the membranes (Santa-Rita et al., 2004). Also, the alcohol sesquiterpene nerolidol possessess inhibitory activity under biosynthesis of isoprenoids (dolichol, ergosterol and ubiquinone) from *Leishmania* species (Arruda et al., 2005). In this context, the alcohol sesquiterpene linalool could exert the same activity based on ultrastructure alterations cited previously and the same functional groups. Parasites of the genera *Leishmania* and *Trypanosoma* have a strict requirement for specific endogenous sterols (ergosterol and analogs) for survival and growth and can not use the cholesterol supply present in their vertebrate hosts. Another example of metabolism inhibition is the growth inhibition of *T. cruzi* epimastigote caused by ajoene (Figure 13), a derivative of garlic that can be attributed to changes in the phospholipid composition of the parasite membrane. One of the evidences for these alterations is the low levels of phosphatidylcholine detected, followed by high levels of its immediate precursor, phosphatidylethanolamine, suggesting that ajoene inhibits the final stage of phosphatidylcholine biosynthesis, altering the phospholipid composition of the cell membrane (Urbina et al., 1993).



(1R, 4S)-1-hydroperoxy-p-menth-2-en-8-ol acetate



(-)-(1S, 4S)-p-mentha-2,8-dien-1-hydroperoxide



Ajoene

Fig. 13. Ajoene, (1R,4S)-1-hydroperoxy-p-menth-2-en-8-ol acetate and (−)-(1S,4S)-p-mentha-2,8-dien-1-hydroperoxide structures.

The peroxide group present in some monoterpenes is important for the activity of these compounds. The transformation of peroxide into the hydroxyl group ends the activity of the substances. For example (1R,4S)-1-hydroperoxy-p-menth-2-en-8-ol acetate isolated from leaves of *Laurus nobilis* showed minimum lethal concentrations at 1.4 μM against

epimastigotes of *T. cruzi* (Uchiyama et al., 2002). Other similar substances isolated from *Chenopodium ambrosioides*, ascaridole and related monoterpenes (−)-(2S,4S)- and (−)-(2R,4S)-p-mentha-1(7),8-dien-2-hydroperoxide, (−)-(1R,4S)- and (−)-(1S,4S)-p-mentha- 2,8-dien-1-hydroperoxide and (−)-(1R,4S)-hydroperoxy-p-menth-2-en-8-ol acetate (Figure 13) were active against epimastigotes at concentrations of 23, 1.2, 1.6, 3.1, 0.8 and 1.4 µM, respectively (Kiuchi et al., 2002). The mode of action of these monoterpene peroxides is associated to reactive oxygen species derived from the hydroperoxy group.

Only a few studies evaluating the activity of essential oils on *T. cruzi* have been presented so far, however many of them with promising results. Besides the features described here, it should be noted that up to the present no resistance or adaptation by parasites has been reported in the literature. This may be due to multiple targets that essential oils can achieve, disabling many of the parasite modes of resistance. Much more can be done along these lines to discover new and effective therapies for the control and treatment of Chagas' disease.

4. Cysteine peptidase: A target for drug development

4.1 Peptidases

Peptidases, also known as proteases or proteinases, are hydrolases able to hydrolyze peptide bonds in proteins or peptides. They act as processing enzymes taking part in regulatory or catabolic processes in the cell as extracellular enzymes and play an important role in the degradation of proteic substrates serving as carbon or energy sources. Peptidases are important in several pathological conditions and one of them, well documented in the literature, is their role in parasitic invasions that occur in parasitic diseases such as Chagas disease. Some *Trypanosoma cruzi* peptidases are thought to play central roles in diverse processes such as, differentiation, cell cycle progression, proteins degradation and evasion of the host immune response (Klemba, 2002; Kosec, 2006; McKerrow, 2008; Vermelho, 2010; Alvarez, 2011).

The MEROPS database is an Internet resource containing information on peptidases, their substrates and inhibitors including details of cleavage positions in substrates, both physiological and non-physiological, natural and synthetic and their mechanism of catalysis. The enzymes are classified using three different approaches including the chemical mechanism of catalysis, the catalytic reaction and the molecular structure and homology. Based on these criteria peptidases are grouped into protein species, which in turn are grouped into families, and then grouped into clans (Rawlings, 2010).

Peptidases are druggable targets and peptidases inhibitors have proven to be effective drugs for hypertension (Hoover, 2010), HIV infection (Moyle, 1998), human cancers (Hoekstra, 2006) and infectious diseases (Renslo, 2006). Especially in infectious diseases peptidases play an important role in the life cycle of protozoan parasites and in the pathogenesis of these diseases, such as in the inactivation of host immune defence mediators, the processing of host or parasite surface proteins for invasion of host cells, as well as in the digestion of host proteins for nutrition (Franke de Cazzulo, 1994; McKerrow, 2008).

Peptidases, such as, serine, threonine, aspartyl, metallo and cysteine peptidases have been detected in *Trypanosoma cruzi* and they have been shown to be involved in numerous roles in the physiology of the parasite. The inhibition of some of these enzymes has shown high anti-*T. cruzi* activity *in vitro* and *in vivo* (Capaci-Rodrigues, 2010). The serine peptidases described in *Trypanosoma cruzi* include oligopeptidase B, a member of the prolyl

oligopeptidase family involved in Ca^{2+} signaling during mammalian cell invasion; a 80-kDa prolyl serine oligopeptidase (POP Tc80), belonging to the prolyl oligopeptidase family (EC 3.4.21.26) against which inhibitors are being developed, and a lysosomal serine carboxypeptidase that probably hydrolyzes human collagen (Types I and IV) and fibronectin that has been implicated in the parasite adhesion to host cells and cell entry (Burleigh, 1995; Burleigh, 1997; Caler, 1998; Grellier, 2001; Bastos, 2005; da Silva-Lopez, 2008). The threonine (proteasome) peptidase in the parasite has properties similar to those of other eukaryotes, and its inhibition by lactacystin blocks some differentiation steps in the life cycle of the parasite (Nkemgu-Njinkeng, 2002; Cardoso, 2008; Gutiérrez, 2009).

Recently, in *T. cruzi*, two aspartic peptidases were isolated from the epimastigote forms, named cruzipain-I and cruzipain-II. One from the water soluble fraction and the second from the 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) fraction. Both peptidase activities were inhibited by the classic pepstatin-A inhibitor and the aspartic active site labelling agent, 1,2- epoxy-3-(phenyl-nitrophenoxy) propane (EPNP). However, no coding genes for aspartic peptidases have yet been observed in the *T. cruzi* genome. The authors explained that they could not rule out the possibility that aspartic peptidase homologues are present in *T. cruzi*, because this genome has not been completely mapped (Pinho, 2009).

Metallopeptidases homologous to the gp63 of *Leishmania* spp. are present in *T. cruzi*, as well as two metallocarboxypeptidases belonging to the M32 family, before found only in prokaryotes (Bonaldo, 1991; Lowndes, 1996; Burleigh, 2002; Cuevas, 2003; Nogueira de Melo, 2004; Gutierrez, 2008; Niemirowicz, 2007; Niemirowicz, 2008; Kulkarni, 2009).

The most abundant among these enzymes is cruzipain (Cz), also known as GP57/51 or cruzain (the recombinant catalytic domain of cruzipain) a cysteine peptidase (CP) expressed as a mixture of isoforms. This CP is located in clan CA, being a member of the papain C1 family of CP with an intermediate specificity between cathepsin L and cathepsin B (Cazzulo, 1989).

Although Cz is found in different compartments, it is expressed in all stages of the *T. cruzi* lifecycle. In the epimastigote stage, cruzipain is probably involved in the degradation of proteins. It may also play an important role in nutrition of the parasite, at least in the gut of the hematophagous insect vector. Cruzipain is stored in the pre lysosomal organelle called reservosome, in the posterior portion of the cell. In the intracellular amastigote stage, this peptidase can still be detected in the lysosome-related compartment, but most cruzipain is found fixed on the surface of the parasite, presumably through a GPI anchor, directly in contact with host cell cytoplasm. Trypomastigotes are able to secret some isoforms into the medium. This is extremely significant for the role of cruzipain as a virulence factor in Chagas disease (Souto-Padron, 1990; Engel, 2000; Vieira, 2005; McKerrow, 2009; Alvarez, 2011).

Cruzipain is involved in a number of cellular processes and in important virulence factors in the pathogenesis of parasitic diseases, such as: in the cell invasion phenomenon, facilitating the proteolytic degradation of host tissues and triggering the evasion mechanism (Franke de Cazzulo, 1994; Costales, 2007; Capaci-Rodrigues, 2010; Nogueira de Melo, 2010).

In addition, this CP has demonstrated an ability to induce the production of the proinflammatory peptide Lys-bradykinin by the proteolysis of kininogen or by activation of plasmatic pre-kallikrein (Del Nery, 1997; Scharfstein, 2000; Benítez-Hernández, 2010; Capaci-Rodrigues, 2010).

Although some other minor CPs have been described in *T. cruzi*, such as 30 kDa cathepsin B-like CPs and more recently *Tc*CPmet (secreted by metacyclic trypomastigotes), it is not yet known how many different enzymes of this type are present and their possible functions (Garcia, 1998; Yong, 2000; Duschak, 2006; Capaci-Rodrigues, 2010).

The structure of the active site, of the cruzipain , has been reported through X-ray crystal structures of the enzyme in complex with reversible and irreversible inhibitors. The enzyme is composed of one polypeptide chain of 215 amino acid residues and the catalytic triad of Cys25, His159, and Asn175 , as well as the highly conserved Trp177 of cruzipain, which is contained within a cleft between the two structural domains of the enzyme (McGrath, 1995). This domain is formed by seven substrate binding sites, four (S4, S3, S2, and S1) on the acyl side and three (S1′, S2′, and S3′) on the amino side of the scissile bond. The interaction of the S2 site of the enzyme with the corresponding P2 inhibitor residue is the key specificity determining factor (Chen, 2010).

Although Cz has a catalytic domain that is highly homologous to human cysteine peptidases (HCP) (Brinen, 2000), studies have indicated that there is a preferential inhibition with cruzipain compared to HCP. The preferential inhibition is possibly due to the fact that human cathepsins are located in the lysosomes, a less accessible sub-compartment. The parasite, Cz is found in the host cell cytoplasm and therefore, more exposed to the action of inhibitors (McKerrow, 1995; McKerrow, 1999; Brak, 2008). Moreover, the parasite has a specific mechanism for the uptake of small molecules such as peptidase inhibitors that could facilitate the entry of these inhibitors into the parasite (McKerrow, 1999; Mallari, 2008; McKerrow,2008).

Recently there has been great interest in developing new cruzipain inhibitors as chemotherapeutic targets. As proof of this more than 50% of the 51 patents in the 2000-2006 period aiming at different targets for the treatment of Chagas disease, were cruzipain inhibitors (targets included: ergosterol biosynthesis inhibitors, inhibitors of polyamine and trypanothione formation) (Duschak, 2007).

In an attempt to obtain a new lead, scaffolds for cruzipain inhibition with good efficacy and minimal toxicity for several classes of cruzipain inhibitors have been developed. Knowledge of the Cz crystal structure has promoted the design of a variety of cysteine peptidase inhibitors including peptidyl and non-peptidyl inhibitors. Among peptidyl inhibitors, the irreversible inhibitor, *N*-acylhydrazone has been studied (Ifa, 2000; Lima, 2009; dos Santos Filho, 2009), as well as the halomethyl ketone based (Ashall, 1990; Harth, 1993), diazomethane ketones (Shaw, 1994; Lalmanach, 1996), vinyl sulfones (Roush, 2001; Engel, 1998; Barr, 2005; Kerr, 2009; Jacobsen, 2000), and reversible inhibitors such as oxadiazoles (Ferreira,2009), and aryl ureas (Du, 2000). In the non-peptidyl group thiosemicarbazones (Du, 2002), triazole, triazine nitriles (Brak, 2010; Mott, 2010) and non-peptidic vinylsulfones (Bryant, 2009) can be found some of which are presented in Table 4.

Among all chemical classes of inhibitors of cruzipain (Cz) researched until now, vinyl sulfone can be highlighted as it is in an advanced stage clinical trial.

Peptidyl vinyl sulfone inhibitor compounds are irreversible inhibitors of cruzipain via a Michael addition by active site Cys25. According to Palmer's (Palmer, 1995) hypothesis, the Michael addition is facilitated through hydrogen bonding between the protonated His159 residue and one of the vinyl sulfone oxygens. Thus, the vinyl group would be polarized, and consequently the β-vinyl carbon would become positively charged. This would then facilitate a nucleophilic attack by the active site thiolate. The other sulfone oxygen could participate in hydrogen bonding with the active site glutamine (Figure 14) (Powers, 2002).

Chemical class of cruzipain inhibitor			Chemical Structure	Reference
Peptidyl inhibitor	Irreversible	*N*-acylhydrazones		Lima, 2009
		Vinyl sulfones		Kerr, 2009
	Reversible	Oxadiazoles		Ferreira, 2009
		Thiosemicarbazones		Du, 2002
Non-peptidyl inhibitors		Triazole, triazine nitriles		Brak, 2010
		Non-peptidic vinyl sulfones		Bryant, 2009

Table 4. Some cruzipain inhibitors

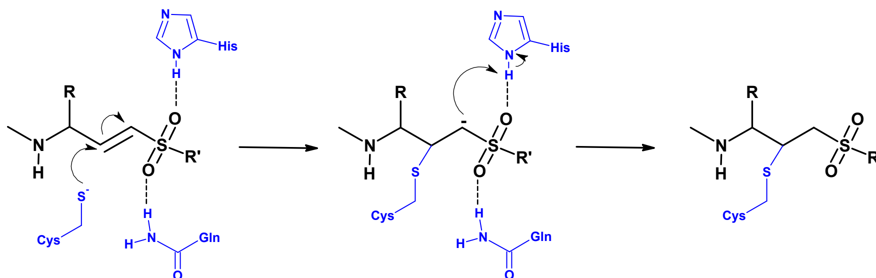


Fig. 14. Mechanism of irreversible inhibition of cysteine peptidase by vinyl sulfones (Palmer, 1995; Powers, 2002; Capaci-Rodrigues, 2010)

The most important peptidyl vinyl sulfone derivative is K11777 (*N*-methyl-piperazine-Phe-homoPhe-vinylsulfone-phenyl), also known as K777 (compound **6**, Figure 15) that is currently in late-stage preclinical trials. K11777 is a second generation vinyl sulfone (Roush, 2001). The compound was developed by Jim McKerrow's team at the Sandler Center for Drug Discovery in San Francisco in the United States(McKerrow, 2009). K777 is non-mutagenic, has an acceptable PK profile, anis the most extensively characterized in efficacy and safety studies in rodents, dogs and primates **(Clayton , 2010)** . Studies with K11777 have proven that blocking cruzipain is enough to cure Chagas disease in all of its stages and disrupts amastigote intracellular development. (McKerrow, 2009, Doyle 2011). This compound has a wide spectrum of activity against different *T. cruzi* strains (e.g., Y, Tulahuén, CL, CA-I/72), even against nifurtimox and benznidazole-resistant *T. cruzi* strains (McKerrow, 2009). In addition, studies have indicated that concomitant use of a cruzipain inhibitor with benznidazole might be beneficial for the treatment of Chagas disease, as well as, the possibility of reducing the benznidazole dosage and thus decrease the side effects of this drug (McKerrow, 2009).K777 has now progressed into IND (Investigation New Drug) -enabling studies in collaboration with the DNDi (Drugs for Neglected Diseases Initiative) **(**Clayton , 2010).

This vinyl sulfone was developed by a rational design aimed to enhance specificity and *in vivo* stability and minimizing toxicity (Engel, 1998) from the predecessor K11002 (compound **7**, Figure 15) by the replacement of a morpholine-urea ring by a *N*-methylpiperazine ring (P3 subsite). This change was intended to increase the oral bioavailability, and increase the solubility in intestinal fluids. The *in vitro* assay showed an improvement of 3 to 19.9% (Jacobsen, 2000) and in murine models of acute and chronic Chagas disease, showed prolonged survival and eradicated the parasite infection with minimal toxicity (Engel, 1998; Barr, 2005; Urbina, 2003; Kerr, 2009; McKerrow, 2009).

Therefore, the P3 substituent of the vinyl sulfones has been of great interest, and the modification of this position has influenced several properties, such as lysosomotropism, hepatotoxicity, and pharmacokinetics (Kerr, 2009; Jacobsen, 2000; Zhang, 1998). Similar to most other papain-like cysteine peptidase, the interaction of the S2 subsite of the enzyme with the corresponding P2 residue is the key specificity determining factor. Cruzipain is a dual-specific peptidase that is able to accommodate phenylalanine or arginine in the P2 residue of the ligand due to the presence of Glu208 found at the base of the S2 subsite, however with a preference for phenylalanine over arginine (Chen, 2010). In this way, a new analogue of K11777 was synthesized, e.g.: WRR-483 (compound 8, Figure 15), replacing

phenylalanine (K11777) with arginine (WRR-483) in the P2 substituent**.** This compound showed sensitivity to pH conditions, being more potent at higher pH levels, but it was still relatively weak when compared to K11777 in the inhibition of cruzipain. Probably the Glu208 in the S2 pocket of the cruzain-WRR-483 is not fully anchored to the arginine residue. However, in the *in vitro* cell assay, this compound showed trypanocidal activity comparable to the lead compound, K11777, and surprisingly was effective in curing acute *T. cruzi* infection in an *in vivo* assay. This difference could be explained by the possibility that WRR-483 is targeting an as yet unknown cathepsin L-like cysteine peptidase in *T. cruzi*. Another possibility is that the WRR-483 inhibits cruzipain either located on the cell membrane or released by the parasite, and not in the lysosome. The inhibitor is hydrophilic in nature, primarily due to the guanidine group, and is more active at physiological pH, making it very favourable in the extracellular environment. In the light of these results, studies have shown that WRR-483 has potential to be developed as a treatment for Chagas' disease (Chen, 2010).

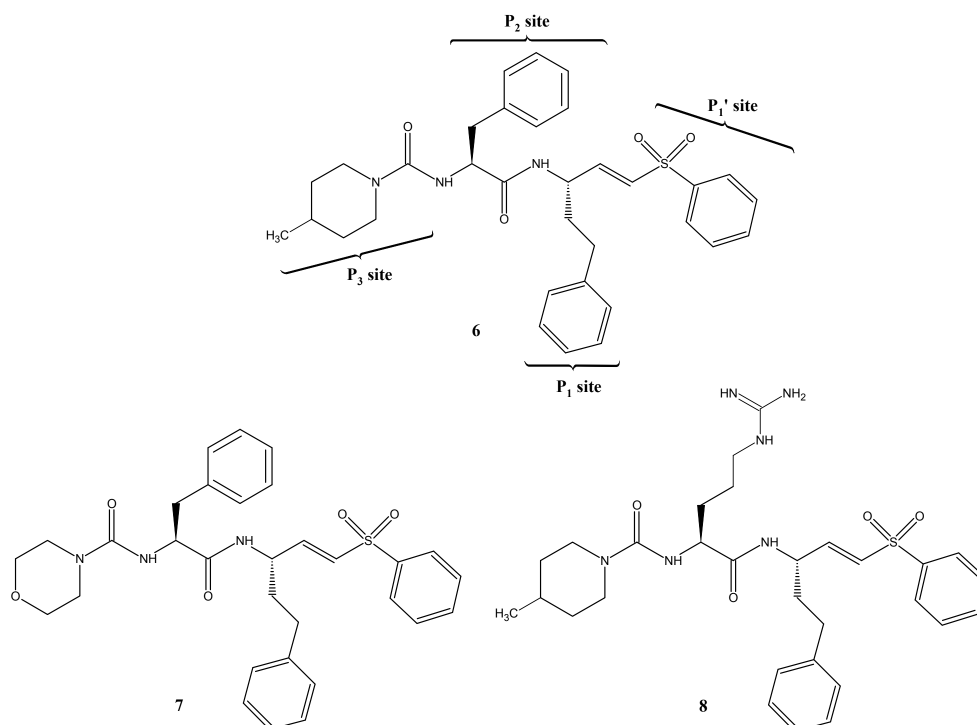


Fig. 15. Chemical structure of K11777 (**6**), K11002 (**7**) and WRR-483(**8**) vinyl sulfone derivatives inhibitors of cruzipain. The P1 –P3 subsites of K11777 are labelled (McKerrow, 1999; Chen, 2010).

5. Conclusion

This chapter focused in the action mechanism and limitations of the drugs currently used for Chagas disease and described the importance of the study of Megazol and natural products development for Chagas disease chemotherapy that it is active against different strains of the parasite and has great effectiveness in monkeys even in the chronic stage. For this reason, megazol was considered as an alternative lead-compound for the treatment of the Chagas' diseas. Megazol is a highly active compound against *Trypanosoma cruzi*, and has become a core structure for the design of new trypanocidal agents. Another point presented is about the use o natural products in Chagas disease. The knowledge of using natural products is of great value, especially the use of medicinal plants, as the countless number of species reflects an inestimable number of biologically active molecules against different illnesses, including infectious diseases. In the last decades the interest in natural compounds extracted from plants with anti-*Trypanosoma cruzi* activity has been renewed, and laboratories across the world have shown promising results. Moreover the use of peptidases, mainly cysteine peptidases as a target for drug development was discussed showing the importance of these enzymes development of new drugs for Chagas disease. A special focus was given for K777 currently in late-stage preclinical trials

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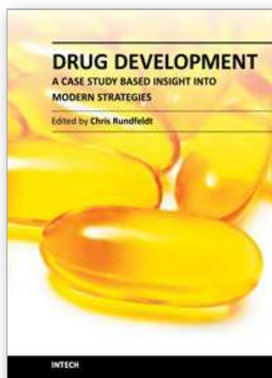

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This book represents a case study based overview of many different aspects of drug development, ranging from target identification and characterization to chemical optimization for efficacy and safety, as well as bioproduction of natural products utilizing for example lichen. In the last section, special aspects of the formal drug development process are discussed. Since drug development is a highly complex multidisciplinary process, case studies are an excellent tool to obtain insight in this field. While each chapter gives specific insight and may be read as an independent source of information, the whole book represents a unique collection of different facets giving insight in the complexity of drug development.

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