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Original article

Synthesis and biological evaluation of some novel 1-indanone thiazolylhydrazone derivatives as anti-*Trypanosoma cruzi* agents

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ABSTRACT

A series of novel 4-arylthiazolylhydrazones (TZHs) derived from 1-indanones were synthesized in good yields (66–92%) in a simple procedure using microwave irradiation and then characterized by spectroscopy studies. The compounds were evaluated for their in vitro anti-*Trypanosoma cruzi* activity against the epimastigote, trypomastigote and amastigote forms of the parasite. Most TZHs displayed excellent activity, and were more potent and selective than the reference drug Benznidazole, used in the current chemotherapy. Analysis of the free sterols from parasite incubated with the compounds showed that inhibition of ergosterol biosynthesis is a possible target for the action of these new TZHs. In particular, TZH **9** emerged as a promising antichagasic compound to be evaluated in animal models.

1. Introduction

Chagas's disease, also called American Trypanosomiasis, is a parasitic disease caused by the kinetoplastid protozoan *Trypanosoma cruzi (T. cruzi)*. Despite recent advances in the control of its vectorial and transfusional transmission [1,2], it is endemic from Southern California to Argentina, and afflicts 24 million people [3]. The parasite has a complex life cycle, which includes obligatory stages in the mammalian host and in the vector. In the host, *T. cruzi* has two forms: an intracellular dividing form (amastigote) and a non-dividing highly infective bloodstream form (trypomastigote) that can invade host cells. Two forms also occur in the vector: one replicative (epimastigote) and one non-replicative (metacyclic trypomastigote) [4,5].

Current specific chemotherapy relies on two compounds: Nifurtimox (Nfx) and Benznidazole (Bnz), associated with long-term treatments that may give rise to severe side effects [6]. In fact, although Nfx and Bnz are able to eliminate patent parasitemia and to reduce serological titers in acute and early chronic infections [7,8], they have significantly low efficacy in long-term chronic infections [9]. The side effects of these drugs result from the oxidative damage in the host tissues and are thus inextricably linked to their anti-parasitic activity [10].

Recently, we have reported the synthesis of new thiosemicarbazones derived from 1-indanones. These compounds possess an important activity against *T. cruzi* with selectivity indexes higher than that of the reference drug Nfx. Some of them are also able to inhibit cruzipain, the major cysteine protease of the parasite [11]. In continuation of our search for bioactive molecules, we envisaged that the transformation of the thiosemicarbazone group into thiazolylhydrazone moiety would generate novel templates which are likely to exhibit anti-*T. cruzi* activity.

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Thiazolylhydrazones (TZHs) have been of interest to medicinal chemists because of their biological activities. They have been described as antibacterial [12,13], antifungal [14] and anti-inflammatory agents [13]. Chimenti and coworkers developed a series of selective inhibitors of the A and B isoforms of monoaminooxidase (MAO) with the thyazolylhydrazone function in their structure [15].

Several studies have shown that commercially available ergosterol biosynthesis inhibitors having a thiazole or triazole nucleus, which are highly successful for the treatment of fungal diseases (such us ketoconazole, itraconazole, posaconazole or ravuconazole) have suppressive effects against *T. cruzi* infections in humans or experimental animals [7,16].

In this work, we present the preparation of seventeen TZHs derived from 1-indanones in a simple manner, by microwave-assisted synthesis. The synthesized compounds were fully characterized by IR, NMR and mass spectral studies. TZHs were assayed for their in vitro anti-*T. cruzi* activity against the epimastigote, trypomastigote and amastigote forms of the parasite. Their cytotoxicity in mammalian cell cultures was also investigated, and the structural requirements for their optimal activity are discussed. Further, investigations on the possible involvement of TZHs with cruzipain activity and ergosterol biosynthesis, as possible therapeutic targets, were performed.

2. Results and discussion

2.1. Chemistry

Hantzsch reaction of α-halocarbonyl compounds with thioamide derivatives provides a useful method for the preparation of the thiazole nucleus [17]. In general, this process requires high temperatures and long reaction times and often produces low yields. Here, we report the successful use of microwave (MW) irradiation to enhance this classical method, by the condensation of α-chloroketones with thiosemicarbazones for the preparation of TZHs (Scheme 1).

With the aim to find the optimal reaction conditions to synthesize these derivatives using MW irradiation, we investigated the preparation of TZH derived from thiosemicarbazone of 5,6-dimethoxy-1-indanone and phenacyl chloride using three different solvents at the same temperature: a) EtOH, b) acetonitrile, and c) DMF. Results were compared with those obtained when the reaction takes place using the conventional method (reflux in EtOH) (Table 1).

When the selected TZH was synthesized using method c, the desired product was achieved in 30 s at 80 °C with very good yields (92%). Taking this result into account, we prepared a series of TZHs by reacting thiosemicarbazones derived from 1-indanones, with different substituents in the aromatic ring, as starting materials, and several phenacyl chlorides in the presence of a few drops of DMF. The TZHs obtained under optimized reaction conditions are summarized in Table 2. Spectroscopic data of the synthesized compounds were in accordance with those of TZHs resulting from cyclic ketone thiosemicarbazone derivatives as the hydrochloride and hydrobromide forms previously described by Chimenti and coworkers [14,15].

Due to the good results obtained when the synthesis was performed in DMF under MW irradiation (method c), we decided to explore the preparation in DMF, but with conventional heating. Surprisingly, the products obtained with this methodology were not the same as those obtained with method c. The new products had different melting points and showed different spectroscopic data; hence, we decided to investigate the structure of the resulting compounds in detail.

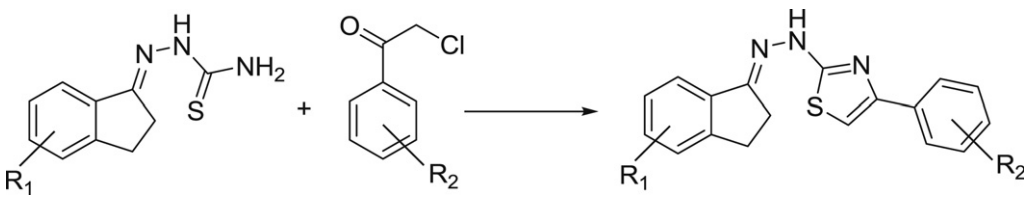
All the compounds synthesized were characterized by spectral studies. None of the compounds obtained gave crystals adequate to carry out an X-ray analysis. For structural elucidation and comparison, we chose TZHs **1** and **4** and the counterpart compounds obtained by conventional heating in DMF (**18** and **19** respectively). To facilitate the structural assignment of the selected compounds, we carried out the cyclohexanone thiosemicarbazone condensation with phenacyl chloride and 4-methyl-phenacyl chloride in both reaction conditions (**20**, **21** by microwave irradiation and **22**, **23** by conventional heating) (Fig. 1). Spectroscopic data of TZHs **20** and **21** were coincident with those of the hydrochloride and hydrobromide forms previously described [15c,18]. In addition, the patterns observed in the IR and ^{1}H and ^{13}C NMR spectra were similar for TZHs **1**, **4**, **20** and **21**.

TZH **21** was obtained in a crystalline form suitable to perform an X-ray crystallographic study. Analysis of the ORTEP diagram confirmed that the compound was forming a hydrochloride, due to the release of HCl (g) in the condensation reaction. The detailed study of the crystal structure demonstrated that C2-N3 and C2-N13 distances are 1.321 Å and 1.343 Å respectively, indicating that a resonance phenomenon occurs between the atoms involved (Fig. 2) [19].

Bearing in mind the similarity observed in the spectroscopic data of TZHs **1**, **4**, **20** and **21**, which correspond to hydrochloride forms, we treated these compounds with $NaHCO_3$10% overnight. The melting points and IR spectra of the resulting products were coincident with those of compounds **18**, **19**, **22** and **23**. This led us to consider the possibility that the compounds from the reaction performed in DMF with conventional heating were the corresponding free bases.

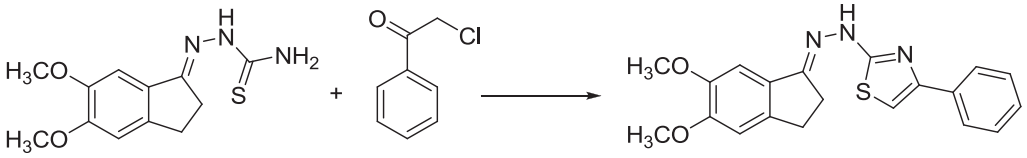
Hydrochloride TZHs and free base TZHs (compounds **1**, **4**, **20**, **21** and **18**, **19**, **22** and **23** respectively) can be discriminated through the analysis of ^{1}H NMR, ^{13}C NMR and IR spectroscopic data. The NMR signals of all compounds considered were similar when the spectra were recorded in DMSO-d_6, but different when they were recorded in $CDCl_3$. Clearly, there is solvent effect acting on the hydrochloride/free base equilibrium. Table 3 reports these data.

In the ^{1}H NMR spectra, there is a broad and low intense signal with one proton integral at 12.92, 12.78, 12.76 and 12.26 ppm for TZHs **1**, **4**, **20** and **21**, respectively. Since the same signal was shifted downfield by about 4 ppm for free base TZHs (**18** and **22**, not observed for compounds **19** and **23**), it was assigned to the hydrazone NH proton (D_2O exchangeable). In addition, an extra signal between 13.20 and 14.25 ppm appeared in the spectra of the hydrochloride TZHs, indicating that **1**, **4**, **20** and **21** are in the salt



Scheme 1. Synthesis of TZHs by condensation of 1-indanone thiosemicarbazone derivatives and phenacyl chlorides.

Table 1
Synthesis of TZH derived from thiosemicarbazone of 5,6-dimethoxy-1-indanone and phenacyl chloride in different reaction conditions.



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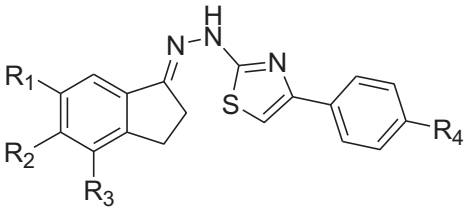
	Time (min)	Yield[b] (%)
Reflux in EtOH	240	45
MW irradiation in EtOH[a] (method a)	8	53
MW irradiation in acetonitrile[a] (method b)	12	51
MW irradiation in DMF[a] (method c)	0.5	92

[a] Temperature: 80 °C.
[b] Yields refer to pure products.

Table 2
TZHs synthesized under MW irradiation in DMF.



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TZHs	R_1	R_2	R_3	R_4	Yield[a] (%)
1	H	H	H	H	71
2	H	H	H	CH_3	76
3	H	H	H	Cl	74
4	OCH_3	OCH_3	H	H	92
5	OCH_3	OCH_3	H	CH_3	77
6	OCH_3	OCH_3	H	Cl	86
7	H	H	CH_3	H	85
8	H	H	CH_3	CH_3	76
9	H	H	CH_3	Cl	83
10	H	H	OCH_3	Cl	68
11	H	H	NO_2	Cl	91
12	H	CH_3	H	Cl	81
13	CH_3	H	H	Cl	67
14	H	CH_3	H	H	66
15	H	CH_3	H	CH_3	73
16	CH_3	H	H	H	84
17	CH_3	H	H	CH_3	79

[a] Time reaction: 30 s. Temperature reaction: 80 °C.

form. Further, IR spectra of **18**, **19**, **22** and **23** exhibited a sharp intense band between 3305 and 3395 cm^{-1}, characteristic for NH stretching, whereas spectra of the compounds in the hydrochloride form showed a weak broad band between 2550 and 2780 cm^{-1}, corresponding to NH^+ vibration of the salt form, in agreement with the literature [20]. The vibration band of the C=N moiety of the thiazole nucleus appeared between 1555 and 1565 cm^{-1}, whereas the band in the salt form was shifted to a higher frequency

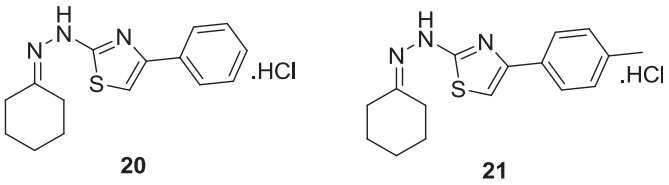


Fig. 1. TZHs derived from the reaction of cyclohexanone under MW irradiation in DMF thiosemicarbazone with phenacyl chloride (**20**) and 4-methyl-phenacyl chloride (**21**).

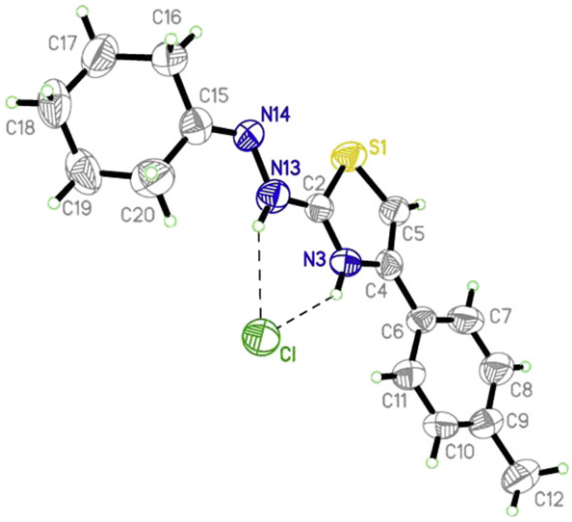


Fig. 2. ORTEP diagram for compound **21**.

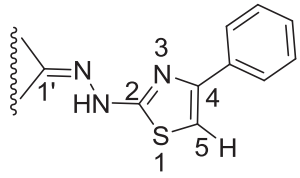
(1615–1630 cm^{-1}). These observations also confirm that the hydrochloride and the free base are the chemical structures that correspond to both series of compounds. The analysis of the allows establishing significant differences in C1′ chemical shifts of both forms, which are deshielded and shielded by over 10 ppm in the hydrochloride and free base TZHs respectively. The marked downfield shift of C1′ (164.5, 165.9, 165.4 and 163.2 ppm) in compounds **1**, **4**, **20** and **21** compared to **18**, **19**, **22** and **23** is due to increased positive charge density on that carbon. Further, the chemical shifts observed for C4 in the ^{13}C NMR spectra of the free base TZHs are in agree with previously assignments [21].

2.2. In vitro anti-T. cruzi activity

The compounds were biologically evaluated in aqueous solutions obtained from stock solutions of the hydrochloride forms of TZHs in DMSO, whose final concentration did not exceed 1% DMSO. As noted previously, under these conditions, both forms (hydrochloride and free base) showed identical spectroscopic characteristics.

The new compounds, **1**–**17**, were initially tested in vitro against the epimastigote form of *T. cruzi*, Tulahuen 2 strain. TZHs were incorporated into the media at 15, 10, 7.5, 5 and 2.5 μM concentrations. Table 4 shows the $IC_{50\ epi}$ (50% inhibitory concentration) value determined for each of them. Bnz was used as the reference

Table 3
^{1}H NMR and ^{13}C NMR spectroscopy data of the hydrochloride TZHs and free base TZHs selected.



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Compound	Solvent	C1′	C2	C5	C4	NH	H5
1	$CDCl_3$	164.5	169.9	100.6	142.3	12.92	6.74
18	$CDCl_3$	155.9	169.3	103.5	151.3	8.75	6.90
1/18	DMSO-d_6	157.5	169.9	104.3	148.4	11.16	7.31
4	$CDCl_3$	165.9	168.6	100.1	140.5	12.78	6.71
19	$CDCl_3$	156.7	169.3	103.3	151.2	-[a]	6.87
4/19	DMSO-d_6	158.7	170.2	103.3	152.3	10.90	7.26
20	$CDCl_3$	165.4	170.4	101.2	140.9	12.76	6.67
22	$CDCl_3$	158.6	170.7	103.0	148.8	8.15	6.70
20/22	DMSO-d_6	156.5	170.4	103.8	149.8	10.85	7.24
21	$CDCl_3$	163.2	170.4	100.8	140.4	12.26	6.60
23	$CDCl_3$	156.7	170.9	102.6	150.9	-[a]	6.75
21/23	DMSO-d_6	155.3	170.0	102.2	150.1	10.91	7.27

[a] Not observed.

trypanosomicidal agent. Compounds **8**, **9**, **14**, **16**, **17** were the most active anti-*T. cruzi* derivatives, with $IC_{50\ epi}$ values between 4.08 and 4.69 μM, which also resulted more active than the reference drug, while TZHs **3**, **6**, **7**, **12**, **13**, **15** displayed moderate activities, with $IC_{50\ epi}$ values between 5.24 and 8.62 μM. Derivatives **1**, **2**, **4**, **5**, **10** and **11** were less potent than Bnz. These data suggest that better trypanocidal activity may be attained when R_1, R_2 or R_3 is a methyl group; on the other hand, the nature of the substituent in the R_4 position is not decisive in the anti-*T. cruzi* activity observed.

The lytic effect on mouse blood trypomastigotes was also evaluated for compounds **1**–**17** (Table 4). When analyzing the relative potency of the compounds tested in comparison to Bnz, we found better activities for TZHs **8**, **9**, **10** and **16**. Compounds **5**, **7**, **13**, **15** and **17** showed moderate activities (around 2 times lower than that of the reference drug). The other derivatives were poorly active against the bloodstream form of the parasite. The results observed in this assay demonstrate that, in general, compounds with a methyl substituent in position R_1 and R_3 are the most active of the series.

TZHs **5**, **7**, **8**, **9**, **10**, **15**, **16**, and **17** were analyzed to determine their activity against the intracellular replicative amastigotes. The compounds selected were the most active against the epimastigote and trypomastigote forms of the parasite, except for compound **15**. The results obtained can be seen in Table 5. The compounds **7**, **8**, **9**, **10**, **15** and **17** showed excellent activity, with $IC_{50\ amas}$ values ranging from 0.09 to 1.35 μM, being TZH **9** the most active compound of this series ($IC_{50\ amas}$ = 0.09 μM). In particular, TZH **9** showed very good correlation of activity against the three forms evaluated. These results make compound **9** an excellent candidate for further biological studies.

Table 4
Biological characterization of TZHs against the epimastigote and trypomastigote forms of *T. cruzi*.

TZHs	$IC_{50\ epi}$[a,c] (μM)	$IC_{50\ trypo}$[b,c] (μM)
1	18.99 ± 2.59	>>500
2	>15	>>500
3	8.62 ± 0.25	308.41 ± 8.66
4	10.14 ± 0.43	195.22 ± 8.36
5	>15	69.57 ± 7.02
6	7.17 ± 0.56	403.69 ± 11.46
7	7.08 ± 0.68	48.91 ± 6.86
8	4.36 ± 0.49	17.24 ± 3.44
9	4.53 ± 0.66	20.01 ± 1.99
10	11.78 ± 0.55	19.72 ± 2.63
11	17.05 ± 0.78	596.95 ± 11.33
12	7.15 ± 0.53	372.16 ± 7.43
13	8.59 ± 0.65	61.10 ± 4.79
14	4.69 ± 0.43	172.42 ± 10.32
15	5.24 ± 0.27	87.01 ± 5.16
16	4.27 ± 0.24	22.74 ± 2.11
17	4.08 ± 0.20	49.53 ± 2.87
Bnz	5.39 ± 0.25	30.26 ± 2.85

[a] $IC_{50\ epi}$: concentration (in μM) that inhibits 50% of epimastigote form of *T. cruzi* growth.
[b] $IC_{50\ trypo}$: concentration (in μM) that inhibits 50% of trypomastigote form of *T. cruzi* lysis.
[c] All data are expressed as means±standard deviations of three separate experiments, running in duplicates or triplicates.

2.3. In vitro unspecific cytotoxicity

The mammal cytotoxicity of the new compounds was studied in vitro using Vero cells as the cellular model. None of the seventeen TZHs caused cell death up to a concentration of 100 μM (Bnz CC_{50} = 82.79 μM).

2.4. Mechanism of action studies

As mentioned above, compounds with antichagasic activity structurally related to TZHs, such as thiosemicarbazones and thiazoles, act by inhibition of cruzipain and sterol biosynthesis respectively. In order to determine a probable mechanism of action, we decided to investigate the effect on these two probable therapeutic targets of the synthesized compounds. The following studies were performed: inhibition of cruzipain and sterol biosynthesis analysis, both studies were done on the epimastigote stage.

2.4.1. Inhibition of T. cruzi cruzipain

TZHs **2**, **5**, **7**, **8**, **9**, **10**, **15**, **16**, and **17** were studied as inhibitors of *T. cruzi* cruzipain, following a previously described procedure [22,23]. E-64 [*L-trans*-epoxy-succinyl-leucyl-amido(4-guanidino) butane] was used as reference drug [23]. None of the assayed compounds resulted to be inhibitors of this enzyme at the studied doses (10, 20 and 50 μM) (Table 6).

2.4.2. Sterol biosynthesis analysis

After a pre-established protocol (2.5×10^7 cells/mL, 24 h of incubation) [24], the controls (untreated, terbinafine-treated) and TZHs-treated parasites were collected and the total lipids were extracted and analyzed as described previously [25,26]. Qualitative analyses of neutral lipid fractions were carried out using TLC. None of the studied compounds were able to accumulate lanosterol (Fig. 3).

Table 5
In vitro activity of the TZHs selected against the amastigote form of *T. cruzi*.

TZHs	$IC_{50\ amas}$[a,b] (μM)
5	7.72 ± 0.68
7	0.68 ± 0.09
8	0.72 ± 0.11
9	0.09 ± 0.02
10	0.84 ± 0.14
15	1.35 ± 0.18
16	3.96 ± 0.32
17	0.27 ± 0.06

[a] $IC_{50\ amas}$: concentration (in μM) that inhibits 50% of amastigote form of *T. cruzi* growth.
[b] All data are expressed as means±standard deviations of three separate experiments, running in duplicates or triplicates.

Table 6
Effect on *T. cruzi* cruzipain activity of selected TZHs.

Compound	Concentration (μM)	Inhibition (%)
None[a]		0
2	10	8
	20	5
	50	5
5	10	0
	20	0
	50	0
7	10	3
	20	0
	50	0
8	10	4
	20	2
	50	0
9	10	3
	20	0
	50	3
10	10	0
	20	0
	50	0
15	10	0
	20	0
	50	0
16	10	3
	20	0
	50	0
17	10	3
	20	0
	50	0
E-64	10	62

[a] None: assay that corresponds to the enzyme incubated in the absence of drug but in presence of the respective amount of DMSO.

Among TZHs studied, compounds **8** and **9** seem to accumulate squalene in different degrees with the concomitant decrease of ergosterol. These results suggest that inhibition of the biosynthesis of ergosterol is a possible target for the action of these TZHs.

3. Conclusions

Seventeen 4-arylthiazolylhydrazones derived from 1-indanones were prepared with good yields in a simple manner by using microwave-assisted synthesis. Most of these new derivatives exhibited promising activity against epimastigotes and against trypomastigote and amastigote forms, which are the mammal stages of *T. cruzi*. We identified compound **9** as an excellent candidate for further molecular structural modifications and biological studies, especially in vivo evaluations. Preliminary results from our laboratory showed that some TZHs could be able to produce the inhibition of the enzymatic system involved in the sterols biosynthetic route. Further studies to confirm the probable mechanism of action of these novel compounds will be performed.

4. Experimental

4.1. Chemistry

Melting points (uncorrected) were determined on a Thomas Hoover apparatus. Thin layer chromatography (TLC) was used to monitor reactions. Reactions were carried out in a Microwave Synthesis Reactor Microwave·300 Anton Paar. 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. High resolution mass spectra were obtained on Bruker micrOTOF-Q II spectrometer. Single crystal X-Ray diffraction data were collected at room temperature, using a Gemini A diffractometer, Oxford Diffraction. Data-collection strategy and data reduction followed standard procedures implemented in the CrystAlisPro software. The structures were solved using program SHELXS-97 and refined using the full-matrix LS procedure with SHELXL-97. Anisotropic displacement parameters were employed for non-hydrogen atoms and H atoms were treated. All H atoms were located at the expected positions and they were refined using a riding model. In the final cycle of refinement, LS weights of the form $w = 1/[\sigma^2(F_o^2) + (a^*P)^2 + b^*P]$, where $P = [(F_o^2) + (2^*F_c^2)]/3$, were employed. All the 1-indanones were commercially purchased, except 5-methyl-1-indanone [27] and 4-nitro-1-indanone which was prepared according to the protocol previously described [11]. Phenacyl chlorides were synthesized as reported in our previous communication [28].

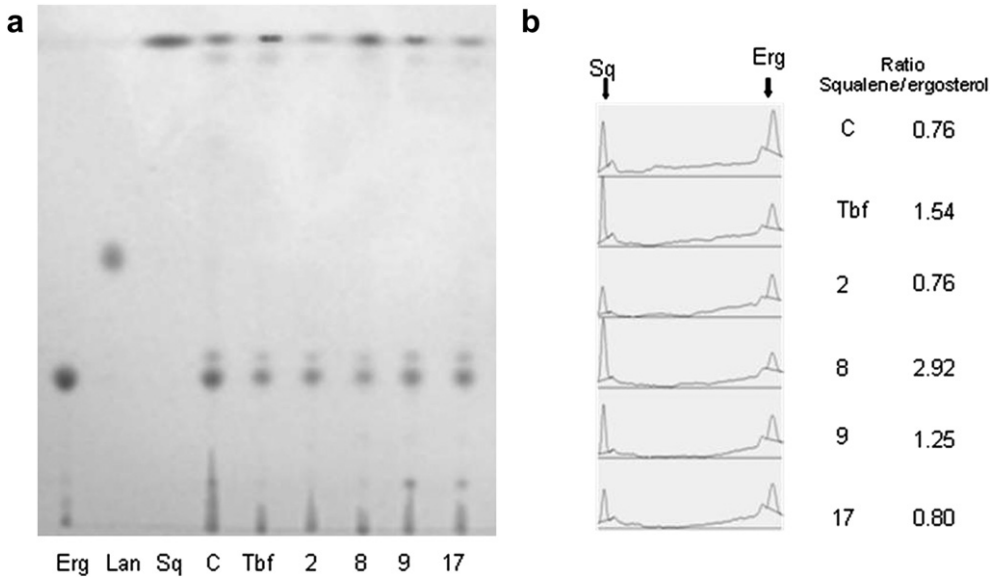

Fig. 3. a) TLC analysis of lipid extracts from epimastigote *T. cruzi* treated with terbinafine (Tbf), **2**, **8**, **9** and with **17**. Erg: ergosterol, Lan: lanosterol, Sq: squalene, C: extract without treatment. b) Relative intensities of bands were quantified by densitometry using Scion image software (Scion). Results are expressed in arbitrary units.

4.1.1. Reaction of 1-indanone thiosemicarbazone derivatives with phenacyl chlorides under microwave irradiation. General procedure

In a typical procedure a mixture of thiosemicarbazone (0.010 mmol), phenacyl chloride (0.013 mmol) and the appropriate solvent (0.15 mL) (method a: EtOH, method b: acetonitrile, method c: DMF) in a borosilicate boiling tube, was placed in a microwave synthesizer at 80 °C. After completion of the reaction (monitored by TLC), the mixture was suspended in water, filtered, and washed with EtOH and hexane. Compounds **1**–**17**, **20** and **21** (hydrochloride forms) were prepared according to method c. All synthesized TZHs were crystallized from EtOH.

*4.1.1.1. 2(2-(2,3-Dihydro-1H-inden-1-ylidene)hydrazinyl)-4-phenylthiazolium chloride (**1**).* Mp: 209–210 °C. IR ν/cm^{-1} (KBr): 2714 (NH^+), 1618 and 1586 (C═N). ^{1}H NMR (500 MHz; $CDCl_3$): δ: 3.13 (m, 2H, CH_2), 3.18 (m, 2H, CH_2), 6.74 (s, 1H, H-thiazole) 7.32 (t, 1H, J = 7.6 Hz, H–Ar), 7.37 (d, 1H, J = 7.6 Hz, H–Ar), 7.41–7.50 (m, 4H, H–Ar), 7.71 (d, 2H, J = 7.1 Hz, H–Ar), 7.79 (d, 1H, J = 7.6 Hz, H–Ar), 12.91 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 27.9, 28.6, 104.3, 121.2, 126.1, 126.3, 127.6, 128.3, 129.2, 130.7, 134.6, 137.9, 148.6, 150.1, 157.7, 169.9. HRMS (ESI) m/z (M + H)$^+$ calcd for $C_{18}H_{16}N_3S$ 306.10594, found 306.10677.

*4.1.1.2. 2(2-(2,3-Dihydro-1H-inden-1-ylidene)hydrazinyl)-4-p-tolylthiazolium chloride (**2**).* Mp: 209–211 °C. IR ν/cm^{-1} (KBr): 2654 (NH^+), 1618 and 1580 (C═N). ^{1}H NMR (500 MHz; $CDCl_3$): δ: 2.38 (s, 3H, CH_3), 3.14 (m, 2H, CH_2), 3.20 (m, 2H, CH_2), 6.66 (s, 1H, H-thiazole), 7.27 (d, 2H, J = 8.0 Hz, H–Ar), 7.32 (m, 1H, H–Ar), 7.37 (d, 1H, J = 7.5 Hz, H–Ar), 7.43 (m, 1H, H–Ar), 7.58 (d, 2H, J = 8.0 Hz, H–Ar), 7.78 (d, 1H, J = 7.8 Hz, H–Ar), 12.88 (s, 1H, NH), 13.52 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 28.0, 28.7, 104.3, 121.3, 126.0, 127.5, 128.2, 129.1, 130.6, 134.6, 138.1, 141.6, 144.6, 148.5, 157.6, 169.6. HRMS (ESI) m/z (M + H)$^+$ calcd for $C_{19}H_{18}N_3S$ 320.12159, found 320.12229.

*4.1.1.3. 4-(4-Chlorophenyl) 2-(2-(2,3-dihydro-1H-inden-1-ylidene) hydrazinyl)thiazolium chloride (**3**).* Mp: 213–215 °C. IR ν/cm^{-1} (KBr): 2627 (NH^+), 1615 and 1583 (C═N). ^{1}H NMR (500 MHz; $CDCl_3$): δ: 3.10 (m, 2H, CH_2), 3.16 (m, 2H, CH_2), 6.74 (s, 1H, H-thiazole), 7.32 (t, 1H, J = 7.7 Hz, H–Ar), 7.37 (d, 1H, J = 7.7 Hz, H–Ar), 7.45 (m, 3H, H–Ar), 7.66 (d, 2H, J = 8.5 Hz, H–Ar), 7.78 (d, 1H, J = 7.7 Hz, H–Ar), 12.68 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 28.1, 28.9, 105.2, 121.4, 126.6, 127.8, 128.0, 129.4, 130.9, 132.7, 134.3, 138.2, 148.7, 149.6, 157.7, 170.4. HRMS (ESI) m/z (M + H)$^+$ calcd for $C_{18}H_{15}ClN_3S$ 340.06752, found 340.06817.

*4.1.1.4. 2-(2-(5,6-Dimethoxy-2,3-dihydro-1H-inden-1-ylidene)hydrazinyl)-4-phenylthiazolium chloride (**4**).* Mp: 219–221 °C. IR ν/cm^{-1} (KBr): 2689 (NH^+), 1615 and 1592 (C═N). ^{1}H NMR (500 MHz; $CDCl_3$): δ: 3.12 (s, 4H, 2× CH_2), 3.92 (s, 3H, OCH_3), 3.95 (s, 3H, OCH_3), 6.71 (s, 1H, H-thiazole), 6.80 (s, 1H, H–Ar), 7.16 (s, 1H, H–Ar), 7.45 (m, 3H, H–Ar), 7.69 (d, 2H, J = 7.3, H–Ar), 12.84 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 28.7, 28.8, 56.2, 56.3, 103.3, 104.2, 108.8, 126.3, 128.5, 129.4, 130.0, 134.8, 142.5, 149.4, 150.2, 152.3, 158.7, 170.2. HRMS (ESI) m/z (M + H)$^+$ calcd for $C_{20}H_{20}N_3O_2S$ 366.12707, found 366.12786.

*4.1.1.5. 2-(2-(5,6-Dimethoxy-2,3-dihydro-1H-inden-1-ylidene)hydrazinyl)-4-p-tolylthiazolium chloride (**5**).* Mp: 221–223 °C. IR ν/cm^{-1} (KBr): 2714 (NH^+), 1627 and 1591 (C═N). ^{1}H NMR (500 MHz; $CDCl_3$): δ: 2.39 (s, 3H, CH_3), 3.12 (s, 4H, 2× CH_2), 3.92 (s, 3H, OCH_3), 3.95 (s, 3H, OCH_3), 6.63 (s, 1H, H-thiazole), 6.82 (s, 1H), 7.16 (s, 1H), 7.27 (d, 2H, J = 8.1 Hz, H–Ar), 7.58 (d, 2H, J = 8.1 Hz, H–Ar), 12.82 (s, 1H, NH), 13.99 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 20.1, 28.0, 28.2, 55.5, 55.6, 102.6, 104.1, 108.1, 125.5, 129.2, 129.6, 131.2, 137.1, 141.6, 148.4, 148.8, 151.7, 158.1, 169.2. HRMS (ESI) m/z (M + H)$^+$ calcd for $C_{21}H_{22}N_3O_2S$ 380.14272, found 380.14330.

*4.1.1.6. 4-(4-Chlorophenyl)-2-(2-(5,6-dimethoxy-2,3-dihydro-1H-inden-1-ylidene)hydrazinyl)thiazolium chloride (**6**).* Mp: 226–228 °C. IR ν/cm^{-1} (KBr): 2710 (NH^+), 1618 and 1588 (C═N). ^{1}H NMR (500 MHz; $CDCl_3$): δ: 3.12 (s, 4H, 2× CH_2), 3.92 (s, 3H, OCH_3), 3.95 (s, 3H, OCH_3), 6.71 (s, 1H, H-thiazole), 6.82 (s, 1H), 7.15 (s, 1H), 7.45 (d, 2H, J = 8.2 Hz, H–Ar), 7.64 (d, 2H, J = 8.2 Hz, H–Ar), 12.68 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 28.0, 28.1, 55.5, 55.6, 102.6, 104.2, 108.1, 127.2, 128.6, 129.4, 131.9, 133.3, 141.4, 148.5, 148.8, 151.6, 157.6, 169.6. HRMS (ESI) m/z (M + H)$^+$ calcd for $C_{20}H_{19}ClN_3O_2S$ 400.08810, found 400.08868.

*4.1.1.7. 2-(2-(4-Methyl-2,3-dihydro-1H-inden-1-ylidene)hydrazinyl)-4-phenylthiazolium chloride (**7**).* Mp: 225–227 °C. IR ν/cm^{-1} (KBr): 2712 (NH^+), 1618 (C═N). ^{1}H NMR (500 MHz; $CDCl_3$): δ: 2.31 (s, 3H, CH_3), 3.08 (m, 2H, CH_2), 3.14 (m, 2H, CH_2), 6.72 (s, 1H, H-thiazole), 7.24 (m, 2H, H–Ar), 7.43–7.49 (m, 3H, H–Ar), 7.62 (t, 1H, J = 4.4 Hz, H–Ar), 7.70 (d, 2H, J = 7.3 Hz, H–Ar), 12.89 (s, 1H, NH), 13.74 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 18.0, 27.0, 27.5, 103.8, 118.2, 125.6, 127.3, 127.7, 128.7, 130.5, 134.3, 134.7, 137.4, 147.1, 149.3, 157.3, 169.5. HRMS (ESI) m/z (M + H)$^+$ calcd for $C_{19}H_{18}N_3S$ 320.12159, found 320.12238.

*4.1.1.8. 2-(2-(4-Methyl-2,3-dihydro-1H-inden-1-ylidene)hydrazinyl)-4-p-tolylthiazolium chloride (**8**).* Mp: 231–233 °C. IR ν/cm^{-1} (KBr): 2719 (NH^+), 1619 and 1591 (C═N). ^{1}H NMR (500 MHz; $CDCl_3$): δ: 2.31 (s, 3H, CH_3), 2.39 (s, 3H, CH_3), 3.07 (m, 2H, CH_2), 3.13 (m, 2H, CH_2), 6.65 (s, 1H, H-thiazole), 7.24 (m, 2H, H–Ar), 7.27 (d, 2H, J = 7.3 Hz, H–Ar) 7.58 (d, 2H, J = 7.3 Hz, H–Ar), 7.62 (t, 1H, J = 4.4 Hz, H–Ar), 12.84 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 18.0, 20.8, 26.7, 27.5, 102.9, 118.2, 125.5, 127.3, 129.2, 130.6, 131.7, 134.7, 137.0, 137.4, 147.1, 148.1, 157.5, 169.3. HRMS (ESI) m/z (M + H)$^+$ calcd for $C_{20}H_{20}N_3S$ 334.13724, found 334.13779.

*4.1.1.9. 4-(4-Chlorophenyl)-2-(2-(4-methyl-2,3-dihydro-1H-inden-1-ylidene)hydrazinyl)thiazolium chloride (**9**).* Mp: 236–238 °C. IR ν/cm^{-1} (KBr): 2694 (NH^+), 1623 and 1590 (C═N). ^{1}H NMR (500 MHz; $CDCl_3$): δ: 2.31 (s, 3H, CH_3), 3.08 (m, 2H, CH_2), 3.13 (m, 2H, CH_2), 6.73 (s, 1H, H-thiazole), 7.24 (m, 2H, H–Ar), 7.45 (d, 2H, J = 7.3 Hz, H–Ar), 7.61 (t, 1H, J = 4.8 Hz, H–Ar), 7.64 (d, 2H, J = 7.3 Hz, H–Ar), 12.74 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 18.0, 27.0, 27.5, 104.6, 118.2, 127.3, 127.4, 128.7, 130.5, 132.0, 133.5, 134.7, 137.4, 147.1, 148.7, 157.2, 169.6. HRMS (ESI) m/z (M + Na)$^+$ calcd for $C_{19}H_{17}ClN_3S$ 354.08262, found 354.08300.

*4.1.1.10. 4-(4-Chlorophenyl)-2-(2-(4-methoxy-2,3-dihydro-1H-inden-1-ylidene)hydrazinyl)thiazolium chloride (**10**).* Mp: 233–235 °C. IR ν/cm^{-1} (KBr): 2702 (NH^+), 1619 (C═N). ^{1}H NMR (500 MHz; $CDCl_3$): δ: 2.87 (m, 2H, CH_2), 3.09 (m, 2H, CH_2), 3.88 (s, 3H, OCH_3), 6.82 (s, 1H, H-thiazole), 6.85 (d, 1H, J = 7.6 Hz, H–Ar), 7.31 (d, 1H, J = 7.6 Hz, H–Ar), 7.39 (m, 3H, H–Ar), 7.71 (d, 2H, J = 8.5 Hz, H–Ar), 12.77 (s, 1H, NH), 13.20 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 25.7, 28.0, 55.6, 105.0, 111.7, 113.3, 127.7, 129.1, 129.3, 132.3, 134.1, 136.0, 139.8, 149.6, 156.7, 156.9, 170.0. HRMS (ESI) m/z (M + Na)$^+$ calcd for $C_{19}H_{16}ClN_3NaOS$ 392.05948, found 392.05835.

*4.1.1.11. 4-(4-Chlorophenyl)-2-(2-(4-nitro-2,3-dihydro-1H-inden-1-ylidene)hydrazinyl)thiazolium chloride (**11**).* Mp: 230–232 °C. IR ν/cm^{-1} (KBr): 2698 (NH^+), 1621 and 1590 (C═N). ^{1}H NMR (500 MHz; $CDCl_3$): δ: 2.97 (m, 2H, CH_2), 3.66 (m, 2H, CH_2), 6.87 (s, 1H, H-thiazole), 7.38 (d, 2H, J = 8.5 Hz, H–Ar), 7.53 (t, 1H, J = 7.6 Hz, H–Ar), 7.70 (d, 2H, J = 8.5 Hz, H–Ar), 8.10 (d, 1H, J = 7.6 Hz, H–Ar), 8.23 (d, 1H, J = 7.6 Hz, H–Ar), 12.81 (s, 1H, NH).

^{13}C NMR (DMSO-d_6) δ: 28.0, 30.0, 105.4, 125.5, 126.9, 127.7, 129.1, 129.5, 132.4, 134.0, 142.0, 143.7, 146.1, 149.8, 154.2, 169.7. HRMS (ESI) m/z (M + H)$^+$ calcd for $C_{18}H_{14}ClN_4O_2S$ 385.05205, found 385.05300.

*4.1.1.12. 4-(4-Chlorophenyl)-2-(2-(5-methyl-2,3-dihydro-1H-inden-1-ylidene)hydrazinyl)thiazolium chloride (**12**).* Mp: 245–246 °C. IR ν/cm^{-1} (KBr): 2681 (NH$^+$), 1619 and 1592 (C=N). ^{1}H NMR (500 MHz; $CDCl_3$): δ: 2.42 (s, 3H, CH_3), 3.14 (m, 4H, 2× CH_2), 6.71 (s, 1H, H-thiazole), 7.13 (d, 1H, J = 7.8 Hz, H–Ar), 7.19 (s, 1H, H–Ar), 7.46 (d, 2H, J = 8.5 Hz, H–Ar), 7.66 (d, 2H, J = 8.5 Hz, H–Ar), 7.69 (d, 1H, J = 7.8 Hz, H–Ar), 12.76 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 21.7, 28.2, 28.4, 104.9, 121.0, 126.6, 127.7, 128.5, 129.1, 134.0, 140.4, 148.7, 149.3, 157.1, 170.1. HRMS (ESI) m/z (M + H)$^+$ calcd for $C_{19}H_{17}ClN_3S$ 354.08262, found 354.08282.

*4.1.1.13. 4-(4-Chlorophenyl)-2-(2-(6-methyl-2,3-dihydro-1H-inden-1-ylidene)hydrazinyl)thiazolium chloride (**13**).* Mp: 225–227 °C. IR ν/cm^{-1} (KBr): 2687 (NH$^+$), 1618 and 1591 (C=N). ^{1}H NMR (500 MHz; $CDCl_3$): δ: 2.41 (s, 3H, CH_3), 3.13 (m, 4H, 2× CH_2), 6.74 (s, 1H, H-thiazole), 7.46 (m, 3H, H–Ar), 7.58 (s,1H, H–Ar), 7.66 (m, 3H, H–Ar), 12.79 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 21.2, 28.1, 28.2, 104.9, 121.1, 125.8, 127.6, 129.0, 131.4, 132.2, 136.6, 138.1, 145.5, 149.2, 157.0, 169.9. HRMS (ESI) m/z (M + H)$^+$ calcd for $C_{19}H_{17}ClN_3S$ 354.08262, found 354.08306.

*4.1.1.14. 2-(2-(5-Methyl-2,3-dihydro-1H-inden-1-ylidene)hydrazinyl)-4-phenylthiazolium chloride (**14**).* Mp: 227–229 °C. IR ν/cm^{-1} (KBr): 2678 (NH$^+$), 1615 and 1591 (C=N). ^{1}H NMR (500 MHz; $CDCl_3$): δ: 2.40 (s, 3H, CH_3), 3.12 (m, 4H, 2× CH_2), 6.72 (s, 1H, H-thiazole), 7.13 (d, 1H, J = 8.0 Hz, H–Ar), 7.17 (s, 1H, H–Ar), 7.46 (m, 3H, H–Ar), 7.66 (d, 1H, J = 8.0 Hz, H–Ar), 7.70 (d, 2H, J = 8.5 Hz, H–Ar), 12.68 (s, 1H, NH), 13.46 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 21.7, 28.2, 28.4, 104.1, 121.0, 126.0, 126.6, 128.1, 128.5, 129.1, 134.7, 135.5, 140.4, 148.8, 149.7, 157.5, 169.9. HRMS (ESI) m/z (M + H)$^+$ calcd for $C_{19}H_{18}N_3S$ 320.12159, found 320.12204.

*4.1.1.15. 2-(2-(5-Methyl-2,3-dihydro-1H-inden-1-ylidene)hydrazinyl)-4-p-tolylthiazolium chloride (**15**).* Mp: decompose before melting. IR ν/cm^{-1} (KBr): 2732 (NH$^+$), 1618 and 1591 (C=N). ^{1}H NMR (500 MHz; $CDCl_3$): δ: 2.38 (s, 3H, CH_3), 2.40 (s, 3H, CH_3), 3.13 (m, 4H, 2× CH_2), 6.64 (s, 1H, H-thiazole), 7.13 (d, 1H, J = 8.0 Hz, H–Ar), 7.17 (s, 1H, H–Ar), 7.27 (d, 2H, J = 8.2 Hz, H–Ar) 7.58 (d, 2H, J = 8.2 Hz, H–Ar), 7.66 (d, 1H, J = 8.0 Hz, H–Ar), 12.81 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 21.3, 21.7, 28.2, 28.5, 103.2, 121.0, 126.0, 126.6, 128.5, 129.7, 135.6, 137.3, 140.3, 148.7, 157.5, 169.9. HRMS (ESI) m/z (M + H)$^+$ calcd for $C_{20}H_{20}N_3S$ 334.13724, found 334.13787.

*4.1.1.16. 2-(2-(6-Methyl-2,3-dihydro-1H-inden-1-ylidene)hydrazinyl)-4-phenylthiazolium chloride (**16**).* Mp: 222–224 °C. IR ν/cm^{-1} (KBr): 2647 (NH$^+$), 1619 and 1592 (C=N). ^{1}H NMR (500 MHz; $CDCl_3$): δ: 2.40 (s, 3H, CH_3), 3.08 (m, 2H, CH_2), 3.14 (m, 2H, CH_2), 6.74 (s, 1H, H-thiazole), 7.24 (m, 2H, H–Ar), 7.43 (m, 1H, H–Ar), 7.47(m, 1H, H–Ar), 7.58 (s, 1H, H–Ar), 7.71 (d, 2H, J = 7.1 Hz, H–Ar), 12.72 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 21.3, 28.2, 28.4, 104.2, 121.4, 125.9, 126.1, 128.2, 129.1, 131.7, 136.7, 138.1, 145.8, 149.3, 157.6, 169.9. HRMS (ESI) m/z (M + H)$^+$ calcd for $C_{19}H_{18}N_3S$ 320.12159, found 320.12187.

*4.1.1.17. 2-(2-(6-Methyl-2,3-dihydro-1H-inden-1-ylidene)hydrazinyl)-4-p-tolylthiazolium chloride (**17**).* Mp: 224–226 °C. IR ν/cm^{-1} (KBr): 2729 (NH$^+$), 1619 and 1592 (C=N). ^{1}H NMR (500 MHz; $CDCl_3$): δ: 2.41 (s, 3H, CH_3), 2.43 (s, 3H, CH_3), 3.16 (m, 4H, 2× CH_2), 6.70 (s, 1H, H-thiazole), 7.28 (m, 2H, H–Ar), 7.30 (d, 2H, J = 8.5 Hz, H–Ar), 7.61 (s, 1H, H–Ar), 7.62 (d, 2H, J = 8.5 Hz, H–Ar), 12.83 (s, 1H, NH), 14.25 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 21.3, 21.4, 28.2, 28.4, 103.3, 121.4, 125.9, 126.0, 129.7, 131.7, 136.7, 137.5, 138.2, 145.8, 149.6, 157.1, 169.8. HRMS (ESI) m/z (M + H)$^+$ calcd for $C_{20}H_{20}N_3S$ 334.13724, found 334.13811.

*4.1.1.18. 2-(2-Cyclohexylidenehydrazinyl)-4-phenylthiazolium chloride (**20**) [18].* Yield: 68%, mp: 170–172 °C. IR ν/cm^{-1} (KBr): 2586 (NH$^+$), 1614 and 1567 (C=N). ^{1}H NMR (500 MHz; $CDCl_3$): δ: 1.62–1.79 (m, 6H, CH_2), 2.38 (m, 2H, CH_2), 2.60 (m, 2H, J = 6.4 Hz, CH_2), 6.67 (s, 1H, H-thiazole), 7.40–7.48 (m, 3H, H–Ar), 7.67 (d, 2H, J = 6.9 Hz, H–Ar), 12.78 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 25.6, 26.0, 27.3, 27.8, 35.2, 103.7, 126.0, 128.0, 129.1, 134.8, 149.8, 156.5, 170.5.

*4.1.1.19. 2-(2-Cyclohexylidenehydrazinyl)-4-p-tolylthiazolium chloride (**21**) [15c].* Yield: 70%, mp: 165–167 °C. IR ν/cm^{-1} (KBr): 2692 (NH$^+$), 1615 and 1589 (C=N). ^{1}H NMR (500 MHz; $CDCl_3$): δ: 1.59–1.79 (m, 6H, CH_2), 2.36 (m, 2H, CH_2), 2.38 (s, 3H, CH_3), 2.60 (m, 2H, CH_2), 6.60 (s, 1H, H-thiazole), 7.27 (d, 2H, J = 8.1 Hz, H–Ar), 7.57 (d, 2H, J = 8.1 Hz, H–Ar), 12.26 (s, 1H, NH), 14.18 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 20.8, 25.1, 25.5, 26.8, 27.1, 34.7, 102.2, 125.4, 129.1, 132.1, 136.6, 150.1, 155.3, 170.0.

4.1.2. Reaction of 1-indanone thiosemicarbazone derivatives with phenacyl chlorides under conventional heating. general procedure

A suspension of thiosemicarbazone (0.5 mmol) and phenacyl chloride (0.5 mmol) in DMF (30 mL) was heated under reflux until completion of the reaction (monitored by TLC). The mixture was poured onto ice and the resulting solid was filtered, washed with EtOH and hexane. Compounds **18**, **19**, **22** and **23** were prepared according to this methodology. All synthesized TZHs were crystallized from EtOH.

*4.1.2.1. 2(2-(2,3-Dihydro-1H-inden-1-ylidene)hydrazinyl)-4-phenylthiazole (**18**).* Yield: 67%, mp: 158–160 °C. IR ν/cm^{-1} (KBr): 3331 (N–H), 1559 (C=N). ^{1}H NMR (500 MHz; $CDCl_3$): δ: 2.76 (m, 2H, CH_2), 3.16 (m, 2H, CH_2), 6.90 (1H, s, H-thiazole) 7.32–7.42 (m, 7H, H–Ar), 7.80 (d, 2H, J = 7.2 Hz, H–Ar), 8.49 (s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ: 26.3, 28.4, 103.7, 121.6, 125.4, 125.9, 127.2, 127.7, 128.6, 130.2, 134.9, 137.7, 147.5, 151.3, 156.0, 169.3. The ^{13}C NMR spectrum was similar to that observed for compound **1** when it was recorded in DMSO-d_6.

*4.1.2.2. 2-(2-(5,6-Dimethoxy-2,3-dihydro-1H-inden-1-ylidene)hydrazinyl)-4-phenylthiazole (**19**).* Yield: 62%, mp: 204–205 °C. IR ν/cm^{-1} (KBr): 3304(N–H), 1558 (C=N). ^{1}H NMR (500 MHz; $CDCl_3$): δ: 2.74 (m, 2H, CH_2), 3.02 (m, 2H, CH_2), 3.90 (s, 3H, OCH_3), 3.96 s, 3H, OCH_3), (6.78 (s, 1H, H–Ar), 6.87 (s, 1H, H-thiazole), 7.21 (s, 1H, H–Ar), 7.30 (m, 1H, H–Ar), 7.38 (m, 2H, H–Ar) 7.80 (d, 2H, J = 7.2 Hz, H–Ar), 8.49 (s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ: 27.0, 28.4, 56.0, 56.1, 103.0, 103.3, 107.3, 125.9, 127.7, 128.6, 129.8, 134.9, 141.0, 149.2, 151.2, 151.8, 156.7, 169.3. The ^{13}C NMR spectrum was similar to that observed for compound **4** when it was recorded in DMSO-d_6

*4.1.2.3. 2-(2-Cyclohexylidenehydrazinyl)-4-phenylthiazole (**22**).* Yield: 55%, mp:128–130 °C. IR ν/cm^{-1} (KBr): 3394 (N–H), 1561 (C=N). ^{1}H NMR (500 MHz; $CDCl_3$): δ: 1.57–1.70 (m, 6H, CH_2), 2.25–2.35 (m, 4H, CH_2), 6.69 (s, 1H, H-thiazole), 7.27–7.36 (m, 3H, H–Ar), 7.66 (d, 2H, J = 7.4 Hz, H–Ar). ^{13}C NMR ($CDCl_3$) δ: 26.1, 26.4, 27.6, 28.0, 35.8, 103.0, 126.5, 129.0, 129.5, 133.6, 148.8, 158.6, 170.7. The ^{13}C NMR spectrum was similar to that observed for compound **20** when it was recorded in DMSO-d_6

*4.1.2.4. 2-(2-Cyclohexylidenehydrazinyl)-4-p-tolylthiazole (**23**).* Yield: 63%, mp: 85–87 °C. IR ν/cm^{-1} (KBr): 3389 (N–H), 1556 (C=N). ^{1}H NMR (500 MHz; $CDCl_3$): δ: 1.62–1.72 (m, 6H, CH_2), 2.26–2.43 (m, 4H, CH_2), 2.36 (s. 3H, CH_3), 6.75 (s, 1H, H-thiazole), 7.18 (d, 2H, J = 8.0 Hz, H–Ar), 7.64 (d, 2H, J = 8.0 Hz, H–Ar), 8.15 (s, 1H, NH). ^{13}C NMR

($CDCl_3$) δ: 21.9, 26.1, 26.2, 27.5, 35.7, 102.6, 126.5, 126.8, 130.0, 131.6, 132.3, 138.3, 150.9, 156.7, 170.9. The ^{13}C NMR spectrum was similar to that observed for compound **21** when it was recorded in DMSO-d_6

4.2. Biology

4.2.1. Parasites and mammalian cells

T. cruzi epimastigotes (Tulahuen strain, Tul 2 stock) were grown at 28 °C in a liquid medium containing 0.3% yeast extract, 0.9% tryptose, 0.4% dextrose, 1% disodium phosphate 2-hydrate, 0.36% sodium chloride, 0.04% potassium chloride, 0.15% powered beef liver, 0.5% brain heart infusion and 0.5–1.0 mg/100 mL hemin. *T. cruzi* bloodstream trypomastigotes from Tulahuen strain, Tul 2 stock were obtained from infected CF1 mice by cardiac puncture, at the peak of parasitemia on day 15 postinfection. Trypomastigotes were routinely maintained by infecting 21-days-old CF1 mice (weighing 23.8 ± 2.6 g).

Vero cells line was cultivated in MEM or RPMI, respectively, supplemented with 10% FBS, 100 U/ml penicillin, 0.1 mg/mL streptomycin and 0.292 mg/mL glutamine.

4.2.2. Animals

Outbred CF1 male and inbred C3H/HeN female mice were nursed at the Departamento de Microbiología, Facultad de Medicina, Universidad de Buenos Aires. Animals received human care and were treated in accordance with guidelines established by the Animal Care and Use Committee of the Argentine Association of Specialists in Laboratory Animals (AADEALC).

4.2.3. In vitro trypanocidal activity assay

To evaluate the growth inhibition of *T. cruzi* epimastigote, parasites from a 5 days-old culture were inoculated into fresh culture medium to reach an initial concentration of 1.5–2.5 × 10^6 cells/mL. Cells were cultured with different concentrations of compounds (usually of 1.50–15 μM or 0.5–2.5 μM) for 4 days. Bnz (2.50–15 μM) was used as the reference trypanocidal drug (positive control). The compounds ability to inhibit growth of the parasite (antiproliferative activity) was evaluated, in triplicate, in comparison to the control without drug. Cells growth was followed by counting the number of cells per mL of culture using a Neubauer chamber and was expressed as cellular density (CD). For the count only the cells showing motility (parasites without motility were dead as demonstrated by positive staining with trypan blue) were taking into account. The percentage of inhibition (%I) was calculated as: $\%I = \{1-[(CD_{5t}-CD_{0t})/(CD_{5c}-CD_{0c})]\} \times 100$, where CD_{5t} is cellular density of treated parasites at day 5; CD_{0t} is cellular density of treated parasites just immediately after adding the drug (day 0); CD_{5c} is cellular density of untreated parasites (control) at day 5; and CD_{0c} is cellular density of untreated parasites at day 0. The $IC_{50\ epi}$ (50% inhibitory concentration on epimastigote forms) was estimated by lineal regression analysis from the %I values and the decimal logarithm (log) of drug concentration.

The trypanocidal effects of different compounds and Bnz were also tested on bloodstream trypomastigotes according to a standard WHO protocol with minor modifications [29]. Briefly, mouse blood containing trypomastigotes was diluted in culture medium to adjust the parasite concentration to 1.5 × 10^6 cells/ml. Parasites were seeded (150 μl/well) in duplicate in a 96-well microplate, and different concentrations of compounds (0.30–350 μM) were added. Plates were incubated for 24 h, and the remaining alive parasites were counted in a Neubauer chamber as previously described [30]. The results were expressed as the percentage of lysed parasites (%I) relative to the number of parasites in the control (without adding the drug): $\%I = [1-(CD_t/(CD_c)] \times 100$, where CD_t is cellular density of treated parasites and CD_c is cellular density of untreated parasites. The $IC_{50\ trypo}$ (50% inhibitory concentration on trypomastigote form) was estimated by lineal regression analysis from the %I values and the decimal logarithm (log) of drug concentration.

4.2.4. Amastigote growth inhibition assay

For the analysis of intracellular parasites (amastigotes form), 96-well microplate were seeded with J774 murine macrophage cells line, at 5 × 10^3 cells/well in 100 μl volumes and incubated 2 h at 37 °C and 5% CO_2. Cells were infected with transfected blood-trypomastigotes expressing the β-galactosidase gene (clone C4) at a parasite:cell ratio 10:1. After 24 h of contact, cell cultures were washed twice with PBS to remove free parasites and the compounds (2–100 μM) were added in 200 μl fresh complete RPMI medium without phenol red (to avoid interference with the assay absorbance readings at 570 nm). Each concentration was tested in duplicate. After 7 days, the assay were developed by addition of the detergent Nonidet P-40 (1% final concentration) and CPRG (100 μM final concentration). Plates were incubated at 37 °C for 4 h. Wells with galactosidase activity turned the media from yellow to red, and this was quantified by absorbance at 570 nm in a Microplate Reader (Bio-Rad Laboratories). Because that assayed compounds are colored and present significant absorbance at 570 nm, controls including uninfected J774 cells with RPMI alone (0% infection control for untreated infected well) and uninfected J774 cells with RPMI and different doses of assayed compounds (0% infection control for treated infected well) were done. The percentage of inhibition was calculated as: $\%I = [1-(A_{it}-A_{nit}/(A_{ic}-A_{nic}] \times 100$, where A_{it} represents the mean A_{570} value recorded for treated infected wells, A_{nit} represents the mean A_{570} value recorded for treated non-infected wells, A_{ic} represents the mean A_{570} value recorded for untreated infected wells and A_{nic} represents the mean A_{570} value recorded for untreated non-infected wells. The $IC_{50\ amas}$ (50% inhibitory concentration on amastigote form) was estimated by lineal regression analysis from the %I values and the decimal logarithm (log) of drug concentration.

4.2.5. Cytotoxicity assay

Cytotoxic activity was evaluated in vitro, using Vero cells. The cells were cultured under the standard conditions described above. Cells 9 × 10^5 cell/mL were seeded in a 24-well plate and after 48 h different concentrations of compounds (12.5–200.0 μM) or Bnz (3.0–3000 μM) were added. After 24 h of incubation, cells were washed twice with PBS and 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) from a stock solution (5 mg/mL) was added at a final concentration of 0.5 mg/mL. Plates were incubated for 1 h at 37 °C. Finally, blue precipitates were dissolved in 0.5 ml of dimethyl sulfoxide (DMSO) and were read on a plate reader (Spectra Count™ BS 10001) at a wavelength of 570 nm. Values from blank plates containing only medium and reagents were subtracted from the values of the samples. The values of absorbance showed a good correlation with viable cells counts using trypan blue. All MTT assays were repeated at least three times by using four samples per assay.

4.2.6. Cruzipain inhibition assay

Cruzipain was isolated from a cell-free extract of *T. cruzi* epimastigotes by ConA-Sepharose affinity chromatography, as previously described [22]. The enzymatic activity was assayed in an incubation system contained Tris-acetate buffer pH 8.2 (50 μmol), 2-mercaptoethanol (10 μmol) and the synthetic chromogenic substrate Bz-Pro-Phe-Arg-pNA (0.3 μmol) in a total volume of 1 ml. The reaction was followed spectrophotometrically at 410 nm on a Hewlett Packard 8452A–Diode Array spectrophotometer. The drugs were added as solutions in DMSO and the controls were prepared with the correspondent amount of the same solvent. E-64

[*L-trans*-epoxy-succinyl-leucyl-amido(4-guanidino)butane] was used as reference drug [23].

4.2.7. Ergosterol biosynthesis analysis

T. cruzi epimastigotes (2.5×10^7 ells/mL) were cultured for 24 h at 28 °C in the presence of the different TZHs at a concentration of 25 μM as an aqueous solution of DMSO 0.4% (vehicle). Control samples received only the vehicle. Parasites were centrifugated at 10,000× *g* during 10 min, washed with 0.05 M sodium phosphate buffer pH 7.4 and centrifugated again at 10,000× *g* during 15 min. The cells were resuspended in 2.0 ml chloroform:methanol (2:1). Lipid extraction was completed after the suspension was sonified in a Soniprep 150, MSE Ultrasonic Power by two cycles of 30 s each and was heated at 50 °C during 30 min. After centrifugation at 500× *g* for 5 min, the organic phase was saved and the extraction was repeated twice with 1 mL of chloroform:methanol (2:1). Then, the organic phases were pooled, washed with 0.25 volume of 0.88% KCl and were evaporated. The residues were dissolved in chloroform and were analyzed by TLC. The silica-gel 60 plates (Merck) were developed in two runs, employing firstly hexane (to separate squalene of ergosterol) and then hexane:ethyl acetate (8:2) as eluents. The chromatograms were obtained by vaporizing the plates with 1% $CuSO_4$ in 8% H_3PO_4 and heating them at 100 °C. For quantification of TLC, relative intensities of bands were quantified by densitometry using Scion Image software (Scion). Results were expressed in arbitrary units.

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Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejmech.2012.07.013.

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