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Original article

Ligand-based discovery of novel trypanosomicidal drug-like compounds: *In silico* identification and experimental support

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ABSTRACT

Two-dimensional bond-based linear indices and linear discriminant analysis are used in this report to perform a quantitative structure–activity relationship study to identify new trypanosomicidal compounds. A database with 143 anti-trypanosomal and 297 compounds having other clinical uses, are utilized to develop the theoretical models. The best discriminant models computed using bond-based linear indices provides accuracies greater than 90 for both training and test sets. Our models identify as anti-trypanosomals five out of nine compounds of a set of already-synthesized substances. The *in vitro* anti-trypanosomal activity of this set against epimastigote forms of *Trypanosoma cruzi* is assayed. Both models show a perfect agreement between theoretical predictions and experimental results. The compounds identified as active ones show more than 98% of anti-epimastigote elimination (AE) at a concentration of 100 μg/mL. Besides, three compounds show more than 70% of AE at a concentration of 10 μg/mL. Finally, compounds with the best "activity against epimastigote forms/unspecific cytotoxicity" ratio are evaluated using an amastigote susceptibility assay. It should be noticed that, compound Va7-71 exhibit a 100% of intracellular amastigote elimination and shows similar activity when compared to a standard trypanosomicidal as nifurtimox. Finally, we can emphasize that, the present algorithm constitutes a step forward in the search for efficient ways of discovering new anti-trypanosomal compounds.

1. Introduction

Trypanosomiasis and leishmaniasis are parasitic diseases that cause severe infections in humans and domestic animals in the tropics. These infections pose a serious health problem for the countries in tropical regions, in terms of the suffering they inflict and the effects on their economies [1,2]. In particular, Chagas' disease (American Trypanosomiasis) caused by the protozoan parasite *Trypanosoma* (*Schyzotrypanum*) *cruzi*, is the largest parasitic disease to burden the American Continent. The morbidity and mortality associated with this disease in America are more than one order of magnitude higher than those caused by malaria, schistosomiasis, or leishmaniasis.

Chagas disease is one of the highest disease burden Neglected Tropical Disease's in Latin America and Caribbe (LAC) [3–7]. Almost all of the 8–9 millions cases of Chagas disease [3,4] (with approximately 50,000 new cases annually [4]) occur in poor rural zones

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and, increasingly, many new urban and peri-urban areas of Latin America. It is estimated that up to 5.4 million people will develop chronic Chagas heart disease [5,8], while 900,000 will develop megaesophagus and megacolon [8]. In LAC, the burden of disease caused by *T. cruzi* infection is between five and ten times greater than malaria [6].

At present, the chemotherapy of this parasitic infection remains undeveloped. The classical treatments are based on old and quite unspecific drugs that have significant activity in only the acute phase of the disease and, when associated with long-term treatments, give rise to severe side effects [9]. The current synthetic drugs such as nifurtimox(a nitrofuran derivative) and benznidazole (a nitroimidazole derivative), are associated to severe side effects, including psychosis, leucopenia, neurotoxicity, peripheral neuropathy lymphadenopathy, agranulocytosis, thrombocytopenic purpura, articular and muscular pain [10–12]. Their efficacy also varies according to geographical areas, mainly because of differences in drug susceptibility of different *Trypanosoma cruzi* strains [13,14]. Once the disease has progressed to later stages, no medication has consistently proven to be effective [15]. That said above showed the need to search new effective chemotherapeutic and chemoprophylactic agents against *T. cruzi*.

With the cost of new drug discovery exceeding $800 million per new chemical entity [16], novel therapeutics for endemic diseases in the third world would seem out of reach. If academia is to play a role in the discovery and development of drugs for socially imperative but financially challenging conditions, new development paradigms must evolve [17]. Since the cost of creating, maintaining, and screening large compound libraries is high, we have chosen as alternative the development of computational approaches based on discriminate functions. These allows the previous *in silico* identification from large chemical libraries of structural subsystems responsible for a given property or biological activity with a considerable reduction in costs, time and efforts in order to select a new drug candidate.

Moreover, a novel scheme for the rational *in silico* molecular design (or selection/identification of chemicals) and for QSAR/QSPR (Quantity Structure Activity/Property Relationship) studies has been introduced in recent years by our research team: the so-called **TO**pological **MO**lecular **COM**puter **D**esign–**C**omputer **A**ided **R**ational **D**rug **D**esign (**TOMOCOMD–CARDD**) [18–23]. This approach, which is based on principles of novel methods in chemical graph and algebraic theories, has been successfully used for the description of different physical, chemo-physical, and chemical properties of organic compounds [20,24,25], as well as to the prediction of, pharmacokinetical [26,27], biological [28–35] and toxicological [36] properties. In addition, this method has been applied to studies in the field of proteomics and nucleic acid–drug interactions [37,38]. Furthermore, these molecular descriptors (MDs) have been extended to consider three-dimensional (3D) features of small/medium-sized molecules based on the trigonometric-3D-chirality-correction factor approach [39–43].

In the present report, bond-based non-stochastic and stochastic linear indices are used to find classification models that allow the discrimination of anti-trypanosomal compounds. This present approach permits the rational identification of those candidates to be evaluated, which have the highest probabilities of being active ones. Therefore, nine already-synthesized compounds were then *in silico* evaluated and, after that, *in vitro* assayed against epimastigote forms of *T. cruzi*. Cytotoxic studies were also conducted, as a selection criterion of compounds for further anti-amastigote *in vivo* assays.

2. Results and discussion

2.1. Development and validation of the discriminant functions

The data set used in this study consists of 440 compounds of great structural variation, 143 actives against trypanosome and 297 with other uses. The general data set was randomly divided into two subsets, training and test set (which have 346 and 94 compounds, respectively), each other containing active and inactive compounds. In order to derive discriminant functions that permit the classification of chemicals as active (anti-trypanosomal) or inactive, we used the Linear discriminant analysis (LDA) [44] in which bond-based non-stochastic and stochastic linear indices were used as independent variables. The LDA was chosen as a statistical technique because of its simplicity and wide application in chemometric studies [22,27,29,32,45–47]. Two LDA-QSAR models were obtained through the LDA module implemented in STATISTICA 6.0 [48], classifying the compounds as either active or inactive.

The best discriminant functions obtained using bond-based non-stochastic and stochastic linear indices as molecular descriptors, together with their statistical parameters are given below, respectively:

$$\begin{aligned}\text{Class} = {} & -6.19 + 7.51 \times 10^{-1\text{p}} f_0^H(\bar{x}) - 2.07 \times 10^{-3p} f_{5L}^H(\bar{x}E) \\ & + 7.64 \times 10^{-2V} f_{0L}(\bar{x}E) + 5.90 \times 10^{-9\text{p}} f_{13L}^H(\bar{x}E) \\ & - 4.96 \times 10^{-4V} f_4^H(\bar{x}) + 3.19 \times 10^{-3K} f_4^H(\bar{x}) \\ & - 3.82 \times 10^{-1K} f_{0L}(\bar{x}E) \end{aligned} \quad (1)$$

$$N = 346 \quad \lambda = 0.40 \quad D^2 = 6.33 \quad F = 69.71 \quad p < 0.0001$$

$$\begin{aligned}\text{Class} = {} & -5.56 + 5.32 \times 10^{-2\text{Ms}} f_0^H(\bar{x}) - 6.49 \times 10^{-5\text{MS}} f_{6L}^{\text{H}}(\bar{x}E) \\ & - 2.16 \times 10^{-5\text{MS}} f_5^{\text{H}}(\bar{x}) + 6.20 \times 10^{-7\text{MS}} f_{9L}^{\text{H}}(\bar{x}E) \\ & + 2.25 \times 10^{-2\text{Ms}} f_{0L}(\bar{x}E) \end{aligned} \quad (2)$$

$$N = 346 \quad \lambda = 0.43 \quad D^2 = 5.61 \quad F = 87.02 \quad p < 0.0001$$

where N is the number of compounds, λ is the Wilks' statistic, D^2 is the square Mahalanobis distance and F is the Fisher ratio. The statistical analysis showed that there exists an appropriate discriminating power to distinguish between each other groups.

The equations appeared statistically significant at $p < 0.001$. The best non-stochastic model (Eq. (1)), which includes non-stochastic indices, has a good overall accuracy of 90.46% for the training set (See Table 1). In addition, this model showed an adequate Matthews' correlation coefficient (MCC) of 0.79; MCC quantifies the strength of the linear relation between the molecular descriptors and the classifications, and usually it may provide a much more balanced evaluation of the prediction than, for instance, the percentages (accuracy). Together with the accuracy, sensitivity, specificity, and false-positive rate (also known as 'false-alarm rate') are among the most commonly used

Table 1
Prediction performances for LDA-based QSAR models for training and test sets.

Models	Matthews Corr. Coefficient (*C*)	Accuracy 'Q_{Total}' (%)	Specificity (%)	Sensitivity 'hit rate' (%)
Training set				
Eq. 1	0.79	90.46	84.25	89.17
Eq. 2	0.82	91.33	84.09	92.50
Test set				
Eq. 1	0.83	92.55	76.67	100.00
Eq. 2	0.79	90.43	71.88	100.00

Table 2
Compounds evaluated in the present study, their classification (ΔP%) according to the obtained models, their anti-trypanosomal activity and cytotoxicity at three different concentrations (100, 10, and 1 μg/mL) and anti-trypanosomal activity of nifurtimox (reference).

Compound[a]	Exp.[b]	[c]ΔP Eq. 1	[d]ΔP Eq. 2	%AE (SD)[e]			%CI[f]			%AA (SD)[g]		
				100 μg/mL	10 μg/mL	1 μg/mL	100 μg/mL	10 μg/mL	1 μg/mL	100 μg/mL	10 μg/mL	1 μg/mL
Vax-12	I	−98.82	−86.28	26.2 ± 2.4	34.0 ± 2.0	27.2 ± 0.1	np	np	np	np	np	np
Vax-14	I	−84.41	−62.33	34.5 ± 0.3	30.1 ± 0.3	17.8 ± 0.1	np	np	np	np	np	np
Va7-34	I	−43.31	−6.73	70.5 ± 0.9	40.8 ± 3.1	21.0 ± 4.7	29.0 ± 0.8	7.4 ± 1	0 ± 3.9	np	np	np
Va7-35	I	−37.50	−1.17	47.5 ± 2.5	42.9 ± 0.6	27.7 ± 1.4	64.5 ± 1.8	32.3 ± 2.7	0 ± 3.8	np	np	np
Va7-37	A	73.03	86.39	100 ± 0.8	96.2 ± 0.9	43.8 ± 2.2	76.6 ± 3.8	75.8 ± 2.7	4.7 ± 5.2	np	np	np
Va7-38	A	60.57	74.32	100 ± 1.5	74.4 ± 1.0	28.1 ± 0.9	95.9 ± 0.1	48.5 ± 0.2	np	np	np	np
Va7-68	A	7.33	49.65	99.0 ± 0.9	69.1 ± 2.1	19.9 ± 5.1	96.8 ± 0.3	0 ± 0.9	np	np	39.33 ± 2.12	np
Va7-70	A	94.84	33.91	100 ± 1.3	51.2 ± 2.5	25.7 ± 1.5	80.7 ± 0.1	63.9 ± 1.3	np	np	np	np
Va7-71	A	82.71	86.79	100 ± 0.8	100 ± 1.1	65.5 ± 0	14.5 ± 1.2	0 ± 1.5	0 ± 0.6	100 ± 1.12	56.21 ± 3.25	35.39 ± 2.12
nifurtimox				98.7 ± 0.5	90.0 ± 1.8	75.5 ± 3.9	25.9 ± 3.9	0.6 ± 3.9	0.0 ± 2.1	100 ± 0.21	94.44 ± 0.42	88.14 ± 0.81

[a] The micromolar concentration (μM) of compounds at 100 μg/mL was the following: Vax-12 (335.9); Vax-14 (482.6); Va7-34 (352.5); Va7-35 (335.9); Va7-37 (289.2); Va7-38 (281.4); Va7-68 (335.9); Va7-70 (306.9); Va7-71 (277.9); nifurtimox(348.1).
[b] Observed activity. A: active and I: inactive.
[c] Results of the classification of compounds obtained from Model 1, ΔP% = [P(active) - P(inactive)] × 100.
[d] Results of the classification of compounds obtained from Model 2, ΔP% = [P(active) - P(inactive)] × 100.
[e] Anti-epimastigotes percentage and standard deviation (SD).
[f] Cytotocicity percentage.
[g] Anti-amastigotes percentage and standard deviation (SD) np: not performed.

parameters in medical statistics. While the sensitivity is the probability of correctly predicting a positive case, the specificity (also known as 'hit rate') is the probability of that a positive prediction is correct [49].

The non-stochastic model showed, for the training set, a good value of sensitivity of 89.17%, a specificity value of 84.25% and a false-positive rate of only 8.85% (Table 1). Nevertheless, the most important criterion, for the acceptance or not of a discriminant model, is based on statistics for the external prediction set. For the test set, the non-stochastic model showed an accuracy of 92.55%, MCC of 0.83, a good value of sensitivity of 100% and a specificity value of 76.67%.

On the other hand, the best stochastic model (Eq. (2)) presents a good overall accuracy of 91.33% with a good value of MCC of 0.82 for the training set. These values are slightly better than those obtained with the non-stochastic model. The achieved values for sensitivity and specificity were 92.50% and 84.09%, respectively, as well as a false-positive rate of only 9.86%. For the test set the results of the stochastic model were an accuracy of 90.43%, MCC of 0.79, sensitivity of 100%, and specificity of 71.88%; these values are acceptable, but lower than those obtained with bond-based non-stochastic linear indices. All these values are reported in Table 1. The results of the classification for compounds in both, training and test, sets achieved with Eqs. 1 and 2 can be seen in the Supporting Information (Tables S1-S4).

2.2. Biosilico identification of novel anti-trypanosomals and experimental corroboration

The entire algorithm, described in the sections above, was made up with the main objective to explore the possibilities of the current *in silico* approach for the identification of 'hits' (pro-lead compounds) from large databases. Therefore, an *in silico* screening of novel compounds was performed looking for the biological activity concerning this work. In order to carry this out, a pool of compounds never described in the literature as anti-trypanosomal agents was chosen. We evaluated nine compounds with the QSAR models developed in this work and, in order to corroborate the theoretical predictions of the previously synthesized chemicals [50] some *in vitro* assays were performed (for details of these assays, see Experimental Section). Moreover, we proceeded to test the compounds in an epimastigote susceptibility (*in vitro*) assay [51]. After this preliminary *in vitro* test, the unspecific cytotoxicity was determined against macrophages at the concentrations that were used in the previous assay [52,53]. Finally, the compounds that showed the best "activity against epimastigote forms/unspecific cytotoxicity" ratio were evaluated in an amastigote susceptibility assay as we described above.

The ΔP% values of the compounds in the data set using all the discriminant functions and the chemical structures are depicted in Table 2 and Fig. 1, respectively. Hence here we can see that it exits

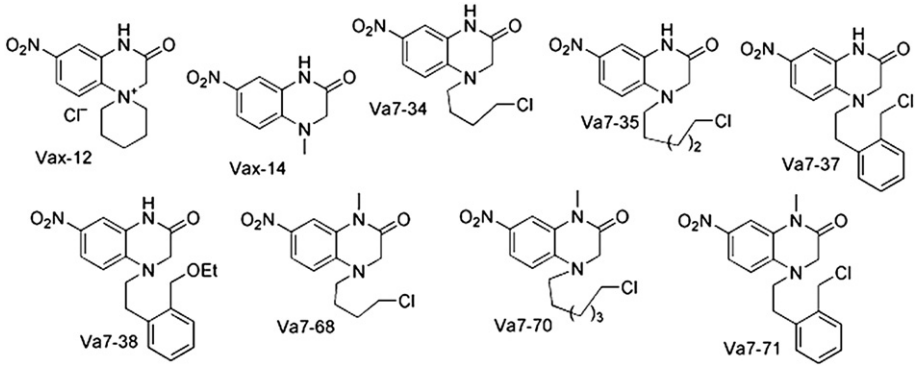

Fig. 1. Molecular structures of experimentally evaluated compounds.

a perfect agreement between the theoretical predictions and the experimental results for all the organic-chemicals. Compound Va7-34 was classified as inactive by the models, although its activity value (at 100 μg/mL) against epimastigote was rather close to the decision margin for this kind of assays. Furthermore, the four compounds (Vax-12, Vax-14, Va7-34 and Va7-35) previously classified by our models as inactive turned out to be experimentally inactive. On the other hand, the five other compounds (Va7-37, Va7-38, Va7-68, Va7-70 and Va7-71) of the data set were classified as active by our two models (Eqs. 1 and 2) showing more than 98% of anti-epimastigote elimination at a concentration of 100 μg/mL (see Table 2 for details). However, only the compound Va7-71 out of these five substances shows a low value (14.5%) of cytotoxicity in macrophages at a concentration of 100 μg/mL. Moreover, three compounds of this group showed more than 70% of anti-epimastigote activity at a concentration of 10 μg/mL (Va7-37, Va7-38, and Va7-71) and substance Va7-68 showed a value near to the cutoff value (69.1% of anti-epimastigote percentage). Compounds Va7-37 and Va7-38 showed high values of cytotoxicity (75.8% and 48.5%, respectively, at 10 μg/mL), while substance Va7-68 was practically no-cytotoxic at this concentration as well as compound Va7-71. It is remarkable that, compound Va7-71 showed similar potency than nifurtimoxat concentrations of 100 μg/mL and 10 μg/mL.

Finally, taking into account the results achieved in the epimastigote susceptibility assay and the unspecific cytotoxicity against macrophages, we select both compounds above to be evaluated an amastigote susceptibility assay. In this assay we only use the concentrations in which they showed the best "activity against epimastigote forms/unspecific cytotoxicity" ratio, for compound Va7-68 it was 10 μg/mL and for the compound Va7-71 the three concentrations (100, 10 and 1 μg/mL) were used. All these results can be seen in Table 2. However in this section we discuss briefly the result obtained with compound Va7-71. This compound shows a 100% of anti-amastigotes elimination at the highest concentration; notice that, in the anti-amastigote assay this compound exhibited similar activity when compared to nifurtimox, which was used as reference structure.

The result above is a rather promising starting point for the future design and optimization of new compounds with anti-trypanosomal activity. Moreover, compound Va7-71 presented similar potency and lesser toxicity in the *in vitro* assays than the reference drug nifurtimox. This outcome opens the door to a virtual study of this structural pattern in order to improve the search for drug-like compounds with anti-trypanosomal activity. Finally, it is important to remark that our aim in this study is to show how the models could be useful for potential drug discovery.

3. Conclusions

In conclusion, the introduction and use of graph-theoretical MDs are attractive and efficient for research in drug design. Herein we present a new set of molecular descriptors, namely bond-based non-stochastic and stochastic linear indices, implemented in the **TOMOCOMD–CARDD** software, and their application to discriminate between active compounds and inactive ones as anti-trypanosomal. This method permits a good prediction of the biological property under consideration, thus increasing the likelihood of an *in silico* discovery of new candidate lead compounds and minimizing the use of resources. Moreover, five out of nine new compounds, subjected to *in silico* screening, were identified with anti-trypanosomal activity. Later, several *in vitro* experiments were performed to corroborate the reliability of the classification functions developed in this report. Finally, we can say that, the present algorithm constitutes a step forward in the search for efficient ways of discovering new anti-trypanosomal compounds.

4. Experimental section

4.1. Data base selection

The general data set used in this study consists of 440 compounds of great structural variation, 143 of which have reported activity against trypanosome; the remaining are inactive. The anti-trypanosomals considered in this study are representative of families with diverse structural patterns and were collected from previous works [33,35,54–71]. The names of compounds in the database together with their experimental data taken from the literature are given in the Supporting Information (Tables S1 and S3). The molecular structures of these 143 anti-trypanosomal agents are listed in Figures S1 and S2 of the Supporting Information. Moreover, it is remarkable that the wide variability of drugs and mechanisms of action of active compounds in the training and prediction sets assures adequate extrapolation power and increases the possibility of the discovery of new lead compounds with novel mechanisms of action of anti-trypanosomal substances, one of the most critical aspects in the construction of non-congeneric data.

On the other hand, 297 compounds having different clinical uses such as antivirals, sedative/hypnotics, diuretics, anticonvulsants, hemostatics, oral hypoglycemics, anti-hypertensives, anti-helminthics and anticancer compounds as well as some other kinds of drugs were selected for the set of inactive compounds through random selection, guaranteeing great structural variability as well. All these compounds were taken from the Negwer Handbook [72] and Merck Index [73] in which their names, synonyms, and structural formulas can be found. The classification of these organic compounds as 'inactive' (non-anti-trypanosomal) does not guarantee that all are truly so; some of them may have trypanomicidal activity that is undetected. This limitation can be reflected in the results of classification for the series of inactive compounds [46].

4.2. Computational strategies

The theory of the bond-based linear indices used in this study was discussed in detail in previous research reports [22,74]. Specifically, the *CARDD* module implemented in the **TOMOCOMD** Software [18] was used in the calculation of bond-based non-stochastic and stochastic linear indices. In this study, the properties used to differentiate the molecular atoms are those previously proposed for the calculation of the DRAGON descriptors [75–77], i.e., atomic mass (M), atomic polarizability (P), atomic Mulliken electronegativity (K), van der Waals atomic volume (V), plus the atomic electronegativity in Pauling scale (G) [78].

The bond-based *linear indices* descriptors computed in this study were the following:

1) kth ($k = 15$) total non-stochastic bond-based linear indices, not considering and considering H-atoms in the molecular graph (G) [$\boldsymbol{f}_k(\overline{w})$ and $\boldsymbol{f}_k{}^{H}(\overline{w})$, respectively].
2) kth ($k = 15$) total stochastic bond-based linear indices, not considering and considering H-atoms in the molecular graph (G) [${}^{S}\boldsymbol{f}_k(\overline{w})$ and ${}^{S}\boldsymbol{f}_k{}^{H}(\overline{w})$, respectively].
3) kth ($k = 15$) bond-type local (group = heteroatoms: S, N, O) non-stochastic linear indices, not considering and considering H-atoms in the molecular graph (G) [$\boldsymbol{f}_{kL}(\overline{w}_{E})$ and $\boldsymbol{f}_{kL}{}^{H}(\overline{w}_{E})$, correspondingly]. These local descriptors are putative molecular charge, dipole moment, and H-bonding acceptors.
4) kth ($k = 15$) bond-type local (group = heteroatoms: S, N, O) stochastic linear indices, not considering and considering H-atoms in the molecular graph (G) [${}^{S}\boldsymbol{f}_{kL}(\overline{w}_{E})$ and ${}^{S}\boldsymbol{f}_{kL}{}^{H}(\overline{w}_{E})$, correspondingly]. These local descriptors are also putative molecular charge, dipole moment, and H-bonding acceptors.

4.3. Chemometric methods

LDA was performed with software package STATISTICA [48]. Forward stepwise was selected as the strategy for variable selection. The quality of the models was determined by examining Wilks' λ parameter (U-statistic), square Mahalanobis distance (D^2), Fisher ratio (F) and the corresponding p-level (p(F)) as well as the percentage in training and test sets of global good classification, Matthews' correlation coefficient (MCC), sensitivity, specificity, negative predictive value (sensitivity of the negative category) and false-positive rate (false alarm rate). Models with a proportion between the number of cases and variables in the equation lower than five were rejected. The statistical robustness and predictive power of the obtained model was assessed using an external prediction (test) set.

4.4. In vitro determination of trypanosomicidal activity and cytotoxicity

4.4.1. Parasites and culture procedure

The CL (clone) strain parasites (CL-B5) stably transfected with the *Escherichia coli* β-galactosidase gene (*LacZ*) were used for the assays. Epimastigotes were grown at 28 °C in liver infusion tryptone (LIT) broth with 10% foetal bovine serum (FBS), penicillin and streptomycin.

4.4.2. Epimastigotes susceptibility assay

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 concentration of 1×10^5 per mL in 200 μL. The plates were then incubated at 28 °C for 72 h with different concentrations of the drugs (100, 10, and 1 μg/mL), at which time 50 μL of CPRG (chlorophenol red-BETA-D-galactopyranoside) solution was added to give a final concentration of 200 μM. The plates were incubated at 37 °C for 6 h and were then spectrophotometrically read at 595 nm. Each concentration was assayed in triplicate. In order to avoid drawbacks, medium, negative and drug controls were used in each test. The anti-epimastigotes percentage (%AE) was calculated as follows: % AE = [(AE−AEB)/(AC−ACB)] × 100, where AE = absorbance of experimental group; AEB = blank of compounds; AC = absorbance of control group; ACB = blank of culture medium. The cutoff value for actives compounds was 70% of AE. Stock solutions of the compounds to be assayed were prepared in DMSO (dimethylsulfoxide), with the final concentration in a mixture water/DMSO never exceeding 0.2% of the latter solvent [51].

4.4.3. Cytotoxicity assays

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) 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. The procedure for cell viability measurement was evaluated with resazurin by a colorimetric method. The J774 macrophages were seeded (5×10^4 cells/well) in 96-well flat-bottom microplates with a volume of 100 μL of RPMI 1640 medium. The cells were allowed to attach for 24 h at 37 °C, 5% CO_2 and the medium was replaced by different concentrations of the drugs in 200 μL of medium, and exposed for another 24 h. Growth controls were also included. Afterwards, a volume 20 μL the 1 mM resazurin solution was added and plates were returned to incubator for another 3 h to evaluate cell viability. The reduction of resazurin was determined by dual-wavelength absorbance measurement at 490 nm and 595 nm. Background was subtracted. Each concentration was assayed in triplicate. Medium and drug controls were used in each test as blanks. Cytotoxicity percentages (%C) were determined as follows:

$$\%c = ((A_{570} \times 117,216 - A_{595} \times 80,586)\text{of test compounds}/(A_{570} \times 117,216 - A_{595} \times 80,586)\text{of untreated positive growth control}) \times 100$$

where A_{570} and A_{595} represent the means values of optical density at 570 and 595 nm, respectively, recorded for wells with macrophages containing different doses of compounds or value recorded for wells with macrophages and no compounds (positive growth controls), 80,586 and 117,216 represents molar extinction coefficients for oxidized resazurin at 570 and 595 nm, respectively.

4.4.4. Amastigotes susceptibility assay

The 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. The 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 solution, 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. After 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 density (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.

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Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejmech.2011.04.057.

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