Design, synthesis and in vitro trypanocidal and leishmanicidal activities of novel semicarbazone derivatives

ARTICLE *in* EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY · JUNE 2015

Impact Factor: 3.45 · DOI: 10.1016/j.ejmech.2015.05.046 · Source: PubMed

10 AUTHORS, INCLUDING:

Hugo Cerecetto
University of the Republic, Uruguay
235 PUBLICATIONS **4,116** CITATIONS
SEE PROFILE

Mercedes Gonzalez
University of the Republic, Uruguay
234 PUBLICATIONS **4,104** CITATIONS
SEE PROFILE

Antonio Carlos Doriguetto
Universidade Federal de Alfenas
118 PUBLICATIONS **870** CITATIONS
SEE PROFILE

Eliezer Barreiro
Federal University of Rio de Janeiro
445 PUBLICATIONS **5,342** CITATIONS
SEE PROFILE

European Journal of Medicinal Chemistry 100 (2015) 24–33

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Research paper

Design, synthesis and *in vitro* trypanocidal and leishmanicidal activities of novel semicarbazone derivatives

Marina A. Alves [a, b], Aline C. de Queiroz [a, c], Magna Suzana Alexandre-Moreira [a, c], Javier Varela [d], Hugo Cerecetto [d], Mercedes González [d], Antonio C. Doriguetto [a, e], Iara M. Landre [a, e], Eliezer J. Barreiro [a, b], Lídia M. Lima [a, b, *]

[a] Instituto Nacional de Ciência e Tecnologia de Fármacos e Medicamentos, Universidade Federal do Rio de Janeiro, Laboratório de Avaliação e Síntese de Substâncias Bioativas, CCS, Cidade Universitária, P.O. Box 68024, 21941-971, Rio de Janeiro, RJ, Brazil[1,2]
[b] Programa de Pós-Graduação em Química, Instituto de Química, Universidade Federal do Rio de Janeiro, 21941-902, Rio de Janeiro, RJ, Brazil
[c] LaFI – Laboratório de Farmacologia e Imunidade, Instituto de Ciências Biológicas e da Saúde, Universidade Federal de Alagoas, Maceió, AL, Brazil
[d] Grupo de Química Medicinal, Laboratorio de Química Orgánica, Facultad de Ciencias, Universidad de la República, Iguá 4225, Montevideo, 11400, Uruguay
[e] Laboratório de Cristalografia, Instituto de Química, Universidade Federal de Alfenas, 37130-000, Alfenas, MG, Brazil

ARTICLE INFO

Article history:
Received 13 November 2014
Received in revised form
28 May 2015
Accepted 30 May 2015
Available online 2 June 2015

Keywords:
Neglected diseases
Trypanosomiasis
Leishmaniasis
Protease
Semicarbazone
Peptide mimetic

ABSTRACT

Trypanosomatids are protozoan parasites that cause various diseases in human, such as leishmaniasis, Chagas disease and sleeping sickness. The highly syntenic genomes of the trypanosomatid species lead the assumption that they can encode similar proteins, indicating the possibility to design new anti-trypanosomatid drugs with dual trypanosomicidal and leishmanicidal activities. In this work a series of compounds (**6a**–**h** and **7a**–**h**), containing a semicarbazone scaffold as a peptide mimetic framework, was designed and synthesized. From this series compound **7g** (LASSBio-1483) highlighted, showing dual *in vitro* trypanosomicidal and leishmanicidal activities, with potency similar to the standard drugs nifurtimox and pentamidine. This data, taken together with its good *in silico* druglikeness profile and its great chemical and plasma stability, make LASSBio-1483 (**7g**) a new antitrypanosomatid lead-candidate.

1. Introduction

Neglected diseases (DN) represent a set of parasitic illnesses that primarily affect poor people in developing countries. Those caused by Trypanosomatidae protozoans include Chagas disease and sleeping sickness, produced by *Trypanosoma* species, and leishmaniasis, caused by different species belonging to the genus *Leishmania* [1]. Affording to World Health Organization (WHO), trypanosomiasis and leishmaniasis are the most challenging among the neglected tropical diseases [2]. A comparative genomics of trypanosomatid parasitic protozoa revealed a conserved core proteome of about 6200 genes among *Leishmania major*, *Trypanosoma cruzi*, and *Trypanosoma brucei* [3]. The highly syntenic genomes of the trypanosomatid species lead the assumption that they can encode similar proteins and drugs designed against conserved core processes should have the advantage of being potentially useful against all three protozoa [4–7]. Among the possible drug targets in trypanosomatids, the peptidases or proteases have concerned attention due their many roles in highly specific functions to the parasites' life cycles [8–10]. Considering the ability of these enzymes to catalyze the hydrolysis of peptide bonds [11–13] compounds containing amide or amide-mimetic frameworks can be designed as proteolytic inhibitors with antitrypanosomatid activity, as exemplified by compounds **1**–**4** (Chart 1) [13–16]. In similar manner, the *ortho*-hydroxyphenyl group linked to the imine subunit of a hydrazone functional group is believed to be an interesting scaffold for cysteine proteases inhibition. This statement is based on a theoretical proposed mechanism involving the nucleophilic attack of sulfhydryl group of a cysteine-protease on a reactive *ortho*-quinonemethyde intermediate, generated from the

* Corresponding author. Universidade Federal do Rio de Janeiro, Centro de Ciências da Saúde, Bloco B, Sala 14, P.O. Box 68024, Rio de Janeiro, RJ, 21941-971, Brazil.
E-mail addresses: lidia@lassbio.icb.ufrj.br, lmlima23@gmail.com (L.M. Lima).
[1] INCT-INOFAR; http://www.inct-inofar.ccs.ufrj.br/.
[2] LASSBio®, http://www.farmacia.ufrj.br/lassbio/.

http://dx.doi.org/10.1016/j.ejmech.2015.05.046
0223-5234/

tautomeric equilibrium of *ortho*-hydroxyarylaldehydehydrazone moiety (*e.g.* compound **5,** Chart 2) [17].

In order to design new peptide mimetic derivatives enclosing frameworks able to be recognized by trypanosomatids proteases, a series of semicarbazone derivatives (**6a**–**h** and **7a**–**h**) were planned by molecular modification on prototype **5** (LASSBio-1022) [18]. These modification were based on ring replacement between quinoxaline nucleus and 1,3-benzodioxole system (**a**, Chart 2); molecular simplification represented by elimination of methyl group (**b**, Chart 2); followed by aza-homologation strategy (**c**, Chart 2), converting the *N*-acylhydrazone subunit in a semicarbazone framework. The congeners series was further designed by classical isosterism replacement on 2-hydroxyphenyl subunit, varying the electronic nature of the monovalent group (**a-f**) and by isosteric ring replacement of phenyl group by a substituted furan system (**g**) and its phenylogous analogue (**h**) (Chart 2) [19]. In this paper we described the synthesis of the designed compounds **6a**–**h** and **7a**–**h** and their trypanosomicidal and leishmanicidal activities.

2. Results and discussion

2.1. Chemistry

Compounds **6a**–**h** and **7a**–**h** were synthesized in three linear steps from the amines **8** and **9**, obtained commercially (Scheme 1). In the first step the amines were condensed with phenyl chloroformate in chloroform at room temperature in order to furnish the carbamates **10** and **11** [20,21]. These compounds were treated with hydrazine monohydrate in ethanol to provide the semicarbazide derivatives **12** and **13** [22]. These key-intermediates were finally condensed with appropriated aldehydes [23], selected based on the design concept depicted in Chart 2, to obtain the semicarbazones **6a**–**h** and **7a**–**h** in good overall yields (Scheme 1). The chemical structure of the compounds **6a**–**h** and **7a**–**h** was elucidated by ^{1}H and ^{13}C NMR, IR and mass spectrometry. The analysis of the ^{1}H and ^{13}C NMR spectra of these compounds revealed the presence of only one signal relative to the hydrogen and carbon of imine double bond (N=CH), suggesting that all compounds were synthesized as a single diastereoisomer. The unequivocal characterization of the relative configuration of imine double bond (*E* or *Z*) was performed using X-ray diffraction study. However, considering the difficulty of getting compounds **6a**–**h** and **7a**–**h** in crystalline form, only derivative **7g** (LASSBio-1483), obtained as crystal solid, was used in X-ray experiment. As shown in Fig. 1, this experiment revealed that compound **7g** was obtained as diastereoisomer *E*. Based on these data and considering the similarity in chemical shifts of imine hydrogen in ^{1}H NMR spectra of compounds **6a**–**h** and **7a**–**h**, is reasonable to propose that all semicarbazone derivatives (**6a**–**h** and **7a**–**h**) were obtained with the same stereochemistry (N=CH; configuration *E*).

2.2. X-ray diffraction analyses

Fig. 1 is a structure representation of **7g** crystallized in the $P2_1/c$ space group**.** Table 1 present its main crystallographic data. The geometric features were studied with the software MOGUL [24] and this analysis showed that all bond lengths and angles were in agreement with the expected statistical values when compared with similar fragments of structures deposited in Cambridge Structural Database (CSD) [25]. The least–square plane through the non-hydrogen atoms of the 5-nitro-2-furaldehyde semicarbazone moiety shows a high planarity (r.m.s = 0.0358). This molecular moiety forms an angle of 10.78(6)^{o} with that one through the benzoic ring (r.m.s = 0.0281).

Information about intermolecular geometry of **7g** and the details of all hydrogen bond contacts involved in its networks can be found in the Supplementary Material (Fig. 29 and Table 1S)

2.3. Cytotoxic studies

Before starting the evaluation of the trypanosomicidal and leishmanicidal activities of semicarbazones **6a**–**h** and **7a**–**h**, the eventual cytotoxic profile of these compounds against mammalian cells was investigated by MTT assay [26]. In this study murine macrophages cell line J774.A1 was treated with compounds **6a**–**h** and **7a**–**h** at serial concentrations (0.1–100 μM) and the half maximal inhibitory concentration (IC_{50}) was determined as illustrated in Table 2. Only compounds **7b** and **7e** showed cytotoxic activity to mammalian cell with IC_{50} = 71.2 and 35.7 μM, respectively (Table 2).

2.4. Trypanosomicidal activity

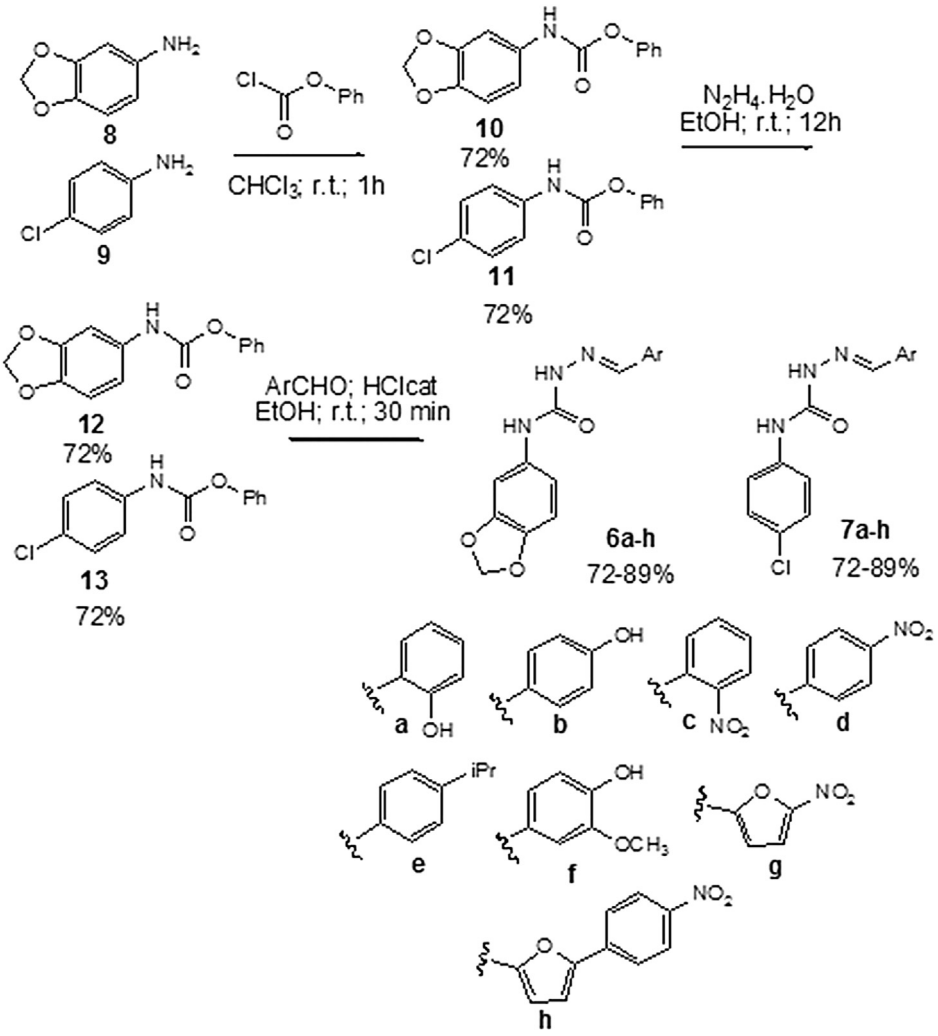
Semicarbazone derivatives **6a**–**h** and **7a**–**h** were evaluated *in vitro* against epimastigote forms of *Trypanosoma cruzi*, Tulahuen 2 strain, discrete typing unit, DTU, Tc VI [27] in a screening concentration of 100 μM. Compounds which presented an inhibition superior than 50% at 100 μM were selected to determine their IC_{50} values, and their ability to inhibit the parasite growth was tested in comparison to the standard drug nifurtimox [28]. As shown in Table 2, compounds **7a** (IC_{50} = 21 μM), **7g** (IC_{50} = 11.9 μM), **6d** (IC_{50} = 8.5 μM) and **6g** (IC_{50} = 11.5 μM) presented the better trypanosomicidal profile being equipotent to the standard drug nifurtimox (IC_{50} = 7.7 μM).

2.5. Leishmanicidal activity

The ability of compounds **6a**–**h** and **7a**–**h** to inhibit the growth of promastigotes forms of *L. major* were investigated, using pentamidine as standard [29]. Compounds with IC_{50} values <100 μM were selected to study their cytotoxic activity against amastigostes forms of *L. major*. As exemplified in Table 2, all compounds containing the 1,3-benzodioxole system (**6a**–**h**) were inactive as leishmanicide. In contrast, compounds **7c**, **7d**, **7f**, **7g** and **7h** showed cytotoxic activity against promastigotes of *L. major*, although with potency inferior than pentamidine. Among these compounds only the semicarbazones **7d** (IC_{50} = 74.0 μM), **7g** (IC_{50} = 1.5 μM) and **7h** (IC_{50} = 0.6 μM) were active against amastigote forms of *L. major*; being compounds **7g** and **7h** more potent than pentamidine (IC_{50} = 17.1 μM).

Considering the aim of identify a new antitrypanosomatid, the analysis of the results depicted in Table 2, allowed the selection of compound **7g** (LASSBio-1483) as a dual trypanosomicidal and leishmanicidal agent. Therefore, the *in silico* prediction of physicochemical, ADME and toxicity properties of LASSBio-1483 (**7g**) were calculated using the ACD/Labs Percepta Platform (License# 56950) and the results were compared to those obtained for nifurtimox and pentamidine.

As demonstrated in Table 3, the druglikeness of compound **7g** was very similar to nifurtimox and different from pentamidine, with no violations of Lipinsky's rule of 5 [30]. Regardless of the poor solubility (predicted in buffer at pH of 6.5), compound **7g** (LASSBio-1483) was showed to be highly permeable based on predicted permeability across Caco-2 monolayers (P_e) and human intestinal absorption (HIA) test. These results were similar to nifurtimox and opposite to pentamidine, that was demonstrated to be a poorly permeable drug ($P_e \leq 1$ * 10-6 cm/s and HIA < 30%) with zero oral bioavailability (F = 0%, Table 3). LASSBio-1483 (**7g**) was expected to have an oral bioavailability (F) of 39% (Table 3). The drug safety profile of these compounds was also projected using Program ACD/



Scheme 1. Synthesis of semicarbazone derivatives **6a**–**h** and **7a**–**h** from the amines **8** and **9**.

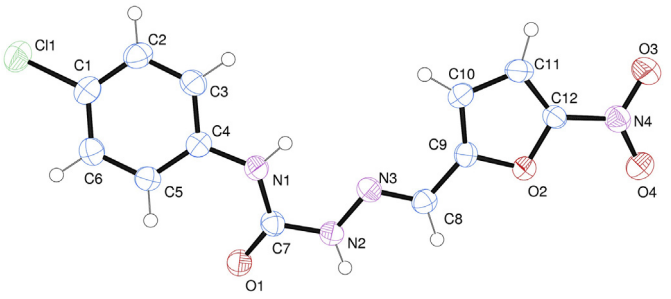


Fig. 1. View of representative semicarbazone derivative **7g** with ellipsoids represent 50%-probability level. H atoms are shown as small spheres of arbitrary radii.

Percepta 14.0.0, based on probabilistic predictors. The metabolic stability in human liver microsomes (HLM), the inhibition of hERG (the **h**uman ***E**ther-à-go-go-**R**elated **G**ene*) and the mutagenic profile (*i.e.* probability of positive Ames test) were calculated and the results converted in the so called classification scores (Table 4). As depicted in Table 3 compound **7g** (LASSBio-1483) was predicted as stable in HLM (≤0.33) and its ability to inhibit hERG was undefined (score > 0.33 and ≤ 0.67). However, similar to nifurtimox, LASSBio-1483 (**7g**) was predictable to be mutagenic (score > 0.67), which is in agreement with the presence of the toxicophoric 5-nitrofuranyl subunit.

Bearing in mind the possibility of the chemical instability of the imine function, present in the semicarbazone framework, the stability profile of compound **7g** (LASSBio-1483) was investigated in buffer solution in pH = 2.0 and 7.4 (Fig. 2A). Moreover, in view of the peptide mimetic profile of semicarbazone scaffold, the plasma stability of LASSBio-1483 was also studied (Fig. 2B).

As demonstrated in Fig. 2A, compound **7g** (LASSBio-1463) presented high stability in buffer solution, either in pH value that simulate gastric juice (pH = 2) or either in pH value that mimic serum content (pH 7.4). The aqueous solubility of LASSBio-1483 (**7g**) was determined using UV-spectroscopic method [31]. Compound **7g** presented a low aqueous solubility, with experimental value (0.0028 mg/mL) similar to that predicted by Program ACD/Percepta 14.0.0 (0.004 mg/mL; Table 3).

The plasma stability of compound **7g** (LASSBio-1483) was determined following the methodology adapted from Konsoula and Jung (2008) [32]. The rat plasma sample was validated using methyl biphenyl-4-carboxylate as standard. As indicated in Fig. 2B, this standard was completely metabolized by plasma enzymes at time of 240 min, resulting in the formation of the biphenyl-4-carboxylic acid (data not shown). In contrast, LASSBio-1483 (**7g**) was not

Table 1
Crystal data and the structure refinement for **7g** (LASSBio-1483).

Parameters	7g (LASSBio-1483)
Empirical formula	$C_{12}H_9ClN_4O_4$
Formula weight	308.68
Wavelength, Å	1.5418
Crystal system	Monoclinic
Space group	$P2_1/c$
Cell parameters, Å and °	$a = 8.8854(2)$, $b = 13.8588(3)$, $c = 13.5620(3)$ $\beta = 129.229(2)$
V, Å^3	1293.65(6)
Z	4
μ, mm^{-1}	2.855
ρ calc., Mg m^{-1}	1.585
θ-range for data collection	5.283–62.270°
Index ranges	$-10 \leq h <= 9$, $-14 \leq k <= 15$, $-15 \leq l <= 15$
Refl. Collected/Unique	3977/2005 [R(int) = 0.0198]
Completeness to theta = 67.680°	85.6%
Data/restraints/parameters	2005/0/190
R indices [I > 2sigma(I)]	R1 = 0.0349, wR2 = 0.0886
R indices (all data)	R1 = 0.0479, wR2 = 0.0964
GooF on F^2	1.042
Residual density, eÅ^{-3}	0.200 and −0.258

metabolized during all the analysis time (0, 30, 60, 120, 180, 240 min). The data revealed the great plasma stability of LASSBio-1483 (Fig. 2B).

In summary a series of semicarbazone derivatives (**6a**–**h** and **7a**–**h**), containing structural modifications on the rings linked to the amine (NH) and imine (N=CH) groups, were designed and synthesized. From this series compound **7g** (LASSBio-1483) highlighted, showing dual *in vitro* trypanosomicidal and leishmanicidal activities with potency similar to the standards drugs nifurtimox and pentamidine. This data, taken together with its good *in silico* druglikeness profile and its great chemical and plasma stabilities, make LASSBio-1483 (**7g**) a new antitrypanosomatid lead-candidate. The *in vivo* trypanosomicidal and leishmanicidal activities of **7g** will be study in our labs.

3. Experimental section

3.1. Chemistry

Reagents and solvents were purchased from commercial suppliers. The reactions were monitored by thin layer chromatography, which was performed on aluminum sheets pre-coated with silica gel 60 (HF-254, Merck) to a thickness of 0.25 mm. The chromatograms were viewed under ultraviolet light (254–265 nm). ^{1}H NMR and ^{13}C NMR spectra were determined in deuterated dimethyl sulfoxide using a Bruker DPX-200 at 200 MHz, Varian Mercury-300 (300 MHz), Varian MR-400 (400 MHz). Signal multiplicities are represented by: s (singlet), d (doublet), t (triplet), q (quadruplet), m (multiplet) and br (broad signal). Infrared (IR) spectra were obtained with a FTLA 2000–100 spectrophotometer using potassium bromide plates. Melting points of final products were determined with a Quimis 340 apparatus and are uncorrected. The purity of compounds were determined by HPLC (>95%) using the Shimadzu –LC20AD apparatus, a Kromasil 100-5C18 (4.6 mm × 250 mm) column and the SPD-M20A detector (Diode Array) at 254 nm for quantification of analyte in a 1 mL/min constant flux. The injector was programmed to inject a volume of 20 μL. The mobile phases used were: CH_3CN:H_2O 1:1; 6:4 and 7:3. The results of elemental analysis were obtained FlashEA 1112 Series instrument (Thermo Scientific) from samples previously dried under vacuum. Ultraviolet spectroscopy was performed using Femto spectrophotometer. The wavelength used in solubility assay was determined by the λ max characteristic of each compound. Spectra were analyzed in FemtoScan software. Mass spectrometry was obtained by positive and negative ionization at Esquire 6000- ESI Ion Trap MSn System Bruker Daltonics and data analyzed in Compass 1.3.SR2 software.

3.1.1. Procedure for the preparation of intermediate carbamates ***10*** *and* ***11*** *(adapted from references [20,21])*

In 30 mL of chloroform was added 7.1 mL (56 mmol) of phenyl chloroformate and allowed to stir. Then it was added slowly with a pipette, a solution of 6g of the functionalized aniline **8** or **9** (47 mmol) in 50 mL of chloroform. The end of the reaction was

Table 2
Determination of the cytotoxicity of semicarbazone derivatives against macrophages (J774.A1), epimastigotes of *T. cruzi* and promastigote and amastigote forms of *L. major*.

Compounds	J774.A1 cell line IC_{50} (μM)[a]	*Epimastigote T. cruzi* IC_{50} (μM)[b]	*Promastigote* L. *major* IC_{50} (μM)[c]	*Amastigote* L. *major*IC_{50} (μM)[c]
Nifurtimox	>100	7.7	0.9	44.1
Pentamidine	>100	N.D.	0.8	17.1
6a (LASSBio-1200)	>100	>100	>100	N.D.
6b (LASSBio-1205)	>100	>100	>100	N.D.
6c (LASSBio-1201)	>100	>100	>100	N.D.
6d (LASSBio-1203)	>100	8.5	>100	N.D.
6e (LASSBio-1206)	>100	>100	>100	N.D.
6f (LASSBio-1210)	>100	>100	>100	N.D.
6g (LASSBio-1302)	>100	11.5	>100	N.D.
6h (LASSBio-1303)	>100	50.0	>100	N.D.
7a (LASSBio-1487)	>100	21.0	>100	N.D.
7b (LASSBio-1701)	71.2	89.1	>100	N.D.
7c (LASSBio-1490)	>100	>100	6.9	>100
7d (LASSBio-1489)	>100	>100	9.4	74.0
7e (LASSBio-1486)	35.7	29.0	>100	N.D.
7f (LASSBio-1488)	>100	86.7	12.6	>100
7g (LASSBio-1483)	>100	11.9	18.5	1.5
7h (LASSBio-1699)	>100	>100	9.7	0.6

N.D. = not determined.
[a] IC_{50} is the concentration required to give 50% death of cells, calculated by linear regression analysis from the Kc values at employed concentrations (100, 10, 1 and 10^{-1} μM).
[b] IC_{50} is the concentration required to give 50% death of *T. cruzi* epimastigotes, calculated by linear regression analysis from the Kc values at employed concentrations (100, 50, 25, 10, 5, 1 μM).
[c] IC_{50} is the concentration required to give 50% death of *L. major* parasites, calculated by linear regression analysis from the Kc values at employed concentrations (100, 10, 1, 10^{-1}, 10^{-2} and 10^{-3} μM).

Table 3
Physico-chemistry properties and ADMET profile of compounds **7g** (LASSBio-1483), nifurtimox and pentamidine calculated using the Program ACD/Percepta 14.0.0.

Predicted properties	Compounds		
	7g (LASSBio-1483)	Nifurtimox	Pentamidine
MW (g/mol)	308.68	287.25	340.42
H-Donors	2	0	6
H-Acceptors	8	8	6
Rotatable Bonds	4	3	10
LogP	3.03	0.04	2.09
Solubility	0.004 mg/ml	2.39 mg/mL	0.61 mg/mL
Caco-2	$P_e = 204 \times 10^{-6}$ cm/s	$P_e = 88 \times 10^{-6}$ cm/s	$P_e = 0.6 \times 10^{-6}$ cm/s
HIA	100%	100%	16%
% F (oral)	39%	99%	0%
HLM	0.33	0.39	0.33
hERG	0.40	0.42	0.34
AMES	0.74	0.86	0.16

Table 4
The meaning of classification score range values for ADMET properties using Program ACD/Percepta 14.0.0.

Classification scores	Predicted ADMET properties		
	HLM	hERG	AMES
≤0.33	Stable	Non-inhibitor	Non-mutagenic
>0.33 and ≤0.67	Undefined	Undefined	Undefined
>0.67	Unstable	Inhibitor	Mutagenic

monitored by TLC after 4 h of reaction, with total consumption of the starting material (eluent: dichloromethane/methanol 5%). 2/3 of the solvent volume was reduced in vacuum being subsequently added 50 mL of hexane and left under stirring for 10 min. The mixture was vacuum filtered, and washed with hexane.

*3.1.1.1. Phenyl benzo[d][1,3]dioxol-5-ylcarbamate (**10**; LASSBio-1213).* Yield: 82%, black solid, m.p. >250 °C; I.R. (KBr) (cm^{-1}): 3390 (vNH), 1717 (vCO); ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): δ 10.59 (s, 1H, Ar–NH) 8.73 (s,1H,C=NH), 7.94 (s, 1H,CONH), 7.82 (d, 2H, H2' & H6′), 7.39 (m, 3H, H3′, H4' & H5′), 7.28 (s, 1H, H4), 7.15 (d, 1H, H6), 6.83 (d, 1H, H7), 5.97 (s, 2H, H2); 99% purity in HPLC (R.T. = 7.3 min, CH_3CN:H_2O (6:1)); MS: m/z = 258.1 $[M+H]^+$.

*3.1.1.2. Phenyl 4-chlorophenylcarbamate (**11**; LASSBio-1481).* Yield: 72%, white solid, m.p. 190–192 °C; I.R. (KBr) (cm^{-1}): 3311 (vNH). 1716 (vC=O); ^{1}H NMR (200 MHz. DMSO-d_6) δ (ppm): δ 10.38 (s, 1H, Ar–NH), 7.54 (d, 2H, H2 & H6), 7.47–7.36 (m, 5H, H2′, H3′, H4′, H5′ & H6′), 7.28 (d, 2H, H3 & H5); ^{13}C NMR (200 MHz. DMSO-d_6) δ (ppm): δ152.2 (C=O), 150.9 (C=NH), 138.1 (C1), 129.9 (C2′, C6′, C3 & C5), 129.3(C3' &C5′), 127.2 (C1′), 126.0 (C4), 122.4 (C2 & C6), 120.5 (C4); purity calculated by elemental analysis: analysis calculated (C 63.04%, H 4.07%, N 5.66%), experimental analysis (C 62.99%, H 4.04%, N 5.50%); MS: m/z = 246.0 $[M-H]^-$.

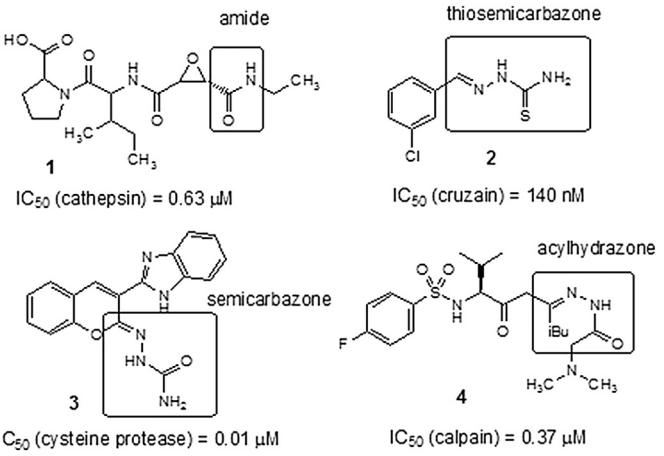


Chart 1. Examples of compound containing amide mimetic framework able to inhibit proteases of parasites.

*3.1.2. Procedure for the preparation of semicarbazides **12** and **13** (adapted from reference [22])*

In a solution containing 1g (4 mmol) of carbamate derivative (10 or 11) and 40 mL of ethanol was added 2.3 mL (15 eq) of hydrazine hydrate 80%. After 12 h at room temperature, it was checked the end of the reaction by TLC (dichloromethane/5% methanol), and the solvent volume was reduced and added ice checking the precipitation of product that was vacuum filtered.

*3.1.2.1. N-(benzo[1,3]dioxol-5- yl) hydrazinecarboxamide (**12**; LASSBio-1212).* Yield: 85%, beige solid, m.p. 215–217 °C; I.R. (KBr) (cm^{-1}): 3356 (vNH_2), 3102–3216 (vNH), 1634 (vCO), 1634 (vC=N); ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): δ 8.50 (s, 1H, ArNH), 7.30 (s, 1H, CONH), 7.25 (s, 1H, H4), 6.84 (d, 1H, H7), 6.78 (d, 1H, H6), 5.90 (s, 2H, H2), 4.29 (s, 1H, NH2), 98% purity in HPLC (R.T. = 2.9 min, CH_3CN:H_2O (6:1)); MS: m/z = 196.1 $[M+H]^+$.

*3.1.2.2. N-(4-chlorophenyl)hydrazinecarboxamide (**13**; LASSBio-1482).* Yield: 61%, white solid, m.p. >250 °C; I.R. (KBr) (cm^{-1}): 3337 (vNH), 1668 (vC=O), 1011 (vC-Cl); ^{1}H NMR (200 MHz. DMSO-d_6) δ (ppm): δ8.76 (s, 1H, Ar–NH), 7.58 (d, 2H, H2 & H6), 7.46 (s, 1H, CONH), 7.25 (d, 2H, H3 & H5), 4,35 (s, 2H, NH_2); ^{13}C NMR (200 MHz. DMSO-d_6) δ (ppm): δ 157.2 (CO), 138.9 (C1), 128.2 (C3 & C5), 124.8(C4), 119.6 (C2 & C6); purity calculated by elemental analysis: calculated (C 45.30%, H 4.34%, N 22.64%), experimental (C 45.36%, H 4.37%, N 22.70%); MS: m/z = 184.0 $[M-H]^-$.

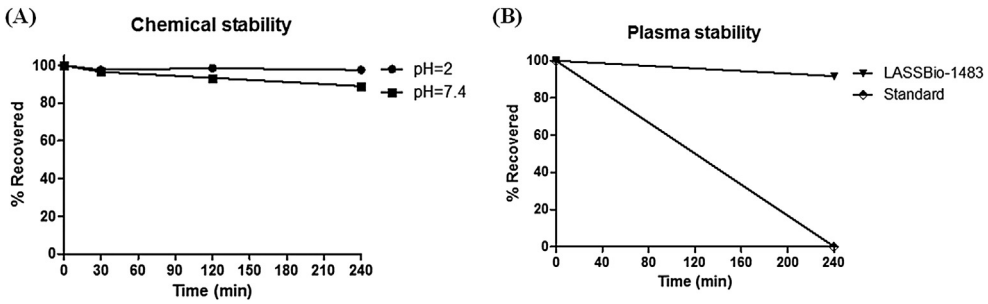


Fig. 2. A) Chemical stability of the compound LASSBio-1483 (**7g**) at pH 2 and 7.4; B) Plasma stability of LASSBio-1483 (**7g**) and the standard methyl biphenyl-4-carboxylate.

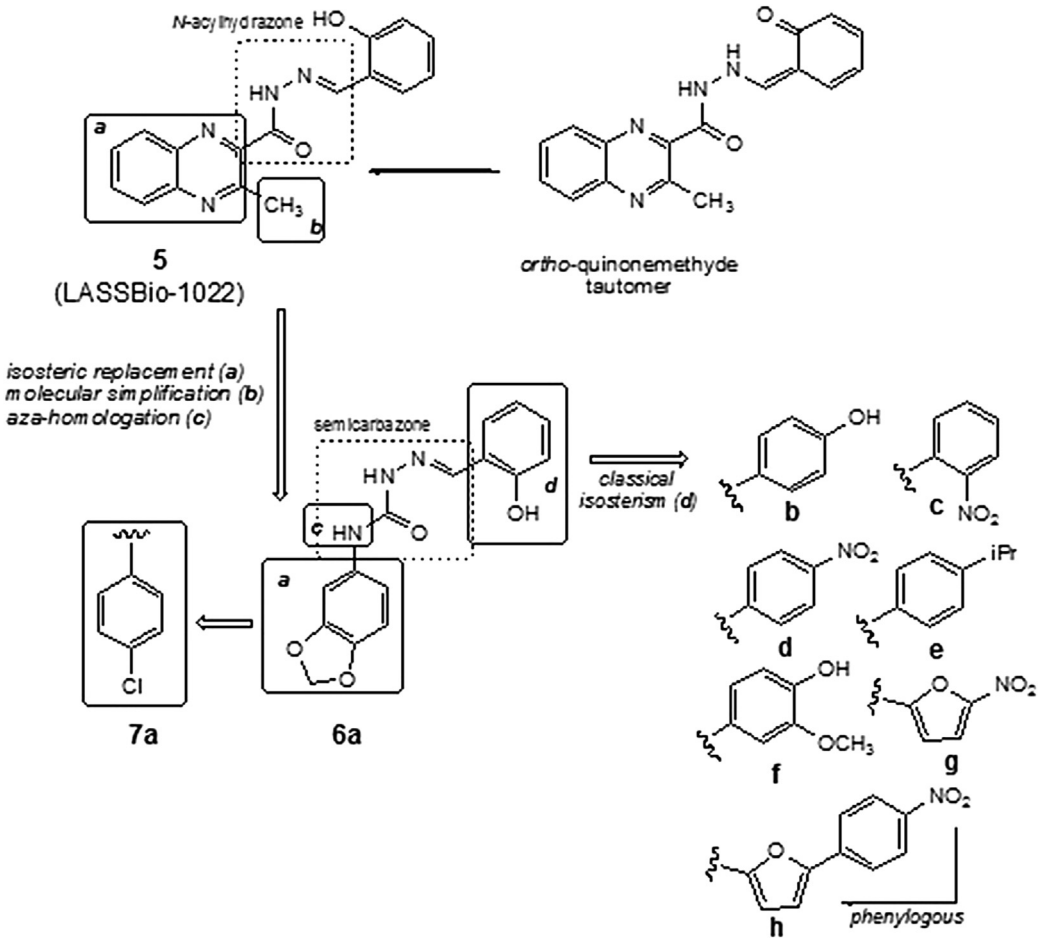


Chart 2. Design concept of semicarbazone derivatives (**6a**–**h** and **7a**–**h**) from molecular modifications on prototype **5.**

*3.1.3. Procedure for the preparation of semicarbazones **6a**–**h** and **7a**–**h** (adapted from reference [23])*

Semicarbazide **12** or **13** (0.25 g, 1.35 mmol) was added in 10 mL ethanol and 1.35 mmol of aldehyde, at room temperature, followed by addition of 1 drop of concentrated HCl. The solution remained under stirring for 30–240 min until TLC (dichloromethane/methanol 5–10%) indicated completion of reaction. The volume of ethanol was reduced, and after addition of ice, was observed precipitation of the product that was filtered and dried under vacuum. Yields and characterization pattern are described below.

*3.1.3.1. (E)-N-(benzo[d][1,3]dioxol-5-yl)-2-(2-hydroxybenzylidene) hydrazine carboxamide (**6a**; LASSBio-1200).* Yield: 95%, brown solid, m.p. 215–217 °C; I.R. (KBr) (cm^{-1}): 3334–2779 (vOH), 3190–3042 (vNH), 1651 (vCO), 1576 (vC=N); ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 10.48 (s, 1H, Ar–NH), 10.02 (s, 1H, Ar–OH), 8.72 (s, 1H, N=CH), 8.25 (s, 1H, CONH),7.87 (d, 1H, H2′), 7.29 (s, 1H, H4), 6.84–7.24 (m, 6H, H4, H6, H7, H3′, H4′& H5′), 5.97 (s, 2H, H2); 99% purity in HPLC (R.T. = 4.8 min, CH_3CN:H_2O (6:1)); MS: m/z = 300.1 $[M+H]^+$.

*3.1.3.2. (E)-N-(benzo[d][1,3]dioxol-5-yl)-2-(4-hydroxybenzylidene) hydrazine-carboxamide (**6b**; LASSBio-1205).* Yield: 84%, beige solid, m.p. 202–204 °C; I.R. (KBr) (cm^{-1}): 3389–2894 (vOH), 3200–3088 (vNH), 1659 (vCO), 1523 (vC=N);^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): δ10.38 (Ar–NH), 9.73 (s, 1H, Ar–OH), 8.64 (s, 1H, N=CH), 7.82 (s, 1H, CONH), 7.63 (d, 2H, H2′& H6′), 7.29 (d, 1H, H6), 7.03 (d, 1H, H7), 6.82 (s, 1H, H4), 6.77 (d, 2H, H3′& H5′), 5.95 (s, 2H, H2); ^{13}C NMR (300 MHz, DMSO-d_6) δ (ppm): δ 159.35 (COH), 153.78 (C=O), 147.50 (N=CH), 142.94 (C3), 141.59 (C1), 134.08 (C5), 129.22 (C2′& C6′), 126.03 (C1′), 116.00 (C3′& C5′), 113.21 (C6), 108.28 (C7), 102.82 (C4), 101.33 (C2); 98% purity in HPLC (R.T. = 3.6 min, CH_3CN:H_2O (6:1)); MS: m/z = 300.0 $[M+H]^+$.

*3.1.3.3. (E)-N-(benzo[d][1,3]dioxol-5-yl)-2-(2-nitrobenzylidene)hydrazine-carboxamide (**6c**; LASSBio-1201).* Yield: 89%, yellow solid, m.p.203–205 °C; I.R. (KBr) (cm^{-1}): 3201–3094 (vNH), 1689 (vCO), 1554 (vC=N), 1522 & 1335 (vNO_2); 1H NMR (300 MHz, DMSO-d_6) δ (ppm): δ 10.96 (s, 1H, Ar–NH), 8.78 (s, 1H, N=CH), 8.44 (d, 1H, H2′), 8.34 (s, 1H, CONH), 8.02 (d, 1H, H5′), 7.77 (t, 1H,H3′), 7.62 (t, 1H, H4′), 7.27 (s, 1H, H4), 7.11 (d, 1H, H6), 6.84 (d, 1H, H7), 5.97 (s, 2H, H2); 99% purity in HPLC (R.T. = 6.0 min, CH_3CN:H_2O (6:1)); MS: m/z = 329.1 $[M+H]^+$.

*3.1.3.4. (E)-N-(benzo[d][1,3]dioxol-5-yl)-2-(4-nitrobenzylidene)hydrazine-carboxamide (**6d**; LASSBio-1203).* Yield: 88%, orange solid, m.p.>250 °C; I.R. (KBr) (cm^{-1}): 3196–3085 (vNH), 1682 (vCO), 1549 (vC=N), 1507 & 1339 (vNO_2), ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): δ5.97 (s, 2H, H2), 6.83 (d, 1H, H7), 7.02 (d, 1H, H6), 7.27 (s, 1H, H4), 8.05 (s, 1H, CONH), 8.09 (d, 2H, H2′& H6′), 8.23 (d, 2H, H3′& H5′), 8.92 (s, 1H, N=CH), 10.96 (s, 1H, Ar–NH); ^{13}C NMR (300 MHz, DMSO-d_6) δ (ppm): δ 101.44 (C2), 103.35 (C4), 108.29 (C7), 113.92 (C6), 124.30 (C2′& C6′), 128.39 (C3′& C5′), 133.64 (C5), 138.73 (C1′), 141.47 (C1), 143.32 (C3), 147.52 (N=CH), 147.90 (C=O), 153.49 (C4′). 99% purity in HPLC (R.T. = 6.0 min, CH_3CN:H_2O (6:1)); MS: m/z = 329.1 $[M+H]^+$.

*3.1.3.5. (E)-N-(benzo[d][1,3]dioxol-5-yl)-2-(4-isopropylbenzylidene) hydrazinecarboxamide (**6e**; LASSBio-1206).* Yield: 75%, beige solid, m.p.136–138 °C; I.R. (KBr) (cm^{-1}): 3193–1117(vNH), 1686 (vCO), 1549 (vC=N), 740 v(iPr); ^1H NMR (300 MHz, DMSO-d_6) δ (ppm): δ 10.50 (s, 1H, Ar–NH), 8.67 (s, 1H, N=CH), 7.89 (s, 1H, CONH), 7.71 (d, 2H, H2′, H6′),7.28 (d, 2H, H3′, H5′), 7.25 (s, 1H H4), 7.03 (d, 1H, H6), 6.80 (d, 1H, H7), 5.95 (s, 2H, H_2), 2.85 (m, 1H,CH), 1.20 (d, 6H, $(CH_3)_2$); ^{13}C NMR (300 MHz, DMSO-d_6) δ (ppm): δ24.25 $(CH_3)_2$, 33.88 (CH), 101.36 (C2), 102.96 (C4), 108.29 (C7), 113.40 (C6), 127.06 (C3′& C5′), 127.62 (C2′& C6′), 132.68 (C5), 133.98 (C1′), 141.30 (C1), 143.04 (N=CH), 147.51 (C3), 150.47 (C4′), 153.72 (C=O); 98% purity in HPLC (R.T. = 13.4 min, CH_3CN:H_2O (6:1)); MS: m/z = 326.2 $[M+H]^+$.

*3.1.3.6. (E)-N-(benzo[d][1,3]dioxol-5-yl)-2-(4-hydroxy-3-methoxy benzylidene)hydrazine carboxamide (**6f**; LASSBio-1210).* Yield: 75%, white solid, m.p.198–200 °C; I.R. (KBr) (cm^{-1}): 3193–3100 (vNH), 3349–2841 (vOH), 1666 (vCO), 1549 (vC=N); ^1H NMR (300 MHz, DMSO-d_6) δ (ppm): δ3.85 (s, 3H, CH_3), 5.96 (s, 2H, H2), 6.78–6.83 (m,2H,H5′&H7), 7.04 (d,1H, H6), 7.13 (d, 1H, H6′), 7.31 (d, 1H, H4), 7.43 (s, 1H, H2′), 7.83 (s, 1H, CONH), 8.68 (s, 1H, N=CH), 9.28 (s, 1H, OH), 10.39 (s, 1H, Ar–NH); ^{13}C NMR (300 MHz, DMSO-d_6) δ (ppm): 56.36 (CH_3), 101.35 (C2), 102.94 (C4), 108.31 (C5′), 110.48 (C6), 113.39 (C7), 115.89 (C2′), 121.95 (C5′), 126.46 (C5), 134.07 (C1′), 141.79 (C1), 142.98 (C=NH), 147.50 (C4′), 148.50 (C3′), 148.9 (C3), 153.80 (C=O); 99% purity in HPLC (R.T. = 3.7 min, CH_3CN:H_2O (6:1)); MS: m/z = 330.2 $[M+H]^+$.

*3.1.3.7. (E)-N-(benzo[d][1,3]dioxol-5-yl)-2-((5-nitrofuran-2-yl)methylene) hydrazine carboxamide (**6g**; LASSBio-1302).* Yield: 90%, orange solid, m.p.221–223 °C; I.R. (KBr) (cm^{-1}): 3155(vNH), 1676 (vCO), 1551 & 1325 (vNO_2); ^1H NMR (300 MHz, DMSO-d_6) δ (ppm): δ5.96 (s, 2H, H2), 6.82 (d, 1H, H7), 6.97 (d, 1H, H6), 7.23 (s, 1H, H4), 7.34 (d, 1H, H4′), 7.78 (d, 1H, H3′), 7.88 (s, 1H, CONH), 8.78 (s, 1H, N=CH), 11.08 (s, 1H, Ar–NH); 98% purity in HPLC (R.T. = 4.4 min, CH_3CN:H_2O (6:1)); MS: m/z = 318.9 $[M+H]^+$.

*3.1.3.8. (E)-N-(benzo[d][1,3]dioxol-5-yl)-2-((5-(4-nitrophenyl)furan-2-yl)methylene)hydrazine carboxamide (**6h**; LASSBio-1303).* Yield: 95%, orange solid, m.p.226–228 °C; I.R. (KBr) (cm^{-1}): 3095(vNH), 1698 (vCO), 1501 & 1355 (vNO_2),^1H NMR (300 MHz, DMSO-d_6) δ (ppm): δ5.96 (s, 2H, H2), 6.83 (d, 1H, H7), 7.03 (d, 1H, H6), 7.12 (d, 1H, H3′), 7.29 (s, 1H, H4), 7.41 (d, 1H, H4′), 7.9 (s, 1H, CONH), 8.02 (d, 2H, H3" & H5″), 8.25 (d, 2H, H2" & H6"), 8.65 (s, 1H, N=CH), 10.77 (s,1H,Ar–NH); 95% purity in HPLC (R.T. = 8.4 min, CH_3CN:H_2O (6:1)); MS: m/z = 395.1 $[M+H]^+$.

*3.1.3.9. (E)-N-(4-chlorophenyl)-2-(2-hydroxybenzylidene)hydrazine carboxamide (**7a**; LASSBio-1487).* Yield: 89%, white solid, m.p. 197–199 °C; I.R. (KBr) (cm^{-1}): 3435 (vNH), 1696 (v C=O), 1492 (vOH), 1013 (vAr-Cl); ^1H NMR (200 MHz. DMSO-d_6) δ (ppm): δ 10.70 (s, 1H, Ar–NH), 10.10 (s, 1H, OH), 9.02 (s, 1H, CONH), 8.21 (s, 1H, N=CH), 7.95 (d, 1H, H2′), 7.68 (d, 2H, H2 & H6), 7.33 (d, 2H, H3 & H5), 7.21 (t, 1H, H4′); 6.86 (m, 2H, H3′ & H5′); ^{13}C NMR (200 MHz. DMSO-d_6) δ (ppm): δ156.1 (COH), 152.9 (CO), 139.0 (CN=CH), 138.2 (C1), 130.7 (C4), 128.3 (C3 & C5), 127.1 (C4′), 126.0 (C2′), 121.3 (C2 & C6), 120.3 (C3′), 119.2 (C1′), 116.0 (C5′); 99% purity in HPLC (R.T. = 11.7 min CH_3CN:H_2O (1:1)), MS: m/z = 288.1 $[M-H]^-$.

*3.1.3.10. (E)-N-(4-chlorophenyl)-2-(4-hydroxybenzylidene)hydrazinecarbox amide (**7b**; LASSBio-1701).* Yield: 67%, white solid, m.p. 204–206 °C; I.R. (KBr) (cm^{-1}): 3301 (vNH), 1617 (vC=O), 1488 (vOH); 1015(vAr-Cl); ^1H NMR (200 MHz. DMSO-d_6) δ (ppm): δ 10.59 (s, 1H, Ar–NH), 9.83 (s, 1H, OH), 8.93 (s, 1H, CONH), 7.86 (s, 1H, N=CH), 7.71 (d, 2H, H2′ & H6′), 7.67 (d, 2H, H2 & H6), 7.33 (d, 2H, H3 & H5), 6.80 (d, 2H, H3′ & H5′); ^{13}C NMR (200 MHz. DMSO-d_6) δ (ppm): δ158.87 (COH), 153.05 (C=O), 141.44 (CN=CH), 138.25 (C1), 128.74 (C4), 128.22 (C3 & C5), 125.89 (C1′), 125.36 (C2′ & C6′), 121.17 (C2 & C6), 115.44 (C3′ & C5′); 98% purity in HPLC (R.T. = 3.9 min CH_3CN:H_2O (7:1)), MS: m/z = 288.1 $[M-H]^-$.

*3.1.3.11. (E)-N-(4-chlorophenyl)-2-(2-nitrobenzylidene)hydrazinecarbox amide (**7c**; LASSBio-1490).* The melting point, ^1H NMR, ^{13}C NMR and IR data are in agreement with previous reports [33]. Yield: 85%, yellow solid, m.p. 200–202 °C; I.R. (KBr) (cm^{-1}): 3390 (vNH), 1709 (vC=O), 1537 e 1344 ($vAr-NO_2$), 1013 (vAr-Cl); ^1H NMR (200 MHz. DMSO-d_6) δ (ppm): δ 11.15 (s, 1H, Ar–NH), 9.08 (s, 1H, CONH), 8.47 (d, 1H, H2′), 8.37 (s, 1H, N=CH), 8.03 (d, 1H, H5′), 7.78–7.58 (m, 2H, H3′ & H4′), 7.69 (d, 2H, H2 & H6), 7.35 (d, 2H, H3 & H5); ^{13}C NMR (200 MHz. DMSO-d_6) δ (ppm): δ152.6 (C=O), 147.8 (C6′), 137.8 (N=CH), 136.0 (C1), 133.2 (C3′), 129.9 (C4), 128.4 (C4′), 128.3 (C2′), 128.2 (C3 & C5), 126.3 (C1′), 124.3 (C5′), 121.4 (C2 & C6); purity calculated by elemental analysis: analysis calculated (C 52.76%, H 3.48%, N 17.58%), experimental analysis (C 52.54%, H 3.41%,N 17.34%); MS: m/z = 317.1 $[M-H]^-$.

*3.1.3.12. (E)-N-(4-chlorophenyl)-2-(4-nitrobenzylidene)hydrazinecarbox amide (**7d**; LASSBio-1489).* The melting point, ^1H NMR, ^{13}C NMR and IR data are in agreement with previous reports [33].Yield: 66%, yellow solid, m.p. 248–250 °C; I.R. (KBr) (cm^{-1}): 3086 (vNH), 1684 (vC=O), 1541 e 1318 ($vAr-NO_2$), 1009 (vAr-Cl); ^1H NMR (200 MHz. DMSO-d_6) δ (ppm): δ11.16 (s, 1H, Ar–NH),9.19 (s, 1H, CONH), 8.25 (d, 2H, H3′ & H5′), 8.15 (s, 1H, N=CH), 8.07 (d, 2H, H2′ & H6′), 7.70 (d, 2H, H2 & 6), 7.36 (d, 2H, H3 & H5); ^{13}C NMR (200 MHz. DMSO-d_6) δ (ppm): δ152.80 (C=O), 147.41 (C4′), 140.78 (N=CH), 138.64 (C1′), 137.91(C1), 128.30 (C3 & C5), 127.91 (C2′ & C6′), 126.40 (C4), 123.74 (C3′ & C5′), 121.67 (C2 & C6); 98% purity in HPLC (R.T. = 5.6 min CH_3CN:H_2O (6:1)), MS: m/z = 317.1 $[M-H]^-$.

*3.1.3.13. (E)-N-(4-chlorophenyl)-2-(4-isopropylbenzylidene)hydrazine carboxamide (**7e**; LASSBio-1486).* Yield: 62%, white solid, m.p. 138–140 °C; I.R. (KBr) (cm^{-1}): 3375 (vNH), 1694 (vC=O), 1012 (vAr-Cl); ^1H NMR (200 MHz. DMSO-d_6) δ (ppm): δ10.77 (s, 1H, Ar–NH), 9.01 (s, 1H, CONH), 7.93 (s, 1H, N=CH), 7.76 (d, 2H, H2 & H6), 7.73 (d, 2H, H3& H5), 7.34 (d, 2H, H2′ & H6′), 7.29 (d, 2H, H3′ & H6′), 2.94 (s, 1H, CH), 1.21 (d, 6H, $(CH_3)_2$); ^{13}C NMR (200 MHz. DMSO-d_6) δ (ppm): δ153.0 (C=O), 150.0 (C4′), 141.1 (N=CH). 138.1 (C1), 132.0 (C4), 128.2 (C3 & C5), 127.1 (C2′ & C6′), 126.5 (C3′ & C5′), 126.0 (C1′), 121.3 (C2 & C6), 33.31 (CH), 23.66 $(CH_3)_2$; purity calculated by elemental analysis: analysis calculated (C 52.72%, H 3.24%, N 12.30%), experimental analysis (C 52.52%, H 2.9%, N 12.31%); MS: m/z = 314.1 $[M-H]^-$.

*3.1.3.14. (E)-N-(4-chlorophenyl)-2-(4-hydroxy-3-methoxybenzylidene)hydrazine carboxamide (**7f**; LASSBio-1488).* Yield: 90%, white solid, m.p. 218–220 °C; I.R. (KBr) (cm^{-1}): 3410–2835 (vAr-OH), 3334 (vNH), 1677 (vC=O), 1092 (vAr-Cl); ^1H NMR (200 MHz. DMSO-d_6) δ (ppm): δ10.63 (s, 1H, Ar–NH), 9.45 (s, 1H,OH), 9.01 (s, 1H, CONH), 7.87 (s, 1H, N=CH), 7.73 (d, 2H, H2 & H6), 7.49 (s, 1H, H2′), 7.35 (d, 2H, H3 & H5), 7.16 (d, 1H, H6′), 6.82 (d, 1H, H5′), 3.37 (s, 3H, OCH_3); ^{13}C NMR (200 MHz. DMSO-d_6) δ (ppm): δ153.6 (C=O), 148.9 (N=CH), 148.5 (C4′), 142.2 (C3′), 138.8 (C1), 128.8 (C3 & C5), 126.52(C4), 126.3 (C1′), 122.1 (C6′), 121.8 (C2 & C6), 115.9 (C2′), 110.5 (C5′), 56.3 (CH_3); purity calculated by elemental analysis: analysis calculated (C 56.35%, H 4.41%, N 13.14%), experimental analysis (56.35%, H 4.67%, N 12.92%); MS: m/z = 318.1 $[M-H]^-$.

*3.1.3.15. (E)-N-(4-chlorophenyl)-2-((5-nitrofuran-2-yl)methylene) hydrazine carboxamide (**7g**; LASSBio-1483).* Yield: 80%, yellow

crystal, m.p. 182–184 °C; I.R. (KBr) (cm^{-1}): 3372 (vNH), 1694 (v C═O), 1532 e 1327 (vAr-NO_2), 1013 (vAr-Cl); ^{1}H NMR (200 MHz. DMSO-d_6) δ (ppm): δ11.28 (s, 1H, Ar–NH), 9.09 (s, 1H, CONH), 7.91 (s, 1H, N═CH), 7.82 (d, 1H, H3′), 7.66 (d, 2H, H2 & H6), 7.35 (m, 3H, H3,H5 & H4′); ^{13}C NMR (200 MHz. DMSO-d_6) δ (ppm): δ152.6 (C═O), 152.3 (C2′), 151.3 (N═CH), 137.6 (C1′), 129.0 (C1), 128.3 (C3 & C5), 126.4 (C4), 121.4 (C2 & C6), 115.1 (C3′), 112.9 (C4′); purity calculated by elemental analysis: analysis calculated (C 46.69%, H 2.94%, N 18.15%), experimental analysis (C 46.93%, 2.95%, N 18.28%); MS: *m/z* = 307.1 [M-H]$^-$.

*3.1.3.16. (E)-N-(4-chlorophenyl)-2-((5-(4-nitrophenyl)furan-2-yl) methylene)hydrazine carboxamide (**7h**; LASSBio-1699).* Yield: 64%, yellow solid, m.p. 248–250 °C; I.R. (KBr) (cm^{-1}): 3365 (vNH),1688 (v C═O), 1532 e 1326 (vAr-NO_2), 1010 (vAr-Cl); ^{1}H NMR (200 MHz. DMSO-d_6) δ (ppm): δ10.98 (s, 1H, Ar–NH), 8.98 (s, 1H, CONH), 8.28 (d, 2H, H3" & H5″), 8.05 (d, 2H, H2" & H6″), 7.92 (s, 1H, N═CH), 7.72 (d, 2H, H2 & H6), 7.45 (d, 1H, H3′), 7.35 (d, 2H, H3 & H5), 7.17 (d, 1H, H4); ^{13}C NMR (200 MHz. DMSO-d_6) δ (ppm): δ152.65 (C═O), 151.80 (C2′), 151.26 (N═CH), 146.13 (C4″), 137.96 (C1′), 135.30 (C1), 130.56 (C1′), 128.32 (C3 & C5), 126.18 (C4), 124.40 (C2″,C6", C3″ & C5″), 121.23 (C2 & C6), 114.31(C3′), 112.40 (C4′); 98% purity in HPLC (R.T. = 8.7 min CH_3CN:H_2O (7:1)), MS: *m/z* = 383.1 [M-H]$^-$.

4. Methodology for single crystal X ray diffraction measurements

Orange plate-like crystals of **7g** were obtained by slow evaporation of methanol at room temperature. A well-shaped and suitably-sized single crystal (0.15 × 0.10 × 0.04 mm) was selected for the X-ray diffraction structure determination experiment. The X-ray intensity data were collected at 150 K on a Gemini-Oxford Diffractometer, using CuKα graphite monochromated radiation. The programs CrysAlis CCD and CrysAlis RED [34] were used for data collection, cell refinement and data reduction. The structure was solved by direct methods using the software Sir-92 [35] and the refinement was carried out using SHELXL-2013 [36]. The non-hydrogen atoms were clearly solved and full matrix least-squares refinement of these atoms with anisotropic thermal parameters was carried on. All hydrogen atoms were positioned stereochemically and were refined with fixed individual displacement parameters [Uiso (H) = 1.2 Ueq] using a riding group model with C–H and N–H bond lengths of 0.95 and 0.88 Å, respectively. WINGX software was used to analyze and prepare the data for publication [37]. Molecular graphics were prepared using ORTEP-3 for Windows [38] and Mercury [39]. Crystallographic data for the structural analysis of the compound discussed here has been deposited at the Cambridge Crystallographic Data Centre as a supplementary publication under number CCDC 1031516. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB12 1EZ, UK [fax: +44 1223 336033 or e-mail: deposit@ccdc.cam.ac.uk].

5. Biology

5.1. Culture of J774.A1 murine macrophages

These adherent-phenotype macrophage line was cultured in Dulbecco's Modified Eagle's medium (DMEM, Sigma) supplemented with 10% FBS at 37 °C with 95% humidity and 5% CO2.

5.2. Culture of Trypanosoma cruzi epimastigotes

T. cruzi epimastigotes (Tulahuen 2 strain) were cultured at 28 °C for 5–7 days (exponential phase of growth) under aerobiosis in axenic BHI-tryptose milieu (33 g/L brain-heart infusion, 3 g/L tryptose, 0.02 g/L hemin, 0.3 g/L D-(+)-glucose, supplemented with 10% (v/v) calf serum, 200,000 units/L penicillin and 0.2 g/L streptomycin).

5.3. Culture of L. major promastigotes of L. major

Promastigotes of *L. major* IOC/L0581 (MHOM/SU/1973/5-ASKH) were obtained from Leishmania collection of the Oswaldo Cruz Institute - Fiocruz. Promastigotes of L. amazonensis (MHOM/BR/77/LTB0016) were obtained from Dr. Eduardo Caio Torres dos Santos at Oswaldo Cruz Institute - Fiocruz. The parasites were maintained *in vitro* in Schneider's medium, supplemented with 10% FBS and 2% human urine at 27 °C in BOD incubator.

5.4. Cytotoxicity against host cells

To evaluate the cytotoxic activity against the J774.A1 cell line, the host cells were plated in 96-well vessels at 2 × 10^5 cells per well in complete culture medium 10% FBS at 37 °C. After 1 h wells were washed with warm HBSS to remove non-adherent cells, leaving approximately 10^5 adherent macrophages. All cultures were done in DMEM complete supplemented with 10% FBS. The compounds and pentamidine were added at serial concentrations (0.1–100 μM). The cells were also cultured with medium free from compounds or vehicle (basal growth control) or in media with DMSO 0.1% (vehicle control). Positive control (dead cells) was obtained by cellular lysis with 1% of Triton 100X in DMEM complete. After 48 h, the cytotoxicity was evaluated by the MTT assay [26]. Data obtained from experiments were expressed as the mean ± standard error of the mean (Mean ± S.E.M.) and statistical differences between the treated and the vehicle groups of experiments were evaluated by ANOVA and Dunnett hoc tests. IC_{50} (concentration required to give 50% death of cells) was calculated by linear regression analysis from the Kc values at employed concentrations.

5.5. In vitro anti-T. cruzi activity

Parasites were harvested in the late log phase, resuspended in fresh medium, counted in a Neubauer chamber, and placed in 24-well plates (3 × 10^6/mL). Parasite growth was followed measuring the absorbance of the culture at 610 nm [28]. Before inoculation, the media were supplemented with the indicated amount of the studied compound from a stock solution in DMSO. The final concentration of DMSO in the culture media never exceeded 0.4% and the control was run in the presence of 0.4% DMSO and in the absence of any compound. No effect on epimastigote growth was observed in the presence of up to 1% DMSO in the culture medium. The percentage of growth inhibition at 100 μM determined at day 5th was calculated as: PGI = {1-[(Ap - A0p)/(Ac - A0c)]} x 100, where Ap = A600 of the culture containing the compound at day 5; A0p = A600 of the culture containing the compound right after addition of the inocula (day 0); Ac = A600 of the culture in the absence of any compound (control) at day 5; A0c = A600 in the absence of the compound at day 0. To determine IC_{50}, the parasite growth was followed in the absence (control) and presence of increasing concentrations of the corresponding compound (1–100 μM). The IC_{50} value was taken as the concentration of compound needed to reduce the absorbance ratio to 50%.

5.6. In vitro activity against promastigote forms of Leishmania major

Stock solutions of novel semicarbazone derivatives as well as pentamidine were prepared in DMSO immediately before use. The cytotoxicity of novel semicarbazone derivatives and pentamidine against promastigotes was determined [29,40,41]. Stationary phase *L. major* promastigotes were plated in 96-well vessels (Nunc) at 10^5 cells per well, in Schneider's medium, supplemented with 10% FBS and 2% human urine. Each derivatives solution was added at increasing concentrations ($10^{-3} - 100$ μM). Cells were also cultured in a medium free of compounds or vehicle (basal growth control) or with DMSO 0.1% (vehicle control). After 48 h, extracellular load of *L. major* promastigotes was estimated by counting the parasites in Schneider's medium in a CELM automatic cell counter (model CC530) [41]. Data obtained from experiments were expressed as the mean ± S.E.M. and statistical differences between the treated and the vehicle groups of experiments were evaluated by ANOVA and Dunnett hoc tests. IC_{50} (concentration required to give 50% death of cells) was calculated by linear regression analysis from the Kc values at employed concentrations.

5.7. In vitro activity against amastigote forms of Leishmania major

To assess the activity of the test derivatives against the amastigote stage of *L. major* were realized model of infection in coverglass [42]. The murine macrophages (J774.A1 cell line) were prepared in 24-well vessels (Corning) at 2×10^5 adherent cells/well, infected with 2×10^6 promastigotes in glass coverslips placed inside 1 ml medium culture. The cultures were cultured or not with the test derivatives or reference drugs (10^{-3} -100 μM), and kept for 24 h at 37 °C, 5% CO_2. After 24 h, coverslips were washed, stained with Giemsa-MayGrünwald, and intracellular amastigotes were counted in 100 macrophages. Data obtained from *in vitro* experiments were expressed as the Mean ± S.E.M. of duplicate cultures of representative assays. Statistical differences between the treated and the control groups were evaluated by ANOVA and Dunnett hoc tests. Differences with a p value <0.05 or lower were considered significant. IC_{50} (concentration required to give 50% death of cells) was calculated by linear regression analysis from the Kc values at employed concentrations.

5.8. Chemical stability assay (adapted from reference [43])

In a 2 mL microfuge tube, were added 1 μL (0.01 mM) from a concentrated solution of the test compound **7g** (concentration = 25 mM stock solubilized in DMSO) and 249 μL acid buffer (0.2 M potassium chloride and 0.2 M HCl, pH = 2) or basic (77 mM phosphate dibasic heptahydrate and 22 mM sodium phosphate monobasic monohydrate, pH = 7.4). After vortexing the mixture was placed in a water bath at 37 °C under vigorous stirring for 0, 30, 60 and 240 min. After each reaction time was added 249 μL basic buffer (20 mM potassium phosphate dibasic anhydrous and 77 mM sodium chloride, pH = 8.4) to neutralize the pH. Extraction of the compound was performed by adding 1 ml of acetonitrile and after vigorous vortexing the medium was filtered (Millipore, 0.45 μm pore size) and analyzed by HPLC-PDA using mobile phase acetonitrile: water, in the equipment Shimadzu LC-20AD, 100-5 Kromasil C18 column (4.6 mm × 250 mm) detector SPD-M20A (Diode Array) and performed to quantify the analyte in wavelength 340 nm, flow rate 1 mL/min with 20 μL injection. Data were acquired by LC solution software, version 4.0, and the standard HPLC solvents used had acquired by TEDIA®. In the chemical stability test pH = 7.4, does not require neutralizing the reaction medium after each time taking place.

5.9. In vitro plasma stability assay (adapted from reference [32])

The *in vitro* plasma stability of compound **7g** (LASSBio-1483) was performed using a pool of rat plasma. Heparinized blood was centrifuged at 2000 rpm for 15 min at 10 °C to obtain plasma. Plasma was diluted to 64% (v/v) with phosphate buffered saline (PBS, pH = 7.4) at 37 °C. The reaction was started by adding 1 μL (0.01 mM), of a stock solution 25 mM in DMSO, of sample to 249 μL of plasma. The plasma samples remained in the water bath at 37 °C under constant agitation at 0 and 240 min. After each reaction time was added 500 μL of cold methanol, 500 μL of acetonitrile and then left microtube on ice for 10 min. After agitation, samples were centrifuged at 13,000 rpm for 15 min at room temperature. The supernatant was analyzed by HPLC-PDA (mobile phase: 60% acetonitrile, 40% water), in the equipment Shimadzu LC-20AD, 100-5 Kromasil C18 column (4.6 mm × 250 mm) detector SPD-M20A (Diode Array) and performed to quantify the analyte in wavelength 340 nm, flow rate 1 ml/min with 20 μL injection. Data were acquired by LC solution software, version 4.0, using as standard methyl biphenyl-4-carboxylate. Experiments were performed with incubation of plasma with 0.5% DMSO without sample (blank) and validating the approach used was performed using standard methyl biphenyl-4-carboxylate using the same conditions described for the analyzed sample (i.e. compound 7g).

Acknowledgment

The authors would like to thank Leishmania collection of the Oswaldo Cruz Institute for having provided L. major IOC/L0581 (MHOM/SU/1973/5-ASKH). The authors would like to thank CNPq (BR), FAPERJ (BR), FAPEAL (BR) and INCT-INOFAR (BR, 573.564/2008-6 and E-26/170.020/2008), PEDECIBA, CSIC-UdelaR (UR, 661) for fellowship and financial support. This work was done under the auspices of the Collaborative Network RIDIMEDCHAG-CYTED.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2015.05.046.

References

[1] K. Nussbaum, J. Honek, C.M. Cadmus, T. Efferth, Trypanosomatid parasites causing neglected diseases, Curr. Med. Chem. 17 (2010) 1594–1617.
[2] http://www.who.int/neglected_diseases/diseases/en/, (accessed 28.10.14.).
[3] N.M. El-Sayed, P.J. Myler, D.C. Bartholomeu, D. Nilsson, G. Aggarwal, A.N. Tran, E. Ghedin, E.A. Worthey, A.L. Delcher, G. Blandin, S.J. Westenberger, E. Caler, G.C. Cerqueira, C. Branche, B. Haas, A. Anupama, E. Arner, L. Aslund, P. Attipoe, E. Bontempi, F. Bringaud, P. Burton, E. Cadag, D.A. Campbell, M. Carrington, J. Crabtree, H. Darban, J.F. da Silveira, P. de Jong, K. Edwards, P.T. Englund, G. Fazelina, T. Feldblyum, M. Ferella, A.C. Frasch, K. Gull, D. Horn, L. Hou, Y. Huang, E. Kindlund, M. Klingbeil, S. Kluge, H. Koo, D. Lacerda, M.J. Levin, H. Lorenzi, T. Louie, C.R. Machado, R. McCulloch, A. McKenna, Y. Mizuno, J.C. Mottram, S. Nelson, S. Ochaya, K. Osoegawa, G. Pai, M. Parsons, M. Pentony, U. Pettersson, M. Pop, J.L. Ramirez, J. Rinta, L. Robertson, S.L. Salzberg, D.O. Sanchez, A. Seyler, R. Sharma, J. Shetty, A.J. Simpson, E. Sisk, M.T. Tammi, R. Tarleton, S. Teixeira, S. Van Aken, C. Vogt, P.N. Ward, B. Wickstead, J. Wortman, O. White, C.M. Fraser, K.D. Stuart, B. Andersson, The genome sequence of *Trypanosoma cruzi*, etiologic agent of Chagas disease, Science 15 (2005) 409–415.
[4] J. Cogo, V. Kaplum, D.P. Sangi, T. Ueda-Nakamura, A.G. Correa, C.V. Nakamura, Synthesis and biological evaluation of novel 2,3-disubstituted quinoxaline derivatives as antileishmanial and antitrypanosomal agents, Eur. J. Med. Chem. 90 (2015) 107–123.
[5] C.S. Graebin, M.de F. Madeira, J.K.U. Yokoyama-Yasunaka, D.C. Miguel, S.R.B. Uliana, D. Benitez, H. Cerecetto, M. Gonzalez, R.G. da Rosa, V.L. Eifler-Lima, Synthesis and in vitro activity of limonene derivatives against Leishmania and Trypanosoma, Eur. J. Med. Chem. 45 (2010) 1524–1528.
[6] A.B. Caballero, A. Rodríguez-Dieguez, M. Quiros, J.M. Salas, O. Huertas, I. Ramírez-Macías, F. Olmo, C. Marín, G. Chaves-Lemaur, R. Gutierrez-Sanchez, M. Sanchez-Moreno, Triazolopyrimidine compounds containing first-row transition metals and their activity against the neglected infectious Chagas

disease and leishmaniasis, Eur. J. Med. Chem. 85 (2014) 526–534.
[7] S. Oh, S. Kim, S. Kong, G. Yang, N. Lee, D. Han, J. Goo, J.L. Siqueira-Neto, L.H. Freitas-Junior, Rita Song, Synthesis and biological evaluation of 2,3-dihydroimidazo[1,2-a]benzimidazole derivatives against Leishmania donovani and Trypanosoma cruzi, Eur. J. Med. Chem. 84 (2014) 395–403.
[8] J.A. Sakanari, S.A. Nadler, V.J. Chan, J.C. Engel, C. Leptak, J. Bouvier, *Leishmania major*: comparison of the cathepsin L- and b-like cysteine protease genes with those of Other trypanosomatids, Exp. Parasitol. 85 (1997) 63–76.
[9] M.H. Branquinha, F.A. Marinho, L.S. Sangenito, S.S.C. Oliveira, K.C. Goncalves, V. Ennes-Vidal, C.M. d'Avila-Levy, A.L.S. Santos, Calpains: potential targets for alternative chemotherapeutic intervention against human pathogenic trypanosomatids, Curr. Med. Chem. 25 (2013) 3174–3185.
[10] C.R. Caffrey, D. Steverding, Kinetoplastid papain-like cysteine peptidases, Mol. Biochem. Parasit. 167 (2009) 12–19.
[11] L.O. Santos, A.S. Garcia-Gomes, M. Catanho, C.L. Sodré, A.L.S. Santos, M.H. Branquinha, C.M. d'Avila-Levy, Aspartic peptidases of human pathogenic trypanosomatids: perspectives and trends for chemotherapy, Curr. Med. Chem. 20 (2013) 3116–3133.
[12] P.C. Lima, F.C.G. Reis, T.F.R. Costa, Cysteine peptidase inhibitors in trypanosomatid parasites, Curr. Med. Chem. 20 (2013) 3152–3173.
[13] D. Steverding, D.W. Sexton, X. Wang, S.S. Gehrke, G.K. Wagner, C.R. Caffrey, Trypanosoma brucei: chemical evidence that cathepsin L is essential for survival and a relevant drug target, Int. J. Parasitol. 42 (2012) 481–488.
[14] X. Du, C. Guo, E. Hansell, P.S. Doyle, C.R. Caffrey, T.P. Holler, J.H. McKerrow, F.E. Cohen, Synthesis and structure-activity relationship study of potent trypanocidal thio semicarbazone inhibitors of the trypanosomal cysteine protease cruzain, J. Med. Chem. 45 (2002) 2695–2707.
[15] J. Schröder, S. Noack, R.J. Marhöfer, J.C. Mottram, G.H. Coombs, P.M. Selzer, Identification of semicarbazones, thiosemicarbazones and Triazine Nitriles as inhibitors of *Leishmania mexicana* cysteine protease CPB, PLoS One 8 (2013) e77460.
[16] N. Raghav, M. Singh, S. Jangra, A. Rohilla, R. Kaur, P. Malik, *In-vitro* studies of various carbonyl derivatives on liver alkaline phosphatase, J. Chem. Pharm. Res. 4 (2010) 801–807.
[17] D.R. Ifa, R.B. de Alencastro, C.A.M. Fraga, E.J. Barreiro, A possible molecular mechanism for the inhibition of cysteine proteases by salicylaldehyde *N*-acylhydrazones and related compounds, J. Mol. Struct. 505 (1999) 11–17.
[18] N.C. Romeiro, G. Aguirre, P. Hernández, M. González, Hugo Cerecetto, I. Aldana, S. Pérez-Silanes, Antonio Monge, E.J. Barreiro, L.M. Lima, Synthesis, trypanocidal activity and docking studies of novel quinoxaline-N-acylhydrazones, designed as cruzain inhibitors candidates, Bioorg. Med. Chem. Lett. 17 (2009) 641–652.
[19] L.M. Lima, E.J. Barreiro, Bioisosterism: a useful strategy for molecular modification and drug design, Curr. Med. Chem. 12 (2005) 23–49.
[20] S.P. Gupte, Process for Preparing Carbamates by the Catalytic Transamidation of Organic Carbonates with Ureas, 2005. WO 2005063698 A1 20050714.
[21] P. Yogeeswari, D. Sriram, R. Thirumurugan, J.V. Raghavendran, K. Sudhan, R.K. Pavana, J. Stables, Discovery of N-(2,6-dimethylphenyl)-substituted semicarbazones as anticonvulsants: Hybrid pharmacophore-based design, J. Med. Chem. 48 (2005) 6202–6211.
[22] C. Sheng, X. Che, W. Wang, S. Wang, Y. Cao, Z. Miao, J. Yao, W. Zhang, Design and synthesis of novel triazole antifungal derivatives by structure-based bioisosterism, Eur. J. Med. Chem. 46 (2011) 5276–5282.
[23] P.C. Lima, L.M. Lima, K.C.M. da Silva, P.H. O Léda, A.L.P. de Miranda, C.A.M. Fraga, E.J. Barreiro, Synthesis and analgesic activity of novel *N* -acylarylhydrazones and isosters, derived from natural safrole, Eur. J. Med. Chem. 35 (2000) 187–203.
[24] I.J. Bruno, J.C. Cole, M. Kessler, J. Luo, W.D.S. Motherwell, L.H. Purkis, B.R. Smith, R. Taylor, R.I. Cooper, S.E. Harris, A.G. Orpen, Retrieval of crystallographically-derived molecular geometry information, J. Chem. Inf. Comput. Sci. 44 (2004) 2133–2144.
[25] F.H. Allen, The cambridge structural database: a quarter of a million crystal structures and rising, Acta Cryst. B 58 (2002) 380–388.
[26] R.F. Hussain, A.M. Nouri, R.T. Oliver, A new approach for measurement of cytotoxicity using colorimetric assay, J. Immunol. Method 160 (1993) 89–96.
[27] R.O. Cosentino, F. Agüero, A simple strain typing assay for *Trypanosoma cruzi*: discrimination of major evolutionary lineages from a single amplification product, PLoS Negl. Trop. Dis. 6 (2012) e1777.
[28] A. Merlino, D. Benítez, S. Chavez, J. Da Cunha, P. Hernández, L.W. Tinoco, N.E. Campillo, J.A. Páez, H. Cerecetto, M. González, Development of second generation amidinohydrazones, thio- and semicarbazones as Trypanosoma cruzi-inhibitors bearing benzofuroxan and benzimidazole 1,3-dioxide core scaffolds, Med. Chem. Comm. 1 (2010) 216–228.
[29] V.S. Amato, J.G. de Paula, R. Imamura, V.N. Amato, M.I. Duarte, M.I. Boulos, M. Boulos, A.C. Nicodemo, J.S. de Mendonça, Treatment of american cutaneous leishmaniasis, with lesions in the mucosa, using pentamidine isethionate, Rev. Soc. Bras. Med. Trop. 29 (1996) 477–481.
[30] C.A. Lipinski, F. Lombardo, B.W. Dominy, P.J. Feeney, Adv. Drug Deliv. Rev. 23 (1997) 3–25.
[31] P. Schneider, S.S. Hosseiny, M. Szczotka, V. Jordan, K. Shlitter, Rapid solubility determination of the triterpenes oleanoic acid and ursolic acid by UV- spectroscopy in different solvents, Phytochem. Lett. 2 (2009) 85–87.
[32] R. Konsoula, M. Jung, *In vitro* plasma stability, permeability and solubility of mercaptoacetamide histone deacetylase inhibitors, Int. J. Pharm. 361 (2008) 19–25.
[33] S.N. Pandeya, V. Mishra, I. Ponnilavarasan, J.P. Stables, Anticonvulsant activity of p-chlorophenyl substituted arylsemicarbazones: the role of primary terminal amino group, Pol. . J. . Pharmacol. 52 (2000) 283–290.
[34] CrysAlisPRO, Oxford Diffraction/Agilent Technologies UK Ltd, Yarnton, England, 2006.
[35] A. Altomare, G. Cascarano, C. Giacovazzo, A. Guagliardi, M.C. Burla, G. Polidori, M. Camalli, SIR92 – a program for automatic solution of crystal structures by direct methods, J. Appl. Cryst. 27 (1994) 435.
[36] G.M. Sheldrick, A short history of SHELX, Acta Cryst. A 64 (2008) 112–122.
[37] L.J. Farrugia, WinGX and ORTEP for windows: an update, J. Appl. Cryst. 45 (2012) 849–854.
[38] L.J. Farrugia, ORTEP-3 for windows – a version of ORTEP-III with a graphical user interface (GUI), J. Appl. Cryst. 30 (1997) 565.
[39] C.F. Macrae, I.J. Bruno, J.A. Chisholm, P.R. Edgington, P. McCabe, E. Pidcock, L. Rodriguez-Monge, R. Taylor, J. Van de StreekWood, P.A. Mercury, CSD 2.0 – new features for the visualization and investigation of crystal structures, J. Appl. Cryst. 41 (2008) 466–470.
[40] C.P.D. Ribeiro, J.H.D. Sampaio, D.R. Cardoso, R.S.N. Ribeiro, A comparative study between the efficacy of pentamidine isothionate given in three doses for one week and N-methil-glucamine in a dose of 20mgSbV/day for 20 days to treat cutaneous leishmaniasis, Rev. Soc. Bras. Med.Trop 36 (2003) 356–371.
[41] H. Rangel, F. Dagger, A. Hernandez, A. Liendo, J.A. Urbina, Naturally Azole-Resistant leishmania braziliensis promastigotes are Rendered Susceptible in the presence of Terbinafine: comparative study with Azole-Susceptible leishmania mexicana promastigotes, Antimicrob. Agents Chemother. 40 (1996) 2785–2791.
[42] M.P. Nunes, L. Cysne-Finkelstein, B.C. Monteiro, D.M. De-Souza, N.A. Gomes, G.A. Dos-Reis, CD40 signaling induces reciprocal outcomes in Leishmania-infected macrophages; roles of host genotype and cytokine milieu, Microb. Infect. 7 (2005) 78–85.
[43] M.D. Perry, E. Carvalho, E. Rosa, J. Iley, Towards an efficient prodrug of the alkylating metabolite monomethyltriazene: synthesis and stability of N-acylamino acid derivatives of triazenes, Eur. J. Med. Chem. 44 (2009) 1049–1056.