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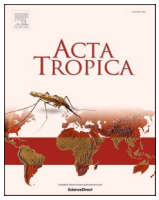

Potential of sulfur-selenium isosteric replacement as a strategy for the development of new anti-chagasic drugs

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ABSTRACT

Current treatment for Chagas disease is based on only two drugs: benznidazole and nifurtimox. Compounds containing sulfur (S) in their structure have shown promising results *in vitro* and *in vivo* against *Trypanosoma cruzi,* the parasite causing Chagas disease. Notably, some reports show that the isosteric replacement of S by selenium (Se) could be an interesting strategy for the development of new compounds for the treatment of Chagas disease. To date, the activity against *T. cruzi* of three Se- containing groups has been compared with their S counterparts: selenosemicarbazones, selenoquinones, and selenocyanates. More studies are needed to confirm the positive results of Se compounds. Therefore, we have investigated S compounds described in the literature tested against *T. cruzi*. We focused on those tested *in vivo* that allowed isosteric replacement to propose their Se counterparts as promising compounds for the future development of new drugs against Chagas disease.

1. Introduction

Chagas disease (CD), also known as American trypanosomiasis, is one of the most prevalent neglected diseases. It is caused by the protozoan parasite *T. cruzi*. This protozoan has an heteroxenic and complex life cycle with different morphological forms. The epimastigote form lives and multiplies in the vector (Reduviidae) and it changes into the infective form (metacyclic trypomastigote) in the midgut of the insect. In the mammalian hosts, including humans, trypomastigote form change for amastigote in the phagocytic vacuole present in the in the cytoplasm of the host cells where multiply and transform into trypomastigotes which are released in the blood stream (Gonçalves et al., 2018). The infective form initiates the acute phase, whereas the intracellular form determines the chronic phase (Echavarría et al., 2021).

Currently, more than 6 million people are infected by this parasite (DNDi, 2022b). This disease is underdiagnosed (less than 10% of the patients are detected), and more than 73 million people are at risk of getting the infection (DNDi, 2022b). Although CD is endemic to Latin America, cases are increasing in other places, such as Europe, the United States, Japan and Australia, due to human infected migration (DNDi, 2022b). In these non-endemic areas the transmission of the infection continues mainly via vertical, blood transfusion and transplants (Antinori et al., 2017). Currently, there are only two available drugs for the treatment of CD: benznidazole (BZ) and nifurtimox (NFX). The severe side effects caused by both drugs make it difficult for patients to adhere to treatment, especially in the chronic phase. Moreover, these drugs are more effective when administered in the acute phase (DNDi, 2022b; WHO, 2021). These facts motivate the development of new treatments for CD. Furthermore, genetic differences between strains of *T. cruzi* also have a notable effect on the efficacy of the treatment (Vela et al., 2021; Zingales et al., 2014). According to molecular genetics, ecological and epidemiological characteristics of *T. cruzi*, the strains are classified into seven groups called discrete typing units (DTUs) (TcI-VI and TcBat). (Zingales et al., 2009; Zingales et al., 2012). TcBat is a recent DTU from bats that has been already detected in humans (Pinto et al., 2015).

Selenium (Se) has recently emerged as an interesting alternative for new therapies. Se is a trace element that plays an important role in several physiological processes. It is mainly present in the form of selenoproteins, but it also participates in human immunity (Alcolea and Pérez-Silanes, 2020; Hou and Xu, 2022; Rashidi et al., 2022).

Compounds containing Se have been useful as treatments for other diseases (Barchielli et al., 2022), such as cancer (Mi et al., 2022),

Abbreviations: ALT, alanine aminotransferase; BZ, benznidazole; CD, Chagas disease; Cz, cruzipain; DTU, discrete typing unit; MZ, megazol; NFX, nifurtimox; S, sulfur; SCN, thiocyanate; Se, selenium; SeCN, selenocyanate; SeSC, selenosemicarbazone; SQS, squalene synthase; TdZ, thiadiazole; TPP, target product profile; TSC, thiosemicarbazone; TZ, thiazole; TZN, thiazolidinone.

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Alzheimer's disease (Barbosa et al., 2022), depression (de Oliveira *et al.*, 2022; Sajjadi et al., 2022), inflammatory states (Forceville et al., 2022), leishmaniasis (Alcolea *et al.*, 2021; Etxebeste-Mitxeltorena et al., 2021), malaria (Nieves et al., 2016), African trypanosomiasis (Eze et al., 2013) and intestinal parasites (Dkhil et al., 2019; Fahmy et al., 2021).

Due to the reduced structural variability in the molecules tested in clinical trials and the fact that current therapy for CD must be improved, Se has been proposed as an interesting element to test against *T. cruzi*. Previously, our group justified the Se interest in CD (Alcolea and Pérez-Silanes, 2020).

In chemistry, Se is considered a classic bioisoster of sulfur (S) (Lima and Barreiro, 2005). Both compounds belong to the same group of the periodic table, which implies small differences in chemical properties. These small changes positively affect the biological activity of Se, which is more active than S (Hou and Xu, 2022). Se is larger than S, so its outer valence electrons are more loosely bound, which is relevant to the antioxidant properties of Se (Se reacts with reactive oxygen species faster than S, for example). Se-X bonds are weaker than S-X analogues, making Se more reactive as an electron acceptor and more electrophilic than the S analogues. Se has low polarity and is more polarizable than S, resulting in stronger nucleophilic power, which is beneficial for Se to coordinate to the metal centre in the catalytic sites of enzymes. Moreover, Se compounds tend to lead to molecules with greater lipophilicity and permeability (Hou and Xu, 2022; Reich and Hondal, 2016; Weekley and Harris, 2013).

For these reasons, the isosteric replacement of S by Se has been proposed as a new strategy for the development of new drugs against CD. The activity of Se- and S-compounds has already been compared in different diseases, such as cancer (Alcolea et al., 2016; Rostán et al., 2021), as well as in several microorganisms, such as *Trichomonas vaginalis* (Ibáñez-Escribano et al., 2022), *Leishmania* spp. (Fernández-Rubio et al., 2019), *Plasmodium falciparum* and *Mycobacterium tuberculosis* (Nieves et al., 2016). The results reported in these papers support the idea that Se is more active than S in those diseases.

Recently, a clinical trial tested the efficacy and safety of Se (as 100 μg/day of sodium selenite) in chronic chagasic cardiomyopathy. The study concluded that Se supplementation for one year was safe and had a beneficial effect on patients with a left ventricular ejection fraction less than 45% and no heart failure (Holanda et al., 2021). Therefore, Se has been proposed as an interesting option for CD (Alcolea and Pérez-Silanes, 2020), and Se compounds evaluated against *T. cruzi* thus far have shown favourable results (Martín-Escolano et al., 2021a; Martín-Escolano et al., 2021b).

Due to these positive results in the replacement of S by Se in other diseases, we decided to explore whether this effect is also observed in CD. With this aim, we present in this review a comparison of Se compounds and their S homologues published to date with anti-*T. cruzi* activity. Furthermore, in the second part of this review, we propose different molecules with S that have already shown *in vivo* activity against *T. cruzi* in the literature as a starting point for the synthesis of their Se analogues in the search for new antichagasic drugs.

2. Comparison of selenium and sulfur compounds in Chagas disease

To date, the activity against *T. cruzi* of three Se-containing groups has been compared with their S counterparts: selenosemicarbazones, selenoquinones, and selenocyanates.

2.1. Thio- and selenosemicarbazones

Thiosemicarbazones (TSCs) are semicarbazone analogues in which the oxygen is replaced by a sulfur atom. TSCs are easily synthesized and have been widely studied, showing a broad profile for biological activity, such as antitumor (Niso et al., 2021), antibacterial (Gaber et al., 2021), antifungal (Pham et al., 2022), antiviral (Francesconi et al., 2020), and antiprotozoal (de Oliveira *et al.*, 2020; Teixeira de Moraes Gomes *et al.*, 2020; Ibáñez-Escribano et al., 2022) activities, among others. Interestingly, TSCs have been demonstrated to be more active than semicarbazones in CD and other trypanosomatids (Magalhaes Moreira *et al.*, 2014; Silva et al., 2019; Silva and Silva, 2017; Soares et al., 2011). According to the literature, TSCs are cruzipain (Cz) inhibitors (Dos Santos Nascimento *et al.*, 2021; Jasinski et al., 2022). This enzyme is a cysteine protease responsible for the main proteolytic activity in all life stages of *T. cruzi* (Santos and Ferreira, 2022; Santos et al., 2021). In addition, these compounds are versatile chelators of an extensive range of metal ions (Silva and Silva, 2017; Tang et al., 2017).

Additionally, selenosemicarbazones have been already described in the literature. These compounds are useful as antitumoral agents (Zec et al., 2014), antiviral (Turk et al., 1986), antifungal (Mautner et al., 1956) and against parasites such as *T. vaginalis* and *T. cruzi* (Ibáñez-Escribano et al., 2022).

Pizzo et al. (2012, 2016) explored whether the isosteric replacement of S by Se in these structures could generate more potent Cz inhibitors and therefore, possibly more effective compounds against *T. cruzi* infection. Accordingly, different selenosemicarbazone (SeSC) derivatives were synthesized (Fig. 1) by a reaction between selenosemicarbazide and the corresponding ketone or aldehyde (Pizzo et al., 2012). In contrast, Compound **7** was obtained from the corresponding semicarbazone via *O-Se* exchange using PCl_5, CH_2Cl_2, and Ishihara reagent (Pizzo et al., 2016).

As shown in Fig. 1, the comparison between TSCs and SeSCs is very difficult with the current data. Each type of structure was evaluated in a different paper with distinct experiments that are not directly comparable. TSCs were evaluated for their IC_{50} against Cz, whereas the activity of SeSCs in that enzyme was only tested at 0.3 μM. In addition, no data on the activity of TSCs in the parasite are available, except for Compound **6**, which was tested on a different parasitic form of its Se counterpart. However, we can compare the inhibitory activity in Cz of Compounds **1** and **2**. At the same dose, the Se compound shows much higher inhibitory activity than the S-analogue (81% vs. 24%). Moreover, it seems that SeSCs synthesized from ketones (Compounds **1** and **5**) were more potent Cz inhibitors than SeSC derived from aldehyde (Compound **3**).

Due to its promising *in vitro* results in terms of activity and selectivity, Compound **1** was tested *in vivo*. Unfortunately, it was toxic to mice at a dose of 50 mg/kg. Nonetheless, at a dose of 10 mg/kg, Compound **1** decreased parasitemia by 50% in comparison with the untreated group. However, Compound **1** was less effective than BZ, which decreases parasitemia by 88% compared to the untreated group (Pizzo et al., 2016). It should be noted that BZ was administered at a higher dose than Compound **1** (50 mg/kg vs. 10 mg/kg). It should be noted that despite its good results in *in vitro* assays, Compound **1** showed not as satisfactory results in *in vivo* models. This fact could be explained by other factors that play an important role in *in vivo* activity such as differences in the response of the *T. cruzi* strain since the mode of action of the drug depends on parasite replication. Furthermore, compounds need to exhibit appropriate pharmacokinetics to allow appropiated systemic drug exposure *in vivo* (Goldenberg, 2022).

Therefore, SeSCs could be an interesting option for *in vivo* studies, but their toxicity should be carefully evaluated. In addition, more studies are required to confirm whether SeSCs are more active than their S analogues.

2.2. Quinones

Selenoquinones described in the literature have shown antitumor activity. On the contrary, thioquinones have not exhibited relevant biological activities so far (Dubarle-Offner et al., 2014; Shaaban et al., 2016).

The group of Jardim et al. (2015) worked on the synthesis of novel derivatives of lapachol using different types of reactions. One of the

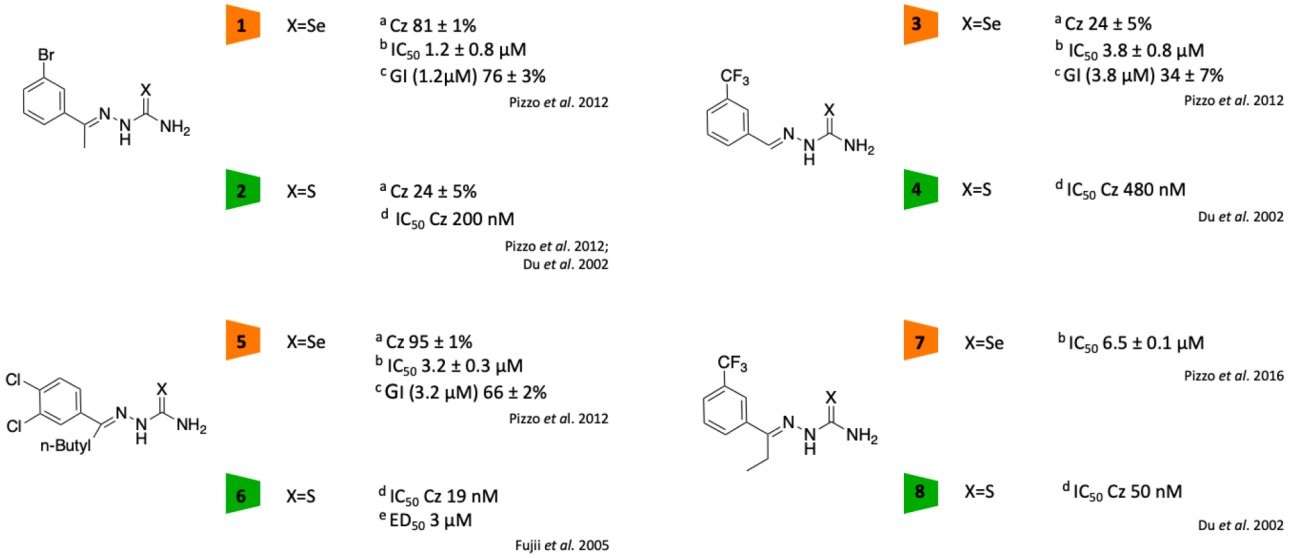


Fig. 1. Selenosemicarbazones synthesized by Pizzo et al. (2012, 2016), and thiosemicarbazones synthesized by Du et al. (2002) and Fujii et al. (2005). (a) Percentage of inhibition of Cz at 0.3 μM (treatment period: 5 min). (b) Activity was assayed in Dm28c T. cruzi epimastigotes (TcI) and expressed as IC50, the concentration that inhibits 50% of growth compared to an untreated control (treatment period: 72 h). (c) GI: Growth inhibition at the IC50 in intracellular Dm28c T. cruzi amastigotes (TcI) (treatment period: 24 h). Vero cells were used for infectivity assays. (d) Activity in Cz using 1 nM purified recombinant protein (treatment period: 5 min). (e) Activity against intracellular T. cruzi amastigotes expressed as ED50, the effective dose that kills 50% of the parasites. Human macrophages were used for infectivity assays. The results are expressed as the mean ± standard deviation of at least three independent experiments.

reactions was chalcogenation, which afforded Se- and S-containing beta-lapachone derivatives (Compounds **9-12** in Fig. 2). The anti-*T. cruzi* activity of those lapachone derivatives was evaluated in Y strain trypomastigotes. Although those compounds were not very interesting in terms of activity, Se compounds (Compounds **9, 11**) turned out to be more active than their S counterparts (Compounds **10, 12**).

As a possible mechanism of action, the authors suggested that quinones and naphthoquinones participate in oxidative processes and that both are able to generate reactive oxygen species. These compounds react with biological molecules such as DNA, lipids, and proteins, causing molecular damage.

2.3. *Thio- and selenocyanates*

The thiocyanate (SCN) moiety has already been described as an active substituent in different parasite-related diseases, such as Chagas (Rodriguez, 2016), leishmaniasis (Cottrell et al., 2004), and malaria (Nieves et al., 2016). Likewise, selenocyanates (SeCNs) have been shown antitumoral (Calvo-Martín et al., 2022), leishmanicidal (Alcolea et al., 2021), trypanocidal (Martín-Escolano et al., 2021a), and antimicrobial activity (Day et al., 2020).

In the work of Chao et al. (2017), thiocyanate WC-9 (4-phenoxyphenoxyethyl thiocyanate) (Fig. 3, Compound **14**) was chosen as the lead structure due to its role as an inhibitor of the enzyme squalene synthase (SQS) of *T. cruzi* (Urbina et al., 2003). It is a membrane-bound enzyme located in the glycosome, an exclusive organelle of trypanosomatids, and mitochondria. SQS plays an important role in *T. cruzi,* as it is responsible for the biosynthesis of sterols. As this parasite cannot use the sterol of the host cell, it has a strict requirement for specific endogenous sterols (Braga et al., 2004; Shang et al., 2014; Urbina et al., 2002). As a consequence, the inhibition of this enzyme is an interesting target for the treatment of CD (Shang et al., 2014; Veiga-Santos et al., 2015). The group of Chao et al. (2017) decided to explore whether the substitution of Se for S could offer compounds with better activity against *T. cruzi.*

This group synthesized several isosteric analogues of Compound **14** combining S and Se (Chao et al., 2017). These compounds can be divided into three groups according to their main structure: diphenyl ether derivatives (Compounds **13-16** in Fig. 3), dihydrobenzofuran derivatives (Compounds **17, 18**), and halogenated diphenyl ether derivatives (Compounds **19, 20**). Afterwards, the synthesized compounds were assayed in *T. cruzi* CL tdTomato amastigotes (TcVI), and their toxicity was evaluated in Vero cells (Fig. 3).

In every group, Se compounds were at least 45 times more active than S compounds. In addition, SeCNs were less cytotoxic to Vero cells and therefore more selective. The most striking case was derivative **15**, which was 130 times more active and 390 times more selective than its counterpart, Compound **16**. To explain this improvement in activity and selectivity, the group of Chao et al. (2017) hypothesized that Se-containing compounds might form a new S-Se bond with a cysteine residue at the binding site of the target, which is supposed to be SQS.

In a later work by Chao et al. (2019), it was demonstrated that the presence of either a Se or a S atom is fundamental for the antiparasitic activity of these compounds.

As a conclusion of the published examples, the presence of Se notably

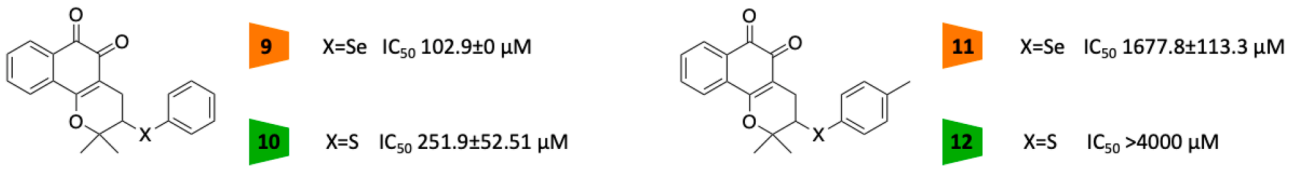


Fig. 2. Some of the compounds with Se and S that were synthesized by Jardim et al. (2015). The activity was assayed in T. cruzi Y strain trypomastigotes (TcII) and expressed as IC50, the concentration that inhibits 50% of growth compared to an untreated control. The results are expressed as the mean ± standard deviation of three independent experiments.

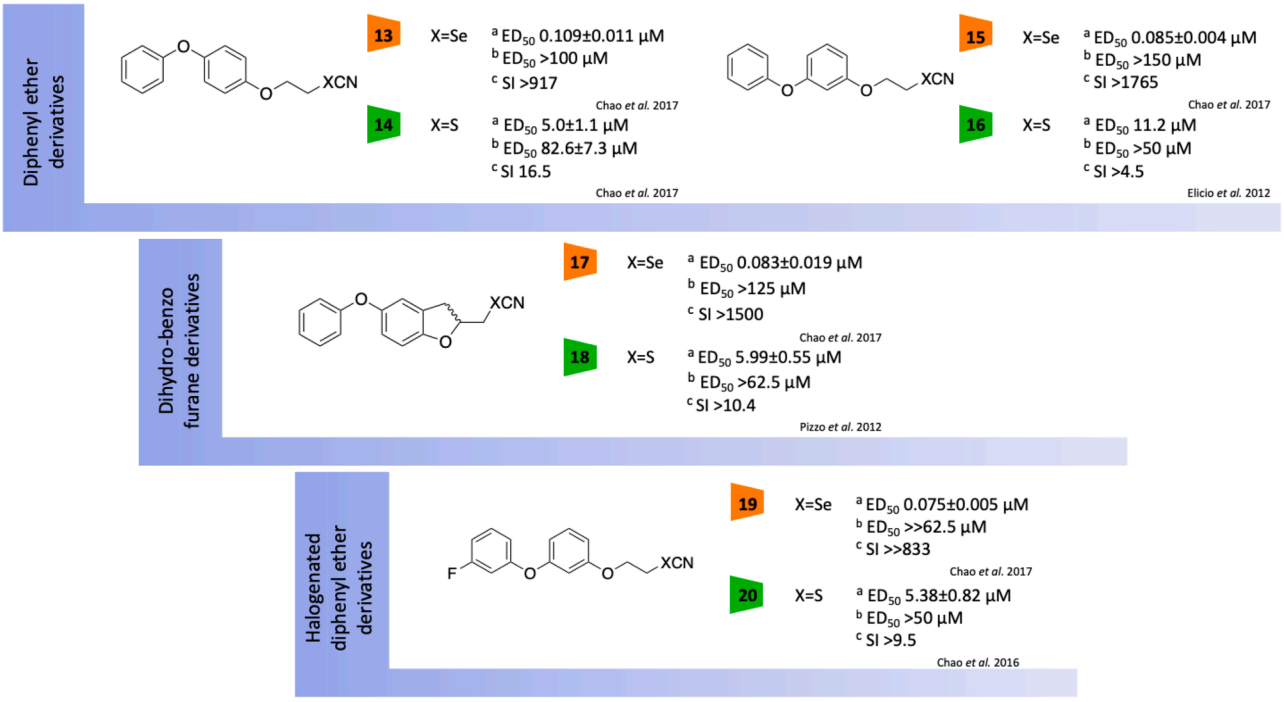


Fig. 3. Comparison of selenocyanates (Chao et al., 2017) and their thiocyanate counterparts (Chao et al., 2016; Elicio et al., 2013). a Activity was evaluated in T. cruzi CL tdTomato strain amastigotes (TcVI) infecting Vero cells. b Cytotoxicity was assayed in Vero cells. Both activity and cytotoxicity were expressed as ED50, the effective dose that kills 50% of the parasites or cells, respectively. c SI: Selectivity index, calculated as the cytotoxicity/activity ratio. The results are expressed as the mean ± standard deviation of three independent experiments.

increased the anti-*T. cruzi* activity of their S analogues in the case of cyanate derivatives. The toxicity data also improved with this substitution in these compounds. However, the effects of the isosteric replacement were not as obvious for the other described derivatives, and more studies are required to confirm the positive results.

3. Thiocompounds tested *in vivo* against *T. cruzi*

In accordance with the urgency for finding a safer and more effective treatment for CD, new molecules have been synthesized over many years. In the first part of this review, we have tried to demonstrate the interest in Se derivatives in the search for new antichagasic drugs. Bearing in mind the S/Se bioisosterism, our intention in this second part of the review is to summarize the known thioderivatives with anti-*T. cruzi* activity, as a good starting point for the synthesis of their Se analogues.

Thioderivatives have played an important role over the years in the search for a new treatment against *T. cruzi*. Among all compounds containing S, TSCs stand out because of the large number of papers addressing them in the literature. Furthermore, modifications to the TSC moiety could generate different bioisosteric heterocycles, such as thiazole (TZ), thiazolidinone (TZN) and thiadiazole (TdZ), which are bioisoters of TSC (Fig. 4) and have also shown interesting results against *T. cruzi* (Bekhit et al., 2015; Costa et al., 2016; de Oliveira Filho *et al.*, 2015). This opens a large number of possible compounds of interest for the treatment of CD.

In an exhaustive bibliographic revision, we can find numerous reviews of TSCs (Benito Scarim and Man Chin, 2019; Dos Santos Nascimento *et al.*, 2021; Leite et al., 2019; Scarim et al., 2019; Silva and Silva, 2017), TZs (Benito Scarim and Man Chin, 2019; Dos Santos Nascimento *et al.*, 2021; Leite et al., 2019; Moreno-Herrera et al., 2021; Scarim et al., 2019), TZNs (Dos Santos Nascimento *et al.*, 2021; Leite et al., 2019; Scarim et al., 2019; Silva Júnior *et al.*, 2022) and TdZs (Camargo et al., 2020) tested against *T. cruzi*. Therefore, we will focus on TSCs, TZs, TZNs and TdZs with interesting *in vitro* activities that were also tested *in vivo* against *T. cruzi* as representatives of the most important compounds to be proposed for Se replacement. Table 1 summarizes the TSCs, TZs, TZNs and TdZs derivatives with the best *in vivo* anti-*T. cruzi* activity.

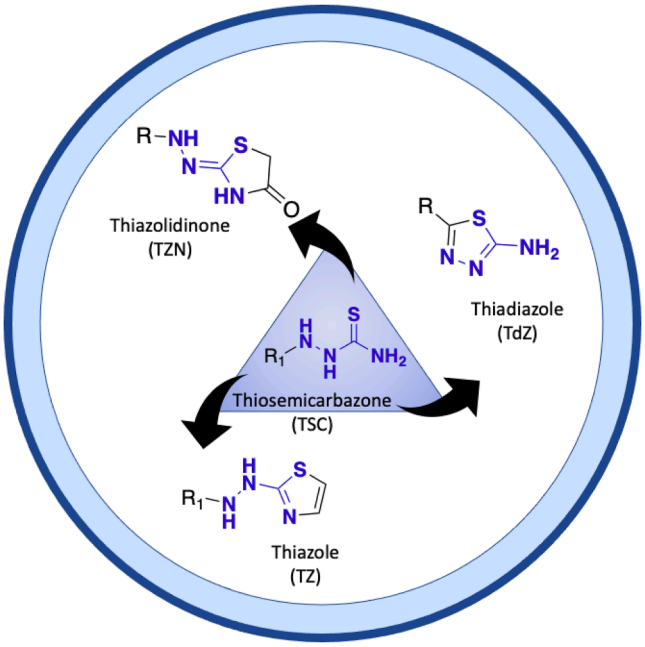
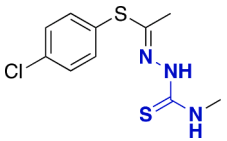
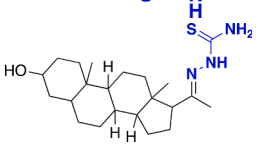
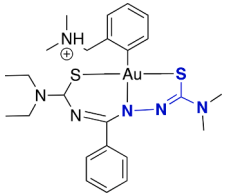
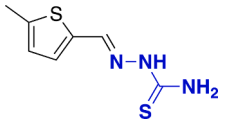
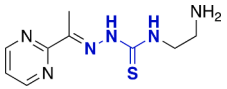
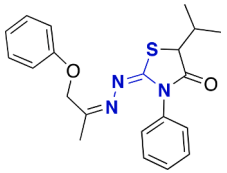
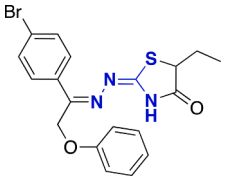


Fig. 4. Bioisosteric heterocycles derived from thiosemicarbazone.

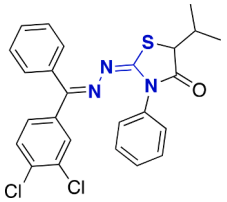
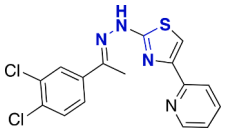
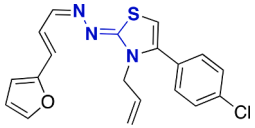
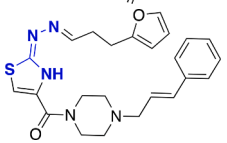
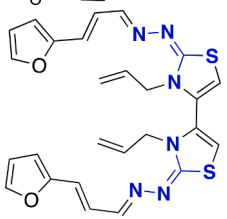
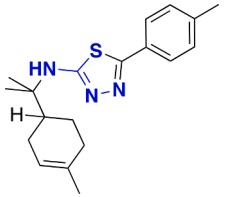
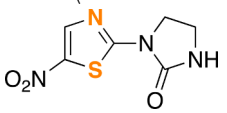
It should be noted that mice models of CD can recapitulate the acute or the chronic phase of this disease. Regarding the acute one, it is characterized by high blood parasitemia and the parasite is found in the trypomastigote form. Simultaneously, it multiplies intensively in the cytoplasm of a great variety of the host cells. Treatment is early

Table 1
Thiocompounds tested *in vivo* against T. cruzi. Compounds are organised according to structure (structures in blue are compounds derived from TSCs, orange structures are heterocycles no TSC-derived and green structures are non cyclic compounds containing S). *In vivo* murine models correspond to the acute phase of CD in all cases.

Comp.	Ref.	Cmpd	*In vitro* IC_{50} (μM)Parasite form and strain (DTU)	SI	*In vivo* Mice (Infective *T. cruzi* strain (DTU))	Dose (mg/kg/day)	Adm.	Treatment duration (start)	Results	Toxicity
21	Rodney Rodrigues de Assis et al. (2021)		ND	ND	Wild type C57BL/6 female (Y (TcII))	**21:** 2, 10 **BZ:** 10	OP/IP	9 days (8 hpi)	Nanoemulsion of **21** (10 mg/kg) was less effective than free-BZ in the reduction of parasitemia, but the survival rate was higher (100% vs. 85%).	Toxicity was not determined *in vivo*.
22	Aguilera et al. (2019)		1.2 ± 0.3 Epimastigotes Tulahuen 2 (TcVI)	42 (J774.1 macrophages/ epimastigotes Y (TcII))	Balb/c male (Y (TcII))	**22:** 50 **BZ:** 50	OP	14 days (6 dpi)	Less reduction in parasitemia than BZ (62% vs. 96%).	No toxic at micronucleus test (150 mg/kg) or in acute oral toxicity assays (>200 mg/kg).
23	Lopes et al. (2019)		3.68 ± 0.09 Amastigotes Tulahuen LacZ (TcVI) in LLC-MK2 cells (Maia et al., 2017)	30.7 (Spleen cells of swiss mice/ amastigotes Tulahuen LacZ (TcVI)) (Maia et al., 2017)	Balb/c female (Y (TcII))	**23:** 2.8 **BZ:** 2.8, 100	OP	10 days (5 dpi)	Better than BZ in parasitemia reduction (61.3% vs 0%) and survival (100% vs 0%) at the same dose.	No alterations in the levels of AST, ALT and CK-MB (2.8 mg/kg).
24	Wilson et al. (1974)		ND	ND	C_3H (Brazilian (Tc not classified))	**24:** 50	SC	5 days (3 dpi)	Significant increase in the mean survival time of infected animals, but cure was not achieved.	Toxicity was not determined *in vivo*.
25	Kinnamon et al. (1997)		ND	ND	Female albino, CF (Brazilian (Tc not classified))	**25:** 0.8125 - 104	OP	14 days (11 dpi)	Almost 50% better than NFX suppressing infection.	Toxicity was not determined *in vivo*.
26	Moreira et al. (2012)		10.1 + 0.09 Amastigotes Y (TcII)	25 (Mouse splenocyte/ amastigotes Y (TcII))	Balb/c female (Y (TcII))	**26:** 95 **BZ:** 65	OP	5 days (5 dpi)	89.6% parasitemia reduction vs. >99% of BZ	No adverse effects observed in mice.
27	Moreira et al. (2014)		5.2 ± 0.54 Amastigotes Y (TcII)	44 (Mouse splenocyte/ amastigotes Y (TcII))	Balb/c female (Y (TcII))	**27:** 55, 110 **BZ:** 65	OP	5 days (5 dpi)	Reduction of parasitemia 75.4% and 97% vs. untreated control. BZ reduction >99%. 100% survival with **27** and BZ.	Potentially hepatotoxic at 600 mg/kg (increase of ALT, amylase, and creatine).
28	de Oliveira Filho et al. (2015)		3.1 ± 0.64 Amastigotes Y (TcII) in J774 macrophages	>58 (J774 macrophages/ trypomastigotes Y (TcII))	Balb/c female (Y (TcII))	**28:** 100 **BZ:** 100	OP	5 days (5 dpi)	86.7% reduction of parasitemia at 10 dpi, vs. BZ >99% at 10 dpi. 100% survival.	No signs of toxicity were seen in mice during the experiment.

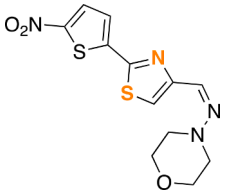
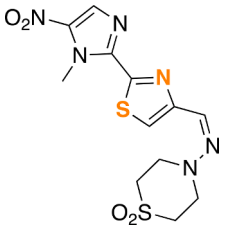
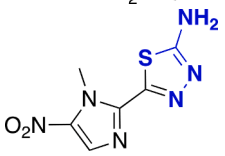
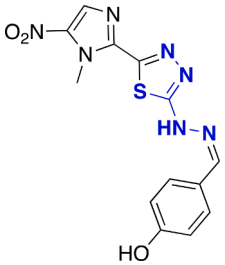
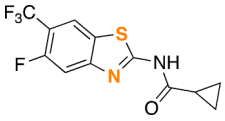
(*continued on next page*)

Table 1 (*continued*)

Comp.	Ref.	Cmpd	*In vitro* IC_{50} (μM)Parasite form and strain (DTU)	SI	*In vivo* Mice (Infective *T. cruzi* strain (DTU))	Dose (mg/kg/day)	Adm.	Treatment duration (start)	Results	Toxicity
										
29	de Oliveira Filho et al. (2017)		0.37 ± 0.1 Trypomastigotes Y (TcII)	27.19 (J774 macrophages/ trypomastigotes Y (TcII))	Balb/c female (Y (TcII))	**29:** 25 **BZ:** 100	OP	5 days (5 dpi)	54.6% reduction of parasitemia at 8 dpi, (BZ >99% from 8 dpi) 83% survival vs. 100% with BZ.	No signs of toxicity in mice
30	Álvarez et al. (2014)		<0.25 ± 0.02 Amastigotes Sylvio X-10 (TcI)	>1000 (Vero cells/ amastigotes Sylvio X-10 (TcI))	Balb/c male (CL Brener, (TcVI))	**30:** 50 **BZ:** 50 **30:** 100 **BZ:** 50	OP	14 days (6 dpi)	Both doses delayed parasitemia peak. 100% survival with all doses.	Orally acute toxicity (100 mg/kg/day) no detected neither mortality nor mutagenicity.
31	Álvarez et al. (2015b)		0.72 ± 0.08 Amastigotes Sylvio X-10 (TcI)	433 (Vero cells/ amastigotes Sylvio X-10 (TcI))	Balb/c male (Y (TcII))	**31:** 50, 100, 200 **BZ:** 50	OP	14 days (6 dpi)	Dose of 200 mg/kg similar to BZ (50 mg/kg). 100% survival at all doses.	Not mutagenic and absence of *in vivo* clastogenic effects. No well tolerated in infected mice.
32	Álvarez et al. (2015a)		1.2 ± 0.4 Amastigotes Sylvio X-10 (TcI)	403 (Vero cells/ amastigotes Sylvio X-10 (TcI))	Balb/c male (CL Brener (TcVI))	**32:** 50 **BZ:** 50	OP	14 days (7 dpi) 19 days (7 dpi; alternating treatment with resting days)	**32** reduced parasitemia, but it did not improve BZ activity. Survival rate improved, especially with the second treatment schedule (30 vs 66.6%) compared to untreated.	No mutagenic. No toxic effects were observed in mice
33	Martins et al. (2016)		1.3 ± 0.3 Amastigotes Y (TcII) in $LLCMK_2$	611.5 ($LLCMK_2$/ amastigotes Y (TcII))	Female Balb/c (Y (TcII))	**33:** 100 **BZ:** 100 **Combination 33+BZ:** 5 each	OP	20 days (1 dpi)	Parasitemia lower with BZ alone than **33** or combination. Lower mortality with combination or BZ alone, compared to **33** alone (35% vs. 80%).	Toxicity was not determined *in vivo*.
34	Velásquez Antich (1969)		ND	ND	Mice strain not specified (Y (TcII))	**34:** 250 (x 3 days) and 125 (x 4 days) **34:** 140	OP	7 days (1 dpi) 14 days	140 mg/kg/day delayed the presence of parasitemia (day 21 vs. day 14). Survival rate improved compared to untreated.	Toxicity was not determined *in vivo*.

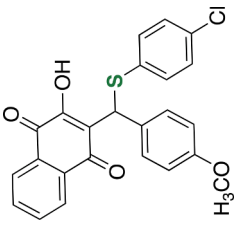
(*continued on next page*)

Table 1 (*continued*)

Comp.	Ref.	Cmpd	*In vitro* IC$_{50}$ (μM)Parasite form and strain (DTU)	SI	*In vivo* Mice (Infective *T. cruzi* strain (DTU))	Dose (mg/kg/day)	Adm.	Treatment duration (start)	Results	Toxicity
35	Verge and Roffey (1975)		ND	ND	CFW (*T. cruzi* strain not specified)	**35:** 25-200 **NFX:** 25-200 **35:** 200-500 **NFX:** 200-500	IP OP	5 days (4 hpi)	IP **35** cured the mice at doses > 100 vs. NFX at all doses. Orally, **34** and NFX cured all mice.	Acute toxicity (LD$_{50}$) >800 mg/kg for IP and >1600 mg/kg for OP.
36	Neville and Verge (1977)		ND	ND	CFW (BH (Tc no classified)) CFW (Y (TcII), Perú (TcII), BHC/10 (Tc not classified))	**36:** 5-100 **NFX:** 5-100 **36:** 5-200 **NFX:** 5-200 **36:** 5-25 **NFX:** 5-25	IP OP OP	5 days (4 hpi) 5 days (4 hpi) 10 days	All mice treated with **36** were cured except animals treated with 5 mg/kg IP and 10, 5 mg/kg OP. **36** was better compared to NFX.	Acute toxicity (LD$_{50}$) IP >800 mg/kg and OP >1600 mg/kg.
37	Filardi and Brener (1982)		ND	ND	Male albino (Y (TcII) and Colombian (TcI))	**37:** 25-100 (Y (TcII)) **NFX:** 50 (Y (TcII)) **37:** 50-100 (Colombian (TcI)) **NFX:** 50 (Colombian (TcI))	OP	20 days (1 dpi)	More effective than NFX against both strains.	Genotoxic and cytotoxic. It was determined *in vivo* by PCEN/NCE ratio and micronuclei assay (Nesslany et al., 2004).
38	Salomão et al. (2010)		2.9 ± 0.6 Amastigotes Y in macrophages	>41 (Macrophages Swiss mice/ amastigotes Y (TcII))	Male albino Swiss (Y (TcII))	**38:** 200 **MZ:** 200 **38:** 100 **MZ:** 50	OP	1 day (5 dpi) 10 days (6 dpi) 10 days (5 dpi)	At 200 mg/kg, parasitemia decreased 50% and mortality was 0%. MZ led to undetectable levels of parasitemia.	A single dose of 400 mg/kg increased GOT 1.3 times compared to untreated. No alteration of GPT or urea.
39	Fleau et al. (2019)		0.079 ± ND Amastigotes CL tdTomato (TcVI)	>630 (Cardiomyocytes H9c2/ amastigotes CL tdTomato (TcVI))	C57BL/6 female (CL tdTomato (TcVI))	**39:** 50 **BZ:** 50	OP	1 day (2 dpi)	A single dose controlled the parasitemia at the site of infection, but it was less effective than BZ	Toxicity was not determined *in vivo*.

(*continued on next page*)

Table 1 (*continued*)

Comp.	Ref.	Cmpd	*In vitro* IC_{50} (μM)Parasite form and strain (DTU)	SI	*In vivo* Mice (Infective *T. cruzi* strain (DTU))	Dose (mg/kg/day)	Adm.	Treatment duration (start)	Results	Toxicity
40	Lara et al. (2021)		6.7 ± 1.8 Amastigotes Dm28c-Luc (Tcl)	14.9 (Vero cells/ amastigotes Dm28c-Luc (Tcl))	Male Swiss Webster (Y (TclI))	**40:** 50 **BZ:** 100	IP OP	5 days (5 dpi)	Parasitemia levels similar to untreated. None of the mice survived.	No adverse effects observed at the cumulative dose of 250 mg/kg. At 500 mg/kg one mouse died after IP administration.

IC_{50}: the concentration that inhibits 50% of the parasite. SI: selectivity index, calculated as a rate between IC_{50} of cell type /IC_{50} in parasite form.
BZ: Benznidazole. NFX: Nifurtimox. ND: Not determined; dpi: days postinfection; hpi: hours postinfection. Adm.: type of administration oral pathway (OP), intraperitoneally (IP) or subcutaneous (SC) pathway. BMM: bone marrow macrophages. MLC: minimum lethal concentration. Aspartate aminotransferase (AST), alanine aminotransferase (ALT) and creatine kinase-MB (CK-Mb). PCE: polychromatic erythrocytes; NCE: normochromatic erythrocytes.

administered after mice infection and the parasitemia peak should be achieved in the first month after inoculation. Symptoms such as anorexia, weight loss and histopathological lesions in the heart and digestive tract, as well as the nervous system associated with these organs, should be evaluated. Also, parasitemia and survival rate are assessed as endpoints. Additionally, mice can be immunosuppressed to determine parasite relapse (de Lana, 2017; Fonseca-Berzal et al., 2018), although hemoculture and PCR can be also used for this purpose. On the contrary, the chronic phase is characterized by low blood parasitemia because the parasite is in the amastigote form inside the cells of different tissues where it multiplies slowly. In this model, mice are treated approximately after three to four months of infection. The beginning of the chronic phase depends on the mice lineage and parasite strain combination. Heart damage is typical from this phase, though its intensity also depends on the parasite strain. In this phase, immunosuppression may be required to assess treatment efficacy because the hemoculture and PCR in blood eluate, usually used for this purpose, may not detect the infection (de Lana, 2017; Fonseca-Berzal et al., 2018). Notably, all the papers in this review analyzed the efficacy of the new compounds in the acute phase.

As mentioned above, mice lineage/parasite strain combination is an important factor to consider when comparing the results of Table 1 as it notably affects the immunity response, parasite load and parasite tropism and the response to treatment (Mateus et al., 2019; Zingales et al., 2014). It has been shown that the same mice lineage has different parasitemia load depending on the strain of *T. cruzi* used for infection (Cruz et al., 2015). Similarly, strains of the same DTU inoculated in different mice lineage cause different parasitemia levels (León et al., 2017). Furthermore, the symptoms and histopathological lesions in the chronic phase are different depending on *T. cruzi* strain and mice lineage (Andersson et al., 2003).

3.1. Thiosemicarbazones

The most recent work (Rodney Rodrigues de Assis *et al.*, 2021) included the synthesis of phenyl-TSC derivatives with a spacer between the aromatic ring and the TSC moiety. After *in vitro* assays of amastigote replication and trypomastigote liberation, two compounds were selected for *in vivo* experiments. Compound **21**, the most active compound, decreased parasitemia in mice when administered orally or intraperitoneally. Thus, Compound **21** and BZ were tested in a nanoemulsion. In this formulation, Compound **21** delayed the peak of parasitemia compared to untreated animals, but it was less effective than a BZ nanoemulsion. Nevertheless, the survival rate with Compound **21** was higher than BZ (Rodney Rodrigues de Assis *et al.*, 2021).

In 2019, Aguilera et al. (2019) showed the synthesis of steroidal TSCs inspired by natural compounds with antimicrobial and antiparasitic activity. Compound **22** was tested *in vivo* because of its interesting activity *in vitro*. It did not show acute toxicity in mice (>200 mg/kg), and it reduced parasitemia, but less so than BZ at the same dose (62% vs. 96%) (Aguilera et al., 2019).

In the same year, Lopes et al. (2019) tested *in vivo* Compound **23**, a gold complex . It was 3 times more effective than BZ in killing trypomastigotes, and it is a more potent agent against amastigote forms of *T. cruzi in vitro*. *In vivo*, Compound **23** showed greater activity than BZ reducing tissue parasitism at a very low dose in addition to protecting the liver and heart from tissue damage, leading to the survival of 100% of the compound **23**-treated mice during the acute phase.

Formerly, other TSC compounds (Compounds **24, 25**) were also tested in mice with different results. TSCs synthesised by Wilson et al. (1974) (Compound **24**) did not cure the infection *in vivo,* and all mice died (Wilson et al., 1974). On the other hand, Compound **25** synthesised by Kinnamon et al. (1997) was two times more potent than NFX against trypomastigotes.

3.2. Thiazolidinones

TZNs are saturated five-membered rings with a carbonyl group at C2, C4 or C5. This heterocycle has shown a wide range of biological activities, such as antitubercular, antimicrobial, anti-inflammatory, antiviral and antidiabetic activities (Nirwan et al., 2019).

In 2012, the group of Moreira et al. (2012) published a SAR study of 2-iminothiazolindin-4-one derivatives on *T. cruzi*; Compound **26** was evaluated *in vivo* because of its good activity and safety *in vitro*. Although it did not completely eradicate the infection in the acute phase, it reduced parasitemia by 89.6% compared to the untreated group. Nevertheless, it was less effective than BZ (>99%) (Moreira et al., 2012).

Two years later, Moreira et al. (2014) used Compound **26** as the starting point for the synthesis of a new series of TZNs. The new compounds had a T-shape, which gave structural restriction and was expected to improve activity. TZN **27** had similar potency to BZ *in vitro* against *T. cruzi* infection and thus was tested *in vivo*. Parasitemia was significantly reduced at all tested doses of Compound **27** compared to the untreated group. Notwithstanding, parasitemia reduction after the highest dose of Compound **27** (110 mg/kg) was very similar to parasitemia after 65 mg/kg of BZ (97% vs. >99%). Although all animals survived and no toxic effects were observed during the experiment, the acute oral toxicity of Compound **27** was assayed. This experiment showed that this compound could be potentially hepatotoxic at 600 mg/kg due to an increase in ALT, amylase and creatinine (Moreira et al., 2014).

de Oliveira Filho et al. (2015) modified one of the most potent TSCs against Cz identified in the literature (3,4-dichlorophenyl thiosemicarbazone) into its bioisoster 4-TZN. With this change, they improved the druggability of TSC (reducing their toxicity) while the antiparasitic activity was maintained. Among all synthesized compounds, Compound **28** was the most selective against trypomastigotes and more active than BZ *in vitro*. Its activity was then evaluated *in vivo*. After treatment with Compound **28** (100 mg/kg), parasitemia was reduced by 86.7% and 97.1% on days 10 and 12 postinfection, respectively, while BZ reduced parasitemia >99% from day 10 postinfection (de Oliveira Filho *et al.*, 2015).

3.3. Thiazoles

Using the aforementioned strategy, de Oliveira Filho et al. (2017) modified 4-TZN into a TZ ring. They used Compound **28** as a starting point for the design of new derivatives. Compound **29** was assayed against intracellular amastigotes and tested in combination with BZ against trypomastigotes, showing a synergistic effect. *In vivo*, the maximum reduction in parasitemia after treatment with Compound **29** was 64.1% on day 12 postinfection, whereas BZ reduced parasitemia >99% from day 8 postinfection. It should be noted that the dose of Compound **29** was a quarter of the dose of BZ (25 vs. 100 mg/kg) (de Oliveira Filho *et al.*, 2017).

The group of Álvarez et al. (2014) synthesized a large group of molecules. The design of these new compounds provides insight into nitro derivatives, with NFX as the reference for structural modifications. Some of these compounds were TSC, TZN and TZ derivatives. From the series of TZs, two compounds attracted attention due to their results in epimastigote and amastigote assays. These compounds, which differed in the radical at position 4 of the TZ ring (4-chlorophenyl and 2-naphthalene), were tested *in vivo*. Compound **30** (4-clorophenyl) stood out because it was able to decrease parasitemia compared to untreated mice. Nevertheless, it was not better than BZ. It must be mentioned that different vehicles for administration and doses (50 and 100 mg/kg) were tested with Compound **30** (Álvarez et al., 2014).

The aforementioned Compound **30** showed solubility problems. Therefore, Álvarez et al. (2015b) synthesized Compound **31** as an improved derivative in terms of solubility. It was tested *in vivo* under conditions similar to those of Compound **30**. Parasitemia levels of both compounds were compared at 50 and 100 mg/kg and Compound **31** turned out to be 1.5 to 4.5 more active than Compound **30**. An extra dose of 200 mg/kg of Compound **31** was administered and showed similar results to BZ (50 mg/kg) in terms of parasitemia levels. Moreover, the highest dose of Compound **31** (200 mg/kg) was not toxic in healthy animals, although it was not well tolerated in infected mice (Álvarez et al., 2015b).

Furthermore, using Compounds **30** and **31** as a starting point, the same group of researchers (Álvarez et al., 2015a) synthesized the bis-thiazole **32**, which showed interesting activity *in vivo*. Bis-thiazole was designed to increase the activity against the target triosephosphate isomerase of *T. cruzi*. This enzyme is a homodimer that belongs to the glycolysis pathway and shows differences between the parasite and human forms, allowing the selective design of molecules for the parasite (Aguirre et al., 2014; Álvarez et al., 2015a; Téllez-Valencia et al., 2004). *In vitro* assays showed that Compound **32** was equipotent to the reference drugs (BZ, NFX). Furthermore, it was 1.3 and 2 times more selective than BZ and NFX, respectively (Álvarez et al., 2015a). In *in vivo* assays, Compound **32** significantly reduced parasitemia levels compared to the untreated group, but it did not improve BZ activity. As a result of a good biological profile, more experiments were performed by changing the dose (75 and 100 mg/kg) and treatment schedule (combining administration with resting days). With the highest dose (100 mg/kg) and the second schedule administration, an increase in survival rate (66.6%) compared to the untreated group was observed. Unfortunately, no comparison to BZ was done this time (Álvarez et al., 2015a).

3.4. Thiadiazoles

One step further is the cyclization of TSCs to form TdZ derivatives (Fig. 4). Martins *et al.* tested *in vivo* Compound **33**, a 1,3,4-thiadiazole (Martins et al., 2016). Compound **33** was assayed alone (100 mg/kg) and in combination with BZ (5 mg/kg each). Although both treatments decreased parasitemia, the combination therapy was better for the survival rate (65% vs. 20%). Notwithstanding, BZ alone achieved the lowest levels of parasitemia, and the survival rate was the same as that of the combination therapy (65%).

3.5. Other heterocycles with sulfur not TSC-derived

Some thiazoles not derived from TSCs were also tested *in vivo* against *T. cruzi* in the 1970s (Compounds **34**-**36**) (Neville and Verge, 1977; Velásquez Antich, 1969; Verge and Roffey, 1975). Most of them were nitroheterocycles. In the 1980s, a nitroimidazole-thiadiazole derivative, currently known as Megazol (MZ, Compound **37**), was assayed in an acute model of CD for the first time. Interestingly, no resistance of treated parasites was detected at 50 mg/kg in the *T. cruzi* Y strain (TcII) (Filardi and Brener, 1982). Although MZ (Compound **37**) eradicated mouse infection caused by Colombian (TcI) and Y strains and was more active than NFX (Filardi and Brener, 1982), it was discarded because of its *in vitro* mutagenicity and genotoxicity (Ferreira and Ferreira, 1986; Nesslany et al., 2004; Poli et al., 2002).

Nevertheless, MZ was explored as the lead compound for the synthesis of new molecules due to its potent activity (Chauvière et al., 2003; da Silva *et al.*, 2013; Salomão et al., 2010; Santiago et al., 2020). In 2010, the group of Salomão et al. (2010) synthesized a new series of compounds using molecular hybridization between MZ and guanylhydrazones. Eight 1,3,4-thiadiazoles were tested *in vivo* at the same dose (200 mg/kg). Afterwards, three compounds were selected for more studies, but no encouraging results were found. Compound **38** and its 4-bromo derivative were evaluated *in vivo* at different doses (50, 100 or 200 mg/kg). Neither of the two compounds showed more activity *in vivo* or less toxicity than MZ, the reference drug in this assay (Salomão et al., 2010).

In 2019, Fleau et al. (2019) performed high-throughput screening, and an acylaminobenzothiazole derivative was selected as the lead

compound. Different modifications were made, and as a result, compound **39** was synthesized and tested *in vivo*. In this case, the efficacy was evaluated by the parasitemia load at the site of injection compared to BZ and the untreated group. Compound **39** controlled the parasitemia load, although it was less effective than BZ (Fleau et al., 2019).

At this moment, in the clinical portfolio of Chagas there are no new molecules in clinical trials, but the study of new regimens of BNZ, NFX, and studies with repositioning drugs are ongoing (DNDi, 2022c). In this line, known antifungal drugs containing TZ have been assayed in *T. cruzi,* and some of them have been included in clinical trials since they have shown interesting results *in vivo*, such as ravuconazole and its prodrug fosravuconazole (Clinical Trials.gov, 2022; DNDi, 2022a; Santos et al., 2020) (Fig. 5).

Fosravuconazole, also known as E1224, was discarded as monotherapy after the results of the clinical trial due to low efficacy. However, the possibility of combination therapy with BZ was proposed (Torrico et al., 2018).

Another TZ that has been combined with BZ was levamisole (Fig. 5). It was tested *in vivo* (levamisole 0.5 + BZ 10 mg/kg), and the coadministration was 5.5 times more effective than BZ alone (100 mg/kg) in reducing parasitemia, although animal survival was compromised (Rocha Simões-Silva *et al.*, 2019).

Finally, nitazoxanide (Fig. 5) was developed as a broad-spectrum antiparasitic drug, mainly used for cryptosporidiosis or giardiasis (Valle-Reyes et al., 2017), and it was also used as an antiviral (Rossignol, 2014). As an antiparasitic drug, its activity against CD was studied *in vitro* (Chan-Bacab et al., 2009) and later *in vivo* (Valle-Reyes et al., 2017). After *in vivo* assays, this compound was discarded due to the increase in parasitemia and tissue damage in animals treated with nitazoxanide (Valle-Reyes et al., 2017).

3.6. Other noncyclic compounds containing sulfur

In addition, other types of S-containing compounds have been tested *in vitro* and *in vivo*. Although they have been less explored, the synthesis of their Se analogues could also be of interest.

Thioethers, also known as sulfides, have been mainly tested *in vitro* against *T. cruzi* (Eberle et al., 2009; Eberle et al., 2011; Parveen et al., 2005). These compounds are supposed to target trypanothione reductase, and the assays have been only performed against this enzyme and not the complete parasite (Stump et al., 2008).

Moreover, thioethers have been combined with other scaffolds, such as benzothiazole (Avila-Sorrosa et al., 2019), biphosphonic acid (Recher et al., 2013) and quinone (Cardoso et al., 2015). As mentioned above, the quinones are an interesting chemical group to investigate in regard to CD. Recently, a naphthoquinone (Compound **40**) and its parent compound (without the methoxy group (Lara et al., 2018), were tested *in vivo*. None of them was able to reduce parasitemia or protect against mortality. However, all mice survived the experiment, and parasitemia was undetectable after treatment with BZ, despite BZ being twice the dose of Compound **40**. Moreover, BZ administration was oral, whereas Compound **40** was administered IP; therefore, direct comparison is not possible (Lara et al., 2021).

Finally, other compounds containing S can be found in the literature, such as thioureas (Du et al., 2000; Plutín et al., 2017), SCNs (Chao et al., 2015; Chao et al., 2017; Elhalem et al., 2002; Liñares et al., 2007; Szajnman et al., 2000) and isothiocyanates (Bernardes et al., 2000; Haraguchi et al., 2011). They have been tested only *in vitro* against CD. Therefore, they are structures to take into account for future studies and modifications, and they should be considered for a possible replacement of S by Se.

Considering all the studies included in *Table 1*, we can conclude that Compounds **23** and **25** were able to improve the activity observed for BZ and NFX, respectively, in *in vivo* studies. In addition, Compounds **35** and **36** were comparable to NFX, and Compounds **26** and **28** showed a reduction in parasitemia similar to BZ. Therefore, these compounds are especially interesting to take into consideration for Se replacement. In order to evaluate the suitability of these S compounds to become drugs for the treatment of CD, they should be compared with the ideal Target Product Profile (TPP). Unfortunately, current available information related to these compounds is not enough to completely evaluate their fulfillment of the ideal TPP. Some points such as their effects on the chronic phase, interactions with other drugs, contraindications or effects on geographic distribution have not been evaluated. Nevertheless, their synthesis is not complicated nor expensive and most of compounds in *Table 1* were orally administered to mice, which means a promising starting point.

It should also be emphasized that all the *in vivo* assays mentioned in this review were performed in murine models of the acute phase of CD, and no studies in the chronic phase were performed. More research into the chronic phase is required since current chemotherapy reduces its efficacy in that stage. Particularly in the treatment of human CD, the cure of the chronic phase remains a challenge for the scientific community. In view of the positive results of Se supplementation in chronic Chagas cardiomyopathy, we encourage the introduction of Se in the treatment of CD and the substitution of Se for S to develop new molecules for this devastating disease.

4. Conclusions

Although the substitution of S by Se has been shown to be a successful strategy in other diseases, this fact cannot be effectively confirmed in CD with current information. Available comparisons are scarce up to now, and most of them have been carried out under different conditions. Our hypothesis is supported by the direct comparison of SeCN and SCN, where Se notably increased the activity and selectivity *in vitro*. In addition, the positive results of Se supplementation in the clinical trial performed in patients with chronic chagasic cardiomyopathy reinforce the idea that this isosteric replacement could be a very interesting strategy for the development of new anti-chagasic drugs.

Despite the many attempts that have been made in the search for a definitive treatment for CD, we have seen in this review that overall, researchers have had very limited success. Moreover, the lack of

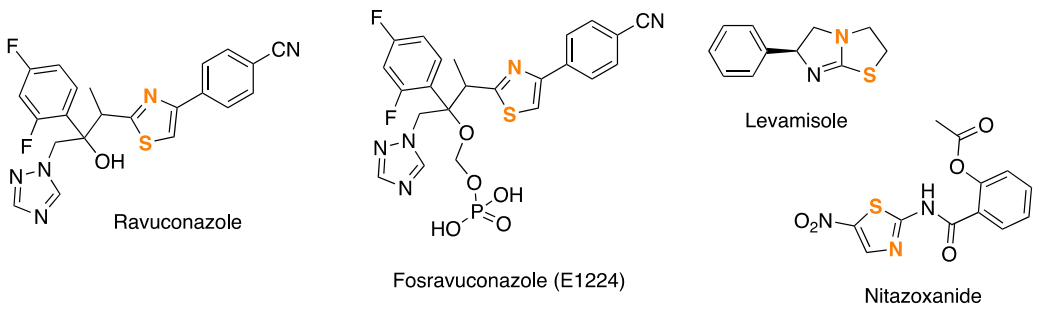


Fig. 5. Repositioning drugs: known azoles tested against T. cruzi.

consensus in *in vivo* models and the variability in CD outcomes depending on the selected mice lineage, inoculum, age of the animal, route of administration, time of infection when treated, treatment duration, the dose of the compound used, besides the phase of the infection and *T. cruzi* strains makes it difficult to directly compare the results shown in Table 1. The age of the animal is more important than the genetic lineage of the mice when the same *T. cruzi* strain is used because is related to immune resistance to infection. However, the *T. cruzi* strain and its resistance/susceptibility to treatment seems to be the most important factor in chemotherapy results. Another bottleneck in Chagas research is the lack of translation from *in vitro* data to *in vivo* model and clinical outcomes, which is a clear cause of concern. Moreover, there are not clinical trials on going with new molecules.

Nevertheless, we can conclude that some TSCs, TZNs, TZs and other S heterocycles have shown similar or even higher efficacy than the reference drugs *in vivo*. Therefore, we propose different S-containing molecules as interesting options for Se replacement. The benefits offered by Se, taking advantage of S derivatives with interesting activity *in vivo*, can lead to the synthesis of novel selenoderivative structures with interesting projections as future antichagasic drugs.

This review once again highlights the difficulties in finding a cure for this devastating disease. To date, only repositioned drugs have been evaluated in clinical trials. Efforts should be made to consider a higher structural variability of compounds in the early stages of the drug discovery process.

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Mercedes Rubio-Hernández: Conceptualization, Writing – original draft, Writing – review & editing. **Verónica Alcolea:** Supervision, Writing – review & editing. **Silvia Pérez-Silanes:** Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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