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New potent 5-nitroindazole derivatives as inhibitors of *Trypanosoma cruzi* growth: Synthesis, biological evaluation, and mechanism of action studies

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ABSTRACT

New 5-nitroindazole derivatives were developed and their antichagasic properties studied. Eight compounds (**14–18**, **20**, **26** and **28**) displayed remarkable in vitro activities against *Trypanosoma cruzi* (*T. cruzi*). Its unspecific cytotoxicity against macrophages was evaluated being not toxic at a concentration at least twice that of *T. cruzi* IC_{50}, for some derivatives. The electrochemical studies, parasite respiration studies and ESR experiment showed that 5-nitroindazole derivatives not be able to yield a redox cycling with molecular oxygen such as occurs with nifurtimox (Nfx). The study on the mechanism of action proves to be related to the production of reduced species of the nitro moiety similar to that observed with benznidazole.

1. Introduction

Chagas disease (American trypanosomiasis) is an insidious, potentially fatal parasitic disease that is widespread in Latin America affecting 10–14 million or more. This disease is caused by the flagellate *Trypanosoma cruzi* (*T. cruzi*). Chagas disease is cause for epidemiologic concern not only in Latin America but also potentially for the United States, Canada, Spain, Italy, Israel, and Australia, due the high rate immigration of them.[1] In fact, the development of new compounds is a clear need for a safe and effective drug for the treatment of chronic Chagas disease. While generally successful when used in acute cases, the current standard therapy benznidazole (Rochagan®, Radanil®, Roche; http://www.roche.com/) or nifurtimox (Nfx, Lampit®, Bayer; http://www.bayer.com/) is, at best, only minimally effective in chronic Chagas disease and is associated with significant side effects.[1] Both compounds have exhibited significant activity in the acute phase, with up to 80% parasitological cures in treated patients. However, in the chronic phase, less than 20% of treated patients are parasitologically cured. These agents function as prodrugs and must undergo enzyme-mediated activation within the pathogen to have cytotoxic effects, reactions which are catalyzed by nitroreductases (NTRs). It is believed that they act through a bioreduction of the nitro group, but their mechanisms of action are different.[2,3] In fact, Nfx acts via the reduction of the nitro group to unstable nitroanion radical, which react to produce highly toxic, reduced oxygen metabolites (superoxide anion, hydrogen peroxide and hydroxyl radical).[4] Benznidazole, acts via reductive stress and involves covalent modification of macromolecules by nitroreduction intermediates.[4]

Despite their toxicity, nitroaromatic compounds are generally very potent against *T. cruzi* and have been considered as important hits for molecular modifications.[5,6] Recently, we have described the synthesis of a series of 5-nitroindazole derivatives (5-NI) and also their antiparasitic activity against *T. cruzi,* showing the remarkable anti-epimastigote and anti-trypomastigotes properties of some of them.[7–9] However, some of these 5-NI did not show significant unspecific cytotoxicity against macrophages.[7] Additionally, we have also demonstrated the effectiveness of some 5-nitroindazoles in an in vivo acute model of Chagas disease.[9]

On the other hand, previous results showed that N-oxidation of ω-tertiary amino moiety and the intramolecular cyclization of side chain, at position 1 of indazole ring, yield completely inactive compounds; affecting drastically the in vitro activity of the substituents at positions 3 and 5 of indazolic ring wherein the 3-hydroxy derivatives were completely inactive.[8] Besides, the 5-nitro moiety of indazole ring seems to be essential for the in vitro trypanocidal activity. Furthermore, ESR experiments employing *T. cruzi* microsomal fraction showed that 5-NI suffer bio-reduction without reactive oxygen species generation.[8]

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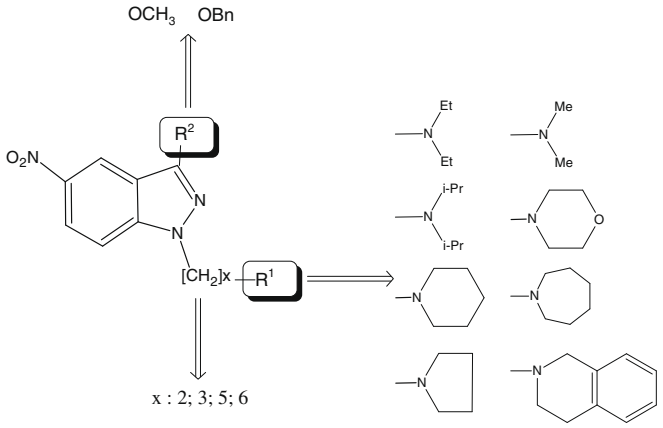


Scheme 1.

In accordance with those findings and considering both the biological significance of the nitro group and the substituent at position 3 of indazole ring, we have devised now a new series of 5-NI (Scheme 1). They have been functionalized with different length of side chain at the position 1, two different 3-alkoxy substituent and differences in the volume of the hydrophilic amino-substituents at the lateral side chain. The performed structural modifications together with their effect on anti-*T. cruzi* activities, respiration inhibition, redox potential, redox cycling properties and ESR studies allow us to extract some structural requirements for adequate trypanocidal of future designed agents.

2. Results and discussion

2.1. Synthesis

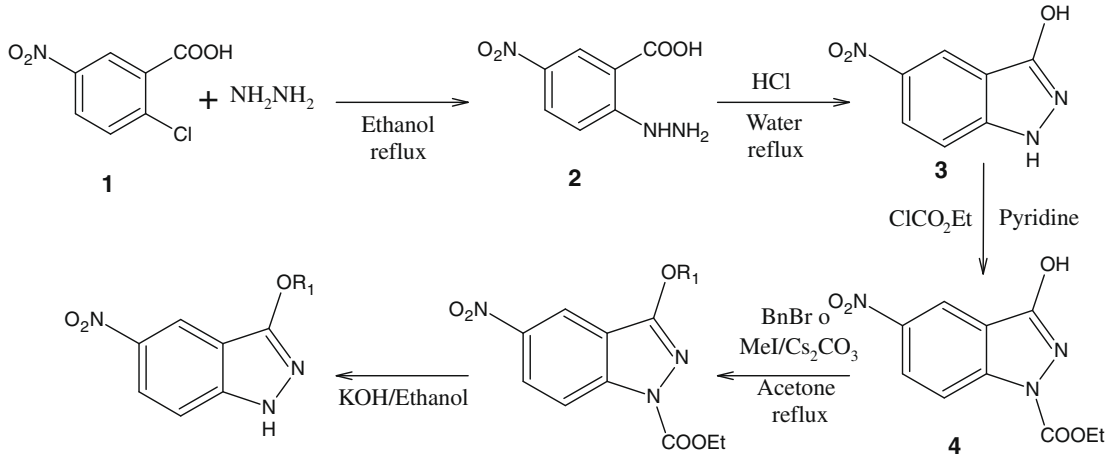
The modifications that were done on the 5-nitroindazole structure are shown in Scheme 1: variations in the length of side chain, inclusion of a hydrophilic amino substituent (morpholino moiety) and lipophilic amino-substituents with different volumes (dimethylamino, diethylamino, diisopropylamino, pyrrolidino, piperidino, homopiperidino and tetrahydroisoquinolinyl moieties). The whole series of derivatives was prepared using 3-alkoxy-5-nitroindazoles (**7** and **8**, Scheme 2) as common starting materials. These compounds were prepared following the pathways previously reported by our research group (see Scheme 2).[10]

Treatment of 3-methoxy-5-nitroindazole **7** with 1,3-dibromopropane and treatment of 3-benzyloxy-5-nitroindazole **8** with 1,5-dibromopentane or 1,6-dibromohexane afforded the respective 3-alkoxy-1-(ω-bromoalkyl)-5-nitroindazoles **9–11** (Scheme 3), in moderate to good yield; some 1,1′-polymethylenebis(3-alkoxy-5-nitroindazole) derivatives also produced in the alkylation reactions were easily separated by chromatography. Using bromides **9**–**11** as starting materials, the reaction with dimethylamine, pyrrolidine, piperidine, homopiperidine and tetrahydroisoquinoline afforded in each case the tertiary amine, **12**–**18**, in good yields (see Scheme 3). On the other hand, direct treatment of 3-alkoxyindazoles **7** and **8** with the required 2-(dialkylamino)ethyl chlorides, afforded tertiary amines **19**–**28** in good yield (see Scheme 3).

2.2. In vitro trypanocidal activity

In order to study the in vitro trypanocidal activity we used the noninfective epimastigote forms, as in previous studies with other nitroheterocyclics which have also proved to be efficient against the trypomastigote and amastigote forms.[6] We reported recently that the 5-NI derivatives showed to be more effective in trypomastigote than in epimastigote forms.[8] Consequently, epimastigote form is a suitable model for studying the mode of action of the compounds employed in the present study.

All the compounds were tested in vitro against CL Brener clone of epimastigote forms of *T. cruzi* at different concentrations. Table 1 shows the percentage of the IC_{50} of the 5-NI. The most actives of the new 5-NI were **14–18**, **20**, **26** and **28** with IC_{50} value in the same order as that of the reference drug (Nfx) being the best derivatives compounds **15**, **17** and **20**. Besides, these results confirm that substituent at positions 3 of indazole ring affects drastically the in vitro activity, such as we reported previously.[8] 3-OBn compounds being always more active than the corresponding 3-OMe derivatives. Moreover, the effect on the in vitro activity of the tested 5-NI derivatives seems to be also related with other additional factors. In this sense, length of the side chain is a factor to be considered; trypanocidal activity increases with the increase



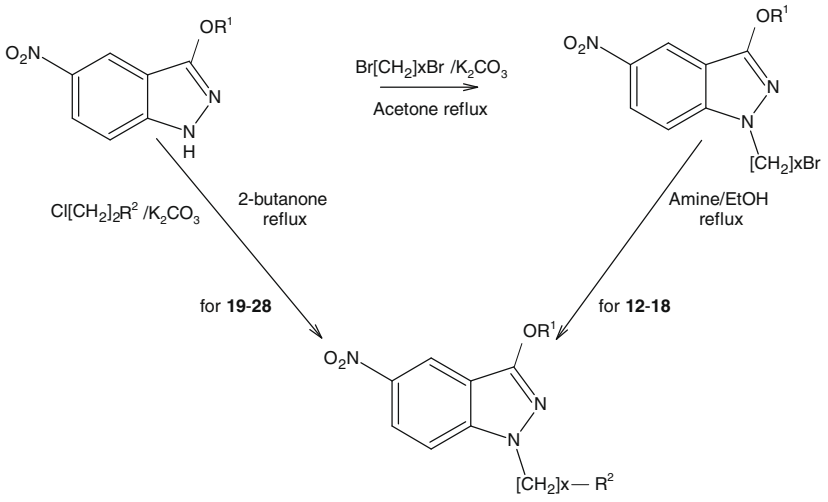
R_1	Compound
Me	**7**
Bn	**8**

R_1	Compound
Me	**5**
Bn	**6**

Scheme 2.

R^1	Compound
Me	**7**
Bn	**8**

R^1	x	Compound
Me	**3**	**9**
Bn	**5**	**10**
Bn	**6**	**11**



R^1	x	R^2	Compound
Me	**3**	dimethylamino	**12**
Me	**3**	piperidino	**13**
Bn	**5**	1,2,3,4-tetrahydroisoquinolyl	**14**
Bn	**5**	pyrrolidino	**15**
Bn	**5**	homopiperidino	**16**
Bn	**6**	piperidino	**17**
Bn	**6**	dimethylamino	**18**
Me	**2**	piperidino	**19**
Bn	**2**	piperidino	**20**
Me	**2**	dimethylamino	**21**
Bn	**2**	dimethylamino	**22**
Me	**2**	morpholino	**23**
Bn	**2**	morpholino	**24**
Me	**2**	diethylamino	**25**
Bn	**2**	diethylamino	**26**
Me	**2**	diisopropylamino	**27**
Bn	**2**	diisopropylamino	**28**

Scheme 3.

of the length chains (compare activity of the pairs **10** and **11**, **12** and **21**, and **18** and **22**). In addition, the amino moiety presence at the end of the side chain tends to improve the activity (compare **10** with **14**, **15** and **16**; **11** with **17** and **18**). However, hydrophilic thiomorpholino[8] and morpholino moieties do not improve the activity in relation to that of piperidino derivatives (compare activities of compounds **20** and **24**). Besides the hydrophilic differences another explanation could be depicted, the electron-withdrawing effect of the oxygen on the morpholine system tends to decrease the electron density at the nitrogen making it less basic. If we compare the activity and pK_a (calculated using the ChemAxon's[11] prediction plugins) of amino moieties of derivatives **20** (piperidine pK_a = 8.63) and compound **24** (morpholine pK_a = 6.46), it is observed that piperidine remains protonated up to physiologic pH and its activity is at least threefold-higher than compound **24**. Furthermore, bulky and branch amino groups tend to improve the activity (compare activities of derivatives **22**, **26** and **28**).

In order to check the specific antichagasic activity, unspecific cytotoxic activity against macrophages was also studied. A viability test such as MTT reduction was carried out using 25 μM as compounds concentrations. Results are exhibit in Table 1, being unspecific activity expressed as cytotoxicity percentage (% C) compounds

Table 1
5-Nitroindazoles in vitro anti-*T. cruzi* activity (epimastigote form) and unspecific cytotoxic activity (% C) against macrophages

Compound	[a]IC_{50}	[b]%C
9	67.8	—
10	20.7	—
11	19.3	—
12	>25.0	29
13	>>25.0	0
14	12.9	15
15	7.5	25
16	10.5	25
17	7.4	33
18	9.4	40
19	>>25.0	10
20	8.4	27
21	>>25.0	15
22	~25.0	12
23	>>25.0	0
24	>>25.0	0
25	>>25.0	0
26	11.3	30
27	>>25.0	15
28	9.2	0
Nfx	3.4	40

[a] IC_{50} = concentration (μM) that inhibits 50% of *T. cruzi* growth (CL-Brener clone).
[b] % C = cytotoxicity percentages, using 25 μM as compounds concentrations.

13, **23–25**, and **28** are the least toxic studied 5-NI. Interestedly, derivatives **14–17**, **20**, **26** and **28** showed cytotoxicity against macrophages lower than the 40%, the values for the anti-*T. cruzi* reference compound (Nfx), being this concentration around twice their epimastigote IC_{50} values.

2.3. Electrochemical studies

The reduction potential in aprotic media for this family of 5-NI was studied using DMF as aprotic solvent. The data in aprotic milieu not only describe the situation in that medium but also permit to obtain biological significance parameters as the E_7^1 It is considered as indicative of nitro radical anion formation in vivo.[12] In this regards, a good correlation between cathodic peak potential, in aprotic medium, with E_7^1 (value obtained by pulse radiolysis) has been previously described. Consequently, we employed the experimental values, in aprotic medium, to calculate the E_7^1 value by interpolation. Table 2 sorts these values, where we can observe that the 5-NI show reduction potentials higher than nifurtimox and benznidazole. This result indicates that reduction could be less favorable in the biological milieu. However, this kind of compounds could be biotransformated via enzymatic reduction, because the reduction potential (E_7^1) is comparable with those obtained for the well-known enzymatic reducible drug metronidazole (Table 2). Additionally, it has previously showed that nitro compounds with reduction potentials lower than E_7^1 <−400 mV or E_{pc} <−1000 mV (in DMF), as it is the case for metronidazole, the rate of superoxide formation by microsomal enzymes was not significant.[13] Interestingly, this indicates that 5-NI and benznidazole could not be able to yield a redox cycling with molecular oxygen such as occurs with Nfx.

On the other hand, we calculated the k_2 dimerization values (see Table 2), which indicate that in general the 5-NI radical anions are more stable than the Nfx radical in aprotic milieu which implies a lower deactivation. In this sense, these compounds are a promising alternative as an antichagasic from the point of view of the electrochemical parameters. Figure 1 shows a linear relationship between E_{pc} and k_2 values. This means < that whereas the 5-NI is more difficult to be reduced, the yielded radical is more stable. Our electrochemical results in aprotic media are contradictory with the results obtained in the trypanocidal activity (see Table 1), in the sense that the substituent at positions 3 of indazole ring affects drastically the in vitro activity. However, if we consider the energy needed to reduce the nitro group to the nitro anion radical and the stabilities of the them, the electrochemical results indicate that these properties are not affected with different substituent in position 3-alcoxy.

Table 2
5-Nitroindazole derivatives electrochemical potential and dimerization ability

Compound	$-E_{Pc}$/mV	$k_2/(M^{-1}\,s^{-1})$	E_7^1/mV (NHE)
12	1273	$178_{\pm 31.4}$	581
13	1266	$167_{\pm 25.1}$	575
14	1212	$281_{\pm 40.3}$	528
15	1238	$248_{\pm 11.1}$	551
16	1258	$226_{\pm 13.1}$	568
17	1232	$225_{\pm 22.0}$	546
18	1274	$209_{\pm 24.1}$	582
19	1162	$447_{\pm 44.1}$	485
20	1155	$444_{\pm 28.7}$	479
21	1200	$419_{\pm 26.4}$	518
22	1182	$174_{\pm 11.4}$	503
23	1216	$444_{\pm 28.7}$	532
24	1214	$322_{\pm 41.3}$	530
25	1275	$229_{\pm 28.3}$	582
26	1233	$383_{\pm 39.5}$	546
27	1205	$218_{\pm 56.5}$	522
28	1227	$250_{\pm 45.2}$	541
[a]Nfx	930	$486_{\pm 36}$	$286_{(286)}$
[a]Bnz	1019	—	$362_{(380)}$
[a]Metronidazole	1160	—	$484_{(486)}$

[a] Refs. 12,13.

2.4. Effect on the parasitic respiration

In order to evaluate if the 5-NI could induce redox cycling, with the subsequent production of superoxide radical anion and hydroxyl radical, oxygen uptake experiments were performed. Table 3 shows Tulahuen epimastigote oxygen uptake in the presence of some selected 5-NI. Some of the assayed derivatives, **10** and **11**, did not show inhibition of oxygen uptake with respect to untreated control cells (Table 3). While that the three derivatives with amino moieties on the side chain, **18**, **20** and **22**, inhibited respiration between 39% and 49% at IC_{50} equivalent concentrations. This result indicates that the respiratory chain could be an important target

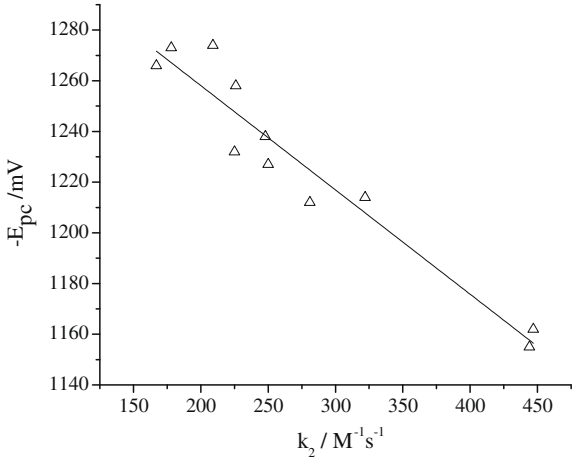


Figure 1. Cathodic peak potential values versus dimerization rate constant values from the one-electron reduction of some selected 5-NI derivatives (**12–20**, **24** and **28**) in non-aqueous medium.

Table 3
5-Nitroindazole derivatives effect upon oxygen uptake

Compound	Concentration in the assay (mM) (times of IC_{50} concentration)	[a,b,c] Oxygen uptake (%)
10	2 (32)	38
	0.83 (13)	100
	0.42 (7)	100
11	2 (34)	100
	0.83 (14)	100
	0.42 (7)	100
18	0.42 (18)	52
	0.208 (9)	59
	0.104 (4)	100
20	0.83 (20)	62
	0.42 (10)	61
	0.208 (5)	100
22	0.42 (8)	51
	0.208 (4)	64

[a] Percent rate of oxygen consumption compared with that of control (C) cells in Tulahuen strain.
[b] Values correspond to means of three independent experiments with a SD less than 5% in all cases.
[c] Control respiration was 31.5 nanoatoms of oxygen per min and per mg of protein.

for this type of compounds. As well they point out that these compounds did not have any effects on redox cycling as previously we reported for other 5-NI.[8,9] This result is in agreement with the high reduction potentials above described. In addition, the effect on the parasitic respiration of the tested 5-NI seems to be related with the type of group at the end of side chain, the two derivatives with a bromine moiety on the side chain have no effect on the parasite respiration. However, similar derivatives with an amino group instead a bromine inhibit the parasitic respiration. Furthermore, the presence of nitro group and length of side chain seems do not have a significant effect on the parasitic respiration.

2.5. ESR spectroscopic studies

2.5.1. ESR evidence for enzymatic reduction

Figure 2 shows that the incubation of 5-NI derivative, **28**, with mammalian cytosolic fraction in presence of NADPH and POBN, as spin-trapping under aerobic conditions, produces a characteristic spectrum that could be identified as the POBN-CH_3 adduct. The six lines have the following hyperfines aN = 15.8 G and aH = 2.6 G consistent with the trapped methyl radical (marked with * in Fig. 2C). The observation here described indicates that 5-NI could be reduced by cytosolic fraction to a nitro anion radical and the reoxidation of which is accompanied by hydroxyl radical formation.[14,15] POBN–CH_3 adduct was observed in presence of dimethylsulfoxide (DMSO) as a results of hydroxyl radical reaction with the solvent.[16] In spite of POBN–CH_3 being a secondary adduct, we have chosen to use DMSO in order to estimate radical yield due to the higher stability of this adduct when compared to those of the POBN–OH or DMPO oxygen radical adduct.[16] When we used DMPO in similar conditions and when we used acetonitrile instead DMSO it was not observed these signals (Fig. 2B).

Attempts to demonstrate 5-NI capacity to generate superoxide or hydroxyl radicals into *T. cruzi*, under similar conditions to those used with cytosolic fraction, were unsuccessful. However, the incubation of 5-NI (in acetonitrile) with *T. cruzi*, in the presence of NADPH and DMPO, generated an ESR spectrum consistent with the trapping of the nitroheterocycle radical (see Fig. 2D). In agreement to these results 5-NI did not stimulate oxygen consumption in *T. cruzi* epimastigote form (see Table 3). Previous reports have shown formation of both hydroxyl and superoxide radicals concomitantly with a increase of the oxygen consumption.[17]

All the compounds with IC_{50} values around o lower than 25 μM were biotransformed via enzymatic reduction in a similar way, independent of the tripanocide activity of them. These results indicate that the bioreduction mechanism is not the only choice to explain the tripanocide activity of these compounds. In this regard, the parasitic respiration showed by the compounds **18**, **20** and **22** confirm this. In addition, these results show that 5-NI *T. cruzi* growth inhibition induction occurs at concentrations that do not stimulate hydroxyl radical generation indicating that the trypanocidal effect does not depend on the effect of oxygen radicals. In this regard, the 5-NI could display a mechanism of action similar to that of benznidazole.[18–20]

2.5.2. Reactivity against dpph and galvinoxyl free radicals

Reactivity of tertiary amines towards free radicals species is a process of high biological interest,[21] the associated mechanism corresponds to an oxidative N-dealkylation of tertiary amines induced by free radicals species.[22] On the other hand, the oxidative N-dealkylation of amines has been reported as the metabolic mechanism of some xenobiotics and endogenous compounds.[21,23] Consequently, information of the reactivity of 5-NI against 2,2-diphenyl-1-picrylhydrazyl (dpph) and galvinoxyl free radicals, two stable lipophilic chromogenic radicals, could be an important point in order to consider the N-dealkylation like a possible metabolic mechanism of 5-NI.

In general, all the studied compounds were able to scavenge both radicals in a percent lower than the 30% (Fig. 3). However, derivatives **16** and **18** show a scavenger percent higher than 40%. Reactivity of these derivatives toward dpph and galvinoxyl free radicals could involve an N-dealkylation mechanism. Scheme 4 shows that N-dealkylation processes maybe proceed directly via α-carbon hydroxylation. The reactivity of the *N*-methylene C–H bond of tertiary amines has been previously reported such as a concerted proton–electron transfer mechanism.[22] The results seem to show that rise in the reactivity is related with large side chain (derivative **18**) and the homopiperidine group (derivative **16**). Furthermore, the low reactivity exhibit by the great majority of 5-NI derivatives indicate that the N-dealkylation induces by free radicals is low. However, these results constitutes a point to be considers for further drug development.

3. Conclusion

The trypanocidal activity of new 5-nitroindazole derivatives has been reported, where some derivatives (**14–18**, **20**, **26** and **28**) showed interesting properties with activities in the same magnitude order that Nfx. Fortunately, some of more active derivatives (**14–17**, **20**, **26** and **28**) do not show significant unspecific cytotoxicity against macrophages. Besides, taking into account the role of amine group volume and length of side chain on tripanocidal activity, new derivatives bearing a quaternized amino moiety and with a length of side chain between 5- and 6-CH_2 could present better activities and they will be synthesized and tested to confirm it. The study on the mechanism of action proves to be related to the production of reduced species of the nitro moiety similar to that observed with benznidazole. The results provide supporting evidence to stimulate further in vivo studies of the new compounds in appropriate animal models of Chagas' disease.

4. Experimental

4.1. Chemistry

Compounds **2**–**8** were prepared according to literature procedures.[10,24] All starting materials were commercially available re-

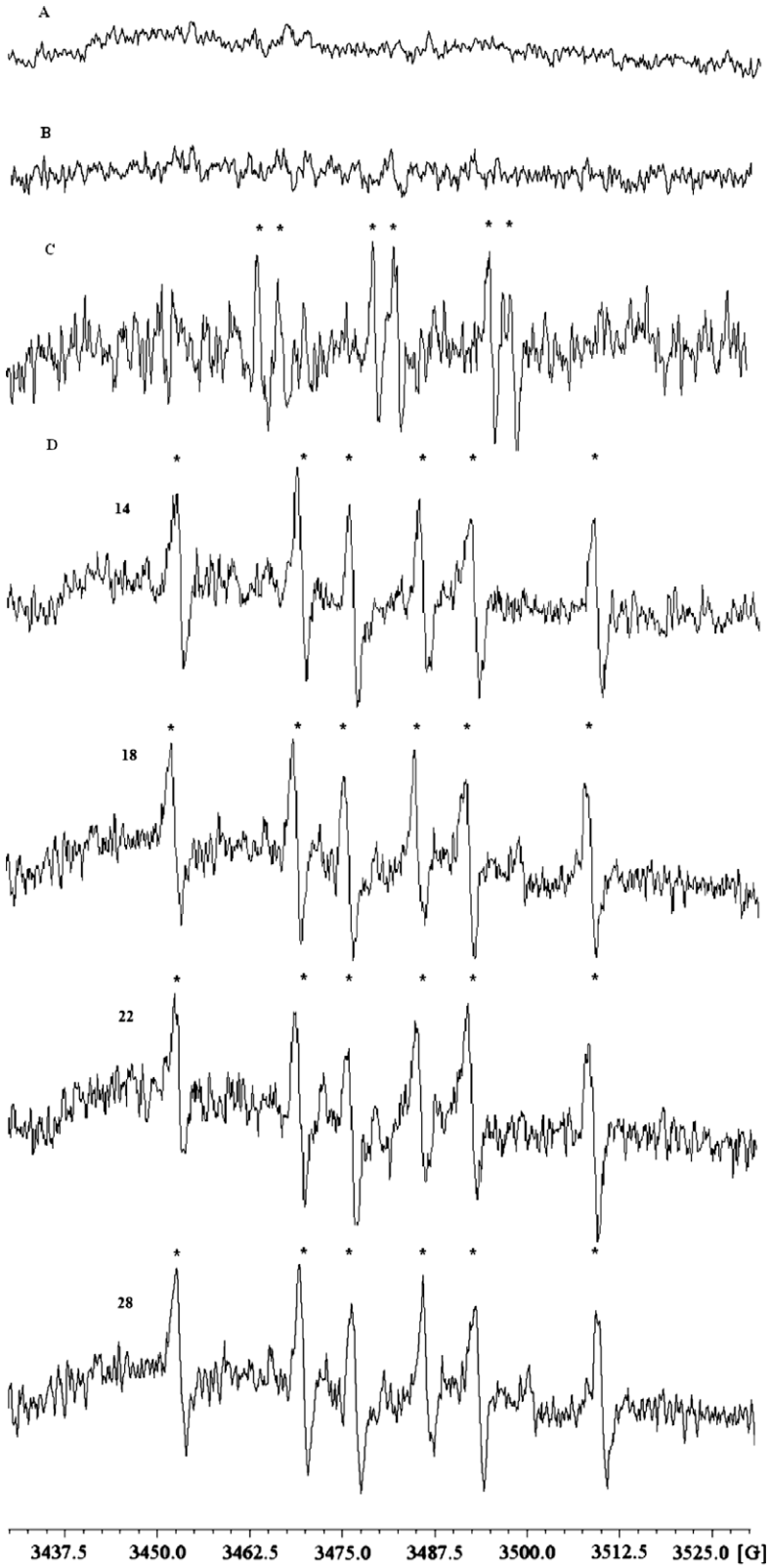


Figure 2. ESR spectra of radical adducts obtained with mammalian cytosolic fraction and *T. cruzi* microsomes. The ESR spectra were observed 5 min after incubation at 37 °C with mammalian cytosolic fraction (1 mg protein/mL), NADPH (1 mM), in phosphate buffer (20 mM), pH 7.4 (A) **28** (1 mM in acetonitrile 10 v/v), (C) **28** (2 mM in acetonitrile 10 v/v) and POBN (100 mM). The ESR spectra were observed 15 min after incubation at 28 °C with *T. cruzi* microsomal fraction (4 mg protein/mL), NADPH (1 mM), in phosphate buffer (20 mM), pH 7.4 (B) **28** (1 mM in acetonitrile 10 v/v), (D) **14**, **18**, **22** and **28**, (2 mM in acetonitrile 10 v/v) and DMPO (100 mM). Spectrometer conditions: microwave frequency 9.75 GHz microwave power 20 mW, modulation amplitude 0.87 G, time constant 0.25 s number scans: 10.

search-grade chemicals and used without further purification. All solvents were dried and distilled prior to use. ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) experiments have been performed on a Bruker AVANCE DRX300 spectrometer equipped with a pulse gradient unit capable of producing magnetic field pulse gradients in the *z*-direction of 53.5 G cm^{-1}. The spectra have been acquired in

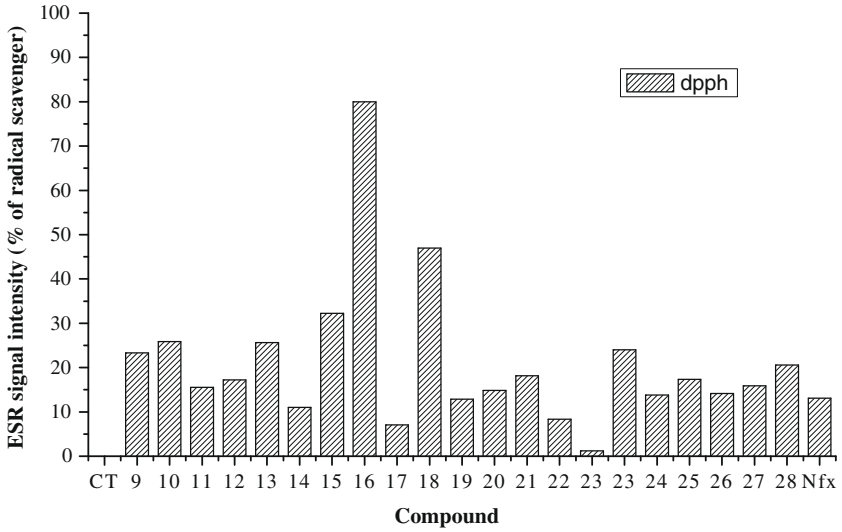
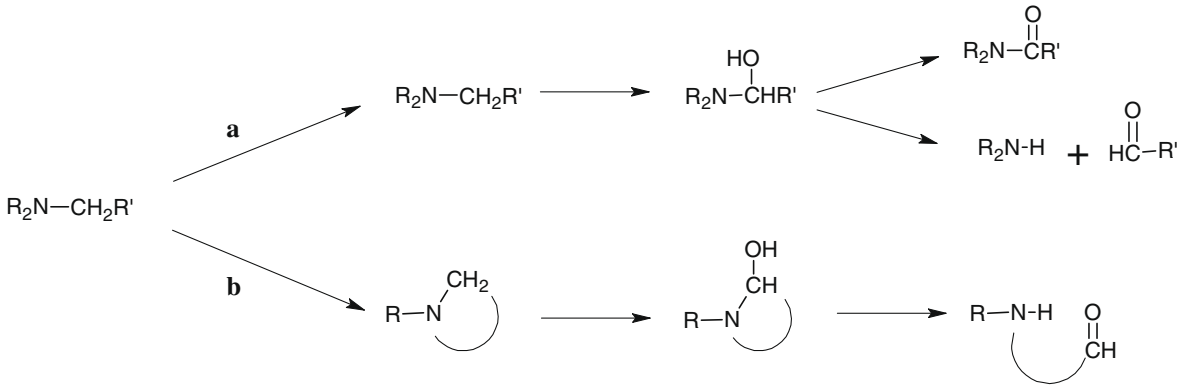


Figure 3. 5-Nitroindazole derivatives reactivity against to dpph free radical. (a) The reaction mixture contained 1.0 mM DPPH in the presence or absence of 1.0 mM concentrations of the compounds under study. ESR signals were recorded 1 min following the start of the reaction. Spectrometer conditions were: microwave frequency, 9.72 GHz; microwave power, 20 mW; modulation amplitude, 0.98 G; receiver gain, 59 db; and sweep time, 20.972 s.



Scheme 4. Proposed mechanism of tertiary amine oxidation by dpph or galvinoxyl free radicals.

an inversed probe-head at 298 K in 5 mm tubes. The chemical shifts are reported in parts per million from TMS (δ scale). *J* values are given in hertz. The assignments have been performed by means of different standard homonuclear and heteronuclear correlation experiments (ROESY, HMQC and HMBC). DC-Alufolien Silica Gel 60 PF254 (Merck, layer thickness 0.2 mm) and Silica Gel 60 (Merck, particle size 0.040–0.063 mm) were used for TLC and flash column chromatography, respectively. Melting points are uncorrected and were determined with a Reichert Galen III hot plate microscope with a DUAL JTEK Dig—Sense thermocouple thermometer. Elemental analyses were obtained from vacuum-dried samples (over phosphorous pentoxide at 3–4 mm Hg, 24 h at room temperature) and performed on a Fisons EA 1108 CHNS-O analyser.

4.2. General method for the preparation of 9–11

These compound were prepared starting from 3-alkoxy-5-nitroindazole derivatives **7** or **8** (1 equiv), potassium carbonate (2 equiv) and the corresponding dibromoalcane (1,3-dibromopropane, 1,5-dibromopentane or 1,6-dibromohexane, 5 equiv) in acetone. The mixture was heated at reflux for 6 h and then evaporated to dryness. The residue was treated with water and extracted with chloroform (3 × 30 mL). The combined organic layers were dried ($MgSO_4$) and evaporated, and the residue applied to a chromatography column (SiO_2, $CHCl_3$/MeOH (95:5)) to afford the intermediates **9–11** which were used in the next step without further purification.

4.3. General method for the preparation of 12–13, 15–18

These compounds were prepared starting from 3-alkoxy-1-bromoalkyl-5-nitroindazole derivatives (**9–11**, 1 equiv) and the corresponding amine (dimethylamine, piperidine, pyrrolidine and homopiperidine, 2 equiv) in ethanol. The mixture was heated at reflux for 24 h and then evaporated to dryness. The residue was treated with aqueous potassium carbonate (5%, 30 mL) and extracted with chloroform (3 × 30 mL). The combined organic layers were dried ($MgSO_4$) and evaporated to dryness to yield the desired tertiary amines as the corresponding free bases.

4.4. General method for the preparation of 19, 21–23, 25

These compound were prepared starting from 3-alkoxy-5-nitroindazole derivatives **7** and **8** (1 equiv), potassium carbonate (5 equiv) and the corresponding 2-(dialkylamino)ethyl chlorides [2-(dimethylamino)ethyl chloride hydrochloride, 2-(diethylamino)ethyl chloride hydrochloride, 1-(2-chloroethyl)piperidine hydrochloride and 4-(2-chloroethyl)morpholine hydrochloride, 2 equiv)] in 2-butanone. The mixture was heated at reflux for 24 h and evaporated to dryness. The residue was then treated with

aqueous potassium carbonate (5%, 30 mL) and extracted with chloroform (3 × 30 mL). The combined organic layers were dried ($MgSO_4$) and evaporated to dryness to afford the desired tertiary amines as the corresponding free bases.

4.5. General method for the preparation of 14, 20, 24, 26–28

For compound **14**, a mixture of 3-alkoxy-1-bromoalkyl-5-nitroindazole derivative (**10**, 1 equiv) and tetrahydroisoquinoline (2 equiv) in ethanol. The mixture was heated at reflux for 24 h. For compounds **20**, **24**, **26–28**, a mixture of 3-alkoxy-5-nitroindazole derivatives **7** and **8** (1 equiv), potassium carbonate (5 equiv) and the corresponding 2-(dialkylamino)ethyl chlorides [2-(diethylamino)ethyl chloride hydrochloride, 2-(diisopropylamino)ethyl chloride hydrochloride, 1-(2-chloroethyl)piperidine hydrochloride and 4-(2-chloroethyl)morpholine hydrochloride, 2 equiv)] in 2-butanone. The mixture was heated at reflux for 24 h. In each case, the mixture was then evaporated to dryness and, after addition of 5% aqueous potassium carbonate (30 mL), extracted with chloroform (3 × 30 mL). The combined organic layers were dried ($MgSO_4$) and evaporated to dryness. The residue was treated with aqueous 0.5 N hydrochloric acid (50 mL) and the insoluble material was removed by extraction with diethyl ether (3 × 50 mL). Evaporation of the acidic layer afforded the desired tertiary amines as the corresponding hydrochlorides.

4.5.1. 1-(3-Dimethylaminopropyl)-3-methoxy-5-nitro-1*H*-indazole (12)

Yield: 75%; mp 57–60 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 1.93 (m, 2H, 2′-H), 2.15 (s, 6H, N(CH_3)$_2$), 2.21 (t, J = 6.9 Hz, 2H, 3′-H), 4.05 (s, 3H, OCH_3), 4.31 (t, J = 6.7 Hz, 2H, 1′-H), 7.68 (d, J = 9.3 Hz, 1H, 7-H), 8.19 (dd, J = 2.0, 9.3 Hz, 1H, 6-H), 8.48 (d, J = 2.0 Hz, 1H, 4-H); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 27.09 (C-2′), 45.15 (NCH_3), 46.38 (C-1′), 55.89 (C-3′), 56.86 (OCH_3), 110.55 (C-7), 110.79 (C-3a), 117.86 (C-4), 122.35 (C-6), 140.59 (C-5), 142.94 (C-7a), 157.86 (C-3). Anal. Calcd for $C_{13}H_{18}N_4O_3$ (278.3): C, 56.10; H, 6.52; N, 20.13. Found: C, 56.28; H, 6.42; N, 20.04.

4.5.2. 3-Methoxy-5-nitro-1-(3-piperidinopropyl)-1*H*-indazole (13)

Yield: 99%; mp 119–122 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 1.44 (m, 6H, piperidino 3-, 4- and 5-H), 1.94 (m, 2H, 2′-H), 2.18 (m, 2H, 3′-H), 4.06 (s, 3H, OCH_3), 4.32 (t, J = 6.5 Hz, 2H, 1′-H), 7.70 (d, J = 9.4 Hz, 1H, 7-H), 8.20 (dd, J = 2.1, 9.4 Hz, 1H, 6-H), 8.51 (d, J = 1.6 Hz, 1H, 4-H); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 24.45 (piperidino C-4), 25.84 (piperidino C-3 and -5), 26.42 (C-2′), 46.55 (C-1′), 54.25 (C-3′), 55.41 (piperidino C-2 and -6), 56.87 (OCH_3), 110.75 (C-7, C-3a), 117.87 (C-4), 122.19 (C-6), 140.55 (C-5), 143.10 (C-7a), 157.89 (C-3). Anal. Calcd for $C_{16}H_{22}N_4O_3$ (318.4): C, 60.36; H, 6.97; N, 17.60. Found: C, 60.28; H, 6.85; N, 17.73.

4.5.3. 3-Benzyloxy-5-nitro-1-[5-(1,2,3,4-tetrahydroisoquinol-2-yl)pentyl]-1*H*-indazol hydrochloride (14·HCl)

Yield: 67%; mp 190–195 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 1.28 (m, 2H, 3′-H), 1.77 (m, 2H, 4′-H), 1.87 (m, 2H, 2′-H), 3.10 (broad signal, 4H, 5′-H, tetrahydroisoquinolyl, 7-H), 3.68 (broad signal, 2H, tetrahydroisoquinolyl, 8-H) 4.36 (t, J = 6.5 Hz, 4H, 1′-H, tetrahydroisoquinolyl, 10-H), 5.46 (s, 2H, Bn–CH_2), 7.26 (m, 4H, tetrahydroisoquinolyl 1-, 2-, 3- and 4-H), 7.40 (m, 3H, Ph 3-, 4- and 5-H), 7.57 (m, 2H, Ph 2- and 6-H), 7.79 (d, J = 9.4 Hz, 1H, 7-H), 8.23 (dd, J = 2.0, 9.4 Hz, 1H, 6-H), 8.57 (d, J = 2.0 Hz, 1H, 4-H), 9.78 (tetrahydroisoquinolyl NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 23.61 (C-3′, tetrahydroisoquinolyl C-7), 25.44 (C-4′), 29.10 (C-2′), 48.39 (C-1′), 49.41 (tetrahydroisoquinolyl, C-3), 52.51 (tetrahydroisoquinolyl, C-10), 55.47 (C-5′), 71.09 (Bn–CH_2), 110.76 (C-7), 111.22 (C-3a), 118.15 (C-4), 122.62 (C-6), 127.08 (tetrahydroisoquinolyl C-2), 127.13 (tetrahydroisoquinolyl C-3), 128.20 (tetrahydroisoquinolyl, C-4), 128.59 (Ph, C-3 and -5), 128.68 (Ph, C-4), 128.92 (Ph, C-2 and -6), 129.04 (tetrahydroisoquinolyl, C-1), 131.83 (tetrahydroisoquinolyl, C-5), 136.76 (Ph, C-1, tetrahydroisoquinolyl, C-6), 142.94 (C-5), 143.25 (C-7a), 157.17 (C-3). Anal. Calcd for $C_{28}H_{31}ClN_4O_3$ (471.6): C, 71.31; H, 6.63; N, 11.88. Found: C, 71.44; H, 6.55; N, 10.99.

4.5.4. 3-Benzyloxy-5-nitro-1-(5-pyrrolidinopentyl)-1*H*-indazole (15)

Yield: 99%; mp 135–139 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 1.23 (m, 2H, 3′-H), 1.59 (m, 2H, 4′-H), 1.85 (m, 6H, 2′-H, pyrrolidino 3 and 4-H), 2.87 (t, J = 7.8 Hz, 2H, 5′-H), 3.02 (broad signal, 4H, pyrrolidino 2- and 5-H), 4.33 (t, J = 6.7 Hz, 2H, 1′-H), 5.45 (s, 2H, Bn–CH_2), 7.42 (m, 3H, Ph 3-, 4- and 5-H), 7.56 (m, 2H, Ph 2- and 6-H), 7.77 (d, J = 9.4 Hz, 1H, 7-H), 8.21 (dd, J = 2.0, 9.4 Hz, 1H, 6-H), 8.55 (d, J = 2.0 Hz, 1H, 4-H); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 23.01 (pyrrolidino C-3 and -4), 23.67 (C-3′), 25.78 (C-4′), 29.02 (C-2′), 48.32 (C-1′), 53.53 (pyrrolidino C-2 and -5), 54.43 (C-5′), 70.95 (Bn–CH_2), 110.64 (C-7), 111.08 (C-3a), 118.02 (C-4), 122.48 (C-6), 128.48 (Ph, C-3 and -5), 128.56 (Ph, C-4), 128.80 (Ph, C-2 and -6), 136.65 (Ph, C-1), 140.75 (C-5), 142.79 (C-7a), 157.03 (C-3). Anal. Calcd for $C_{23}H_{28}N_4O_3$ (408.5): C, 67.63; H, 6.91; N, 13.72. Found: C, 67.72; H, 6.83; N, 13.64.

4.5.5. 1-(5-Azepanylpentyl)-3-benzyloxy-5-nitro-1*H*-indazole (16)

Yield: 98%; mp 79–81 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 1.25 (m, 2H, 3′-H), 1.45 (broad signal, 10H, 4′-H and azepanyl 3-, 4-, 5- and 6-H), 1.87 (m, 2H, 2′-H), 2.85 (broad signal, 6H, 1′-H and azepanyl, 2- and 7-H), 4.34 (t, J = 6.8 Hz, 2H, 5′-H), 5.45 (s, 2H, Bn–CH_2), 7.41 (m, 3H, Ph 3-, 4- and 5-H), 7.57 (m, 2H, Ph 2- and 6-H), 7.75 (d, J = 9.3 Hz, 1H, 7-H), 8.22 (dd, J = 2.1, 9.3 Hz, 1H, 6-H), 8.54 (d, J = 2.1 Hz, 1H, 4-H); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 23.12 (azepanyl, C-4 and -5), 23.67 (C-3′), 24.50 (azepanyl, C-3 and -6), 25.80 (C-2′), 29.21 (C-4′), 48.30 (C-5′), 53.25 (azepanyl, C-2 and -7), 54.43 (C-1′), 70.95 (Bn–CH_2), 110.62 (C-7), 111.09 (C-3a), 118.01 (C-4), 122.50 (C-6), 128.48 (Ph, C-3 and -5), 128.56 (Ph, C-4), 128.81 (Ph, C-2 and -6), 136.63 (Ph, C-1), 140.73 (C-5), 142.77 (C-7a), 157.01 (C-3). Anal. Calcd for $C_{25}H_{32}N_4O_3$ (436.5): C, 68.78; H, 7.39; N, 12.83. Found: C, 68.88; H, 7.30; N, 12.96.

4.5.6. 3-Benzyloxy-5-nitro-1-(6-piperidinohexyl)-1*H*-indazole (17)

Yield: 99%; mp 127–130 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 1.23 (m, 4H, 4′-H, 3′-H), 1.45 (m, 4H, piperidino 3- and 5-H), 1.59 (m, 4H, 5′-H, piperidino 4-H), 1.79 (m, 2H, 2′-H), 2.58 (broad signal, 2H, 6′-H), 2.70 (broad signal, 4H, piperidino 2- and 6-H), 4.31 (t, J = 6.7 Hz, 2H, 1′-H), 5.44 (s, 2H, Bn–CH_2), 7.41 (m, 3H, Ph 3-, 4- and 5-H), 7.56 (m, 2H, Ph 2- and 6-H), 7.75 (d, J = 9.5 Hz, 1H, 7-H), 8.20 (dd, J = 1.9, 9.5 Hz, 1H, 6-H), 8.54 (d, J = 1.9 Hz, 1H, 4-H); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 22.93 (piperidino C-4), 24.20 (piperidino C-3 and -5), 24.78 (C-4′), 26.07 (C-3′), 26.40 (C-5′), 29.28 (C-2′), 48.46 (C-1′), 53.23 (piperidino C-2 and -6), 57.34 (C-6′), 70.93 (Bn–CH_2), 110.61 (C-7), 111.04 (C-3a), 117.99 (C-4), 122.42 (C-6), 128.49 (Ph C-3 and -5), 128.52 (Ph C-4), 128.77 (Ph C-2 and -6), 136.67 (Ph C-1), 140.69 (C-5), 142.77 (C-7a), 156.99 (C-3). Anal. Calcd for $C_{25}H_{32}N_4O_3$ (436.5): C, 68.78; H, 7.39; N, 12.83. Found: C, 68.89; H, 7.52; N, 12.70.

4.5.7. 3-Benzyloxy-1-(6-dimethylaminohexyl)-5-nitro-1*H*-indazole (18)

Yield: 85%: mp 45–48 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 1.21 (m, 4H, 4′-H, 3′-H), 1.29 (broad signal, 2H, 5′-H), 1.78 (m, 2H, 2′-H), 2.07 (s, 6H, N(CH_3)$_2$), 2.12 (broad signal, 2H, 6′-H), 4.29 (t, J = 6.7 Hz, 2H, 1′-H), 5.44 (s, 2H, Bn–CH_2), 7.39 (m, 3H, Ph 3-, 4-

and 5-H), 7.56 (m, 2H, Ph 2- and 6-H), 7.74 (d, J = 9.5 Hz, 1H, 7-H), 8.19 (dd, J = 1.9, 9.5 Hz, 1H, 6-H), 8.53 (d, J = 1.9 Hz, 1H, 4-H); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 26.57 (C-3′, -4′ and -5′), 29.23 (C-2′), 45.14 (N(CH_3)$_2$), 48.43 (C-1′), 59.23 (C-6′), 70.93 (Bn–CH_2), 110.53 (C-7 and -3a), 117.97 (C-4), 122.34 (C-6), 128.37 (Ph C-3 and -5), 128.60 (Ph C-4), 128.84 (Ph C-2 and -6), 136.77 (Ph C-1), 140.73 (C-5), 142.82 (C-7a), 156.99 (C-3). Anal. Calcd for $C_{22}H_{28}N_4O_3$ (396.5): C, 66.64; H, 7.12; N, 14.13. Found: C, 66.50; H, 7.27; N, 14.25.

4.5.8. 3-Methoxy-5-nitro-1-(2-piperidinoethyl)-1*H*-indazole (19)

Yield: 79%; mp 78–79 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 1.46 (m, 6H, piperidino 3-, 4- and 5-H), 2.46 (m, 4H, piperidino 2- and 6-H), 2.76 (t, J = 6.3 Hz, 2H, 2′-H), 4.15 (s, 3H, OCH_3), 4.48 (t, J = 6.3 Hz, 2H, 1′-H), 7.83 (d, J = 9.5 Hz, 1H, 7-H), 8.28 (dd, J = 1.0, 9.5 Hz, 1H, 6-H), 8.58 (d, J = 1.0 Hz, 1H, 4-H); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 24.31 (piperidino C-4), 26.04 (piperidino C-3 and -5), 46.87 (C-1′), 54.52 (piperidino C-2 and -6), 56.95 (C-2′), 57.85 (OCH_3), 110.93 (C-7), 111.02 (C-3a), 117.89 (C-4), 122.25 (C-6), 140.63 (C-5), 143.46 (C-7a), 157.88 (C-3). Anal. Calcd for $C_{15}H_{20}N_4O_3$ (304.3): C, 59.20; H, 6.62; N, 18.41. Found: C, 59.12; H, 6.72; N, 18.50.

4.5.9. 3-Benzyloxy-5-nitro-1-(2-piperidinoethyl)-1*H*-indazol hydrochloride (20·HCl)

Yield: 86%; mp 196–203 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 1.77 (m, 6H, piperidino 3-, 4- and 5-H), 2.94 (m, 2H, piperidino 1- and 6-H$_{eq}$), 3.51 (m, 2H, 2′-H, 2H, piperidino 1- and 6-H$_{ax}$), 4.83 (t, J = 6.5 Hz, 2H, 1′-H), 5.47 (s, 2H, Bn–CH_2), 7.40 (m, 4H, 7-H and Ph 3-, 4- and 5-H), 7.57 (m, 2H, Ph 2- and 6-H), 7.89 (d, J = 9.3 Hz, 1H, 7-H), 8.30 (dd, J = 2.1, 9.3 Hz, 1H, 6-H), 8.60 (d, J = 2.1 Hz, 1H, 4-H), 10.49 (sa, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 21.70 (piperidino C-4), 22.81 (piperidino C-3 and -5), 43.29 (C-1′), 52.80 (piperidino C-2 and -6), 54.32 (C-2′), 71.21 (Bn–CH_2), 111.03 (C-7), 111.90 (C-3a), 118.09 (C-4), 123.05 (C-6), 128.57 (Ph C-3 and -5), 128.73 (Ph C-4), 128.97 (Ph C-2 and -6), 136.60 (Ph C-1), 141.38 (C-5), 143.25 (C-7a), 157.62 (C-3). Anal. Calcd for $C_{21}H_{25}ClN_4O_3$ (416.9): C, 60.50; H, 6.04; N, 13.44. Found: C, 60.44; H, 6.14; N, 13.36.

4.5.10. 1-(2-Dimethylaminoethyl)-3-methoxy-5-nitro-1*H*-indazole (21)

Yield: 83%; mp 73–76 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 2.26 (s, 6H, N(CH_3)$_2$), 2.78 (t, J = 6.4 Hz, 2H, 2′-H), 4.16 (s, 3H, OCH_3), 4.49 (t, J = 6.4 Hz, 2H, 1′-H), 7.86 (d, J = 9.3 Hz, 1H, 7-H), 8.30 (dd, J = 2.1, 9.3 Hz, 1H, 6-H), 8.61 (d, J = 2.1 Hz, 1H, 4-H); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 45.69 (N(CH_3)$_2$), 47.06 (C-1′), 56.98 (C-2′), 58.30 (OCH_3), 110.85 (C-7), 110.96 (C-3a), 117.97 (C-4), 122.45 (C-6), 140.68 (C-5), 143.30 (C-7a), 157.88 (C-3). Anal. Calcd for $C_{12}H_{16}N_4O_3$ (264.3): C, 54.54; H, 6.10; N, 21.20. Found: C, 54.61; H, 6.19; N, 21.33.

4.5.11. 3-Benzyloxy-1-(2-dimethylaminoethyl)-5-nitro-1*H*-indazole (22)

Yield: 93%; mp 93–96 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 2.24 (s, 6H, N(CH_3)$_2$), 2.78 (t, J = 6.2 Hz, 2H, 2′-H), 4.50 (t, J = 6.2 Hz, 2H, 1′-H), 5.56 (s, 2H, Bn–CH_2), 7.51 (m, 3H, Ph 3-, 4- and 5-H), 7.67 (m, 2H, Ph 2- and 6-H), 7.87 (d, J = 9.3 Hz, 1H, 7-H), 8.30 (dd, J = 2.1, 9.3 Hz, 1H, 6-H), 8.64 (d, J = 2.1 Hz, 1H, 4-H); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 45.66 (N(CH_3)$_2$), 47.06 (C-1′), 56.31 (C-2′), 71.01 (Bn–CH_2), 110.88 (C-7), 111.18 (C-3a), 118.02 (C-4), 122.46 (C-6), 128.62 (Ph C-3 and -5), 128.67 (Ph C-4), 128.86 (Ph C-2 and -6), 136.79 (Ph C-1), 140.76 (C-5), 143.28 (C-7a), 157.06 (C-3). Anal. Calcd for $C_{18}H_{20}N_4O_3$ (340.4): C, 63.52; H, 5.92; N, 16.46. Found: C, 63.42; H, 5.85; N, 16.53.

4.5.12. 3-Methoxy-1-(2-morpholinoethyl)-5-nitro-1*H*-indazole (23)

Yield: 79%; mp 112–114 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 2.41 (m, 4H, morpholino 3- and 5-H), 2.71 (t, J = 6.3 Hz, 2H, 2′-H), 3.46 (m, 4H, morpholino 2- and 6-H), 4.05 (s, 3H, OCH_3), 4.41 (t, J = 6.3 Hz, 2H, 1′-H), 7.75 (d, J = 9.5 Hz, 1H, 7-H), 8.20 (dd, J = 2.1, 9.5 Hz, 1H, 6-H), 8.51 (d, J = 2.1 Hz, 1H, 4-H); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 46.44 (C-1′), 53.69 (morpholino C-3 and -5), 56.98 (C-2′), 57.49 (OCH_3), 66.64 (morpholino C-2 and -6), 110.99 (C-7), 111.04 (C-3a), 117.95 (C-4), 122.38 (C-6), 140.72 (C-5), 143.49 (C-7a), 157.94 (C-3). Anal. Calcd for $C_{14}H_{18}N_4O_4$ (306.3): C, 54.89; H, 5.92; N, 18.29. Found: C, 54.77; H, 5.79; N, 18.41.

4.5.13. 3-Benzyloxy-1-(2-morpholinoethyl)-5-nitro-1*H*-indazol hydrochloride (24·HCl)

Yield: 95%; mp 71–74 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 3.15 (d, J = 7.8 Hz, 2H, morpholino 3- and 5-H$_{eq}$), 3.47 (d, J = 12.0 Hz, 2H, morpholino 3- and 5-H$_{ax}$), 3.62 (d, J = 7.8 Hz, 2H, morpholino 2- and 6-H$_{eq}$), 3.76 (t, J = 11.50 2H, 2′-H), 3.95 (d, J = 12.0 Hz, 2H, morpholino 2- and 6-H$_{eq}$), 4.84 (t, J = 6.5 Hz, 2H, 1′-H), 5.48 (s, 2H, Bn–CH_2), 7.43 (m, 3H, Ph 3-, 4- and 5-H), 7.58 (m, 2H, Ph 2- and 6-H), 7.88 (d, J = 9.3 Hz, 1H, 7-H), 8.31 (dd, J = 2.0, 9.3 Hz, 1H, 6-H), 8.61 (d, J = 2.0 Hz, 1H, 4-H), 10.49 (sa, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 43.09 (C-1′), 51.81 (morpholino C-3 and -5), 54.45 (C-2′), 63.67 (morpholino C-2 and -6), 71.22 (Bn–CH_2), 111.04 (C-7), 112.01 (C-3a), 118.11 (C-4), 123.07 (C-6), 128.58 (Ph C-3 and -5), 128.74 (Ph C-4), 128.97 (Ph C-2 and -6), 136.61 (Ph C-1), 141.40 (C-5), 143.27 (C-7a), 157.64 (C-3). Anal. Calcd for $C_{20}H_{23}ClN_4O_4$ (418.9): C, 57.35; H, 5.53; N, 13.38. Found: C, 57.47; H, 5.46; N, 13.50.

4.5.14. 1-(2-Diethylaminoethyl)-3-methoxy-5-nitro-1*H*-indazole (25)

Yield: 81%; mp 35–37 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 1.46 (t, J = 7.1, 6H, N(CH_2CH_3)$_2$), 2.41 (q, J = 7.1, 4H, N(CH_2CH_3)$_2$), 2.77 (t, J = 6.0 Hz, 2H, 2′-H), 4.05 (s, 3H, OCH_3), 4.32 (t, J = 6.0 Hz, 2H, 1′-H), 7.71 (d, J = 9.5 Hz, 1H, 7-H), 8.18 (dd, J = 2.1, 9.5 Hz, 1H, 6-H), 8.49 (d, J = 2.1 Hz, 1H, 4-H); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 12.42 (N(CH_2CH_3)$_2$), 47.06 (N(CH_2CH_3)$_2$), 47.73(C-1′), 52.07 (C-2′), 56.95 (OCH_3), 110.83 (C-7), 111.10 (C-3a), 117.91 (C-4), 122.23 (C-6), 140.58 (C-5), 143.70 (C-7a), 157.91 (C-3). Anal. Calcd for $C_{14}H_{20}N_4O_3$ (292.3): C, 57.52; H, 6.90; N, 19.17. Found: C, 57.70; H, 7.05; N, 19.05.

4.5.15. 3-Benzyloxy-1(2-diethylaminoethyl)-5-nitro-1*H*-indazol hydrochloride (26·HCl)

Yield: 91%; mp 176–178 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 1.20 (t, J = 7.2, 6H, N(CH_2CH_3)$_2$), 3.16 (t, J = 7.2, 4H, N(CH_2CH_3)$_2$), 3.55 (t, J = 5.2 Hz, 2H, 2′-H), 4.83 (t, J = 6.5 Hz, 2H, 1′-H), 5.48 (s, 2H, Bn–CH_2), 7.43 (m, 3H, Ph 3-, 4- and 5-H), 7.58 (m, 2H, Ph 2- and 6-H), 7.93 (d, J = 9.5 Hz, 1H, 7-H), 8.31 (dd, J = 2.1, 9.3 Hz, 1H, 6-H), 8.60 (d, J = 2.1 Hz, 1H, 4-H), 10.77 (sa, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 8.82 (N(CH_2CH_3)$_2$), 43.40 (C-2′), 46.98 (N(CH_2CH_3)$_2$), 49.36 (C-1′), 71.21 (Bn–CH_2), 111.08 (C-7), 111.91 (C-3a), 118.08 (C-4), 123.07 (C-6), 128.58 (Ph C-3 and -5), 128.72 (Ph C-4), 128.96 (Ph C-2 and -6), 136.63 (Ph, C-1), 141.38 (C-5), 143.40 (C-7a), 157.64 (C-3). Anal. Calcd for $C_{20}H_{25}ClN_4O_3$ (404.9): C, 59.33; H, 6.22; N, 13.84. Found: C, 59.49; H, 6.07; N, 13.67.

4.5.16. 1-(2-Diisopropylaminoethyl)-3-methoxy-5-nitro-1*H*-indazol hydrochloride (27·HCl)

Yield: 74%; mp 171–173 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 1.33 (d, J = 6.65, 6H, N($CHCH_3CH_3$)$_2$), 1.36 (d, J = 6.65, 6H, N($CHCH_3CH_3$)$_2$), 3.56 (m, 2H, N($CHCH_3CH_3$)$_2$), 3.72 (t, J = 7.0 Hz, 2H, 2′-H), 4.09 (s, 3H, OCH_3), 4.81 (t, J = 7.0 Hz, 2H, 1′-H), 7.89 (d,

J = 9.3 Hz, 1H, 7-H), 8.29 (dd, J = 2.1, 9.3 Hz, 1H, 6-H), 8.55 (d, J = 2.2 Hz, 1H, 4-H), 10.02 (sa, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 17.19 (N(CHCH$_3$CH$_3$)$_2$), 18.47 (N(CHCH$_3$CH$_3$)$_2$), 45.14 (C-1′ and -2′), 54.98 (N(CHCH$_3$CH$_3$)$_2$), 57.17 (OCH$_3$), 111.27 (C-7), 111.61 (C-3a), 117.95 (C-4), 122.99 (C-6), 141.33 (C-5), 143.54 (C-7a), 158.47 (C-3). Anal. Calcd for $C_{16}H_{25}ClN_4O_3$ (356.9): C, 53.85; H, 7.06; N, 15.70. Found: C, 53.69; H, 7.19; N, 15.80.

4.5.17. 3-Benzyloxy-1-(2-diisopropylaminoethyl)-5-nitro-1*H*-indazol hydrochloride (28·HCl)

Yield: 94%; mp 205–207 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 1.32 (d, J = 6.65, 6H, N(CHCH$_3$C*H*$_3$)$_2$), 1.34 (d, J = 6.65, 6H, N(CHC*H*$_3$-CH$_3$)$_2$), 3.56 (m, 2H, N(C*H*CH$_3$CH$_3$)$_2$), 3.72 (t, J = 6.9 Hz, 2H, 2′-H), 4.81 (t, J = 6.9 Hz, 2H, 1′-H), 5.49 (s, 2H, Bn–CH$_2$), 7.42 (m, 3H, Ph 3-, 4- and 5-H), 7.57 (m, 2H, Ph 2- and 6-H), 7.90 (d, J = 9.3 Hz, 1H, 7-H), 8.30 (dd, J = 2.1, 9.3 Hz, 1H, 6-H), 8.59 (d, J = 2.1 Hz, 1H, 4-H), 10.01 (sa, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 17.19 (N(CHCH$_3$CH$_3$)$_2$), 18.46 (N(CHCH$_3$CH$_3$)$_2$), 44.09 (C-1′), 45.13 (C-2′), 54.95 (N(CHCH$_3$CH$_3$)$_2$), 71.22 (Bn–CH$_2$), 111.35 (C-7), 111.68 (C-3a), 118.02 (C-4), 123.01 (C-6), 128.60 (Ph, C-3 and -5), 128.73 (Ph, C-4), 128.95 (Ph, C-2 and -6), 136.66 (Ph, C-1), 141.39 (C-5), 143.48 (C-7a), 157.67 (C-3). Anal. Calcd for $C_{22}H_{29}ClN_4O_3$ (332.9): C, 61.03; H, 6.75; N, 12.94. Found: C, 61.21; H, 6.59; N, 12.81.

4.6. Biology

4.6.1. Epimastigote culture and growth inhibition assays

T. cruzi epimastigotes, CL Brener clone, were grown at 28 °C in an axenic medium (BHI–Tryptose) as previously described, complemented with 5% fetal bovine serum (FBS)[7,25–30] Cells from a 10-day old culture (stationary phase) 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 measuring everyday the absorbance of the culture at 600 nm. Before inoculation, the media was supplemented with the indicated amount of the drug from a stock solution in DMSO. The final concentration of DMSO in the culture media never exceeded 0.4% and the control was run in the presence of 0.4% DMSO and in the absence of any drug. No effect on epimastigotes growth was observed by the presence of up to 1% DMSO in the culture media. The percentage of inhibition was calculated as follows: $\% = \{1 - [(A_p - A_{0p})/(A_c - A_{0c})]\} \times 100$, 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. To determine IC_{50} values, 50% inhibitory concentrations, parasite growth was followed in the absence (control) and presence of increasing concentrations of the corresponding drug. At day 5, the absorbance of the culture was measured and related to the control. The IC_{50} value was taken as the concentration of drug needed to reduce the absorbance ratio to 50%.

4.6.2. Cell culture

Human intestinal epithelial cells (Caco-2 ATCC accession HTB-37) were maintained in DMEM-F12, supplemented with 10% fetal bovine serum, penicillin (100 unit/mL), and streptomycin (0.1 mg/mL), at 37 °C, in a humidified incubator with 5% CO_2/95% air atmosphere. 5×10^4 cells/mL of culture medium were plated in 96-well plates.

4.6.3. MTT reduction assay

Cell viability was measured by MTT assay, based on the ability of live cells to convert thiazolyl blue into a dark blue formazan. Cells were treated with PBS containing the 5-NI derivatives, **12–28** (25 μM) for 2 h. After washing, 0.5 mg/mL MTT were added to each well and cells were further incubated for 120 min at 37 °C. The supernatant was discarded and DMSO was added to dissolve the formazan. After 10 min. incubation at 37 °C, the absorbance at 540 nm was measured.

4.7. Oxygen uptake

Tulahuen strain *T. cruzi* epimastigotes were harvested by 500*g* centrifugation, followed by washing and re-suspension in 0.05 M sodium phosphate buffer, pH 7.4, and containing 0.107 M sodium chloride. Respiration measurements were carried out polarographically with a Clark no. 5331 electrode (Yellow Springs Instruments) in a 53 YSI model (Simpson Electric Co). The chamber volume was 2 mL and the temperature was 28 °C. The amount of parasite used was equivalent to 2 mg of protein. The IC_{50} equivalent concentration corresponds to the final concentration used in the oxygen uptake experiments. This concentration was calculated considering that the IC_{50} (culture growth experiments) was determined using 3×10^6 parasites/mL, equivalent to 0.0375 mg protein/mL as the initial parasite mass; 80×10^6 parasites/mL, equivalent to 1 mg protein/mL, was used in the oxygen uptake experiments. In order to maintain the parasite masse drug ratio constant in these two types of experiments, the original IC_{50} was corrected by this 26-fold parasite mass increase in the oxygen uptake experiment. Values are expressed as mean ± SD for three independent experiments. No effect of DMSO alone was observed.

4.8. Preparation of the rat liver cytosolic fractions

Livers were obtained from male Sprague-Dawley rats (198–202 g) from 'Centro de Investigaciones Nucleares' (UdelaR, Montevideo, Uruguay). The animals were allowed food and water ad libitum. The experimental protocols with animals were adhered to the Principles of Laboratory Animal Care. The animals were sacrificed by cervical dislocation and the livers, maintained in an ice bath, were perfused in situ with an ice-cold KCl (0.9%) solution and washed with 3 volumes of Tris–HCl (0.05 M)–sucrose (0.25 M) pH 7.4 in a Potter–Elvehjem glass–Teflon homogeniser. The homogenates were centrifuged for 30 min at 900*g* at 4 °C and the supernatant fraction was centrifuged at 10,000*g* for 1 h at 4 °C. The pellet was discarded and the supernatant fraction was further centrifuged at 100,000*g* for 1 h at 4 °C. Metabolic assays were carried out with cytosol either fresh or frozen in Tris–HCl buffer and stored at −80 °C.

4.9. Cyclic voltammetry

DMSO (spectroscopy grade) was obtained from Aldrich. Tetrabutylammonium perchlorate (TBAP), used as supporting electrolyte, was obtained from Fluka. CV was carried out using a Metrohm 693VA instrument with a 694VA Stand convertor and a 693VA Processor, in DMSO (ca. 1.0×10^{-3} mol L^{-1}), under a nitrogen atmosphere at room temperature, with TBAP (ca. 0.1 mol L^{-1}), using a three-electrode cell. A hanging mercury drop electrode was used as the working electrode, a platinum wire as the auxiliary electrode and saturated calomel electrode (SCE) as the reference.

Using the Nicholson's procedure[31] the I_{pa}/I_{pc} values (RNO_2/$RNO_2^{-\cdot}$) was measured from each cyclic voltammogram with scan rate between 0.1 and 5 V/s. Employing the theoretical approaches of Olmstead et al. for dimerization,[32] the I_{pa}/I_{pc} values were inserted into a working curve to determine the parameter ω, which is described by the equation too

$$\omega = k_2 C_0 \tau$$

where k_2 is the second-order rate constant for the dimerization of $RNO_2^{-\cdot}$, C_0 is the nitrocompound concentration and $\tau = (E_\lambda - E_{1/2})/v$, where E_λ is the switching potential, $E_{1/2}$ is the half-wave potential and v is the scan rate.

4.10. ESR experiments

ESR spectra were recorded in the X-band (9.7 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. The ESR spectra were simulated using the program WINEPR Simphonia 1.25 version.

4.10.1. Parasites for ESR studies

T. cruzi epimastigotes (Tulahuen strain), from our collection, were grown at 28 °C in Diamond's monophasic medium as reported earlier, with blood replaced by 4 mM hemin.[33] Fetal calf serum was added to a final concentration of 4%. Parasites: 8×10^7 cells correspond to 1 mg protein or 12 mg of fresh weight. ESR spectra were produced using a microsomal fraction (4 mg protein/mL) obtained from *T. cruzi*, in a reaction medium containing 1 mM NADPH, and 100 mM DMPO, in 20 mM phosphate buffer, pH 7.4. All experiments were done after of 15 min of incubation, to 28 °C, of 5-NI with *T. cruzi* microsomal fraction, NADPH and DMPO in an aerobic environment.

4.10.2. Rat cytosolic fractions for ESR studies

The standard incubation mixture, in aerobic conditions, was: 1 mM NADPH in a 0.1 M potassium phosphate buffer (pH 7.4) containing EDTA (1.5 mM) and the corresponding 5-NI (40 μM) dissolved in DMSO or acetonitrile and 100 mM of DMPO or POBN as spin-trapping. The experiments were performed after pre-equilibration of the mixture at 37 °C, appropriate volume of cytosolic suspension was added to give a final protein concentration of 1 mg/mL.

4.10.3. Assay for Dpph radical

The dpph radical-scavenging capacity of individual selected 5-NI derivatives was determined with an ESR spectrometry method.[34] Each 5-NI solution was mixed with dpph stock solution to initiate the 5-NI–radical reaction. All reaction mixtures contained 1.0 mM dpph and 1.0 mM 5-NI, and the control solution contained no 5-NI. Both dpph and 5-NI solutions were prepared in acetonitrile. ESR signals were recorded 1 min following the start of the reaction. Spectrometer conditions were: microwave frequency, 9.72 GHz; microwave power, 20 mW; modulation amplitude, 0.98 G; receiver gain, 59 db; and sweep time, 20.972 s.

The scavenging activity of each compound was estimated by comparing the dpph signals in the 5-NI–radical reaction mixture and the control reaction at the same reaction time, and expressed as percentage dpp remaining. The dpph radical-scavenging rate of test compounds was calculated using the formula scavenging rate = $[(A_0 - A_x)/A_0] \times 100\%$, where A_x and A_0 are the double-integral ESR for the first line of samples in the presence and absence of test compounds, respectively.

4.10.4. Assay for galvinoxyl radical

The galvinoxyl radical-scavenging capacity of individual selected 5-NI was determined with an ESR spectrometry method. The 5-NI derivatives (1 mM) were mixed with 1 mM galvinoxyl in all reaction mixtures, and the control solution contained no 5-NI. Both galvinoxyl and 5-NI solutions were prepared in acetonitrile. ESR signals were recorded 1 min following the start of the reaction. Spectrometer conditions were the same as described above.

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