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In vitro activity and mechanism of action against the protozoan parasite *Trypanosoma cruzi* of 5-nitrofuryl containing thiosemicarbazones

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Abstract—The in vitro growth inhibition activity of new thiosemicarbazone derivatives against the protozoan parasite *Trypanosoma cruzi*, the causative agent of American trypanosomiasis, are described. The designed compounds combine in the same molecule the thiosemicarbazone function, recently described as a potent cruzain-inhibitor moiety, and the recognised 5-nitrofuryl group, an oxidative stress promoter. Some of the derivatives were found to be very active against the cultured (epimastigote) form of the parasite, being 1.5–1.7-fold more active than the reference compound, Nifurtimox. Free radicals production was detected when the compounds were incubated in presence of mammalian-liver microsomes. The thiosemicarbazones' capacity to act as pharmacophore in the cruzain inhibition process was theoretically analysed. Frontier molecular orbital HOMO was found as an adequate descriptor in this process. Acute in vivo toxicity of two of the more active derivatives was evaluated. The results showed that these compounds are among the most potent 5-nitrofuryl derivatives tested against this parasite thus support further in vivo studies of some of these thiosemicarbazones.

1. Introduction

Chagas' disease or American trypanosomiasis is an important health problem that affects around 20 million people in Central and South America. Around 2–3 million individuals develop the typical symptoms of this disease that results in 50,000 yearly deaths.[1] The causative agent of this disease is the haemoflagellate pro tozoan *Trypanosoma cruzi (T. cruzi)*, which is transmitted in rural areas to humans and other mammals by reduviid bugs such as *Rhodnius prolixus* and *Triatoma infestans*.[2]

The main route of transmission is the result of blood-sucking activity of Chagas' disease vectors on mammals when feeding in a cyclic process. The parasite presents three main morphological forms in a complex life cycle. It replicates within the crop and midgut of Chagas' disease vectors as the epimastigote form, it is released with the insect excrements as the nondividing highly infective metacyclic trypomastigotes that invade mammalian tissues via wounds provoked by blood-sucking action. The parasite multiplies intracellularly as amastigote form, which is released as the nondividing bloodstream

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trypomastigote form that invades other tissues. Recently, the existence of the epimastigote form as an obligate mammalian intracellular stage has been revisited[3] and confirmed.[4] Despite the progress achieved in the study of *T. cruzi* biochemistry and physiology, in which several crucial enzymes for parasite survival, absent in the host, have been identified as potential targets for the design of new drugs,[5] the chemotherapy to control this parasitic infection remains undeveloped. The pharmacology is based on old and quite unspecific drugs associated with long term treatments that rise to severe side effects. In fact, although Nifurtimox {4-[(5-nitrofurfurylidene)-amino]-3-methylthio morpholine-1,1-dioxide, Nfx, Fig. 1} and Benznidazole (*N*-benzyl-2-nitro-1-imidazoleacetamide, Bnz), the only two drugs currently in use for clinical treatment of this disease, are able to wipe out parasitaemia and to reduce serological titters, they are not specific enough to all *T. cruzi* strains and they do not guarantee complete cure. Both drugs act via the reduction of the nitro group. In the case of Nfx, reduction generates an unstable nitro anion radical, which produces highly toxic reduced oxygen species. Whereas, Bnz involves covalent modification of macromolecules by nitro reduction intermediates.[6] The side effects of these drugs result from the oxidative or reductive damage in the host's tissues and are thus inextricably linked to its anti-parasitic activity. Despite these limitations, some studies involving nitroimidazole derivatives have been recently described.[7]

Besides, we have recently shown that new 5-nitrofuryl derivatives such as **1** and **2** (Fig. 1) posses high anti-*T. cruzi* activity in vitro and in vivo[8] Moreover, we have demonstrated that this family of compounds produces oxidative stress into the parasite as the main mechanism of action.[9]

Cruzain is the major cysteine protease of *T. cruzi*. The protease is expressed in all life cycle stages of the parasite and it is essential for replication of the intracellular form. Cruzain inhibition is currently one of the most advanced and widely studied strategies in the design of new drugs for the treatment of American trypanosomiasis.[10] Recently, Cohen co-workers have described that some thiosemicarbazone derivatives exhibit potent activity against cruzain as well as trypanocidal activity against parasites in cell culture.[11] The dock studies into the active site of cruzain suggest that the covalent attack of the Cys25 on one of the most active derivatives is directed towards the thiocarbonyl carbon assisted by the transfer of the His159 proton to the thiocarbonyl sulfur (as shown in Fig. 2).

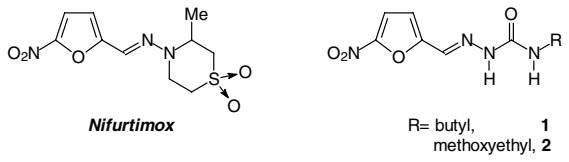


Figure 1. Nifurtimox and parent compounds **1** and **2**.

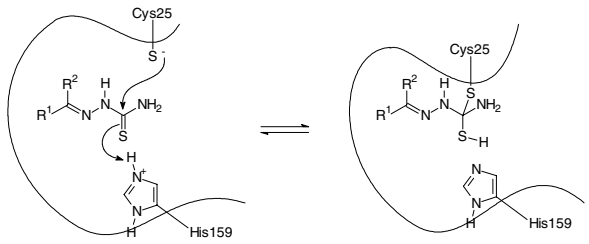
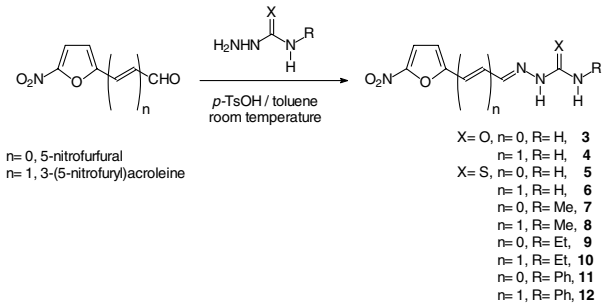


Figure 2. Mechanism of cruzain inhibition of thiosemicarbazone derivatives proposed by Cohen and co-workers.



Scheme 1. Synthetic procedure for the preparation of the develop thiosemicarbazone and oxygenated analogues.

In this paper, we describe the development of 5-nitrofuryl containing thiosemicarbazone compounds (**5**–**12**, Scheme 1) that could act against *T. cruzi* by a dual mechanism of action, oxidative stress and inhibition of cruzain. We present a study, including derivatives with different linkage between 5-nitrofuryl and thiosemicarbazone moieties and with different N^4-substituents covering a wide range of physicochemical properties (Scheme 1). The in vitro anti-parasitic activity of these compounds and an approach to understand their mode of action is also studied. For some selected derivatives, with an adequate in vitro bioactivity profile, the acute toxicity on mice was evaluated.

2. Methods and results

2.1. Synthesis

The 5-nitrofuryl derivatives **4**–**12** were prepared, with excellent yields, using the methodology indicated in Scheme 1. A typical procedure consisted in the reaction between 5-nitrofurfural or 3-(5-nitrofuryl)acroleine (1 equiv) and the thiosemicarbazide derivative (1 equiv), in presence of catalytic amounts of *p*-toluenesulfonic acid (*p*-TsOH) and dried toluene as solvent, at room temperature. Semicarbazones **3** (nitrofurazone^R), and **4** were used as oxygenated analogues. All new compounds were characterised by NMR (^{1}H, ^{13}C, NOE-diff and HETCOR experiments), IR and MS. The purity was established by TLC and microanalysis.

2.2. Biological characterisation

2.2.1. In vitro studies. All 5-nitrofuryl derivatives were tested in vitro against two strain of *T. cruzi*, Tulahuen

2 and Brener strain, as previously described.[8,12] The compounds were incorporated into the media at 5 μM and their ability to inhibit growth of the parasite, percentage of growth inhibition (PGI), was evaluated in comparison to the control (no drug added to the media) Nfx was used as the reference trypanocidal drug and the activity of compounds was expressed as $rPGI_{Nfx}$, ratio of percentage of growth inhibition respect to Nfx taking $PGI_{Nfx} = 1.0$ (Table 1). The ID_{50} concentration (50% inhibitory dose), for Tulahuen strain, was assessed for compounds presenting higher trypanocidal activity and Nfx (Table 1, Fig. 3).

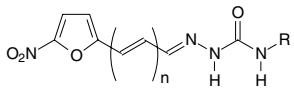
2.2.2. Acute toxicity in vivo. As a first approach to know the in vivo behaviour of new thiosemicarbazones, we compare the acute toxicity of two of them with the reference and parents compounds. So, with compounds **1**, **2**, **9**, **10** and Nfx acute toxicity was studied in healthy animals, working with a single oral and a single intraperitoneal dose of compound, 7.5- and 5.0-fold the pharmacological dose of Nfx (60 mg/kg/day),[8b] respectively. Daily the animals were weighted and observed and at the end of the trial (day 3) blood and dissected organs were used for biochemical and histological studies.

The thiosemicarbazones **9** and **10** showed nontoxic effects in vivo in the conditions assayed. So, the body weight, clinical biochemistry, haematology, macroscopic observations in the dissection process and the histological studies of organs showed that there did not exist relevant differences in the analysed parameters between treated, with **1**, **2**, thiosemicarbazones **9** and **10** and Nfx, and nontreated alive animals. However, the number of surviving animals treated with the different compounds indicate different grade of acute toxicity.[13] According to the results, the parent compounds **1** and **2** resulted more toxic than the other studied compounds when they were administered intraperitoneally (100% of death at second day of trial). However, the new developed derivatives, such as **9**, and **10**, showed the same animal's survival behaviour of Nfx in both via of administrations (100% of animal's survival at third day of trial).

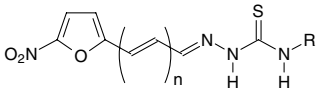
2.3. Free radical production experiments

The biological free radical production capacity of the new 5-nitrofuryl containing thiosemicarbazones was assessed by ESR using mammalian-liver microsomes as biological reductant system.[9c,d,14] All the 5-nitrofuryl thiosemicarbazone derivatives (**5**–**12**) were capable to produce free radicals in biological medium. So, the microsomal incubations of all the compounds gave an ESR spectrum after a brief induction period of 1–2 min,

Table 1. In vitro biological characterisation of developed 5-nitrofuryl derivatives



Compound	*n*	–R	Tulahuen 2 strain		Brener strain
			$rPGI_{Nfx}$[a]	ID_{50} (μM) (±0.5)	$rPGI_{Nfx}$[a]
1	0	–*n*-Butyl	0.7	8.3	ND[b]
2	0	$–CH_2CH_2OMe$	0.4	ND	ND
3	0	–H	1.3	3.4	0.4
4	1	–H	1.4	3.9	0.6



Compound	*n*	–R	Tulahuen 2 strain		Brener strain
			$RPGI_{Nfx}$[a]	ID_{50} (μM) (±0.5)	$rPGI_{Nfx}$[a]
5	0	–H	1.5	2.7	0.8
6	1	–H	1.6	3.5	1.3
7	0	–Me	1.1	5.0	0.5
8	1	–Me	1.3	4.5	0.8
9	0	–Et	1.1	4.9	0.8
10	1	–Et	1.6	4.1	1.6
11	0	–Ph	0.3	ND	0.3
12	1	–Ph	1.7	3.6	1.7
Nfx			PGI[c] = 46%	6.1	PGI = 50%

[a] $rPGI_{Nfx}$: Ratio of percentage of growth inhibition at 5 μM respect to Nfx (PGI of Nfx was taken 1.0).
[b] ND: Not determined.
[c] PGI: Percentage of growth inhibition at 5 μM.

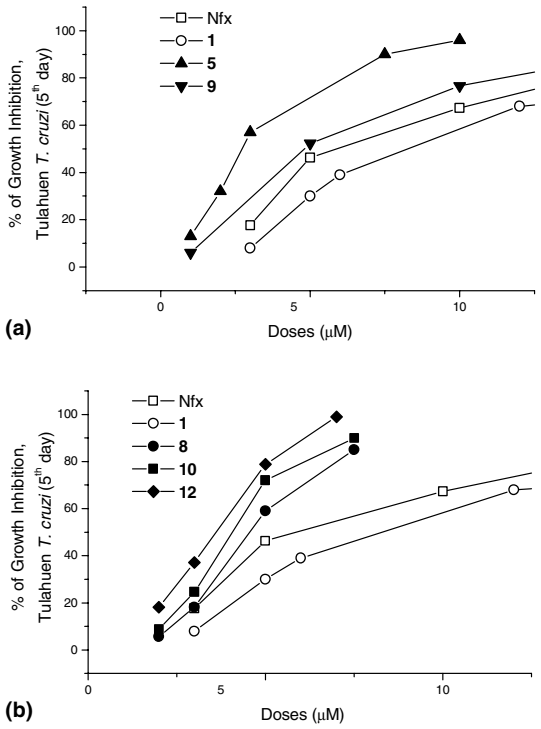


Figure 3. Curves dose–response of Nfx and derivatives **1**, **5**, **8**–**10** and **12**.

which was required for the incubation to become anaerobic. Figure 4 shows, as an example, the ESR spectra of derivative **9** and its corresponding simulated spectrum.

The hyperfine splitting pattern of the biochemical anaerobic free radicals generated was the same as that obtained by electrochemical reduction (data not shown). For example, for derivative **9** (Fig. 4) the hyperfine constants were a_N (NO_2) = 8.4 G, a_{H1} = 5.5 G, a_{H2} = 3.0 G and a_{H3} = 0.5 G and a_N (ylidenic) = 2.2 G, demonstrating that the free radical generated in the NO_2 group is delocalised up to the ylidenic nitrogen.

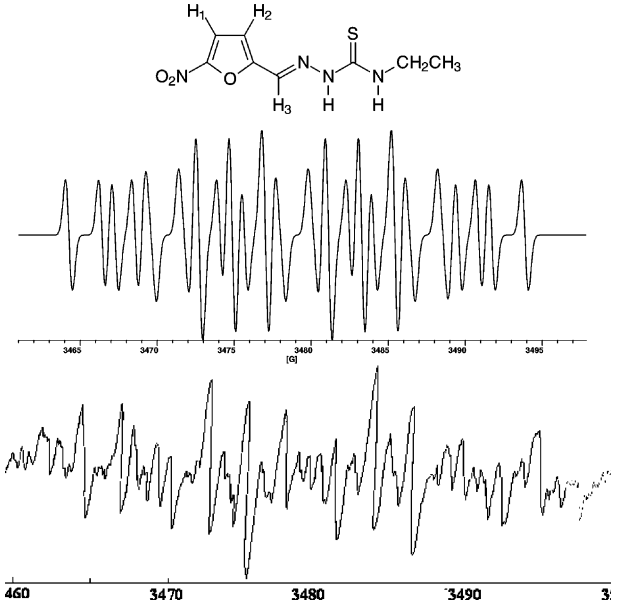


Figure 4. Down: ESR experimental spectrum of the radical of **9** generated by microsomal-anaerobic system; Up: computer simulation of the same spectrum according to the hyperfine constants indicated in the text. Spectrometer conditions: microwave frequency 9.68 GHz, microwave power 20 mW, modulation amplitude 0.2 G, scan rate 1.25 G/s, time constant 0.5 s, number of scans: 15.

2.4. Molecular modelling studies

Molecular modelling studies were performed on the developed thiosemicarbazones for calculating the stereoelectronic properties of electron surface density, HOMO maps and charges in order to understand the mechanism of action. The stereoelectronic properties were determined using B3LYP/6-31G*//6-31G* calculation.[15] A detailed conformational search for each of the molecules was performed, using MM methods (Conformational Distribution module implemented in PC SPARTAN *Pro*package),[16] to find the minimum energy and highest abundance conformer. The geometry of this conformer was fully optimised by applying ab initio 6-31G* basis in gas phase that allow to obtain acute results with low time of computational calculi.[15] Then, single point B3LYP/6-31G* density functional calculation was applied. The properties determined and examined in this study were HOMO's and LUMO's energies, atomic charges (as natural charges) on thiocarbonyl's atoms and nitro's nitrogen atom, gap (*E*_LUMO−*E*_HOMO) and the logarithm of the partition coefficient of the non-ionised molecules (Log*P*) (Table 2). Theoretical Log*P* (cLog*P*) was calculated using Villar method, implemented in PC SPARTAN *Pro*package,[16] at AM1 semiempirical level.

3. Discussion

The data presented in Table 1 show the effects of the 5-nitrofuryl derivatives on the growth of the epimastigote form of *T. cruzi* Tulahuen and Brener strains, at 5 μM at day 5 of exposure. These derivatives resulted near to 1.5–1.7-fold more active than Nfx at 5 μM towards both strains of *T. cruzi.* ID_{50} values, for Tulahuen strain, were obtained from the dose–response curves (Table 1). These nitrofuran thiosemicarbazone derivatives displayed ID_{50} from 1.7- to 3.1-fold respect to the parent compound **1**.

ESR studies showed that thiosemicarbazone derivatives (**5**–**12**) produced free radicals in biological medium, indicating that these could maintain the mode of action of 5-nitrofuryl pharmacophore. However, when we analysed the dose–response plots (Fig. 3) it could be observed that the most active compounds, thiosemicarbazones derivates from 3-(5-nitrofuryl)acroleine (i.e., **6**, **8**, **10** and **12**), presented different slopes from that of Nfx, nitrofurazone^R (**3**) and parent compound **1** (Fig. 3) indicating that they could act in a different manner of the last compounds. So, theoretically we analysed the biological behaviour of the studied compounds. All derivatives showed similar values of atomic charge on NO_2 nitrogen, included derivatives **3** and **4** (data not shown). This fact confirmed the results obtained in the microsomal-ESR experiments. The compounds possess simi-

Table 2. Physicochemical properties theoretically determined, of the developed 5-nitrofuryl derivatives

Compd	cLog*P*	Atomic charge[a]			E_{LUMO}[b]	E_{HOMO}[b]	GAP[b,c]
		NO_2 nitrogen	C=S carbon	C=S sulfur			
5	2.79	0.505	0.242	−0.237	−2.70	−6.01	3.31
6	3.32	0.504	0.247	−0.253	−2.72	−5.82	3.10
7	2.70	0.505	0.248	−0.244	−2.63	−5.94	3.31
8	3.59	0.503	0.252	−0.258	−2.66	−5.75	3.09
9	2.17	0.471	0.242	−0.222	−2.94	−6.09	3.15
10	3.65	0.504	0.258	−0.268	−2.66	−5.73	3.07
11	4.41	0.505	0.249	−0.216	−2.70	−6.08	3.37
12	5.31	0.504	0.253	−0.231	−2.73	−5.89	3.16

[a] Atomic charges as natural charges (electron).
[b] eV.
[c] $GAP = E_{LUMO} - E_{HOMO}$.

lar electrochemical behaviour, so they could act biologically in an initial redox pathway. Regression analysis of $rPGI_{Nfx}$, for both strains, versus the different calculated physicochemical properties were performed and poor correlation between $rPGI_{Nfx}$ and lipophilicity, atomic charge on NO_2 nitrogen, atomic charge on C=S carbon, and LUMO energy were obtained. However, discrete linear correlations between activity and the other physicochemical descriptors, atomic charge on C=S sulfur, HOMO energy and GAP, were obtained.[17] These relationships indicate that when the charge on thiocarbonyl sulfur is more negative, or the HOMO energy is less negative, or the GAP is lower, the activity increases. The relationships between activity and the atomic sulfur charge and HOMO energy are in accordance with the proposed Cohen's thiosemicarbazone mechanism of action (Fig. 2),[11b] where the thiocarbonyl sulfur in the active site of cruzain acts as a basic moiety. To gain insight into the atomic contribution on the frontier orbital HOMO, we displayed three-dimensional electron density isosurface with HOMO isosurface. It was possible to note that the more active compounds in both strains, such as derivatives **6**, **10** or **12**, showed the highest HOMO's contribution on thiocarbonyl sulfur while semicarbazone derivative, **4**, showed a very different atomic HOMO contribution with a diminished HOMO's molecular orbital semicarbazone–carbonyl oxygen contribution (Fig. 5).

Besides, thiosemicarbazone derivates from 3-(5-nitrofuryl)acroleine resulted more potent trypanocidal compounds in both strains assayed than the corresponding 5-nitrofuryl analogues. This difference was studied

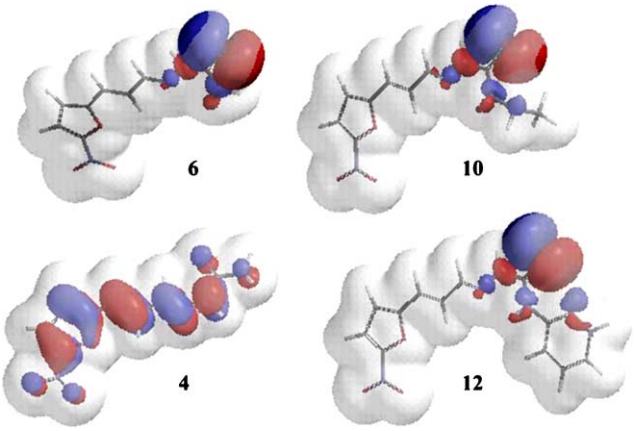


Figure 5. Electron density (transparent, isovalue = 0.002) and HOMO (solid, isovalue = 0.025) isosurfaces for derivatives **4**, **6**, **10** and **12**.

dividing the population of thiosemicarbazones in two groups of compounds, the 3-(5-nitrofuryl)acroleine derivatives and the 5-nitrofurfural derivatives. Then, the calculated theoretical properties were submitted to a *t*-Test analysis. Table 3 shows the descriptors whose means resulted significantly different, at a level lower than 0.05, for both pre-defined populations of compounds (atomic charge C=S sulfur means resulted significantly different, for both, at a 0.07 level). The HOMO energy means of both data sets resulted significantly different at a 0.004 level (Two-populations *t*-Test analysis), which indicates the importance of this descriptor in the different bioactivity observed in both population of compounds. Once again, the results were in

Table 3. Results of the *t*-Test analysis performed on the two populations of thiosemicarbazones, 5-nitrofuryl (**5**, **7**, **9** and **11**) and 3-(5-nitrofuryl)acroleinyl (**6**, **8**, **10** and **12**) derivatives

Compd	Atomic charge C=S Carbon		E_{HOMO}		GAP	
5	Mean = 0.245		Mean = −6.03		Mean = 3.29	
7						
9						
11		$t = -2.47$		$t = -4.61$		$t = 3.48$
6	Mean = 0.253	$p = 0.0487$	Mean = −5.80	$p = 0.0036$	Mean = 3.10	$p = 0.013$
8						
10						
12						

Table 4. Physiochemical properties and Lipinski's 'rule of 5'

Compd	CLog*P*	H-bond donors	H-bond acceptors	Molecular weight	'Rule of 5' criteria met
Rule	<5	⩽5	⩽10	<500	3
5	2.79	3	9	214	All
6	3.32	3	9	240	All
7	2.70	2	9	228	All
8	3.59	2	9	254	All
9	2.17	2	9	242	All
10	3.65	2	9	268	All
11	4.41	2	9	290	All
12	5.31	2	9	316	3

accordance to the thiosemicarbazone moiety as determinant for the trypanocidal activity.

4. Conclusions

The results presented above indicate that the in vitro activity of new 5-nitrofuryl containing thiosemicarbazones (derivatives **6**, **8**, **10** and **12**) against *T. cruzi*, Tulahuen 2 and Brener strains, is superior to that of the nitrofuran commercially used in the past, Nfx. All new developed thiosemicarbazones have shown to be able to produce free radicals in biological medium.

The more active products, 3-(5-nitrofuryl)acroleinyl derivatives, have shown different physicochemical properties from those of 5-nitrofuryl analogues related to their thiocarbonyl group. Selected derivatives, **9** and **10**, showed acute toxicity similar to that of Nfx. This fact, along with their predicted pharmacokinetic properties (compounds fulfil Lipinski's 'rule of 5',[18] Table 4), provides supporting evidence for further in vivo studies of these compounds in appropriate animal models for Chagas' disease.

5. Experimental

5.1. Chemistry

All starting materials were commercially available research-grade chemicals and used without further purification. Compounds **1**, **2**, **3** (nitrofurazone^{R}) and **4**–**6** were prepared following literature procedures.[8a,19] All solvents were dried and distilled prior to use. All the reactions were carried out in a nitrogen atmosphere. Melting points were determined using a Leitz Microscope Heating Stage Model 350 apparatus and are uncorrected. Elemental analyses were obtained from vacuum-dried samples (over phosphorous pentoxide at 3–4 mmHg, 24 h at room temperature) and performed on a Fisons EA 1108 CHNS–O analyser. Infrared spectra were recorder on a Perkin Elmer 1310 apparatus, using potassium bromide tablets; the frequencies are expressed in cm^{-1}. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker DPX-400 (at 400 and 100 MHz) instrument, with tetramethylsilane as the internal reference and in $CDCl_3$; the chemical shifts are reported in ppm. *J* values are given in hertz. Mass spectra were recorded on a Shimadzu GC–MS QP 1100 EX instrument at 70 eV.

5.2. General procedure for the synthesis of 7–12

A mixture of the corresponding aldehyde (5-nitro-2-furaldehyde or 3-(5-nitrofuryl)acroleine) (1 equiv), the corresponding thiosemicarbazide reactant (1 equiv), *p*-TsOH (catalytic amounts) and toluene as solvent was stirred at room temperature until the carbonyl compound was not present (SiO_2, 1% MeOH in CH_2Cl_2). The resulting precipitate was collected by filtration and was crystallised from EtOH–H_2O.

5.2.1. 4-(Methyl)-1-(5-nitrofurfurylidene)thiosemicarbazide (7). Yellow powder (75%). Mp 210.0–212.0 °C, IR ν_{max}: 3611.2 (NH), 3302.5 (NH), 1236.5 (C=S), 962.6 (furan). δ_H 1.05 (3H, t, –C*H*$_3$, EtOH crystallisation), 3.02 (3H, s, –C*H*$_3$), 3.44 (2H, qi, –C*H*$_2$, EtOH crystallisation), 4.31 (1H, t, –O*H*, EtOH crystallisation), 7.31 (1H, d, *J* 4.0, furan–*H*), 7.79 (1H, d, *J* 4.0, furan–*H*), 7.98 (1H, s, —C*H*=N), 8.52 (1H, br s, N*H*), 11.87 (1H, br s, N*H*). δ_C (HMQC, HMBC) 19.39 (–*C*H$_3$, EtOH), 31.86 (–*C*H$_3$), 56.89 (–*C*H$_2$, EtOH), 114.21 (furan–*C*), 115.97 (furan–*C*), 130.21 (H—*C*=N), 152.42 (furan–*C*), 153.44 (furan–*C*), 178.63 (—*C*=S). *m*/*z* 228 (M^{+}, 100.0%), 197 (5.6), 182 (16.4). Anal. Calcd for $C_7H_8N_4O_3S{\cdot}C_2H_6O$: C, 39.41; H, 5.14; N, 20.43; S, 11.60. Found: C, 39.34; H, 5.55; N, 20.08; S, 11.22.

5.2.2. 4-(Methyl)-1-[3-(5-nitrofuryl)propenilidene]thiosemicarbazide (8). Orange powder (68%). Mp 198.0–200.0 °C, IR ν_{max}: 3335.3 (NH), 1244.2 (C=S), 964.5 (furan). δ_H 2.97 (3H, s, –C*H*$_3$), 6.98 (1H, d, *J* 4.0, furan–*H*), 7.00 (2H, d + d, *J* 15.5, —C*H*=C*H*—), 7.72 (1H, d, *J* 4.0, furan–*H*), 7.87 (1H, d, *J* 7.6, —C*H*=N), 8.46 (1H, br s, N*H*), 11.61 (1H, br s, N*H*). δ_C (HMQC, HMBC) 31.68 (–*C*$_3$), 114.46 (furan–*C*), 116.22 (furan–*C*), 123.33 (—*C*H=), 130.95 (—*C*H=), 142.31 (H—*C*=N), 152.16 (furan–*C*), 155.62 (furan–*C*), 178.59 (—*C*=S). *m*/*z* 254 (M^{+}, 100.0%), 223 (7.0), 208 (8.3). Anal. Calcd for $C_9H_{10}N_4O_3S$: C, 42.51; H, 3.96; N, 22.03; S, 12.61. Found: C, 42.40; H, 4.03; N, 21.87; S, 12.28.

5.2.3. 4-(Ethyl)-1-(5-nitrofurfurylidene)thiosemicarbazide (9). Orange-red needles (89%). Mp 186.0–188.0 °C, IR ν_{max}: 3360.4 (NH), 1242.3 (C=S), 964.5 (furan). δ_H 1.07 (3H, t, *J* 6.9, –C*H*$_3$), 3.60 (2H, m, –C*H*$_2$), 7.31 (1H, d, *J* 3.9, furan–*H*), 7.79 (1H, d, *J* 3.8, furan–*H*), 7.98 (1H, s, —C*H*=N), 8.54 (1H, br s, N*H*), 11.82 (1H, br s, N*H*). δ_C (HMQC, HMBC) 15.14 (–*C*H$_3$), 39.38 (–*C*H$_2$), 114.34 (furan–*C*), 115.95 (furan–*C*), 130.29 (H—*C*=N), 152.44 (furan–*C*), 153.39 (furan–*C*), 177.68 (—*C*=S). *m*/*z* 242 (M^{+}, 55.2%), 197 (5.8), 155 (14.9). Anal. Calcd for $C_8H_{10}N_4O_3S$: C, 39.66; H, 4.16; N, 23.13; S, 13.23. Found: C, 39.60; H, 4.30; N, 22.99; S, 12.90.

5.2.4. 4-(Ethyl)-1-[3-(5-nitrofuryl)propenilidene]thiosemicarbazide (10). Orange powder (79%). Mp 218.0–220.0 °C, IR ν_{max}: 3327.6 (NH), 1232.7 (C=S), 958.7 (furan). δ_H 1.12 (3H, t, *J* 6.8, –C*H*$_3$), 3.54 (2H, m, –C*H*$_2$), 7.00 (3H, m, furan–*H* + —C*H*=C*H*—), 7.72 (1H, d, *J*

4.1, furan–*H*), 7.87 (1H, m, —C*H*=N), 8.47 (1H, br s, N*H*), 11.57 (1H, br s, N*H*). δ_C (HMQC, HMBC) 15.18 (–*C*H$_3$), 39.24 (–*C*H$_2$), 114.43 (furan–*C*), 116.23 (furan–*C*), 123.39 (–*C*H=), 131.02 (–*C*H=), 142.37 (H—*C*=N), 152.15 (furan–*C*), 155.65 (furan–*C*), 177.53 (—*C*=S). *m/z* 268 ($M^{\cdot+}$, 100.0%), 223 (5.8), 180 (9.4). Anal. Calcd for $C_{10}H_{12}N_4O_3S$: C, 44.77; H, 4.51; N, 20.88; S, 11.95. Found: C, 44.49; H, 4.63; N, 20.97; S, 11.67.

5.2.5. 4-(Phenyl)-1-(5-nitrofurfurylidene)thiosemicarbazide (11). Yellow powder (80%). Mp 208.0–210.0 C, IR ν_{max}: 3314.1 (NH), 1250.0 (C=S), 964.5 (furan). δ_H 7.23 (1H, t, *J* 7.5, phenyl–*H*), 7.39 (2H, t, *J* 7.7, phenyl–*H*), 7.49 (1H, d, *J* 4.0, furan–*H*), 7.55 (2H, d, *J* 7.9, phenyl–*H*), 7.82 (1H, d, *J* 4.0, furan–*H*), 8.08 (1H, s, —C*H*=N), 10.16 (1H, br s, N*H*), 12.20 (1H, br s, N*H*). δ_C (HMQC, HMBC) 114.41 (furan–*C*), 115.98 (furan-*C*), 126.57, 126.65, 129.09 (phenyl–*C*), 131.05 (H—*C*=N), 139.57 (phenyl–*C*), 152.52 (furan–*C*), 153.40 (furan–*C*), 177.21 (—*C*=S). *m/z* 290 ($M^{\cdot+}$, 27.0%), 197 (47.8), 93 (100.0). Anal. Calcd for $C_{12}H_{10}N_4O_3S$: C, 49.65; H, 3.47; N, 19.30; S, 11.04. Found: C, 49.44; H, 3.38; N, 19.08; S, 10.87.

5.2.6. 4-(Phenyl)-1-[3-(5-nitrofuryl)propenilidene]thiosemicarbazide (12). Red needles (64%). Mp 192.0–194.0 °C, IR ν_{max}: 3294.0 (NH), 1235.8 (C=S), 960.7 (furan). δ_H 7.02 (1H, d, *J* 3.9, furan–*H*), 7.08 (2H, m, C*H*=C*H*), 7.18 (1 H, t, *J* 7.4, phenyl–*H*), 7.35 (2H, t, *J* 7.9, phenyl–*H*), 7.62 (2H, d, *J* 8.0, phenyl–*H*), 7.73 (1H, d, *J* 3.9, furan–*H*), 7.97 (1H, t, *J* 4.4, —C*H*=N), 10.08 (1H, br s, N*H*), 11.96 (1H, br s, N*H*). δ_C (HMQC, HMBC) 114.76 (furan–*C*), 116.21 (furan–*C*), 124.12 (—*C*H=), 125.75, 126.03, 128.95 (phenyl–*C*), 130.83 (–*C*H=), 139.70 (phenyl–*C*), 143.18 (H—*C*=N), 152.25 (furan–*C*), 155.56 (furan–*C*), 176.60 (—*C*=S). *m/z* 316 ($M^{\cdot+}$, 12.3%), 223 (11.8), 93 (100.0). Anal. Calcd for $C_{14}H_{12}N_4O_3S$: C, 53.16; H, 3.82; N, 17.71; S, 10.13. Found: C, 52.94; H, 3.71; N, 17.70; S, 10.01.

5.3. ESR experiments

The rat liver microsomes were prepared by published methods[20] and kept frozen at −70 °C until used. The ESR experiments were run in Tris buffer (150 mM KCl, 50 mM Tris·HCl, pH 7.36 at 25 °C) treated with Chelex ion exchange resin before use to remove adventitiously present metal ions. NADPH, NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were obtained from Sigma. All chemicals were dissolved in buffer (adding minimal quantities of DMSO, if necessary, for the thiosemicarbazone derivatives). Incubations consisted of homogenised microsomes in buffer (typically 3 mg of protein/mL), thiosemicarbazone derivatives (5.0 mM), glucose-6-phosphate (10 mM), with glucose-6-phosphate dehydrogenase as an NADPH-generated system, and NADPH (1 mM). Buffer was added if necessary to maintain a constant total volume for all experiments. In all cases, NADPH was added immediately before the ESR spectrum was recorded. ESR spectra were recorded in the X band (9.85 GHz) using a Bruker ECS 106 spectrometer with a rectangular cavity and 50 kHz field modulation. The hyperfine splitting constants were estimated to be accurate within 0.05 G.

5.4. Molecular modelling

First, the compounds were built with standard bond lengths and angles using the PC SPARTAN *Pro*molecular modelling program. A detailed conformational distribution study was performed by using MM methods (Conformational Distribution module-PC SPARTAN *Pro*package). The geometry of each minimum energy conformer was fully optimised by applying ab initio 6-31G* basis in gas phase. Then, single point B3LYP/6-31G* density functional calculation was applied. Theoretical Log*P* (cLog*P*) was calculated using Villar method, at AM1 semiempirical level.

5.5. Biology

5.5.1. In vitro evaluation. The Tulahuen 2 and Brener strain stocks of *T. cruzi* were used in this study. Handling of live *T. cruzi* was done according to established guidelines.[21] The epimastigote form of the parasite was grown at 28 °C in an axenic medium (BHI-Tryptose), complemented with 10% foetal calf serum. Cells from a 5-day old culture were inoculated into 50 mL of fresh culture medium to give an initial concentration of 1×10^6 cells/mL. Cell growth was followed by daily measuring the absorbance of the culture at 600 nm for 11 days. Before inoculation, the media was supplemented with 5 μM solutions of compounds from a stock DMSO solution. The final DMSO concentration in the culture media never exceeded 0.4% (vol/vol) and had no effect by itself on the proliferation of the parasites (no effect on epimastigote growth was observed by the presence of up to 1% DMSO in the culture media). The compounds ability to inhibit growth of the parasite was evaluated, in triplicate, in comparison to the control (no drug added to the media). The control was run in the presence of 0.4% DMSO and in the absence of any drug. The percentage of inhibition was calculated as follows: $\% = \{1-[(A_p-A_{0p})/(A_c-A_{0c})]\}\times100$, where $A_p = A_{600}$ of the culture containing the drug at day 5; $A_{0p} = A_{600}$ of the culture containing the drug just after addition of the inocula (day 0); $A_c = A_{600}$ of the culture in the absence of any drug (control) at day 5; $A_{0c} = A_{600}$ in the absence of the drug at day 0.[22] The 50% effective concentrations (ID_{50}) were obtained. Nifurtimox (Lampit^R, Bay 2502, obtained from Bayer) was used as the reference trypanocidal drug.

5.5.2. In vivo evaluation. Compounds **1**, **2**, **9**, **10** and Nfx were suspended in sterile physiological saline:Tween 80 (4:1) solution (vehicle solution) immediately prior to use. These preparations were made in aseptic conditions (laminar flux) and in all cases a complete suspension was obtained by shaking in ultrasound conditions. The experiments were carried out on two months CD1 female mice (20–22 g) bred under specific pathogen-free conditions. Animals were housed in wire mesh cages in a room at 20 ± 2 °C with natural light-dark cycles. The animals allow 'ad libitum' to standard pellet diet and

water, and were used after a minimum of 3 days acclimation to the housing conditions.[23] Control and experimental group consisted of 5–10 animals each. The experimental protocols were evaluated and supervised by the local ethic committee. All time the animals were evaluated by supervision according international protocols. The animals were anaesthetised with ethyl ether and sacrificed by cervical dislocation. All animals were dealt with in a humane way in accordance with recognised guidelines on experimentation.[24] The compounds were administered to healthy animals with a single oral and a single intraperitoneal dose, 450 and 300 mg/kg, respectively. The volume of the oral administration was 0.2 mL and the intraperitoneal was 0.5 mL. In the experiments were included animals that received 0.2 mL of vehicle solution orally or 0.5 mL of vehicle solution intraperitoneally as negative control. Post-application animals were weighted and observed daily (i.e., general movement and behaviour, skin affection and lesion apparitions) and also microenvironment was examined (i.e., apparition of blood, aspect of faecal material). At day 3 the animals were sacrificed and dissected and the organs and blood were submitted for further studies. Organs (lung, kidney, liver, spleen, brain, heart, adrenal gland, intestine, uteri and ovary) obtained by dissection process were measured and weighted and they were maintained in aqueous formalin solution (10%) for further histological studies. Blood for biochemical and haematological studies was obtained by aortic extraction and maintained in EDTA or heparin anti-coagulant, respectively, at 0 °C. The determinations were carried out no more than 24 h post-extraction.

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13. Animal's survival:

	Percentage of animal survival at day 3 of a single administration of				
	1	**2**	**9**	**10**	Nfx
Oral administration[a]	100	100	100	100	100
Intraperitoneal administration[b]	0	0	100	100	100

[a]Doses = 450 mg/kg.
[b]Doses = 300 mg/kg.

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17. Statistic parameters in the linear correlation analysed between $rPGI_{Nfx}$ and different calculated physicochemical properties:

	Atomic charge C=S sulfur	E_{HOMO}	GAP
$rPGI_{Nfx}$ (Tulahuen 2 strain)	$r=-0.616$	$r=0.624$	$r=-0.672$
	$s=0.015$	$s=0.119$	$s=0.095$
	$p=0.104$	$p=0.098$	$p=0.068$
$rPGI_{Nfx}$ (Brener strain)	$r=-0.468$	$r=0.614$	$r=-0.728$
	$s=0.017$	$s=0.120$	$s=0.088$
	$p=0.242$	$p=0.105$	$p=0.041$

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