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Structural design, synthesis and pharmacological evaluation of 4-thiazolidinones against *Trypanosoma cruzi*

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ABSTRACT

Chagas disease is an infection caused by protozoan *Trypanosoma cruzi*, which affects approximately 8–10 million people worldwide. Benznidazole is the only drug approved for treatment during the acute and asymptomatic chronic phases of Chagas disease; however, it has poor efficacy during the symptomatic chronic phase. Therefore, the development of new pharmaceuticals is needed. Here, we employed the bioisosterism to modify a potent antiparasitic and cruzain-inhibitor aryl thiosemicarbazone (**4**) into 4-thiazolidinones (**7**–**21**). Compounds (**7**–**21**) were prepared by using a straightforward synthesis and enabled good to excellent yields. As a chemical elucidation tool, X-ray diffraction of compound (**10**) revealed the geometry and conformation of this class compounds. The screening against cruzain showed that 4-thiazolidinones were less active than thiosemicarbazone (**4**). However, the antiparasitic activity in Y strain trypomastigotes and host cell cytotoxicity in J774 macrophages revealed that compounds (**10** and **18**–**21**) are stronger and more selective antiparasitic agents than thiosemicarbazone (**4**). Specifically, compounds (**18**–**20**), which carry a phenyl at position N3 of heterocyclic ring, were the most active ones, suggesting that this is a structural determinant for activity. In infected macrophages, compounds (**18**–**20**) reduced intracellular amastigotes, whereas Benznidazole did not. In *T. cruzi*-infected mice treated orally with 100 mg/kg of compound (**20**), a decreased of parasitemia was observed. In conclusion, we demonstrated that the conversation of thiosemicarbazones into 4-thiazolidinones retains pharmacological property while enhances selectivity.

1. Introduction

It is estimated that 5–10% of the Latin American population has Chagas disease, which is caused by the protozoan *Trypanosoma cruzi*.[1] This situation is alarming, because there are no vaccines available and the current treatment using Benznidazole is of low efficacy and followed by several side effects.[2,3] Benznidazole has antiparasitic effects against bloodstream parasites, but its activity is limited against parasites in tissues.[4,5] Additionally, many patients experience drug intolerance and adverse effects.[6] Thus, new drugs to treat Chagas disease are necessary.

To address this need, a number of molecular targets for anti-*T. cruzi* drugs have been investigated, increasing the quality of drug identification for Chagas disease treatment. Examples of such targets are the lanosterol 14 α-demethylase,[7] trypanothione reductase,[8] cruzain,[9] *trans*-sialidase,[10] phosphatidylinositol 3-kinase[11] and cytochrome *b*.[12] By using structure-based drug design, two small-molecules were developed and are considered strong drug candidates: **K11777**, a vinyl sulfone peptide that inhibits cruzain,[13] and **VNI**, an oxadiazole derivative inhibitor of 14-α-demethylase activity.[14,15]

More recently, compound **VFV** reveals a broader antiprotozoal spectrum of action. It has stronger antiparasitic activity in cellular

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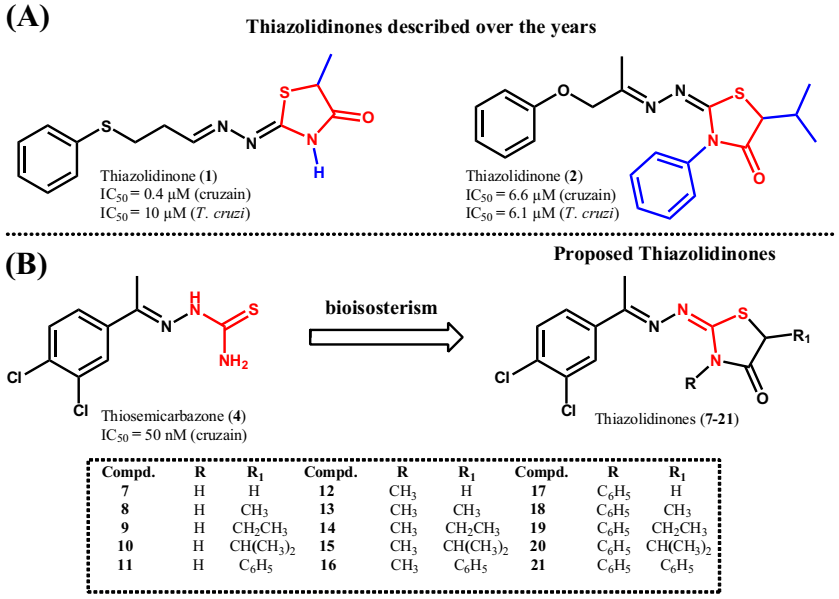


Compd.	R	R_1	Compd.	R	R_1	Compd.	R	R_1
7	H	H	**12**	CH_3	H	**17**	C_6H_5	H
8	H	CH_3	**13**	CH_3	CH_3	**18**	C_6H_5	CH_3
9	H	CH_2CH_3	**14**	CH_3	CH_2CH_3	**19**	C_6H_5	CH_2CH_3
10	H	$CH(CH_3)_2$	**15**	CH_3	$CH(CH_3)_2$	**20**	C_6H_5	$CH(CH_3)_2$
11	H	C_6H_5	**16**	CH_3	C_6H_5	**21**	C_6H_5	C_6H_5

Figure 1. Bioisosterism-based design of novel thiazolidinones as potential anti-parasitic compounds. (A) Thiazolidinones described over the years (compounds **1**[35] and **2**[38] were previously investigated), (B) thiazolidinones proposed here.

experiments, and cures the experimental Chagas disease with 100% of efficacy, and suppresses visceral leishmaniasis by 89% (vs 60% for **VNI**).[16] These compounds are likely to enter into clinical trials; however, the chances of any drug being approved in the clinical stage are very low. Given the outcomes observed for **K11777**, **VNI** and **VFV** as anti-*T. cruzi* agents, the design of compounds to inhibit cruzain and the 14-α-demethylase activity have received special attention.[17,18]

Additionally, with the goal of avoiding the development of resistant *T. cruzi* strains, an enhanced of the number of potential drug candidates by combined drug therapy is necessary.[19,20]

Thiosemicarbazones have been largely investigated as anti-*T. cruzi* agents.[21–27] Among this class, the 3,4-dichlorophenyl thiosemicarbazone (**4**) was identified as one of the most potent cruzain inhibitor. The structure–activity relationships studies identified that the 3,4-dichlorophenyl is an important structural determinant for trypanocidal activity.[28]

Due to the poor drugability of thiosemicarbazones, many attempts have been made to identify thiosemicarbazone-like compounds with improved antiparasitic effects.[29–34] Following this line of research, our research group demonstrated an existing bioisosteric relationship between thiosemicarbazones and thiazolidinones, which led to the identification of strong antiparasitic thiazolidinones.[35–38]

Based on these findings, here new thiazolidinones (**7**–**21**) were planned by employing the thiosemicarbazone (**4**) as a structural prototype (Fig. 1). We synthesized compounds (**7**–**21**) and evaluated their activity against cruzain and *T. cruzi*. The choice of substituents attached in compounds (**7**–**21**) was oriented by observing previously described structure–activity relationships.[38]

2. Results

2.1. Synthesis and chemical characterization

The general route to preparing thiazolidinones is shown in Scheme 1. Firstly, thiosemicarbazones (**4**–**6**) were prepared by reacting commercially available 3′,4′-dichloroacetophenone (**3**) with the appropriate thiosemicarbazide in an ultrasound bath in the presence of catalytic H_2SO_4 or HCl (thiosemicarbazone **6**). Thiazolidinones (**7**–**21**) were prepared by reacting the respective aryl thiosemicarbazone with commercially available ethyl 2-bromoacetate or by preparing the desired 2-substituted-2-bromoacetates. These reactions were carried out in the presence of an excess of anhydrous NaOAc under reflux. This afforded compounds (**7**–**21**) in variable yields (38–84%) and acceptable purity (>95%).

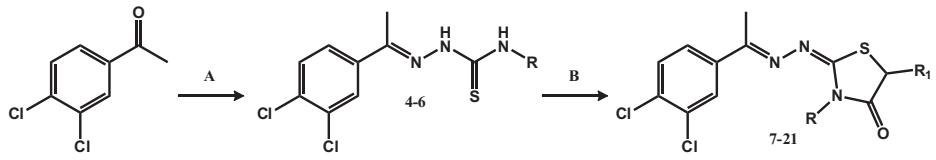
Structures of compounds were determined by nuclear magnetic resonance (NMR, ^1H and ^{13}C), infrared (IR) and mass (HR-MS) spectral, while purity was determined by elemental analysis (E.A.). To define the relative configuration of the iminic bond, we executed X-ray diffraction of single crystals of compound (**10**). As observed in Figure 2, this compound adopts an *E*-geometry. In addition, it was observed that iminic bond in C1 is located in an exocyclic position in regard to the heterocyclic ring.

2.2. Pharmacological evaluation

We assessed the host cell cytotoxicity in J774 macrophages, while the in vitro anti-*T. cruzi* activity was determined against bloodstream trypomastigotes of Y strain (Table 1). Compounds that showed IC_{50} values comparable to Benznidazole were considered active.

The inhibitory activity for thiazolidinones (**5**–**19**) against cruzain was also investigated. We measured cruzain mediated enzymatic activity inhibition by using an assay based on competition with the substrate Z-Phe-Arg-aminomethylcoumarin (Z-FR-AMC). Compounds were screened at 100 μM, the maximum concentration at which they were soluble in the assay buffer. However, cruzain inhibition by these compounds was not observed (Table S1).[39]

The structure–activity relationship study began with two modifications on 4-thiazolidinone ring: (a) substituents at C5 position and (b) attachment of a methyl or phenyl group at nitrogen N3. These modifications yielded compounds (**7**–**21**). Among the tested compounds, compound (**20**) was the most potent antiparasitic, with an IC_{50} value of 1.7 ± 0.17 μM for bloodstream trypomastigotes. This compound presented better antiparasitic properties than Benznidazole. Importantly, this did not affect host cells viability in concentrations up to 100 μM.



Tiosemicarbazones 4-6

Compd.	R
4	H
5	CH_3
6	C_6H_5

Thiazolidinones 7-21

Compd.	R	R_1	Compd.	R	R_1	Compd.	R	R_1
7	H	H	12	CH_3	H	17	C_6H_5	H
8	H	CH_3	13	CH_3	CH_3	18	C_6H_5	CH_3
9	H	CH_2CH_3	14	CH_3	CH_2CH_3	19	C_6H_5	CH_2CH_3
10	H	$CH(CH_3)_2$	15	CH_3	$CH(CH_3)_2$	20	C_6H_5	$CH(CH_3)_2$
11	H	C_6H_5	16	CH_3	C_6H_5	21	C_6H_5	C_6H_5

Scheme 1. Synthetic procedures for thiazolidinones (**7**–**21**). Reagents and conditions: (A) thiosemicarbazides, EtOH, H_2SO_4, or HCl, ultrasound bath, rt, 1–2 h, yields of 80–89%; (B) ethyl 2-bromoacetate or ethyl 2-substituted-2-bromoacetates; NaOAc, EtOH, reflux, overnight, yields of 38–84%.

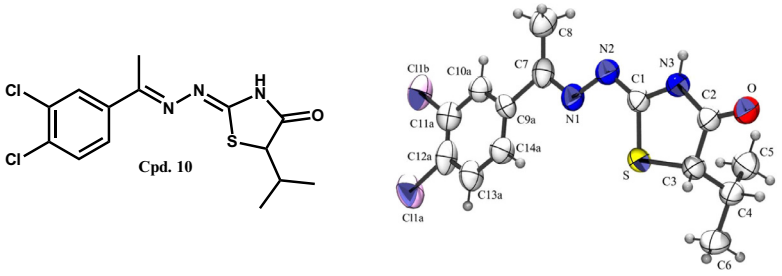


Figure 2. (Left) chemical structure of compound (**10**). (Right) ORTEP-3 projection of compound (**10**) showing atom-numbering and displacement ellipsoids at the 50% probability level.

Comparing thiosemicarbazones (**4**–**6**), it is observed that with the increase of lipophilicity in substituents at N3, with methyl (**5**) or phenyl (**6**), the trypanocidal activity decrease with an improvement in toxicity for macrophages. This trend is not observed for cyclic derivatives (**7**–**21**), otherwise an improvement in toxicity profile is observed with the cyclization of the thioamide into thiazolidinone moiety.

The good trypanocidal activity observed in unsubstituted thiosemicarbazone (**4**) was not kept in their cyclic derivatives (**7**, **12** and **17**), however, their present lower cytotoxicity for macrophages, in opposition to (**4**). Among cyclic derivatives, only compounds (**18**), (**19**) and (**20**) present better trypanocidal activity than thiosemicarbazone (**4**).

In general, substitutions at N3 improved the antiparasitic activity, with *N*-phenyl compounds (**17**–**21**) exhibiting the strongest activity among all tested compounds. For instance, the methyl (**18**), ethyl (**19**), and *iso*propyl (**20**) compounds were, respectively, 12-, 16- and 25-times more potent antiparasitic agents than unsubstituted compound (**17**).

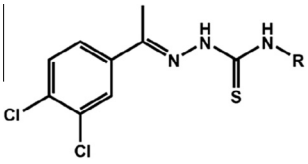
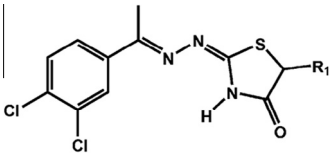
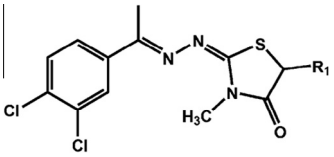
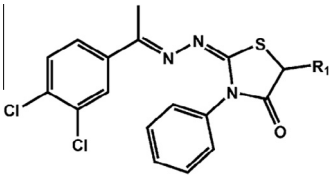
Given the selectivity of these compounds against bloodstream trypomastigotes of *T. cruzi*, we next examined their activity against intracellular parasites. To this end, we assessed an in vitro model of parasite infection using mouse macrophages infected with Y strain trypomastigotes. Three days after infection, 35–40% of the untreated macrophages were infected, and a high number of amastigotes per 100 macrophages were observed. Treatment with 50 μM Benznidazole reduced the number of infected cells and the number of intracellular amastigotes (P <0.001). We then tested the three most potent compounds, thiazolidinones (**18**), (**19**), and (**20**) at concentration of 50 μM. As shown in Figure 3, these compounds inhibited *T. cruzi* infection with potency higher than Benznidazole (P <0.001). When tested at different concentrations, it was possible to calculate the IC_{50} values for compounds (**18**), (**19**) and (**20**) against intracellular amastigotes. IC_{50} values of 5.9 ± 0.52, 3.2 ± 0.3, and 3.1 ± 0.64 μM for compounds (**18**), (**19**) and (**20**) were found, respectively. These values were lower than Benznidazole (IC_{50} = 13.9 ± 0.39 μM).

Finally, we evaluated the in vivo efficacy of compound (**20**). Starting on day 5 post-infection (dpi), compound (**20**) was administered at 100 mg/kg orally once per day for five consecutive days in BALB/c mice infected with 10^4 Y strain trypomastigotes. Controls included untreated and Benznidazole-treated mice. The course of infection was monitored by counting blood parasites and animal survival was followed for 1 month. As seen in Figure 4, compound (**20**) reduced blood parasitemia (P <0.001) compared to untreated control. At dose of 100 mg/kg, administration of (**20**) caused a reduction in blood parasitaemia of 86.7 (10 days post-infection (dpi)) and 97.1% (12 dpi) (Table 2). In the group treated with Benznidazole, it was observed >99% of inhibition of blood parasitaemia, indicating that eradication of infection was achieved. Treatment with (**20**), similar to the treatment with Benznidazole, had a protective effect on mortality. No signs of toxicity or body weight loss were observed in animals in the treatment group (data not shown).

3. Discussion

Here, we examined the chemistry and pharmacology of thiazolidinones derived from a thiosemicarbazone which is a highly potent cruzain inhibitor but a poor selective antiparasitic agent. The employed route to preparing thiazolidinones was efficient to produce compounds in acceptable yield and purity. X-ray diffraction revealed the conformation and geometry of iminic bound as well as the tautomerism of heterocyclic ring. By varying substituents attached at heterocyclic ring, it was possible to screen active compounds and to identify the main structural determi-

Table 1
Anti-*T. cruzi* activity of thiazolidinones (**7**–**21**)

Compd	R_1	Trypomastigotes, Y strain *T. cruzi* IC_{50} ± SD[a] (μM)	Macrophages CC_{50} ± SD[b] (μM)	Selectivity index[c]
				
4	H	7.1 ± 0.76	13.4 ± 0.82	1.7
5	Methyl	11.2 ± 1.62	>100	8.8
6	Phenyl	>50	>100	—
				
7	H	>50	76.5 ± 3.18	—
8	Methyl	49.5 ± 0.51	>100	2.0
9	Ethyl	>50	76.7 ± 0.46	—
10	Isopropyl	16.9	>100	5.9
11	Phenyl	>50	96.9 ± 1.37	—
				
12	H	>50	>100	—
13	Methyl	>50	>100	—
14	Ethyl	48.5	>100	2.0
15	Isopropyl	>50	>100	—
16	Phenyl	41.5	97.6	—
				
17	H	>50	>100	—
18	Methyl	4.2 ± 0.28	26.1 ± 0.90	6
19	Ethyl	2.9 ± 0.91	23.3 ± 0.96	8
20	Isopropyl	1.7 ± 0.17	>100	>58
21	Phenyl	17.7 ± 0.09	27.1 ± 0.51	1.5
Bdz	—	10.6 ± 0.87	>100	>9.4
GV	—	—	0.45 ± 0.04	—

ND = not determined. Bdz = Benznidazole, GV = violet gentian. SD = standard deviation.
[a] Determined after 24 h of incubation in the presence of compounds.
[b] Determined in J774 cells for 72 h after incubation.
[c] (CC_{50} macrophages)/(IC_{50} trypomastigotes).

nants of thiazolidinones for antiparasitic activity. We observed that the nonsubstituted or those containing a methyl at N3 presented poor antiparasitic activity. In contrast, thiazolidinones containing a phenyl group at N3 exhibited trypanocidal properties and were active against intracellular parasites, corroborating with a previously work of our group.[38] In addition, it is worth mentioning that these compounds presented low cytotoxicity in host cell viability, therefore showing good selectivity indexes.

Compounds (**1**) (Fig. 1) and (**2**) were previously synthesized by our group, with good trypanocidal activities (10 μM and 6.1 μM, respectively). The compounds with similar structure are compounds (**8**) and (**20**), with the same substituents at N3 and C5, respectively. The main structural difference between these compounds (**1**–**2** and **8**, **20**) are the substituents linked at N1 (phenylthiopropyl for (**1**), phenoxypropan-2-yl for (**2**) and 3,4-dichlorophenyl for (**8**) and (**20**). Comparing compound **1** with compound (**8**), it was not observed an improvement in trypanocidal activity. However, an improvement in trypanocidal activity was observed for compound (**20**), being 3-times more potent than compound (**2**), the lead compound identified by Moreira et al.[38] (6.1 μM vs 1.7 μM). This result highlight the importance of the 3,4-dichloro moiety for the trypanocidal activity (Fig. 5).

Regarding the mechanism of action, the thiazolidinones did not inhibit cruzain activity, while the thiosemicarbazone prototype did. These findings show that the mechanism of action of these antiparasitic thiazolidinones is different from thiosemicarbazones-inhibiting cruzain. In addition to displaying activity against axenic parasite, the thiazolidinones exhibited strong activity against intracellular amastigotes. In comparison to untreated infected macrophages, the compounds not only reduced the in vitro infection but also the parasite burden. Importantly, this activity was superior to Benznidazole-treated cells. Compound (**20**), the most potent in vitro thiazolidinone, was efficient in reducing the blood parasitemia in acutely-infected mice.

In overall, the substitution at N3 with phenyl and C5 with alkyl groups were benefic for the trypanocidal activity, generating compounds (**18**), (**19**) and (**20**), the most potent compounds presented in this study. It is worth to mention that compounds (**18**) (4.2 ± 0.28 μM), (**19**) (2.9 ± 0.91 μM) and (**20**) (1.7 ± 0.17 μM) were, respectively, 2-, 3- and 6-times more potent than the reference drug, Benznidazole (10.6 ± 0.87 μM) for trypomastigote form of the parasite. About intracellular amastigotes, compounds (**18**), (**19**) and (**20**) also present good trypanocidal activity, being 2-, 4- and 4-times more potent than Benznidazole, the unique drug available for treatment.

4. Conclusions

The search for antiparasitic compounds against *T. cruzi* based on heterocyclic chemistry led us to develop a new series of thiazolidinones. This study enabled molecular modifications at positions N3 and C5 on the thiazolidinic ring. The X-ray diffraction of compound (**10**) revealed that an *E*-geometry for iminic bond is observed. The in vitro screening showed that the presence of a phenyl group at position N3 improves the antiparasitic activity. This led to the identification of the potent *N*-phenyl substituted compounds (**18**–**20**), which were more potent trypanocidal agents than Benznidazole and previously described thiazolidinones (**1**–**2**). Compound (**20**) reduced parasitemia in both in vitro and in an acutely-infected mice model, thereby corroborating to the general notion that this class of heterocyclic compounds are strong antiparasitic agents.

5. Experimental section

5.1. Reagents and spectra analysis

All reagents were used as purchased from commercial sources (Sigma–Aldrich, Vetec, or Fluka). Progress of the reactions was followed by thin-layer chromatography (silica gel 60 F_{254} in aluminum foil). Purity of the target compounds was confirmed by combustion analysis (for C, H, N and S) performed by a Carlo-Erba instrument (model EA 1110). IR was determined in KBr pellets. For NMR, we used a Varian UnityPlus 400 MHz (400 MHz for ^1H and 100 MHz for ^{13}C) and Bruker AMX-300 MHz (300 MHz for ^1H and 75.5 MHz for ^{13}C) instruments. DMSO-d_6, acetone-d_6, or D_2O were purchased from CIL. Chemical shifts are reported in ppm, and multiplicities are given as s (singlet),

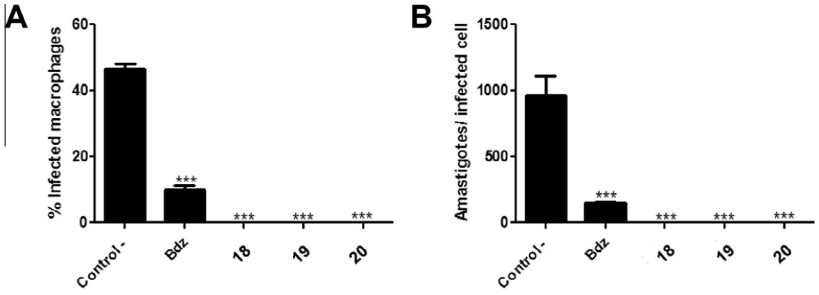


Figure 3. Thiazolidinones (**18**), (**19**), and (**20**) affect intracellular parasite development. The percentage of infected macrophages (A) and the relative number of amastigotes per 100 macrophages (B) are higher in untreated controls than in cultures treated with test compounds (**18**), (**19**), and (**20**), or Bdz. Macrophages were infected with Y strain trypomastigotes for 2 h, and test inhibitors were then added at 50 μM. Cell cultures were incubated for 3 days. $^{***}P$ <0.001 compared to untreated controls. All compounds were tested in triplicate, and two independent experiments were performed. Bars indicate standard deviation. Bdz was used as a positive control.

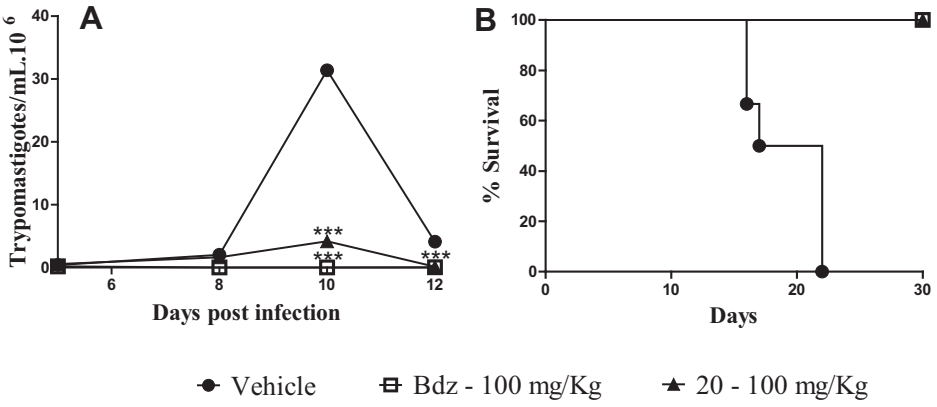


Figure 4. (A) Compound (**20**) substantially reduced parasitemia in mice. Female BALB/c mice were infected with 10^4 Y strain trypomastigotes. Five days after infection, mice were treated orally with compound (**20**) or Bdz with one daily dose (100 mg/kg) for five consecutive days. Parasitemia was monitored by counting the number of trypomastigotes in fresh blood samples. Values represent the mean ± SEM of 6 mice per group. $^{***}P$ <0.001 compared to untreated-infected group (vehicle). (B) Treatment with compound (**20**) increases survival rates in infected mice. The animals were monitored for 30 days after infection. Results are from one experiment done with 6 mice per group.

Table 2
Parasitemia and mortality evaluation in mice infected with Y strain *T. cruzi* and treated daily with (**20**) or Benznidazole for 5 days

Compounds	Dose (mg/kg)	% Blood parasitemia reduction in mice[a]		Mortality[b]
		10 dpi	12 dpi	
20	100	86.7	97.1	0/6
Bdz	100	>99	>99	0/6
Vehicle	–	–	–	6/6

dpi = days post-infection. Bdz = Benznidazole. Vehicle = untreated and infected group.

[a] Calculated as ([average vehicle group − average treated group)/average vehicle group] × 100%).

[b] Mortality was monitored until 30 days after treatment.

d (doublet), t (triplet), q (quartet), m (multiplet), and dd (double doublet), and coupling constants (*J*) in hertz. NH signals were localized in each spectrum after the addition of a few drops of D_2O. Structural assignments were corroborated by DEPT analysis. Mass spectrometry experiments were performed on a Q-TOF spectrometer LC-IT-TOF (Shimadzu). When otherwise specified, ESI was carried out in the positive ion mode. Reactions in an ultrasound bath were carried out under frequency of 40 kHz (180 W) and without external heating.

5.1.1. General procedure for the synthesis of thiosemicarbazones (4–6). Example for compound (4): Synthesis of 1-(3,4-dichlorophenyl)-ethylidenethiosemicarbazone (4)

Under magnetic stirring: in a 100 mL round-bottom flask, 1.06 mmol (0.097 g) of thiosemicarbazide, 1.06 mmol (0.2 g) of 3′,4′-dichloroacetophenone (**3**), 4 drops of H_2SO_4 and 20 mL of ethanol were added and maintained under magnetic stirring and reflux for 20 h. After cooling back to rt, the precipitate was filtered in a Büchner funnel with a sintered disc filter, washed with cold ethanol, and then dried over SiO_2. Product was purified by recrystallization in hot toluol. Colorless crystals, mp: 187–189 °C; yield: 0.10 g (38%).

Under ultrasound irradiation: in a 10 mL round-bottom flask, 1.06 mmol (0.097 g) of thiosemicarbazide, 1.06 mmol (0.2 g) of 3′,4′-dichloroacetophenone (**3**), 4 drops of H_2SO_4 and 20 mL of ethanol were added and maintained in a ultrasound bath for 30 min. After cooling back to rt, the precipitate was filtered in a Büchner funnel with a sintered disc filter, washed with cold ethanol, and then dried over SiO_2. Product was purified by recrystallization in hot toluol. Colorless crystals, mp: 187–189 °C; yield: 0.22 g (80%); R_f = 0.56 (toluol/ethyl acetate 6:4). IR (KBr): 3427 (NH_2), 3399 (NH), 3138 (C—H), 1591 (C=N) cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6): δ 2.27 (s, 3H, CH_3), 7.58 (d, 1H, *J* 9.37 Hz, Ar), 7.86 (dd, 1H, *J* 9.37 and 3.75 Hz, Ar), 8.18 (s, 1H, NH_2), 8.26 (d, 1H, *J* 3.75 Hz, Ar), 8.36 (s, 1H, NH_2), 10.29 (s, 1H, NH). Signals at δ

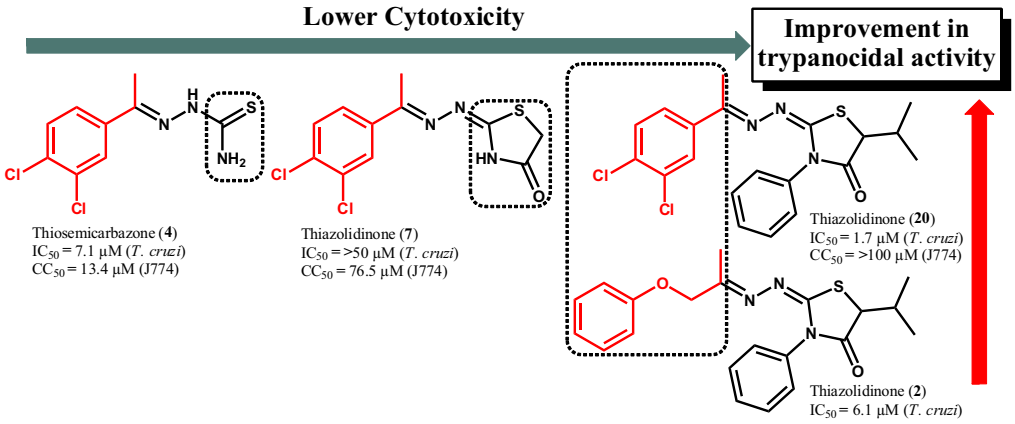


Figure 5. Structural improvements of proposed compounds.

8.18, 8.36 and 10.29 ppm disappear after adding D_2O. ^{13}C NMR (75.5 MHz, DMSO-d_6): δ 13.8 (CH_3), 126.7 (CH, Ar), 128.2 (CH, Ar), 130.2 (CH, Ar), 131.3 (C, Ar), 131.6 (3ClC, Ar), 138.2 (4ClC, Ar), 145.312 (C=N), 179.0 (C=S). HRMS (ESI): 259.9565 $[M-H]^+$. Anal. calcd for $C_9H_9Cl_2N_3S$: C, 41.23; H, 3.46; N, 16.03; S, 12.23. Found: C, 41.21; H, 3.61; N, 16.36; S, 12.78.

5.1.1.1. 1-(3,4-Dichlorophenyl)ethylideno-4-methylthiosemicarbazone (5). Recrystallization in hot toluol afforded colorless crystal, mp: 195–197 °C; yield: 0.24 g (84%); R_f = 0.7 (toluol/ethyl acetate 6:4). IR (KBr): 3343 and 3219 (NH), 1547 (C=N) cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6): δ 2.28 (s, 3H, CH_3), 3.04 (d, 3H, *J* 6 Hz, N—CH_3, coupling with N—H), 7.63 (d, 1H, *J* 8.57 Hz, Ar), 7.88 (dd, 1H, *J* 8.57 and 2.14 Hz, Ar), 8.23 (d, 1H, *J* 2.14 Hz, Ar), 8.61 (broad d, 1H, *J* 6 Hz, CH_3—N*H*), 10.33 (s, 1H, NH). ^{13}C NMR (75.5 MHz, DMSO-d_6): δ 13.8 (CH_3), 31.1 (N—CH_3), 126.7 (CH, Ar), 128.1 (CH, Ar), 130.2 (CH, Ar), 131.3 (C, Ar), 131.5 (3ClC, Ar), 138.3 (4ClC, Ar), 145.0 (C=N), 178.6 (C=S). HRMS (ESI): 275.9475 $[M+H]^+$. Anal. calcd for $C_{10}H_{11}Cl_2N_3S$: C, 43.49; H, 4.01; N, 15.21; S, 11.61. Found: C, 43.34; H, 3.98; N, 15.55; S, 11.74.

5.1.1.2. 1-(3,4-Dichlorophenyl)ethylidene)-4-phenylthiosemicarbazone (6). Recrystallization in hot toluol afforded colorless crystal, mp: 200–202 °C; yield: 0.32 g (89%); R_f = 0.73 (toluol/ethyl acetate 6:4). IR (KBr): 3309 and 3239 (NH), 3046 (C—H), 1523 (C=N) cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6): δ 2.36 (s, 3H, CH_3), 7.23 (t, 1H, *J* 7.21 Hz, Ar), 7.38 (t, 2H, *J* 7.21 Hz, Ar), 7.51 (d, 2H, *J* 7.21 Hz, Ar), 7.64 (d, 1H, *J* 9.09 Hz, Ar), 7.97 (dd, 1H, *J* 9.09 and 2.72 Hz, Ar), 8.34 (d, 1H, *J* 2.72 Hz, Ar), 10.18 (s, 1H, NH), 10.66 (s, 1H, ArNH). ^{13}C NMR (75.5 MHz, DMSO-d_6): δ 14.2 (CH_3), 125.6 (CH, Ar), 126.5 (CH, Ar), 127.0 (CH, Ar), 128.1 (CH, Ar), 128.4 (CH, Ar), 130.2 (CH, Ar), 131.3 (C, Ar), 131.8 (3ClC, Ar), 138.1 (4ClC, Ar), 139.2 (C—N, Ar), 146.3 (C=N), 177.3 (C=S). HRMS (ESI): 337.9678 $[M+H]^+$. Anal. calcd for $C_{15}H_{13}Cl_2N_3S$: C, 53.26; H, 3.87; N, 12.42; S, 9.48. Found: C, 53.23; H, 3.84; N, 12.31; S, 9.32.

5.1.2. General procedure for the synthesis of thiazolidinones 7–21. Example for compound (7)

Synthesis of 2-[1-(3,4-dichlorophenyl)ethylidenohydrazone]thiazolidin-4-one (**7**). in a 100 mL round-bottom flask, 3.81 mmol (1.0 g) of thiosemicarbazone (**4**) was dissolved in 30 mL of ethanol, followed by adding 15.24 mmol (1.25 g) anhydrous sodium acetate under magnetic stirring and warming. After 15 min, 5.73 mmol (0.8 g) of ethyl 2-bromoacetate was added in portions and the mixture was maintained under reflux for 20 h. After cooling back to rt, the precipitate was filtered in a Büchner funnel with a sintered disc filter, washed with cold ethanol, and then dried over SiO_2. Product was purified by recrystallization in hot cyclohexane. Colorless crystals were obtained. Mp: 234–236 °C; yield: 0.75 g (65%); R_f: 0.53 (toluol/ethyl acetate 6:4). IR (KBr): 3143 (NH), 2985 (C—H), 1715 (C=O), 1628 (C=N) cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6): δ 2.36 (s, 3H, CH_3), 3.89 (s, 2H, S—CH_2), 7.69 (d, 1H, *J* 10 Hz, Ar), 7.80 (d, 1H, *J* 10 Hz, Ar), 8.00 (s, 1H, Ar), 12.06 (s, 1H, NH). ^{13}C NMR and DEPT (75.5 MHz, DMSO-d_6): δ 14.8 (CH_3), 33.3 (CH_2, heterocycle), 126.8 (CH, Ar), 128.4 (CH, Ar), 131.0 (CH, Ar), 131.8 (C, Ar), 132.7 (3ClC, Ar), 138.7 (4ClC, Ar), 158.6 (C=N), 166.0 (S—C=N), 174.3 (C=O). HRMS (ESI): 301.9364 $[M+H]^+$. Anal. calcd for $C_{11}H_9Cl_2N_3OS$: C, 43.72; H, 3.00; N, 13.91; S, 10.61. Found: C, 44.00; H, 3.20; N, 13.47; S, 10.11.

5.1.2.1. 2-[1-(3,4-Dichlorophenyl)ethylidenohydrazone]-5-methylthiazolidin-4-one (8). Recrystallization in toluol/cyclohexane 2:1 afforded colorless. Mp: 208–210 °C; yield: 0.68 g (56%); R_f: 0.52 (toluol/ethyl acetate 6:4). IR (KBr): 3422 (NH), 3139 (C—H), 1704 (C=O), 1627 (C=N) cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6): δ 1.48 (d, 3H, *J* 7.5 Hz, CH_3 heterocycle), 2.27 and 2.35 (s, 3H, CH_3), 4.15 (q, 1H, *J* 7.5 Hz, CH heterocycle), 7.60 and 7.68 (d, 1H, *J* 8.75 Hz, Ar), 7.78 and 7.87 (d, 1H, *J* 8.75 Hz, Ar), 7.99 and 8.26 (s, 1H, Ar), 8.36 and 10.30 (s, 1H, NH). ^{13}C NMR (75.5 MHz, DMSO-d_6): δ 13.8 and 14.3 (CH_3 heterocycle), 18.8 (CH_3), 41.0 (CH, heterocycle), 126.4 and 126.8 (CH, Ar), 127.8 and 128.2 (CH, Ar), 130.2 (CH, Ar), 131.3 and 131.6 (C, Ar), 132.2 (3ClC, Ar), 138.2 and 138.3 (4ClC, Ar), 145.3 (C=N), 157.9 (S—C=N), 177.3 (C=O). HRMS (ESI): 315.9491 $[M+H]^+$. Anal. calcd for $C_{12}H_{11}Cl_2N_3OS$: C, 45.58; H, 3.51; N, 13.29; S, 10.14. Found: C, 45.86; H, 3.68; N, 12.90; S, 10.00.

5.1.2.2. 2-[1-(3,4-Dichlorophenyl)ethylidenohydrazone]-5-ethylthiazolidin-4-one (9). Recrystallization in hot toluol afforded colorless. Mp: 175–177 °C; yield: 0.94 g (75%); R_f: 0.63 (toluol/ethyl acetate 6:4). IR (KBr): 2975 (C—H), 1704 (C=O), 1623 (C=N) cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6): δ 0.95 (broad s, 3H, CH_3), 1.88 (m, 2H, CH_2), 2.36 (s, 3H, CH_3), 4.20 (broad s, 1H, CH heterocycle), 7.69 (d, 1H, *J* 8 Hz, Ar), 7.80 (d, 1H, *J* 8 Hz, Ar), 7.99 (s, 1H, Ar), 12.05 (s, 1H, NH). ^{13}C NMR (75.5 MHz, DMSO-d_6): δ 10.3 (CH_3), 14.3 (CH_3), 25.4 (CH_2), 49.0 (CH, heterocycle), 126.4 (CH, Ar), 127.9 (CH, Ar), 130.6 (CH, Ar), 131.3 (C, Ar), 132.2 (3ClC, Ar), 138.3 (4ClC, Ar), 158.2 (C=N), 164.1 (S—C=N), 176.0 (C=O). HRMS (ESI): 329.9624 $[M+H]^+$. Anal. calcd for $C_{13}H_{13}Cl_2N_3OS$: C, 47.28; H, 3.97; N, 12.72; S, 9.71. Found: C, 47.20; H, 3.60; N, 12.34; S, 9.55.

5.1.2.3. 2-[1-(3,4-Dichlorophenyl)ethylidenohydrazone]-5-isopropylthiazolidin-4-one (10). Recrystallization in toluol/cyclohexane 2:1 afforded colorless. Mp: 193–195 °C; yield: 0.73 g (56%); R_f: 0.6 (toluol/ethyl acetate 6:4). IR (KBr): 2953 (C—H), 1707 (C=O), 1616 (C=N) cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6): δ 0.87 (d, 3H, *J* 6 Hz, CH_3), 0.98 (d, 3H, *J* 6 Hz, CH_3), 2.36 (s, 3H, CH_3), 2.50 (m, 1H, CH), 4.30 (d, 1H, *J* 3 Hz, CH heterocycle), 7.69 (d, 1H, *J* 10 Hz, Ar), 7.81 (d, 1H, *J* 10 Hz, Ar), 7.99 (s, 1H, Ar), 12.06 (s, 1H, NH). ^{13}C NMR (75.5 MHz, DMSO-d_6): δ 14.4 (CH_3), 16.4 (CH_3), 20.3 (CH_3), 29.9 (CH), 54.7 (CH, heterocycle), 126.4 (CH, Ar), 128.0 (CH, Ar), 130.6 (CH, Ar), 131.3 (C, Ar), 132.3 (3ClC, Ar), 138.3 (4ClC, Ar), 158.6 (C=N), 163.7 (S—C=N), 175.4 (C=O). HRMS (ESI): 343.9749 $[M-H]^+$. Anal. calcd for $C_{14}H_{15}Cl_2N_3OS$: C, 48.84; H, 4.39; N, 12.21; S, 9.31. Found: C, 49.19; H, 4.56; N, 12.16; S, 9.49.

5.1.2.4. 2-[1-(3,4-Dichlorophenyl)ethylidenohydrazone]-5-phenylthiazolidin-4-one (11). Recrystallization in hot toluol afforded colorless. Mp: 245–247 °C; yield: 0.95 g (65%); R_f: 0.57 (toluol/ethyl acetate 6:4). IR (KBr): 1711 (C=O), 1618 (C=N) cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6): δ 2.41 (s, 3H, CH_3), 5.49 (s, 1H, CH heterocycle), 7.39 (m, 5H, Ar), 7.68 (d, 1H, *J* 9 Hz, Ar), 7.81 (dd, 1H, *J* 9 and 2.25 Hz, Ar), 8.01 (d, 1H, *J* 2.25 Hz, Ar), 12.35 (s, 1H, NH). ^{13}C NMR (75.5 MHz, DMSO-d_6): δ 14.9 (CH_3), 51.4 (CH, heterocycle), 126.9 (CH, Ar), 128.4 (CH, Ar), 128.7 (CH, Ar), 128.9 (CH, Ar), 129.3 (CH, Ar), 131.1 (CH, Ar), 131.8 (C, Ar), 132.8 (C, Ar), 137.2 (3ClC, Ar), 138.6 (4ClC, Ar), 159.2 (C=N), 162.0 (S—C=N), 175.0 (C=O). HRMS (ESI): 378.0310 $[M+H]^+$. Anal. calcd for $C_{17}H_{13}Cl_2N_3OS$: C, 53.98; H, 3.46; N, 11.11; S, 8.48. Found: C, 53.75; H, 3.53; N, 11.12; S, 8.78.

5.1.2.5. 2-[1-(3,4-Dichlorophenyl)ethylidenohydrazone]-3-methylthiazolidin-4-one (12). Recrystallization in hot toluol afforded colorless. Mp: 195–197 °C; yield: 0.70 g (61%); R_f: 0.73 (toluol/ethyl acetate 6:4). IR (KBr): 2942 (C—H), 1717 (C=O), 1613 and 1570 (C=N) cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6): δ 2.43 (s, 3H, CH_3), 3.20 (s, 3H, N—CH_3), 3.95 (s, CH_2 heterocycle), 7.72 (d, 1H, *J* 11.42 Hz, Ar), 7.83 (dd, 1H, *J* 11.42 and 1.43 Hz, Ar), 8.03 (d, 1H, *J* 1.43 Hz, Ar). ^{13}C NMR (100 MHz, DMSO-d_6): δ 14.2 (CH_3), 29.2 (N—CH_3), 32.0 (CH_2 heterocycle), 126.3 (CH, Ar), 127.9 (CH, Ar), 130.5 (CH, Ar), 131.2 (C, Ar), 132.3 (3ClC, Ar), 138.1 (4ClC, Ar), 159.4 (C=N), 164.5 (S—C=N), 172.0 (C=O). HRMS (ESI): 315.9498 $[M+H]^+$. Anal. calcd for $C_{12}H_{11}Cl_2N_3OS$: C, 45.58; H, 3.51; N, 13.29; S, 10.14. Found: C, 45.48; H, 3.33; N, 13.18; S, 9.90.

5.1.2.6. 2-[1-(3,4-Dichlorophenyl)ethylidenohydrazone]-3,5-dimethylthiazolidin-4-one (13). Recrystallization in cyclohexane/toluol 3:1 afforded colorless. Mp: 178–180 °C; yield: 0.90 g (75%); R_f: 0.8 (toluol/ethyl acetate 6:4). IR (KBr): 1704 (C=O), 1606 and 1570 (C=N) cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6): δ 1.53 (d, 3H, *J* 7.14 Hz, CH_3), 2.42 (s, 3H, CH_3), 3.22 (s, 3H, N—CH_3), 4.24 (q, *J* 7.14 Hz, CH heterocycle), 7.70 (d, 1H, *J* 8.51 Hz, Ar), 7.82 (dd, 1H, *J* 8.51 and 2.12 Hz, Ar), 8.02 (d, 1H, *J* 2.12 Hz, Ar). ^{13}C NMR (100 MHz, DMSO-d_6): δ 14.1 (CH_3, position 5 of the heterocycle), 18.5 (CH_3), 29.3 (N—CH_3), 41.1 (CH heterocycle), 126.3 (CH, Ar), 127.8 (CH, Ar), 130.4 (CH, Ar), 131.2 (C, Ar), 132.2 (3ClC, Ar), 138.1 (4ClC, Ar), 159.3 (C=N), 163.0 (S—C=N), 175.0 (C=O). HRMS (ESI): 329.9630 $[M+H]^+$. Anal. calcd for $C_{13}H_{13}Cl_2N_3OS$: C, 47.28; H, 3.97; N, 12.72; S, 9.71. Found: C, 47.33; H, 3.81; N, 12.63; S, 9.69.

5.1.2.7. 2-[1-(3,4-Dichlorophenyl)ethylidenohydrazone]-5-ethyl-3-methylthiazolidin-4-one (14). Recrystallization in hot cyclohexane afforded colorless. Mp: 167–168 °C; yield: 0.80 g (64%); R_f: 0.81 (toluol/ethyl acetate 6:4). IR (KBr): 2970 (C—H), 1716 (C=O), 1610 and 1567 (C=N) cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6): δ 0.95 (t, 3H, CH_3), 1.83 (m, 1H, CH_2), 2.02 (m, 1H, CH_2), 2.42 (s, 3H, CH_3), 3.19 (s, 3H, N—CH_3), 4.27 (m, 1H, CH heterocycle), 7.70 (d, 1H, *J* 8 Hz, Ar), 7.83 (dd, 1H, *J* 8 and 2 Hz, Ar), 8.02 (d, 1H, *J* 2 Hz, Ar). ^{13}C NMR (100 MHz, DMSO-d_6): δ 10.1 (CH_3), 14.2 (CH_3), 25.3 (CH_2), 29.2 (N—CH_3), 48.1 (CH heterocycle), 126.3 (CH, Ar), 127.9 (CH, Ar), 130.4 (CH, Ar), 131.2 (C, Ar), 132.3 (3ClC, Ar), 138.1 (4ClC, Ar), 159.5 (C=N), 162.9 (S—C=N), 174.0 (C=O). Anal. calcd for $C_{14}H_{15}Cl_2N_3OS$: C, 48.84; H, 4.39; N, 12.21; S, 9.31. Found: C, 48.83; H, 4.09; N, 12.15; S, 9.58.

5.1.2.8. 2-[1-(3,4-Dichlorophenyl)ethylidenohydrazone]-5-isopropyl-3-methylthiazolidin-4-one (15). Recrystallization in cyclohexane/toluol 3:1 afforded colorless. Mp: 137–140 °C; yield: 0.70 g (53%); R_f: 0.84 (toluol/ethyl acetate 6:4). IR (KBr): 2947 (C—H), 1713 (C=O), 1610 and 1563 (C=N) cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6): δ 0.84 (d, 3H, *J* 6.42 Hz, CH_3), 1.00 (d, 3H, *J* 6.42 Hz, CH_3), 2.43 (s, 3H, CH_3), 2.45 (m, 1H, CH), 3.21 (s, 3H, N—CH_3), 4.38 (d, 1H, *J* 2.5 Hz, CH heterocycle), 7.72 (d, 1H, *J* 7.5 Hz, Ar), 7.85 (dd, 1H, *J* 7.5 and 1.25 Hz, Ar), 8.03 (d, 1H, *J* 1.25 Hz, Ar). ^{13}C NMR (75.5 MHz, DMSO-d_6): δ 14.4 (CH_3), 16.4 (CH_3), 20.2 (CH_3), 29.3 (CH), 30.1 (N—CH_3), 53.9 (CH heterocycle), 126.5 (CH, Ar), 128.1 (CH, Ar), 130.7 (CH, Ar), 131.3 (C, Ar), 132.4 (3ClC, Ar), 138.1 (4ClC, Ar), 159.9 (C=N), 163.2 (S—C=N), 173.8 (C=O). HRMS (ESI): 357.9853 $[M+H]^+$. Anal. calcd for $C_{15}H_{17}Cl_2N_3OS$: C, 50.28; H, 4.78; N, 11.73; S, 8.95. Found: C, 50.29; H, 4.64; N, 11.71; S, 9.19.

5.1.2.9. 2-[1-(3,4-Dichlorophenyl)ethylidenohydrazone]-3-methyl-5-phenylthiazolidin-4-one (16). Recrystallization in hot toluol afforded colorless. Mp: 194–196 °C; yield: 0.91 g (87%); R_f: 0.82 (toluol/ethyl acetate 6:4). IR (KBr): 1709 (C=O), 1603 and 1565 (C=N) cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6): δ 2.47 (s, 3H, CH_3), 2.50 (s, 3H, N—CH_3), 5.55 (s, 1H, CH heterocycle), 7.39 (m, 5H, Ar), 7.71 (d, 1H, *J* 8.57 Hz, Ar), 7.84 (dd, 1H, *J* 8.57 and 2.14 Hz, Ar), 8.03 (d, 1H, *J* 2.14 Hz, Ar). ^{13}C NMR (75.5 MHz, DMSO-d_6): δ 14.4 (CH_3), 29.9 (N—CH_3), 50.1 (CH heterocycle), 126.5 (CH, Ar), 128.0 (CH, Ar), 128.3 (CH, Ar), 128.5 (CH, Ar), 128.8 (CH, Ar), 130.6 (CH, Ar), 131.3 (C, Ar), 136.5 (3ClC, Ar), 138.0 (4ClC, Ar), 160.0 (C=N), 163.0 (S—C=N), 173.0 (C=O). HRMS (ESI): 392.0450 $[M+H]^+$. Anal. calcd for $C_{18}H_{15}Cl_2N_3OS$: C, 55.11; H, 3.85; N, 10.71; S, 8.17. Found: C, 55.37; H, 3.92; N, 10.58; S, 8.44.

5.1.2.10. 2-[1-(3,4-Dichlorophenyl)ethylidenohydrazone]-3-phenylthiazolidin-4-one (17). Recrystallization in hot toluol afforded colorless. Mp: 232–233 °C; yield: 0.8 g (71%); R_f: 0.79 (toluol/ethyl acetate 6:4). IR (KBr): 2970 (C—H), 1728 (C=O), 1606 and 1562 (C=N) cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6): δ 2.16 (s, 3H, CH_3), 4.10 (s, 2H, CH_2 heterocycle), 7.41 (d, 2H, *J* 7.54 Hz, Ar), 7.45 (t, 1H, *J* 7.54 Hz, Ar), 7.53 (t, 2H, *J* 7.54 Hz, Ar), 7.70 (d, 1H, *J* 9.05 Hz, Ar), 7.79 (d, 1H, *J* 9.05 Hz, Ar), 7.99 (s, 1H, Ar). ^{13}C NMR (100 MHz, DMSO-d_6): δ 14.3 (CH_3), 32.2 (CH_2), 126.3 (CH, Ar), 127.8 (CH, Ar), 128.4 (CH, Ar), 128.7 (CH, Ar), 130.5 (CH, Ar), 134.9 (C, Ar), 136.8 (C, Ar), 163.0 (C=N), 171.6 (S—C=N), 198.6 (C=O). HRMS (ESI): 377.9950 $[M+H]^+$. Anal. calcd for $C_{17}H_{13}Cl_2N_3OS$: C, 53.98; H, 3.46; N, 11.11; S, 8.48. Found: C, 53.68; H, 3.42; N, 10.89; S, 7.94.

5.1.2.11. 2-[1-(3,4-Dichlorophenyl)ethylidenohydrazone]-3-phenyl-5-methylthiazolidin-4-one (18). Recrystallization in cyclohexane/toluol 3:1 afforded colorless. Mp: 208–209 °C; yield: 0.78 g (67%); R_f: 0.83 (toluol/ethyl acetate 6:4). IR (KBr): 1712 (C=O), 1609 and 1571 (C=N) cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6): δ 1.63 (d, 3H, *J* 6.9 Hz, CH_3), 2.16 (s, 3H, CH_3), 4.41 (q, 1H, *J* 6.9 Hz, CH heterocycle), 7.41–7.55 (m, 5H, Ar), 7.70 (d, 1H, *J* 8.4 Hz, Ar), 7.79 (dd, 1H, *J* 8.4 and 1.8 Hz, Ar), 7.99 (d, 1H, *J* 1.8 Hz, Ar). ^{13}C NMR (75.5 MHz, DMSO-d_6): δ 14.3 (CH_3), 18.7

(CH_3), 41.0 (CH heterocycle), 126.4 (CH, Ar), 127.9 (CH, Ar), 128.5 (CH, Ar), 128.8 (CH, Ar), 130.6 (CH, Ar), 131.3 (C, Ar), 132.4 (3ClC, Ar), 135.0 (C, Ar), 138.0 (4ClC, Ar), 159.7 (C=N), 163.2 (S—C=N), 174.8 (C=O). Anal. calcd for $C_{18}H_{15}Cl_2N_3OS$: C, 55.11; H, 3.85; N, 10.71; S, 8.17. Found: C, 55.36; H, 4.01; N, 10.44; S, 7.64.

5.1.2.12. 2-[1-(3,4-Dichlorophenyl)ethylidenohydrazone]-3-phenyl-5-ethylthiazolidin-4-one (19). Recrystallization in hot cyclohexane afforded colorless. Mp: 154 °C; yield: 0.42 g (35%); R_f: 0.86 (toluol/ethyl acetate 6:4). IR (KBr): 1708 (C=O), 1607 and 1568 (C=N) cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6): δ 1.03 (t, 3H, CH_3), 1.98 (m, 1H, CH_2), 2.08 (m, 1H, CH_2), 2.16 (s, 3H, CH_3), 4.45 (m, 1H, CH heterocycle), 7.39–7.55 (m, 5H, Ar), 7.71 (d, 1H, *J* 8.7 Hz, Ar), 7.80 (dd, 1H, *J* 8.7 and 1.8 Hz, Ar), 7.99 (d, 1H, *J* 1.8 Hz, Ar). ^{13}C NMR (75.5 MHz, DMSO-d_6): δ 10.1 (CH_3), 14.4 (CH_3), 25.5 (CH_2), 48.2 (CH heterocycle), 126.4 (CH, Ar), 127.9 (CH, Ar), 128.5 (CH, Ar), 128.9 (CH, Ar), 130.6 (CH, Ar), 131.3 (C, Ar), 132.4 (3ClC, Ar), 134.9 (C, Ar), 138.0 (4ClC, Ar), 160.0 (C=N), 163.0 (S—C=N), 173.9 (C=O). HRMS (ESI): 405.9517 $[M+H]^+$. Anal. calcd for $C_{19}H_{17}Cl_2N_3OS$: C, 56.16; H, 4.22; N, 10.34; S, 7.89. Found: C, 55.80; H, 4.23; N, 9.96; S, 7.97.

5.1.2.13. 2-[1-(3,4-Dichlorophenyl)ethylidenohydrazone]-3-phenyl-5-isopropylthiazolidin-4-one (20). Recrystallization in hot cyclohexane afforded colorless. Mp: 158–160 °C; yield: 0.76 g (61%); R_f: 0.9 (toluol/ethyl acetate 6:4). IR (KBr): 2964 (C—H), 1720 (C=O), 1603 and 1564 (C=N) cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6): δ 0.98 (d, 3H, CH_3), 1.06 (d, 3H, CH_3), 2.16 (s, 3H, CH_3), 2.36 (m, 1H, CH), 4.53 (d, 1H, CH heterocycle), 7.36–7.56 (m, 5H, Ar), 7.71 (d, 1H, *J* 8.4 Hz, Ar), 7.81 (dd, 1H, *J* 8.4 and 1.8 Hz, Ar), 7.99 (d, 1H, *J* 1.8 Hz, Ar). ^{13}C NMR (75.5 MHz, DMSO-d_6): δ 14.5 (CH_3), 16.5 (CH_3), 20.1 (CH_3), 30.5 (CH), 53.8 (CH heterocycle), 126.5 (CH, Ar), 127.9 (CH, Ar), 128.4 (CH, Ar), 128.7 (CH, Ar), 130.2 (CH, Ar), 131.3 (C, Ar), 132.5 (3ClC, Ar), 134.8 (C, Ar), 138.0 (4ClC, Ar), 160.3 (C=N), 163.1 (S—C=N), 173.5 (C=O). HRMS (ESI): 419.9888 $[M+H]^+$. Anal. calcd for $C_{20}H_{19}Cl_2N_3OS$: C, 57.15; H, 4.56; N, 10.00; S, 7.63. Found: C, 57.16; H, 4.49; N, 9.90; S, 7.68.

5.1.2.14. 2-[1-(3,4-Dichlorophenyl)ethylidenohydrazone]-3,5-diphenylthiazolidin-4-one (21). Recrystallization in hot toluol afforded colorless. Mp: 210–212 °C; yield: 0.77 g (58%); R_f: 0.88 (toluol/ethyl acetate 6:4). IR (KBr): 3053 (C—H), 1715 (C=O), 1605 and 1566 (C=N) cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6): δ 2.20 (s, 3H, CH_3), 5.70 (s, 1H, CH heterocycle), 7.36–7.58 (m, 10H, Ar), 7.69 (d, 1H, *J* 8.4 Hz, Ar), 7.80 (dd, 1H, *J* 8.4 and 2.1 Hz, Ar), 7.99 (d, 1H, *J* 2.1 Hz, Ar). ^{13}C NMR (75.5 MHz, DMSO-d_6): δ 14.0 (CH_3), 50.1 (CH heterocycle), 126.3 (CH, Ar), 127.8 (CH, Ar), 128.0 (CH, Ar), 128.1 (CH, Ar), 128.4 (CH, Ar), 128.7 (CH, Ar), 129.2 (CH, Ar), 129.6 (CH, Ar), 130.5 (CH, Ar), 133.5 (C, Ar), 133.7 (C, Ar), 134.8 (C, Ar), 134.9 (C, Ar), 136.8 (C, Ar), 163.0 (C=N), 171.2 (S—C=N), 198.0 (C=O). HRMS (ESI): 452.0380 $[M-H]^+$. Anal. calcd for $C_{23}H_{17}Cl_2N_3OS$: C, 60.80; H, 3.77; N, 9.25; S, 7.06. Found: C, 60.68; H, 3.74; N, 9.26; S, 7.08.

5.3. Cruzain inhibition

Cruzain activity was measured by monitoring the cleavage of the fluorogenic substrate Z-FR-AMC. Assays were performed in 0.1 M sodium acetate buffer pH 5.5, in the presence of 1 mM beta-mercaptoethanol and 0.01% Triton X-100. The final concentrations of cruzain was 0.5 nM, and the Z-FR-AMC substrate concentration was 2.5 μM (K_m = 1 μM) to a final volume of 200 μL and at 25 °C. In all assays, the enzyme was pre-incubated with the compounds for 10 min. before adding a solution containing the Z-FR-AMC substrate. Enzyme kinetic was followed by continuous reading for 5 min at 12 s intervals and readings were determined in a Synergy 2 (Biotek), from the Center of Flow Cytometry and Fluorimetry at the Biochemistry and Immunology Department (UFMG). The filters employed were 340 nM for excitation and 440 nM for emission. Activity was calculated based on initial velocity rates, compared to a DMSO control, since all compound stocks were prepared in DMSO. All compounds were evaluated at 10 μM and inhibition was measured in at least two independent experiments, each case in triplicate.

4.3. Animals

Female BALB/c mice (6–8 weeks old) were supplied by the animal breeding facility at Centro de Pesquisas Gonçalo Moniz (Fundação Oswaldo Cruz, Bahia, Brazil) and maintained in sterilized cages under a controlled environment, receiving a balanced diet for rodents and water ad libitum. All experiments were carried out in accordance with the recommendations of Ethical Issues Guidelines, and were approved by the local Animal Ethics Committee.

4.4. Parasites

Bloodstream trypomastigotes forms of *T. cruzi* were obtained from supernatants of LLC-MK2 cells previously infected and maintained in RPMI-1640 medium (Sigma–Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Cultilab, Campinas, Brazil), and 50 μg/mL gentamycin (Novafarma, Anápolis, Brazil) at 37 °C and 5% CO_2.

4.5. Host cell toxicity

J774 macrophages were dispensed on 96-well plates at a cell number of 5×10^4 cells/mL in 200 μL of RPMI medium supplemented with 10% of FBS and 50 μg/mL of gentamycin and incubated for 24 h at 37 °C and 5% CO_2. After that time each compound, dissolved in DMSO was added at six concentrations (0.41–100 μM) in triplicate and incubated for 72 h. Cell viability was determined by AlamarBlue assay (Invitrogen, Carlsbad, CA, USA) according to the manufacturer instructions. Colorimetric readings were performed after 6 h at 570 and 600 nm. Cytotoxic concentration to 50% (CC_{50}) was calculated using data-points gathered from three independent experiments. Gentian violet (Synth, São Paulo, Brazil) was used as positive control. The final concentration of DMSO was less than 1% in all in vitro experiments.

4.6. Toxicity for Y strain trypomastigotes

Trypomastigotes collected from the supernatant of LLC-MK2 cells were dispensed into 96-well plates at a cell density of 4×10^5 cells/well. Test inhibitors, were diluted into five different concentrations and added into their respective wells, and the plate was incubated for 24 h at 37 °C and 5% of CO_2. Aliquots of each well were collected and the number of viable parasites, based on parasite motility, was assessed in a Neubauer chamber. The percentage of inhibition was calculated in relation to untreated cultures. IC_{50} calculation was also carried out using non-linear regression with Prism 4.0 GraphPad software. Benznidazole was used as the reference drug.

4.7. Intracellular parasite development

Peritoneal exudate macrophages were obtained by washing, with cold RPMI medium, the peritoneal cavity of BALB/c mice 4–5 days after injection of 3% thioglycolate (Sigma) in saline (1.5 mL per mice). Then, cells were seeded at a cell density of 2×10^5 cells/well in a 24 well-plate with rounded coverslips on

the bottom in RPMI supplemented with 10% FBS and incubated for 24 h. Cells were then infected with Y strain trypomastigotes at a ratio of 10 parasites per macrophage for 2 h. Free trypomastigotes were removed by successive washes using saline solution. Next, cultures were incubated in complete medium alone or with the test inhibitors at 50 μM for 6 h. The medium was replaced by a fresh medium and the plate was incubated for 3 days. Cells were fixed in methanol and the percentage of infected macrophages and the relative number of amastigotes per 100 macrophages were determined by manual counting after Giemsa staining in an optical microscope (Olympus, Tokyo, Japan). The percentage of infected macrophages and the number of amastigotes per 100 macrophages was determined by counting 100 cells per slide. To estimate IC_{50} values, compounds were tested at five concentrations (1–50 μM).

4.8. Infection in mice

Female BALB/c mice (6–8 weeks old) were infected with bloodstream trypomastigotes by intraperitoneal injection of 10^4 parasites in 100 μL of saline solution. Mice were then randomly divided in groups (six mice per group). After five days post-infection, treatment with 100 mg/kg weight of compound (**20**) was given orally once a day for five consecutive days. For the control group, Benznidazole was also given orally at dose of 100 mg/kg weight. Infection was monitored by counting the number of motile parasites in 5 μL of fresh blood sample drawn from the lateral tail veins as recommended by standard protocols.[40] Survival was monitored for 30 days after treatment. The one-way ANOVA and Bonferroni for multiple comparisons were used to determine the statistical significance of the group comparisons.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2015.10.048. These data include MOL files and InChiKeys of the most important compounds described in this article.

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