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New ligand-based approach for the discovery of antitrypanosomal compounds

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Abstract—The antitrypanosomal activity of 10 already synthesized compounds was in silico predicted as well as in vitro and in vivo explored against *Trypanosoma cruzi*. For the computational study, an approach based on non-stochastic linear fingerprints to the identification of potential antichagasic compounds is introduced. Molecular structures of 66 organic compounds, 28 with antitrypanosomal activity and 38 having other clinical uses, were parameterized by means of the *TOMOCOMD-CARDD* software. A linear classification function was derived allowing the discrimination between active and inactive compounds with a confidence of 95%. As predicted, seven compounds showed antitrypanosomal activity (%AE > 70) against epimastigotic forms of *T. cruzi* at a concentration of 100 μg/mL. After an unspecific cytotoxic assay, three compounds were evaluated against amastigote forms of the parasite. An in vivo test was carried out for one of the studied compounds.

Annually, an estimate of 50,000 people in Central and South America die affected by American trypanosomiasis, while ca. 18 million are living infected by its causal parasite and about 100 million are at risk of infection in 21 countries.[1] The etiological agent, *Trypanosoma cruzi*, is transmitted to humans by blood-sucking insects, blood transfusions, and from mother to child during pregnancy.[1] Despite the progresses made in *T. cruzi* biochemistry and physiology, the chemotherapy of Chagas' disease (trypanosomiasis) remains deficient. An early medication during the acute stage of infection is usually effective; nevertheless, no medication has proven to be adequate in the chronic phase.[2,3]

On the other hand, effective therapy has not been consistent among distinct geographical areas, due to naturally resistant *T. cruzi* strains.[1] In addition as a serious drawback, Nifurtimox, and Benznidazole, the current antitrypanosomal drugs, present severe side effects such as peripheral neuropathy, anorexia, vomiting, allergy, and dermopathy, as well as considerable cardiac and renal toxicity.[2,3]

In this sense, it is clear that there is urgency in discovering new effective and less toxic antichagasic compounds. However, the great costs associated with the development of new drugs and the small economic size of the market for antiprotozoals make this development slow.[4]

As promising tools, powerful computational methods for *rational* drug design and lead-like dataset screening are now available as interesting alternatives to the expensive and time-consuming in vitro and in vivo tests.[5–7] In this way, one of our research groups has recently developed various molecular descriptors' families based on the algebraic theory.[8–10] A number of applications of these descriptors in the prediction of physical, physicochemical, chemical as well as pharmacokinetic properties of organic compounds have been reported in the literature.[9–14]

In the present study, linear non-stochastic fingerprints were used in the development of a new model based

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on Linear Discriminant Analysis for the prediction of antitrypanosomal activity. This kind of approach permits the rational identification of those candidates to be evaluated, which have the highest probabilities of being active ones. Following this idea, 10 already synthetized compounds were then in silico evaluated and, after that, in vitro assayed against epimastigote forms of *Trypanosoma cruzi*. Cytotoxic studies were also conducted, as selection criterion of compounds to be evaluated in further antiamastigote and in vivo assays.

The molecular structures of 88 compounds, 38 of them with antitrypanosomal activity[15–24] and the remaining ones (50 non-antitrypanosomal compounds) with other pharmacological uses (such as antivirals, sedatives/hypnotics, diuretics, anticonvulsivants, hemostatics, antihypertensives, antihelminthics, antibiotics, anticancer compounds, etc.),[25] were parameterized by means of the non-stochastic linear indices implemented in the *TOMOCOMD-CARDD* Software.[8] The mathematical basis and the methodological explanation about the use of this approach have been described in earlier publications [8,11–18] and Supplementary Data. In this report, the *k*th total linear indices [$^{k}f(x)$ and $^{k}f^{H}(x)$] and the *k*th local ones (atom-type = heteroatoms: S, N, O) [$^{k}f_{L}(x_E)$ and $^{k}f_{L}{}^{H}(x_E)$] without and with consideration of H-atoms, respectively, were computed using Pauling electronegativities[28] as atomic weights. After that, the selected series were split according to two *k*-means cluster analysis (*k*-MCAs)[26,27] into training and test groups (including 66 and 22 compounds, respectively).

Using the forward stepwise procedure for LDA (Linear Discriminant Analysis) implemented in the *STATISTICA* Software,[29] and taking into account the principle of parsimony (Occam's razor)[30] the following model was generated:

$$\begin{aligned} \textbf{\textit{Class}} = {} & -5.33 + 9.90 \times 10^{-4} {}^{7}f(x) \\ & - 5.27 \times 10^{-4} {}^{8}f(x) + 7.85 \times 10^{-5} {}^{9}f(x) \\ & + 3.74 \times 10^{-7} {}^{14L}f(x_E) - 1.13 \\ & \times 10^{-7} {}^{15L}f(x_E) - 1.15 \times 10^{-3} {}^{4}f^{H}(x) \end{aligned} \quad (1)$$

$$N = 66, \quad \lambda = 0.32, \quad D^2 = 8.27,$$

$$F(6, 59) = 20.48, \quad p < 0.0001.$$

The quality of the model was determined by examination of the corresponding statistical parameters (Wilks' λ, square Mahalanobis distance (D^2), Fisher ratio (F), and the significance level (p-value)). In addition, the recommended overall measures of accuracy were calculated for both training and test sets (Matthews correlation coefficient, sensitivity, specificity, false positive rate (also known as 'false alarm rate'), and sensitivity for the negative category) (see Table 1).[31]

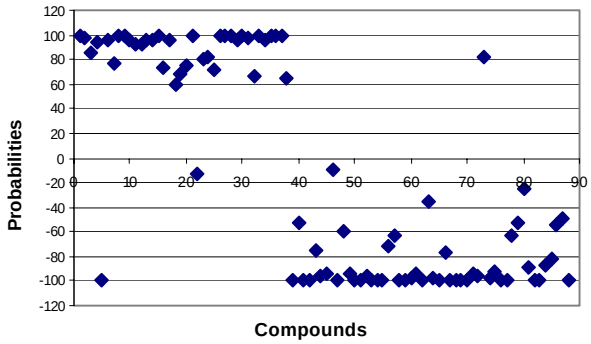

Figure 1. Plot of compounds in training and test sets and their probabilities to be active, computed using model **1** ($\Delta P\% = [P(\text{Active}) - P(\text{Inactive})] \times 100$; P(Active) and P(Inactive) are the posterior probabilities). Compounds **1**–**38** are active and compounds **39**–**88** inactive ones.

It is noticeable that the presence of bulky moieties plays a role in the manifestation of biological activity. The descriptors selected in model **1** include information of fragments of medium and large sizes (for total and local ones, respectively). However, a more detailed analysis of the structure–activity relationships should be conducted in further works considering a more extense sample. Therefore, the aim of the present report is only to show the potentialities of the non-stochastic linear indices to be used in antitrypanosomals design.

By using the discriminant functions, each compound is classified as either active if $\Delta P\% > 0$ or as inactive otherwise. These results are depicted in Figure 1. A correct selection of the training dataset can reduce the magnitude of the error range of the model. Following this idea, we built a training dataset choosing compounds with as much structural variability as possible. In certain cases, despite this precaution, the combination of some structural patterns can result in mathematical values closer to those obtained from the combination of structural fragments in molecules of the opposite group. In such a case, the model will not recognize the true class of the observation. As it can be seen in Table 2, only one inactive and two active compounds were misclassified using our approach.

As internal validation experiment, a LOO (leave one out) procedure was carried out. An overall mean of correct classification equal to 95.15% with standard deviation of 0.67 was obtained for the training group, indicating a good level of robustness and stability. In addition, in order to prove the ability of our algorithm to perform well on novel data from the same domain, the previously prepared external prediction set (22 compounds) was evaluated. The results of this validation experiment are shown in Table 3.

Table 1. Overall measures of accuracy obtained for the training and prediction sets using model 1

	Matthews corr. coefficient	Accuracy (%)	Sensitivity (hit rate %)	Specificity (%)	False alarm rate (%)	Predictive value (−) (%)
Training set	0.91	95.45	92.86	96.30	2.63	97.37
Test set	1.00	100.00	100.00	100.00	0.00	100.00

Table 2. Classification of compounds in the training set by use of the discriminant functions obtained by LDA

Compound	$\Delta P\%$[a]	Cl.
Training active group		
2,6-Dimethylphenoxy-ethyl thiocyanate	96	+
3,5-Dimethylphenoxy-ethyl thiocyanate	95	+
3-(3,4-Dichlorophenyl)-4-methyl-2-pyrazoline-1-thiocarboxamide	81	+
4-Phenoxyphenoxy-ethyl thiocyanate	100	+
2-(2-Thiocyanate-ethoxy)-9*H*-carbazole	100	+
9-Amino-1,2,3,4-tetrahydroacridine	86	+
Nifurtimox	−99	−
9-Aminoacridine	99	+
Mecaprine	98	+
6-Chlorotacrine	94	+
2-Chlorophenoxyethyl thiocyanate	96	+
2-Methylphenoxyethyl thiocyanate	93	+
3-(3-Trifluoromethylphenyl)-4-methyl-2-pyrazoline-1-thiocarboxamide	72	+
4-Bromomethyl-3-nitro benzoic acid	60	+
4-Methylphenoxyethyl thiocyanate	92	+
2-(*E*-[Prop-1-en-1-yl])-4-henoxyphenoxyethyl thiocyanate	100	+
Tubercidin	76	+
3-Deazoguanosine	96	+
3-Deazaguanine	73	+
Methyl 4-bromomethyl-3-nitro-benzoate	67	+
1-((5-Nitrofuran-2-yl)methyle-ne)-4-phenethylsemicarbazide	76	+
Formicin B	96	+
1-(1-(3-Bromophenyl) propylidene)thiosemicarbazide	−14	−
1-(1-(3,5-Bis(trifluoromethyl) phenyl)propylidene)thiosemicarbazide	80	+
2-Nitroneocryptolepine	100	+
2-Methoxyneocryptolepine	100	+
Neocryptolepine	100	+
3-benzyl-1-(2-(dimethylamino) ethyl)-6-(trifluoromethyl)-4,5-dihydro-4-(4-methoxyphenyl)-1*H*-benzo[*b*]azepin-2(3*H*)-one	99	+
Training inactive group		
3-Episiostatin B	−99	−
Thiacetazone	−53	−
Cloral betaine	−99	−
Vernelan	−99	−
Cetohexazine	−75	−
Carbavin	−95	−
Zonisamide	−94	−
Orotonsan Fe	−9	−
Ferrocholinate	−100	−
Ferrosi ascorbas	−59	−
Arecoline	−94	−
Butanolum	−100	−
Perchloroethane	−98	−
Spironolactone	−96	−
Glycerol	−100	−
Propamine"soviet	−100	−
Cystamine	−100	−
Adeturon	−71	−
Glisolamide	−63	−
Bromcholine	−100	−
Mebetide	−100	−
Minoxidil	−97	−
Thiouracil	−35	−
Pancuronium bromide	−100	−
Ganglefene hydrochloride	−94	−
Besunide	−100	−
Ascaridole	−100	−
Fentanyl	−77	−
Tenalidine tartrate	−99	−
Gentamicin A1	−100	−
Norgamem	−99	−
Furtrethonium iodide	−99	−
Isofenefrine	−94	−

Table 2 (*continued*)

Compound	$\Delta P\%$[a]	Cl.
Phenylethanolamine	−96	–
Cefalexin	83	+
Streptomycin	−98	–
Azirinomycin	−93	–
2-Hydroxypropyl trimethylammonium hydroxide	−100	–

[a] Results of classification obtained from Eq. 1. $\Delta P\% = P(\text{Active}) - P(\text{Inactive})] \times 100$.

Table 3. Classification of compounds in the test set by use of the discriminant function obtained by LDA

Compound	$\Delta P\%$[a]	Cl.
Test active group		
N-(5-(diethylamino) pentan-2-yl)-2-methoxy-acridin-9-amine	97	+
2-(6-Chloro-2-methoxy-acridin-9-ylthio)-*N*,*N*-diethylethanamine	99	+
Megazol	98	+
Benznidazole	67	+
2,4-Dichlorophenoxyethyl thiocyanate	99	+
2-Bromophenoxyethyl thiocyanate	96	+
4-(Phenylamino)phenoxyethyl thiocyanate	100	+
4-Phenylsulfinylphenoxyethyl thiocyanate	100	+
Brazilizone A	100	+
3-(3-Trifluoromethylphenyl)-2-pyrazoline-1-thiocarboxamide	66	+
Test inactive group		
Amantadine	−100	–
Nevanide	−63	–
Cinromide	−53	–
Adrenalone	−26	–
Canrenone	−89	–
Acetazolamide	−100	–
Hydrochlorothiazide	−100	–
Geroquinol	−87	–
Glyprothiazol	−82	–
Nicopholine	−54	–
Carbimazole	−49	–
Kanamycin A	−100	–

[a] Results of classification obtained from Eq. 1. $\Delta P\% = [P(\text{Active}) - P(\text{Inactive})] \times 100$.

Also with the aim of testing the potential of the present *TOMOCOMD-CARDD* approach for the detection of novel lead compounds, we predicted the biological activity of 10 compounds recently obtained by some of our research groups (Figure 2).[32,33] After applying the LDA-based QSAR model to this selected family, we proceeded to test the compounds in an epimastigote inhibition (in vitro) assay.[34,35] As Table 4 shows, the

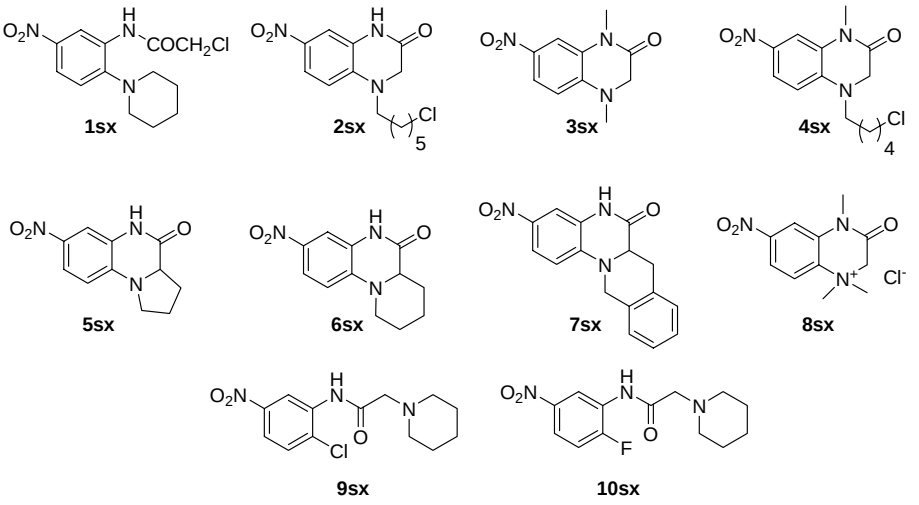

Figure 2. Chemical structures of the assayed compounds.

Table 4. Computational and biological results of 10 screened compounds

Compound	(ΔP%)[a]	Concentration		In vitro results		
		μg/mL	μM	%AE_{sd}[b]	%C_{sd}[c]	%AA_{sd}[d]
1sx	20	100	336.00	$84.0_{2.3}$	$24.9_{4.4}$	nd
		10	33.60	$34.8_{3.2}$	$6.3_{1.2}$	nd
		1	3.36	$7.1_{3.3}$	$8.1_{0.6}$	nd
2sx	2	100	158.12	$90.9_{2.3}$	$97.4_{2.1}$	nd
		10	15.81	$72.1_{4.1}$	$32.9_{1.4}$	$100_{0.6}$
		1	1.58	$23.2_{1.7}$	$16.6_{0.5}$	nd
3sx	73	100	452.06	$72.7_{3.3}$	$0.0_{7.8}$	$85.4_{2.3}$
		10	45.20	$21.6_{5.8}$	$0.0_{1.0}$	nd
		1	4.52	$22.5_{4.9}$	$0.0_{0.3}$	nd
4sx	15	100	158.38	$83.3_{2.5}$	$99.5_{0.1}$	nd
		10	15.84	$82.2_{1.2}$	$17.8_{1.9}$	nd
		1	1.58	$15.0_{3.7}$	$0.0_{1.8}$	nd
5sx	72	100	428.78	$90.5_{0.9}$	$23.6_{0.1}$	$30.2_{4.1}$
		10	42.88	$70.3_{0.5}$	$0.0_{3.6}$	$13.3_{1.2}$
		1	4.29	$25.6_{3.0}$	$0.0_{3.6}$	nd
6sx	64	100	404.45	$77.0_{1.2}$	$34.2_{0.4}$	nd
		10	40.44	$30.9_{1.0}$	$5.6_{0.2}$	nd
		1	4.04	$3.9_{1.9}$	$2.4_{1.8}$	nd
7sx	99	100	338.68	$72.1_{4.3}$	$50.4_{0.1}$	nd
		10	33.87	$29.4_{2.8}$	$0.0_{2.3}$	nd
		1	3.39	$19.4_{1.9}$	$0.0_{1.2}$	nd
8sx	63	100	423.28	$67.2_{0.4}$	$64.8_{3.3}$	nd
		10	42.32	$9.0_{0.5}$	$10.5_{2.0}$	nd
		1	4.23	$0.9_{0.8}$	$9.8_{1.2}$	nd
9sx	−17	100	164.37	$11.0_{3.3}$	nd	nd
		10	16.44	$1.3_{5.7}$	nd	nd
		1	1.64	$21.9_{0.6}$	nd	nd
10sx	−13	100	173.77	$14.3_{0.2}$	nd	nd
		10	17.38	$4.4_{0.5}$	nd	nd
		1	1.74	$0.0_{2.2}$	nd	nd
Nifurtimox		100	348.08	$98.7_{0.5}$	$25.9_{3.9}$	$100_{0.2}$
		10	34.80	$90.0_{1.8}$	$0.6_{3.9}$	$94.4_{0.4}$
		1	3.48	$75.5_{3.9}$	$0.0_{2.1}$	$88.1_{0.8}$
Benznidazole		100	384.24	nd	$1.4_{1.8}$	$96.6_{0.2}$
		10	38.42	nd	$1.6_{2.1}$	$9.2_{2.1}$
		1	3.84	nd	$1.9_{1.2}$	$83.2_{1.1}$

nd, not determined.

[a] Probabilities computed from model 1.

[b] Activity against epimastigote forms (% inhibition) and standard deviation (sd).

[c] Unspecific cytotoxicity (%) and standard deviation (sd).

[d] Activity against amastigote forms (% inhibition) and standard deviation (sd).

in vitro results were in agreement with the theoretical predictions. Three compounds (2sx, 4sx, and 5sx) showed more than 70% of epimastigote inhibition at a concentration of 10 μg/mL (15.8, 15.84, and 42.88 μM, respectively). Even though none of them resulted more active than Nifurtimox, the current results constitute a step forward in the search for efficient ways to discover new lead antitrypanosomals. After this preliminary in vitro test, the unspecific cytotoxicity was determined against macrophages at the concentrations that were used in the previous assay.[36,37] Nifurtimox and Benznidazole cytotoxicities were also determined. In a next step, compounds 2sx, 3sx, and 5sx were selected to carry out an amastigote susceptibility assay.[38] Compound 3sx, which showed the best rate ''activity against amastigote forms/unspecific cytotoxicity'', was evaluated in an in vivo test.[39] In this case, a suppressive activity of 60.7% on the peak of parasitemia was determined in 10 mice using a dose of 50 mg/kg/day. The activity of Nifurtimox was determined as reference (100% of suppressive activity). The mortality of animals was null in both cases. The experiments were carried out according to the European Council, published in the Guidelines 86/609ED, and controlled in Spain by Royal Decree 223/1988 of 14 March, on the protection of animals used for experimental and other scientific purposes.

Analyzing all these in vitro and in vivo results, it is clear that further refinement algorithms are needed to identify the ways in which the activity and the toxicity of the present chemical core can be optimized; however, the current results demonstrate the potentialities of the non-stochastic linear indices implemented in the *TOMOCOMD-CARDD* software to be used in the rational design of novel antichagastic drugs. At the same time this work presents a new family of organic compounds, as promising source of new lead antitrypanosomals. Research in this direction is now in progress.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2005.12.087.

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35. CL strain parasites (clone CL-B5) stably transfected with the *Escherichia coli* β-galactosidase gene (*LacZ*) were used. Epimastigotic forms were grown at 28 °C in liver infusion tryptose (LIT) broth with 10% fetal bovine serum (FBS), penicillin, and streptomycin. The screening assay was performed in 96-well microplates with culture that had not reached the stationary phase. Epimastigote forms, CL strain, were seeded at a concentration of 1×10^5 per mL in 200 μL. The plates were then incubated at 28 °C for 72 h with various concentrations of the drugs (100, 10, and 1 μg/mL), at which time 50 μL chlorophenol red-β-D-galactopyranoside (CPRG) solution was added to give a final concentration of 200 μM. The plates were incubated at 37 °C for 6 h and absorbances were then read at 595 nm. Each concentration was assayed in triplicate. In order to avoid drawback, medium, negative, and drug controls were used in each test. The inhibition percentage (%AE) was calculated as follows: $\%AE = [(AE - AEB)/(AC - ACB)] \times 100$, where AE = absorbance of experimental group; AEB = blank of compounds; AC = absorbance of control group; ACB = blank of culture medium. Stock solutions of the compounds to be assayed were prepared in dimethylsulfoxide, with the final concentration in a water/DMSO mixture never exceeding 0.2% of the later solvent.
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37. Murine J774 macrophages were grown in plastic 25 cm^2 flasks in Roswell Park Memorial Institute (RPMI)-1640 medium (Sigma) supplemented with 20% heat inactivated (30 min, 56 °C) fetal bovine serum (FBS) and 100 IU penicillin/mL + 100 μg/mL streptomycin, in a humidified 5% CO_2/95% air atmosphere at 37 °C and subpassaged once a week. J774 macrophages were seeded (70,000 cells/well) in 96-well flat-bottomed microplates (Nunc) with 200 μL of medium. The cells were allowed to attach for 24 h at 37 °C and then exposed to the compounds (dissolved in DMSO, maximal final concentration of solvent was 0.2%)for another 24 h. Afterwards, the cells were washed with phosphate-buffered saline (PBS) and incubated (37 °C) with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) 0.4 mg/mL for 60 min. MTT solution was removed and the cells were solubilized in DMSO (100 μL). The extent of reduction of MTT to formazan within cells was quantified by measurement of OD595. Each concentration was assayed three times and six cell growth controls were used in each test. The assays were performed in duplicate. Cytotoxicity percentages (%C) were determined as follows: $\%C = [1 - (OD_p - OD_{pm})/(OD_c - OD_m)] \times 100$, where OD_p represents the mean value of optical density at 595 nm (OD595) recorded for wells with macrophages containing different doses of product; OD_{pm} represents the mean OD595 value recorded for different concentrations of product in medium; OD_c represents the mean OD595 value recorded for wells with macrophages and no product (growth controls), and OD_m represents the mean OD595 value recorded for medium/control wells.
38. NCTC-929 (The National Collection of Type Cultures Clone 929) fibroblasts were established in 24-well tissue culture plates at a previously determined optimal concentration of 2.5×10^3 cells/well. NCTC-929-derived trypomastigotes were added to the monolayers at parasite:cell ratio of 1:8 and incubated for 24 h at 33 °C with 5% CO_2. The infected cells were then washed twice with PBS, so removing extracellular trypomastigotes. The drugs were added in triplicate, to give a final volume of 900 μL/well. The plates were incubated for 7 days at 33 °C. At this time, 100 μL chlorophenol red-β-D-galactopyranoside (CPRG; Roche, Indianapolis, Ind.) solution (final concentration of 400 μM) in 0.3% Triton X-100 (pH 7.4) was added. After 4 h of incubation at 37 °C, the colorimetric reaction was quantified as optical densities (OD) at 595 nm. The amastigote inhibition percentage (%AA) was calculated as follows: %AA = 100 − (OD experimental wells/OD control wells) × 100. Background controls (only NCTC-929 cells) were subtracted from all the values.
39. 10 female NMRI (Naval Medical Research Institute) mice, weighting 18–20 g, were inoculated intraperitoneally with a 2×10^4 trypomastigote Y strain of *T. cruzi* harvested from infected murine cardiac blood. After three days, tail blood was examined for the presence of parasites. Mice were treated, in the in vivo experiment three days postinfection, when positive parasitemia was microscopically detected. Only those mice with positive parasitemia were included in the experiments. All compounds were suspended in carboxymethylcellulose and each animal received 0.25 mL of drug suspension daily by gavage feeding. Mice received a dose of 50 mg/kg. One group of control mice was treated orally with 5 consecutive doses of nifurtimox (50 mg/kg). The groups were checked daily and tail blood level parasitemia was checked between days 7 and 22 postinfection (number of parasites per 10 microscope fields, 40× magnification) to give an indication of the level of infection. Infected mice with the same quantity of parasites were used as controls and received only the vehicle as treatment.