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Synthesis and biological properties of new 5-nitroindazole derivatives

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Abstract—A series of new 3-alkoxy- or 3-hydroxy-1-[ω-(dialkylamino)alkyl]-5-nitroindazoles have been synthesized and their trichomonacidal, antichagasic and antineoplastic properties studied. Five derivatives (**5**, **6**, **8**, **9** and **17**) showed remarkable trichomonacidal activity against *Trichomonas vaginalis* at 10 μg/mL concentration. Three compounds (**8**, **10**, **11**) exhibited interesting antichagasic activity and these same compounds moderate antineoplastic activity against TK-10 and HT-29 cell lines. Unspecific cytotoxicity against macrophages has also been evaluated and only compounds **9**, **10** and **11** resulted cytotoxic at the higher dose evaluated (100 μg/mL), loosing cytotoxicity at lower doses. QSAR studies have been carried out. X-ray crystallographic study of compound **8** has been performed.

1. Introduction

Parasitic infections caused by pathogenic protozoa affect more than three billion people worldwide and impose a substantial health and economic burden.[1] Many of the compounds showing antiprotozoal activity bear a nitro group in their heterocyclic structure, such as nifurtimox and benznidazole for treatment of Chagas' disease or metronidazole in trichomoniasis chemotherapy. The ability to produce radical species capable to induce a cascade of reduced materials, which are toxic towards the parasite, has been the proposed mechanism of action of these nitro compounds.[2] These reduced intermediates can act as toxic agents against protozoa as well as malign cells of solid tumours. So, 5-nitroazoles such as metronidazole, tinidazole, nifurtimox or niridazole are effective antiprotozoal agents, whilst 2-nitroazoles as misonidazole and benznidazole act also as antitumour agents. There are other examples of compounds showing antiparasitic properties having also antineoplastic activity. Thus, suramin, trimetrexate, emetine and cycloheximide, among others, act as antiparasitic agents and show also antineoplastic properties.

We had previously studied the in vitro and in vivo anti-*Trypanosoma cruzi* activity of some nitroheterocycles,[3,4] which showed remarkable anti-epimastigote properties, and also presented cytotoxic activity against TK-10 tumoural cells.[5] Besides, we described the cytostatic activity against HeLa cells of a series of nitroindazole and nitroindole derivatives.[6,7]

Keywords: 5-Nitroindazoles; Synthesis; Antiprotozoal and antineoplastic activities; QSAR.
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For all these reasons, we have now synthesized a series of new 5-nitroindazoles with the aim of testing their trichomonacidal, antichagasic and antineoplastic activities.

2. Results and discussion

2.1. Chemistry

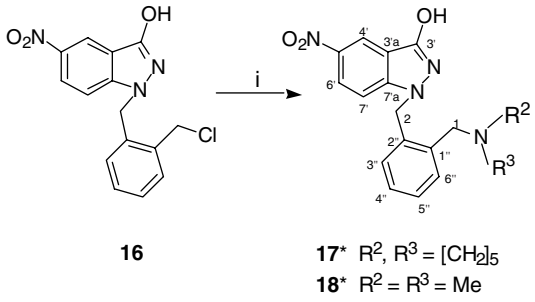
The new 5-nitroindazoles were prepared following the pathways shown in Schemes 1 and 2. As starting materials we used indazole-derived alkyl halides **1**,[8] **2**,[8] **3**,[9] **4**[8] and **16**,[10] previously prepared by our research group taking advantage of our studies on the synthesis and reactivity of 1,1-disubstituted indazolium-3-olates[10–12] and 1-substituted indazol-3-ols.[7,9–11]

Treatment of 3-alkoxyindazole derivatives **1**–**4** with piperidine or dimethylamine afforded tertiary amines **5**–**11** in good yield (Scheme 1). As expected, iodo (**1**) and bromo (**2**, **4**) derivatives are much more reactive than the corresponding chloro derivative (**3**); so, the latter required longer reaction time, but in spite of this fact, final good yields were achieved in all cases.

Analogously, compounds **17** and **18** were obtained from 3-hydroxyindazole derivative **16** and the mentioned secondary amines (Scheme 2). However, in this case noticeable amounts of the indazolo[1,2-*b*]phthalazinone arising from intramolecular cyclization[10] of **16** could be detected (TLC, ^{1}H NMR) in the crude reaction products.

On the other hand, accordingly to the previously observed sensitivity of 3-OBn substituted indazoles to acids,[7] compounds **7**, **8**, **10** and **11** afforded the corresponding indazol-3-ol derivatives **12**–**15** after treatment with hydrobromic acid (Scheme 1).

3-Benzyloxyindazoles **7**, **8**, **10** and **11** were obtained as the free bases, while 3-methoxy derivatives **5**, **6**, **9** were isolated as hydrochlorides, by treatment of the corresponding free bases with hydrochloric acid. This treatment was necessary due to the physical properties (mainly low mps and high solubility) of these bases. 3-Hydroxy derivatives **12**–**15**, **17** and **18** were isolated as the corresponding hydrohalides owing to the synthetic method used.



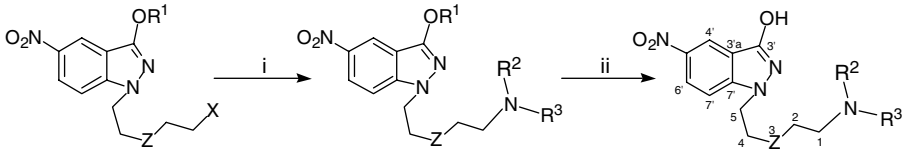
16 **17*** R^2, R^3 = $[CH_2]_5$ **18*** R^2 = R^3 = Me

Scheme 2. Reagents and conditions: (i) R^2R^3NH/EtOH, rt. (*)Compounds isolated as hydrochlorides.

Derivative **8** was studied by X-ray diffraction methods. The terminal nitro group is nearly coplanar with the indazole ring and the benzyl group subtends a dihedral angle of 68.0(1)° with this plane. The nitrogen on the lateral chain, dimethylamine moiety, shows a sp^3 bonding structure. Figure 1 shows the ORTEP drawing of derivative **8** (atomic coordinates, bond distances and angles, crystal data, data collection procedure, structure determination method and refinement results are given as Supplementary information).

2.2. Trichomonacidal and unspecific cytotoxic activity

The in vitro efficacy against *Trichomonas vaginalis* at 24 and 48 h of contact together with unspecific cytotoxicity to macrophages were tested for new compounds **5**–**15**, **17** and **18**. The cytostatic and cytocidal activities against *T. vaginalis* are expressed as percentage of growth inhibition (cytostatic activity, % GI) and percentage of reduction respect to control (cytocidal activity, % *R*), respectively. Compounds were assayed at 100, 10 and 1 μg/mL and the results are gathered in Table 1. Cyto-



	R^1	X	Z
1	Me	I	O
2	Bn	Br	O
3	Me	Cl	CH_2
4	Bn	Br	CH_2

	R^1	R^2, R^3	Z
5*	Me	$[CH_2]_5$	O
6*	Me	Me, Me	O
7	Bn	$[CH_2]_5$	O
8	Bn	Me, Me	O
9*	Me	$[CH_2]_5$	CH_2
10	Bn	$[CH_2]_5$	CH_2
11	Bn	Me, Me	CH_2

	R^2, R^3	Z
12**	$[CH_2]_5$	O
13**	Me, Me	O
14**	$[CH_2]_5$	CH_2
15**	Me, Me	CH_2

Scheme 1. Reagents and conditions: (i) R^2R^3NH/EtOH, reflux or heating in autoclave at 80–90 °C; (ii) from **7**, **8**, **10** and **11**, 48% aq HBr, reflux. (*)Compounds isolated as the hydrochlorides. (**)Compounds isolated as hydrobromides.

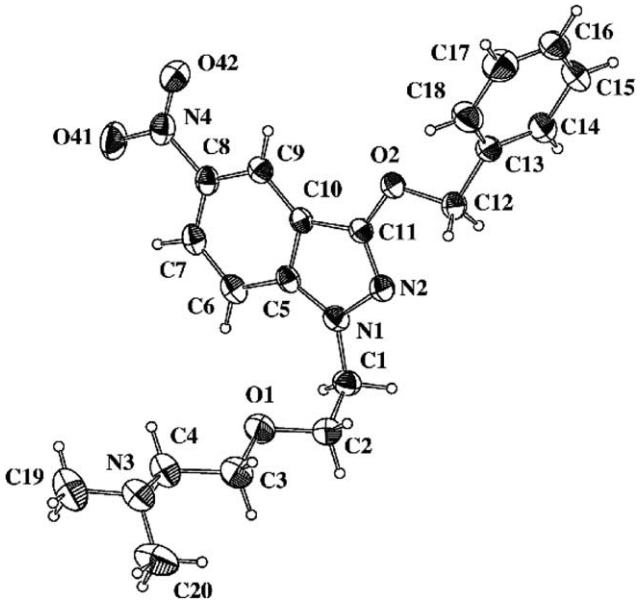


Figure 1. Molecular plot of derivative **8** showing the labelling of the non-H atoms and their displacement ellipsoids at the 30% probability level.

cidal activity is shown in brackets. Metronidazole (Mtz) was used as trichomonacidal reference drug.

In order to check the specific antiprotozoal activity, unspecific cytotoxic activity against macrophages was also studied at the same concentrations as trichomonacidal activity was measured. Results are gathered in Table 2, unspecific activity being expressed as cytotoxicity percentage (% *C*).

Except for compounds **7**, **12**, **13** and **15**, which only exhibited a moderate cytostatic activity against *T. vaginalis*, at 24 h of contact (83% ⩾ % GI ⩾ 35%), at the higher concentration assayed (100 μg/mL), the compounds tested (9 at 24 h of contact and 10 at 48 h) showed a very high trichomonacidal activity (% $R = 100\%$). Compounds **5**, **6**, **8**, **9** and **17** retained a high trichomonacidal activity (100% ⩾ % R ⩾ 92%) at 48 h of contact at the dose of 10 μg/mL. This trichomonacidal activity is not due to unspecific cytotoxicity since at this concentration only compounds **9**, **10** and **11** showed a slight unspecific cytotoxic activity (12% ⩾ % C ⩾ 6%, see Table 2). Unfortunately, trichomonacidal activity disappeared at the lowest dose assayed of 1 μg/mL, although **5**, **6** and **17** retained some cytostatic activity (55% ⩾ % GI ⩾ 25%) at this concentration.

Respect to unspecific cytotoxicity, only derivatives **9**, **10** and **11** showed appreciable cytotoxicity against macrophages at the higher dose assayed. This cytotoxicity dramatically diminished at concentration of 10 μg/mL.

2.3. Trypanocidal activity

The existence of the epimastigote form as an obligate mammalian intracellular stage has been revisited[13,14] and confirmed recently.[15] For this reason, compounds were tested in vitro against epimastigote forms of *Trypanosoma cruzi* (Brener strain) at 25 μM concentration, as indicated in Section 4.22. Table 3 shows the percentage of growth inhibition (% GI) for the evaluated derivatives and nifurtimox (Nfx) used as standard. Compounds **8**, **10** and **11** exhibited high anti-epimastigote activity (100% ⩾ % GI ⩾ 80%) at 25 μM concentration. This activity is not due to unspecific cytotoxicity, since at this concentration (10 μg/mL, approximately 25 μM) cytotoxicity percentages against macrophages are 0%, 6% and 10%, respectively (see Table 2). Compounds exhibiting a percentage of growth inhibition higher than 80% were tested at lower concentrations to determine their IC_{50}. Results are shown in Table 3 and Figure 2. Although IC_{50} of compounds

Table 1. Anti-*Trichomonas vaginalis* activity

Compd	% GI_{24} (% R_{24})			% GI_{48} (% R_{48})		
	100 μg/mL	10 μg/mL	1 μg/mL	100 μg/mL	10 μg/mL	1 μg/mL
5	(100)	(95)	49	(100)	(100)	35
6	(100)	(96)	67	(100)	(100)	55
7	83	49	13	(100)	36	18
8	(100)	(86)	20	(100)	(92)	17
9	(100)	87	18	(100)	(98)	10
10	(100)	50	2	(100)	68	0
11	(100)	75	26	(100)	77	6
12	41	14	7	37.4	26	0
13	35	3	0	9.5	0	0
14	(100)	0	0	(100)	9	0
15	58	7	0	62.6	2	0
17	(100)	(96)	0	(100)	(98)	25
18	(100)	66	18	(100)	(42)	7
Mtz	(100)	(99)	(98)	(100)	(100)	(99)

Mtz = metronidazole: 2, 1 and 0.5 μg/mL.
% GI_{24} = percentages of growth inhibition at 24 h.
% GI_{48} = percentages of growth inhibition at 48 h.
(% R_{24}) = percentage of reduction at 24 h.
(% R_{48}) = percentage of reduction at 48 h.

Table 2. Unspecific cytotoxic activity (% *C*) against macrophages

Compd	100 μg/mL	10 μg/mL	1 μg/mL
5	0	0	0
6	0	0	0
7	0	0	0
8	0	0	0
9	97	12	3
10	99	6	0
11	100	10	12
12	0	0	0
13	0	0	0
14	0	0	0
15	0	0	0
17	0	0	0
18	0	0	0

% *C* = cytotoxicity percentages.

Table 3. Antichagasic activity[a]

Compd	% GI	IC_{50}
5	25	—
6	17	—
7	24	—
8	80	16.4
9	33	—
10	100	6.6
11	100	12.5
12	9	—
13	3	—
14	8	—
15	6	—
17	11	—
18	10	—
Nfx	100	3.4

% GI = growth inhibition percentages of *T. cruzi* epimastigotes at 25 μM concentration of compounds.
IC_{50} = concentration (μM) that inhibits 50% of *T. cruzi* growth.
[a] Results are the means of three different experiments with a SD less than 10% in all cases.

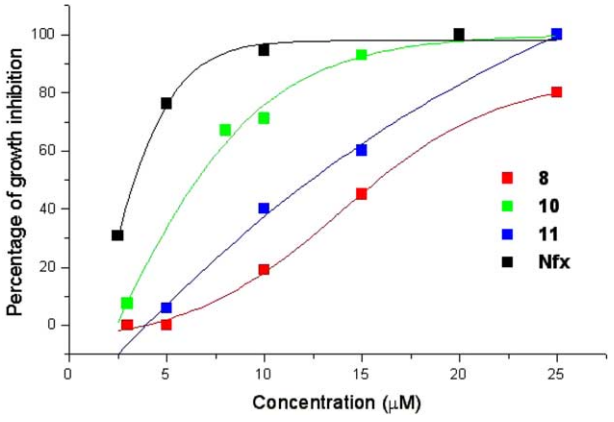


Figure 2. Curves dose–response as anti-epimastigote *T. Cruzi* for derivatives **8**, **10**, **11** and nifurtimox (Nfx).

8, **10** and **11** are higher than that of standard, compound **10** shows similar behaviour as nifurtimox as can be seen in the plot of curves dose–response shown in Figure 2.

2.4. Antineoplastic activity

For a primary screening, to select active samples, three tumour cell lines were employed, human colon adenocarcinoma (HT-29), human mammary adenocarcinoma (MCF-7) and human kidney carcinoma (TK-10). Compounds were tested at 100 μM concentration and cell survival percentages (SP %) are gathered in Table 4. Most compounds do not show antineoplastic activity, since their SP % were very high in the three cell lines used. Only compounds **8**, **10** and **11** exhibited some antitumour activity at this concentration and their IC_{50} (values in brackets) were calculated, results being shown in Table 4. The three compounds resulted not much active against MCF-7 cell line (95 μM $\geqslant$ IC_{50} $\geqslant$ 90 μM), some more against TK-10 (79 μM $\geqslant$ IC_{50} $\geqslant$ 49 μM) and HT-29 (75 μM $\geqslant$ IC_{50} $\geqslant$ 30 μM). However, the unspecific cytotoxicity of these compounds is much lower. Compound **11** presents the most interesting antitumour activity against HT-29 cell line with a IC_{50} = 30 μM being its unspecific cytotoxicity five times lower at similar concentration (% *C* = 10% at 10 μg/mL, 26 μM).

2.5. Structure–activity relationships

Molecular modeling studies were performed on the developed 5-nitroindazole derivatives calculating the stereoelectronic properties in order to understand the mechanism of action. The stereoelectronic properties were determined using pBP/DN* density functional calculation.[16,17] The crystallographic structure of derivative **8** was used as template to compare the theoretical level used for the calculi. As shown in Table 5, AM1 geometry described adequately bonds, angles and dihedral angles. The properties determined and examined in this study were total energy, solvatation (water) energy, magnitude of dipolar moment, volume, HOMO's

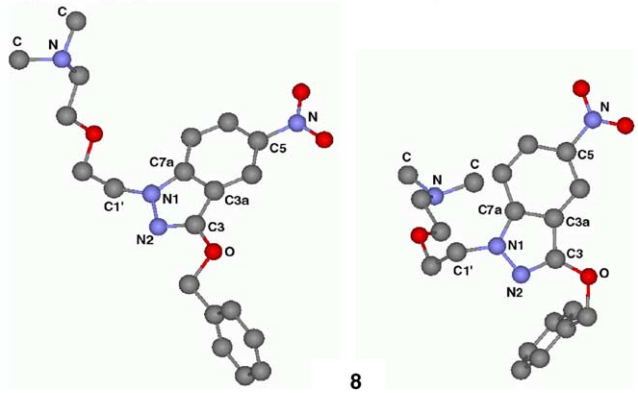
Table 4. Cytotoxicity to MCF-7, TK-10 and HT-29 cell lines

Compd	% SP (IC_{50}, μM)		
	MCF-7	TK-10	HT-29
5	78	80	92
6	86	74	97
7	90	103	99
8	3 (90)	1 (79)	1 (75)
9	84	91 —	99 —
10	12 (95)	1 (49)	40 (60)
11	6 (94)	1 (55)	2 (30)
12	81	97	96
13	83	98	105
14	63	104	99
15	85	105	98
17	75	97	101
18	79	101	93

% SP = cell survival percentages at 100 μM concentration.
IC_{50} = concentration (μM) that causes 50% cell growth. This value is shown in brackets.

Table 5. Crystallographic and AM1 optimized structure of compound **8**

Crystallographic structure AM1 optimised structure



8

Distances, angles and dihedrals[a,b]	Crystallographic data	AM1 optimized geometry	Relative error $\|(d_{AM1} - d_{crist})/d_{crist}\|$
N1–N2	1.389	1.360	0.02
N2–C3	1.306	1.359	0.04
C5–N	1.446	1.449	0.00
C3–O	1.322	1.364	0.03
C5–N	1.459	1.482	0.02
C7a–N1–N2	111.1	111.7	0.01
N1–N2–C3	105.1	107.3	0.02
N2–C3–C3a	111.0	110.9	0.00
O–N–O (nitro group)	123.0	121.9	0.01
C–N–C (dimethylamino group)	110.3	112.8	0.02
C3–N2–N1–C1′	2.2	8.6	2.91
N1–N2–C3–O	179.1	177.5	0.01

[a] Distances in Å, angles and dihedrals in °.
[b] Numbers according to figure.

and LUMO's energies, gap ($E_{LUMO} - E_{HOMO}$) and the logarithm of the partition coefficient of the non-ionized molecules (log *P*). Theoretical log *P* (cLog*P*) was calculated using Villar method, implemented in PC SPARTAN *Pro* package,[17] at AM1 semiempirical level.

Activities used in the structure–activity relationship studies were cytostatic activity (compounds **7**, **9**–**13**, **15** and **18**) against *T. vaginalis*, at 24 h of contact and at 10 μg/mL, $GI_{24,10}$, and the inhibitory effect on the growth of *T. cruzi* expressed as percentage of growth inhibition at day 5 and at 25 μM, % GI (for all compounds). Unspecific cytotoxicity against macrophages and cytotoxicities to MCF-7, TK-10 and HT-29 were not studied because the corresponding values did not cover homogenously all the range of activity (0–100%). As the dependent variables in the linearization procedure, we used $\log_{10}(GI_{24,10})$ and $\log_{10}(\%\ GI)$ values. In the equations and models, *n* represents the number of data points, r^2 is the correlation coefficient, *s* is the standard deviation of the regression equation, the *F* value is related to the *F*-statistic analysis (Fischer test) and r^2_{adj} defines the cross-validated correlation coefficient.

One-variable and multi-variable regressions between the activities and the physicochemical properties were studied. In the case of trichomonacidal activity the best equation (Eq. 1) was obtained when we analyzed the correlation between activity and the independent variable volume, *V*.

$$\log_{10}(GI_{24,10}) = -19.8(\pm 6.9) + 9.9(\pm 3.4)V - 1.1(\pm 0.4)V^2$$
$$r^2 = 0.8875 \quad r^2_{adj} = 0.7027 \quad s = 0.2914$$
$$F = 9.272 \quad n = 8 \quad \text{(derivatives } \mathbf{7},\ \mathbf{9}\text{–}\mathbf{13},\ \mathbf{15},\ \mathbf{18}\text{)} \tag{1}$$

In Figure 3a the plot of experimental values of *T. vaginalis* cytostatic activity versus the values calculated from Eq. 1 is represented; this equation adequately predicts the experimental activities of the eight compounds used in the study. The plot of experimental cytostatic activity versus molecular volume is represented in Figure 3b; the results clearly reveal that exists an optimum volume for maximal trichomonacidal activity of the studied 5-nitroindazole derivatives. This fact could be indicating a specific accommodation in an active pocket of some biological receptor.

On the other hand, for the trypanocidal activity, the best equation was obtained when we analyzed the correlation between activity and the independent variables cLog*P* and HOMO energy, E_{HOMO} (Eq. 2). Eq. 2 adequately predicts the experimental activities of the 13 compounds used in the study (Fig. 3c).

$$\log_{10}(\%\ GI) = 3.87(\pm 1.97) + 0.69(\pm 0.38)E_{HOMO} + 0.31(\pm 0.07)\text{cLog}P$$
$$r^2 = 0.8534 \quad r^2_{adj} = 0.6739 \quad s = 0.27312$$
$$F = 13.40154 \quad n = 13 \tag{2}$$

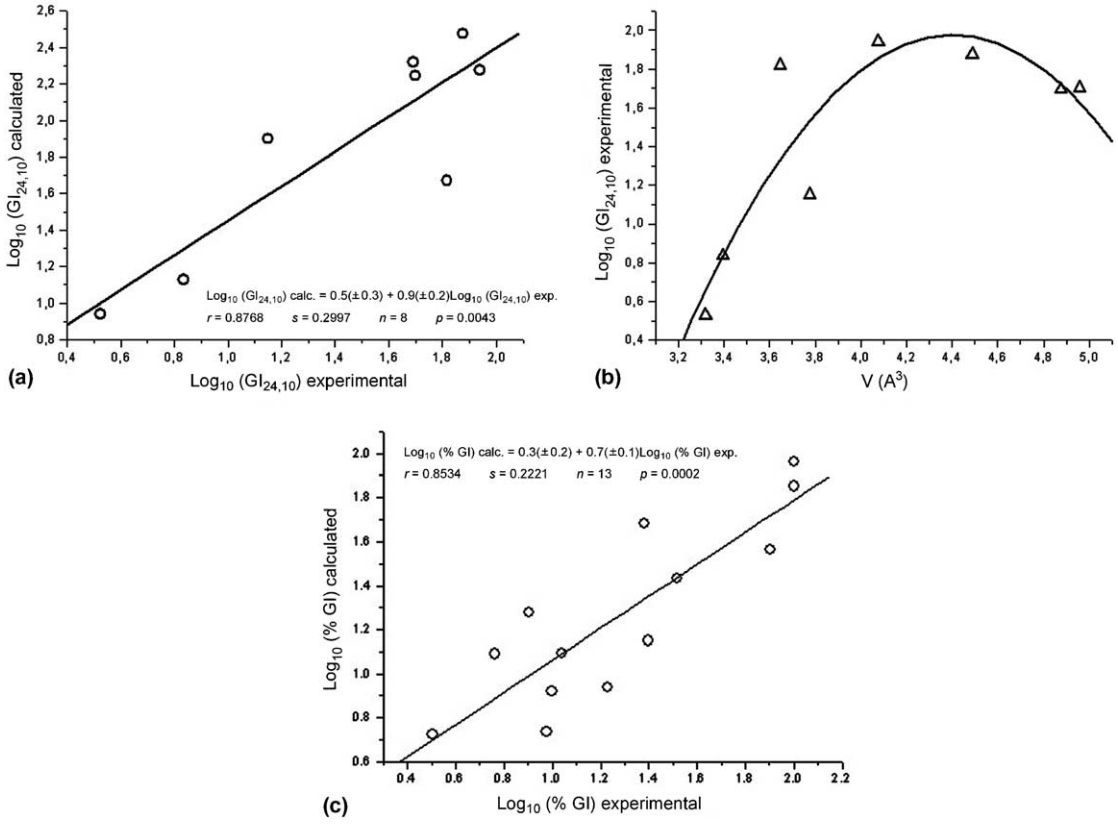


Figure 3. (a) Plot of log of percentage of trichomone growth inhibition at dosage of 10 μg/mL at 24 h of contact ($\log_{10}GI_{24,10}$) calculated from Eq. 1 against experimental values. (b) Plot of log of experimental percentage of trichomone growth inhibition at dosage of 10 μg/mL at 24 h of contact ($\log_{10}GI_{24,10}$) against molecular volume. (c) Plot of log of percentage of trypanosome growth inhibition at dosage of 25 μM ($\log_{10}$% GI) calculated from Eq. 2 against experimental values.

These results showed that lipophilic properties and, in more extension, HOMO energies could be playing a role in the trypanocidal activity of the studied 5-nitroindazole derivatives. Studying the HOMO distribution it was possible to observe, in all cases, that these frontier orbitals were located in the tertiary amine of the indazole lateral chain.

Otherwise, the correlation matrixes for the used physicochemical properties were performed and cross-correlations between the descriptors were not found. Thus, these parameters are orthogonal, allowing the safely use of these in the multilinear regression.[18]

In any case, we know that the number of data points in Eqs. 1 and 2 are not numerous enough and more compounds should be tested in order to check if the obtained results are significant.

3. Conclusions

Fortunately, the 5-nitroindazoles tested do not show significant unspecific cytotoxicity against macrophages. Some of the compounds studied exhibited remarkable trichomonacidal activity at 10 μg/mL concentration, although this activity is lower than that of standard metronidazole. However, taking into account the role of 5-nitroindazole volume on trichomonacidal activity, new derivatives bearing 3-OEt moiety, with a volume between OMe and OBn, could present better activities and they will be synthesized and tested to confirm it.

The trypanocidal activity of 5-nitroindazole family has been reported for the first time. Three of the studied derivatives (**8**, **10** and **11**) showed interesting properties. These new compounds are a start point for further chemical modification in order to improve the antichagasic activity. Compounds **8**, **10** and **11** displayed also certain antineoplastic activity confirming the parallelism that has previously been observed between antichagasic and anticancer activities.

QSAR studies demonstrate the importance of the volume for the trichomonacidal activity. Besides, for the anti-*T. cruzi* activity it was observed the significance of the lipophilic properties and, in more extension, the energy of HOMO.

4. Experimental

4.1. Chemistry

Mps were determined in a Gallenkamp capillary apparatus. ^{1}H (300 or 400 MHz) and ^{13}C (75 or 100 MHz) NMR spectra were recorded on Varian Unity 300 or Varian Inova 400 spectrometers. The chemical shifts

are reported in ppm from TMS (δ scale) but were measured against the solvent signal. The assignments have been performed by means of different standard homonuclear and heteronuclear correlation experiments (NOE, HMQC and HMBC). Numbering used in the description of spectra is given in Schemes 1 and 2. Electron impact (EI) and electrospray (ES+) mass spectra were obtained at 70 eV on a Hewlett Packard 5973 MSD spectrometer or on a Hewlett Packard 1100 MSD spectrometer, respectively. DC-Alufolien silica gel 60 PF_{254} (Merck, layer thickness 0.2 mm) and silica gel 60 (Merck, particle size 0.040–0.063 mm) were used, respectively, for TLC and flash column chromatography. Microanalyses were performed by the Centro de Microanálisis Elemental, Universidad Complutense, Madrid, Spain; crystallized samples for analysis were dried under vacuum over phosphorus(V) oxide, at 25 °C the compounds isolated as the free bases and at 60 °C those isolated as the corresponding hydrochlorides or hydrobromides.

4.2. General method A (applied to compounds 5, 6 and 9)

For compounds **5** and **6**, a mixture of 5-(3-methoxyindazol-1-yl)alkyl iodide **1** (1.03 g, 3.0 mmol) and piperidine (0.53 g, 6.2 mmol) or dimethylamine [0.30 g, 6.7 mmol (1.20 mL of a 5.6 M solution in ethanol)] in ethanol (12 mL) was refluxed or heated at 80–90 °C in an autoclave, respectively, for 4 h. For compound **9**, a mixture of 5-(3-methoxyindazol-1-yl)alkyl chloride **3** (0.89 g, 3.0 mmol) and piperidine (0.77 g, 9.0 mmol) was refluxed for 3 days. In each case, the mixture was then evaporated to dryness and, after addition of 5% aq 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 aq 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.

4.3. General method B (applied to compounds 7, 8, 10 and 11)

Following a procedure similar to that described in method A, a mixture of the corresponding 5-(3-benzyloxyindazol-1-yl)alkyl bromide (**2** or **4**) (3.0 mmol) and piperidine (6.2 mmol) or dimethylamine (6.7 mmol) in ethanol (10 mL) was, respectively, refluxed or heated in an autoclave and then concentrated to ca. 6 mL. In both cases, the obtained suspension or solution was allowed to stand at 4 °C overnight and then the crystallized tertiary amine was collected by filtration, washed with cold ethanol (2×5 mL) and air dried.

Yields of crystallized materials thus obtained range from 79% (**8**) to 94% (**7**). If desired, ca. 90% yield can be reached in all cases following this simple chromatographic isolation procedure: after collection of crystallized material, the corresponding filtrate was evaporated to dryness and, after addition of 5% aq potassium carbonate (30 mL), the mixture was extracted with chloroform (3×10 mL). The organic layers were separated, dried ($MgSO_4$), concentrated and applied to the top of a chromatography column; after elution of some high R_f by-products with a 30:1 chloroform–methanol mixture, the corresponding tertiary amines were eluted with 10:1–5:1 chloroform–methanol mixtures.

4.4. General method C (applied to compounds 12–15)

A mixture of the corresponding 3-benzyloxyindazole derivative (**7**, **8**, **10** or **11**) (2 mmol) and 48% aq hydrobromic acid (10 mL) was refluxed for 1 h. After cooling, the solution was extracted with hexane (3×20 mL) to remove benzyl bromide and the aqueous layer evaporated to dryness to afford the desired 3-hydroxyindazole-derived tertiary amines as the corresponding hydrobromides.

4.5. General method D (applied to compounds 17 and 18)

A mixture of compound **16** (0.48, 1.5 mmol) and piperidine (0.27 g, 3.2 mmol) or dimethylamine [0.15 g, 3.3 mmol (0.59 mL of a 5.6 M solution in ethanol)] in ethanol (50 mL) was stirred at room temperature for 48 h and then evaporated to dryness. For compound **17**, the residue was treated with 10% aq hydrochloric acid (25 mL) and the precipitated product collected by filtration and air dried. The obtained solid was triturated with chloroform (25 mL) and the insoluble tertiary amine hydrochloride was gathered by filtration, washed with chloroform (3×10 mL) and air dried. For compound **18**, the residue was treated with 0.25 N sodium hydroxide (50 mL) and extracted with chloroform (4×25 mL). The aqueous layer was evaporated to dryness and, after addition of 10% aq hydrochloric acid (25 mL), the precipitated yellow hydrochloride was collected by filtration and air dried.

4.6. *N*-[5-(3-Methoxy-5-nitro-1*H*-indazol-1-yl)-3-oxapentyl]piperidine hydrochloride (5·HCl)

Yield: 1.10 g (93%) (method A); mp 186–188 °C (decomp., 2-propanol). ^{1}H NMR (DMSO-d_6): δ 10.17 (br s, 1H, NH), 8.50 (d, $J = 2.1$ Hz, 1H, 4′-H), 8.20 (dd, $J = 9.4$, 2.1 Hz, 1H, 6′-H), 7.77 (d, $J = 9.4$ Hz, 1H, 7′-H), 4.51 (t, $J = 5.0$ Hz, 2H, 5-H), 4.06 (s, 3H, OCH_3), 3.82 (t, $J = 5.0$ Hz, 2H, 4-H), 3.74 (t, $J = 4.7$ Hz, 2H, 2-H), 3.13 (br d, $J = -11.7$ Hz, 2H, piperidine 2- and 6-H_{eq}), 3.06 (m, 2H, 1-H), 2.63 (m, 2H, piperidine 2- and 6-H_{ax}), 1.57 (m, 5H, piperidine 3-, 5-H and 4-H_A), 1.17 (m, 1H, piperidine 4-H_B); ^{13}C NMR (DMSO-d_6): δ 157.59 (C-3′), 143.26 (C-7′a), 140.33 (C-5′), 122.09 (C-6′), 117.46 (C-4′), 110.69 (C-7′), 110.57 (C-3′a), 68.57 (C-4), 64.74 (C-2), 56.59 (OCH_3), 55.01 (C-1), 52.31 (piperidine C-2 and -6), 48.15 (C-5), 22.14 (piperidine C-3 and -5), 21.05 (piperidine C-4); MS (ES+): *m*/*z* (%) 350 (52) [$MH^+ - Cl$], 349 (100) [$M^+ - Cl$]. Anal. Calcd for $C_{17}H_{25}ClN_4O_4 \times 1/2H_2O$ (393.9): C, 51.84; H, 6.65; N, 14.22. Found: C, 51.80; H, 6.41; N, 14.01.

4.7. *N*,*N*-Dimethyl-5-(3-methoxy-5-nitro-1*H*-indazol-1-yl)-3-oxapentylamine hydrochloride (6·HCl)

Yield: 0.94 g (91%) (method A); mp 206–208 °C (decomp., 2-propanol). ^{1}H NMR (DMSO-d_6): δ 10.21 (br s, 1H, NH), 8.49 (d, J = 2.2 Hz, 1H, 4′-H), 8.19 (dd, J = 9.3, 2.2 Hz, 1H, 6′-H), 7.79 (d, J = 9.3 Hz, 1H, 7′-H), 4.52 (t, J = 4.9 Hz, 2H, 5-H), 4.05 (s, 3H, OCH_3), 3.82 (t, J = 4.9 Hz, 2H, 4-H), 3.70 (t, J = 5.0 Hz, 2H, 2-H), 3.10 (t, J = 5.0 Hz, 2H, 1-H), 2.58 (s, 6H, NCH_3); ^{13}C NMR (DMSO-d_6): δ 157.57 (C-3′), 143.27 (C-7′a), 140.33 (C-5′), 122.07 (C-6′), 117.45 (C-4′), 110.80 (C-7′), 110.59 (C-3′a), 68.64 (C-4), 64.69 (C-2), 56.58 (OCH_3), 55.41 (C-1), 48.22 (C-5), 42.43 (NCH_3); MS (ES+): *m*/*z* (%) 310 (43) [MH^+-Cl], 309 (100) [M^+-Cl]. Anal. Calcd for $C_{14}H_{21}ClN_4O_4$ (344.8): C, 48.77; H, 6.14; N, 16.25. Found: C, 48.58; H, 6.40; N, 16.07.

4.8. *N*-[5-(3-Benzyloxy-5-nitro-1*H*-indazol-1-yl)-3-oxapentyl]piperidine (7)

Yield: 1.20 g (94%) of crystallized material (method B); mp 152–154 °C (ethanol). ^{1}H NMR ($CDCl_3$): δ 8.64 (d, J = 2.2 Hz, 1H, 4′-H), 8.18 (dd, J = 9.3, 2.2 Hz, 1H, 6′-H), 7.50 (m, 2H, Ph 2- and 6-H), 7.45–7.28 (m, 4H, Ph 3-, 4- and 5-H, and 7′-H), 5.41 (s, 2H, 3′-OCH_2), 4.36 (t, J = 5.1 Hz, 2H, 5-H), 3.81 (t, J = 5.1 Hz, 2H, 4-H), 3.45 (t, J = 5.8 Hz, 2H, 2-H), 2.35 (t, J = 5.8 Hz, 2H, 1-H), 2.19 (m, 4H, piperidine 2- and 6-H), 1.45 (m, 4H, piperidine 3- and 5-H), 1.34 (m, 2H, piperidine 4-H); ^{13}C NMR ($CDCl_3$): δ 157.57 (C-3′), 143.56 (C-7′a), 140.76 (C-5′), 136.14 (Ph C-1), 128.53 (Ph C-3 and -5), 128.32 (Ph C-4), 128.11 (Ph C-2 and -6), 122.26 (C-6′), 118.40 (C-4′), 111.91 (C-3′a), 109.51 (C-7′), 70.97 (3′-OCH_2), 69.51 (C-2), 69.40 (C-4), 58.42 (C-1), 54.89 (piperidine C-2 and -6), 49.29 (C-5), 25.77 (piperidine C-3 and -5), 24.06 (piperidine C-4); MS (EI): *m*/*z* (%) 423 (2) [M^+-1], 333 (17), 111 (45), 98 (100), 91 (64). Anal. Calcd for $C_{23}H_{28}N_4O_4$ (424.5): C, 65.08; H, 6.65; N, 13.20. Found: C, 64.85; H, 6.90; N, 12.98.

4.9. *N*,*N*-Dimethyl-5-(3-benzyloxy-5-nitro-1*H*-indazol-1-yl)-3-oxapentylamine (8)

Yield: 0.91 g (79%) of crystallized material, 1.04 g (90%) after additional chromatographic isolation (method B); mp 95–97 °C (ethanol). ^{1}H NMR (DMSO-d_6): δ 8.52 (d, J = 2.2 Hz, 1H, 4′-H), 8.18 (dd, J = 9.3, 2.2 Hz, 1H, 6′-H), 7.72 (d, J = 9.3 Hz, 1H, 7′-H), 7.55 (m, 2H, Ph 2- and 6-H), 7.40 (m, 3H, Ph 3-, 4- and 5-H), 5.44 (s, 2H, 3′-OCH_2), 4.46 (t, J = 5.1 Hz, 2H, 5-H), 3.75 (t, J = 5.1 Hz, 2H, 4-H), 3.39 (t, J = 5.9 Hz, 2H, 2-H), 2.19 (t, J = 5.9 Hz, 2H, 1-H), 1.96 (s, 6H, NCH_3); ^{13}C NMR ($CDCl_3$): δ 157.62 (C-3′), 143.57 (C-7′a), 140.89 (C-5′), 136.21 (Ph C-1), 128.54 (Ph C-3 and -5), 128.33 (Ph C-4), 128.11 (Ph C-2 and -6), 122.30 (C-6′), 118.44 (C-4′), 112.02 (C-3′a), 109.51 (C-7′), 71.02 (3′-OCH_2), 69.68 (C-2), 69.49 (C-4), 58.81 (C-1), 49.30 (C-5), 48.53 (NCH_3); MS (EI): *m*/*z* (%) 383 (1) [M^+-1], 295 (3), 293 (3), 103 (4), 91 (79), 71 (59), 58 (100). Anal. Calcd for $C_{20}H_{24}N_4O_4$ (384.4): C, 62.49; H, 6.29; N, 14.57. Found: C, 62.35; H, 6.40; N, 14.55.

4.10. *N*-[5-(3-Methoxy-5-nitro-1*H*-indazol-1-yl)pentyl]-piperidine hydrochloride (9·HCl)

Yield: 1.09 g (95%) (method A); mp 179–181 °C (decomp., ethanol/diethyl ether). ^{1}H NMR (DMSO-d_6): δ 10.60 (br s, 1H, NH), 8.47 (d, J = 2.4 Hz, 1H, 4′-H), 8.18 (dd, J = 9.3, 2.4 Hz, 1H, 6′-H), 7.78 (d, J = 9.3 Hz, 1H, 7′-H), 4.31 (t, J = 6.8 Hz, 2H, 5-H), 4.04 (s, 3H, OCH_3), 3.34 (br d, J = −11.6 Hz, 2H, piperidine 2- and 6-H_{eq}), 2.88 (m, 2H, 1-H), 2.76 (m, 2H, piperidine 2- and 6-H_{ax}), 1.86–1.62 (m, 9H, 2- and 4-H, piperidine 3-, 5-H and 4-H_A), 1.34 (m, 1H, piperidine 4-H_B), 1.23 (m, 2H, 3-H); ^{13}C NMR (DMSO-d_6): δ 157.42 (C-3′), 142.46 (C-7′a), 140.18 (C-5′), 122.03 (C-6′), 117.48 (C-4′), 110.39 (C-3′a), 110.23 (C-7′), 56.52 (OCH_3), 55.33 (C-1), 51.64 (piperidine C-2 and -6), 47.79 (C-5), 28.52 (C-4), 23.17 (C-3), 22.43 (C-2), 22.15 (piperidine C-3 and -5), 21.45 (piperidine C-4); MS (ES+): *m*/*z* (%) 348 (25) [MH^+-Cl], 347 (100) [M^+-Cl]. Anal. Calcd for $C_{18}H_{27}ClN_4O_3$ (382.9): C, 56.46; H, 7.11; N, 14.63. Found: C, 56.12; H, 6.92; N, 14.35.

4.11. *N*-[5-(3-Benzyloxy-5-nitro-1*H*-indazol-1-yl)pentyl]-piperidine (10)

Yield: 1.03 g (81%) of crystallized material, 1.18 g (93%) after additional chromatographic isolation (method B); mp 101–103 °C (ethanol). ^{1}H NMR (DMSO-d_6): δ 8.53 (d, J = 2.0 Hz, 1H, 4′-H), 8.19 (dd, J = 9.3, 2.0 Hz, 1H, 6′-H), 7.74 (d, J = 9.3 Hz, 1H, 7′-H), 7.54 (m, 2H, Ph 2- and 6-H), 7.38 (m, 3H, Ph 3-, 4- and 5-H), 5.43 (s, 2H, 3′-OCH_2), 4.29 (t, J = 6.8 Hz, 2H, 5-H), 2.20 (m, 4H, piperidine 2- and 6-H), 2.10 (t, J = 7.1 Hz, 2H, 1-H), 1.78 (m, 2H, 4-H), 1.35 (m, 8H, 2-H, piperidine 3-, 4- and 5-H), 1.15 (m, 2H, 3-H); ^{13}C NMR ($CDCl_3$): δ 157.37 (C-3′), 142.46 (C-7′a), 140.67 (C-5′), 136.19 (Ph C-1), 128.50 (Ph C-3 and -5), 128.28 (Ph C-4), 128.12 (Ph C-2 and -6), 122.35 (C-6′), 118.70 (C-4′), 111.81 (C-3′a), 108.43 (C-7′), 70.98 (3′-OCH_2), 59.22 (C-1), 54.63 (piperidine C-2 and -6), 48.79 (C-5), 29.45 (C-4), 26.53 (C-2), 25.93 (piperidine C-3 and -5), 24.83 (C-3), 24.40 (piperidine C-4); MS (EI): *m*/*z* (%) 421 (2) [M^+-1], 331 (59), 315 (4), 246 (17), 154 (4), 124 (11), 110 (6), 98 (100), 91 (52), 84 (7). Anal. Calcd for $C_{24}H_{30}N_4O_3$ (422.5): C, 68.22; H, 7.16; N, 13.26. Found: C, 68.31; H, 6.98; N, 13.29.

4.12. *N*,*N*-Dimethyl-5-(3-benzyloxy-5-nitro-1*H*-indazol-1-yl)pentylamine (11)

Yield: 0.94 g (82%) of crystallized material, 1.06 g (92%) after additional chromatographic isolation (method B); mp 104–106 °C (ethanol). ^{1}H NMR (DMSO-d_6): δ 8.52 (d, J = 2.0 Hz, 1H, 4′-H), 8.18 (dd, J = 9.3, 2.0 Hz, 1H, 6′-H), 7.74 (d, J = 9.3 Hz, 1H, 7′-H), 7.54 (m, 2H, Ph 2- and 6-H), 7.40 (m, 3H, Ph 3-, 4- and 5-H), 5.43 (s, 2H, 3′-OCH_2), 4.29 (t, J = 7.0 Hz, 2H, 5-H), 2.08 (t, J = 6.8 Hz, 2H, 1-H), 2.03 (s, 6H, NCH_3), 1.78 (m, 2H, 4-H), 1.35 (m, 2H, 2-H), 1.17 (m, 2H, 3-

H); ^{13}C NMR ($CDCl_3$): δ 157.40 (C-3′), 142.49 (C-7′a), 140.68 (C-5′), 136.20 (Ph C-1), 128.52 (Ph C-3 and -5), 128.31 (Ph C-4), 128.13 (Ph C-2 and -6), 122.39 (C-6′), 118.73 (C-4′), 111.83 (C-3′a), 108.45 (C-7′), 70.99 (3′-OCH_2), 59.53 (C-1), 48.80 (C-5), 45.50 (NCH_3), 29.47 (C-4), 27.32 (C-2), 24.59 (C-3); MS (EI): *m/z* (%) 291 (21) [M^+−Bn], 246 (25), 91 (62), 58 (100). Anal. Calcd for $C_{21}H_{26}N_4O_3$ (382.5): C, 65.95; H, 6.85; N, 14.65. Found: C, 66.12; H, 6.66; N, 14.51.

4.13. *N*-[5-(3-Hydroxy-5-nitro-1*H*-indazol-1-yl)-3-oxapentyl]piperidine hydrobromide (12·HBr)

Yield: 0.74 g (89%) (method C); 143–145 °C (decomp., ethanol). ^{1}H NMR (DMSO-d_6): δ 11.42 (br s, 1H, OH), 9.22 (br s, 1H, NH), 8.68 (d, *J* = 2.2 Hz, 1H, 4′-H), 8.15 (dd, *J* = 9.3, 2.2 Hz, 1H, 6′-H), 7.70 (d, *J* = 9.3, 1H, 7′-H), 4.45 (t, *J* = 5.0 Hz, 2H, 5-H), 3.82 (t, *J* = 5.0 Hz, 2H, 4-H), 3.72 (t, *J* = 4.8 Hz, 2H, 2-H), 3.18 (br d, *J* = −12.0 Hz, 2H, piperidine 2- and 6-H_{eq}), 3.13 (m, 2H, 1-H), 2.70 (m, 2H, piperidine 2- and 6-H_{ax}), 1.56 (m, 5H, piperidine 3-, 5-H and 4-H_A), 1.20 (m, 1H, piperidine 4-H_B); ^{13}C NMR (DMSO-d_6): δ 156.44 (C-3′), 142.63 (C-7′a), 139.81 (C-5′), 121.54 (C-6′), 118.55 (C-4′), 111.37 (C-3′a), 110.30 (C-7′), 68.60 (C-4), 64.54 (C-2), 55.07 (C-1), 52.41 (piperidine C-2 and -6), 47.78 (C-5), 22.22 (piperidine C-3 and -5), 20.97 (piperidine C-4); MS (ES+): *m/z* (%) 336 (32) [MH^+−Br], 335 (100) [M^+−Br]. Anal. Calcd for $C_{16}H_{23}BrN_4O_4$ (415.3): C, 46.28; H, 5.58; N, 13.49. Found: C, 46.13; H, 5.58; N, 13.06.

4.14. *N*,*N*-Dimethyl-[5-(3-hydroxy-5-nitro-1*H*-indazol-1-yl)-3-oxapentyl]amine hydrobromide (13·HBr)

Yield: 0.69 g (92%) (method C); mp 192–194 °C (decomp., 2-propanol). ^{1}H NMR (DMSO-d_6): δ 11.44 (br s, 1H, OH), 9.24 (br s, 1H, NH), 8.67 (d, *J* = 2.2 Hz, 1H, 4′-H), 8.15 (dd, *J* = 9.3, 2.2 Hz, 1H, 6′-H), 7.68 (d, *J* = 9.3, 1H, 7′-H), 4.43 (t, *J* = 5.1 Hz, 2H, 5-H), 3.82 (t, *J* = 5.1 Hz, 2H, 4-H), 3.66 (t, *J* = 5.0 Hz, 2H, 2-H), 3.15 (br t, *J* = 5.0 Hz, 2H, 1-H), 2.63 (s, 6H, NCH_3); ^{13}C NMR (DMSO-d_6): δ 156.45 (C-3′), 142.64 (C-7′a), 139.84 (C-5′), 121.55 (C-6′), 118.57 (C-4′), 111.41 (C-3′a), 110.37 (C-7′), 68.66 (C-4), 64.46 (C-2), 55.64 (C-1), 47.83 (C-5), 42.59 (NCH_3); MS (ES+): *m/z* (%) 296 (26) [MH^+−Br], 295 (100) [M^+−Br]. Anal. Calcd for $C_{13}H_{19}BrN_4O_4$ (375.2): C, 41.61; H, 5.10; N, 14.93. Found: C, 41.88; H, 5.01; N, 14.77.

4.15. *N*-[5-(3-Hydroxy-5-nitro-1*H*-indazol-1-yl)pentyl]-piperidine hydrobromide (14·HBr)

Yield: 0.75 g (91%) (method C); mp 166–168 °C (decomp., ethanol). ^{1}H NMR (DMSO-d_6): δ 11.41 (br s, 1H, OH), 9.03 (br s, 1H, NH), 8.67 (d, *J* = 2.0 Hz, 1H, 4′-H), 8.15 (dd, *J* = 9.3, 2.0 Hz, 1H, 6′-H), 7.69 (d, *J* = 9.3 Hz, 1H, 7′-H), 4.24 (t, *J* = 6.7 Hz, 2H, 5-H), 3.38 (br d, *J* = −12.4 Hz, 2H, piperidine 2- and 6-H_{eq}), 2.92 (m, 2H, 1-H), 2.80 (m, 2H, piperidine 2- and 6-H_{ax}), 1.90–1.50 (m, 9H, 2- and 4-H, piperidine 3-, 5-H and 4-H_A), 1.35 (m, 1H, piperidine 4-H_B), 1.20 (m, 2H, 3-H); ^{13}C NMR (DMSO-d_6): δ 156.34 (C-3′), 141.98 (C-7′a), 139.74 (C-5′), 121.54 (C-6′), 118.67 (C-4′), 111.21 (C-3′a), 109.92 (C-7′), 55.47 (C-1), 51.87 (piperidine C-2 and -6), 47.50 (C-5), 28.54 (C-4), 23.17 (C-3), 22.68 (C-2), 22.35 (piperidine C-3 and -5), 21.29 (piperidine C-4); MS (ES+): *m/z* (%) 334 (26) [MH^+−Br], 333 (100) [M^+−Br]. Anal. Calcd for $C_{17}H_{25}BrN_4O_3$ (413.3): C, 49.40; H, 6.10; N, 13.56. Found: C, 49.54; H, 5.99; N, 13.38.

4.16. *N*,*N*-Dimethyl-[5-(3-hydroxy-5-nitro-1*H*-indazol-1-yl)pentyl]amine hydrobromide (15·HBr)

Yield: 0.72 g (94%) (method C); mp 144–147 °C (decomp., ethanol). ^{1}H NMR (DMSO-d_6): δ 11.30 (br s, 1H, OH), 9.35 (br s, 1H, NH), 8.67 (d, *J* = 2.0 Hz, 1H, 4′-H), 8.14 (dd, *J* = 9.3, 2.0 Hz, 1H, 6′-H), 7.68 (d, *J* = 9.3 Hz, 1H, 7′-H), 4.24 (t, *J* = 6.7 Hz, 2H, 5-H), 2.95 (m, 2H, 1-H), 2.71 (d, *J* = 4.9 Hz, 6H, NCH_3), 1.79 (m, 2H, 4-H), 1.61 (m, 2H, 2-H), 1.19 (m, 2H, 3-H); ^{13}C NMR (DMSO-d_6): δ 156.33 (C-3′), 141.97 (C-7′a), 139.73 (C-5′), 121.53 (C-6′), 118.69 (C-4′), 111.22 (C-3′a), 109.93 (C-7′), 56.21 (C-1), 47.52 (C-5), 42.00 (NCH_3), 28.50 (C-4), 23.18 (C-2), 22.97 (C-3); MS (ES+): *m/z* (%) 294 (28) [MH^+−Br], 293 (100) [M^+−Br]. Anal. Calcd for $C_{14}H_{21}BrN_4O_3 \times 1/2H_2O$ (382.3): C, 43.99; H, 5.80; N, 14.66. Found: C, 44.05; H, 5.64; N, 14.58.

4.17. *N*-{2-[(3-Hydroxy-5-nitro-1*H*-indazol-1-yl)methyl]-benzyl}piperidine hydrochloride (17·HCl)

Yield: 0.52 g (82%) (method D); mp 225–227 °C (decomp., methanol/10% aq hydrochloric acid). ^{1}H NMR (DMSO-d_6): δ 11.79 (br s, 1H, OH), 10.41 (br s, 1H, NH), 8.82 (d, *J* = 2.2 Hz, 1H, 4′-H), 8.22 (dd, *J* = 9.3, 2.2 Hz, 1H, 6′-H), 7.97 (d, *J* = 9.3 Hz, 1H, 7′-H), 7.68 (m, 1H, 6″-H), 7.37 (m, 2H, 4″- and 5″-H), 7.01 (m, 1H, 3″-H), 5.82 (s, 2H, 2-H), 4.51 (d, *J* = 5.5 Hz, 2H, 1-H), 3.40 (br d, *J* = −11.5 Hz, 2H, piperidine 2- and 6-H_{eq}), 3.07 (m, 2H, piperidine 2- and 6-H_{ax}), 1.83 (m, 5H, piperidine 3-, 5-H and 4-H_A), 1.42 (m, 1H, piperidine 4-H_B); ^{13}C NMR (DMSO-d_6): δ 156.90 (C-3′), 142.45 (C-7′a), 140.22 (C-5′), 137.58 (C-2″), 133.21 (C-6″), 130.03 (C-4″), 128.87 (C-3″), 128.18 (C-1″), 127.99 (C-5″), 122.23 (C-6′), 119.32 (C-4′), 111.52 (C-3′a), 110.38 (C-7′), 55.89 (C-1), 52.04 (piperidine C-2 and -6), 49.09 (C-2), 22.20 (piperidine C-3 and -5), 21.58 (piperidine C-4); MS (ES+): *m/z* (%) 368 (50) [MH^+−Cl], 367 (100) [M^+−Cl]. Anal. Calcd for $C_{20}H_{23}ClN_4O_3 \times H_2O$ (420.9): C, 57.07; H, 5.99; N, 13.31. Found: C, 57.18; H, 5.87; N, 13.24.

4.18. *N*,*N*-Dimethyl-2-[(3-hydroxy-5-nitro-1*H*-indazol-1-yl)methyl]benzylamine hydrochloride (18·HCl)

Yield: 0.49 g (86%) (method D); mp 220–223 °C (decomp., ethanol/10% aq hydrochloric acid). ^{1}H NMR (DMSO-d_6): δ 11.76 (br s, 1H, OH), 10.70 (br s, 1H, NH), 8.73 (d, *J* = 2.2 Hz, 1H, 4′-H), 8.20 (dd, *J* = 9.3, 2.2 Hz, 1H, 6′-H), 7.93 (d, *J* = 9.3 Hz, 1H, 7′-H), 7.63 (m, 1H, 6″-H), 7.36 (m, 2H, 4″- and 5″-H), 6.90 (m, 1H, 3″-H), 5.82 (s, 2H, 2-H), 4.53 (s, 2H, 1-H), 2.81 (s, 6H, NCH_3); ^{13}C NMR (DMSO-d_6): δ 156.88 (C-3′),

142.52 (C-7′a), 140.24 (C-5′), 137.43 (C-2″), 132.71 (C-6″), 130.07 (C-4″), 128.75 (C-1″), 128.54 (C-3″), 128.06 (C-5″), 122.19 (C-6′), 118.94 (C-4′), 111.62 (C-3′a), 110.33 (C-7′), 56.26 (C-1), 48.98 (C-2), 41.90 (NCH_3); MS (ES+): *m/z* (%) 328 (50) [MH^+-Cl], 327 (100) [M^+-Cl]. Anal. Calcd for $C_{17}H_{19}ClN_4O_3 \times H_2O$ (380.8): C, 53.62; H, 5.56; N, 14.71. Found: C, 53.78; H, 5.47; N, 14.67.

4.19. X-ray diffraction

The molecular structure of derivative **8**, $C_{20}H_{24}N_4O_4$, was determined by X-ray diffraction methods. The substance crystallizes in the monoclinic $P2_1/n$ space group with $a = 6.817(3)$, $b = 13.090(3)$, $c = 22.672(3)$ Å, $\beta = 96.00(3)°$, and $Z = 4$. The structure was solved from 1817 reflections with $I > 2\sigma(I)$ and refined to an agreement $R1$ factor of 0.0663. The hydrogen atoms were positioned stereochemically and refined with the riding model. The methyl H-atoms positions were optimized in the refinement by treating them as rigid bodies allowed to rotate around the corresponding C–N bond. As expected, these refined positions converged to a staggered methyl configuration. Intramolecular bond distances and angles, along with other crystallographic data, are given as Supporting information. The structure of compound **8** has been deposited in the Cambridge Crystallographic Data Centre, reference number CCDC-247713.

4.20. Trichomonacidal in vitro test

T. vaginalis strain JH31A no. 4 was cultured at 37 °C with 5% CO_2 in TYM (trypticase–yeast extract–maltose) medium supplemented with 10% of heat inactivated equine serum. The assays were carried out using glass tubes containing 100,000 protozoa/mL in a final volume of 2 mL. Compounds were added to the cultures 6 h after seeding. For each concentration assayed, there were three experimental and six growth controls. Viable protozoa were assessed at 24 and 48 h after incubation in the presence of the compounds by counting in an haemocytometer. Results are expressed both as percentages of inhibition growth (cytostatic activity) and percentages of reduction (cytocidal activity) respect to controls and calculated as previously described.[19] Metronidazole was used as reference drug.

4.21. Cytotoxicity to macrophages test[20]

J774 Macrophages were seeded (70,000 cells/well) in 96 well flat bottom microplates (Nunclon) with 200 μL of RPMI 1640 medium supplemented with 20% heat inactivated foetal calf serum. Cells were allowed to attach for 24 h in a humidified 5% CO_2/95% air atmosphere at 37 °C. Then, cells were exposed to the compounds (100, 10 and 1 μg/mL) for 24 h. Afterwards, the cells were washed with PBS and incubated (37 °C) with MTT 0.4 mg/mL for 60 min. Then, formazan was dissolved with DMSO (100 μL) and optical densities were measured as above. Each concentration was assayed three times and six growth controls were used in each test. Cytotoxicity percentages (% *C*) were determined as

$$\%\ C = 100–(\mathrm{ODd}–\mathrm{ODdm})/(\mathrm{ODc}–\mathrm{ODcm}) \times 100$$

where ODd is the mean of OD595 of wells with macrophages and different concentrations of the compounds; ODdm is the mean of OD595 of wells with different compounds concentrations in medium; ODc is the growth control and ODcm is the mean of OD595 of wells with only medium.

4.22. Trypanocidal in vitro test

Trypanosoma cruzi epimastigotes (Brener strain) were grown at 28 °C in an axenic medium (BHI-tryptose) complemented with 5% foetal calf serum. 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: $\% = \{1 - [(A_p - A_{0p})/(A_c - A_{0c})]\}100$, 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 doses, 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%.

4.23. Antineoplastic assay

The compounds were tested at 10^{-4} M in the following tumour cell lines: MCF-7 (human mammary adenocarcinoma) (ATCC HTB-38), TK-10 (human kidney carcinoma) (NCI) and HT-29 (human colon adenocarcinoma) (ATCC HTB-38). The cytotoxicity test was carried out according to previously described procedures[21] with some modifications. *Cells*: an adequate cell suspension was prepared for each cell line (MCF-7, TK-10 or HT-29) in RPMI medium, supplemented with L-glutamine (1%), penicillin/streptomycin (1%), non-essential amino acids (1%) and 10% (v/v) foetal bovine serum (FBS). Then, 225 μL were added into 96-well plates. The cultures were maintained at 37 °C and 5% CO_2 for 48 h. *Treatment*: compound solutions were prepared just before dosing. Stock solutions, 1 mM, were prepared in 10% DMSO and 25 μL (final concentration 10^{-4} M) were added to each well. The cells were exposed for 24 h at 37 °C in 5% CO_2 atmosphere. *Measurement*: after exposure to the compound,

the medium was eliminated and the cells were washed with PBS. The cells were fixed with 50 μL of trichloroacetic acid (50%) and 200 μL of culture medium (without FBS) for 1 h at 4 °C. Then, the cells were washed with purified water and treated with sulforhodamine B (0.4% wt/vol in 1% acetic acid) for 10 min at room temperature. The plates were washed with 1% acetic acid and dried overnight. Finally, 100 μL of Tris buffer (pH = 10.0) was added and absorbance at 540 nm was determined. *Calculations*: the cell survival percentage (SP) was calculated for all of the compounds as [(absorbance of cells post-treatment with product − absorbance of blank)/(absorbance of cells post-treatment with solvent − absorbance of blank)]100.

4.24. Molecular modeling

A detailed conformational search for each of the neutral molecules was performed, using MM methods (MMFF94, Conformer Distribution module implemented in PC SPARTAN *Pro* package), to find the minimum energy and highest abundance conformer. The geometry of this conformer was fully optimized by applying semiempirical AM1 method in gas phase, that allow obtaining acute results with low time of computational calculi. All of these minimum of energies were confirmed by the absence of negative frequencies (throughout vibrational analysis). Then, single point pBP/DN* density functional calculation was applied.

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Supplementary data

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