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New carbohydrazide derivatives of 1*H*-pyrazolo[3,4-*b*]pyridine and trypanocidal activity

RAQUEL R.S. SALVADOR[1], MURILO L. BELLO[2], IGOR R.L. BARRETO[1], MARIA A.F. VERA[1], ESTELA M.F. MURI[1], SÉRGIO DE ALBUQUERQUE[3] and LUIZA R.S. DIAS[1]

[1]Laboratório de Química Medicinal/LQMed, Faculdade de Farmácia, Universidade Federal Fluminense/UFF, Rua Mário Viana, 523, 24241-000 Santa Rosa, Niterói, RJ, Brazil
[2]Laboratório de Modelagem Molecular & QSAR/ModMolQSAR, Faculdade de Farmácia, Universidade Federal do Rio de Janeiro/UFRJ/CCS, Av. Carlos Chagas Filho, 373, 21941-599 Rio de Janeiro, RJ, Brazil
[3]Departamento de Análises Clínicas, Toxicológicas e Bromatológicas, Universidade de São Paulo/USP, Faculdade de Ciências Farmacêuticas de Ribeirão Preto/FCFRP, Av. do Café, s/n, 14040-903 Ribeirão Preto, SP, Brazil

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ABSTRACT

This paper reports the *in vitro* trypanocidal activity evaluation of new carbohydrazide derivatives from 3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*b*]pyridine, substituted at C-6 position by phenyl, methyl or trifluoromethyl group. These compounds were evaluated in order to identify the antiparasitic profile against trypomastigote and amastigote forms of *Trypanosoma cruzi*. The 4-carbohydrazide derivatives presented different profiles of activity. In the investigation of the chemical structure influence in the trypanocidal activity, the results indicated there are large lipophilicity and volume differences among these derivatives. The complementarities of their stereoelectronic and physical-chemical aspects seem to be relevant for the biological activity against *T. cruzi*.

Key words: 1*H*-pyrazolo[3,4-*b*]pyridine, carbohydrazide derivatives, biological activities, trypanocidal activity, molecular modeling.

INTRODUCTION

Chagas disease is endemic in 21 countries in the Americas, but it is currently considered a global human parasitic infection due to the growing migration of populations from areas where these infections are highly endemic to settings where they are not endemic. The World Health Organization (WHO) estimates that there are about 7 million infected people globally, being a public health problem with serious social and economic impacts (Steverding 2014, Chatelain 2015, WHO 2016).

This disease is considered as a neglected tropical disease, which means that it is associated with poverty and neglected by socio-economic system and by policy makers (Schmunis and Yadon 2010, Nunes et al. 2013, Martins-Melo et al. 2014, Chatelain 2015). It is estimated an annual incidence of 28,000 cases in the region of the Americas and causes about 12 thousand deaths annually. Of these, approximately 6000 deaths occur in Brazil among more than 4 million infected, and about 70-90% of cases are detected in urban areas (PAHO-WHO

Correspondence to: Luiza Rosaria Sousa Dias
E-mail: lrsdias@id.uff.br

2014, WHO 2016). Although Chagas disease affects mostly the Latin American population, it has been increasingly detected outside of Latin America and three quarters of these cases are concentrated in the USA (Steverding 2014, Chatelain 2015).

The clinical manifestations result from the infective cycle. Blood trypomastigotes and intracellular amastigotes are the *T. cruzi* forms encountered in mammalian hosts. During the acute phase prevails bloodstream trypomastigotes. The parasite and the host can reach an immunological balance and the disease enters the chronic phase. In this phase, the parasitaemia is greatly reduced and the patients become asymptomatic but about one third of infected people will enter the symptomatic chronic stage of the disease associated with the manifestation of organ damage. High mortality rates have been recorded for the chronic phase due to Chagas' heart disease (de Souza et al. 2010, Hidron et al. 2010, Coura and Borges-Pereira 2012).

The only available drugs for the etiological treatment of Chagas disease, Nifurtimox and Benznidazole, are effective in curing 80% of acute cases and less than 20% of chronic cases, and cause severe side effects. Resistance and cross-resistance to both drugs have also been observed (Guedes et al. 2012, Coura and Borges-Pereira 2012). Within this framework, the demand for better drugs drives the interest in finding new chemical entities for chemotherapy of this disease.

Despite the large number of substances investigated against the *T. cruzi* parasite, since the introduction of the drugs used in Chagas disease therapy, only few drugs has reached the clinical screening. However, they were unable to complementing chagasic therapy (Wingeter et al. 2007, Hidron et al. 2010, Coura and Borges-Pereira 2012, Dias and Salvador 2012).

Previously we developed 4-carbohydrazide derivatives of 1*H*-pyrazolo[3,4-*b*]pyridine as new potent antichagasic agents. In order to investigate the effect of substituents at C-6 position on the interaction profile with the receptor site of the *T. cruzi*, the phenyl group investigated in previous works (Dias et al. 2007), was replaced by $-CH_3$ and $-CF_3$ groups in the new carbohydrazide derivatives (Figure 1).

MATERIALS AND METHODS

GENERAL EXPERIMENTAL PROCEDURE

The reactions were monitored by TLC performed on 2.0–6.0 cm aluminum sheets precoated with silica gel 60 (HF-254, Merck) to a thickness of 0.25 mm using the mixture of ethanol (5%) in chloroform as eluent and the developed chromatograms were viewed under ultraviolet light (254-365 mm). Melting points were determined on a Thomas Hoover PC03296 apparatus and are uncorrected. IR spectra were recorded on a Varian 660-IR, FT-IR spectrophotometer by using KBr pellet. NMR spectra were determined in $CDCl_3$ containing 1% TMS as an internal standard with a Varian VNMRS 500 MHz (500 MHz for ^{1}H and 125 MHz for ^{13}C) spectrometer. The identification of the compounds on ^{13}CNMR was aided by APT spectra. High-Resolution Mass Spectra (HRMS) were obtained using a Bruker Micro-Q-TOF instrument.

GENERAL PROCEDURE FOR CARBOHYDRAZIDE DERIVATIVES (**4a**– **4c**)

Hydrazine monohydrate (3 mL) was added to a solution of the carboxylate **1-3** (1.19 mmol) in methanol (10 mL). The reaction mixture was refluxed for 2-3 h under stirring. The solvent was removed by rota evaporation and poured into cold water to obtain a solid precipitated. It was filtered, washed with water, and dried to furnish the carbohydrazide derivatives.

3,6-dimethyl-1-phenyl-1*H*-pyrazolo[3,4-*b*] pyridine-4-carbohydrazide: light yellow solid, 90% yield, m.p. 185-186 °C. IR (cm^{-1}): 3277 (N-H); 1647 (C=O); 1507 (C=C).^{1}H NMR (500 MHz)

$CDCl_3$/TMS (δ-ppm): 8.21-23 (m, 2H); 7.48-51 (m, 2H); 7.26-30 (m, 1H) H-2' – H-6'; 7.03 (s, 1H, H-5); 4.20 (s, 1H, NH); 2.68 (s, 3H, H-9); 2.61 (s, 3H, H-8). ^{13}CNMR-APT (125MHz) DMSO-d_6/TMS (δ-ppm): 110.73 (C-3a); 138.37 (C-1'); 139.01; 141.99 (C-4); 150.75 (C-7a); 158.82(C-6); 164.75 (C=O); 115.79 (C-5); 120.41(C-2' and C-6'); 125.48 (C-4'); 129.00 (C-3' and C-5'); 14.12 (C-8); 24.51 (C-9). HRMS: 282.1358 (theoretical: 282.134937).

3-methyl-1-phenyl-6-trifluoromethyl-1*H*-pyrazolo[3,4-*b*]pyridine-4-carbohydrazide: yellow solid, 94% yield, m.p. 190 °C. IR (cm^{-1}): 2921.97 (N-H), 1675.81 (C=O), 1505.03 (C=C), 1138.28 (C-F_3). ^{1}H NMR (500 MHz) $CDCl_3$/TMS (δ-ppm): 8.82 (s, 1H, NH); 8.36 (s, 1H, H-5); 8.01-03 (m, 2H); 7.54-57 (m, 2H); 7.38-41 (m, 1H) H-2' – H-6'; 4.18 (s, 2H, NH_2); 2.75 (s, 3H, H-8). ^{13}CNMR-APT (125MHz) DMSO/TMS (δ-ppm): 163.58 (C=O); 150.07 (C-7a); 148.16 (C-3); 141.63(C-6); 138.25 (C-4); 133.24 (C-1'); 129.30 (C-3' and C-5'); 127.05 (C-4'); 123.43 ($\underline{C}F_3$); 122.09 (C-2' and C-6'); 113.39 (C-3a); 112.06 (C-5); 14.40 (CH_3). HRMS: 336.1068 (theoretical: 336.106671).

TRYPANOCIDAL ACTIVITY

The assays were evaluated against both trypomastigotes and intracellular amastigotes forms of *Trypanosoma cruzi*, clone B5 from CL Brener strain (Brener 1962).

The trypomastigotes forms were obtained from supernatant of previous infected cellular culture. Then, the number of parasites was adjusted to 10^6 forms/mL in RPMI medium, supplemented by antibiotics and fetal bovine serum, being distributed in a 96 well microplate. The compounds were added at concentrations of 0.5, 2.0, 8.0 and 32.0 μM and the microplate was incubated for 24 hours at 4 °C. All the compounds were evaluated in triplicate.

Thus, it was added 50 μL of chlorophenol red β-D-galactopyranoside (CPRG) solution (400 μM in 0.3% Triton X-100, pH 7.4) and the plates were incubated at 37 ºC for 6 h. The colorimetric reaction was quantified at 595 nm and the results expressed as percentage activity (% AA) (Buckner et al. 1996).

The assays against intracellular amastigotes forms were performed in $LLCMK_2$ cells. Previously, the cells were cultivated in 96 wells microplate (10^3 cells/well) in RPMI medium. After 2 hours, trypomastigotes forms, obtained from a previous cellular culture, were added in a 1:10 ratio and incubated for 4 hours. Then, the wells were washed with PBS (phosphate buffered saline) to remove the extracellular parasites and the tested compounds were added at the final concentrations of 0.5, 2.0, 8.0 and 32.0 μM. After this step, the microplate was incubated at 37 °C, in CO_2 atmosphere, for 72 hours.

After that, the process of quantification of the parasites number was realized as previously described for the trypomastigotes forms assay. We used RPMI culture medium with DMSO for negative control and Benznidazole drug in the same concentrations for positive control. The results were expressed as IC_{50}, calculated by linear regression method.

CYTOTOXICITY ASSAYS

$LLCMK_2$ cell cytotoxicity was performed in microplates of 96 wells using Trypan blue exclusion test. Cells were added to microplates (10^3 cell wells) and maintained in the CO_2 incubator at 37° C for 2 hours. The cytotoxicity profile of the derivatives was evaluated at 0.5, 2.0, 8.0 and 32.0 μM under the same conditions as above for 72 hours. Afterward the cells were removed by adding trypsin solution 0.25%. Trypan blue 1% solution was added to cellular system and cell viability was determined on an automatic counter of cells (CEDEX XS - Roche). 10% (wt/vol) Triton X solution was used as positive control and 0.6% DMSO solution was used as a negative control.

MOLECULAR MODELING

Molecular modeling was performed using SPARTAN´10 software (Wavefunction Inc. Irvine, CA, 2000). Three dimensional molecular structure of the carbohydrazide derivatives (**4a**, **4b** and **4c**) (non-ionized state according with the predominant molecular population in the physiological environment) and benznidazole (ionized state) were prepared and submitted to the calculations simulating the vacuum without any geometric constraint (Bello et al. 2011). Values of LogP and volume of the compounds were obtained using the ChemAxon software available at http://www.chemicalize.org, as well as the predominant molecular states of the compounds in the physiological environment (Funk and Krise 2012, Bello et al. 2015).

Conformational analysis for the selection of the lowest energy conformer was performed by the molecular mechanics method using force field MMFF (Merck Molecular Force Field) (Halgren 1996). Thereafter, conformers were subjected to geometry optimization using the semi-empirical method RM1 (Rocha et al. 2006). The conformer with the lowest energy was subjected to single point calculation by the quantum mechanics Hartree-Fock method, with 6-31G* basis set. Subsequently, in order to observe the stereoelectronics displacement caused by substituents at C-6 of 1*H*-pyrazolo[3,4-*b*] pyridine system, the Electrostatic Potential Maps (EPM) and dipole moment were calculated.

RESULTS AND DISCUSSION

Summing up, carbohydrazide compounds (**4a - 4c**) were synthesized by nucleophilic substitution reaction in the C=O group of the 4-carboxylate ester (**1-3**) (Dias et al. 2000, Volochnyuk et al. 2010) with unsubstituted hydrazine monohydrate, in yields exceeding 90% (Figure 1). The structural characterization and degrees of purity were analyzed by spectroscopic methods (IR, NMR and HRMS). Afterwards, these compounds were subjected to *in vitro* biological evaluation against clone CL Brener strain B5 of *T. cruzi* parasite (trypomastigote and amastigote forms), using the Benznidazole drug as positive control (Table I) (Brener 1962, Vega et al. 2005).

The 4-carbohydrazide derivatives of 1*H*-pyrazolo[3,4-*b*]pyridine (**4a-4c**) presented different profiles of activity (Table I). The compounds **4a** and **4b** showed some level of antiparasitic activity against trypomastigote form, but **4c** was inactive. Thus, **4a** and **4b** were testing against intracellular amastigote form. The evaluation of these compounds just showed the phenyl derivative (**4b**) as active against amastigote form (IC_{50}= 10.47 μM).

In order to evaluate the relation of the carbohydrazide moiety with the activity, the ester derivatives precursors (**1-3**) have been tested and were not active, which leads to deduct the requirement of this moiety for this biological profile.

The data shows no significant cytotoxicity of 4-carbohydrazide derivatives (**4a** and **4b**) when tested on LLC-MK_2 cells. No cytotoxicity was showed by **4a** derivative, while the **4b** derivative (CC_{50}= 12106 μM) showed to be 38 times less cytotoxic than benznidazole, used as control in this study (Table I).

The **4b** was more selective against the intracellular amastigotes (IC_{50}= 10.47 μM) than the mammalian cells LLC-MK_2 (CC_{50}= 12106 μM), and it showed 38 times less cytotoxic than benznidazole (Table I). It is generally considered that biological efficacy is not due to *in vitro* cytotoxicity. The low toxicity against mammalian cells is an important criterion in the search for active compounds with antiprotozoal activity (Duran-Rehbein et al. 2014).

Since the amastigote stage, *T. cruzi* can escape from the phagocytic vacuole of human monocytes, efficient drugs should be able to cross the phagosomol membrane and be taken up by amastigote (Ley et al. 1990, Touret et al. 2005). Then the lipophilicity of the compounds **4a** – **4c** was investigated.

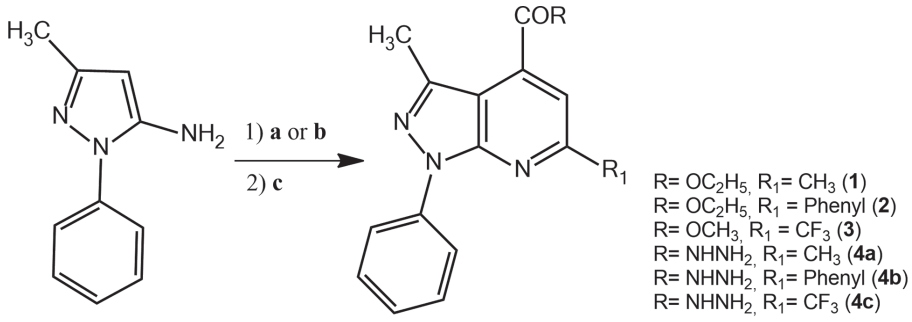

Figure 1 - Reagents and conditions: (**a**) Ethyl 2,4-dioxovalerate or ethyl-2,4-dioxobutanoate, reflux, furnished (**1-2**) in 90% yield; (**b**) Sequence of reactions involving: ethyl 4,4,4-trifluroacetoacetate; Dowtherm A; $POBr_3$; CuCN; 20% v/v NaOH; $SOCl_2$, MeOH, furnished (**3**) in 80 % yield; (**c**) $NH_2NH_2.H_2O$, MeOH, under reflux, furnished (**4a-4c**) in 90-94% yield.

TABLE I
The *in vitro* trypanocidal activity of the 4-carbohydrazide derivatives of 3-methyl-1-phenyl-6-substituted-1*H*-pyrazolo[3,4-*b*]pyridine and benznidazole drug.

Compound	Trypomastigote IC_{50} (μM)	Amastigote IC_{50} (μM)	$LLCMK_2$ cell CC50 (μM)	LogP	Dipole moment (D)	Volume ($Å^3$)
Benznidazole	73	3.90	312.9	1.64	4.61	219.16
4a (CH_3)	227.9	NA	not converged	1.04	3.57	247.27
4b (phenyl)	$2.27x10^3$	10.47	12106	2.95	3.63	301.17
4c (CF_3)	NA	ND	ND	2.18	2.55	262.17

NA = not active, ND = not determinate.

Lipophilicity, expressed as logP, has been recognized as molecular descriptor considered in studies of absorption, permeability, hydrophobic interactions with proteins and distribution of a molecule in the biological environment (Van der Waterbeemd et al. 1996, Eros et al. 2002). Replacing the phenyl group (**4b**) by methyl (**4a**) or trifluoromethyl group (**4c**) decreases lipophilicity and volume values of these molecules (Table I). This feature may have contributed to activity from **4b** against amastigote form. Thus, it is possible that a lipophilic character allows better penetration of the compound **4b** through the plasma membrane of the parasite (de Souza et al. 2010).

In order to gain some insight into the influence of the chemical structure in the trypanocidal activity we analyzed the stable conformation and the three-dimensional frontier orbital coefficient contribution from the carbohydrazide derivatives (**4a - 4c**). The computations were performed using SPARTAN´10 (Wavefunction Inc. Irvine, CA, 2000).

According with the conformational analysis and structural superposition of **4a-4c** derivatives, in the most stable conformation, they differ only in the shape of the C-6 substituent of 1*H*-pyrazolo[3,4-*b*]pyridine system. The similar backbone conformation is probably by virtue of their structural rigidity (Figure 2).

In addition to the shapes of compounds, the electronic properties also played influence on the biological activity of the carbohydrazide derivatives. The lack of trypanocidal activity of the **4c** was caused by the trifluoromethyl group insertion at C-6 of 1*H*-pyrazolo[3,4-*b*]pyridine system, since fluorine atom is an electron withdrawing group from systems containing aromatic ring (Swain and Lupton 1968). The EPM show the electron withdrawn from 1*H*-pyrazolo[3,4-*b*]

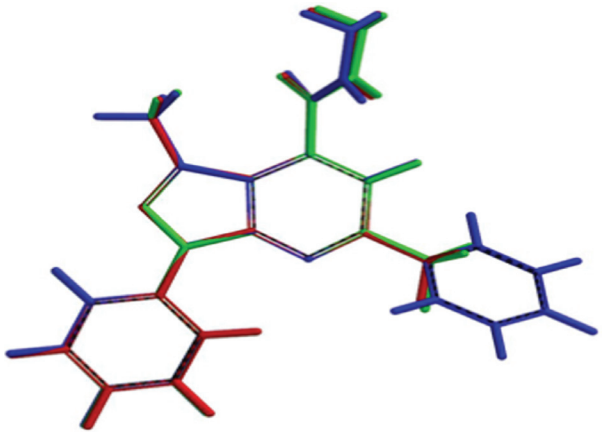

Figure 2 - The most stable conformations superposition. **4a** (red), **4b** (blue) and **4c** (green).

pyridine system caused by trifluoromethyl group in the compound **4c**, where this property shifted the electron density affecting the trypanocidal activity of the carbohydrazide moiety.

Electron density displacement was not observed in **4a** and **4b** compounds (Figure 3). In line with the electrons density displacement, the dipole moment shows the importance of maintaining a more polarized region in the compound to the trypanocidal activity.

Since the trypomastigotes are the infective form of the parasite and it will have to go through different stages in the parasite life cycle until being released into the bloodstream, the compound **4b** active on intracellular amastigotes proliferation form can provide information on the capacity of the drugs to target intracellular organisms.

Replacement at C-6 of 1*H*-pyrazolo[3,4-*b*] pyridine system by CF_3 or CH_3 group promoted significant stereoelectronic and lipophilic changes that affected the trypanocidal activity. These results suggest that changes of stereoelectronic and physical-chemical parameters may affect the trypanocidal activity profile implemented by carbohydrazide moiety as showed by derivatives of 1*H*-pyrazolo[3,4-*b*]pyridine (**4a** and **4b**) contributing to the planning of new active molecules for this target.

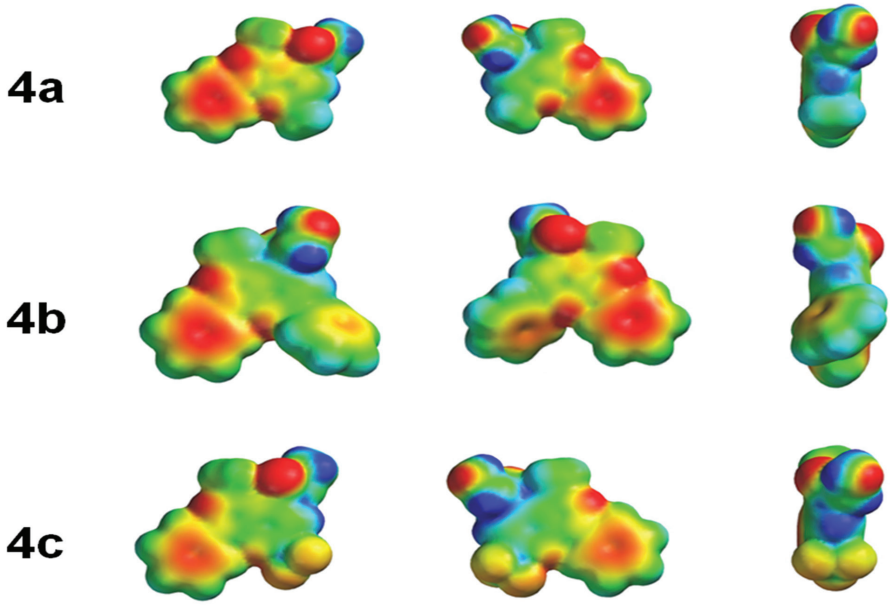

Figure 3 - Electrostatic Potential Maps (EPM) of the most stable conformations of the three carbohydrizide derivatives (**4a**, **4b** and **4c**), shown in different positions. The different values of electrostatic potential at the surface are represented by different colors ranging from deepest red (electronegative regions) to deepest blue (electropositive regions).

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REFERENCES

BELLO ML ET AL. 2011. Trimethoxy-chalcone derivatives inhibit growth of *Leishmania braziliensis*: synthesis, biological evaluation, molecular modeling and structure-activity relationship (SAR). Bioorg Med Chem 19: 5046-5052.

BELLO ML, JUNIOR AM, VIEIRA BA, DIAS LRS, SOUSA VP, CASTRO HC, RODRIGUES CR AND CABRAL LM. 2015. Sodium Montmorillonite/Amine-Containing Drugs Complexes: New Insights on Intercalated Drugs Arrangement into Layered Carrier Material. PLoS One 10(3): 1-20.

BRENER Z. 1962. Therapeutic activity and criterion of cure in mice experimentally infected with *Trypanosoma cruzi*. Rev Inst Med Trop S Paulo 4: 389-396.

BUCKNER FS, VERLINDE CL, LA FLAMME AC AND VAN VOORHIS WC. 1996. Efficient technique for screening drugs for activity against *Trypanosoma cruzi* using parasites expressing beta-galactosidase. Antimicrob Agents Chemother 40: 2592-2597.

CHATELAIN E. 2015. Chagas Disease Drug Discovery: Toward a New Era. J Biomol Screen 20: 22-35.

COURA JR AND BORGES-PEREIRA J. 2012. What is known and what should be improved: A systemic review. Rev Soc Bras Med Trop 45: 286-296.

DE SOUZA W, DE CARVALHO TMU AND BARRIAS ES. 2010. Review on *Trypanosoma cruzi*: Host Cell Interaction. Int J Cell Biol 2010: 1-14.

DIAS LRS, MCCHESNEY JD, FREITAS ACC AND BARREIRO EJ. 2000. Synthesis and biological activity of new potential antimalarial: pyrazolopyridine derivatives. Boll Chim Farmac 139: 14-20.

DIAS LRS AND SALVADOR RRS. 2012. Pyrazole carbohydrazide derivatives of pharmaceutical interest. Pharmaceuticals 5: 317-324.

DIAS LRS, SANTOS MB, DE ALBUQUERQUE S, CASTRO HC, DE SOUZA AMT, FREITAS ACC, DIVAIO MAV, CABRAL LM AND RODRIGUES CR. 2007. Synthesis, in vitro evaluation and SAR studies of a potencial antichagasic 1*H*-pyrazolo[3,4-*b*]pyridine series. Bioorg Med Chem 15: 211-219.

DURAN-REHBEIN GA, VARGAS-ZAMBRANO JC, CUÉLLAR A, PUERTA CJ AND GONZALEZ JM. 2014. Mammalian cellular culture models of *Trypanosoma cruzi* infection: a review of the published literature. Parasite 21(38): 1-9.

EROS D, KÖVESDI I, ORFI L, TAKÁCS-NOVÁK K, ACSÁDY G AND KÉRI G. 2002. Reliability of LogP Predictions Based on Calculated Molecular Descriptors: A Critical Review. Curr Med Chem 9: 1819-1829.

FUNK RS AND KRISE JP. 2012. Cationic Amphiphilic Drugs Cause a Marked Expansion of Apparent Lysosomal Volume: Implications for an Intracellular Distribution-Based Drug Interaction. Mol Pharmaceutics 9: 1384-1395.

HALGREN TA. 1996. Merck Molecular Force Field I. Basis, Form, Scope, Parameterization, and Performance of MMFF94. J Comp Chem 17: 490-519.

HIDRON A, VOGENTHALER N, SANTOS-PRECIADO J I, RODRIGUEZ-MORALES AJ, FRANCO-PAREDES C AND RASSI JR A. 2010. Cardiac Involvement with Parasitic Infections. Clin Microbiol Rev 23(2): 324-349.

LEY V, ROBBINS ES, NUSSENZWEIG V AND ANDREWS NW 1990. The exit of *Trypanosoma cruzi* from the phagosome is inhibited by raising the pH of acidic compartments. J Exp Med 171(2): 401-413.

MARTINS-MELO FR, RAMOS JR AN, ALENCAR CH AND HEUKELBACH J. 2014. Prevalence of Chagas disease in Brazil: A systematic review and meta-analysis. Acta Trop 130: 167-174.

NUNES MCP, DONES W, MORILLO CA, ENCINA JJ AND RIBEIRO AL. 2013. An Overview of Clinical and Epidemiological Aspects. J Am Coll Cardiol 2: 767-776.

PAHO/WHO - PAN AMERICAN HEALTH ORGANIZATION / WORLD HEALTH ORGANIZATION. 2014. General Information - Chagas Disease (updated April 2014). (http://www.paho.org/hq/index.php?option=com_content&view=article&id=5856&Itemid=41506&lang=en (accessed August 04, 2016)

ROCHA GB, FREIRE RO, SIMAS AM AND STEWART JJP. 2006. RM1: A Reparameterization of AM1 for H, C, N, O, P, S, F, Cl, Br and I. J Comp Chem 27: 1101-1111.

SCHMUNIS GA AND YADON ZE. 2010. Chagas disease: a Latin American health problem becoming a world health problem. Acta Trop 115: 14-21.

STEVERDING D. 2014. The development of drugs for treatment of sleeping sickness: a historical review. Parasit Vectors 7: 317.

SWAIN CG AND LUPTON EC. 1968. Field and Resonance Components of Substituent Effects. J Am Chem Soc 90(16): 4328-4337.

TOURET N, PAROUTIS P AND GRINSTEIN S. 2005. The nature of the phagosomal membrane: endoplasmic reticulum versus plasmalemma. J Leukoc Biol 77: 878-885

VAN DER WATERBEEMD H, CAMENISCH G, FOLKERS G AND RAEVSKY OA. 1996. Estimation of Caco-2 Cell Permeability using Calculated Molecular Descriptors. Quant Struct Act Relat 15: 480-490.

VEGA C, ROLÓN M, MARTÍNEZ-FERNÁNDEZ AR, ESCARIO JA AND GÓMEZ-BARRIO A. 2005. A new pharmacological screening assay with *Trypanosoma cruzi* epimastigotes expressing ß-galactosidase. Parasitol Res 95: 296-298.

VOLOCHNYUK DM, RYABUKHIN SV, PLASKON AS, DMYTRIV YV, GRYGORENKO OO, MYKHAILIUK PK, KROTKO DG, PUSHECHNIKOV A AND TOLMACHEV AA. 2010. Approach to the library of fused pyridine-4-carboxylic acid by combes-type reaction of acyl pyruvates and electron-rich amino heterocycles. J Comb Chem 12: 510-517.

WHO - WORLD HEALTH ORGANIZATION. 2016. Chagas disease (American trypanosomiasis) fact sheet (updated March 2016) http://www.who.int/mediacentre/factsheets/fs340/en/ (accessed August 04, 2016).

WINGETER MA, GUILHERMETTI E, SHINOBU CS, TAKAKI I AND SVIDZINSKI TIE. 2007. Identificação microbiológica e sensibilidade *in vitro* de Candida isoladas da cavidade oral de indivíduos HIV positivos. Rev Soc Bras Med Trop 40: 272-276.