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Synthesis, Cruzain Docking, and in vitro Studies of Aryl-4-Oxothiazolylhydrazones Against *Trypanosoma cruzi*

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Research in recent years has demonstrated that the Trypanosoma cruzi *cysteine protease cruzain (TCC) is a valid chemotherapeutic target. Herein we describe a small library of aryl-4-oxothiazolylhydrazones that have been tested in assays against* T. cruzi *cell cultures. The docking studies carried out suggest that these compounds are potential ligands for the TCC enzyme. The most promising compound of this series,* N-*(4-oxo-5-ethyl-2′-thiazolin-2-yl)*-N′-*phenylthio-(Z)-ethylidenehydrazone (**6 f**), was shown to be very active at non-cytotoxic concentrations in in vitro assays with mammalian cells and has a potency comparable with reference drugs such as nifurtimox (Nfx) and benznidazole (Bdz).*

Introduction

According to the World Health Organization (WHO), 16–18 million people are infected with *T. cruzi*, 2–3 million individuals have clinical symptoms of the chronic phase of Chagas' disease, and 63 000 people die of the disease and 300 000 new cases are reported each year.[1] Moreover, Chagas' disease is transmitted not only by Triatomine insects, but also by blood transfusion, the latter being responsible for the occurrence of Chagas' disease in regions to which this disease is not endemic.[2] Currently, this pathology is treated with nitroheterocyclic agents such as nifurtimox (Nfx) and benznidazole (Bdz). These two drugs act against the circulating form of the parasite (trypomastigotes) and are effective when administered during the acute phase of the disease, but not during the chronic phase. Clearly there is an urgent need for new selective drugs with different mechanisms of action for the treatment of Chagas' disease.[3]

Study of the basic biochemistry of *T. cruzi* has allowed the identification of new targets for chemotherapy.[4] *T. cruzi* cruzain (TCC) is the major cysteine protease of this parasite and is released during all stages of its life cycle, although it is delivered to different cell compartments at each stage. This enzyme is essential for replication of the intracellular parasite and appears to have potential as a target for the development of new anti-trypanosomal chemotherapies.[5]

A number of reports in recent years have described the inhibitory activity of several families of compounds such as *N*-acylhydrazides, chalcones, ureas, thioureas, and thiosemicarbazones toward TCC, and the relationship with their in vitro trypanocidal activity (Figure 1).[6–8] For example, Greenbaum et al. described 3′-bromopropiophenone and 3-trifluormethylphenyl thiosemicarbazone as the most effective cruzain inhibitors, with trypanocidal activity at levels that are nontoxic to mammalian cells.[9] To compare these findings, Fujii et al.[10] investigated thiosemicarbazones that possess larger alkyl groups at the adjoining hydrazone and reported that they are most active with an *n*-butyl group linked to the 3,4-dichlorobenzoyl moiety. Siles et al.[11] subsequently synthesized a series of thiosemicarbazone derivatives bearing fused rings and discovered new lead compounds that are more potent than non-fused-ring analogues, which are known to be the most active thiosemicarbazone-based inhibitors of TCC. These early SAR studies

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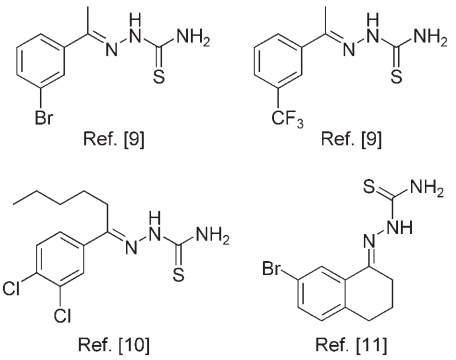


Figure 1. Representative thiosemicarbazones that are potent inhibitors of TCC.

did not include investigations of replacing the thioamide terminal amino group of thiosemicarbazones with a heterocyclic ring system.

Thiosemicarbazones can be viewed as noncyclic nonclassical bioisosteres of 4-oxothiazolylhydrazones and noncyclic classical bioisosteres for 2-pyrazoline-1-thiocarboxamides (also described as potent TCC inhibitors),[12] as depicted in Figure 2.[13] Furthermore, in addition to possessing other properties, 4-thiazolidone derivatives have been found to be active antitumor,[14] antibacterial,[15] antifungal,[16] and antiprotozoal[17] agents.

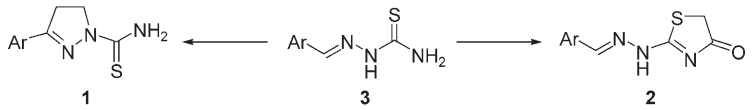


Figure 2. Thiosemicarbazone (center) and the classical bioisostere pyrazoline **1**, and the nonclassical bioisostere aryl-4-oxothiazolylhydrazone **2**.

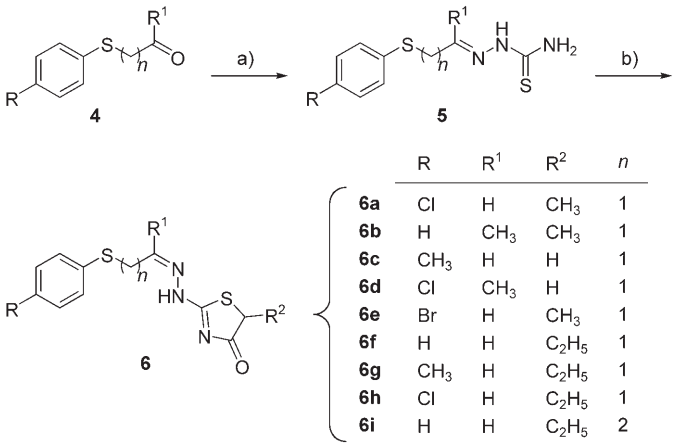
As part of our ongoing search for mechanism-based small-molecule inhibitors for these parasite proteases, a series of acylhydrazines, aryl-4-oxothiazolylhydrazones, and acylthiazolidones were recently evaluated for their ability to inhibit the growth of epimastigote and trypomastigote forms of *T. cruzi*, and a docking analysis with TCC was also conducted. Subsequently, the same aryl-4-oxothiazolylhydrazone derivatives were found to be potent anti-*T. cruzi* agents, and reported as possible ligands for TCC. It was also found that the 4-oxothiazolyl ring is an important requirement for interaction with TCC.[18]

For the study presented herein, we synthesized a series of aryl-4-oxothiazolylhydrazones with a view to further investigate the substituents on the aromatic ring and various linkages between the aromatic ring and the 4-oxothiazolylhydrazone scaffold. Substituents at the 5-position of the heterocyclic ring were also included to cover a range of physicochemical properties. The resulting derivatives were tested for their ability to inhibit the growth of epimastigote and trypomastigote forms of *T. cruzi*, and docking analysis with TCC was also carried out. The compounds thus designed were subsequently analyzed as potential ligands for cruzain.

Chemistry

Synthesis

The aryl-4-oxothiazolylhydrazones **6a–i** were prepared essentially as reported previously,[17,18] with moderate yields in the range of 30–56% (Scheme 1). All new compounds were identified by IR and ^{1}H NMR spectroscopy, and their purity was established by TLC and microanalysis. In theory, two configurations (*E* and *Z*) around the imine (C=N) double bond are possible for the above aryl-4-oxothiazolylhydrazones.[19] However, analysis of the ^{1}H NMR spectra and NOESY contour map (for compound **6b**) indicates that only an isomer with the *Z* configuration was obtained (Supporting Information); the ^{1}H NMR spectra upon addition of the chiral shift reagent Eu(hfc)$_3$ reveal that two enantiomers are present for all chiral products (hfc = 3-(heptafluoropropylhydroxymethylene)-*d*-camphorate). Hence, for compounds **6c** and **6d**, the diagnostic S–CH_2 (heterocyclic ring) showed a singlet around $\delta = 3.90$–3.70 ppm. Compounds **6a–b** and **6e**, which possess a 5-methyl group, showed a doublet around $\delta = 3.90$–3.70 ppm and a multiplet around $\delta = 4.20$–3.09 ppm. For compounds **6f–i**, the 5-ethyl group showed signs of being two multiplets (diastereotopic protons in the proximity of the chiral center) at $\delta = 2.09$–1.70 ppm (ethylenic) and at around $\delta = 0.90$–0.73 ppm (methylenic). The CH=N signal occurs as a triplet at $\delta = 7.85$–6.92 ppm.



	R	R^1	R^2	*n*
6a	Cl	H	CH_3	1
6b	H	CH_3	CH_3	1
6c	CH_3	H	H	1
6d	Cl	CH_3	H	1
6e	Br	H	CH_3	1
6f	H	H	C_2H_5	1
6g	CH_3	H	C_2H_5	1
6h	Cl	H	C_2H_5	1
6i	H	H	C_2H_5	2

Scheme 1. Synthesis of the (*Z*)-aryl-4-oxothiazolylhydrazone derivatives: a) **4**, thiosemicarbazide, MeOH, AcOH, reflux, 12 h (70–90%); b) **5**, α-chlorocarboxylic acids, NaOAc, EtOH, reflux, 8 h (30–56%).

Molecular modeling

The structures and conformational analysis of compounds **6a–i** and Bdz were obtained through the application of the AM1[20] method available as part of the BioMedCAChe software package,[21] using internal default settings for convergence criteria. The most stable conformers were selected for docking studies.

The *Z* configuration around the imine (C=N) double bond was considered in all cases, as determined by NMR results.

Molecules **6a**, **6b**, and **6e**–**f** were synthesized and tested as racemic mixtures, so that the molecular modeling treated the two enantiomers (*R* and *S*) independently, and the docking procedure used both isomers separately for each compound. The docking analysis was carried out on the TCC binding site (PDB code: 1U9Q),[22] where the enzyme's residues are in close proximity to the ligand known as "186", co-crystallized in complex with cruzain. This crystal structure is the monomeric catalytic domain termed X. The active site was defined as all atoms within a radius of 6.5 Å from the co-crystallized ligand, and the FlexX[23] module, available as part of the Sybyl 7.2 package,[24] was used for docking calculations.

The theoretical binding profile proposed for these ligands with TCC was determined as the highest (most negative) scored among 30 possible solutions generated according to the FlexX scoring function. The initial structures were optimized using the Tripos force field before docking.

Pharmacology

Cytotoxicity

The compounds were tested at various concentrations in mouse spleen cell cultures as described in the Experimental Section. The highest non-cytotoxic concentration of each compound was then used in subsequent assays to evaluate their anti-*T. cruzi* activity.

Anti-*T. cruzi* activity

The compounds were tested in vitro against epimastigote (Y and Colombian strains) and trypomastigote (Y strain) forms of *T. cruzi* at non-cytotoxic concentrations as outlined in the Experimental Section. Table 1 lists the percent inhibition at compound concentration of 40 μM and the IC_{50} values against the epimastigote forms of the Y and Colombian strains. The most active compounds, **6f** and **6i**, were also evaluated in time-dependent experiments at 40 μM after application of drugs for 15 h. Bdz and Nfx were used as references for the epimastigote assay, and gentian violet (GV) for the trypomastigote assay.

Table 1. In vitro characterization of aryl-4-oxothiazolylhydrazone derivatives.

Compd	Epimastigote			
	Y Strain		Colombian Strain	
	Inhibition [%][a]	IC_{50} [μM][b]	Inhibition [%][a]	IC_{50} [μM][b]
6a	69	9.1	56	23.6
6b	71	36.6	69	33.9
6c	32	63.4	33	87.6
6d	78	24.0	85	9.6
6e	56	4.5	80	19.7
6f	75	0.3	85	18.7
6g	73	20.9	52	33.0
6h	62	9.1	60	22.6
6i	100	3.2	48	44.1
Bdz[c]	100	1.8	60	26.8
Nfx[c]	100	1.9	NT[d]	NT[d]

[a] Percent growth inhibition determined for each compound at 40 μM. [b] Values are ±0.5 SD and were calculated from seven non-cytotoxic concentrations using data obtained from at least three independent experiments. [c] Bdz = benznidazole; Nfx = nifurtimox. [d] NT = not tested.

Results and Discussion

The 4-thiazolidone scaffolds have beneficial properties: a convenient log *P*, good hydrogen bond donating and accepting capabilities (compatible with Lipinski's "rule of five"), and are amenable to easy and economical synthetic routes. Therefore, to explore the activity based on this scaffold, a library of nine compounds was put together as potential inhibitors against the cysteine protease of *T. cruzi* (TCC). Analysis of docking into the TCC structure was also carried out.

All compounds were screened for inhibitory activity against the epimastigote form (Y and Colombian strains). As Table 1 shows, *N*-(4-oxo-5-ethyl-2′-thiazolin-2-yl)-*N*′-phenylthio-(*Z*)-ethylidenehydrazone (**6f**) is the most potent antitrypanosomal agent of this series, with a mean IC_{50} value of 0.3 μM against the Y strain. The simple extension of the linker between the heterocycle and the aromatic ring (compounds **6f** to **6i**) gives a tenfold decrease in potency against the parasite. The aryl-4-oxothiazolylhydrazone derivative **6e** with a *p*-bromo substituent is about twofold more potent than analogue **6a** (which possesses a *p*-chloro substituent). Our results are in accordance with those of Du et al.,[12] who reported that monosubstitution with bromine is better than chloro or trifluoromethyl bioisosteres. A similar trend was observed with analogues **6c** and **6g**. The potency of **6g**, in which an ethyl substituent is attached to the 4-thiazolidone ring, is threefold higher than that of the parent compound **6c**.

To better understand the kinetic aspects of the biological activity of this class of derivatives, their time-dependent effects on the growth of the epimastigote form (a suitable model for such time-dependent studies, as this form has a logarithmic growth profile) were determined on the Y strain of *T. cruzi* (Table 2). When the parasites were incubated for only 15 h after the addition of compounds **6f** and **6i** (40 μM), 62 and 64% growth inhibition was observed, respectively. In contrast, both Bdz and Nfx proved to be weak inhibitors after 15 h incubation. Indeed, Bdz and Nfx only decrease the growth of

Table 2. Time-dependent growth inhibition of the *T. cruzi* Y strain.

Compd	Inhibition [%][a]		
	Epimastigote	Trypomastigote	
	15 h	15 h	24 h
6f	62	16	32
6i	64	96	100
Bdz	53	84	98
Nfx	49	92	100
GV[b]	NT[c]	100	100

[a] Percent growth inhibition determined for each compound at 40 μM. [b] GV = gentian violet at 37.8 μg mL^{-1}. [c] NT = not tested.

T. cruzi culture after 24 h incubation, and these relevant effects may be associated with pharmacokinetic parameters. These observations suggest that the aryl-4-oxothiazolylhydrazone compounds could be endowed with an interesting pharmacokinetic profile in vitro. In tests of derivatives **6f** and **6i** (40 μM) against the trypomastigote (Y strain) form of *T. cruzi*, **6i** also shows excellent levels of inhibition, similar to the reference drugs for this assay. Unexpectedly, **6f** does not show significant activity against the trypomastigote form, at least at the doses tested herein.

Previous SAR results have shown that a thioamide terminal substitution (N^1 alkylation) or cyclization (to form the 2-pyrazoline-1-thiocarboxamide ring) in arylthiosemicarbazones are generally good for anti-*T. cruzi* activity.[12] Our results also revealed that if the thioamide amino group is cyclized to afford the 4-thiazolidone ring, the trypanocidal activity of the resulting compounds is significantly increased.

Although the well-known mechanism for Bdz depends on a reductive process,[25] we decided to evaluate, at least from the theoretical (docking) point of view, the Bdz model as a potential ligand of TCC to compare it with the other nine molecules of the series presented herein. The most stable docking solutions for the complexes between TCC and compounds **6a–i** and Bdz are presented in Table 3. For possible associations between experimental and theoretical data, the FlexX score was plotted against pIC_{50} (determined from IC_{50} values against the epimastigote Y strain; Figure 3).

Table 3. Docking results for compounds **6a–i** and Bdz.

Compd	pIC_{50}[a]	FlexX Score [kJ mol^{-1}]	
		R isomer	*S* isomer
6a	5.04	−11.35	−9.66
6b	4.44	−11.34	−9.15
6c	4.20	−11.09	
6d	4.62	−9.15	
6e	5.34	−9.98	−9.86
6f	6.47	−13.87	−11.61
6g	4.68	−12.85	−10.11
6h	5.04	−12.19	−11.40
6i	5.49	−11.50	−9.25
Bdz	5.74	−12.00	

[a] Calculated ($-\log IC_{50}$) from IC_{50} values [M] determined with Y strain epimastigotes (see Table 1).

Figure 3 provides evidence of an association between theoretical binding (docking) and the IC_{50} data; in general, the most potent compounds or those with greater pIC_{50} values, are those with the most negative FlexX docking scores, showing that the compounds with more stable or negative binding energies are also the most active (they have greater affinity for TCC), at least toward the epimastigote Y strain. It is clear that the best comparison would be between the FlexX docking score and the enzyme binding affinity data, but the purpose of Figure 3 is merely to call attention to this observed association between theoretical and experimental data, even though it does not constitute a strong statistical correlation. It is also important to point out that in each case the *R* enantiomer gives a better docking result than the *S* enantiomer, although the relative differences between the FlexX docking energies for *R* and *S* enantiomers are not systematic, as can be observed in Table 3.

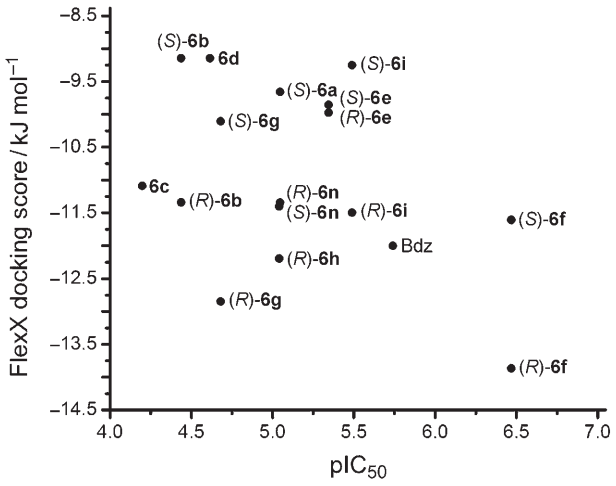


Figure 3. Relationship between the FlexX docking score [kJ mol^{-1}] and the pIC_{50} values (for IC_{50} values in M) of the *R* and *S* enantiomers of compounds **6a–i** and Bdz.

To compare the binding pattern of these molecules (**6a–i** and Bdz) with the crystallographic ligand ("186"), Figure 4 shows the conformations of the docking solutions presented in Table 3 alongside the co-crystallized ligand "186". The compound that produced the best result (the most negative docking score) in docking analysis, (*R*)-**6f**, was analyzed in detail along with the crystallographic structure of ligand "186" in TCC, and the results of this comparison are illustrated in Figure 5. (Figures 4, 5a, and 6 were generated using PyMOL v0.99).[26]

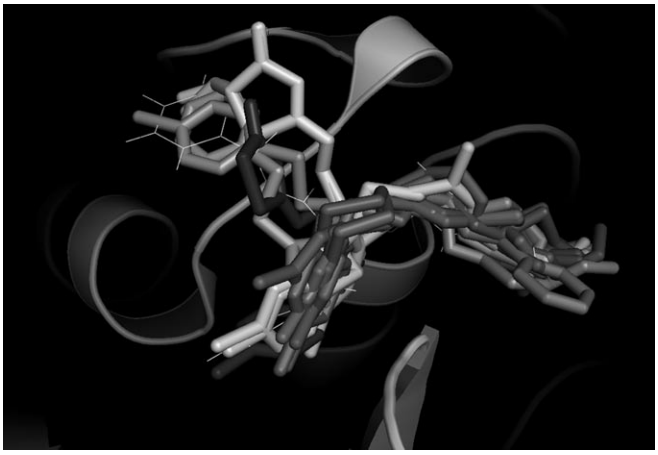


Figure 4. Superimposed docking conformations of compounds **6a–i** and Bdz, alongside the co-crystallized ligand "186" (represented by a thin line).

Figure 5a shows that (*R*)-**6f** forms hydrogen bonds with Gln 19, Asp 158, and Trp 177 in the TCC binding site with mea-

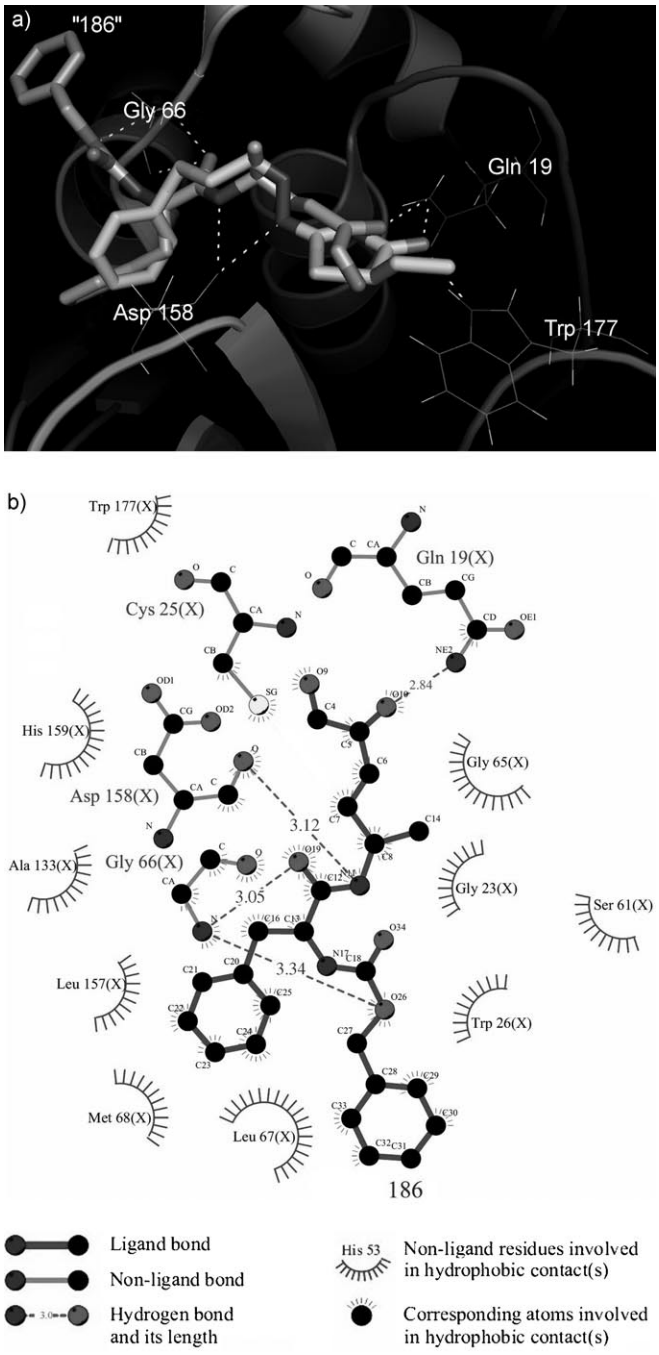


Figure 5. a) Docking solution for compound (*R*)-**6 f** alongside the co-crystallized ligand "186". b) Schematic representation of the crystallographic structure of ligand "186" in complex with TCC produced by the LIGPLOT program;[27] hydrogen bonds and hydrophobic interactions with specific residues are shown.

sured distances of 2.21, 2.92, and 2.75 Å, respectively. Notably, "186", co-crystallized with TCC, also forms important hydrogen bonds with Gln 19, Gly 66, and Asp 158, whereas the reference drug Bdz forms important hydrogen bonds with Gly 66 and Asp 158 (Figure 6). As shown in Figure 5 b, additional hydrophobic interactions (omitted from Figure 5 a for clarity) are present, mainly with the compounds' phenyl rings, and can be

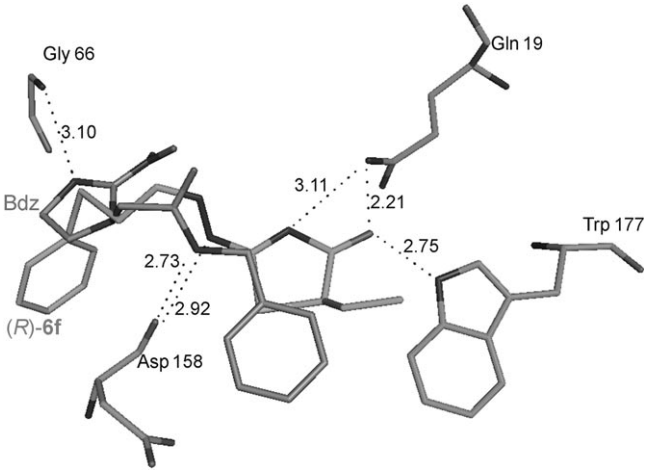


Figure 6. Docking conformations for compounds (*R*)-**6 f** and Bdz. The most important polar interactions (hydrogen bonds) are shown in dotted lines with the respective distances (Å) to the residues involved.

observed in a detailed inspection of the docking conformations.

Conclusions

Our results suggest that aryl-4-oxothiazolylhydrazones constitute validated lead compounds (along with the bioisosteres arylthiosemicarbazones and 2-pyrazoline-1-thiocarboxamides) capable of inhibiting the growth of *T. cruzi* at non-cytotoxic levels. This is the case with **6 f**, which is more potent than Bdz and Nfx, and has pharmacokinetic advantages. The docking model generated with the TCC structure[22] seems consistent with the experimental results, showing an interesting association with pIC_{50} values. The binding patterns observed for all docked molecules, particularly for (*R*)-**6 f** and Bdz, are quite similar to those of the "186" co-crystallized molecule, showing important polar interactions with the same critical residues in the active site. Asp 158 seems to play an important role in the main hydrogen bonds between TCC and the most active molecules (*R*)-**6 f** and Bdz.

Experimental Section

Chemistry: All melting points were determined with a Thomas Hoover apparatus and are uncorrected. IR spectra were obtained using KBr pellets. ^{1}H NMR spectra and NOESY contour map (for compound **6 b**) were measured on a Varian UNITY plus 300 MHz NMR spectrometer, with [D_6]DMSO as solvent and tetramethylsilane as an internal standard. Elemental analyses were performed on a PerkinElmer 2400 instrument, and the results were within acceptable range. Thin-layer chromatography (TLC) was carried out on silica gel plates with fluorescence indicator F_{254} (0.2 mm, Merck); the spots were visualized under UV light. Column chromatography was performed on silica with Kieselgel 60 (230–400 Mesh, Merck). All reagents used were analytical grade. The aldehydes or ketones were prepared as described in reference [17].

Syntheses: General procedure for preparation of compounds **6 a–i**; example: *N*-(4-oxo-5-ethyl-2′-thiazolin-2-yl)-*N*′-phenylthio-(*Z*)-ethyli-

denehydrazone (**6 f**): The aldehyde (1.0 g, 6.5 mmol) was dissolved in MeOH (10 mL), and AcOH (1 mL) was then added to solution at room temperature. Thiosemicarbazide (0.62 g, 6.5 mmol) was added 10 min later, and the mixture was held at reflux for 12 h. The product was filtered and treated with water (50 mL) and washed with brine (20 mL) to furnish pure arylthiosemicarbazone (1.2 g, 91%). This intermediate (1.0 g, 4.3 mmol) was diluted with EtOH (20 mL), anhydrous NaOAc (0.7 g, 8.5 mmol) was added, and the mixture was stirred for 15 min. Then α-chlorobutyric acid (0.62 g, 5.0 mmol) was added, and the reaction was stirred at reflux for 8 h. Ice (15 g) was then added, and the precipitate formed was filtered and washed with 0.1 M $KHSO_4$, brine, and water to obtain pure **6 f** (0.61 g, 51%).

Yield, melting point, recrystallization solvent, IR and H NMR spectroscopic data, and analytical results are listed for each of the following compounds:

***N*-(4-Oxo-5-methyl-2′-thiazolin-2-yl)-*N*′-*p*-chlorophenylthio-(*Z*)-ethylidenehydrazone (6 a):** 56% yield; mp: 146–7 °C (from 95% EtOH); IR (KBr): $\tilde{\nu}$ = 1100 (C–S heterocyclic); 1600 (C=N heterocyclic), 1640 (C=N hydrazone), 1720 (C=O), 2990 cm^{-1} (N–H); ^{1}H NMR ([D_6]DMSO): δ = 1.50 (d, J = 12 Hz, 3H, CH_3), 3.90 (d, J = 10 Hz, 2H, CH_2), 4.20 (m, 1H, CH–S), 7.44 (m, 4H, Ar), 7.70 ppm (t, J = 12 Hz, 1H, CH=N); anal. calcd for $C_{12}H_{12}N_3S_2OCl$: C 45.93, H 3.85, N 13.39; found: C 45.76, H 3.79, N 12.98.

***N*-(4-Oxo-5-methyl-2′-thiazolin-2-yl)-*N*′-phenylthio-2-(*Z*)-propylidenehydrazone (6 b):** 44% yield; mp: 138–9 °C (from abs EtOH); IR (KBr): $\tilde{\nu}$ = 1090 (C–S heterocyclic), 1590 (C=N heterocyclic), 1645 (C=N hydrazone), 1725 (C=O), 3090 cm^{-1} (N–H); ^{1}H NMR (see Supporting Information); anal. calcd for $C_{13}H_{15}N_3S_2O$: C 53.22, H 5.15, N 14.32; found: C 53.31, H 5.15, N 14.39.

***N*-(4-Oxo-2′-thiazolin-2-yl)-*N*′-*p*-methylthio-(*Z*)-ethylidenehydrazone (6 c):** 30% yield; mp: 155–156 °C (from 95% EtOH); IR (KBr): $\tilde{\nu}$ = 1090 (C–S heterocyclic), 1595 (C=N heterocyclic), 1635 (C=N hydrazone), 1730 (C=O), 2990 cm^{-1} (N–H); ^{1}H NMR ([D_6]DMSO): δ = 2.21 (s, 3H, Ar–CH_3), 3.70 (s, 2H, CH_2 heterocyclic), 3.72 (d, J = 12 Hz, 2H, CH_2), 7.20 (m, 4H, Ar), 7.61 ppm (t, J = 12 Hz, 1H, CH=N); anal. calcd for $C_{12}H_{13}N_3S_2O$: C 51.59, H 4.69, N 15.04; found: C 51.70, H 4.50, N 15.05.

***N*-(4-Oxo-2′-thiazolin-2-yl)-*N*′-*p*-chlorophenylthio-2-(*Z*)-propylidenehydrazone (6 d):** 30% yield; mp: 155–156 °C (from 95% EtOH); IR (KBr): $\tilde{\nu}$ = 1100 (C–S heterocyclic), 1600 (C=N heterocyclic), 1640 (C=N hydrazone), 1710 (C=O), 2990 cm^{-1} (N–H), ^{1}H NMR ([D_6]DMSO): δ = 1.90 (s, 3H, CH_3), 3.70 (s, 2H, CH_2), 3.76 (s, 2H, CH_2), 7.39 (d, J = 8.9 Hz, 2H, H′-3 and H′-5); 7.60 ppm (d, J = 8.9 Hz, 2H, H′-2′ and H′-6′); anal. calcd for $C_{12}H_{12}N_3S_2OCl$: C 45.93, H 3.85, N 13.39; found: C 46.00, H 3.90, N 13.38.

***N*-(4-Oxo-5-methyl-2′-thiazolin-2-yl)-*N*′-*p*-bromophenylthio-(*Z*)-ethylidenehydrazone (6 e):** 33% yield; mp: 155 °C (from 95% EtOH); IR (KBr): $\tilde{\nu}$ = 1100 (C–S heterocyclic), 1620 (C=N heterocyclic), 1660 (C=N hydrazone), 1730 (C=O), 2990 cm^{-1} (N–H); ^{1}H NMR ([D_6]DMSO): δ = 1.38 (d, J = 12 Hz, 3H, CH_3), 3.78 (d, J = 6.7 Hz, 2H, CH_2), 4.10 (m, 1H, CH), 6.95 (t, J = 6.5 Hz, 1H, CH=N), 7.16 (d, J = 8.3 Hz, 2H, H′-3 and H′-5); 7.30 ppm (d, J = 8.3 Hz, 2H, H′-2′ and H′-6′); anal. calcd for $C_{12}H_{12}N_3S_2OBr$: C 40.23, H 3.38, N 11.73; found: C 40.00, H 3.15, N 11.21.

***N*-(4-Oxo-5-ethyl-2′-thiazolin-2-yl)-*N*′-phenylthio-(*Z*)-ethylidenehydrazone (6 f):** 51% yield; mp: 120 °C (from 95% EtOH); IR (KBr):$\tilde{\nu}$ = 1100 (C–S heterocyclic), 1585 (C=N heterocyclic), 1640 (C=N hydrazone), 1720 (C=O), 3090 cm^{-1} (N–H); ^{1}H NMR ([D_6]DMSO): δ = 0.77 (t, J = 7.0 Hz, 3H, CH_3), 1.71 (m, 1H, CH_2), 1.93 (m, 1H, CH_2′), 3.09 (m, 1H, CH-S), 3.70 (m, 2H, CH_2-S), 6.92 (t, J = 7.0 Hz, 1H, CH=N), 7.20 ppm (m, 5H, Ar); anal. calcd for $C_{13}H_{15}N_3S_2O$: C 53.22, H 5.15, N 14.32; found: C 52.95, H 4.98, N 14.00.

***N*-(4-Oxo-5-ethyl-2′-thiazolin-2-yl)-*N*′-*p*-methylphenylthio-(*Z*)-ethylidenehydrazone (6 g):** 36% yield; mp: 115 °C (from 95% EtOH); IR (KBr): $\tilde{\nu}$ = 1090 (C–S heterocyclic), 1605 (C=N heterocyclic), 1640 (C=N hydrazone), 1725 (C=O), 3020 (N–H); ^{1}H NMR ([D_6]DMSO): δ = 0.80 (t, J = 7.4 Hz, 3H, CH_3), 1.81 (m, 2H, CH_2), 2.20 (s, 3H, CH_3), 3.20 (m, 1H, CH), 3.53 (m, 2H, CH_2), 7.20 (m, 4H, Ar), 7.58 ppm (t, J = 6.6 Hz, 1H, CH=N); anal. calcd for $C_{14}H_{17}N_3S_2O$: C 54.70, H 5.57, N 13.67; found: C 53.90, H 5.60, N 14.00.

***N*-(4-Oxo-5-ethyl-2′-thiazolin-2-yl)-*N*′-*p*-chlorophenylthio-(*Z*)-ethylidenehydrazone (6 h):** 36% yield; mp: 155 °C (from 95% EtOH); IR (KBr):$\tilde{\nu}$ = 1100 (C–S heterocyclic), 1585 (C=N heterocyclic), 1640 (C=N hydrazone), 1720 (C=O), 3090 cm^{-1} (N–H); ^{1}H NMR ([D_6]DMSO): δ = 0.85 (t, J = 7.4 Hz, 3H, CH_3), 1.80 (m, 2H, CH_2), 3.26 (m, 1H, CH), 3.75 (m, 2H, CH_2), 7.30 (m, 4H, Ar), 7.55 ppm (t, J = 6.6 Hz, 1H, CH=N); anal. calcd for $C_{13}H_{14}N_3S_2OCl$: C 47.63, H 4.30, N 12.82; found: C 48.00, H 4.30, N 12.90.

***N*-(4-Oxo-5-ethyl-2′-thiazolin-2-yl)-*N*′-phenylthio-(*Z*)-*n*-propylidenehydrazone (6 i):** 56% yield; mp: 125 °C (from abs EtOH); IR (KBr/cm^{-1}): $\tilde{\nu}$ = 1100 (C–S heterocyclic), 1615 (C=N heterocyclic), 1650 (C=N hydrazone), 1710 (C=O), 3030 cm^{-1} (N–H); ^{1}H NMR ([D_6]DMSO): δ = 0.80 (t, J = 6.9 Hz, 3H, CH_3), 1.85 (m, 2H, CH_2), 2.60 (m, 2H, CH_2), 3.25 (t, J = 6.9 Hz, 2H, CH_2), 3.53 (m, 1H, CH), 7.40 (m, 5H, Ar), 7.85 ppm (t, J = 6.6 Hz,1H, CH=N); anal. calcd for $C_{15}H_{20}N_3S_2O$: C 55.87, H 6.25, N, 13.03; found: C 55.80, H 6.00, N 12.89.

Cytotoxicity assay: The cytotoxicity of the compounds was determined using Balb/c mice splenocytes (5×10^6 cells $well^{-1}$) cultured in 96-well plates in Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO, USA) supplemented with 10% fetal calf serum (FCS; Cultilab, Campinas, SP, Brazil) and 50 $\mu g\,mL^{-1}$ gentamycin (Novafarma, Anápolis, GO, Brazil). Each compound was evaluated at three concentrations (1, 10, and 100 $\mu g\,mL^{-1}$) in triplicate. Considering an average molecular weight of 300 $g\,mol^{-1}$, these concentrations respectively correspond with 3.25, 32.5, and 325 μM. Cultures were incubated in the presence of [^{3}H]thymidine (1 μCi $well^{-1}$) for 24 h at 37 °C and 5% CO_2. After this period, the content of the plate was harvested to determine the amount of [^{3}H]thymidine incorporated using a β-emission counter (β-matrix 9600, Packard). The toxicity of the compounds was determined by comparing the percent [^{3}H]thymidine incorporated (as indicator of cell viability) in drug-treated wells relative to untreated wells. Non-toxic concentrations were defined as those causing a decrease in [^{3}H]thymidine incorporation $<$ 10% relative to untreated controls.

Anti-*T. cruzi* assay: Epimastigotes of *T. cruzi* (Y and Colombian strains) were cultivated at 26 °C in liver infusion tryptose medium (LIT) supplemented with 10% FCS, 1% hemin, 1% R9 medium, and 50 $\mu g\,mL^{-1}$ gentamycin. Parasites (10^6 cells mL^{-1}) were cultured in fresh medium in the absence or presence of the compounds tested, or 0.01 $mg\,mL^{-1}$ benznidazole (Rochagan, Roche). Cell growth was determined after culture for 11 days and also 15 h (for compounds **6 f** and **6 i**) by counting viable forms in a hemocytometer. The compounds were prepared from a stock solution in DMSO. To determine IC_{50} values, cultures of Y strain epimastigotes in the presence of various compound concentrations were evaluated after 11 days as described above. IC_{50} calculation was carried out using nonlinear regression on Prism 4.0 GraphPad software. Y strain *T. cruzi* trypomastigotes were obtained from culture supernatants of the LCC-MK2 cell line at 37 °C and placed in 96-well

plates (4×10^5 well^{-1}) in DMEM supplemented with 10% FCS and 50 μg mL^{-1} gentamycin. Compounds were added at nontoxic concentrations in triplicate. Viable parasites were counted in a hemocytometer 15 and 24 h after addition of compounds by way of trypan blue exclusion. Percent inhibition was calculated relative to untreated cultures.

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