Exploring the antileishmanial activity of $N^1,N^2$-disubstituted-benzoylguanidines: synthesis and molecular modeling studies

Kaio Maciel de Santiago-Silva$^a$, Bruna Taciane da Silva Bortoleti$^b,c$, Tiago de Oliveira Brito$^d$, Ivete Conchon Costa$^b$, Camilo Henrique da Silva Lima$^a$, Fernando Macedo, Jr.$^d$, Milena Menegazzo Miranda-Sapla$^b$, Wander Rogério Pavanelli$^b$ and Marcelle de Lima Ferreira Bispo$^a$

$^a$Laboratório de Síntese de Moléculas Medicinais (LaSMMed), Departamento de Química, Centro de Ciências Exatas, Universidade Estadual de Londrina, Londrina, PR, Brazil; $^b$Laboratório de Imunoparasitologia das Doenças Negligenciadas e Câncer (LIDNC), Departamento de Ciências Patológicas, Centro de Ciências Biológicas, Universidade Estadual de Londrina, Londrina, PR, Brazil; $^c$Programa de Pós-Graduação em Biotecnologia, Instituto Carlos Chagas (ICC), Fiocruz, Curitiba, PR, Brazil; $^d$Laboratório de Pesquisa em Moléculas Bioativas (LPMB), Departamento de Química, Centro de Ciências Exatas, Universidade Estadual de Londrina, Londrina, PR, Brazil; $^e$Universidade Federal do Rio de Janeiro, Instituto de Química, Rio de Janeiro, RJ, Brasil

Communicated by Ramaswamy H. Sarma

ABSTRACT

In this report, we describe the synthesis and evaluation of nine $N^1,N^2$-disubstituted-benzoylguanidines against promastigotes and amastigotes forms of Leishmania amazonensis. The derivatives 2g and 2i showed low IC$_{50}$ values against promastigote form (90.8 ± 0.05 μM and 68.4 ± 0.03 μM, respectively), low cytotoxicity profile (CC$_{50}$ 396 ± 0.02 μM and 857.9 ± 0.06 μM) for peritoneal macrophages cells and SI of 5.5 and 12.5, respectively. Investigations about the mechanism of action of 2g and 2i showed that both compounds cause mitochondrial depolarization, increase in ROS levels, and generation of autophagic vacuoles on free promastigotes forms. These compounds were also capable of reducing the number of infected macrophages with amastigotes forms (59.5% ± 0.08% and 98.1% ± 0.46%) and the number of amastigotes/macrophages (79.80% ± 0.05% and 96.0% ± 0.16%), through increasing induction of microbiocide molecule NO. Additionally, ADMET-Tox in silico predictions showed drug-like features and free of toxicological risks. The molecular docking studies with arginase and gp63 showed that relevant intermolecular interactions could explain the experimental results. Therefore, these results reinforce that benzoylguanidines could be a starting scaffold for the search for new antileishmanial drugs.

CONTACT Marcelle de Lima Ferreira Bispo mlfbispo@uel.br Laboratório de Síntese de Moléculas Medicinais (LaSMMed), Departamento de Química, Centro de Ciências Exatas, Universidade Estadual de Londrina, Londrina, PR, Brazil

ARTICLE HISTORY
Received 9 January 2021
Accepted 19 July 2021

KEYWORDS
Arginase; gp63; guanidine; in silico studies; trypanosomatids

JOURNAL OF BIOMOLECULAR STRUCTURE AND DYNAMICS
2022, VOL. 40, NO. 22, 11495–11510
https://doi.org/10.1080/07391102.2021.1959403

© 2021 Informa UK Limited, trading as Taylor & Francis Group
1. Introduction

Leishmaniasis is a neglected tropical and subtropical disease caused by protozoan species of the genus *Leishmania* (World Health Organization, 2021). This disease displays a broad spectrum of clinical manifestations, including cutaneous, mucocutaneous, and visceral leishmaniasis or kala-azar, the more lethal and dangerous form (Burza et al., 2018). It is considered a leading public health problem being present in Central and South American countries, Southern Europe, North and Eastern Africa, the Middle East, and the Indian subcontinent (Croft et al., 2006). Estimations indicate 700,000 to 1 million new cases and some 26,000 to 65,000 deaths every year worldwide (World Health Organization, 2021).

Despite the high toxicity and several related adverse effects, the first-line treatment of leishmaniasis is still based primarily on the use of pentavalent antimonials. When it fails, the indicated treatment is second-line drugs such as amphotericin B, pentamidine, miltefosine, and paromomycin. However, besides the toxicity and clinical resistance, most of these drugs are expensive and require parenteral administration for several days, except for miltefosine, which is orally active. These drawbacks could complicate access to treatment, mainly in underdeveloped countries, which are more affected by this disease. The association of these disadvantages can be responsible for the emergence of parasite resistance (Sundar & Singh, 2018). Therefore, the search for new therapeutic agents is required.

In this context, literature data in several studies suggest that guanidine derivatives have a wide range of pharmacological activities such as antimicrobial (Mo et al., 2009), antiviral (Cheng et al., 2012), anti-inflammatory (Hirsch et al., 2008), antidiabetic agents (Coxon et al., 2009) antitumoral (Brożewicz & Sławinski, 2012), antifungal (Manetti et al., 2009) and antiprotozoal (Arafa et al., 2011; McKeever et al., 2013). Moreover, the literature reports that guanidine is a relevant pharmacophoric group used in the treatment of neglected tropical diseases (Espirito Santo et al., 2014), including leishmaniasis (Berlinck et al., 2012; do Espírito Santo et al., 2019; El-Demerdash et al., 1975; Giulianiotti et al., 2017; Gonzalez et al., 2007; Khomutov et al., 2010; Martins et al., 2016; Murtaza et al., 2011; Stephens et al., 2003).

Therefore, considering the potential of guanidine as antileishmanial agents, in this work, we synthesized and evaluated the antileishmanial activity of nine, including two new benzoylguanidines (BGNs 2a-i, Figure 1). The design concept of these derivatives aims to perform a structure-activity relationship study with have different aliphatic or aromatic substituent at N². Among the aromatic substituent, groups with different steric and electronic features attached at 4 – position of the benzene ring were evaluated to verify their influence on the antipromastigote activity of *L. amazonensis*. Besides, we assayed the cytotoxicity of the compounds on peritoneal macrophages of BALB/c mice, and based on their selectivity index (SI), we identified the most potent compounds. Therefore, we evaluated the selected hits against amastigote forms and conducted biological assays, such as mitochondrial depolarization, generation of reactive oxygen species (ROS), formation of autophagic vacuoles, cell size change, and nitric oxide (NO) production, to understand the mechanisms of action in promastigote and amastigote forms of this parasite. Finally, we performed molecular docking on two molecular targets, arginase and gp63 (leishmanolysin) of *L. amazonensis*. These are promising targets for the new anti-leishmanial agents’ development because affecting the survival and/or virulence of this parasite.

2. Materials and methods

2.1. General information

Solvents and reagents for synthetic purposes were treated, distilled, and dried when required following the processes described by Armarego (Armarego, 2017). Melting points (MP) were determined on a Microquímica MQAPF 302 hot plate apparatus and are uncorrected. HRMS spectra of the title compounds methanol (MS degree) solutions (1 mg/mL) with acetic acid 0.1% were obtained on a high-resolution quadrupole-TOF electrospray mass spectroscopy (Bruker, model MaXis). The spectra were recorded in positive mode at 3800 V of capillary energy, with the nebulizer pressure of 0.3 Bar at 180 °C for the heater and the dry gas at 4.0 L/min. The scan range was between 50 to 1500 m/z and calibrated with acetate standard. Infrared spectra (400–4000 cm⁻¹) were recorded as KBr discs on a Shimadzu FT-IR Model 8300 instrument. NMR spectra were obtained on a Bruker spectrometer Model Avance III operating at 400 MHz for ¹H and 100 MHz for ¹³C using a 5 mm broadband probe. NMR resonances were registered using CDCl₃ (Merck, Darmstadt, Germany) or DMSO-d₆ (Merck, Darmstadt, Germany) as solvents and TMS as the internal standard in CDCl₃. Chemical shifts (δ in ppm) were referenced to the DMSO residual solvent signal in DMSO-d₆ at δ 2.50 or the TMS signal in CDCl₃ at δ 0.00. The splitting of proton resonances in the reported ¹H NMR spectra is defined as singlet (s), doublet (d), triplet (t), quadruplet (qua), quintuplet (qui), and complex pattern (m). Coupling constants (J) are reported in Hz (Hertz).

2.2. Chemistry

2.2.1. Synthesis of N′-substituted benzoylguanidines (BGNs)

Benzoylthioureas (BTUs, 1a-i) were synthesized according to our previous work (Brito et al., 2015). These compounds were used as precursors for the preparation of the series of BGNs (2a-i) using the methodology described by Cunha and co-workers (Cunha & Rodrigues, 2006), with some modifications. Briefly, a solution of BTU (1a-i) (2 mmol) in 30 mL CH₂CN (Synth, Diadema, Brazil) maintained under vigorous magnetic stirring were added 40% aqueous NH₄OH (10 mmol) (Hexis, Jundiaí, Brazil), Et₃N (4 mmol) (Anidrol, Diadema, Brazil), NaBiO₃ (2 mmol) (Anidrol, Diadema, Brazil) and BiI₃ (0.1 mmol) (Sigma-Aldrich, St. Louis, USA). The resulting mixtures were stirred for five hours at room temperature. After the consumption of the starting material, the solvent was removed at reduced pressure. Then, the resulting solid was resuspended in CH₂Cl₂ (Synth, Diadema, Brazil) and filtered through a celite pad. The organic phase was dried with...
anhydrous MgSO₄ (Merck, Darmstadt, Germany), and the solvent was removed at reduced pressure in a rotary evaporator. The crude product was then solubilized in Et₂O/petroleum ether (1:1 ratio) (Synth, Diadema, Brazil) and cooled in a refrigerator for two days. After that, the resulting solid (BGNs 2a–i) was filtered and washed with petroleum ether (2 x 10 mL).

Melting points and ¹H NMR data for all synthesized compounds are in line with the reported in the literature. The complete set of data for the new BGNs (2b and 2h) are listed below.

**N-(N°-phenylcarbamimidoyl) benzamide (2a):** (87% yield, 96% purity). MP: 90–91 °C (Lit. MP. 90–91 °C; (Cusmano et al., 1955). ¹H NMR (400 MHz, DMSO-d₆) δ 9.33 (1H, s), 8.09 (2H, d, J = 6.8 Hz), 7.54 (2H, d, J = 8.5 Hz), 7.50–7.36 (5H, m), 7.11 (1H, t, J = 7.3 Hz) (Gu et al., 2013).

**N-(N°-(4-hydroxyphenyl) carboximidoyl) benzamide (2b):** (59% yield, 98% purity). MP: 175–176 °C. ¹H NMR (400 MHz, DMSO-d₆) δ 9.33 (1H, s), 8.09 (2H, d, d, J = 7.0 Hz), 7.22 (2H, d, J = 8.0 Hz), 6.80 (2H, d, J = 8.7 Hz). ¹³C NMR (DEPT-135 phase) (100 MHz, DMSO-d₆) δ 115.1 (+) (CH), 124.7 (C), 127.3 (+) (CH), 128.1 (+) (CH), 130.3 (+) (CH), 138.5 (C), 154.2 (C), 159.7 (N = O), 175.4 (C = O). HRMS (ESI): Calcld for C₁₄H₁₃N₃O₃ (M+H⁺): 256.1008; Found: 256.1085.

**N-(N°-(4-methoxyphenyl) carboximidoyl) benzamide (2c):** (81% yield, 99% purity). MP: 136 °C (Lit. MP. 136–137 °C; (Ito, 1961). ¹H NMR (400 MHz, CDCl₃) δ 8.18 (2H, d, J = 6.8), 7.48–7.38 (3H, m), 7.15 (2H, d, J = 8.7 Hz), 6.92 (2H, d, J = 9.6 Hz), 3.81 (3H, s) (Pape et al., 2015).

**N-(N°-(4-chlorophenyl) carboximidoyl) benzamide (2d):** (83% yield, 99% purity). MP: 84–85 °C. ¹H NMR (400 MHz, CDCl₃) δ 8.08 (2H, d, J = 8.4 Hz), 7.50–7.34 (5H, m), 7.10 (2H, d, J = 8.6 Hz) (vali Shaik et al., 2017).

**N-(N°-(4-bromophenyl) carboximidoyl) benzamide (2e):** (41% yield, 98% purity). MP: 175–176 °C. ¹H NMR (400 MHz, CDCl₃) δ 8.07 (2H, d, J = 7.3 Hz), 7.48–7.39 (5H, m), 7.14 (2H, d, J = 8.6 Hz) (Åkerbladh et al., 2017).

**N-(N°-(4-nitrophenyl) carboximidoyl) benzamide (2f):** (83% yield, 99% purity). MP: 84–85 °C. ¹H NMR (400 MHz, CDCl₃) δ 8.07 (2H, d, J = 7.3 Hz), 7.51–7.47 (3H, m), 7.43–7.40 (2H, m), 7.05 (2H, d, J = 8.5 Hz) (Åkerbladh et al., 2017).

**N-(N°-(4-(tert-butyl) phenyl) carboximidoyl) benzamide (2g):** (53% yield, 99% purity). MP: 118–119 °C. ¹H NMR (400 MHz, CDCl₃) δ 8.17 (2H, d, J = 7.0 Hz), 7.48–7.38 (3H, m), 7.20 (2H, d, J = 8.0 Hz), 2.35 (3H, s) (vali Shaik et al., 2017).

**N-(N°-(4-(tert-butyl) phenyl) carboximidoyl) benzamide (2h):** (44% yield, 95% purity). MP: 214–215 °C. FT-IR (KBr, cm⁻¹): 3468 (amide N–H), 3336 (amine N–H), 1630 (C = O). ¹H NMR (400 MHz, CDCl₃) δ 8.17 (2H, d, J = 7.0 Hz), 7.48–7.39 (3H, m), 7.14 (2H, d, J = 8.6 Hz), 1.32 (9H, s). ¹³C NMR (DEPT-135 phase) (100 MHz, DMSO-d₆) δ 31.00 (+) (CH₃), 33.85 (C), 121.39 (+) (CH), 125.39 (+) (CH), 127.66 (+) (CH), 128.43 (+) (CH), 130.3 (+) (CH), 138.5 (C), 154.2 (C), 159.7 (N = O), 175.4 (C = O). HRMS (ESI): Calcld for C₁₄H₁₃N₃O₃ (M+H⁺): 256.1008; Found: 256.1085.
(CH), 130.71 (+) (CH), 135.39 (C), 138.59 (C), 145.89 (C), 159.26 (N = C), 176.00 (C = O). HR-MS (ESI): Calc'd for C18H21N3O (M + H+): 296.1685; Found: 296.1760.

**N-(Butylcarbamimidoyl) benzamide (2i):** (62% yield, 96% purity). MP: 113–114 °C (Lit. MP. 114°C). 1H NMR (400 MHz, CDCl3) δ 8.13 (2H, d, J = 7.0 Hz), 7.44–7.35 (3H, m), 3.43–3.11 (2H, m), 1.54 (2H, d, J = 7.2 Hz), 1.38–1.33 (2H, m), 0.9 (3H, t, J = 7.3) (Åkerbladh et al., 2017).

### 2.3. Biological assays

#### 2.3.1. Culture of Leishmania (Leishmania) amazonensis

Promastigotes forms of *L. amazonensis* (MHOM/BR/1989/166MJO) were maintained in culture medium 199 (GIBCO, Invitrogen, NY, USA) pH 7.18–7.22 supplemented with 10% fetal bovine serum (FBS) (GIBCO, Invitrogen, NY, USA), 1 M HEPEs buffer, 1% human urine, 1% L-glutamine, 10% fetal bovine serum (FBS) (GIBCO, Invitrogen, NY, USA) pH 7.18–7.22 and 10% sodium bicarbonate. The parasites culture was maintained in a B.O.D incubator at 24 °C.

#### 2.3.2. Animals and ethics committee

Male BALB/c mice were kindly provided from Carlos Chagas Institute/Fiocruz-PR, Curitiba, Brazil. The animals weighing approximately 25–30 g and aged 6–8 weeks under sterile conditions and were used according to protocols approved by the Ethics Committee for Animal Experimentation of Universidade Estadual de Londrina (UEL) (8685.2018.13). The animals were euthanized by Ketamine- Xylazine overdose (100 mg/kg–10 mg/kg, i.p) followed by cervical dislocation.

#### 2.3.3. BNGs (2a-1) activity against *L. amazonensis* promastigotes

Briefly, *L. amazonensis* promastigotes forms (10⁶ cells/mL) were treated with nine BNGs (2a-1) at different concentrations (100, 200, and 400 μM) and maintained in a B.O.D incubator at 24 °C. Viable parasites were counted in the Neubauer chamber after 24 h of treatment. *L. amazonensis* promastigotes maintained in culture M199 medium without treatment or with 0.01% DMSO (HPLC grade, Sigma-Aldrich, St. Louis, USA) served as negative controls, and promastigotes treated with 1 μM amphotericin B (Amb) (Sigma-Aldrich, St. Louis, USA) as the positive control. The results of antipromastigote activities were expressed by the calculation of half-maximal inhibitory concentration for 50% of parasites (IC₅₀) in μM.

#### 2.3.4. Viability of peritoneal macrophages

The cytotoxic effects of BGNs on peritoneal macrophages were tested based on mitochondrial oxidation by MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, St. Louis, USA) (Fanti et al., 2018). Peritoneal macrophages (5 × 10⁵ cell/mL) from BALB/c mice were incubated with the BGNs (2a-i) at different concentrations for 24 h (37°C, 5% CO₂). The cells were washed, and MTT (5 mg/mL) was added, followed by another incubation for 4 h. The oxidation product of MTT (formazan crystals) was diluted with 300 μL of DMSO (Sigma-Aldrich, St. Louis, USA) transferred to 96-well plates and read in a spectrophotometer (Thermo Fisher Scientific, Multiskan Go, Waltham, USA) using wavelengths of 550 nm (test) and 630 nm (reference). The results were expressed as the percentage of viable cells compared to the control group calculated with the following formula: % (viable macrophages) = (treated samples/OD untreated sample) x 100.

#### 2.3.5. Selectivity index (SI)

The half-maximal inhibitory concentration for 50% of parasites (IC₅₀) was determined on *L. amazonensis* promastigotes forms treated with the nine compounds and the cytotoxic concentration that causes the death of to 50% of cells (CC₅₀) on peritoneal macrophages. IC₅₀ and CC₅₀ were calculated by non-linear regression (GraphPad Software, Inc., USA, 500.288). The selectivity index of the tested compounds was expressed as SI = CC₅₀ on peritoneal macrophages/IC₅₀ on promastigotes forms.

#### 2.3.6. In silico ADME-Tox predictions

Free online platform SwissADME (http://www.swissadme.ch) was used to predict Absorption, Distribution, Metabolism, Elimination (ADME) properties and also molecular descriptors related to Lipinski’s “Rule of Five” (Lipinski et al., 1997) and Veber extensions (Veber et al., 2002), such as molecular weight (MW), octanol/water partition coefficient (clog P), number of hydrogen bond acceptor (HBA) and hydrogen bond donor (HBD), number of rotating connections (RB) and topological polar surface area (tPSA). Prediction of toxicity parameters was performed in OSIRIS DataWarrior property calculation software (Version 5.2.1).

#### 2.3.7. Determination of mitochondrial membrane potential (Δψm)

To assess the inner mitochondrial membrane potential, we conducted an assay using a tetramethylrhodamine ethyl ester (TMRE) staining (Sigma-Aldrich, St. Louis, USA) previously described by (Fanti et al., 2018). For this purpose, promastigotes (10⁶ cell/mL) treated with 2g and 2i (IC₅₀ and 2x IC₅₀) for 24 h and incubated with 25 nM of TMRE for 30 min at 24 °C and analyzed in Perkin-Elmer Victor X3 fluorimeter (PerkinElmer, Turku, Finland), using excitation/emission wavelength of 480/580 nm. In addition, 100 μM of carbonyl cyanide m-chlorophenylhydrazone (CCCP) (Sigma-Aldrich, St. Louis, USA) was used as a positive control, and the vehicle (DMSO 0.01%) was used as a negative control.

#### 2.3.8. Reactive oxygen species (ROS) generation in *L. amazonensis* promastigotes

To evaluate the ROS generation in promastigotes forms of *L. amazonensis*, parasites (10⁶ cells/mL) were treated with the
compounds 2g and 2i (IC50 and 2x IC50) for 24 h at 24 °C and incubated with 10 μM of a permeant probe diacetate 2′, 7′- dichlorodihydrofluorescein (H2DCFDA) (Sigma Aldrich, St. Louis, USA) diluted in DMSO in the dark for 45 min, 24 °C. As a positive control, hydrogen peroxide (H2O2 0.4%) (Merck, Darmstadt, Germany) was used, and the vehicle (DMSO 0.01%) was used for negative control. ROS was measured as an increase in fluorescence caused by the conversion of the non-fluorescent dye to the fluorescent 2.7-dichlorodihydrofluorescein, with an excitation wavelength of 488 nm and emission of 530 nm in Perkin-Elmer Victor X3 fluorimeter (PerkinElmer, Turku, Finland).

2.3.9. Quantification of autophagic vacuoles
Promastigote forms (10⁶ cells/ml) were treated with the compounds 2g and 2i (IC50 and 2x IC50) for 24 h, subsequently were labeled with monodansylcadaverine (50 μM) (MDC) (Sigma-Aldrich, St. Louis, USA) for 1 h at 24 °C according to Bortoleti et al., 2019; Machado et al., 2017 and analyzed in Perkin-Elmer Victor X3 fluorimeter using 380 nm and 525 nm wavelengths for excitation and emission, respectively.

2.3.10. Determination of parasites cell size
Promastigotes (10⁶ cells/ml) untreated or treated with 2g and 2i (IC50 and 2x IC50) and incubated for 24 h at 25 °C. Subsequently, the parasites were analyzed using a BD Accuri™ C6 Plus flow cytometer (BD 27 Biosciences, San Jose, USA) according to Bortoleti (Bortoleti et al., 2019, 2018). Histograms were generated, and FSC-A represented the cell size. 10,000 events were acquired in the region corresponding to the parasites. Control and vehicle (DMSO 0.01%) were used as a negative control.

2.3.11. Antiamastigote assay
Peritoneal macrophages (5 × 10⁵ cell/ml) were cultivated in 24-well plates (Fanti et al., 2018). After infection, the non-internalized promastigotes were removed by washing with PBS, and the adherent macrophages infected were treated with the 2g and 2i in non-cytotoxic concentrations (100 μM and 200 μM) for 24 h (37 °C, 5% CO2). RPMI 1640 medium, DMSO (0.01%), and AmB (1 μM) were used as negative control, vehicle, and positive control, respectively. Then, cells were stained with Giemsa (Laborclin, Pinhais, Brazil), and 20 fields were analyzed by increased immersion (1000x magnification), using an optical microscope (Olympus BX41, Olympus Optical Co., Ltd., Tokyo, Japan) to determine the % of infected macrophages and the number of amastigotes/cell after 24 h of treatment. The supernatant was stored for the measurement of nitric oxide.

2.3.12. Determination of nitrite as estimative of NO levels
Nitric oxide (NO) was determined by the Griess method according to Gonçalves et al., 2018; Miranda-Sapla et al., 2019. Supernatant samples (60 μL) of antiamastigote assay were placed in 96-well microplates and added 50 μL of Griess reagent (sulfanilamide 1% and N-(1-Naphthyl) ethylenediamine dihydrochloride 0.1% in orthophosphoric acid (H3PO4 5%) (Sigma-Aldrich, St. Louis, USA). After 10 min incubation at room temperature, the samples were read at 550 nm on a microplate reader (Thermo Fisher Scientific, Multiskan GO, Waltham, USA). A calibration curve was made using serial dilutions of NaNO2.

2.3.13. Statistical analysis
Data were expressed as mean ± standard error of the mean (SEM). Three independent experiments were performed, each with duplicate datasets. Data were analyzed using the GraphPad Prism statistical software (GraphPad Software, Inc., USA, 500.288). Significant difference between the groups were determined by T-test and one-way ANOVA, followed by Tukey’s test for multiple comparisons. Differences were considered statistically significant when p ≤ 0.05.

2.4. Molecular docking
2.4.1. Proteins and ligands preparation for docking
The structural models of arginase and gp63 (leishmanolysin) from L. amazonensis were built by homology modeling with the aid of the SwissModel server (https://swissmodel.expasy.org/) (Biasini et al., 2014). The protein sequence of LaARG and LaGP63 was obtained from the UniProt database (accession number: O96394 and Q27673, respectively) (Ilari et al., 2018). The 3D structures used as a template were arginase of Leishmania mexicana (D’Antonio et al., 2013) (PDB: 4ITY, resolution: 1.80 Å) and gp63 of Leishmania major (Schlagenhauf et al., 1998) (PDB: 1LML, resolution: 1.86 Å), obtained from the Protein Data Bank (PDB).

The 3D structures of BGNs (2g and 2i) were built up in ChemDraw. Then, the geometry optimization was performed using the MM2 force field in ChemBio3D v.12.0 (PerkinElmer Informatics) (Evans, 2014).

2.4.2. Consensus molecular docking
Molecular docking was performed using AutoDock v. 4.2 (Morris et al., 1998) and AutoDock Vina (Trott & Olson, 2010). The protein and ligand preparation were performed using AutoDockTools (ADT) v.1.5.6 (Morris et al., 2009). All water molecules were removed, polar hydrogen atoms were added, and atoms charges of protein and ligand are assigned by Kollman and Gasteiger methods. The center of the grid box from LaARG for AutoDock was at x: 15.141, y: −15.1248, z: −5.40, size 40 × 50 × 38 Å³ points and spacing of 0.375 Å; for the AutoDock Vina same coordinates were set, with a size of 15 × 18 × 14 Å³. About LaGP63, the center of the grid box for AutoDock was at x: 17.017, y: 45.928, z: 14.824, size 35 × 35 × 35 Å³ points, and spacing of 0.375 Å; for AutoDock Vina size was set to 13 × 13 × 13 Å³.

Molecular docking calculations were carried out considering the hybrid scoring function, implemented in Vina, and Genetic (GA) and Lamarckian Genetic algorithm (LGA) (Morris et al., 1998), implemented in Autodock. Ligands were performed 10 iterative runs and the best-scored pose for which
docking results were considered to root-mean-square-deviation (RMSD) calculation.

Intermolecular interactions analysis and the RMSD calculations were carried out using Discovery Studio Visualizer (Dassault Systèmes BIOVIA, Discovery Studio Modeling Environment, Release 2017, San Diego: Dassault Systèmes, 2016).

3. Results and discussion

3.1. Chemistry

The guanidine derivatives can be synthesized by the stoichiometric reaction of an amine with an electrophilic guanlylation reagent (Zhang et al., 2015). The more common reaction involves the use of an electrophilic thiourea reagent, which is required for prior activation (Alonso-Moreno et al., 2014; Katritzky & Rogovoy, 1976). In these reactions, thiophiles are used as activation agents, commonly, Hg II (Kim & Qian, 1993), Cu II (Kelly & Rozas, 2013) or Bi(III) (Cunha & Rodrigues, 2006) salts, Mukaiyama’s reagent (Shibanuma et al., 1978), N-iodosuccinimide (NIS) (Ohara et al., 2009), cyanuric chloride (TCT) (Porcheddu et al., 2009).

The use of Bi (III) salts is a breakthrough in green chemistry since it replaces salts of highly toxic heavy metals such as Hg II (Shaw et al., 2015). Therefore, we decided to prepare the BGNs derivatives (2a-i) in a single step by the reaction of NH2OH with different benzoylthioureas (1a-i) in the presence of catalytic BiI3 and NaBiO3 as a co-oxidizing agent, as summarized in Scheme 1. In this methodology, the thiourea guanlylation occurs in an elimination-addition process. First, the thiourea is activated (Shaw et al., 2015) by the formation of a complex of the sulfur atom with the catalytic bismuth (Bi3+), which acts as a desulfurization agent. Thus, the elimination of the sulfur atom provides the formation of a carbodiimide. In the addition step, the aqueous NH2OH reacts with the highly electrophilic carbon sp of carbodiimide resulting in the BGNs (Cunha & Rodrigues, 2006).

All benzoylguanidines were obtained in moderate to high yields (41%-87%). The measured melting points and/or spectroscopic data (IR, 1H and 13C NMR) for the nine derivatives herein synthesized were compared to those reported in the literature when available. The two previously unreported compounds (2b and 2h) were fully characterized by spectroscopic techniques (Supporting Information).

In general, 1H NMR spectra of the benzoylguanidines showed the signals related to aromatic hydrogen of both benzoyl and the p-substituted N-phenyl rings around 6.7–8.2 ppm. The signals corresponding to the N-H protons were observed only in the spectra of the compounds 2a, 2b, and 2f, as a broad singlet with low intensity, around 9.3–9.7 ppm. Possibly, it occurs due to the amino-imino tautomerism, since at room temperature, the guanidine group rapidly interconvert between three possible tautomers (O’Donovan et al., 2013). The characteristic signals for the carbonyl moieties (C=O) and N=C were observed as expected in the 13C NMR spectra at 175.4–178.1 ppm and 156.2–162.1 ppm, respectively (Murtaza et al., 2011). Furthermore, all detected 1H and 13C NMR signals of the corresponding aliphatic or aromatic group in benzoylguanidines were assigned and compared with the reported values (Akerbladh et al., 2017; Gu et al., 2013; Pape et al., 2015; vali Shaik et al., 2017). Complementarily, strong C=O stretching absorption was observed for all compounds in IR spectra between 1640–1598 cm⁻¹, as well as in region 3400–3100 cm⁻¹, are observed N-H stretch bands of primary amine (–NH2) and monosubstituted amides (R-CO-NH-R). No characteristic C=S stretching band was observed between 1291 and 1236 cm⁻¹ in the IR spectra of the guanylation products (Brito et al., 2015). Novel compounds (2b and 2h) showed similar IR and NMR spectroscopic parameters, and their molecular formulas were unequivocally confirmed by HR-MS (Supplementary material).

3.2. Biological evaluation

3.2.1. Antipromastigote and cytotoxicity activity in vitro of the BGNs series (2a-i): SAR study

The antileishmanial effect of BGNs (2a-i) on promastigotes forms of L. amazonensis was evaluated in vitro by determining the proliferation of the parasites. Besides, since macrophages are the critical host cells for Leishmania spp, the cytotoxicity on mammalian cells was evaluated by the method developed by Mosmann (Mosmann, 1983) based on the mitochondrial oxidation of peritoneal macrophages of mice. The tested compounds showed antipromastigote activity ranging from 54.9 ± 0.04 to 734.6 ± 0.09 μM and cytotoxicity ranging from 51.3 ± 0.10 to > 1672.9 ± 0.15 μM, according to Table 1.

Among the aromatic derivatives of BGNs (2a-h), the presence of electron-releasing groups (ERG) such as methoxyl (2c) and t-butyl (2h) did not contribute to improving the biological activity since they presented IC₅₀ values higher than the compound 2a (Table 1). However, a comparison between the strong ERGs, such as hydroxyl (2b) and methoxyl (2c),

| BNGs | R  | BNGs | R  | BNGs | R  |
|------|----|------|----|------|----|
| 2a   | Ph | 2d   | 4-Cl-Ph | 2g   | 4-Me-Ph |
| 2b   | 4-OH-Ph | 2e   | 4-Br-Ph | 2h   | 4-t-Bu-Ph |
| 2c   | 4-OMe-Ph | 2f   | 4-NO₂-Ph | 2i   | n-Bu |

Scheme 1. Reagents and conditions: (a) BiI₃ (5 mols %), NaBiO₃, NH₄OH, Et₃N, MeCN, r.t., 5 h.
suggests that the electron donation capacity enhances the antileishmanial activity. In contrast, the addition of weak ERGs, such as methyl \((2g)\) and \(t\)-butyl \((2h)\), indicates that the steric effects could decrease the antipromastigote activity. Regarding the halogenated substituent, the chlorinated derivative \((2d)\) is slightly more active than the brominated derivative \((2e)\), indicating again that steric effects negatively affect biological activity. However, these effects are less pronounced when compared to the observed subset of ERGs \((2b, 2c, 2g, \text{ and } 2h)\). The presence of a strong electron-withdrawing group (EWG) such as NO₂ \((2f)\) attached at the benzene ring seems to have a positive effect on the antileishmanial activity since the derivative \(2f\) is the third more active compound among the aromatic series. However, this derivative showed higher cytotoxicity, which can be related to the presence of the nitro group since there is evidence that nitro compounds can induce severe toxicity (Nepali et al., 2019).

Finally, among the aromatic subset, the derivative \(2g\) can be considered the most potent compound due to its relevant IC₅₀ and SI values.

Furthermore, an aliphatic derivative \((R = n\text{-Bu}; \; 2i)\) was identified as the most potent compound because its IC₅₀ value \((68.4 ± 0.03 \mu M)\) was comparable to the aromatic series \((54.9–66.9 \mu M)\) and the selectivity index \((12.5)\) was the highest among BNGs.

As shown in Table 1, all BGNs derivatives showed low activity against promastigotes of \(L. \text{amazonensis}\), with IC₅₀ values ranging from \(54.9 ± 0.04\) to \(734.6 ± 0.09 \mu M\). However, we decided to explore the biological and molecular aspects of two of our best compounds, because knowledge of these aspects it is possible to carry out structural modifications to obtain more potent and selective compounds in further work. Therefore, the most potent compounds identified \((2g\) and \(2i)\) submitted herein to more specific biological assays, such as mechanisms of action in promastigote forms, evaluation of antiamastigote activity, and molecular docking studies, aiming to exploit their activities in \(L. \text{amazonensis}\).

### 3.2.2. Mechanisms of action in promastigotes of \(L. \text{amazonensis}\)

Parasites of the genus \(Leishmania\) have a single mitochondrion, which is essential in metabolic pathways, synthesis of fatty acids, electron transport chain, and energy metabolism of the parasite. Therefore, the dysfunction of this vital organelle should have irreversible damage compromising its survival (Fidalgo & Gille, 2011; Monzote & Gille, 2010). Thus, any change in mitochondrial transmembrane potential may cause an increase in the production of ROS, which are the main microbicidal molecules that fight infection by pathogens and, consequently, induces protozoan death, being one of the fundamental components to trigger death by apoptosis (Smirlis et al., 2010). In promastigotes, ROS generation followed by mitochondrial depolarization membrane and morphological changes such as cell size reduction is a phenomenon that leads to parasite death by apoptosis-like mechanism (Bortoleti et al., 2018; Miranda-Sapla et al., 2019). Besides, ROS increases cause some consequences for parasites, such as cellular stress. This survival response could be involved in the formation of the autophagic vacuoles that can be activated by ROS (Costa et al., 2019; Scariot et al., 2017).

Therefore, we investigate the mechanisms involved in the elimination of promastigote forms of \(L. \text{amazonensis}\) induced by the presence of the most potent compounds \(2g\) and \(2i\), such as mitochondrial depolarization through TMRE (Figures 2A and B), ROS by H₂DCFDA probe (Figures 2C and 2D), autophagic vacuoles through MDC (Figures 2E and 2F) and cell size by flow cytometry (Figures 3A and 3B).

As shown in Figure 2, the treatment with the most potent compounds \(2g\) (Figure 2A) and \(2i\) (Figure 2B) decreased the total fluorescence intensity of TMRE compared to the control, indicating a reduction in the potential of the mitochondrial membrane (ΔΨm). However, there was no difference between the tested concentrations. Besides, the treatment with both compounds induced oxidative stress, with an increase in ROS production in promastigotes when compared to control (Figures 2C and 2D). Also increased vacuoles autophagic formation concerning MDC, used as control (Figures 2E and 2F), and reduce the cell size of treated parasites (Figures 3A e 3B).

Our results suggest that compounds \(2g\) and \(2i\) cause mitochondrial dysfunction due to the loss of transmembrane potential and thus elevates ROS levels, causing changes in cellular redox status in promastigotes. The interruption of mitochondrial functions associated with morphological changes and autophagic vacuoles formation by ROS

### Table 1. In vitro antileishmanial activity in promastigotes forms (IC₅₀), cytotoxicity (CC₅₀), and selectivity index (SI) of BGNs (2a-i).

| #  | R        | IC₅₀ (μM)¹ | CC₅₀ (μM)² | SI³ |
|----|----------|------------|------------|-----|
| 2a | Ph       | 246.7 ± 0.03⁴ | 1672.9 ± 0.15 | 6.8 |
| 2b | 4-OH-Ph  | 239.0 ± 0.10⁴ | 740.4 ± 0.05 | 3.1 |
| 2c | 4-OMe-Ph | 587.1 ± 0.11⁵ | 442.2 ± 0.03 | 0.8 |
| 2d | 4-Cl-Ph  | 54.9 ± 0.04⁶ | 51.3 ± 0.10 | 0.9 |
| 2e | 4-Br-Ph  | 59.1 ± 0.06⁷ | 190 ± 0.90  | 3.2 |
| 2f | 4-NO₂-Ph | 66.9 ± 0.06⁸ | 105.6 ± 0.05 | 1.6 |
| 2g | 4-Me-Ph  | 90.8 ± 0.05⁹ | 396.0 ± 0.02 | 5.5 |
| 2h | 4-tert-Bu-Ph | 734.6 ± 0.09⁵ | 843.0 ± 0.11 | 1.1 |
| 2i | n-Bu     | 68.4 ± 0.03⁴ | 857.9 ± 0.06 | 12.5 |
| AmB⁴ | —     | 0.068 ± 0.21⁴ | 49.72 ± 0.00 | 731.17 |

¹Values of an inhibitory concentration of 50% of parasites (IC₅₀); IC₅₀ ± SEM.
²Values of cytotoxicity concentration of 50% of macrophages (CC₅₀); IC₅₀ ± SEM.
³SI = selectivity index (CC₅₀/IC₅₀).
⁴Amphotericin B was used as reference drug.

Values followed by different lower-case letters within a column are significantly different at \(p < 0.0001\).
increasing can induce apoptosis-like programmed cell death. Similar results were described in *L. amazonensis* promastigotes treated with other compounds (Bortoleti et al., 2019, 2018; Gonçalves et al., 2018). Although to confirm this hypothesis are necessary to further studies.

### 3.2.3. Evaluation of antileishmanial activity on intracellular amastigotes forms of *L. amazonensis*

The antimastigote effect of the most potent compounds (2g and 2i) in *L. amazonensis*-infected macrophages was also investigated (Figure 4).

---

**Figure 2.** Mechanism of action induced by benzoylguanidines 2g and 2i in promastigote forms of *L. amazonensis*. Parasites were treated for 24 h with the compounds 2g and 2i (IC50 e 2x IC50). (A) TMRE assay for evaluation of mitochondrial membrane potential of the 2g compound and (B) 2i; (C) reactive oxygen species measurement through the H2DCFDA probe of the 2g compound and (D) 2i; (E) vacuoles autophagic quantification by monodansylcadaverine of 2g and 2i (F). Data represent the mean ± SEM of three independent experiments performed in duplicate. * Significance difference compared to control (*p* < 0.05), ** * (p < 0.01) *** (p < 0.001), **** (p < 0.0001).
amastigotes by macrophages, when treated with 100 μM and 50.9 ± 0.4% and 79.7 ± 0.16% the number of amastigotes/macrophages. Besides, amphotericin B, reducing by approximately 100 ± 0.1% the concentration of cellular amastigotes of *L. amazonensis* confirms that benzoylguanidines were active against intracellular amastigotes compared to untreated control (Martins et al., 2016). Furthermore, according to the hit-and-lead criteria for discovering and developing new drugs for tegumentary leishmaniases, a compound is considered promising when it reduces > 99% intracellular amastigotes compared to untreated control considering *in vitro* assays (Caridha et al., 2019). Compound *2g* had a strong *in vitro* effect against amastigote forms of *L. amazonensis*, being close to the criteria for the discovery and development of new antileishmanial agents, reducing the % of infected macrophages and the number of amastigotes/macrophages in a dependent concentration without caused cytotoxicity in the peritoneal macrophages. Our results encourage future structural modifications and procedures to assess their effectiveness *in vivo*.

### 3.2.4. Determination of nitrite as estimates of NO levels

After the antiamastigote assay, the next step was to verify whether *2g* and *2i* could induce the production of nitric oxide (NO) in macrophages infected with *L. amazonensis* (Figure 5). NO develops a pivotal role in biological systems, mainly in antileishmanial infections. Activated macrophages can produce this substance via inducible nitric oxide synthase (iNOS), which catalyzes the oxidation of the guanidine nitrogen of L-arginine to generate NO that kills the pathogens such as *Leishmania*, which is an established mechanism for intracellular infection resolution (Hibbs et al., 1988; Horta et al., 2012).

Concerning NO levels, our results showed that infected macrophages when treated with the compounds *2g* and *2i*, increase their NO levels when compared to the control (Figure 5). NO is a crucial component against invading parasites. However, to survive and propagate in the macrophages, most *Leishmania* species have developed strategies to inhibit several host cells’ functions during the early moments of infection, including the production of NO (Olivier et al., 2012). Besides, the efficient response of macrophages against *Leishmania* spp involves the production of toxic mediators, such as NO. It is a microbicide molecule for a crucial mechanism to eliminate *Leishmania* spp, leading to the resolution of infection (Fang, 2004; Iles & Forman, 2002). In this study, we demonstrated that *L. amazonensis*-macrophages produced *in vitro* elevated NO concentrations, which contributes to eliminating these parasites, suggesting that this mechanism can be involved in activity antileishmanial.

### 3.2.5. *In silico* study to predict pharmacokinetic and toxicity parameters ADME-Tox prediction

Owing to the antileishmanial potential of *2g* and *2i*, we decided to carry out calculations of ADME-Tox parameters using the SwissADME platform (Daina et al., 2017). We investigate the three possible amino-imino tautomeric forms of guanidine (O’Donovan et al., 2013) since these structure changes could affect their physicochemical properties (Table 2).

Table 2 demonstrates that the physicochemical parameters of tautomers of both compounds did not violate any of Lipinski’s and Veber’s rules, indicating that the compounds are likely to have good bioavailability and gastrointestinal absorption, thus suitable to be administered orally (Lipinski et al., 1997; Veber et al., 2002). Besides, the tautomers of compounds *2g* and *2i* have a great capacity to permeate biological membranes, presenting a high gastrointestinal absorption and BBB permeation. This last property is not desirable since it could be responsible for some CNS side effects (Gao et al., 2016).

From this data, we can see that the compounds do not act as a substrate for glycoprotein-P. In the intestine, this
Figure 4. Effect of benzoylguanidines 2g and 2i on L. amazonensis-infected macrophages. L. amazonensis-infected macrophages were treated for 24 h. The percentage of infected macrophages of 2g (A), the number of amastigotes per macrophage of 2g (B), the percentage of infected macrophages of 2i (C), and the number of amastigotes per macrophage of 2i (D) were evaluated and (E) Optical microscopy images showing L. amazonensis-infected macrophages treated with 2g and 2i stained Giemsa. N = nucleus; ▲ = amastigote forms; DMSO 0.01% (vehicle) and AmB 1 μM (positive control). The values represent the mean ± SEM of three independent experiments performed in triplicate. Significant difference in relation to control **** (p ≤ 0.0001). ###### Significant difference in relation to treatments (p ≤ 0.0001).
transporter acts as a transmembrane efflux pump, pumping its substrates back into the lumen, decreasing its absorption. So, drugs that induce glycoprotein-P may reduce the bioavailability of some other ones (Finch & Pillans, 2014).

The interaction analysis of compound 2g with cytochrome P450 isoforms (CYP) showed that the tautomer I could inhibit the CYP1A2 isoform and tautomer II the CYP1A2 and CYP2C9 isoforms. Few commercialized drugs are substrates for CYP1A2 and, therefore, important drug interactions are unlikely to occur in patients with leishmaniasis (Gopinath et al., 2014). However, the CYP2C9 isoform is related to the metabolic clearance of up to 15%–20% of all drugs submitted to phase I metabolism (Van Booven et al., 2010). A drug interaction can occur when an inhibitor such as 2g is added to a therapeutic regimen that includes drugs metabolized by the CYP2C9 isoform, such as non-steroidal anti-inflammatory drugs that may be important in the treatment of cutaneous leishmaniasis. Analysis of compound 2i showed that no tautomers are capable of inhibiting CYP isoforms. This result is relevant because cases of leishmaniasis-HIV coinfection are common (Sundar & Singh, 2018), and the inhibition of CYP isoforms can impair the bioavailability of antiretrovirals that are metabolized by these enzymes.

Besides, we performed an in silico prediction of toxicity using the OSIRIS DataWarrior software (Sander et al., 2015). This software can estimate mutagenic, carcinogenic, teratogenic, and irritant effects. These predictions indicated that all tautomers did not exhibit toxicity risks, except a low irritant risk for the tautomer I of 2g. These results corroborate with the in vitro cytotoxicity assays in murine macrophages, suggesting that 2g and 2i have suitable safety profiles.

### 3.3. Molecular docking

In addition to the mechanisms of action to obtain more information about the antileishmanial activity, we carried out molecular docking simulations of the tautomers of compounds 2g and 2i in two of the main enzymatic targets of L. amazonensis, arginase (D’Antonio et al., 2013) and gp63 (Schlagenhauf et al., 1998). These enzymes are necessary for the survival and/or virulence of Leishmania, so these targets are relevant for new antileishmanial agents development.

In Leishmania spp, the first enzyme in the polyamine biosynthetic pathway is named arginase. It is essential for the parasite’s survival since it is involved in the control of reactive oxygen species induced apoptosis. Therefore, its inhibition

![Figure 5. NO production by L. amazonensis-infected macrophages treated with 2g and 2i.](image)

**Table 2. In silico prediction of physicochemical (Lipinski 5 Rule and Veber extensions) and pharmacokinetic properties of the tautomers of 2g and 2i.**

| Parameters                          | Compound (R) | 2g (4-Me-Ph) | 2i (n-Bu) |
|-------------------------------------|--------------|--------------|-----------|
|                                    | I            | II           | III       | I          | II          | III         |
| MW                                  | 253.3        | 253.3        | 253.3     | 219.2      | 219.2       | 219.2       |
| cLog P                              | 2.66         | 2.96         | 2.62      | 1.84       | 2.09        | 1.93        |
| HBA                                 | 2            | 2            | Yes       | 2          | 2           | Yes         |
| HBD                                 | 2            | 3            | 2         | 2          | 3           | 2           |
| RB                                  | 4            | 5            | 4         | 6          | 7           | 6           |
| tPSA                                | 67.48        | 64.98        | 67.48     | 67.48      | 64.98       | 67.48       |
| Absorption Gl                       | High         | High         | High      | High       | High        | High        |
| BBB permeability                    | Yes          | Yes          | Yes       | Yes        | Yes         | Yes         |
| P-glycoprotein substrate            | No           | No           | No        | No         | No          | No          |
| CYP1A2 inhibitor                    | Yes          | Yes          | No        | No         | No          | No          |
| CYP2C19 inhibitor                   | No           | No           | No        | No         | No          | No          |
| CYP2C9 inhibitor                    | No           | Yes          | No        | No         | No          | No          |
| CYP2D6 inhibitor                    | No           | No           | No        | No         | No          | No          |
| CYP3A4 inhibitor                    | No           | No           | No        | No         | No          | No          |

MW, molecular weight; cLog P, partition coefficient log; HBA, number of hydrogen bonding acceptors; HBD, number of hydrogen bonding donors; RB, number of rotating connections; tPSA, topological polar surface area; Gl, gastrointestinal; BBB, blood-brain barrier, CYP, isoforms of cytochrome P450 enzymes.
leads to mitochondrial dysfunction and the death of the parasite (Reigada et al., 2016; Vannier-Santos et al., 2008). Another important enzyme is gp63 (leishmanolysin), zinc metallopeptidase found in the parasite surface, which through cleavage and/or degradation of substances, helping the parasite to adhere to macrophages, contributing to a well-established function for *Leishmania* interact with the macrophages and mammalian host (Gomez et al., 2009; Olivier et al., 2012).

**Figure 6.** Interaction diagrams of the consensual pose of compound 2g: tautomer I (A), tautomer II (B), tautomer III (C), and compound 2i: tautomer I (D), tautomer II (E), tautomer III (F) in the active site of LaARG. Interacting residues are represented by a stick model. The dashed black lines represent the hydrogen bonding interactions with residues.

**Figure 7.** Interaction diagrams of the consensual pose of compound 2g: tautomer I (A), tautomer II (B), tautomer III (C), and compound 2i: tautomer I (D), tautomer II (E), tautomer III (F) in the active site of LaGP63. Interacting residues are represented by a stick model. The dashed black lines represent the hydrogen bonding interactions with residues.
Therefore, the 3D models of LaARG and LaGP63 were built by homology modeling considering the structure of the arginase from L. Mexicana and gp63 from L. major, respectively, since their sequences share 95.4% and 62.8% of identity with the sequence of arginase and gp63 from L. amazonensis.

Consensus docking is a useful validation method since it improves the fitting performance of the scoring and prediction of the pose (Tuccinardi et al., 2014). Therefore, we decided to apply it to predict and visualize the most favorable interactions between the compounds and the active site, considering the consensus between at least two scoring functions (RMSD < 2.0).

Consensus docking simulation of the arginase complexes with the tautomers of compound 2g exhibited the lowest binding affinity due to the exclusive presence of hydrophobic interactions (Figures 6A-C). Tautomers I and III of compound 2i presented the same interaction pattern, being able to interact by a hydrogen bond between the carbonyl oxygen atoms and the nitrogen of the guanidinium group with three amino acid residues Asp141, Asp143 and Glu197 (Figures 6D and 6F). We also observed hydrogen bond interactions of the tautomer II of 2i with two amino acid residues Asp141 and Asp143 (Figure 6E). In all cases, there are no interactions with the cofactors. These results have shown that the hydrogen bond interactions with the residue Asp141 presented by three tautomers of compound 2i are relevant for the activity because this amino acid residue is responsible for coordinating the Mn²⁺ cofactor on the active site of arginase (da Silva et al., 2002).

Figure 7 exhibits that tautomers I and II of compound 2g have the same pattern of interactions with the active site of gp63. They interact by hydrogen bonds with two amino acid residues Glu264 and Pro344 (Figure 7A and 7B). However, for tautomer III (Figure 7C), we observe that the hydrogen bonds with these residues are lost. In the case of compound 2i, we observe again a similar pattern of the three tautomers, which interact only by hydrogen bonds with the residue Pro344 (Figure 7C-D).

Overall, these results indicate that all tautomers of compound 2i interact more efficiently with critical residues at the active site of arginase than compound 2g. On the other hand, in the case of gp63, compound 2g showed more interactions by hydrogen bond with amino acid residues at the active site.

4. Conclusions

In this study, we identified the two most potent compounds (2g and 2i) against promastigote forms of L. amazonensis due to their highest activity and SI. Studies on the mechanisms of action in promastigote forms demonstrated that these compounds cause mitochondrial depolarization, an increase of intracellular ROS levels, and autophagic vacuoles generation in non-toxic concentrations for mammalian cells. These compounds also were capable of reduces the % infected macrophages and the number of amastigotes/cells through increases induction of microbicide molecule NO. Furthermore, ADME-Tox in silico predictions indicated high oral bioavailability and intestinal absorption, including the absence of toxicological risks. The results of the molecular docking study suggest that the targets chosen in the present study may be associated with the biological activity observed in the face of the presented interactions between 2g and 2i and the active site of these enzymes. Therefore, our results demonstrated that these compounds could be a good starting point in the search for new drugs for the therapy of leishmaniasis.

Acknowledgements

The authors would like to thank the Laboratório Multiusuário de Análises Químicas do Instituto de Química - Unesp Araraquara for HR-MS analysis, as well the Multiuser Nuclear Magnetic Resonance Laboratory-RMNUEL/FINEP for NMR experiments.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001. Besides, it was supported by Conselho Nacional de Pesquisa (CNPq, Brazil).

ORCID

Kaio Maciel de Santiago-Silva http://orcid.org/0000-0001-6550-7820
Bruna Taciane da Silva Bortoletti http://orcid.org/0000-0003-2631-6719
Tiago de Oliveira Brito http://orcid.org/0000-0002-5036-9995
Ivete Conchon Costa http://orcid.org/0000-0002-9800-2068
Camilo Henrique da Silva Lima http://orcid.org/0000-0003-3579-7809
Fernanda Macedo http://orcid.org/0000-0002-4230-1309
Mônica Menegazzo Miranda-Sapla http://orcid.org/0000-0001-8324-9642
Wander Rogério Pavanelli http://orcid.org/0000-0001-7778-8404
Marcelle de Lima Ferreira Bispo http://orcid.org/0000-0002-6001-360X

References

Åkerbladh, L., Schembri, L. S., Larhed, M., & Odell, L. R. (2017). Palladium(0)-catalyzed carbonylative one-pot synthesis of N-acylguanidines. The Journal of Organic Chemistry, 82(23), 12520–12529. https://doi.org/10.1021/acs.joc.7b02294
Alonso-Moreno, C., Antíñolo, A., Carrillo-Hermosilla, F., & Otero, A. (2014). Guanidines: From classical approaches to efficient catalytic syntheses. Chemical Society Reviews, 43(10), 3406–3425. https://doi.org/10.1039/c4cs00013g
Arafa, R. K., Wenzler, T., Brun, R., Chai, Y., & David Wilson, W. (2011). Molecular modeling study and synthesis of novel dicationic flexible triaryl guanidines and imidamides as antiprotozoal agents. European Journal of Medicinal Chemistry, 46(12), 5852–5860. https://doi.org/10.1016/j.ejmech.2011.09.047
Armarego, W. L. F. (2017). Purification of laboratory chemicals (8th ed.). Butterworth-Heinemann.
Berlinc, R. G. S., Trindade-Silva, A. E., & Santos, M. F. C. (2012). The chemistry and biology of organic guanidine derivatives. Natural Product Reports, 29(12), 1382–1406. https://doi.org/10.1039/c2np20071f
Sander, T., Freyss, J., von Koff, M., & Rufener, C. (2015). DataWarrior: An open-source program for chemistry aware data visualization and analysis. Journal of Chemical Information and Modeling, 55(2), 460–473. https://doi.org/10.1021/ci500588j

Scariot, D. B., Britta, E. A., Moreira, A. L., Falzirolli, H., Silva, C. C., Ueda-Nakamura, T., Dias-Filho, B. P., & Nakamura, C. V. (2017). Induction of early autophagic process on leishmania amazonensis by synergistic effect of miltefosine and innovative semi-synthetic thiosemicarbazone. Frontiers in Microbiology, 8, 255. https://doi.org/10.3389/fmicb.2017.00255

Schlagenhauf, E., Etges, R., & Metcalf, P. (1998). The crystal structure of the Leishmania major surface proteinase leishmanolysin (gp63). Structure (London, England: 1993), 6(8), 1035–1046. https://doi.org/10.1016/S0969-2126(98)00104-X

Scott, F. L. (1957). Studies in the pyrazole series. VIII. 1 aminolyses of some 3,5-dimethyl-1-acylguanylpyrazoles. The Journal of Organic Chemistry, 22(12), 1568–1575. https://doi.org/10.1021/jo01363a008

Shaw, J. W., Grayson, D. H., & Rozas, I. (2015). Synthesis of guanidines and some of their biological applications. In P. Selig (Ed.), Guanidines as reagents and catalysts I. Topics in heterocyclic chemistry (Vol. 50, pp. 1–51). Springer. https://doi.org/10.1007/978_2015_174

Shibanuma, T., Shiono, M., & Mukaiyama, T. (1978). Cheminform Abstract: A convenient method for the preparation of carbodiimides using 2-chloropropiridinium salt. Chem. Informationons, 9(6), 06161. https://doi.org/10.1002/chin.197806161

Smirlis, D., Duszenko, M., Ruiz, A., Scoulca, E., Bastien, P., Fasel, N., & Soteriadou, K. (2010). Targeting essential pathways in trypanosomids gives insights into protozoan mechanisms of cell death. Parasites & Vectors, 3, 107. https://doi.org/10.1186/1756-3305-3-107

Stephens, C. E., Brun, R., Salem, M. M., Werbovetz, K. A., Taniou, F., Wilson, W. D., & Boykin, D. W. (2003). The activity of diguanidino and “reversed” diamidino 2,5-diarylfurans versus Trypanosoma cruzi and Leishmania donovani. Bioorganic & Medicinal Chemistry Letters, 13(12), 2065–2069. https://doi.org/10.1016/S0960-894X(03)00319-6

Trott, O., & Olson, A. J. (2010). AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. Journal of Computational Chemistry, 31(2), 455–461. https://doi.org/10.1002/jcc.21334

Veber, D. F., Johnson, S. R., Cheng, H.-Y., Smith, B. R., Ward, K. W., & Kopple, K. D. (2002). Molecular properties that influence the oral bioavailability of drug candidates. Journal of Medicinal Chemistry, 45(12), 2615–2623. https://doi.org/10.1021/jm020001n

World Health Organization. (2021). Trypanosomiasis, human African (sleeping sickness) [WWW Document]. https://www.who.int/en/news-room/fact-sheets/detail/trypanosomiasis-human-african-(sleeping-sickness)

Zhang, W.-X., Xu, L., & Xi, Z. (2015). Recent development of synthetic preparation methods for guanidines via transition metal catalysis. Chemical Communications (Cambridge, England), 51(2), 254–265. https://doi.org/10.1039/c4cc05291a