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Thiosemicarbazones derived from 1-indanones as new anti-*Trypanosoma cruzi* agents

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ABSTRACT

In the present work, we synthesized a series of thiosemicarbazones derived from 1-indanones with good anti-*Trypanosoma cruzi* activity. Most of them displayed remarkable trypanosomicidal activity. All the compounds showed nonspecific cytotoxicity on human erythrocytes. The ability of the new compounds to inhibit cruzipain, the major cysteine protease of *T. cruzi*, was also explored. Thiosemicarbazones **12** and **24** inhibited this enzyme at the dose assayed. This interaction was also studied in terms of molecular docking.

1. Introduction

Chagas' disease or American Trypanosomiasis is a parasitic disease endemic to Latin America, where it affects about 20 million people. Mortality rates range from 8% to 12%, depending on the age and physiological state of the patient.[1] The causative agent of this disease is the flagellate protozoan *Trypanosoma cruzi* (*T. cruzi*), which is transmitted to humans either by transfusion of infected blood, from an infected mother to her child, by the feces of several species of triatomine bugs that are strictly hematophages, or orally by contaminated food.

The disease is controlled at present through the elimination of the vectors with insecticides and serological screening of blood. Better housing and educational campaigns are also fruitful approaches. Like other parasitic diseases, Chagas' disease is associated with poverty and low educational levels. The development of vaccines has thus far been unsuccessful.[2] Chemotherapy to control this parasite infection is very limited and is based on the nitroderivatives Nifurtimox (Nfx) and Benznidazole; these drugs have variable therapeutic effects according to the geographical region, and require long term treatment, besides they frequently have toxic side effects.[3] Therefore, it is necessary to find a more effective and safer therapy against Chagas' disease.

In this regard, thiosemicarbazones (TSCs) have been described to present parasitocidal action against *T. cruzi*.[4] TSCs are a class of small molecules that have been evaluated over the last 50 years as antiviral,[5] antibacterial[6] and anticancer compounds.[7] Du and co-workers first introduced the thiosemicarbazone functionality into compounds designed to inhibit cruzipain,[8] the major cysteine protease expressed in all the life cycle stages of the parasite. This endoproteinase is the most abundant member among the cysteine-, serine-, threonine-, and metallo-proteinases, and it is expressed as a complex mixture of isoforms. The bulk of the enzyme is lysosomal and is also present in an epimastigote-specific pre-lysosomal organelle called 'reservosome'; in addition, some plasma membrane-bound isoforms and cruzipain forms released into the medium have been reported. Cruzipain is essential for infection host cells, replication and metabolism of the parasite and plays multiple roles in disease pathogenesis, therefore has been emerged as one of the best targets for the development of vaccines and drugs with anti-*T. Cruzi* activity.[8]

In the search for a pharmacological control of Chagas' disease, new 5-nitrofurane derivatives containing thiosemicarbazone moiety and their metallic complexes have been designed and synthesized, showing significant anti-*T. cruzi* activity.[9]

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Previously, we have reported the evaluation of several TSCs derived from 1-indanones with different patterns of substitution in the aromatic ring against bovine viral diarrhea virus (BVDV) as a surrogate model of hepatitis C virus (HCV).[10] The TSC derived from 5,6-dimethoxy-1-indanone showed the most potent anti-BVDV activity, with a selectivity index higher than that of the reference drug Ribavirin.

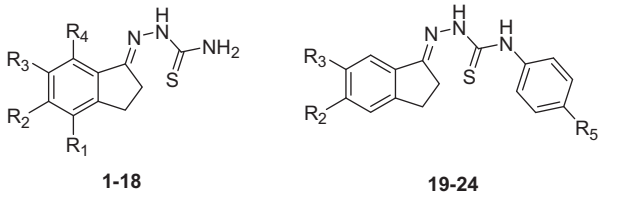
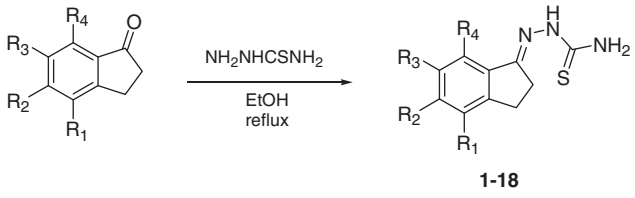
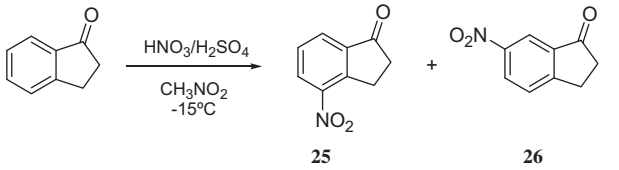


Figure 1. General chemical structures of the TSCs and N^4-TSCs synthesized.



Scheme 1. Synthetic procedure for obtaining TSCs **1**–**18**.



Scheme 2. Synthesis of 4-nitro- and 6-nitro-1-indanone.

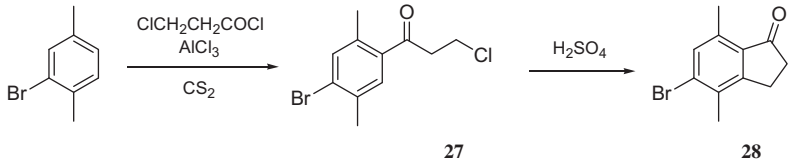
Taking into account the anti-*T. cruzi* activity presented by compounds possessing a thiosemicarbazone moiety, we decide to investigate the potential antichagasic action of TSCs derived from 1-indanones previously synthesized (**1**–**14**). The promising antichagasic activity showed by these compounds led us to prepare new TSCs with other substituents on the aromatic ring of the indanic nucleus and different substitution patterns, as well as N^4-substituted TSCs with phenyl groups (N^4-TSCs). In this work, we present the in vitro anti-proliferative activity of twenty-four TSCs (**1**–**24**) (Fig. 1), including the synthesis of four new TSCs and six new N^4-TSCs, all derived from 1-indanones, on the epimastigote form of *T. cruzi*. We developed a microwave-assisted synthesis that comprises a three-component coupling reaction for N^4-TSCs preparation in good yields. A structure–activity relationship is also discussed to highlight the structural requirements for optimal activity. Docking analysis with cruzipain was performed in order to explore the capacity of these TSCs to inhibit this enzyme. Also, experimental cruzipain inhibition capacity was experimentally investigated for some selected features.

2. Results and discussion

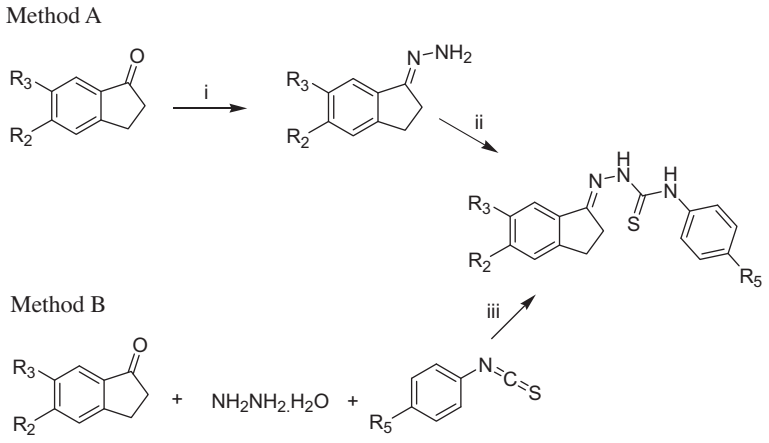
2.1. Chemistry

TSCs **1**–**18** were synthesized in high yield from the corresponding 1-indanones by treatment with thiosemicarbazide, according to the general procedure previously described[10] (Scheme 1).

TSCs **15**–**18** are new compounds. 1-Indanone precursors were available from different sources: 5-fluoro-1-indanone is a commercial reagent; 4-nitro-1-indanone (**25**) and 6-nitro-1-indanone (**26**) were prepared by nitration of 1-indanone, as shown in Scheme 2;



Scheme 3. Synthesis of 5-bromo-4,7-dimethyl-1-indanone.

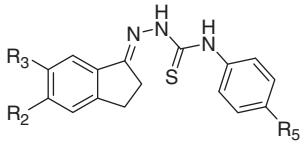


Scheme 4. Synthetic routes for obtaining N^4-TSCs. Reaction conditions: (i) hydrazine hydrate 85%/isopropanol, reflux; (ii) isothiocyanate/benzene, room temperature; (iii) acetic acid, isopropanol, MW.

and, finally, 5-bromo-4,7-dimethyl-1-indanone (**28**) was obtained following the protocol presented in Scheme 3.

The N^4-TSCs were obtained by two different synthetic methodologies. The first approach (Method A, Scheme 4) involves a procedure that comprises two steps: (1) synthesis of hydrazone from the corresponding 1-indanone and hydrazine hydrate, (2) preparation of the desired N^4-TSCs by reaction of hydrazone with the suitable isothiocyanate. This method includes the isolation of each prepared intermediate, which are time, solvent and energy consuming procedure.

Table 1
Comparative results obtained in the synthesis of N^4-TSCs by Methods A and B



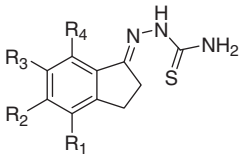
Compound	R_2	R_3	R_5	Method A		Method B	
				Time (h)	Yield (%)	Time (min)	Yield (%)
19	H	H	H	25	89	20	89
20	H	H	CH_3	48	72	10	78
21	H	H	Cl	4	79	30	73
22	OCH_3	OCH_3	H	25	77	15	74
23	OCH_3	OCH_3	CH_3	72	53	20	69
24	OCH_3	OCH_3	Cl	48	79	20	84

The second methodology (Method B, Scheme 4) is a one-pot synthesis via a multicomponent coupling reaction, under microwave irradiation (MW). For this environmentally friendly synthesis toward N^4-TSCs, in the same pot we mixed one equivalent of the corresponding 1-indanone and an excess of 15% of isothiocyanate and hydrazine hydrate in isopropanol and a catalytic amount of acetic acid. This route afforded N^4-TSCs in good yields and short reaction times. Comparative results obtained in both synthetic processes are shown in Table 1.

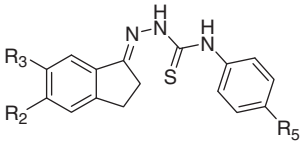
2.2. Anti-*T. cruzi* evaluation

TSCs **1**–**18** and N^4-TSCs **19**–**24** were evaluated against the epimastigote form of *T. cruzi*, Tulahuen 2 strain (Table 2). The occurrence of the epimastigote form of *T. cruzi* as an obligate mammalian intracellular stage has been reevaluated and confirmed.[11] Furthermore, it should be noted that a good correlation between the anti-proliferative epimastigote activity and the in vivo anti-*T. cruzi* activity was observed with compounds from our chemical library.[12] Firstly, the mentioned compounds were assayed at 25 μM concentration; all the TSCs were incorporated into the growth medium at 25 μM and its ability to inhibit the growth of the parasite was evaluated by comparison with untreated controls on day 5 and using Nfx as reference trypanosomicidal drug.[12] The 50% inhibitory concentration (IC_{50}) was determined. TSCs derived from 1-indanones **1**–**18** exhibited, in some cases, relevant anti-*T. cruzi* activities, the most active being the 4,5-dimethoxy-subtituted

Table 2
In vitro activity of synthesized TSCs



1-18



19-24

Compound	R_1	R_2	R_3	R_4	R_5	IC_{50}[a] (μM)	PGI[b]	% Hemolysis[c] (25 μM)	SI[d]
1	H	H	H	H		18.6	80.8	0.2	404.0
2	H	CH_3	H	H		3.6	100.0	0.5	200.0
3	CH_3	H	H	H		4.0	98.5	0.1	985.0
4	H	CH_3	H	CH_3		4.3	66.5	0.1	665.0
5	Br	CH_3	H	CH_3		5.7	100.0	7.7	12.9
6	H	CH_3	Cl	CH_3		>50	14.4	2.8	5.1
7	CH_3	Cl	CH_3	H		7.8	83.7	0.1	837
8	OCH_3	H	H	H		6.2	84.2	1.0	84.2
9	H	OCH_3	H	H		7.5	92.1	0.8	115.1
10	H	H	OCH_3	H		7.5	100.0	7.5	13.3
11	H	OCH_3	OCH_3	H		>50	19.0	0.1	190.0
12	OCH_3	OCH_3	H	H		1.8	98.9	0.1	989.0
13	H	Cl	H	H		>50	1.7	0.1	170.0
14	H	Br	H	H		2.3	98.8	2.4	41.2
15	NO_2	H	H	H		>50	22.0	4.5	4.9
16	H	H	NO_2	H		>50	3.1	0.1	310.0
17	H	F	H	H		8.3	84.2	0.2	421.0
18	CH_3	Br	H	CH_3		25.0	51.0	2.4	21.2
19	H	H	H	H	H	7.7	69.6	2.7	25.8
20	H	H	H	H	CH_3	5.9	76.0	1.6	47.5
21	H	H	H	H	Cl	25	50.8	0.1	508.0
22	H	OCH_3	OCH_3	H	H	1.0	67.5	0.4	168.8
23	H	OCH_3	OCH_3	H	CH_3	3.0	77.7	4.2	18.5
24	H	OCH_3	OCH_3	H	Cl	1.0	77.0	0.1	770
Nfx						7.7	100.0	–	–
Amphotericin B						0.152[e]	100.0[e]	100.0[e]	1.0[e]

[a] IC_{50}: concentration that produces 50% reduction in parasite growth. All the values are the mean of three different experiments.
[b] PGI: percentage of parasite growth inhibition at 25 μM. All the values are the mean of three different experiments.
[c] % Hemolysis: percentage of erythrocytes lysis at 25 μM.
[d] SI: selectivity index, ratio between PGI and % hemolysis.
[e] Taken from Ref. 9h.

TSC **12** (Table 2). Despite the interesting biological properties of some of these molecules, it is difficult to highlight in detail their structure–activity relationship. Nevertheless, some general aspects merit to be commented: chloro and nitro TSCs were not active against *T. cruzi* in culture (**6**, **13**, **15** and **16**), except compound **7** with moderate activity. TSCs with a methyl substituent in the aromatic ring showed an increased anti-*T. cruzi* activity (**2**, **3** and **4**). Also, it should be noted that the relative position of the methoxyl group is not significant when the TSC is monosubstituted (**8**, **9** and **10**), however, when the TSC is disubstituted, there is a remarkable difference in activity (**11** and **12**). All N^4-TSCs (**19**–**24**) displayed very good activities, being more active than the reference drug Nfx, with the only exception of derivative **21**. The increase in the anti-*T. cruzi* activity when N^4-substitution was performed is clearly noted. Moreover, the 5,6-dimethoxy substitution gives rise to some of the best anti-*T. cruzi* agents (compare compounds **22**, **23** and **24,** with an IC_{50} of 1.0, 3.0 and 1.0 μM, respectively, with compound **11,** with an IC_{50} >50 μM).

2.3. Unspecific mammalian cell cytotoxicity

TSCs were evaluated in terms of the non-specific cytotoxicity using human erythrocytes as a mammalian cell model.[9h] In these experiments, Amphotericin B was used as a reference drug due to its recognized hemolytic effects. All the TSCs were evaluated at 25 μM and compared with the parasite growth inhibition at the same concentration (Table 2). None of the TSCs studied showed remarkable unspecific mammalian cytotoxicity, with percentages of erythrocyte lysis between 0.1 and 7.7. Therefore, the selectivity indexes (SI), defined as the ratio between the percentages of parasite growth inhibition at 25 μM (PGI) and of hemolysis, showed values much higher than for Amphotericin B. These results demonstrate that these TSCs could be considered excellent lead compounds as anti-*T. cruzi* agents.

2.4. Molecular docking studies

Molecular docking models have been shown to adequately predict the binding mode of different anti-*T. cruzi* thiosemicarbazone derivatives to cruzipain.[9g,13] Therefore, in order to gain more insights into the nature of the trypanosomicidal activity displayed by the novel TSCs reported in this work, molecular docking studies with cruzipain were performed. The theoretical dissociation constants for the compounds studied as potential cruzipain inhibitors (K_d CP) range from 10^{-4} to 10^{-2} M, indicating that these molecules could be moderate inhibitors of the enzyme (Figure 2). It is worth noticing that TSCs **12**, **22** and **24**, the most active compounds, are those with best cruzipain inhibition capacity, with K_d values in the order of 10^{-4} M. On the other hand, TSCs **2**, **3**, **4** and **23** showed K_d values one order of magnitude higher, in agreement with their good anti-*T. cruzi* activities. In the same sense, TSC **15,** an inactive agent, showed the highest K_d, demonstrating that, in general terms, this molecular model is able to adequately predict the experimental data regarding the trypanosomicidal profile of these TSCs. An exception to this model would be compounds **11** and **16** (inactive compounds) with K_d values in the order of 10^{-4} M.

2.5. Inhibition of cruzipain

Some TSCs showing variable activity against *T. cruzi* were selected to evaluate their capacity to inhibit *T. cruzi* cruzipain. The selection was based on the values of the dissociation constants of the TSCs studied. The compounds selected were **8**, **10**, **11**, **12**, **16**, **22** and **24** and the results are shown in Table 3. (Note: the reference compound in the experimental assay against cruzipain has a theoretical K_d of 6.2×10^{-4} M.)[9h] In a previous work, we investigated for the first time the mechanism governing the self aggregation of TSCs in water (in particular compound **11**). Overall information indicated the fast initial formation of negatively-charged nano-aggregates that gradually grew in size to generate larger clusters, these structures serving as nuclei for the later crystallization and precipitation of the compound in water.[14] We extended the study of aggregation to the compounds that were selected to be evaluated as inhibitors of cruzipain, and we observed that these derivatives have a similar tendency to form such aggregates, therefore the differences in activity should not be attributed to this phenomenon. The most active anti-proliferative compounds were those with the highest inhibitory action against cruzipain (see derivatives **12** and **24**). An exception was compound **22,** which is an excellent anti-*T. cruzi* agent, without capacity to inhibit cruzipain, this result suggests that this compound could be acting by another mechanism of action. The lack of activity against cruzipain of derivative **22** emphasizes the importance of the chlorine-substitution in position 4 of the N^4-phenyl ring, being this the only structural difference between compounds **22** and **24**. By comparing the cruzipain inhibitory profile of compounds **11** and **12** (1.7% vs 61%), it is clear that 4,5-dimethoxy substitution significantly improves the inhibitory activity with respect to the 5,6-dimethoxy substitution against cruzipain. TSCs **8** and **10**, with moderate activity, are not capable to inhibit cruzipain. The best inhibitor evaluated against cruzipain was N^4-TSC **24**, with 67% inhibition. These results indicate that compounds **12** and **24**

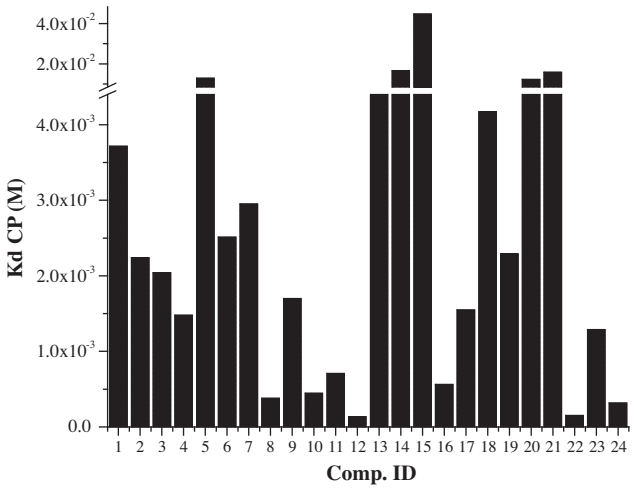


Figure 2. Dissociation constants of the TSCs studied.

Table 3
Cruzipain inhibition activities of selected TSC compared to IC_{50} against *T. cruzi* epimastigotes

Compound	IC_{50}[a] (μM)	%Inh CP[b] (100 μM)
8	6.2	9.8
10	7.5	0.0
11	>50	1.7
12	1.8	61.0
16	>50	24.0
22	1.0	0.0
24	1.0	67.0
3′-Bromopropiophenone thiosemicarbazone[c]	—	100.0

[a] IC_{50}: concentration that produces 50% reduction in parasite growth. All the values are the mean of three different experiments.
[b] % Inh CP: percentage of cruzipain inhibition at 100 μM.
[c] Reference compound.[8]

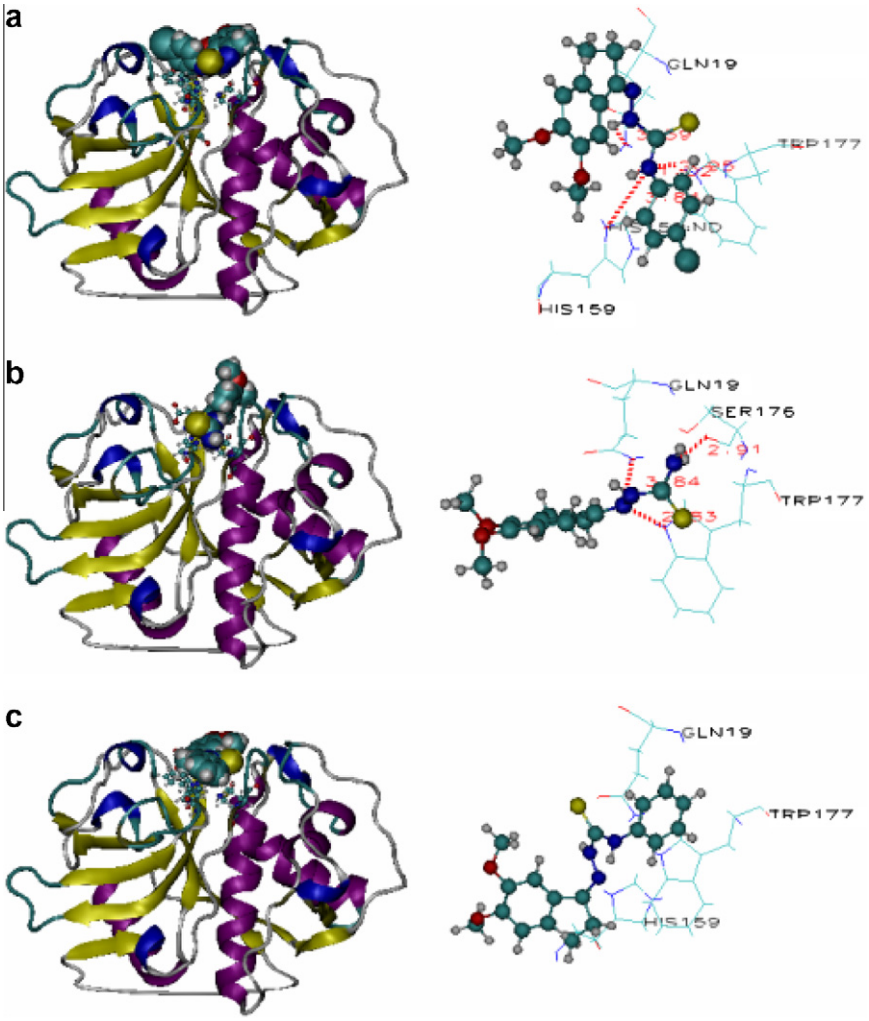


Figure 3. Best conformations of TSCs **24** (a), **12** (b) and **22** (c) into cruzipain active-site cleft.

emerge as cruzipain inhibitor lead structures for future chemical optimization based on rational drug design.

The mode of interaction of the best experimental inhibitors **12** and **24** with cruzipain was studied using theoretical models. As illustrated in Figure 3, both compounds occupy the substrate binding site by establishing interactions with different residues lining the cleft. The Nε1 hydrogen of Trp177 makes hydrogen bonds to the thiosemicarbazone N^4 of inhibitor **24** and N^1 of inhibitor **12** (Fig. 3a and b, respectively). Nε2 of Gln19 forms a hydrogen bond to the thiosemicarbazone N^2 hydrogen of **24** and interacts with the thiosemicarbazone N^1 of **12**. The Nδ1 hydrogen of His159 makes hydrogen bonds to the thiosemicarbazone N^4 of **24**. The thiosemicarbazone N^4 in compound **12** forms a hydrogen bond with the CO oxygen of Ser176. Moreover, the 4-chlorophenyl moiety of **24** makes a large hydrophobic contact with the aromatic side chain of Trp177. This aromatic interaction and the hydrogen bond to His159 are lost in compound **12**, which is less stabilized than **24** in the binding cleft. This behavior is in accordance with our experimental results. It is worth noting that the methoxy groups in the 4,5-position of compound **12** are oriented toward the solvent (Fig. 3b). According to the docking results, compounds **24** and **22** (Fig. 3a and c, respectively) bind to the enzyme in a similar fashion. Therefore, the lack of activity against cruzipain displayed by the latter could be related to solubility problems in the conditions of the assay.

3. Conclusions

Here, we developed a microwave-assisted one-pot three-component synthesis for a rapid preparation of N^4-TSCs with good yields. The main advantages of this method are its simple purification process and its short experimental time for the reaction to be completed. Among all the twenty-four TSCs prepared in this work, derivatives **12**, **22** and **24** were excellent anti-*T. cruzi* agents with excellent selectivity indexes higher than 150, thus indicating that these TSCs can be lead trypanosomicidals. Also, TSC **12** and **24** displayed inhibitory activity against cruzipain, the major cysteine protease of *T. cruzi*, pointing this as the target of action. The mode of action was explained using molecular docking studies.

4. Experimental

4.1. Chemistry

Melting points (uncorrected) were determined on a Thomas Hoover apparatus. Thin layer chromatography (TLC) was used to monitor reactions. Flash chromatography was performed with silica gel (Merck silica gel 60, 230–400 mesh). IR spectra were recorded as KBr pellets using a Perkin Elmer Spectrum One FT-IR spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker 500 MHz spectrometer (see the NMR spectra in the

Supplementary data). High resolution mass spectra were obtained on Bruker micrOTOF-Q II spectrometer. Micro-analyses were carried out on a Carlo Erba elemental analyzer (Model 1106) and were within 0.4% of the theoretical values. Microwave-assisted reactions were carried out in a Microwave Synthesis Reactor Monowave 300 Anton Paar. Absorbance readings were made in a Flex Station 3 Multi-mode Microplate Reader.

4.1.1. 4-Nitro-indan-1-one (25) and 6-nitro-indan-1-one (26)

To a well-stirred mixture of H_2SO_4 (13.9 mL of a 98% solution) and HNO_3 (2.6 mL of a 65% solution) was added at −10 °C a solution of 1-indanone (1.0 g, 7.6 mmol) in nitromethane (1.1 mL). The addition rate was carefully adjusted to raise the temperature from −10 to −5 °C during 30 min. The reaction mixture was stirred further for 15 min at this temperature (attention has to be paid to temperature and reaction time!). After ice-water hydrolysis (200 mL), the yellow precipitate was collected and extracted with CH_2Cl_2 (2 × 20 mL). The organic phase was washed with a $KHCO_3$ solution 5% (2 × 15 mL) washed with saturated brine (10 mL), dried over anhydrous Na_2SO_4 and evaporated in vacuo. The residue was separated by flash chromatography using hexane–ethyl acetate (1:0.2) as eluent. Compound **25**, yield: 0.94 g, 80%, mp: 98–100 °C (mp lit.[15] 100–101 °C) ^{1}H NMR ($CDCl_3$) δ ppm: 2.80 (m, 2H, CH_2), 3.53 (m, 2H, CH_2), 7.61 (m, 1H, H-Ar), 8.08 (d, J = 8.5 Hz, 1H, H-Ar), 8.47 (d, J = 8.3 Hz, 1H, H-Ar). Compound **26**, yield: 0.23 g, 20%, mp: 73–74 °C (mp lit.[16] 72–73 °C). ^{1}H NMR ($CDCl_3$) δ ppm: 2.83 (m, 2H, CH_2), 3.28 (m, 2H, CH_2), 7.61 (d, J = 8.5 Hz, 1H, H-Ar), 8.44 (dd, J = 2.2 Hz, J = 8.5 Hz, 1H, H-Ar), 8.56 (d, J = 2.2 Hz, 1H, H-Ar).

4.1.2. 1-(4-Bromo-2,5-dimethyl-phenyl)-3-chloro-propan-1-one (27)

$AlCl_3$ (2.60 g, 19.5 mmol) was added to a magnetically stirred solution of 2-bromo-1,4-dimethylbenzene (1 mL, 7.2 mmol) and β-chloro-propionyl chloride (0.92 mL, 9.6 mmol) in CS_2 (7 mL) at 0 °C over a period of 30 min. The reaction mixture was heated under reflux for a further 30 min. The resulting dark-brown solution was cooled at room temperature and carefully poured onto ice and was extracted with CH_2Cl_2 (20 mL), washed with water (2 × 10 mL), dried over anhydrous Na_2SO_4, and evaporated in vacuo. Yield: 1.22 g, 61%, mp: 82–83 °C (mp lit.[17] 84–85 °C). ^{1}H NMR ($CDCl_3$) δ ppm: 2.40 (s, 3H, CH_3), 2.44 (s, 3H, CH_3), 3.34 (t, J = 6.5 Hz, 2H, CH_2), 3.87 (t, J = 6.5 Hz, 2H, CH_2), 7.44 (s, 1H, H-Ar), 7.49 (s, 1H, H-Ar).

4.1.3. 5-Bromo-4,7-dimethyl-indan-1-one (28)

Chloroketone **27** (1.00 g, 3.6 mmol) was added in small portions with swirling to concentrated H_2SO_4 (14 mL). The resulting solution was heated on a silicon bath at 90 °C. After 1 h, the reaction mixture was cautiously poured onto ice and then extracted with ethyl acetate (50 mL). The organic layer was washed with a solution of 10% NaOH (2· × 20 mL), water (20 mL), dried over anhydrous Na_2SO_4, and evaporated in vacuo. The residue was purified by flash chromatography using CH_2Cl_2–hexane (1:1) as eluent. Yield: 0.75 g, 75%, mp: 102–104 °C (mp lit.[18] 105–106). ^{1}H NMR ($CDCl_3$) δ ppm: 2.35 (s, 3H, CH_3), 2.56 (s, 3H, CH_3), 2.66 (t, J = 6.0 Hz, 2H, CH_2), 2.99 (t, J = 6.0 Hz, 2H, CH_2), 7.32 (s, 1H, H-Ar).

4.1.4. General procedure for the synthesis of TSCs 15–18[10]

A suspension of corresponding 1-indanone (1.2 mmol) and thiosemicarbazide (2.7 mmol) in absolute ethanol (20 mL) was heated under reflux for 30 min, then concentrated H_2SO_4 (0.10 mL) was added and the heating was continued until the 1-indanone was consumed. The conversion of 1-indanone in the corresponding TSC was monitored by TLC on silica gel 60 F_{254} using chloroform–ethanol (1:0.1) as eluent. The solvent was removed in vacuo and the solid was suspended in water (20 mL), filtered and washed with water (2 × 5 mL), EtOH (5 mL), CH_2Cl_2 (5 mL) and finally hexane (2 × 5 mL). TSCs were recrystallized from ethanol.

4.1.4.1. 4-Nitro-indan-1-one thiosemicarbazone (15). Yield: 89%, mp: decompose before melting. IR ν/cm^{-1} (KBr): 3410 (N–H), 3225 (N–H), 3137 (N–H), 1586 (C=N), 1098 (C=S). ^{1}H NMR (DMSO-d_6) δ ppm: 2.95 (m, 2H, CH_2), 3.49 (m, 2H, CH_2), 7.60 (m, 1H, H-Ar),), 8.14 (s, 1H, NH), 8.20 (d, J = 8.1 Hz, 1H, H-Ar), 8.31 (s, 1H, NH), 8.36 (d, J = 7.8 Hz, 1H, H-Ar), 10.49 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ ppm: 27.2, 29.7, 125.5, 128.0, 128.7, 141.9, 143.8, 145.5, 154.1, 178.9. HRMS (ESI) m/z (M+H)$^+$ calcd for $C_{10}H_{11}N_4O_2S$ 251.05972, found 251.05961. Anal. Calcd for $C_{10}H_{10}N_4O_2S$: C, 47.99; H, 4.03; N, 22.39; S, 12.81. Found: C, 48.06; H, 4.04; N, 22.34; S, 12.78.

4.1.4.2. 6-Nitro-indan-1-one thiosemicarbazone (16). Yield: 78%, mp: decompose before melting. IR ν/cm^{-1} (KBr): 3414 (N–H), 3306 (N–H), 3160 (N–H), 1582 (C=N), 1082 (C=S). ^{1}H NMR (DMSO-d_6) δ ppm: 2.96 (m, 2H, CH_2), 3.17 (m, 2H, CH_2), 7.60 (d, J = 8.5 Hz, 1H, H-Ar), 8.18 (d, J = 8.5 Hz, 1H, H-Ar), 8.27 (s, 1H, NH), 8.38 (s, 1H, NH), 8.79 (s, 1H, H-Ar), 10.42 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ ppm: 27.8, 28.8, 117.3, 125.0, 126.8, 140.0, 147.7, 154.1, 155.7, 179.0. HRMS (ESI) m/z (M+H)$^+$ calcd for $C_{10}H_{11}N_4O_2S$ 251.05972, found 251.05928. Anal. Calcd for $C_{10}H_{10}N_4O_2S$: C, 47.99; H, 4.03; N, 22.39; S, 12.81. Found: C, 48.04; H, 4.04; N, 22.41; S, 12.83.

4.1.4.3. 5-Fluor-indan-1-one thiosemicarbazone (17). Yield: 90%, mp: 229–230 °C. IR ν/cm^{-1} (KBr): 3415 (N–H), 3226 (N–H), 3140 (N–H), 1594 (C=N), 1085 (C=S). ^{1}H NMR (DMSO-d_6) δ ppm: 2.90 (m, 2H, CH_2), 3.06 (m, 2H, CH_2), 7.14 (m, 1H, H-Ar), 7.20 (dd, J = 1.6 Hz, J = 9.2 Hz, 1H, H-Ar), 7.93 (dd, J = 5.7 Hz, J = 7.5 Hz, 1H, H-Ar), 7.98 (s, 1H, NH), 8.19 (s, 1H, NH), 10.25 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ ppm: 27.7, 28.4, 112.4 (d, J = 18.0 Hz), 114.8 (d, J = 19.2 Hz), 123.9, 134.5, 151.3 (d, J = 7.3 Hz), 155.6, 164.0 (d, J = 197.6 Hz), 178.6. HRMS (ESI) m/z (M+H)$^+$ calcd for $C_{10}H_{10}FN_3NaS$ 246.04717, found 246.04706. Anal. Calcd for $C_{10}H_{10}FN_3S$: C, 53.79; H, 4.51; F, 8.51; N, 18.82; S, 14.36. Found: C, 53.87; H, 4.54; F, 8.48; N, 18.80; S, 14.40.

4.1.4.4. 5-Bromo-4,7-dimethyl-indan-1-one thiosemicarbazone (18). Yield: 92%, mp: 274–275 °C. IR ν/cm^{-1} (KBr): 3426 (N–H), 3247 (N–H), 3199 (N–H), 1588 (C=N), 1083 (C=S). ^{1}H NMR (DMSO-d_6) δ ppm: 2.25 (s, 3H, CH_3), 2.49 (s, 3H, CH_3), 2.93 (m, 2H, CH_2), 2.98 (m, 2H, CH_2), 7.22 (s, 1H, NH), 7.35 (s, 1H, H-Ar), 8.31 (s, 1H, NH), 10.21 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ ppm: 21.9, 22.3, 27.9, 28.6, 122.2, 126.6, 128.6, 135.4, 141.4, 149.7, 158.3, 178.6. HRMS (ESI) m/z (M+H)$^+$ calcd for $C_{12}H_{15}BrN_3S$ 312.01646, found 246.01530. Anal. Calcd for $C_{12}H_{14}BrN_3S$: C, 46.16; H, 4.52; Br, 25.29; N, 13.46; S, 10.27. Found: C, 46.22; H, 4.53; Br, 25.56; N, 13.44; S, 10.30.

4.1.5. General procedure for the synthesis of N^4-TSCs (19–24)

Method A: In the first step, a mixture of 1-indanone or 5,6-dimethoxy-1-indanone (3.8 mmol) and hydrazine hydrate 85% (0.9 mL) in isopropanol (5 mL) was heated under reflux for 1 h. After cooling, the solvent was evaporated and the residue was dissolved in CH_2Cl_2 (20 mL), dried over anhydrous Na_2SO_4 and evaporated again to render the crude derivative. Hydrazones were recrystallized from methanol, the physical data were in according with those reported in the literature.[19] In the second step, to a solution of the hydrazone (2.5 mmol) in benzene (10 mL) was added dropwise a solution of the corresponding isothiocyanate (3.0 mmol) in benzene (5 mL). The resulting mixture was left stirring at room temperature monitored by TLC until the hydrazone was consumed.

The solid obtained was filtered, washed with benzene (5 mL) and with ethanol (2 mL).

Method B: A mixture of 1-indanone or 5,6-dimethoxy-1-indanone (0.38 mmol), hydrazine hydrate 85% (21 μL), the corresponding isothiocyanate (0.43 mmol), acetic acid (20 μL) and ethanol (0.5 mL) in a glass tube equipped with a screw cap and magnetic agitation, was placed in a microwave synthesizer at 90 °C (30 W, 2.5 bar). After completion of the reaction, monitored by TLC, the obtained mixture was suspended in water (5 mL), filtered and washed with ethanol (2 mL). N^4-TSCs were recystallized from ethanol.

4.1.5.1. Indan-1-one *N*-phenylthiosemicarbazone (19)[20]. Mp: 202–204 °C. IR ν/cm^{-1} (KBr): 3267 (N–H), 3193 (N–H), 1595 (C=N), 1097 (C=S). ^1H NMR ($CDCl_3$) δ ppm: 2.84 (m, 2H, CH_2), 3.21 (m, 2H, CH_2), 7.29–7.43 (m, 6H, H-Ar), 7.69 (d, J = 7.7 Hz, 2H, H-Ar), 7.78 (d, J = 7.8 Hz, 1H, H-Ar), 8.53 (s, 1H, NH), 9.67 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ ppm: 27.9, 28.7, 1229, 125.6, 126.0, 127.3, 128.5, 129.2, 131.2, 138.1, 139.6, 149.4, 158.3, 176.8. HRMS (ESI) m/z $(M+H)^+$ calcd for $C_{16}H_{16}N_3S$ 282.10594, found 282.10569. Anal. Calcd for $C_{16}H_{15}N_3S$: C, 68.30; H, 5.37; N, 14.93; S, 11.40. Found: C, 68.39; H, 5.35; N, 14.90; S, 11.43.

4.1.5.2. Indan-1-one *N*-(4-methylphenyl)thiosemicarbazone (20)[20]. Mp: 173-175 °C. IR ν/cm^{-1} (KBr): 3301 (N–H), 3197 (N–H), 1589 (C=N), 1102 (C=S). ^1H NMR ($CDCl_3$) δ ppm: 2.36 (s, 3H, CH_3), 2.84 (m, 2H, CH_2), 3.20 (m, 2H, CH_2), 7.20 (d, J = 8.2 Hz, 2H, H-Ar), 7.31 (m, 1H, H-Ar), 7.37–7.42 (m, 2H, H-Ar), 7.52 (d, J = 8.2 Hz, 2H, H-Ar), 7.76 (d, J = 7.9 Hz, 1H, H-Ar), 8.56 (s, 1H, NH), 9.27 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ ppm: 21.0, 27.9, 28.7, 122.9, 126.0, 127.3, 129.0, 129.1, 131.1, 134.8, 137.0, 138, 1, 149.3, 158.1, 176.9. HRMS (ESI) m/z $(M+Na)^+$ calcd for $C_{17}H_{17}N_3NaS$ 318.10354, found 318.10263. Anal. Calcd for $C_{17}H_{17}N_3S$: C, 69.12; H, 5.80; N, 14.22; S, 10.85. Found: C, 69.04; H, 5.79; N, 14.18; S, 10.88.

4.1.5.3. Indan-1-one *N*-(4-chlorophenyl)thiosemicarbazone (21). Mp: 192–194 °C. IR ν/cm^{-1} (KBr): 3261 (N–H), 3224 (N–H), 1589 (C=N), 1083 (C=S). ^1H NMR ($CDCl_3$) δ ppm: 2.84 (m, 2H, CH_2), 3.20 (m, 2H, CH_2), 7.23 (m, 1H, H-Ar), 7.25–7.37 (m, 4H, H-Ar), 7.65 (d, J = 7.8 Hz, 2H, H-Ar), 7.76 (d, J = 7.8 Hz, 1H, H-Ar), 8.56 (s, 1H, NH), 9.32 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ ppm: 28.0, 28.7, 122.9, 126.0, 127.2, 127.7, 128.4, 129.6, 131.2, 138.1, 138.6, 149.4, 158.6, 176.8. HRMS (ESI) m/z $(M+Na)^+$ calcd for $C_{16}H_{14}ClN_3NaS$ 338.04892, found 338.04925. Anal. Calcd for $C_{16}H_{14}ClN_3S$: C, 60.85; H, 4.47; Cl, 11.23; N, 13.31; S, 10.15. Found: C, 68.39; H, 5.35; N, 14.90; S, 11.43.

4.1.5.4. 5,6-Dimethoxyindan-1-one *N*-phenylthiosemicarbazone (22). Mp: 204–205 °C. IR ν/cm^{-1} (KBr): 3248 (N–H), 3201 (N–H), 1596 (C=N), 1078 (C=S). ^1H NMR ($CDCl_3$) δ ppm: 2.83 (m, 2H, CH_2), 3.12 (m, 2H, CH_2), 3.92 (s, 3H, OCH_3), 3.94 (s, 3H, OCH_3), 6.83 (s, 1H, H-Ar), 7.16 (s, 1H, H-Ar), 7.40 (m, 3H, H-Ar), 7.66 (d, J = 7.6 Hz, 2H, H-Ar), 8.48 (s, 1H, NH), 9.27 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ ppm: 28.4, 28.5, 56.0, 56.2, 104.8, 108.2, 125.7, 108.2, 125.7, 126.5, 128.6, 129.8, 139.7, 143.0, 149.3, 152.6, 158.9, 176.6. HRMS (ESI) m/z $(M+H)^+$ calcd for $C_{18}H_{20}N_3O_2S$ 342.12707, found 342.12578. Anal. Calcd for $C_{18}H_{19}N_3O_2S$: C, 63.32; H, 5.61; N, 12.31; S, 9.39. Found: C, 63.37; H, 5.59; N, 12.34; S, 9.41.

4.1.5.5. 5,6-Dimethoxyindan-1-one *N*-(4-methylphenyl)thiosemicarbazone (23). Mp: 179–181 °C. IR ν/cm^{-1} (KBr): 3262 (N–H), 3192 (N–H), 1593 (C=N), 1084 (C=S). ^1H NMR ($CDCl_3$) δ ppm: 2.35 (s, 3H, CH_3), 2.83 (m, 2H, CH_2), 3.11 (m, 2H, CH_2), 3.92 (s, 3H, OCH_3), 3.93 (s, 3H, OCH_3), 6.82 (s, 1H, H-Ar), 7.15 (s, 1H, H-Ar), 7.20 (d, J = 7.9 Hz, 2H, H-Ar), 7.49 (d, J = 7.9 Hz, 2H, H-Ar), 8.53 (s, 1H, NH), 9.18 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ ppm: 21.1, 28.3, 28.5, 56.0, 56.2, 104.8, 108.2, 126.4, 129.0, 129.9, 134.9, 137.2, 142.9, 149.3, 152.5, 158.8, 176.6. HRMS (ESI) m/z $(M+H)^+$ calcd for $C_{19}H_{22}N_3O_2S$ 356.14272, found 356.14177. Anal. Calcd for $C_{19}H_{21}N_3O_2S$: C, 64.20; H, 5.95; N, 11.82; S, 9.02. Found: C, 64.12; H, 5.97; N, 11.79; S, 9.04.

4.1.5.6. 5,6-Dimethoxyindan-1-one *N*-(4-chlorophenyl)thiosemicarbazone (24). Mp: 190–192 °C. IR ν/cm^{-1} (KBr): 3313 (N–H), 3194 (N–H), 1587 (C=N), 1079 (C=S). ^1H NMR ($CDCl_3$) δ ppm: 2.82 (m, 2H, CH_2), 3.12 (m, 2H, CH_2), 3.93 (s, 3H, OCH_3), 3.94 (s, 3H, OCH_3), 6.83 (s, 1H, H-Ar), 7.15 (s, 1H, H-Ar), 7.35 (d, J = 8.7 Hz, 2H, H-Ar), 7.63 (d, J = 8.7 Hz, 2H, H-Ar), 8.48 (s, 1H, NH), 9.22 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ ppm: 28.3, 28.5, 56.1, 56.2, 104.9, 108.2, 128.0, 128.4, 129.7, 129.8, 138.7, 143.1, 149.2, 152.6, 159.3, 176.5. HRMS (ESI) m/z $(M+Na)^+$ calcd for $C_{18}H_{18}ClN_3NaO_2S$ 398.07005, found 398.06860. Anal. Calcd for $C_{18}H_{18}ClN_3O_2S$: C, 57.52; H, 4.83; Cl, 9.43; N, 11.18; S, 8.53. Found: C, 57.58; H, 4.82; N, 11.14; S, 8.51.

4.2. Biological evaluation

4.2.1. In vitro anti-trypanosomal activity

T. cruzi epimastigotes (Tulahuen 2 strain) were grown at 28 °C in an axenic medium (BHI-Tryptose) as previously described,[9,12] complemented with 5% fetal calf serum. Cells were harvested in the late log phase, re-suspended in fresh medium, counted in Neubauer's chamber and placed in 24-well plates (2×10^6/mL). Cell growth was measured as the absorbance of the culture at 590 nm, which was proved to be proportional to the number of cells. Before inoculation, the media were supplemented with the indicated amount of the studied compound from a stock solution in DMSO. The final concentration of DMSO in the culture media never exceeded 1% and the control was run in the presence of 1% DMSO and in the absence of any compound. No effect on epimastigotes growth was observed by the presence of up to 1% DMSO in the culture media. Nfx and Amphotericin B were used as the reference trypanosomicidal drugs. The percentage of growth inhibition was calculated as follows $\{1 - [(Ap - A0p)/(Ac - A0c)]\} \times 100$, where $Ap = A_{590}$ of the culture containing the studied compound at day 5; $A0p = A_{590}$ of the culture containing the studied compound right after addition of the inocula (day 0); $Ac = A_{590}$ of the culture in the absence of any compound (control) at day 5; $A0c = A_{590}$ in the absence of the compound at day 0. To determine IC_{50} values, parasite growth was followed in the absence (control) and presence of increasing concentrations of the corresponding compound. The IC_{50} values were determined as the drug concentrations required to reduce by half the absorbance of that of the control (without compound).

4.2.2. Unspecific mammalian cytotoxicity

Red blood cell lysis assay.[9h] Human blood collected in sodium citrate solution (3.8%) was centrifuged at 1500 rpm for 10 min at 4 °C. The plasma supernatant was removed and the erythrocytes were suspended in ice cold PBS. The cells were again centrifuged at 1500 rpm for 10 min at 4 °C. This procedure was repeated two more times to ensure the removal of any released hemoglobin. Once the supernatant was removed after the last wash, the cells were suspended in PBS to get a 2% w/v red blood cell solution. A volume of 400 μL of studied compounds, in PBS (final concentration 50, 100 and 200 μM), negative control (solution of PBS), or Amphotericin B (final concentration 1.5 μM) were added to 400 μL of the 2% w/v red blood cell solution in ten microcentrifuge tubes for each concentration and incubated for 24 h at 37 °C. Complete hemolysis was attained using neat water yielding the

100% control value (positive control). After incubation, the tubes were centrifuged and the supernatants were transferred to new tubes. The release of hemoglobin was determined by spectrophotometric analysis of the supernatant at 405 nm. Results were expressed as percentage of the total amount of hemoglobin released by action of the compounds. This percentage is calculated using the equation: Hemolysis percentage (%) = [(A1 − A0)/A1 water] × 100, where A1 is the absorbance at 405 nm of the test sample at t = 24 h, A0 is the absorbance at 405 nm of the test sample at t = 0 h, and A1 water is the absorbance at 405 nm of the positive control (water) at t = 24 h. The experiments were done by quintuplicate.

4.2.3. Cruzipain inhibitory activity[9h]

Cruzipain (6 μM) was incubated in a reaction mixture containing 50 mM PBS pH 7.3 or acetate buffer pH 5.3, 5 mM DTT and 100 μM compound for 5 min at room temperature. Fluorogenic substrate *Z*-Phe-Arg-AMC (K_M = 1.8 μM) was added to a concentration of 10 μM, and increase in fluorescence (excitation 380 nm and emission 460 nm) was monitored for 10 min at room temperature in a 96 well-microplate Varioskan spectrofluorometer and spectrophotometer. Compounds were added as solutions in DMSO and positive controls contained only buffered solvent. The final assay volume was 100 μL and the final DMSO concentration never exceeded 10%. The cruzipain inhibitor reference compound 3'-bromopropiophenone thiosemicarbazone,[8] was included in the analysis as a control, 100% at 100 μM. The values represent means of at least three experiments.

4.3. Docking studies

TSCs **1**–**24** were virtually screened for their capability of acting as cruzipain inhibitors using SurflexDock[21] method implemented in the molecular modeling package Sybyl 8.1.[22] SurflexDock is a new docking methodology that combines Hammerhead's empirical scoring function[23] with a molecular similarity method to generate putative alignment of ligands. SurflexDock employs an idealized active site ligand (called a protomol) as a target to generate putative alignments of molecules or molecules fragments.[24] These putative poses are achieved using the Hammerhead scoring function. Before performing docking, all compounds were minimized using the Conjugate Gradient algorithm with a conjugated gradient of <0.001 kcal/mol convergent criteria provided by the MMFF94 force field[25] and MMFF94 electrostatic charges. TSCs **1**–**24** were docked into the binding site of cruzipain (PDB ID 1F29). Using 1F29 as reference complex, we generated a protomol, a computational representation of the intended binding site to which putative ligands are aligned. SurflexDock's protomol use CH_4, C═O and NH fragments and its purpose is to direct the initial placement of the ligands during the docking process. Protomol construction was based on protein residues present in the active site of the enzyme. Each docking of putative ligands returned up to 50 scored poses, with the score consisting of an affinity score expressed as $-\log_{10}(K_d)$.

Besides, molecular docking of compounds **12**, **22** and **24** into the three-dimensional X-ray structure of *T. cruzi* cruzipain (PDB code 1EWL) was carried out using Autodock 4.2 software, as implemented through the graphical user interface (GUI) AutoDockTools (ADT 1.5.4).[26] ADT was employed to setup the enzymes: all hydrogen atoms were added, Gasteiger charges were calculated, and nonpolar hydrogens were merged to carbon atoms. The 3D structures of ligand molecules were obtained from molecular mechanics calculation using the MMFF94 force filed[25] as implemented in the molecular modeling package Sybyl 8.1.[22] ADT was used to generate docking input files. In all docking experiments a grid box size of 60 × 60 × 66 points in x, y, and z directions was used, and the maps were centered on S atom of the catalytic cysteine 25 residue. A grid spacing of 0.375 Å (approximately one fourth of the length of carbon–carbon covalent bond) and a distance-dependent function of the dielectric constant[27] were used for the calculation of the energetic map. Fifty runs were generated by using Lamarckian Genetic Algorithm searches. Default settings were used with an initial population of 150 randomly placed individuals, a maximum number of 7.0×10^5 energy evaluations, and a maximum number of 2.7×10^4 generations. A mutation rate of 0.02 and a crossover rate of 0.8 were chosen. In all cases the pose with best score from multiple docking of the same ligand were chosen.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.09.037.

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