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CONCISE ARTICLE

Preliminary *in vitro* evaluation of *N′*-(benzofuroxan-5-yl)methylene benzohydrazide derivatives as potential anti-*Trypanosoma cruzi* agents†

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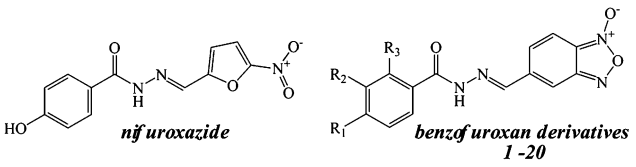
A set of benzofuroxan derivatives was tested *in vitro* against *Trypanosoma cruzi* epimastigote forms. The influence of physicochemical properties on these benzofuroxan derivatives' activity was observed, and the presence of electron-withdrawing and hydrophobic groups attached to the benzene ring seems to make a favorable contribution at lower concentrations.

The search for new therapeutic options is a continuous need when the so-called neglected diseases are considered. One of the main reasons for this designation is the lack of interest from major pharmaceutical companies in investing money into the research and development (R&D) for new drugs concerning these diseases, since the investments would hardly be returned as profits.[1,2] Neglected diseases primarily affect underdeveloped countries in Africa, Asia, and Latin America, especially in areas where sanitation and housing are precarious, with little or no access to health care.[1,3] According to the World Health Organization (WHO), it is estimated that 1 billion people, almost one sixth of the global population, suffer from one or more neglected diseases.[4]

Chagas disease (CD) is considered one of the thirteen "most" neglected diseases,[1,5] being part of a group that receives less than 5% investments into R&D programs.[6] This parasitic disease, caused by the protozoal hemoflagellate *Trypanosoma cruzi*, is endemic in twenty one Latin American countries and presents a death rate of approximately 14 000 people per year, which is higher than any other vector-transmitted disease, including malaria.[6]

Since the 1970s, the clinical treatment for CD is dependent on only two drugs, benznidazole (Rochagan®, Roche) and nifurtimox (Lampit®, Roche). Furthermore, the use of these two drugs in clinical practice is controversial, since neither promotes healing in the chronic phase and both show several adverse effects in infected patients.[7] The high toxicity of these two drugs, which is represented by the side effects, joined with the emergence of *T. cruzi* resistant strains, reinforces the need for new therapeutic options with better pharmacological profiles.

Previous studies of benzofuroxan derivatives, structurally analogous to nifuroxazide (see Fig. 1), demonstrated the influence of physicochemical properties on the antibacterial activity against multidrug-resistant *Staphylococcus aureus* strains.[8,9] In order to continue the research on the potential of these compounds, in this study the anti-*T. cruzi* activity of a series of twenty *N′*-(benzofuroxan-5-yl)methylenebenzohydrazides was investigated *in vitro*. The use of the benzofuroxan system as a bioreductive pharmacophore and also as a cruzipain inhibitor has been described as an alternative in the search for new anti-chagasic drugs.[10] The series was designed to explore the influence of physicochemical properties such as the electronic effect (σ) and hydrophobicity (π), based on the Craig diagram.[11]



	R_1	R_2	R_3
1	H	H	H
2	CH_3	H	H
3	NH_2	H	H
4	OH	H	H
5	F	H	H
6	CN	H	H
7	CH_2CH_3	H	H
8	OCH_3	H	H
9	Cl	H	H
10	$COCH_3$	H	H
11	$N(CH_3)_2$	H	H
12	OCH_2CH_3	H	H
13	NO_2	H	H
14	CF_3	H	H
15	Br	H	H
16	SO_2NH_2	H	H
17	I	H	H
18	Cl	H	Cl
19	Cl	Cl	H
20	NO_2	CF_3	H

Fig. 1 Chemical structural of nifuroxazide and a set of benzofuroxan derivatives.

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† Electronic supplementary information (ESI) available: Complete profile of control solutions, compounds and benznidazole, and the number of parasites per mL during every test day, for each compound concentration used and also for the PGI. See DOI: 10.1039/c2md20019h

Table 1 Biological activity after 72 hours of compounds **1–20** and BZN exposure[a]

				0 hours	10 μg mL^{-1}		20 μg mL^{-1}		50 μg mL^{-1}	
Compound	R_1	R_2	R_3	Cells per mL[b]	Cells per mL[b]	PGI[c]	Cells per mL[b]	PGI[c]	Cells per mL[b]	PGI[c]
1	H	H	H	10.72	21.41	46	6.97	82	2.10	95
2	CH_3	H	H	10.75	26.20	34	14.98	62	9.65	76
3	NH_2	H	H	11.53	36.75	7	30.29	24	23.00	42
4	OH	H	H	12.62	40.63	0	38.36	3	20.80	47
5	F	H	H	10.99	21.95	45	8.29	79	8.85	78
6	CN	H	H	11.26	36.37	8	24.04	39	22.12	44
7	CH_2CH_3	H	H	10.09	21.04	47	9.49	76	5.94	85
8	OCH_3	H	H	10.97	25.54	36	12.09	69	9.59	76
9	Cl	H	H	10.97	18.05	54	6.66	83	15.00	62
10	$COCH_3$	H	H	11.01	34.25	14	25.97	34	6.72	83
11	$N(CH_3)_2$	H	H	10.19	38.13	4	36.70	7	35.51	10
12	OCH_2CH_3	H	H	10.52	26.90	32	12.83	68	13.39	66
13	NO_2	H	H	11.01	34.63	13	10.57	73	10.77	73
14	CF_3	H	H	11.22	13.92	65	5.53	86	6.99	82
15	Br	H	H	11.00	22.00	44	9.52	76	16.56	58
16	SO_2NH_2	H	H	10.08	37.80	5	36.17	9	34.83	12
17	I	H	H	10.21	18.15	54	7.61	81	15.88	60
18	Cl	H	Cl	10.48	23.15	42	11.12	72	4.45	89
19	Cl	Cl	H	10.66	19.43	51	7.46	81	9.86	75
20	NO_2	CF_3	H	9.87	18.10	54	7.06	82	7.10	82
BZN				10.33	22.03	44	13.47	66	9.08	77
Positive control				10.53	39.60	0	39.60	0	39.60	0

[a] Values corresponding to the average of triplicate (standard deviation less than 10% for all cases). [b] $\times 10^6$ mL^{-1}. [c] PGI (%) = $[(Ac_3 - Ap_3)/Ac_3] \times 100$, where $Ac_3 = A_{580}$ of the culture in the absence of the drug at day 3, and $Ap_3 = A_{580}$ of the culture containing the drug at day 3.

The σ constant, defined by Hammett, characterizes the electronic behavior of a substituent group. The absolute values of σ reflect the magnitude of the electronic effect, whether inductive or resonance, conveyed the substituent on the reaction center or on the physicochemical measure. Positive values of σ are observed for electron withdrawing substituent groups, while negative values are observed in electron-donating substituents.[12,13] The π constant, defined by Hansch–Fujita, has been widely used as a measure of the hydrophobic contribution of substituent groups, and measures the individual contribution of these groups to the partition coefficient of a molecule. The position of the substituent group has a major influence on the π constant's value and it is possible to verify different values for the same substituent group according to its position in the molecule. By definition, positive values of π are found for groups with lipophilic behavior and negative values for hydrophilic groups.[13,14]

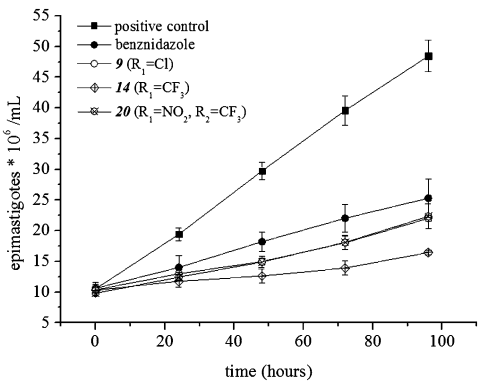

Fig. 2 Number of epimastigote forms per mL after exposure to the compounds 4-Cl (**9**), 4-CF_3 (**14**), 3-CF_3,4-NO_2 (**20**) and BZN. The values represent the mean of three experiments ± SD (standard deviation).

Eighteen derivatives have already been synthesized and identified in previous studies,[8,15] and 4-ethyl (**7**) and 4-ethoxy (**12**) substituted compounds were included in this investigation to complete the series. The synthesis of these two new compounds followed the synthetic path described in the literature.[15] The structure of these new compounds was confirmed by ^{1}H and ^{13}C NMR spectra (recorded on a Bruker ADPX Advanced spectrometer, 300 MHz employing DMSO-d_6 solutions with tetramethylsilane as internal standard), elemental analysis (performed on a Perkin-Elmer 24013 CHN Elemental Analyzer) and melting point (determined using Micro-Química MQAPF-301 apparatus). The identification data for both compounds are shown in the "Notes and references" section.[16]

In vitro anti-*T. cruzi* activity assays were performed against the epimastigote form of *T. cruzi* Y strain,‡ isolated by Silva and Nussenzweig in 1953,[17] cultured at 28 °C in liver infusion tryptose (LIT) medium, and supplemented with 10% heat-inactivated fetal calf serum. The Y strain is classified as biodeme type I, which consists of parasites of a slender shape with interstitial tissue tropism in the acute phase, and a slender shape with tropism for muscle tissue in the chronic phase. The parasites multiply rapidly, with a high parasitemia and parasitemic peak around 10 days, and mortality of the infected animals between 7 and 12 days.[18] Furthermore, the use of *T. cruzi* epimastigote forms is relevant in a preliminary evaluation of the antiproliferative effect of potential antichagasic agents, since those forms are present in mammalian cells.[19,20] Parasites in the logarithmic growth phase (from an initial culture with 1.0 × 10^7

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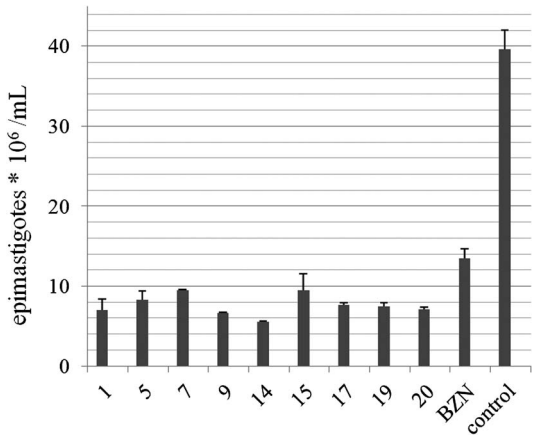

Fig. 3 Number of epimastigote forms per mL after 72 hours exposure to 20 μg mL^{-1} of compounds 4-H (**1**), 4-F (**5**), 4-CH_2CH_3 (**7**), 4-Cl (**9**), 4-CF_3 (**14**), 4-Br (**15**), 4-I (**17**), 3,4-Cl_2 (**19**), 3-CF_3,4-NO_2 (**20**) and BZN drug. The values represent the mean of three experiments ± SD.

epimastigotes per mL) were incubated with increasing concentrations of the test compounds **1–20** (10.0, 20.0 and 50.0 μg mL^{-1}) in DMSO (0.5 and 1.0% final concentration) for 24, 48, 72 and 96 hours.[21] Benznidazole (BZN) was used as control drug in the same concentrations and conditions as the tested compounds. No effect on epimastigotes' growth was observed in the presence of up to 1% (v/v) DMSO in the cultured media. Parasite viability and growth response were determined by absorbance readings every 24 h at 580 nm (A_{580}) in a spectrophotometer. All assays were carried out in triplicate. The activity was determined by calculating the percentage of growth inhibition (PGI) in the treated samples against the control in the absence of any drug. The PGI was calculated as follows: PGI (%) = $[(Ac_n - Ap_n)/Ac_n] \times 100$, where $Ac_n = A_{580}$ of the culture in the absence of the drug at day n ($n = 1, 2, 3$ or 4), and $Ap_n = A_{580}$ of the culture containing the drug at day n ($n = 1, 2, 3$ or 4).[22,23] The absorbance readings after 72 hours were considered as biological endpoints,[23] and Table 1 presents the results regarding the number of parasites per mL of the culture after 72 hours of compounds **1–20** and BZN exposure.

The complete profile for the control, compounds **1–20**, and BZN solutions, the findings regarding the number of parasites per mL at every reading time for each compound concentration, and, also, the PGI of the compounds are properly reported in the ESI†.

The compounds 4-Cl (**9**), 4-CF_3 (**14**), and 3-CF_3,4-NO_2 (**20**) showed the best activities at 10 μg mL^{-1} concentration. They were more active than BZN (see Fig. 2). After 72 hours, compound **14** inhibited around 65% of the parasite growth.

The compounds 4-NH_2 (**3**), 4-OH (**4**), 4-CN (**6**), 4-$N(CH_3)_2$ (**11**) and 4-SO_2NH_2 (**16**) did not show good activities, and after 72 hours no activity on the parasite growth was observed for compound **4**.

For 20 μg mL^{-1} concentration, the compounds substituted with hydrophobic ($\pi > 0$) and electron-withdrawing ($\sigma > 0$) groups, such as 4-F (**5**), 4-Cl (**9**), 4-CF_3 (**14**), 4-Br (**15**), 4-I (**17**), 3,4-Cl_2 (**19**) and 3-CF_3,4-NO_2 (**20**), eliminated the epimastigote forms inoculated at day 0 and decreased the parasite density over more than 10^7 epimastigotes per mL in cultures (see Fig. 3). The same profile was observed for compounds 4-H (**1**) and 4-CH_2CH_3 (**7**).

Compound **14** showed the best activity at 20 μg mL^{-1} concentration, which represents a reduction of 86% over the normal parasitary growth. Under these conditions, BZN inhibits the parasite growth around 66%; it was also observed that compounds 4-OH (**4**), 4-$N(CH_3)_2$ (**11**) and 4-SO_2NH_2 (**16**) showed minor activities.

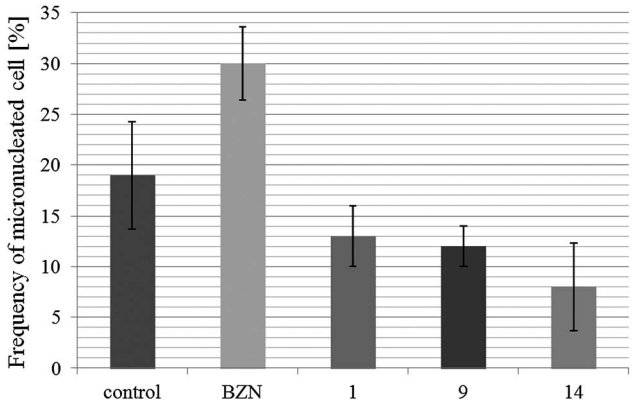

Fig. 5 Frequency of micronucleated HBEC incubated with benznidazole (BZN), compounds 4-H (**1**), 4-Cl (**9**), and 4-CF_3 (**14**), at 50 μg mL^{-1}. The values represent the mean of three experiments ± SD (standard deviation).

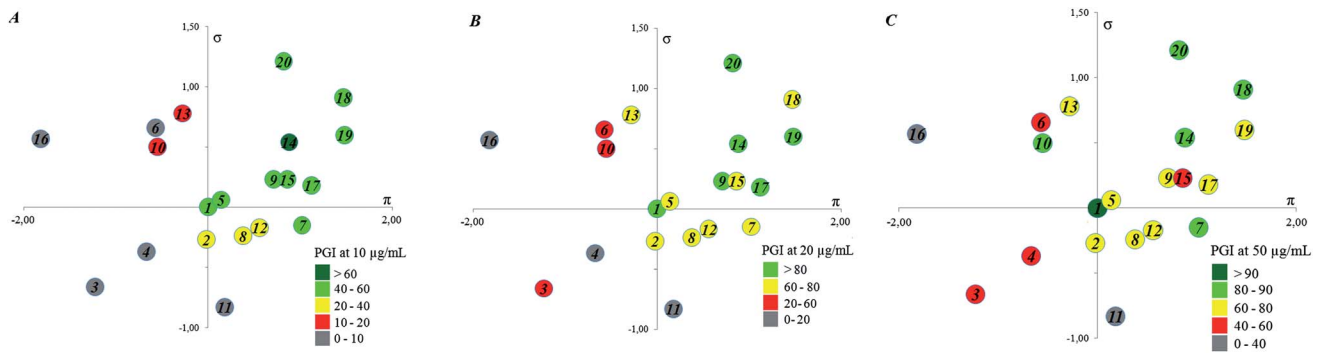

Fig. 4 The activity of the compounds after 72 hours according to the substituent groups selected employing the Craig diagram. (A) At 10 μg mL^{-1}; (B) at 20 μg mL^{-1}; (C) at 50 μg mL^{-1}. [Adapted with permission from P. N. Craig, Interdependence between physical parameters and selection of substituent groups for correlation studies, *J. Med. Chem.*, **14**, 680–684, Copyright (1971) American Chemical Society].

When the compounds were assayed at 50 μg mL^{-1} concentration, compound 4-H (**1**) demonstrated the best activity after 72 hours of exposure, reducing the concentration of epimastigote forms to 95% over the culture in the absence of any drug.

Regarding the plots of the PGI values on the Craig diagram (see Fig. 4), when compounds were tested at 10 μg mL^{-1} better activity values were found for those containing hydrophobic and electron-withdrawing groups. However, as soon as the compounds' concentration increases, the hydrophobicity becomes less important to activity, suggesting that there is a greater availability of hydrophilic compounds in the culture medium to be absorbed by the parasitic forms, which indicates the need for a lipo/hydrophilic balance in the design of new molecules.

The monosubstituted compounds with better activities at 10 μg mL^{-1}, 4-Cl (**9**), 4-CF_3 (**14**) at 50 μg mL^{-1} 4-H (**1**), and BZN were selected to investigate genotoxicity.[24] The materials and methods for this procedure are mentioned in the "Notes and references" section.[25] The frequency of micronucleus on Human Bronchial Epithelial Cells (HBEC) treated for 24 hours was analyzed (see Fig. 5), and BZN increased the number of micronucleated cells (30.0 ± 3.6) in comparison with the control (19.0 ± 5.2). This finding is correlated with genotoxic effects. Otherwise, the compounds 4-H (**1**), 4-Cl (**9**), and 4-CF_3 (**14**) did not significantly induce the increase of the number of micronucleated cells, and their frequency values were 13.0 (±3.0), 12.0 (±2.1) and 8.0 (±4.3), respectively.

These preliminary results pointed out the relevance of the investigated class of compounds. However, aiming at a better understanding of the influence of physicochemical properties on these derivatives' activity, new protocols to measure the biological data (IC_{50} values) are already being carried out, and will be reported soon. The IC_{50} values will be used as dependent variables in a quantitative structure–activity relationship (QSAR) approach, which can provide more helpful data for designing novel potential anti-*T. cruzi* analogues.

Conclusions

In this study, a set of twenty benzofuroxan derivatives were biologically assayed against *T. cruzi* in order to obtain a preliminary action profile for these compounds. The derivatives presented activity against epimastigote *T. cruzi* forms, except for the 4-$N(CH_3)_2$ (**11**) and 4-SO_2NH_2 (**16**) compounds at the three investigated concentrations. The findings indicated that the anti-*T. cruzi* activity is influenced by the physicochemical properties of the substituent group attached to the benzene ring. Dissimilar from BZN, the compounds 4-H (**1**), 4-Cl (**9**), and 4-CF_3 (**14**) did not show genotoxic effects. The results highlighted the importance of these benzofuroxan derivatives as potential leads for designing novel anti-*T. cruzi* drug candidates.

Acknowledgements

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16 *4-Ethyl-[N′-(benzofuroxan-5-yl)methylene]benzohydrazide(**7**)*: yellow solid (88%); mp 195.0–197.0 °C. ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 11.68 (s, 1H, H10), 8.50 (s, 1H, H8), 7.92 (d, 1H, J = 9.5 Hz, H6), 7.85 (d, 2H, J = 8.0 Hz, H13, H17), 7.75 (s, 1H, H4), 7.66 (d, 1H, J = 9.5 Hz, H7), 7.34 (d, 2H, J = 8.0 Hz, H14, H16), 2.69 (q, 2H, $J_{(Ha,Hb)}$ = 7.5 Hz, CH_2), 1.22 (t, 3H, $J_{(Hb,Ha)}$ = 7.5 Hz, CH_3); ^{13}C NMR {H} (DMSO-d_6, 75 MHz): δ (ppm) 164.5 (C11), 148.5 (C8), 144.8 (C15), 138.2 (C5), 132.5 (C12), 131.1 (C7a), 129.5 (C3a), 128.4 (C14, C16), 128.3 (C6), 128.1 (C13, C17), 116.5 (C7), 114.9 (C4), 28.5 (CH_2), 15.3 (CH_3). Anal. Calcd for ($C_{14}H_8C_{l2}N_4O_3$): C, 61.93%; H, 4.55%; N, 18.96%. Found: C, 60.53%; H, 4.30%; N, 18.00%. *4-Ethoxy-[N′-(benzofuroxan-5-yl)methylene]benzohydrazide(**12**)*: yellow solid (89%); mp 209.0–211.0 °C. ^{1}H NMR (DMSO-d_6, 300 MHz): δ (ppm) 11.80 (s, 1H, H10), 8.48 (s, 1H, H8), 7.92 (d, 1H, J = 9.4 Hz, H6), 7.89 (d, 2H, J = 8.6 Hz, H13, H17), 7.78 (s, 1H, H4), 7.68 (d, 1H, J = 9.5 Hz, H7), 7.02 (d, 2H, J = 8.6 Hz, H14, H16), 4.13 (q, 2H, $J_{(Ha,Hb)}$ = 6.9 Hz, CH_2), 1.35 (t, 3H, $J_{(Hb,Ha)}$ = 6.9 Hz, CH_3); ^{13}C NMR {H} (DMSO-d_6, 75 MHz): δ (ppm) 164.0 (C15), 162.0 (C11), 144.5 (C8), 138.2 (C5), 130.4 (C7a), 130.3 (C13, C17), 129.8 (C3a), 129.5 (C6), 125.6 (C12), 116.6 (C7), 114.8 (C4), 114.7 (C14, C16), 64.0 (CH_2), 14.9 (CH_3). Anal. Calcd for ($C_{14}H_8C_{l2}N_4O_3$): C, 58.89%; H, 4.32%; N, 17.17%. Found: C, 58.54%; H, 4.12%; N, 16.87%.
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25 Human Bronchial Epithelial Cells (HBEC) obtained from Oncology Department by Dra Maria Mitizi Brentani were cultured in RPMI-1640 medium, supplemented with 10% fetal bovine serum and 100 units per mL penicillin, 100 mg mL^{-1} streptomycin at 37 °C in a humidified atmosphere with 5% CO_2 (Gibco, Karlsruhe, Germany). 1×10^5 cells were plated in triplicate in 6-well plate one day before treatment. After 24 h of incubation the cells were treated with benznidazole, 4-H (**1**), 4-Cl (**9**) and 4-CF_3 (**14**) derivatives at 50 μg mL^{-1} concentration. After treatment, the cells were trypsinized, centrifuged and resuspended in 0.075 M KCl incubated for 5 min at 37 °C. After incubation, the cells were then fixed in methanol–glacial acetic acid (3 : 1), washed with methanol containing 1% acetic acid and resuspended in methanol containing 1% acetic acid. The cells were dropped over slides and the nuclei were stained by 40 μg mL^{-1} acridine orange (Nacalai Tesque). The frequency of micronucleus was observed by fluorescence microscopy (Carl Zeiss LSM 700; Leica, Mannheim, Germany). Quantification of micronuclei was recorded based on observation by counting at least 300 cells.

