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Research paper

Desing and synthesis of potent *anti-Trypanosoma cruzi* agents new thiazoles derivatives which induce apoptotic parasite death

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ABSTRACT

Chagas disease, caused by the kinetoplastid protozoan parasite *Trypanosoma cruzi*, remains a relevant cause of illness and premature death and it is estimated that 6 million to 7 million people are infected worldwide. Although chemotherapy options are limited presenting serious problems, such as low efficacy and high toxicity. *T. cruzi* is susceptible to thiazoles, making this class of compounds appealing for drug development. Previously, thiazoles resulted in an increase in *anti-T. cruzi* activity in comparison to thiosemicarbazones. Here, we report the structural planning, synthesis and *anti-T. cruzi* evaluation of new thiazoles derivatives (**3a-m** and **4a-m**), designed from molecular hybridization associated with nonclassical bioisosterism. By varying substituents attached to the phenyl and thiazole rings, substituents were observed to retain, enhance or greatly increase their *anti-T. cruzi* activity, in comparison to the corresponding thiosemicarbazones. In most cases, electron-withdrawing substituents, such as bromine, 3,4-*di*chloro and nitro groups, greatly increased antiparasitic activity. Specifically, new thiazoles were identified that inhibit the epimastigote proliferation and were toxic for trypomastigotes without affecting macrophages viability. These compounds were also evaluated against cruzain. However, inhibition of this enzyme was not observed, suggesting that the compounds work through another mechanism. In addition, examination of *T. cruzi* cell death showed that these molecules induce apoptosis. In conclusion, except for compounds **3h** and **3k**, all thiazoles derivatives evaluated exhibited higher cytotoxic activity against the trypomastigote forms than the reference medicament benznidazole, without affecting macrophages viability. Compounds **4d** and **4k** were highlights, CC50 = 1.2 e 1.6 μM, respectively. Mechanistically, these compounds do not inhibit the cruzain, but induce *T. cruzi* cell death by an apoptotic process, being considered a good starting point for the development of new *anti*-Chagas drug candidates.

1. Introduction

Chagas disease, caused by the kinetoplastid protozoan parasite *Trypanosoma cruzi* (*T. cruzi*), remains a relevant cause of illness and premature death with 6 million to 7 million people estimated to be

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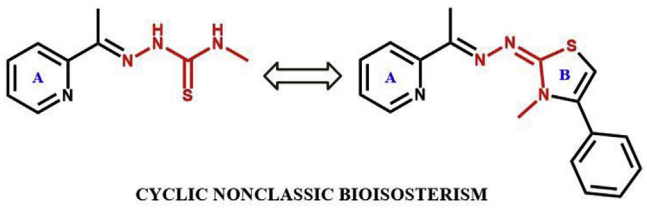
infected worldwide, mainly in Latin America. Thereby it is considered the most important parasitic disease in the Western Hemisphere [1,2]. Chemotherapy options are limited, with only two trypanocidal drugs available: nifurtimox (Nfx) and benznidazole (Bdz). Moreover, these drugs present serious problems, such as low efficacy and high toxicity. Only benznidazole is in common use, due to the risk of serious central nervous system and peripheral neurotoxicity with nifurtimox [3]. On the other hand, benznidazole is effective against the circulating form of the parasite (trypomastigotes) in the acute phase of the disease, but its efficacy during the chronic stage is debatable [4]. This situation has spurred the search for more effective and better tolerated therapeutics [5–7].

Several molecular targets for designing new drugs have been investigated, among which cruzain, the major cysteine protease expressed in all the life cycle stages of the parasite. This enzyme plays an important role in differentiation, cell invasion, intracellular multiplication, and immune evasion [8,9]. Furthermore, studies have demonstrated that cysteine proteinase inhibitors have trypanocidal activity with negligible mammalian toxicity [10,11].

Heterocyclic thiazole derivatives are considered a privileged structure in Medicinal Chemistry, considering their potential interaction with different biological targets and anti-parasitic activity demonstrated both by *in vitro* [12–14] and *in vivo* studies [15–17]. In the same away, pyridine derivatives have been shown to be potent agents *anti-T. cruzi* [18,19] and able to inhibit cruzain catalytic activity [20]. In our continuing effort to develop potent trypanocidal compounds our research group has exploited thiosemicarbazones and their heterocyclic bioisosters, 2-imino-1,3-thiazoles and 2-iminothiazolidin-4-ones. Thereby we identified new compounds with potent activity against cruzain and *T. cruzi* [7,10,21,22]. In addition, based on molecular hybridization of the pyridine group **A** with the heterocyclic ring thiazole **B** drawn from the non-classical cyclic bioisosterism of thiosemicarbazone (Scheme 1) we obtained non-toxic and potent inhibitors of *T. cruzi* and cruzain [20].

In light of these findings, we turned our attention towards the structural optimization and further identification new *anti-T. cruzi* 2-(pyridin-2-yl)thiazoles. Structural modifications were performed by insertion of substituents on the N3 position of the thiazole ring due to previous results showing derivatives with phenyl or methyl groups in N3 as potent trypanocidal agents [15,17,23]. Here, we prepared twenty four new thiazoles. In this synthetic design a range of substituents were considered for the phenyl ring attached to the thiazole moiety to examine their role to the antiparasitic activity and rationally the observed trends in terms of electronic and steric contributions.

Evaluation of the *anti-T. cruzi* activity for compounds (**3a-m**, **4a-m**), which possess different substituents in phenyl ring in the scaffold shown in Scheme 2, allowed to establish structure-activity relationships (SAR) regarding the trypomastigote form. Besides, it was possible the identification of new compounds equally or more potent than benznidazole.



Scheme 1. Design of the 2-(pyridin-2-yl)-1,3-thiazoles derivatives through strategy of cyclic non-classic biososterism.

2. Results and discussion

2.1. Synthesis

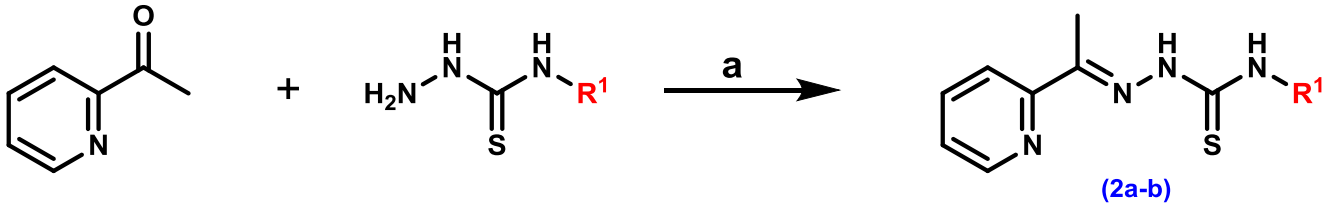
The synthetic procedures employed in 2-(pyridin-2-yl)-1,3-thiazoles (**3a-m**, **4a-m**) preparation is shown in Scheme 2. First, 2-acetyl-2-pyridine (**1**) reacted with thiosemicarbazides (methyl and phenyl-thiosemicarbazide) via Schiff base condensations in acidic conditions at room temperature. After 2 h of reaction, thiosemicarbazones (**2a-b**) compounds were obtained with higher yields than 80%. Compounds (**2a-b**) were then reacted with halo-substituted acetophenones via Hantsch cyclization in an ultrasound bath using propanol as a solvent and short times, similar to a protocol previously described [20]. After 1 h, 2-(pyridin-2-yl)-1,3-thiazoles (**3a-m**, **4a-m**) precipitated in the reaction mixture and were collected by simple filtration. Some thiazoles were obtained pure and those with impurities (**3d**, **3e**, **3f**, **3g**, **3h**, **3i**, **3j**, **3l**, **4b**, **4e**, **4h**, **4i**, **4l** and **4m**) were recrystallized with overall yields ranging from 30 to 100% while **3k** was purified for column chromatography with 50% yield.

The purified 2-(pyridin-2-yl)-1,3-thiazoles were characterized by usual spectroscopy. As exemplified with the ^{1}H NMR analysis of 4-(4-fluorophenyl)-3-methyl-2-(1-(pyridin-2-yl)ethylidene)hydrazono)-2,3-dihydrothiazole (**3e**), the singlet peak at δ 2.42 corresponds to the methyl group in the imine carbon and a second singlet peak at δ 3.35 to the methyl group in the thiazole ring. The aromatic protons occurred as doublets, triplets or multiplet. For the pyridyl ring, peaks were observed at δ 7.58, 7.80, 8.09 and 8.56. For the aromatic ring coupled to the thiazole ring, multiplet peak were found at δ 7.33. For the thiazole ring, a singlet at δ 6.46 was observed. The ^{13}C NMR spectrum of **3e** indicates disappearance of the ^{13}C = S resonance from the parent thiosemicarbazone while a new ^{13}C-H resonance appeared at 100 ppm, confirming cyclization. Quaternary carbon peaks were confirmed by DEPT experiments to appear at δ 126, 139, 148, 155, 156, 161 and 169. Peaks of the pyridine aromatic carbons were found at δ 119, 123, 136, 148 and 155. Resonances from the phenyl ring coupled to the thiazole ring were observed at δ 115.68, 115.89, 126, 131.07, 131.16 and 161. A combination of ^{1}H and ^{13}C NMR, DEPT, IR and HRMS confirmed the purity and identity of all the compounds. Crystallization was achieved only for compound **2a** (Fig. 1).

The ^{1}H NMR spectra of **4b** and **4d** compounds showed that they are composed by diastereomers. For this series, it was not possible to obtain crystals able to make crystallographic assays. Based on previous crystallized analoguess by our group, we suggest that the major isomer formed present the *E-Z* configuration (Supplementary Material). Indeed, hydrazine double-bond C2=N2 is commonly assigned as *E* configuration [20,34,35]. Concerning the exocyclic double-bond N3=C3, we suggest that the predominant configuration is in *Z*-configuration [20,23,24]. Besides, a representative ^{1}H NMR spectrum of compound **4b** is presented as Supplementary Material.

2.2. Pharmacological evaluation

After structural characterization of 2-(pyridin-2-yl)-1,3-thiazoles (**3a-m** and **4a-m**), the antiparasitic and host cell cytotoxicity was determined. First, compounds were evaluated concerning their ability to inhibit the epimastigote proliferation of *T. cruzi* Dm28 strain, as well as their toxicity against Y strain trypomastigotes. Results were respectively expressed in terms of IC_{50} and CC_{50} values. Following this, cytotoxicity was determined in J774A.1 macrophages and results were expressed as the highest non-cytotoxic concentration (HNC) and given in μM. Benznidazole (Bdz) was used as a reference antiparasitic drug and exhibited CC_{50}



Comp.	R^1	R^2	R^3	Comp.	R^1	R^2	R^3
3a		Ph	H	**4a**		Ph	H
3b		Ph	Me	**4b**		Ph	Me
3c		4-Ph-Ph	H	**4c**		4-Ph-Ph	H
3d		4-MeO-Ph	H	**4d**		4-MeO-Ph	H
3e		4-F-Ph	H	**4e**		4-F-Ph	H
3f		4-Cl-Ph	H	**4f**		4-Cl-Ph	H
3g	Me	4-Br-Ph	H	**4g**	Ph	4-Br-Ph	H
3h		4-NO_2-Ph	H	**4h**		4-NO_2-Ph	H
3i		3-NO_2-Ph	H	**4i**		3-NO_2-Ph	H
3j		3,4-*di*Cl-Ph	H	**4j**		3,4-*di*Cl-Ph	H
3k		2,4-*di*Cl-Ph	H	**4k**		2,4-*di*Cl-Ph	H
3l		4-Br-Ph	Me	**4l**		4-Br-Ph	Me
3m		2-Naph	H	**4m**		2-Naph	H

Scheme 2. Synthetic procedures for thiosemicarbazones (**2a-b**) and 2-(pyridin-2-yl)-1,3-thiazoles (**3a-m**; **4a-m**). Reagents and conditions: (a) 4-methyl-3-thiosemicarbazide or 4-phenyl-3-thiosemicarbazide, ethanol, acetic acid (5 drops), rt, 120 min; (b) halo-substituted acetophenones, 2-propanol, ultrasound irradiation, r. t., 60 min; R^3 = H for all compounds, except to (**3b**), (**3l**), (**4b**) and (**4l**) where R_3 = Me.

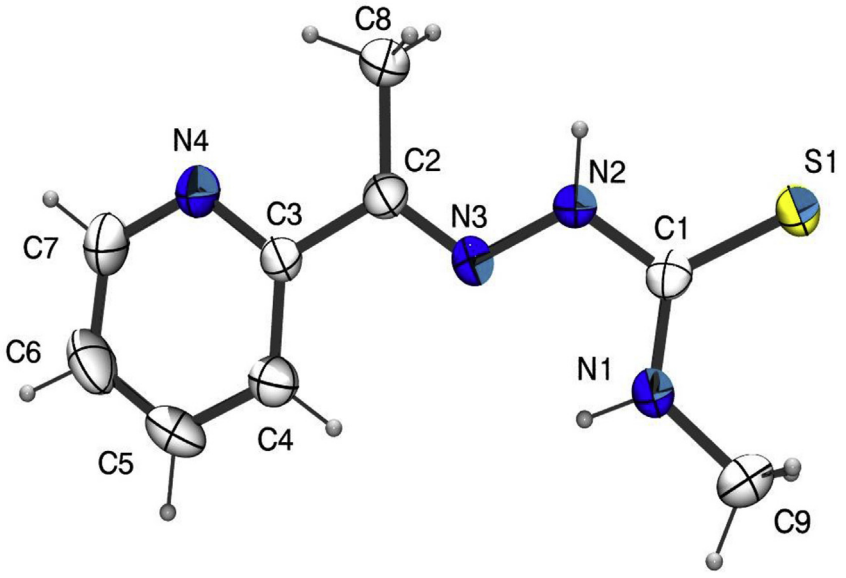
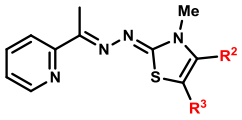


Fig. 1. ORTEP-3 projection of compound (**2a**) showing atom-numbering and displacement ellipsoids at the 50% probability level.

value of 6.2 μM against trypomastigotes. Compounds which exhibited lower CC_{50} than Bdz in this assay were considered active *anti-T. cruzi* agents [25].

Table 1 shows results of *anti-T. cruzi* activity of the compounds **3a-m**. Almost all of the thirteen compounds tested presented trypanocidal properties higher than Bdz, with the exception of compounds **2a** (intermediary thiosemicarbazone), **3h** and **3k**. In the **3a-m** series, we highlight compounds **3g, 3j** and **3i**, which present CC_{50} values of 2.2, 2.3 and 2.4 μM respectively, against trypomastigotes. Based on these results the effect of the substitutions on the phenyl attached to the thiazole ring was analyzed. Concerning the activity against trypomastigotes, nonsubstituted thiazole (**3a**) was slightly more active (CC_{50} = 4.0 μM) than Bdz (CC_{50} = 6.2 μM). We evaluated the trypanocidal activity of thiazoles containing halogen atoms attached to *para*-position of the phenyl ring. This peculiar substitution produced active compounds, since substituents 4-bromophenyl (**3g**), 4-chlorophenyl (**3f**) and 4-fluorophenyl (**3e**) presented CC_{50} values of 2.2, 4.5 and 5.6 μM, respectively. Regarding the thiazoles containing two chlorines attached to the phenyl ring, a divergence in their trypanocidal capabilities was

Table 1
Anti-T. cruzi activities of 2-(pyridin-2-yl)-1,3-thiazoles **3a-m**.

Compd.	R^2	R^3	*T. cruzi*		Cytotoxicity HNC [μM][c]	SI[d]
			Trypomastigotes CC_{50} [μM][a]	Epimastigotes CC_{50} [μM][b]		
						
2a	–	–	8.8	ND	22	2.5
3a	Ph	H	4	56	4	1
3b	Ph	Me	2.7	6.2	97	36
3c	4-PhPh	H	2.2	66.6	6	3
3d	4-OMePh	H	4.6	4.5	20	4
3e	4-FPh	H	5.6	8.5	12	2
3f	4-ClPh	H	4.5	ND	20	4
3g	4-BrPh	H	2.2	3.2	33	15
3h	4-NO_2Ph	H	7.0	13.7	36	5
3i	3-NO_2Ph	H	2.4	7.8	82	34
3j	3,4-*di*ClPh	H	2.3	6.9	11	5
3k	2,4-*di*ClPh	H	13.0	31.6	14	1
3l	4-BrPh	Me	3.4	2.9	68	20
3m	2-Naph	H	3.0	8.2	30	10
Bdz	–	–	6.2	48.8	44.7	7.2

HNC = highest non-cytotoxic concentration.
Bdz = benznidazole.
ND = not determined.
IC_{50} = inhibitory concentration for 50%. CC50 = cytotoxic concentration for 50%. CC_{50} and IC_{50} values were calculated using concentrations in triplicate and experiment was repeated, only values with a standard deviation < 10% mean were considered.
[a] Determined 24 h after incubation with compounds, using Y strain trypomastigotes.
[b] Determined 5 days after incubation with compounds, using Dm28c epimastigotes.
[c] Cell viability of J774A.1 macrophages determined 24 h after treatment.
[d] Selectivity index (SI) is the ratio of macrophages viability (HNC) to the IC_{50} on trypomastigotes.

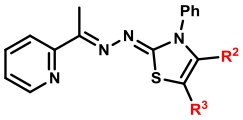
observed, with 3,4-*di*chlorophenyl derivative (**3j**) being three times more active than the Bdz (CC_{50} = 2.3 μM) while 2,4-*di*chlorophenyl derivative (**3k**) was less active with an CC_{50} value of 13 μM. Likewise, the 4-nitrophenyl derivative (**3h**) showed similar activity than Bdz (CC_{50} = 7.0 μM), while the 3-nitrophenyl derivative (**3i**) was also three times more active than Bdz (CC_{50} = 2.4 μM), suggesting that substitutions at the *meta* position of the aromatic ring were beneficial for the trypanocidal activity. In addition, compounds containing electron-donor substituents attached to the phenyl ring were also investigated. The 4-biphenylyl (**3c**) and 2-naphthalene (**3m**) derivatives were more active than Bdz showing CC_{50} values of 2.2 and 3.0 μM, respectively. On the other hand, the 4-methoxyphenyl derivative (**3d**) was also active with a CC_{50} value of 4.6 μM as well the nonsubstituted derivative (**3a**) with CC_{50} value of 4.0 μM. Another estimated effect was the insertion of methyl group attached to C4 of thiazole. This change resulted in minor changes of trypanocidal activity its addition leads to slightly decreased potency for the 4-bromophenyl derivative (compare **3g**, CC_{50} = 2.2 μM and **3l**, CC_{50} = 3.4 μM) but slightly higher potency if we consider the nonsubstituted derivative (**3a**, CC_{50} = 4.0 μM vs **3b**, CC_{50} = 2.7 μM).

Having ascertained the antiparasitic activity for trypomastigotes, we analyzed the antiproliferative activity against epimastigotes. Excluding (**3c**), all compounds were more active than benznidazole and able to inhibit epimastigote proliferation. The compounds (**3g**) and (**3j**), the more active against trypomastigote forms, were 15 and 7 times more potent than Bdz, respectively, to inhibit epimastigote proliferation. Regarding cytotoxicity in macrophages, some of the thiazoles exhibited low cytotoxicity. For instance, compounds (**3a**), (**3b**), (**3g**), (**3i**) and (**3l**) present $CC_{50} \leq 4.0$ μM against trypomastigotes, while they were non-toxic for macrophages at concentrations up to 30 μM. We determined the selectivity index (CC_{50} macrophages/CC_{50} trypomastigotes) for compounds (**3b**), (**3g**), (**3i**) and (**3l**) to be 36, 15, 34 and 20, respectively.

We also assayed the inhibitory activity for thiazoles (**3a-m**) against the enzyme cruzain, based on kinetic assays in which the fluorescence generated by the cleavage of substrate Z-FR-AMC is monitored [14]. Compounds were screened at 50 μM and the maximum perceptual of inhibition observed was 70 and 73% for compounds (**3a**) and (**3b**), respectively (data not shown). Based on these results, cruzain inhibition does not seem the mechanism of action of the trypanocidal compounds.

Next, we evaluated the *anti-T. cruzi* activity for compounds **4a-m**. In this series, all compounds presented trypanocidal properties superior to Bdz, except compounds **4a** and **4m** that showed similar activity (Table 2). Compounds **4d** and **4k** were the most active within this series, being 4–5 fold more potent than Bdz against trypomastigotes (CC_{50} = 1.2 and 1.6 μM). Concerning the activity against trypomastigotes, the nonsubstituted thiazole (**4a**) showed activity similar than Bdz (CC_{50} value of 6.3 μM). We also analyzed the effect of the substitutions on the phenyl ring attached to the thiazole in this series. As observed for the series **3a-m**, halogens attached to 4-position of the phenyl ring produced potent compounds, since substituents 4-bromophenyl (**4g**), 4-chlorophenyl (**4f**) and 4-fluorophenyl (**4e**) presented CC_{50} values of 1.8, 2.0 and 3.9 μM, respectively. The *di*-substitutions with chlorine atoms provided further promising compounds with CC_{50} values of 1.6 and 1.9 μM for 2,4-*di*chlorophenyl derivative (**4k**) and 3,4-*di*chlorophenyl derivative (**4j**), respectively. These results corroborate with the literature data that suggest that halogen atoms cause conformational changes, which allow favorable interactions between bulky groups and the target site. Typically the strength of the interaction decreases with the size of the atomic radius, according to the following order I > Br > Cl [7,10,26] similar to that was observed for the two series here described.

Table 2
Anti-T. cruzi activities of 2-(pyridin-2-yl)-1,3-thiazoles **4a-m**.

Compd.	R^2	R^3	*T. cruzi*		Cytotoxicity HNC [μM][c]	SI [d]
			Trypomastigote CC_{50} [μM][a]s	Epimastigotes CC_{50} [μM][b]		
						
2b	–	–	2.5	15.4	21	8.4
4a	Ph	H	6.3	93.4	51	8
4b	Ph	Me	2.3	15.5	43	19
4c	4-Ph	H	2.0	47.3	7	4
4d	4-OMePh	H	1.2	5.0	477	398
4e	4-FPh	H	3.9	3.1	35	9
4f	4-ClPh	H	2.0	9.1	13	7
4g	4-BrPh	H	1.8	6.3	10	6
4h	4-NO_2Ph	H	3.1	87.4	23	7
4i	3-NO_2Ph	H	1.9	11.0	10	5
4j	3,4-*di*ClPh	H	1.9	13.9	8	4
4k	2,4-*di*ClPh	H	1.6	2.7	81	51
4l	4-BrPh	Me	3.1	17.1	30	10
4m	2-Naph	H	6.1	1.9	51	8
Bdz	–	–	6.2	48.8	44.7	7.2

HNC = highest non-cytotoxic concentration.
Bdz = benznidazole.
ND = not determined.
IC_{50} = inhibitory concentration for 50%. CC50 = cytotoxic concentration for 50%. CC_{50} and IC_{50} values were calculated using concentrations in triplicate and experiment was repeated, only values with a standard deviation < 10% mean were considered.
[a] Determined 24 h after incubation with compounds, using Y strain trypomastigotes.
[b] Determined 5 days after incubation with compounds, using Dm28c epimastigotes.
[c] Cell viability of J774A.1 macrophages determined 24 h after treatment.
[d] Selectivity index (SI) is the ratio of macrophages viability (HNC) to the IC_{50} on trypomastigotes.

The derivatives containing nitro group also showed high potency presenting CC_{50} values of 3.1 and 1.9 μM for 4-nitrophenyl (**4h**) and 3-nitrophenyl (**4i**), respectively. Nitro substituent is a well-known antiparasitic pharmacophoric group [27–29] and in this work this activity was once again confirmed. Moreover, some potent compounds found here with electron-withdrawing substituents in phenyl attached in thiazole exhibit higher activity (**3i**, **3f**, **4i** and **4f**). These results are in accordance with a previous work which a CoMFA model built by HTS-ready assay that predicts the biological activity for similar molecules [29]. In contrast to the series **3a-m**, both 4-biphenyl (**4c**) and 4-methoxyphenyl (**4d**) derivatives were potent trypanocidal agents with CC_{50} values of 2.0 and 1.2 μM, respectively, whereas 2-naphthalene derivative (**4m**) caused a decrease in the trypanocidal activity, compared to other compounds in the series **4a-m**, presenting CC_{50} value of 6.1 μM, however still equipotent to benznidazole. As the same manner in the series 3a-m, the insertion of methyl group attached to C4 of bromine derivatives did not influence the trypanocidal activity since 4-bromophenyl derivative (4 g) has a CC50 value of 1.8 μM, whereas insertion of methyl group attached to C4 of thiazole generate the compound 4 l with CC50 value of 3.1 μM. However, insertion of methyl group attached to C4 of nonsubstituted derivative (**4a**) with CC_{50} value of 6.3 μM generate the compound **4b** with CC_{50} value of 2.3 μM.

A trend toward trypanocidal potency with polar substituents on the phenyl was observed. These substituents led to some of the most potent thiazole variants examined, such as compounds **3g** and **4g** (4-bromine), **3j** and **4j** (3,4-*di*chloro), **3i** and **4i** (3-nitro), and **4d** (4-methoxy). These results have been observed in thiazole derivatives of previous studies [15,17,20].

Antiproliferative activity against epimastigotes was also verified for series **4a-m**. Excluding **4a** and **4h**, all compounds were more active than benznidazole and able to inhibit epimastigote proliferation. Compounds **4d** and **4k**, the most active against blood trypomastigote forms, were 10 and 18-times more potent than Bdz, respectively, in inhibiting epimastigotes proliferation. Concerning cytotoxicity in macrophages, some of the thiazoles exhibited low cytotoxicity. For instance, compounds **4b**, **4d**, **4e**, **4k** and **4l** had CC_{50} < 4.0 μM against trypomastigotes, while they were non-toxic for macrophages at concentrations up to 30 μM. Selectivity indexes (SI = CC_{50} macrophage/CC_{50} trypomastigote) were determined for compounds **4b**, **4d**, **4e**, **4k** and **4l**, to be 19, 397, 9, 50 and 10, respectively. Likewise, thiazoles derivatives already were related as potent and selective inhibitors of *T. cruzi* besides showing absence of *in vitro* mutagenic and *in vivo* toxicity effects [16,17]. It is noteworthy that the compounds **4d** and **4k** presented SI values greater than 10, then they can be considered candidates for new trypanocidal drugs being recommended for *in vivo* tests [25].

We also assayed the inhibitory activity for thiazoles (**4a-m**) against cruzain. Compounds were screened at 50 μM and the maximum perceptual inhibition observed was 58 and 48% for compounds **4e** and **4d**, respectively (data not shown). Despite 2-(pyridin-2-yl)-1,3-thiazoles derivatives being potent *anti-T. cruzi* agents, it was not observed significant inhibition against cruzain as already observed for previously analogues 2-(pyridin-2-yl)-1,3-thiazoles studied [20], suggesting that these compounds act in a different target than cruzain. However our findings can corroborate with previous data describing that 2-imino-1,3-thiazoles are trypanocidal agents by altering the ergosterol biosynthesis instead of inhibiting the catalytic activity of cruzain [30]. In sum, the bioisosterism strategy employed herein led to bioactive compounds more selective than the thiosemicarbazones of origin **2a** and **2b**, SI of 2.5 and 8.4 respectively. The introduction of methyl and phenyl groups at N3 of thiazole ring provided some molecules more active than compounds without such substituents [20] as observed for other thiazole derivatives [17,23].

To understand the parasite death process caused by pyridin-2-yl derivatives, untreated and treated trypomastigotes were incubated for 24 h and then double labeled with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) [31]. The most active compounds in each series (**2b**, **3g**, **4d** and **4k**) were selected for this assay and evaluated in concentrations equal to their CC_{50} and CC_{100}. The data were acquired and analyzed by flow cytometry and results are show in Table 3. In comparison to untreated parasites, treatment with pyridin-2-yl derivatives (**2b**, **3g**, **4d** and **4k**) decreased parasite cell viability. All compounds assayed were more efficient in inducing parasite cell death showing positively stained for PI, PI + FITC and FITC (Table 3). Parasite cells treated with compounds at their CC_{50} were approximately 10 times more positively stained for PI and PI + Annexin V and approximately 40 times more stained for Annexin V, when compared with the control. Parasites treated with the most active compound (**4d**) at 1.2 μM presented 46.91% positively stained cells, of which 38.60% were early apoptotic (annexin V), 4.78% were late apoptotic (PI + annexin V) and 3.53% were necrotic (PI) (Fig. 2). Therefore, we suggest that pyridin-2-yl derivatives based treatment causes parasite cell death through apoptosis, suggesting that these compounds have more effects on cytoplasm and cell nucleus than in the cell membrane.

Finally we evaluated if the compounds synthesized that properties within the Lipinski's Rule of Five, which describes desired intervals for certain properties which are important for pharmacokinetics and drug development. Compound having at least three of the four criteria adheres to the Lipinski rule [32]. Other interest property is the polar surface area (PSA); since compounds with a low PSA ($\leq$140 $Å^2$) tend to have higher oral bioavailability [33]. All compounds synthetized are compatible with Lipinski rule and present appropriate PSA (Table 4).

3. Conclusions

The 2-(pyridin-2-yl)-1,3-thiazoles were structurally designed by employing a molecular hybridization of the pyridine group with the heterocyclic ring thiazole associated with the non-classical bioisosterism strategy. This led to the synthesis and chemical characterization of compounds **3a-m** and **4a-m**, which were evaluated their *anti-T. cruzi*, cytotoxicity and cruzain inhibition activities. The pharmacological evaluation led to the identification of potent and selective thiazoles (**4d**) and (**4k**) as *anti-T. cruzi* agents. Concerning their mechanism of action, these compounds did not inhibit cruzain and were observed to induce parasite cell death through an apoptotic process. The data argue that the strategies used are feasible to obtain novel potent and selective antiparasitic agents.

4. Experimental section

4.1. General

All reagents were used as purchased from commercial sources (Sigma-Aldrich, Acros Organics, Vetec or Fluka). Progress of the reactions was followed by thin-layer chromatography (silica gel 60 F_{254} in aluminum foil). Chemical identity was confirmed by NMR and IR spectroscopy and accurate mass. IR was determined in KBr pellets. For NMR, we used a Varian Unity Plus 400 MHz (400 MHz for ^1H and 100 MHz for ^{13}C) and Bruker AMX-300 MHz (300 MHz for ^1H and 75.5 MHz for ^{13}C) instruments. DMSO-d_6 and $CDCl_3$-d_6 were purchased from CIL or Sigma-Aldrich. Chemical shifts are reported by ppm and multiplicities are given as: s (singlet), d (doublet), t (triplet),q (quartet), m (multiplet) integration, and coupling constants (*J*) in hertz. Structural assignments corroborated by DEPT experiments. Mass spectrometry experiments were performed on a Q-TOF spectrometer (nanoUPLC-Xevo G2 Tof, Waters) or LC-IT-TOF (Shimadzu). When otherwise specified, ESI was carried out in the positive ion mode. Typical conditions were: capillary voltage of 3 kV and cone voltage of 30 V, and peak scan between 50 and 1000 m/z.

4.2. General procedure for the synthesis of thiosemicarbazones (2a-b)

To the solution of acetyl-2-pyridine (0.9 g, 8 mmol) in ethanol (15 mL) was added 4-methyl-3-thiosemicarbazide (0.84 g, 7.98 mmol) or 4-phenyl-3-thiosemicarbazide (1.3 g, 8 mmol) and 5 drops of acetic acid. The reaction mixture was then maintained under stirring for 120 min, at r. t. The precipitate was filtered off, washed with ethanol then dried in desiccator under vacuum. Additional amount of desired compound could be recovered from the filtrate after cooling.

4.2.1. N-methyl-2-[1-(pyridin-2-yl)ethylidene]hydrazine-1-carbothioamide (2a)

White crystals, yield: 94%. M. p. (°C): 172–174. IR (KBr, cm^{-1}): 3287 and 3238 (NH), 1537 (C=N). ^1H NMR (400 MHz, DMSO-d_6): δ 2.38 (s, 3H, CH_3), 3.05 (d, 3H, J = 4.5 Hz, CH_3), 7.37 (t, 1H, J = 4.8 and 11.1 Hz, CH, thiazole), 7.81 (t, 1H, J = 7.5 and 17.1 Hz, CH, Heterocycle), 8.42 (d, 1H, J = 8.4 Hz, CH, Heterocycle), 8.60 (m, 2H, NH, CH, Heterocycle), 10.36 (s, 1H, NH). ^{13}C NMR (75.5 MHz, DMSO-d_6): 12.1 (CH_3), 31.2 (CH_3), 120.8 (CH, Heterocycle), 123.9 (CH, Ar), 136.3 (CH, Heterocycle), 147.8 (Cq = N), 148.4 (Cq-N, Heterocycle), 154.7 (Cq-N, Heterocycle), 178.7 (Cq).

Table 3
Analysis of parasite death process caused by 2-(pyridin-2-yl)-1,3-thiazoles derivatives.

Compd.	Concentration (μM)	% PI- positively stained cells[a]	% PI and annexin V double positively stained cells[a]	% Annexin V positively stained cells[a]
Triton X − 100 (10 μL)	–	67.8		<10
Bdz	5.0	4.2		
Bdz	25	56.4		
2b	2.5	3.6	5.4	36.8
2b	5.0	3.55	4.8	36
3g	2.2	3	4	38
3g	4.4	3.15	4.7	35
4d	1.2	3.5	4.8	38.6
4d	2.4	2.9	4.5	36.4
4k	1.6	2.9	4	39.5
4k	3.2	3.2	4.3	39.4

[a] Values were taken from two different readings of at least 10,000 events 24 h after incubation with Y strain trypomastigotes.

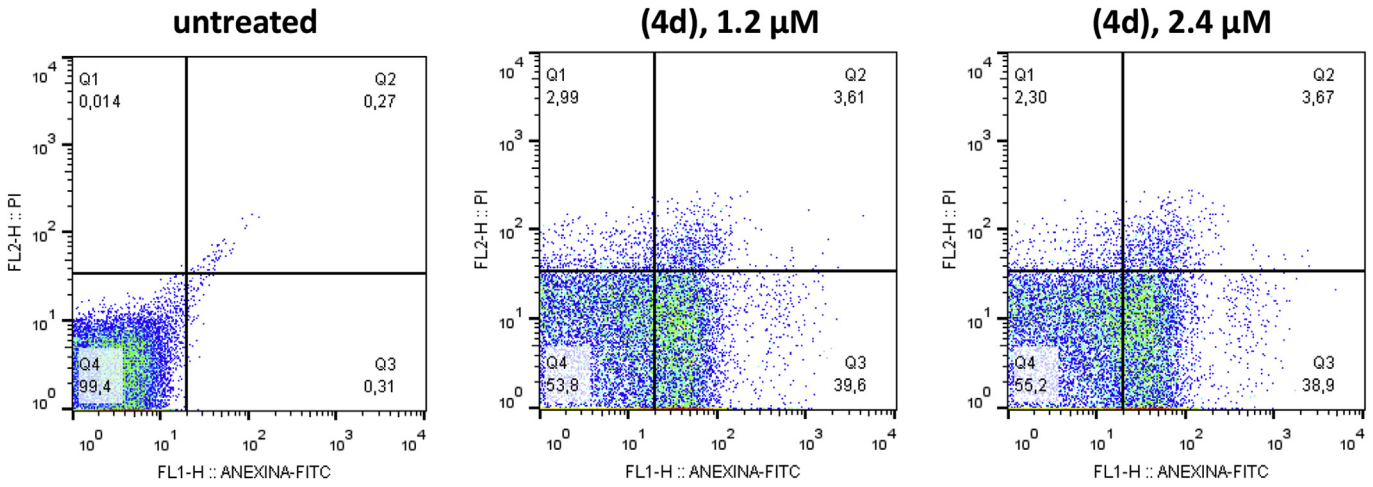


Fig. 2. 2-(pyridin-2-yl)-1,3-thiazoles (**4d**) - based treatment causes parasite death through apoptosis induction. Trypomastigotes were treated with compound (**4d**) for 24 h. Parasites were examined by flow cytometry with annexin V and PI staining. The percentage of cells in each quadrant represent the following: lower left, double negative; upper left, PI single positive; lower right, annexin V single positive; upper right, PI and annexin V double positive.

Table 4
Physicochemical properties calculated for the most potent 2-(pyridin-2-yl)-1,3-thiazoles.

Compd.	MW (g/mol)	C log P	H bond donos	H bond acceptors	Criteria met	PSA (Å^2)
Desirable value	<500	<5	<5	<10	3 at least	≤140
3g	386.02	3.57	0	4	All	40.85
3j	376.03	4.01	0	4	All	40.85
4d	400.13	4.31	0	5	All	50.08
4g	448.03	5.24	0	4	3	40.85
4i	415.11	4.41	0	6	All	86.67
4k	438.04	5.68	0	4	3	40.85

4.2.2. N-phenyl-2-[1-(pyridin-2-yl)ethylidene]hydrazine-1-carbothioamide (2b)

White crystals, yield: 89%. M. p. (°C): 182–184. IR (KBr, cm^{-1}): 3299 and 3240 (NH), 1522 (C=N). ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.87 (s, 3H, CH_3), 7.63 (t, 1H, J = 7.8 and 14.7 Hz, CH, Ar), 7.79 (m, 3H, CH Ar, CH Ar, CH Heterocycle), 7.95 (d, 2H, J = 7.8 Hz, CH Ar, CH Ar), 8.21 (t, 1H, J = 7.8 and 14.7 Hz, CH, Heterocycle), 8.95 (d, 1H, J = 7.8 Hz, CH, Heterocycle), 9.00 (d, 1H, J = 4.2 Hz, CH-N, Heterocycle), 10.61 (s, 1H, NH), 11.10 (s, 1H, NH). ^{13}C NMR (75.5 MHz, DMSO-d_6): δ 12.5 (CH_3), 121.2 (CH, Ar), 124.1 (CH, Ar), 125.6 (CH, Ar), 126.2 (CH, Ar), 128.1 (CH, Ar), 136.4 (CH, Ar), 139.1 (CH, Ar), 148.5 (Cq = N), 149.2 (Cq-N, Ar), 154.5 (Cq-N, Ar), 177.3 (Cq = S). HRMS (ESI): 271.148 [M+H].

4.3. General procedure for the synthesis of 2-imino-1,3-thiazoles 3a-m and 4a-m. example for compound 3a

2-bromoacetophenone (0.47 g, 2.4 mmol) dissolved in 2-propanol (15 mL) was placed in an ultrasound bath (40 MHz, 180 V). Then thiosemicarbazone **2a** (0.5 g, 2.3 mmol) was added to the mixture and kept until the consumption of the starting materials (60 min). The reaction was cooled and the colorful precipitate was separated in funnel with sintered disc filter and washed with cold 2-propanol, and then dried in SiO_2 glass dissector under vacuum. Most of the compounds were shown to be pure in thin layer chromatography. For the compounds **3d**, **3e**, **3f**, **3g**, **3h**, **3i**, **3j**, **3l**, **4b**, **4e**, **4h**, **4i**, **4l** and **4m** were recrystallized from hot ethanol.

4.3.1. N-(4-phenyl-3-methyl-3H-thiazol-2-ylidene)-N'-(1-pyridin-2-yl-ethylidene)-hydrazine (3a)

Reddish crystals, yield: 85% (. M. p. (°C): 204–206. IR (KBr, cm^{-1}): 1581 and 1555 (C=N), 1491 (C=C). ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.45 (s, 3H, CH_3), 3.43 (s, 3H, N-CH_3), 6.69 (s, 1H, CH, Thiazole), 7.52 (m, 5H, CH, Ar), 7.77 (t, 1H, J = 4.0 and 12.0 Hz, CH, Heterocycle), 8.27 (d, 1H, J = 8.0 Hz, CH, Heterocycle), 8.38 (t, 1H, J = 8.0 and 12.0 Hz, CH, Heterocycle), 8.71 (d, 1H, J = 4.0 Hz, CH=N, Heterocycle). ^{13}C NMR and DEPT (100 MHz, DMSO-d_6): 13.1 (CH_3), 34.0 (CH_3), 102.3 (CH, thiazole), 123.1 (CH), 124.8 (CH), 128.88 (2CH, Ar), 128.91 (CH, Heterocycle), 129.5 (CH, Heterocycle), 130.0 (Cq), 141.1 (Cq, Heterocycle), 143.8 (CH=N, Heterocycle), 150.4 (Cq), 172.3 (Cq). HRMS (ESI): 309.14 [M+H].

4.3.2. N-(5-methyl-4-methyl-3-phenyl-3H-thiazol-2-ylidene)-N'-(1-pyridin-2-yl-ethylidene)-hydrazine (3b)

Reddish crystals, yield: 40%. M. p. (°C): 234–236. IR (KBr, cm^{-1}): 1523 and 1495 (C=N), 1464 (C=C). ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.06, 2.45 and 2.49 (s, 3H, CH_3), 7.46 (m, 2H, CH, Ar), 7.56 (m, 3H, CH, Ar), 7.75 (t, 1H, J = 6.0 and 12.6 Hz, CH, Heterocycle), 8.27 (d, 1H, J = 8.1 Hz, CH, Heterocycle), 8.35 (t, 1H, J = 8.1 and 15.0 Hz, CH, Heterocycle), 8.70 (d, 1H, J = 5.1 Hz, CH=N, Heterocycle). ^{13}C NMR and DEPT (75.5 MHz, DMSO-d_6): 12.0 (CH_3); 13.1 (CH_3); 34.1 (CH_3); 112.0 (CH, thiazole); 123.4 (CH, Ar); 125.3 (CH, Ar); 129.3 (CH, Ar); 129.4 (CH, Heterocycle); 129.9 (Cq); 130.0 (CH, Heterocycle); 136.2 (Cq); 144.4 (CH, Heterocycle); 170.9 (CH, Heterocycle). HRMS (ESI): 322.12 [M - H].

4.3.3. N-{[4-(1,1'-biphenyl)-4-yl]-3-methyl-3H-thiazol-2-ylidene}-N'-(1-pyridin-2-yl-ethylidene)-hydrazine (3c)

Reddish crystals, yield: 53%. M. p. (°C): 111–114. IR (KBr, cm^{-1}): 1599 (C=N), 1481 (C=C). ^1H NMR (300 MHz, DMSO-d_6): δ 2.22 and 2.48 (s, 3H, CH_3), 6.91 (s, 1H, CH of thiazole), 7.39 (m, 5H, CH, Ar), 7.59 (m, 4H, CH, Ar), 7.69 (t, 1H, CH, Heterocycle), 8.24 (m, 2H, CH, Heterocycle), 8.68 (d, 1H, J = 4.5 Hz, CH=N, Heterocycle). ^{13}C NMR and DEPT (75.5 MHz, DMSO-d_6): 13.8 (CH_3), 21.3 (CH_3), 103.9 (CH, Thiazole), 107.7 (CH, Ar), 124.2 (CH, Ar), 125.2 (CH, Ar), 126.8 (CH, Ar), 127.0 (CH, Ar), 128.9 (CH, Heterocycle), 129.3 (CH, Heterocycle), 129.4 (CH, Heterocycle), 137.8 (CH=N, Heterocycle), 139.3 (Cq, Heterocycle), 140.5 (Cq), 145.4 (Cq). HRMS (ESI): 384.08 [M - H].

4.3.4. N-[4-(4-methoxy-phenyl)-3-methyl-3H-thiazol-2-ylidene]-N'-(1-pyridin-2-yl-ethylidene)-hydrazine (3d)

Recrystallization from ethanol afforded reddish crystals, yield: 38%. M. p. (°C): 153–156. IR (KBr, cm^{-1}): 1588 and 1521 (C=N), 1420 (C=C), 1251 (*C*-*O*-C). ^1H NMR (300 MHz, DMSO-d_6): δ 2.43 (s, 3H, CH_3), 3.34 (s, 3H, CH_3-N), 3.81 (s, 3H, CH_3-O), 6.35 (s, 1H, CH of thiazole), 7.05 (d, 2H, CH, Ar), 7.39 (m, 3H, CH, Ar), 7.80 (d, 1H, CH, Heterocycle), 8.09 (t, 1H, CH, Heterocycle), 8.57 (d, 1H, CH, Heterocycle). ^{13}C NMR and DEPT (75.5 MHz, DMSO-d_6): 12.9 (CH_3), 33.3 (CH_3-N_3), 55.2 (CH_3-O, Ar), 99.2 (CH, thiazole), 114.1 (2CH, Ar), 119.6 (CH, Ar), 122.6 (Cq, Ar), 123.0 (CH, Ar), 130.1 (CH, Ar), 136.0 (CH, Ar), 140.4 (CH-N, Ar), 148.4 (CH=N, Heterocycle), 154.9 (Cq-N, Heterocycle), 156.0 (Cq-O, Ar), 159.8 (Cq = N), 169.9 (Cq). HRMS (ESI): 339.17 [M+H].

4.3.5. N-[4-(4-fluor-phenyl)-3-methyl-3H-thiazol-2-ylidene]-N'-(1-pyridin-2-yl-ethylidene)-hydrazine (3e)

Recrystallization from ethanol afforded yellowish crystals, **y**ield: 32%. M. p. (°C): 174–176. IR (KBr, cm^{-1}): 1583 and 1520 (C=N), 1419 (C=C). ^1H NMR (400 MHz, DMSO-d_6): δ 2.42 (s, 3H, CH_3), 3.35 (s, 3H, CH_3), 6.46 (s, 1H, CH, Thiazole), 7.33 (m, 4H, CH, Ar), 7.58 (t, 1H, J = 3.0 and 12.0 Hz, CH, Heterocycle), 7.80 (t, 1H, J = 6.0 and 12.0 Hz, CH, Heterocycle), 8.09 (d, 1H, J = 6.0 Hz, CH, Heterocycle), 8.56 (d, 1H, J = 3.0 Hz, CH=N, Heterocycle). ^{13}C NMR and DEPT (100 MHz, DMSO-d_6): 13.1 (CH_3), 33.5 (N-CH_3), 100.5 (CH, Thiazole), 115.7 (CH, Ar), 115.9 (CH, Ar), 119.8 (CH, Ar), 123.3 (CH, Ar), 126.9 (C, Ar), 131.1 (CH, Ar), 131.2 (CH, Ar), 136.2 (CH, Ar), 139.6 (C, Ar), 148.6 (C=N, Heterocycle), 155.2 (*C*-N, Heterocycle), 156 (C=N), 161.3 (*C*-F, Ar), 170 (N-C-*S*). HRMS (ESI): 325.19 [M+H].

4.3.6. N-[4-(4-chloro-phenyl)-3-methyl-3H-thiazol-2-ylidene]-N'-(1-pyridin-2-yl-ethylidene)-hydrazine (3f)

Recrystallization from ethanol afforded yellowish crystals, yield: 39%. M. p. (°C): 154–156. IR (KBr, cm^{-1}): 1584 and 1558 (C=N), 1463 (C=C). ^1H NMR (400 MHz, DMSO-d_6): δ 2.44 (s, 3H, CH_3), 3.37 (s, 3H, N-CH_3), 6.50 (s, 1H, CH Thiazole), 7.36 (t, 1H, J = 6.0 and 12.0 Hz, CH, Ar), 7.56 (m, 4H, CH, Ar), 7.84 (t, 1H, J = 7.6 and 12.0 Hz, CH, Heterocycle), 8.11 (d, 1H, J = 8.0 Hz, CH, Heterocycle), 8.58 (d, 1H, J = 6.8 Hz, CH=N, Heterocycle). ^{13}C NMR and DEPT (100 MHz, DMSO-d_6): 13.0 (CH_3), 33.4 (N-CH_3), 101.0 (CH, Thiazole), 120.0 (2CH, Ar), 123.2 (2CH, Ar), 128.7 (CH, Ar), 129.2 (CH, Ar), 130.4 (C, Ar), 133.9 (CH, Ar), 136.5 (*C*-Cl), 139.4 (C, Ar), 148.1 (C=N, Heterocycle), 154.8 (*C*-N, Heterocycle), 155.6 (C=N), 170.0 (N-C-S, Ar). HRMS (ESI): 343.082 [M - H].

4.3.7. N-[4-(4-bromo-phenyl)-3-methyl-3H-thiazol-2-ylidene]-N'-(1-pyridin-2-yl-ethylidene)-hydrazine (3 g)

Recrystallization from ethanol afforded reddish crystals, yield: 54%. M. p. (°C): 160–162. IR (KBr, cm^{-1}): 1583 and 1525 (C=N), 1420 (C=C). ^1H NMR (300 MHz, DMSO-d_6): δ 2.44 (s, 3H, CH_3), 3.37 (s, 3H, N-CH_3), 6.54 (s, 1H, CH, Thiazol), 7.46 (m, 3H, CH, Ar), 7.70 (d, 2H, CH, Ar), 7.89 (d, 1H, CH, Heterocycle), 8.12 (d, 1H, CH, Heterocycle), 8.42 (t, 1H, CH, Heterocycle), 8.59 (d, 1H, CH, Heterocycle). ^{13}C NMR and DEPT (75 MHz, DMSO-d_6): 13.1 (CH_3), 33.6 (N-CH_3), 101.3 (CH, Thiazole), 120.2 (CH, Ar), 122.6 (C, Ar), 123.5 (CH, Ar), 124.0 (CH, Ar), 129.5 (Cq-N,Ar), 130.8 (CH, Ar), 131.7 (CH, Ar), 137.2 (CH, Ar), 139.6 (C=N, Heterocycle), 147.8 (C=N), 155.0 (N-C-S). HRMS (ESI): 387.063 [M - H].

4.3.8. N-[4-(4-nitro-phenyl)-3-methyl-3H-thiazol-2-ylidene]-N'-(1-pyridin-2-yl-ethylidene)-hydrazine (3 h)

Recrystallization from ethanol afforded yellowish crystals, yield: 43%. M. p. (°C): 200–203. IR (KBr, cm^{-1}): 1598 and 1564 (C=N), 1462 (C=C), 1344 (NO_2). ^1H NMR (300 MHz, DMSO-d_6): δ 2.45 (s, 3H, CH_3), 3.42 (s, 3H, N-CH_3), 6.75 (s, 1H, CH, Thiazole), 7.38 (t, 1H, CH, Ar), 7.83 (m, 3H, CH, Ar), 8.11 (d, 1H, CH, Heterocycle), 8.33 (t, 2H, CH, Heterocycle), 8.59 (d, 1H, CH=N, Heterocycle). ^{13}C NMR (75.5 MHz, DMSO-d_6): 18.4 (CH_3), 39.1 (N-CH_3), 108.8 (CH, Thiazole), 125.2 (CH, Ar), 128.7 (CH, Ar), 129.1 (CH, Ar), 135.0 (C, Ar), 141.8 (Cq, Ar), 144.1 (*C*-NO_2), 152.7 (C=N, Heterocycle), 153.5 (*C*-N, Heterocycle), 160.9 (C=N), 175.3 (N-C-S). HRMS (ESI): 354.10 [M+H].

4.3.9. N-[4-(3-nitro-phenyl)-3-methyl-3H-thiazol-2-ylidene]-N'-(1-pyridin-2-yl-ethylidene)-hydrazine (3i)

Recrystallization from ethanol afforded orange crystals, yield: 93%. M. p. (°C): 242–244. IR (KBr, cm^{-1}): 1588 and 1533 (C=N), 1486 (C=C), 1352 (NO_2). ^1H NMR (300 MHz, DMSO-d_6): δ 2.23 and 2.48 (s, 3H, CH_3), 7.16 (s, 1H, CH, Thiazole), 7.57 (m, 3H, CH, Ar), 7.79 (s, 1H, CH, Ar), 8.06 (d, 1H, J = 24.0 Hz, CH, Heterocycle), 8.32 (m, 2H, CH, Heterocycle), 8.72 (d, 1H, CH, Heterocycle). ^{13}C NMR (75.5 MHz, DMSO-d_6): 13.9 (CH_3), 106.2 (CH, Thiazole), 123.5 (CH, Ar), 123.6 (CH, Ar), 123.8 (CH, Ar), 125.7 (CH, Ar), 128.9 (CH, Ar), 129.5 (CH, Ar), 130.4 (CH, Ar), 132.2 (CH, Ar), 135.2 (CH, Ar), 137.2 (CH, Heterocycle), 138.2 (CH, Heterocycle), 143.9 (CH=N, Heterocycle), 144.5 (CH, Heterocycle), 147.9 (Cq, Heterocycle), 150.7 (Cq), 172.5 (Cq). HRMS (ESI): 352.85 [M - H].

4.3.10. N-[4-(3,4-dichloro-phenyl)-3-methyl-3H-thiazol-2-ylidene]-N'-(1-pyridin-2-yl-ethylidene)-hydrazine (3j)

Recrystallization from ethanol afforded orange crystals, yield: 83%. M. p. (°C): 195–197. IR (KBr, cm^{-1}): 1595 and 1526 (C=N), 1492 (C=C). ^1H NMR (300 MHz, $CHCl_3$-d_6): δ 2.59 (s, 3H, CH_3), 3.43 (s, 3H, CH_3), 6.24 (s, 1H, CH, Thiazole), 7.21 (d, 1H, J = 2.4 Hz, CH, Ar), 7.44 (d, 1H, J = 1.8 Hz, CH, Ar), 7.18 (d, 1H, J = 8.4 Hz, CH, Ar), 7.61 (m, 1H, CH, Heterocycle), 8.20 (m, 2H, CH, Heterocycle), 9.02 (d, 1H, J = 6.0 Hz, CH, Heterocycle). ^{13}C NMR (75.5 MHz, $CHCl_3$-d_6): 13.7 (CH_3), 34.1 (CH_3), 104.2 (CH, Thiazole), 123.0 (CH, Ar), 123.6 (CH, Ar) 128.0 (CH, Ar), 129.8 (CH, Ar), 130.6 (*C*-Cl, Ar), 131.0 (*C*-Cl, Ar), 133.3 (Cq, Ar), 134.1 (CH, Ar), 142.6 (Cq, Heterocycle), 143.4 (C=N, Heterocycle), 146.4 (*C*-N, Heterocycle), 150.6 (C=N), 173.8 (N-C-S). HRMS (ESI): 377.11 [M - H].

4.3.11. N-[4-(2,4-dichloro-phenyl)-3-methyl-3H-thiazol-2-ylidene]-N'-(1-pyridin-2-yl-ethylidene)-hydrazine (3k)

Column chromatography afforded yellowish crystals, yield: 50%. M. p. (°C): 130–132. IR (KBr, cm^{-1}): 1601 (C=N), 1495 (C=C). ^1H NMR (300 MHz, DMSO-d_6): δ 2.60 (s, 3H, CH_3), 3.30 (s, 3H, CH_3), 6.07 (s, 1H, CH, Thiazole), 7.30 (m, 3H, CH, Ar), 7.54 (d, 1H, J = 18.0 Hz, CH, Heterocycle), 7.72 (t, 1H, J = 17.1 and 9.0 Hz, CH, Heterocycle), 8.27 (d, 1H, J = 8.4 Hz, CH, Heterocycle), 8.63 (d, 1H, J = 7.2 Hz, CH=N, Heterocycle). ^{13}C NMR (75.5 MHz, DMSO-d_6): 13.2 (CH_3), 32.5 (CH_3), 101.9 (CH, Thiazole), 120.8 (CH, Ar), 122.9 (CH, Ar), 127.6 (CH, Ar), 128.7 (Cq, Ar), 129.9 (CH, Ar), 132.9 (CH, Ar), 135.6 (Cq, Ar), 136.2 (CH, Heterocycle), 136.5 (Cq, Ar), 136.6 (Cq, Ar), 148.0 (CH, Heterocycle), 156.3 (Cq, Heterocycle), 156.5 (Cq), 169.9 (Cq). HRMS (ESI): 377.02 [M - H].

4.3.12. N-[5-methyl-4-(4-bromo-phenyl)-3-methyl-3H-thiazol-2-ylidene]-N'-(1-pyridin-2-yl-ethylidene)-hydrazine (3 l)

Recrystallization from ethanol afforded yellowish crystals, yield: 31%. M. p. (°C): 150–152. IR (KBr, cm^{-1}): 1555 and 1527 (C=N), 1494 (C=C). ^{1}H NMR (300 MHz, DMSO-d_6): δ 3.43 (s, 3H, CH_3), 3.84 (s, 3H, CH_3), 4.65 (s, 3H, CH_3), 8.76 (t, 1H, J = 6.0 and 12.0 Hz, CH, Heterocycle), 8.84 (d, 1H, J = 7.8 Hz, CH, Ar), 9.20 (m, 2H, CH, Heterocycle), 9.40 (d, 2H, J = 8.1 Hz, CH, Ar), 9.53 (d, 1H, J = 8.1 Hz, CH, Ar), 10.00 (d, 1H, J = 3.9 Hz, CH=N, Heterocycle). ^{13}C NMR (75.5 MHz, DMSO-d_6): 20.2 (CH_3), 33 (CH_3), 43.6 (CH_3), 120.2 (Cq, Thiazole), 123.6 (CH, Heterocycle), 129.0 (CH, Heterocycle), 131.2 (Cq, Ar), 132.4 (2CH, Ar), 132.7 (2CH, Ar), 134.5 (Cq, Ar), 136.6 (CH, Heterocycle), 149.0 (CH=N, Heterocycle), 155.2 (Cq, Heterocycle), 156.6 (Cq), 193.2 (Cq). HRMS (ESI): 401.98 [M - H].

4.3.13. N-[4-(naphthalen-2-yl)-3-methyl-3H-thiazol-2-ylidene]-N'-(1-pyridin-2-yl-ethylidene)-hydrazine (3m)

Orange crystals, yield: 53%. M. p. (°C): 112–125. IR (KBr, cm^{-1}): 1585 and 1524 (C=N), 1417 (C=C). ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.46 (s, 3H, CH_3), 3.29 (s, 3H, CH_3-N), 6.80 (s, 1H, CH of Thiazole), 7.62 (m, 2H, CH, Ar), 7.78 (t, 1H, CH, Ar), 8.02 (m, 4H, CH, Ar), 8.13 (d, 1H, CH, Heterocycle), 8.30 (t, 1H, CH, Heterocycle), 8.38 (t, 1H, CH, Heterocycle), 8.71 (d, 1H, CH, Heterocycle). ^{13}C NMR and DEPT (100 MHz, DMSO-d_6): 13.0 (CH_3), 33.6 (N-CH_3), 100.7 (CH, Thiazole), 119.7 (2CH, Ar); 123.1 (2CH, Ar), 125.9 (C, Ar), 126.7 (CH, Ar), 126.9 (CH, Ar), 127.6 (CH, Ar), 128.0 (CH, Ar), 128.2 (C, Ar), 132.7 (CH, Ar), 136.0 (*C*-N, Ar), 140.6 (C=N, Heterocycle), 148.5 (*C*-N, Heterocycle), 155.3 (C=N), 156.1 (N-C-S, Ar).

4.3.14. N-(4-phenyl-3-phenyl-3H-thiazol-2-ylidene)-N'-(1-pyridin-2-yl-ethylidene)-hydrazine (4a)

Orange crystals, yield: 99%. M. p. (°C): 232–234. IR (KBr, cm^{-1}): 1599 (C=N); 1481 (C=C). ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.23 (s, 3H, CH_3), 6.90 (s, 1H, CH, Thiazole), 7.30 (m, 10H, CH, Ar), 7.81 (t, 1H, J = 4.0 and 12.0 Hz, CH, Heterocycle), 8.26 (d, 1H, J = 8.0 Hz, CH, Heterocycle), 8.39 (t, 1H, J = 8.0 and 12.0 Hz, CH, Heterocycle), 8.73 (d, 1H, J = 4.0 Hz, CH=N, Heterocycle). ^{13}C NMR and DEPT (100 MHz, DMSO-d_6): 13.3 (CH_3), 103.4 (CH, Thiazole), 123.2 (CH, Ar), 125.1 (CH, Ar), 128.2 (CH, Ar), 128.3 (CH, Ar), 128.38 (2CH, Ar), 128.41 (CH, Ar), 128.6 (CH, Ar), 128.8 (CH, Heterocycle), 130.2 (Cq), 137.2 (Cq), 140.1 (Cq), 143.8 (CH=N, Heterocycle), 149.5 (Cq, Heterocycle), 150.1 (Cq), 172.5 (Cq). HRMS (ESI): 371.10 [M+H].

4.3.15. N-(5-methyl-4-phenyl-3-phenyl-3H-thiazol-2-ylidene)-N'-(1-pyridin-2-yl-ethylidene)-hydrazine (4b)

Recrystallization from ethanol afforded yellowish crystals, yield: 52%. M. p. (°C): 267–269. IR (KBr, cm^{-1}): 1523 and 1494 (C=N), 1464 (C=C). ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.08 (s, 3H, CH_3), 2.18 (s, 3H, CH_3), 7.22 (m, 11H, CH, Ar), 7.79 (t, 1H, J = 7.8 and 17.1 Hz, CH Heterocycle), 8.09 (d, 1H, J = 7.8 Hz, CH, Heterocycle), 8.54 (d, 1H, J = 4.8 Hz, CH=N, Heterocycle). ^{13}C NMR and DEPT (75 MHz, DMSO-d_6): 13.1 (CH_3), 13.7 (CH_3), 111.9 (Cq, Thiazole), 120.3 (CH, Ar), 123.9 (CH, Ar), 127.9 (CH, Ar), 128.7 (2CH, Ar), 128.79 (2CH, Ar), 128.94 (2CH, Ar), 128.99 (2CH, Ar), 130.2 (CH, Heterocycle), 130.5 (Cq, Ar), 135.0 (CH, Heterocycle), 136.6 (Cq, Ar), 138.4 (Cq, Ar), 149.1 (CH, Heterocycle), 156.3 (Cq, Heterocycle), 156.6 (Cq), 169.0 (Cq Ar). HRMS (ESI): 384.14 [M - H].

4.3.16. N-{[4-(1,1'-biphenyl)-4-yl]-3-phenyl-3H-thiazol-2-ylidene}-N'-(1-pyridin-2-yl-ethylidene)-hydrazine (4c)

Orange crystals, yield: 100%. M. p. (°C): 202–204. IR (KBr, cm^{-1}): 1616 (C=N), 1438 and 1478 (C=C). ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.24 (s, 3H, CH_3), 6.98 (s, 1H, CH, Thiazole), 7.27 (m, 8H, CH, Ar), 7.39 (m, 8H, CH, Ar), 7.57 (d, 2H, CH, Ar), 7.62 (d, 2H, CH, Ar), 7.82 (t, 1H, CH, Heterocycle), 8.27 (d, 1H, CH, Heterocycle), 8.40 (t, 1H, CH, Heterocycle), 8.74 (d, 1H, CH=N, Heterocycle). ^{13}C NMR and DEPT (100 MHz, DMSO-d_6): 13.3 (CH_3), 103.7 (CH, Thiazole); 123.2 (CH, Ar); 125.1 (CH, Ar); 126.4 (3CH, Ar); 127.8 (C, Ar); 128.3 (3CH, Ar); 128.4 (2CH, Ar); 128.8 (2CH, Ar); 128.9 (2CH, Heterocycle); 129.3 (CH=N, Heterocycle); 137.2 (C, Ar); 138.8 (C, Ar); 139.7 (Cq, Heterocycle); 140.0 (*C*-N, Heterocycle); 143.8 (Cq = N); 172 (N-Cq-S). HRMS (ESI): 447.26 [M - H].

4.3.17. N-[4-(4-methoxy-phenyl)-3-phenyl-3H-thiazol-2-ylidene]-N'-(1-pyridin-2-yl-ethylidene)-hydrazine (4d)

Reddish crystals, yield: 60%. M. p. (°C): 217–219. IR (KBr, cm^{-1}): 1601 (C=N), 1477 and 1438 (C=C), 1252 (*C*-*O*-C). ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.22 (s, 3H, CH_3), 3.70 (s, 3H, CH_3-O), 6.80 (d, 3H, CH, Ar), 7.11 (d, 2H, CH, Ar), 7.35 (m, 5H, CH, Ar), 7.81 (t, 1H, CH, Ar), 8.25 (d, 1H, CH, Heterocycle), 8.40 (t, 1H, CH, Heterocycle), 8.72 (t, 1H, CH, Heterocycle). ^{13}C NMR and DEPT (100 MHz, DMSO-d_6): 13.3 (CH_3), 55.2 (CH_3), 102.2 (CH, Ar), 113.7 (CH, Ar), 122.5 (CH, Ar), 123.2 (Cq, Ar), 125.1 (CH, Ar), 126.4 (CH, Ar), 128.2 (CH, Ar), 128.3 (CH, Ar), 128.5 (CH, Ar), 128.9 (CH, Ar), 129.8 (CH, Heterocycle), 137.25 (Cq, Ar), 140.0 (CH, Heterocycle), 143.7 (CH, Heterocycle), 159.3 (Cq, Ar), 172.6 (CH=N, Heterocycle). HRMS (ESI): 401.14 [M - H].

4.3.18. N-[4-(4-fluor-phenyl)-3-phenyl-3H-thiazol-2-ylidene]-N'-(1-pyridin-2-yl-ethylidene)-hydrazine (4e)

Recrystallization from ethanol afforded yellowish crystals, yield: 31%. M. p. (°C): 171–173. IR (KBr, cm^{-1}): 1597 (C=N), 1524 and 1502 (C=C). ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.22 (s, 3H, CH_3), 6.70 (s, 1H, CH, Thiazole), 7.10 (t, 2H, CH, Heterocycle), 7.35 (m, 7H, CH, Ar), 7.83 (td, 1H, CH, Ar), 7.83 (dt, 2H, CH, Heterocycle), 8.10 (d, 1H, CH, Heterocycle), 8.56 (td, 2H, CH=N, Heterocycle). ^{13}C NMR and DEPT (75 MHz, DMSO-d_6): 13.4 (CH_3), 102.2 (CH, Thiazole), 123.5 (CH, Ar), 124.1 (CH, Ar), 125.5 (CH, Ar), 126.2 (CH, Ar), 127.2 (Cq, Ar), 127.8 (Cq, Ar), 128.1 (CH, Ar), 128.5 (CH, Ar), 128.8 (CH, Ar), 130.5 (CH, Heterocycle), 130.6 (CH, Heterocycle), 136.3 (CH, Heterocycle), 137.4 (Cq), 138.6 (Cq, Ar), 148.5 (C=N, Heterocycle), 155.7 (Cq-N, Heterocycle), 156.7 (Cq = N), 169.8 (Cq, Ar), 177.3 (Cq-F). HRMS (ESI): 389.15 [M - H].

4.3.19. N-[4-(4-chloro-phenyl)-3-phenyl-3H-thiazol-2-ylidene]-N'-(1-pyridin-2-yl-ethylidene)-hydrazine (4f)

Orange crystals, yield: 99%. M. p. (°C): 246–248. IR (KBr, cm^{-1}): 1616 (C=N), 1480 and 1444 (C=C). ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.23 (s, 3H, CH_3), 6.95 (s, 1H, CH, Thiazole), 7.21 (d, 2H, CH, Ar), 7.37 (m, 7H, CH, Ar), 7.79 (t, 1H, CH, Heterocycle), 8.25 (d, 1H, CH, Heterocycle), 8.37 (t, 1H, CH, Heterocycle), 8.73 (d, 1H, CH, Heterocycle). ^{13}C NMR and DEPT (100 MHz, DMSO-d_6): 13.3 (CH_3), 104.1 (CH, Ar), 123.1 (CH, Ar), 125.1 (CH, Ar), 128.3 (CH, Ar), 128.4 (CH, Ar), 128.9 (CH, Ar), 129.1 (Cq, Ar), 130.2 (CH, Heterocycle), 133.4 (Cq, Ar), 137.0 (Cq, Ar), 138.8 (Cq, Heterocycle), 143.4 (CH, Heterocycle), 144.1 (CH=N, Heterocycle), 150.3 (Cq), 172.2 (Cq, Ar). HRMS (ESI): 405.09 [M - H].

4.3.20. N-[4-(4-bromo-phenyl)-3-phenyl-3H-thiazol-2-ylidene]-N'-(1-pyridin-2-yl-ethylidene)-hydrazine (4 g)

Orange crystals, yield: 94%. M. p. (°C): 243–245. IR (KBr, cm^{-1}): 1615 (C=N), 1479 and 1444 (C=C). ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.23 (s, 3H, CH_3), 6.96 (s, 1H, CH, Thiazole), 7.13 (d, 2H, CH, Ar), 7.40 (m, 7H, CH, Ar), 7.80 (t, 1H, CH, Heterocycle), 8.25 (d, 1H, CH, Heterocycle), 8.38 (t, 1H, CH, Heterocycle), 8.73 (d, 1H, CH, Heterocycle). ^{13}C NMR and DEPT (100 MHz, DMSO-d_6): 13.8 (CH_3), 104.6 (CH, Thiazole), 122.6 (Cq, Ar), 123.6 (CH, Ar), 125.6 (CH, Ar), 128.8 (CH, Ar), 128.9 (CH, Ar), 129.4 (CH, Ar), 129.9 (Cq, Ar), 130.9 (CH, Ar), 131.8 (CH, Heterocycle), 137.4 (Cq, Ar), 139.4 (Cq, Heterocycle), 143.9 (CH, Heterocycle), 144.5 (CH=N, Heterocycle), 150.7

(Cq, Ar), 172.7 (Cq, Ar). HRMS (ESI): 449.04 [M - H].

4.3.21. N-[4-(4-nitro-phenyl)-3-phenyl-3H-thiazol-2-ylidene]-N'-(1-pyridin-2-yl-ethylidene)-hydrazine (4 h)

Recrystallization from ethanol afforded reddish crystals, yield: 68%. M. p. (°C): 243–246. IR (KBr, cm^{-1}): 1606 (C=N), 1532 and 1515 (C=C), 1341 (NO_2). ^1H NMR (300 MHz, DMSO-d_6): δ 2.23 (s, 3H, CH_3), 7.01 (s, 1H, CH, Thiazole), 7.40 (m, 8H, CH, Ar), 7.82 (t, 1H, $J = 7.0$ and 14.09 Hz, CH, Heterocycle), 8.09 (d, 3H, $J = 8.4$ Hz, CH, Ar), 8.56 (d, 1H, $J = 4.5$ Hz, CH=N, Heterocycle). ^{13}C NMR and DEPT (75 MHz, DMSO-d_6): 13.5 (CH_3), 106.1 (CH, Thiazole), 119.9 (CH, Ar), 123.5 (CH, Ar), 123.7 (CH, Ar), 128.1 (2CH, Ar), 128.3 (2CH, Ar), 128.9 (CH, Heterocycle), 129.1 (2CH, Ar), 136.4 (2CH, Ar), 136.9 (CH, Heterocycle), 137.3 (Cq-N, Ar), 137.7 (*C*-NO_2), 146.8 (Cq, Ar), 148.7 (C=N, Heterocycle), 155.6 (Cq-N, Heterocycle), 157.5 (Cq = N), 169.6 (N-Cq-S). HRMS (ESI): 416.11 [M+H].

4.3.22. N-[4-(3-nitro-phenyl)-3-phenyl-3H-thiazol-2-ylidene]-N'-(1-pyridin-2-yl-ethylidene)-hydrazine (4i)

Recrystallization from ethanol afforded orange crystals, yield: 100%. M. p. (°C): 150–152. IR (KBr, cm^{-1}): 1601 (C=N), 1524 (C=C), 1347 (NO_2). ^1H NMR (400 MHz, DMSO-d_6): δ 2.23 (s, 3H, CH_3), 7.00 (s, 1H, CH Thiazole), 7.38 (m, 5H, CH, Ar), 7.59 (m, 3H, CH, Ar), 7.86 (s, 1H, CH, Ar), 8.06 (m, 3H, CH, Heterocycle), 8.57 (d, 1H, CH=N, Heterocycle). ^{13}C NMR and DEPT (100 MHz, DMSO-d_6): 13.5 (CH_3), 104.7 (CH, Thiazole), 119.9 (CH, Ar), 122.8 (CH, Ar), 123.1 (CH, Ar), 123.7 (CH, Ar), 128.1 (CH, Ar), 128.5 (CH, Ar), 128.9 (CH, Ar), 129.8 (CH, Ar), 132.1 (CH, Ar), 134.4 (Cq, Ar), 136.4 (CH, Heterocycle), 137.2 (CH, Heterocycle), 137.4 (CH, Heterocycle), 147.4 (Cq), 148.6 (CH, Heterocycle), 155.6 (Cq, Heterocycle), 157.1 (Cq), 169.6 (Cq). HRMS (ESI): 416.04 [M+H].

4.3.23. N-[4-(3,4-dichloro-phenyl)-3-phenyl-3H-thiazol-2-ylidene]-N'-(1-pyridin-2-yl-ethylidene)-hydrazine (4j)

Orange crystals, yield: 100%. M. p. (°C): 227–229. IR (KBr, cm^{-1}): 1616 and 1587 (C=N), 1482 and 1442 (C=C). ^1H NMR (400 MHz, DMSO-d_6): δ 2.23 (s, 3H, CH_3), 7.05 (s, 1H, CH, Thiazole), 7.12 (d, 1H, CH, Ar), 7.45 (m, 7H, CH, Ar), 7.74 (t, 1H, CH, Heterocycle), 8.23 (d, 1H, CH, Heterocycle), 8.30 (t, 1H, CH, Heterocycle), 8.70 (d, 1H, CH, Heterocycle). ^{13}C NMR and DEPT (100 MHz, DMSO-d_6): 13.4 (CH_3), 105.1 (CH, Thiazole), 122.7 (CH, Ar), 124.9 (CH, Ar), 128.4 (CH, Ar), 129.0 (CH, Ar), 130.3 (CH, Ar), 130.4 (CH, Ar), 130.8 (Cq, Ar), 131.0 (Cq, Ar), 131.3 (Cq, Heterocycle), 136.9 (Cq, Ar), 137.4 (Cq), 142.6 (CH, Heterocycle), 144.6 (CH, Heterocycle), 150.9 (CH, Heterocycle), 171.7 (CH=N, Heterocycle). HRMS (ESI): 439.01 [M - H].

4.3.24. N-[4-(2,4-dichloro-phenyl)-3-phenyl-3H-thiazol-2-ylidene]-N'-(1-pyridin-2-yl-ethylidene)-hydrazine (4k)

Brownish crystals, yield: 30%. M. p. (°C): 177–179. IR (KBr, cm^{-1}): 1604 (C=N), 1583 and 1522 (C=C). ^1H NMR (400 MHz, DMSO-d_6): δ 2.21 (s, 3H, CH_3), 6.72 (s, 1H, CH, Thiazole), 7.31 (m, 6H, CH, Ar), 7.42 (d, 1H, CH, Ar), 7.57 (d, 2H, CH, Ar), 7.83 (t, 1H, CH, Heterocycle), 8.10 (d, 1H, CH, Heterocycle), 8.56 (d, 1H, CH, Heterocycle). ^{13}C NMR and DEPT (100 MHz, DMSO-d_6): 13.4 (CH_3), 104.1 (CH, Thiazole), 119.9 (CH, Ar), 123.6 (CH, Ar), 127.3 (CH, Ar), 127.9 (CH, Ar), 128.2 (CH, Ar), 128.5 (CH, Ar), 128.9 (CH, Ar), 129.0 (Cq, Ar), 134.1 (CH, Heterocycle), 134.2 (Cq, Ar), 134.9 (Cq, Ar), 135.0 (Cq, Ar), 136.4 (Cq, Ar), 136.6 (CH, Heterocycle), 148.6 (CH=N, Heterocycle), 155.6 (Cq, Heterocycle), 156.8 (Cq), 169.1 (Cq, Ar). HRMS (ESI): 439.05 [M - H].

4.3.25. N-[5-methyl-4-(4-bromo-phenyl)-3-phenyl-3H-thiazol-2-ylidene]-N'-(1-pyridin-2-yl-ethylidene)-hydrazine (4 l)

Recrystallization from ethanol afforded yellowish crystals, yield: 30%. M. p. (°C): 174–176. IR (KBr, cm^{-1}): 1599 and 1525 (C=N), 1464 (C=C). ^1H NMR (300 MHz, DMSO-d_6): δ 2.09 (s, 3H, CH_3), 2.19 (s, 3H, CH_3), 7.15 (d, 2H, CH, Ar), 7.30 (m, 6H, CH, Ar), 7.48 (d, 2H, CH, Ar), 7.81 (t, 1H, CH, Heterocycle), 8.09 (d, 1H, CH, Heterocycle), 8.55 (d, 1H, CH=N, Heterocycle). ^{13}C NMR and DEPT (75 MHz, DMSO-d_6): 12.5 (CH_3), 13.2 (CH_3), 95.8 (CH, Thiazole), 119.8 (CH, Ar), 121.7 (CH, Ar), 123.6 (CH, Ar), 127.6 (CH, Ar), 128.4 (CH, Heterocycle), 128.6 (CH, Heterocycle), 131.2 (CH, Heterocycle), 132.0 (CH=N, Heterocycle), 136.1 (Cq), 148.5 (Cq). HRMS (ESI): 463.02 [M - H].

4.3.26. N-[4-(naphthalen-2-yl)-3-phenyl-3H-thiazol-2-ylidene]-N'-(1-pyridin-2-yl-ethylidene)-hydrazine (4m)

Recrystallization from ethanol afforded orange crystals, yield: 98%. M. p. (°C): 166–168. IR (KBr, cm^{-1}): 1619 (C=N), 1484 and 1442 (C=C). ^1H NMR (300 MHz, DMSO-d_6): δ 2.23 (s, 3H, CH_3), 6.80 (s, 1H, CH, Thiazole), 7.17 (d, 1H, $J = 8.1$ Hz, CH, Ar), 7.32 (m, 5H, CH, Ar), 7.51 (m, 2H, CH, Ar), 7.77 (m, 6H, CH, Ar), 8.10 (d, 1H, $J = 8.1$ Hz, CH, Heterocycle), 8.55 (d, 1H, $J = 4.2$ Hz, CH=N, Heterocycle). ^{13}C NMR and DEPT (75 MHz, DMSO-d_6): 13.2 (CH_3), 103.0 (CH, Thiazole), 123.1 (CH, Ar), 125.1 (CH, Ar), 125.5 (CH, Ar), 126.7 (CH, Ar), 126.9 (CH, Ar), 127.5 (CH, Ar), 127.6 (CH, Ar), 127.8 (CH, Ar), 127.9 (Cq, Ar), 128 (C, Ar), 128.2 (CH, Ar), 128.4 (CH, Heterocycle), 128.9 (CH, Heterocycle), 132.1 (Cq, Ar), 137.3 (Cq, Ar), 140.0 (Cq, Heterocycle), 143.6 (CH, Heterocycle), 143.9 (CH=N, Heterocycle), 150 (Cq = N), 172 (Cq). HRMS (ESI): 421.14 [M - H].

4.4. Cells

J774A.1 macrophage cell line was cultured in DMEM medium (Cultilab, São Paulo, Brazil) containing 10% heat-inactivated fetal bovine serum (FBS) (Cultilab, São Paulo, Brazil), 100U/mL penicillin G, and 2mMl-glutamine in a humidified atmosphere of 5% CO_2 in air at 37 °C. Culture medium was changed 2–3 days and subcultured when cell population density reached to 70–80% confluence. This cell line was obtained from the cell bank of Rio de Janeiro (BCRJ). T. cruzi Dm28c epimastigotes, cloned derived from Dm28 strain, were maintained at 27 °C in LIT (Liver Infusion Tryptose) medium supplemented with 10% FBS, 1% hemin (Sigma–Aldrich, St. Louis, USA), and 50 μg/mL gentamycin (Novafarma, Anápolis, Brazil). Y strain trypomastigotes were obtained from the supernatant of infected LLC-MK2 cells and were maintained in RPMI-1640 medium (Sigma–Aldrich, St. Louis, USA) supplemented with 10% FBS, and 50 μg/mL gentamycin at 37 °C and 5% CO_2.

4.5. Cytotoxicity in J774A.1 macrophages

MTT-tetrazolium reduction assay was used to evaluate effects of compounds against J774A.1 cells. Briefly, 1×10^5 cells/well were added in 96-well plates and incubated for 24 h (37 °C and 5% CO_2). Compounds were then added in different concentrations (1–100 μg/mL) and incubated for 72 h. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide, 5 mg/mL in PBS) was added to each well and incubated again for 2 h. Culture medium and MTT not reduced was removed and 100 μL of DMSO were added. The amount of formazan was determined by measuring the absorbance at 570 nm. Concentration leading to 50% inhibition of viability (CC_{50}) was calculated by regression analysis with GraphPad Prism Software.

4.6. Anti-T. cruzi activity (epimastigotes)

Epimastigotes were distributed into a 96 well plate to a final density of 10^6 cells per well. Each compound was dissolved in the respective wells, in triplicate. Benznidazole was used as positive control in this assay. Plate was then cultivated for 4 days at 27 °C. After this time, aliquots from each well were collected, and the

number of parasites was calculated in a Neubauer chamber. Epimastigotes not treated with compounds (negative control) were assumed as 100% the number of parasites. Dose-response curves were determined, and the IC50 values were calculated using at least five concentrations and a nonlinear regression (Prism, version 5.0).

4.7. Anti- T. cruzi activity (trypomastigotes)

Trypomastigotes were collected from LLC-MK2 cells supernatants and distributed in a 96 well plate to a final density of 4×10^5 cells per well. Each compound was added to the wells, in triplicate. Benznidazole was used as positive controls in this assay. Plate was then cultivated for 24 h at 37 C and 5% CO_2. After this time, aliquots from each well were collected, and the number of viable parasites (i.e., with apparent motility) was counted in a Neubauer chamber. Wells that did not receive compound were assumed as 100% of viable parasites. Dose-response curves were determined, and the IC50 values were calculated by nonlinear regression (Prism, version 5.0) using at least seven concentrations.

4.8. Assay against cruzain

Cruzain activity was measured by monitoring the cleavage of the fluorescent substrate Z-Phe-Arg-aminomethylcoumarin (Z-FR-AMC). The release of fluorescent 4-amino-7-methylcoumarin was measured at 340nm/440 nm wavelengths for excitation/emission, on a Synergy 2 (Biotek) fluorometer of the Centre for Flow Cytometry of the Department of Biochemistry and Immunology at UFMG. All assays were performed in 96-well plate format, in a final volume of 200 μL of 0.1 M sodium acetate buffer, pH 5.5, in the presence of 0.1 mM betamercaptoethanol, 0.01% Triton X-100, 0.5 nM cruzain and 2.5 μM of substrate [36]. The assay was performed after a 10-min pre-incubation of the compounds with the enzyme. Initial screening was performed with 50 μM of inhibitors, except for **5j**, **6g** and **6c** were assayed with 25 μM. For each assay, two independent experiments were performed, each in triplicates and monitored for 5 min. Enzymatic activities were calculated by comparison to initial rates of reaction of a DMSO control.

4.9. Flow cytometry analysis

Trypomastigotes (4×10^5 cells/mL) were resuspended in RPMI-1640 medium and treated with compounds **3b**, **5g**, **6d** and **6k** (1 and 2xIC_{50}) for 24 h at 37 °C with 5% CO_2. Parasites were labeled with propidium iodide (PI) and annexin V using the annexin V-FITC apoptosis detection kit (Ebioscience, San Diego, USA) according to the manufacturer instructions. Experiment was performed using a BD FACSCalibur flow cytometer (San Jose, USA) by acquiring 20,000 events of the parasite region. Data were analyzed using FlowJo (Tree Star Inc©, Ashland, USA) and expressed as the percentage of cells in each population phenotype (unstained, stained only with PI, stained only with AV or stained with both markers) compared to the total number of cells analyzed. Two independent experiments, in duplicate, were performed.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2017.02.026.

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