ARTICLE TYPE

Synthesis, Antileishmanial Activity and *in silico* Studies of Aminoguanidine Hydrazones (AGH) and Thiosemicarbazones (TSC) Against *Leishmania chagasi* Amastigotes

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Abstract: Background: Leishmaniasis is a worldwide health problem, highly endemic in developing countries. Among the four main clinical forms of the disease, visceral leishmaniasis is the most severe, fatal in 95% of cases. The undesired side-effects from first-line chemotherapy and the reported drug resistance search for effective drugs that can replace or supplement those currently used an urgent need. Aminoguanidine hydrazones (AGH's) have been explored for exhibiting a diverse spectrum of biological activities, in particular the antileishmanial activity of MGBG. The bioisosteres thiosemicarbazones (TSC's) offer a similar biological activity diversity, including antiprotozoal effects against *Leishmania* species and *Trypanosoma cruzi*. **Objective:** Considering the impact of leishmaniasis worldwide, this work aimed to design, synthesize, and perform a screening upon *L. chagasi* amastigotes and for the cytotoxicity of the small "in-house" library of both AGH and TSC derivatives and their structurally-related compounds. **Method:** A set of AGH's (**3-7**), TSC's (**9, 10**), and semicarbazones (**11**) were initially synthesized. Subsequently, different semi-constrained analogs were designed and also prepared, including thiazolidines (**12**), dihydrothiazines (**13**), imidazolines (**15**), pyrimidines (**16, 18**) azines (**19, 20**), and benzotriazepinones (**23-25**). All intermediates and target compounds were obtained with satisfactory yields and exhibited spectral data consistent with their structures. All final compounds were evaluated against *L. chagasi* amastigotes and J774.A1 cell line. Molecular docking was performed towards trypanothione reductase using GOLD® software. **Result:** The AGH's **3i**, **4a**, and **5d**, and the TSC's **9i**, **9k**, and **9o** were selected as valuable hits. These compounds presented antileishmanial activity compared with pentamidine, showing IC₅₀ values ranged from 0.6 to 7.27 µM, maximal effects up to 55.3%, and satisfactory SI values (ranged from 11 to 87). On the other hand, most of the resulting semi-constrained analogs were found cytotoxic or presented reduced antileishmanial activity. In general, TSC class is more promising than its isosteric AGH analogs, and the beneficial aromatic substituent effects are not similar in both series. *In silico* studies have suggested that these hits are capable of inhibiting the trypanothione reductase from the amastigote forms. **Conclusion:** The promising antileishmanial activity of three AGH's and three TSC's was characterized. These compounds presented antileishmanial activity compared with PTD, showing IC₅₀ values ranged from 0.6 to 7.27 µM, and satisfactory SI values. Further pharmacological assays involving other *Leishmania* strains are under progress, which will help to choose the best hits for *in vivo* experiments.

Keywords: Aminoguanidine hydrazone, thiosemicarbazone, antileishmanial activity, *Leishmania chagasi*, molecular docking, structure-activity relationship.

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1. INTRODUCTION

Neglected Tropical Diseases (NTD's) represent a significant public health problem worldwide, contributing to a total of 1 billion people affected, caused by some kind of bacterial, viral, or parasitic infection [1]. Among these, trypanosome parasites are unicellular protozoa belonging to the family *Trypanosomatidae* family (order: *Kinetoplastidae*, *Excavata*). This family is composed of three different types of Kinetoplastids: *Leishmania* spp. (Leishmaniasis), *Trypanosoma brucei* (Human African Trypanosomiasis - HAT), and *T. cruzi* (Chagas disease) [2–4]. However, *Leishmania* genus deserve more attention, since it is related to poverty, unsatisfactory sanitary conditions, and the vector presence in underdeveloped countries [1,5]. The Introduction section should include the background and aims of the research in a comprehensive manner.

Leishmaniasis is a group of zoonotic diseases transmitted by mosquitoes (Diptera: *Psychodidae*: *Phlebotominae*) to humans and animals [6–8]. These are responsible for causing extensive morbidity and mortality in Sub- and Tropical countries, being classified among the most neglected diseases [5,9–11]. Also, it is present in 98 countries, infecting more than 13 million people, with an average of 300,000 cases reported *per year* [5,12,13]. Typically, the

Leishmaniasis can be found as four different clinic aspects: cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), visceral leishmaniasis (LV), and dermal post-Kala-azar leishmaniasis (LPKL) [14]. Among these clinical forms, LV is considered as the most severe clinical aspect of this disease [15]. In sense, *L. donovani* and *L. infantum* are known as agent causatives of LV in the Old-World, while *L. chagasi* in the New-World [16,17]. The *L. chagasi* demonstrates distinct symptomatology, characterized by prolonged and irregular fever, hepatomegaly, splenomegaly, anemia, and weight loss [5,14]. Moreover, this disease affects approximately 500,000 people worldwide and, if not treated, can lead to death in 95% of cases [14,17]. As a zoonotic infection, the transmission is difficult to interrupt, although some attempts to reduce vector and mammalian reservoir populations have been successful [18,19].

In general, the first-line chemotherapy employed against leishmaniasis is based on pentavalent antimonials, such as sodium stibogluconate (Pentostan®) and meglumine antimoniate (Glucantime) (Figure 1, A). Their uses are limited, due to cardiotoxicity, hepatotoxicity, pancreatitis, treatment failure, and combination therapy could develop drug resistance [15,20,21]. Concerning the adverse effects, these drugs cannot be administrated in pregnant and elderly patients [15]. Additionally, pentamidine (Pentam®), oral miltefosine (Miltex®), various amphotericin B formulations (AmBisome®), and paromomycin sulfate (Aminosidine®)

(Figure 1, B) are utilized as agents of second-line in the leishmaniasis treatment. Indeed, these are indicated in cases of intolerance, resistance, and contraindications to antimonial drugs [11,12,22]. The problems exposed emphasize the importance of the development of new drugs

against leishmaniasis. In recent years, it has been seen a significant increase in our fundamental knowledge of parasite, as well as a revolution in chemical techniques and several advances in bioinformatics tools that have motivated the search of new antileishmanial agents [23].

A) First-line drugs:

B) Second-line drugs:

Fig. 1. First- and Second-line drugs utilized in the leishmaniasis treatment.

Aminoguanidine hydrazones (AGH's, **1**) constitute an original chemotype opening to a large avenue of structural diversity and design of novels structurally-related compounds. Basically, this chemical class is represented by a simple pharmacophore comprising a cationic head (guanidine) at specific conditions, involving bond angles and lengths (geometric aspects), which generally allow insertion of various substituents (*i.e.* halogenated, oxygenated, other groups) at *ortho*, *meta* and/or *para* positions [24]. Besides, AGH's represent a class of compounds with promising pharmacological activities on several levels; they derive from the same aminoguanidine chemotype with mixed hydrogen bond acceptor and donor properties, as well as being able to establish electrostatic interactions. AGH derivatives are known to possess a broad range of biological activities, including antiarrhythmic (G-256 and Selohin-1), oncolytic, and anti-obesity. Guanabenz is the AGH prototype, which is used in humans for the treatment of

cardiovascular dysfunction. Also, when checking carefully the literature dealing with novel therapies against protozoal diseases, we highlight the JC-I-127, which presents a 2-amino-3,4,5,6-tetrahydropyrimidine moiety in its AGH core (Figure 2) [25–27].

Different studies reported the antileishmanial activity of an AGH derivative, the methylglyoxal bis(guanyldiazide) – MGBG (Figure 2). In this context, Bachrach *et al.*[28] reported the activity of MGBG on growth and macromolecular syntheses in *L. tropica*, *L. mexicana*, and *L. donovani* species, where it was verified that the MGBG inhibited the synthesis of leishmanial DNA and RNA at growth-inhibiting concentrations. Also, Roberts *et al.*[29] observed that MGBG is toxic towards *L. donovani*, and Mukhopadhyay *et al.*[30] demonstrated that the same derivative inhibits the growth of *L. donovani* promastigotes (strain UR6), in a dose-dependent manner.

Fig. 2. Important AGH drugs/prototypes and their pharmacological activities.

Based on bioisosterism rules, the N→S replacement within the AGH series led to the corresponding thiosemicarbazones (TSC's, **2**) and other structurally related compounds, which offer a similar chemical and biological activity diversity, as illustrated in Figure 3 [31–34]. The biological activity of thiosemicarbazones upon different species of *Leishmania* and *Trypanosoma* have frequently been reported in several studies involving this chemical class. However, it has presented a low bioavailability profile, via oral administration [35–37].

Fig. 3. Important TSC derivatives found in the literature and their pharmacological activities.

Considering the impact of *L. chagasi* worldwide, this work aimed to design, synthesize, and perform a screening of the small "in-house" library of both AGH and TSC derivatives, as well as, their structurally-related compounds. We provide a deeply Structure-Activity Relationship (SAR) analysis resulting from the (i) structural modifications on the AGH moiety (*i.e.* introduction of chemical diversity employing different types of substitution, search for additional interactions, homology, and isosterism) versus their biological evaluation; and (ii) building novel scaffolds, which may replace and mimic the chemotype (aminoguanidine) present in AGH analogs.

For this purpose, a set of AGH (**3-7**), TSC (**9, 10**), and semicarbazones (**11**) were initially synthesized and, subsequently, different semi-constrained analogs were designed and also synthesized (**12-16, 19, 20, 23-25**) (Chart 1). Formally, they replace the prototypical set of H-bond interactions (aminoguanidine as chemotype) by a ring system able to mimic the chemotype in a specific conformation (*novel scaffolds*). After synthesis of all designed compounds, the screening was performed upon *L. chagasi* amastigotes, since these parasitic forms are responsible to provoke a chronic state of the disease (visceral

leishmaniasis). Wherefore, the screening was performed at 10 μ M concentration, and significantly active compounds (parasite killing $\geq 30\%$) were further characterized by means of their IC₅₀ values. In parallel, cytotoxic effects of these compounds were determined by using MTT method. For the most active compounds, the selectivity indexes (SI) were calculated.

Chart 1. "In-house" library of AGH, TSC, and related compounds.

2. EXPERIMENTAL PROTOCOLS

2.1. Compound synthesis

See Supplementary data.

2.2. Biological evaluation

2.2.1. Parasite culture

The promastigotes of *L. chagasi* (MCAN/BR/89/BA262) were maintained *in vitro* in Schneider's medium supplemented with 10% Fetal Bovine Serum (FBS) and 2% male human urine filtered at 27°C on greenhouse biochemical Oxygen demand (BOD). At the time of use, the parasites were put into falcon tubes and centrifuged at 3500 rpm for 10 minutes. Then, the supernatant was discarded and the pellet formed was resuspended in Schneider's medium. There was then the counting of parasites using a Neubauer chamber for later plating and carrying out of experiments.

2.2. Maintenance macrophages

Macrophages from *J774.A1* strain were maintained in culture bottles containing 10 mL RPMI supplemented with 10% FBS and 2 mM *L*-glutamine, non-essential amino acids, and pyruvate. At the time of use, the cells were counted, adjusted in RPMI supplemented with 10% FBS and seeded.

2.3. Determination of cell viability

The study of cellular viability was performed using the MTT reduction method (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide) [38,39]. In this assay, the *J774.A1* macrophage cell line were plated in 96-well plates overnight (1×10^5 /well) and exposed to different concentrations of compounds diluted in supplemented RPMI

medium (0.1-100 μ M) for a period of 24h at 37 °C in a humid atmosphere containing 5% CO₂. The control wells contained cells cultured only with culture medium or cultured cells in the presence of substances diluent (0.1% DMSO). After the incubation period, the supernatant was discarded and then was added 100 μ L of MTT solution (5 mg/10 mL). The plates were re-incubated for a period of 1 hour at 37 °C and 5% CO₂ after the supernatant was removed and added to 100 μ L of DMSO and place in spectrophotometer reading at 550 nm. The viability of the cultures treated with the derivatives was compared to the pattern of death obtained in control cultures. Selectivity index of each treatment was calculated by dividing the 50% cytotoxicity concentration (CC₅₀) observed for *J774.A1* macrophage by the half-maximal inhibitory concentration (IC₅₀).

2.4. Macrophage infection with *L. chagasi* test

The macrophages *J774.A1* cell line were plated in a proportion of 5×10^4 cells/24-well plate well with a coverslip for one hour with RPMI supplemented. The macrophages were infected with *L. chagasi* promastigote forms, parasites a ratio of 10:1 macrophage. The plate was incubated for 6h at 37 °C with a humid atmosphere containing 5% CO₂. After 6 hours of infection, the macrophages were washed with saline solution (PBS, pH 7.2) to remove the non-internalized parasites. The macrophages were cultured with RPMI supplemented with 10% FBS, 2 mM *L*-glutamine, 2 mM nonessential amino acids and 2 mM pyruvate in the presence or not of derivatives in different concentrations (0.1, 1, 10, and 100 μ M) and maintained at 37 °C in a humid atmosphere containing 5% CO₂ for 24 hours. After this culture period, the wells containing coverslip were washed with PBS, cells were fixed with methanol and then stained with May-Giemsa-Grünswald and mounted on glass slides. The assessment of the number of infected macrophages and the number of amastigotes in 100 macrophages were taken with an optical microscope with assistance of 100x objective (immersion) [40].

2.5. Statistical analysis

Significance levels between experimental groups and control were made using analysis of variance (ANOVA) followed by Dunnett's test using GraphPad Prism 5.0 program. The values were considered significant when $p < 0.05$, when compared to the control group.

2.6. In silico analyses

2.6.1. Computational details

All *in silico* experiments involving molecular docking were performed in a Dell® notebook, model 5500U, with an Intel® Core™ 4th generation *i*-7 processor, CPU 2.4 GHz, 16 GB RAM, and running at Windows® 8.1 platform.

2.6.2. Molecular Docking

Initially, the 6 hit analogs (AGH: **3i**, **4a**, and **5d**; and TSC: **9i**, **9k**, and **9o**) were drawn, converted into three-dimensional structures, and energetically minimized by the application of the semi-empiric method Austin Model 1

(AM1), by using ArgusLab® software, v. 4.0.1 (<http://www.arguslab.com>) [41]. Subsequently, the three-dimensional structure of trypanothione reductase (TryR) was obtained at the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB), under PDB ID: 2JK6 (<https://www.rcsb.org/structure/2JK6>). Pretreatment of this macromolecule and molecular docking simulations were performed using GOLD® software, v 5.8.1 (<https://www.ccdc.cam.ac.uk/solutions/csd-discovery/components/gold/>) [42]. Cocrystallized molecules from TryR structure were removed, including water molecules, ligands, and ions. Then, H-bond acceptor and donor atoms were assumed as solvent accessible. Also, the Chemical Piecewise Linear Potential (CHEMPLP) scoring function from GOLD® software was applied for docking simulations. Furthermore, all energetic contributions and interactions (H-bond, hydrophobic, and van der Waals) were individually analyzed. All illustrations were generated by using PyMol® software, v. 0.99 (<https://pymol.org/2/>). Lastly, all procedures performed in this study are in accordance with recently published works by our research team [43–49].

3. RESULTS AND DISCUSSION

3.1 Chemistry

All chemical methodologies are illustrated in Schemes 1 to 7. Different AGH analog, **3–6** (Tables 1–3), could be easily obtained by reaction using the appropriate aldehyde or ketone with aminoguanidine hydrochloride in refluxing ethanol [24,50]. Substitution at the ω -position (**7**) was performed as illustrated in Scheme 1. Thiosemicarbazide was quantitatively methylated by using iodomethane in DMF, at room temperature. The resulting *S*-methyl thiosemicarbazide **1** was then reacted with various aliphatic or aromatic amines, where the resulting aminoguanidines (**2b–e**) were further condensed with corresponding aldehydes (Table 4) [51]. TSC derivatives **9–10** were obtained by condensation of thiosemicarbazide **8** with aldehydes in the presence of catalytic amounts of acetic acid [52]. Substituted TSC's (**10**) were obtained in two-steps reaction using hydrazine with ethyl or benzyl isothiocyanate followed by condensation with the corresponding aldehyde (Table 5) [53]. In this context, the reaction of semicarbazide hydrochloride with different aromatic aldehydes led to the *para*-substituted benzaldehyde semicarbazones (**11**) [51,54,55].

Scheme 1. Synthetic route for preparation of AGH derivatives **3–7**. Reagents and conditions: (a) iodomethane, DMF, r.t, 12h, 87%; (b) R_3NH_2 , *i*-PrOH, reflux, 12h, 45–87%; (c) AcOH cat., EtOH, reflux, 12h, yields: 25–100%.

Scheme 2. Synthetic route for preparation of TSC derivatives **9–10** and semicarbazone **11**. Reagents and conditions: (a) NH_2NH_2 , EtOH, reflux, 12h, yields: 70–74%; (b) AcOH cat., EtOH, reflux, 12h, yields: 58–99%.

Cyclic TSC derivatives **12** and **13** (rigidification performed according to pathway a, chart 1) were synthesized by condensation of **9** with dibromoethane or dibromopropane. Dihydroimidazoles **15** and tetrahydropyrimidines **16** were

prepared in three-steps reaction sequence starting from commercially available cyclic thioureas after condensation of required aldehydes with 2-hydrazinoimidazolines **14** [52]. The semi-constrained AGH (**15, 16**) and TSC analogs (**12,**

13) were designed replacing the prototypical set of H-bond interactions by a ring system able to mimetize the chemotype (aminoguanidine or thiosemicarbazone) in a specific conformation. Particularly, AGH cyclic derivatives

connecting the imine carbon to the terminal guanidine nitrogen were synthesized (rigidification according to pathway *b*, chart 1).

Scheme 3. Preparation of cyclic TSC's derivatives **12-13**. Reagents and conditions: (a) MeOH or *i*-ProH, AcONa, reflux, 12h, yields: 60-80%.

Scheme 4. Preparation of dihydroimidazole and tetrahydropyrimidines derivatives **15** and **16**. Reagents and conditions: (a) MeI, EtOH, reflux, 12h; (b) NH_2NH_2 , EtOH, reflux 12h, yields: 85-87%; (c) AcOH cat., EtOH, reflux, 12h, yields: 54-92%.

Following the pathway *c* in Chart 1, the 5-aryl-2-aminothiadiazines **20** were prepared by one-step reaction, using thiosemicarbazide with a substituted phenylacetyl chloride in methanol (Scheme 6) [52]. Reacting the 5-aryl-2-aminopyrimidines **17** with different arylboronic acids afforded the corresponding 5-aryl-2-amino dihydropyrimidine **18**. The key-step was a careful reduction of **18** into the corresponding dihydropyrimidines **19** by means of triethylsilane in the presence of TFA.

The preparation of benzotriazepinones **23a-b** (rigidification according to pathway *c*, chart 4) is outlined in scheme 7. Addition of benzonitrile to aniline, *via* Sugawasa

reaction, produced the desired 2-aminobenzophenone **21**. This derivative was then involved in a condensation reaction with the help of trichlorophosgene and *N*-PMB-*tert*-butylcarbazate followed by a cyclization step in TFA leading to **23**, within 67% of yield (overall yield starting from **21**). Compound **23** was then converted into the corresponding thiocarbonyl derivative **24** by treatment with Lawesson's reagent under microwave irradiations. Finally, conducting the reaction of **24** with *p*-MeO benzylamine in the presence of HgCl_2 led to the target amino triazepine **25** after the removal of the PMB group. All target compounds exhibited satisfactory spectral data and mass spectra consistent with their structures.

Scheme 5. Synthetic route for preparation of 5-aryl-2-aminodihydropyrimidine **19**. Reagents and conditions: (a) ArB(OH)_2 , Pd(OAc)_2 , SPhos, K_2CO_3 , MeCN/ H_2O , 95 °C, 12h; (b) TES, TFA, DCM, r.t, 24h, yields: 68-88%.

Scheme 6. Synthetic route for the preparation of 5-aryl-2-aminothiadiazine **20**. Reagents and conditions: (a) MeOH, reflux, 15 min.

Scheme 7. Synthetic route for preparation of benzotriazepinone **23** and related compounds **24-25**. *Reagents and conditions:* (a) PhNH_2 , BCl_3 , AlCl_3 , toluene; (b) Triphosgene, Et_3N , DCM, 15 min. then BocNHNHPMB , 18h; (c) TFA, DCM, reflux, 12h, yield: 77%; (d) Lawesson's reagent, benzotrifluoride, MW irradiation, 130 °C, 30 min., yield: 38%; (e) DMB-NH_2 , Et_3N , HgCl_2 , DMF, 4h, 49%; (f) TFA, DCM, MW irradiation, 5 min., yield: 92%.

3.2. Biological evaluation and Structure-Activity Relationship (SAR) Analysis

The cytotoxicity of all synthesized compounds was determined using the MTT method [38,39] performed on the *J774.A1* cell line, and compared to pentamidine (gold-standard drug). The host cells were treated with AGH analogs and compared with the vehicle (0.1% DMSO). The MTT assay measures the metabolic activity of cells and quantifies the metabolic reduction of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) carried by the mitochondrial enzyme succinate dehydrogenase associated with NADPH and NADH, which results in the production of formazan crystals, which are quantified by spectrophotometry. Non-toxic compounds were subjected to the amastigote assay (at 10 μM concentration) [54], considered as a well-known method agreed by the scientific community for *in vitro* *Leishmania* drug discovery research and for evaluation of the resistance of field strains, as well as this assay is capable of evaluating host cell-mediated effects [11]. Both maximum effect (E_{max}) and IC_{50} values against *L. chagasi* amastigotes were used as parameters for antileishmanial activity. For cytotoxicity, % of the kill of *J774.A1* cells at 10 μM and CC_{50} values (only for the most active compounds) were analyzed. Selectivity index (SI) of each treatment was calculated by dividing the CC_{50} observed upon *J774.A1* macrophage *per* IC_{50} value. All biological results are given in Tables 1-4 (AGH), 5 (TSC) and 6-7 (semi-constrained AGH and TSC analogs)..

The non-substituted AGH derivative **3a** was considered as a starting point for a hit-to-lead workflow. This compound showed some antileishmanial activity (E_{max} : 48.3% at 10 μM) and poor cytotoxic effects (17.5% of *J774.A1* killed). SAR analysis showed that the introduction of either electron-withdrawing or electron-donating substituents in *ortho*, *meta* and/or *para* positions of the phenyl ring (Table 1), substitution of the phenyl by cycloalkyl, naphthalene, phenethyl, styryl and heteroaryl groups (Table 2), as well as the presence of cycloalkyl-fused rings (Table 3) or

substituents at the *N*-position (Table 4) affect the cytotoxicity and antileishmanial activity. Deeming these facts, many AGH compounds showed high cytotoxicity (**3l**, **3z**, **5g**, **5h**, **6a**, **7b-e**, and **7g-h**; >50% of *J774.A1* killed) or moderate cytotoxicity (**3e**, **3j-l**, **3o-p**, **3w-x**, **4b-c**, and **5f**; 50% < *J774.A1* killed > 30%), which were immediately discarded from the first screening. The vehicle used to solubilize all substances (0.1% DMSO) showed no significant toxic effects to cells when compared to the negative control (cells were grown in culture medium). Thus, the toxicity of the substances cannot be attributed to the presence of DMSO in culture (data not shown). Some others compounds were non-toxic or less toxic (**3b-d**, **3f-h**, **3n**, **3q-v**, **3y**, **4d-g**, **5a-c**, **5e**, **5i-o**, **6b**, **6d-f**, **7a**, and **7f**; $\leq 17.5\%$ of *J774.A1* killed) when compared with **3a**, but showed less activity or were not active against the parasite ($E_{\text{max}} < 48.3\%$).

Among the different substitution introduced at the phenyl ring, a beneficial *ortho* substitution effect was particularly interesting with AGH **3i** (*o,m*- Cl_2) and **4a** (*o*-Ph). These two compounds were selected due to their non-toxic and antileishmanial effects, with IC_{50} values of 7.27 and 4.97 μM , SI values of 11 and 15, and maximal effect of 63.5 and 63.8%, respectively. In addition, replacement of the phenyl by various heterocycles such as monocycles (furan, thiophene, pyridine), or bicycles (indole, quinolone, benzofuran) led to inactive or cytotoxic compounds. However, the introduction of a quinoline moiety leading to **5d** was interesting: this compound showed a submicromolar IC_{50} value (0.6 μM), better ratio of efficacy to toxicity (SI of 87) and an efficient E_{max} (55%). Interestingly, shifting the position of the aminoguanidine hydrazone fragment from position 2 to position 4 or replacing the quinoline ring with a mimetic benzofuran led to significant loss of activity (comparing **5d** and **5c**). Further substitution of the terminal nitrogen ($\text{R}_3 \neq \text{H}$, **7a-h**) dramatically increases the cytotoxicity of the molecules. It may highlight the importance of the free NH_2 group present in the AGH series. This hypothesis was further

supported by the inactivity of imidazolines (**15a-c**) and tetrahydropyrimidines (**16a-c**).

Table 1. Antileishmanial activity and cytotoxicity of AGH derivatives against *L. chagasi* amastigotes.

Code	R ₁ or Ar	IC ₅₀ (μM)	Maximal effect (%)	% Cytotoxicity	CC ₅₀ /SI

3a	Ph	>10	48.3 ± 3.2***	17.5 ± 4***	—
3b	<i>p</i> -F	>10	—	nt	—
3c	<i>p</i> -Cl	>10	—	nt	—
3d	<i>m</i> -Cl	>10	—	nt	—
3e	<i>o</i> -Cl	—	—	46 ± 1***	—
3f	<i>m,m'</i> -Cl ₂	>10	—	17.5 ± 3***	—
3g	<i>p</i> -Br	>10	—	nt	—
3h	<i>o,p</i> -Cl ₂	—	—	14.4 ± 1.7**	—
3i	<i>o,m</i> -Cl ₂	7.27 ± 0.3	63.5 ± 1***	nt	81.06 ± 0.8/11
3j	<i>o,o'</i> -Cl ₂	—	—	32.4 ± 3***	—
3k	<i>m,p</i> -Cl ₂	—	—	35.5 ± 5***	—
3l	<i>o</i> -CF ₃	—	—	51.9 ± 0.6***	—
3m	<i>p</i> -CF ₃	—	—	20.7 ± 5	—
3n	<i>p</i> -CN	>10	—	nt	—
3o	<i>p</i> -CO ₂ Me	—	—	30.7 ± 14***	—
3p	<i>p</i> -CO ₂ H	—	—	33.2 ± 0.5***	—
3q	<i>p</i> -OMe	>10	—	nt	—
3r	<i>m</i> -OMe	>10	—	nt	—
3s	<i>o</i> -OMe	>10	—	nt	—
3t	<i>p</i> -OH	>10	29.0 ± 1.3**	16.8 ± 4**	—
3u	<i>p</i> -OH, <i>m</i> -OMe	>10	—	nt	—
3v	<i>p</i> -OMe, <i>m</i> -OH	>10	—	nt	—
3w	<i>m,p</i> -(OMe) ₂	—	—	31.5 ± 5*	—
3x	<i>p</i> -OH, <i>m,m'</i> -(<i>t</i> -Bu) ₂	—	—	32.6 ± 4*	—
3y	<i>m,p</i> -(OCH ₂ O)	>10	—	nt	—
3z	<i>p</i> -Ph	—	—	63.1 ± 2***	—
4a	<i>o</i> -Ph	4.97 ± 0.8	63.8 ± 0.4***	nt	75.4 ± 1.03/15

4b		—	—	42 ± 5**	—
4c		—	—	32 ± 5**	—
4d	Ph-(CH ₂) ₂ -	>10	—	nt	—
4e	Ph-CH=CH-	>10	—	nt	—
4f		>10	—	nt	—
4g		>10	30.7 ± 4**	nt	—
Pentamidine		4.4 ± 0.9	67.3 ± 2***	nt	—

IC₅₀: inhibitory concentration 50% of amastigotes forms, calculated by concentration-response curves toxic and expressed as mean ± standard error of the mean; Maximum effect: mean of the maximum toxicity ± standard error of the mean in triplicates of a representative experiment; Cytotoxicity: mean of the maximum cytotoxicity ± standard error of the mean cytotoxicity at the concentration of 10 μM in duplicates of a representative experiment. The maximum effect and cytotoxicity values were considered significant when * *p* < 0.05, ** *p* < 0.01 and *** *p* < 0.001 compared to the 0.1% DMSO group; nt: non-toxic, the substance did not present significant lethal activity for macrophages J774.A1 to the concentration of 10 μM in relation to the DMSO group; —: not determined (for IC₅₀ and maximal effect, when the compound was toxic against macrophages J774.A1; only for maximal effect, when IC₅₀ > 10 μM; for CC₅₀ and SI, when the compound was inactive against amastigotes forms); SI (selectivity index) = CC₅₀/IC₅₀; Pentamidine was used as positive control.

Table 2. Antileishmanial activity and cytotoxicity of heteroaryl AGH derivatives against *L. chagasi* amastigotes.

Code	Het	IC ₅₀ (μM)	Maximal effect (%)	% Cytotoxicity	CC ₅₀ /SI

5a		>10	—	nt	—
5b		>10	—	nt	—
5c		>10	31.9 ± 2**	nt	—
5d		0.6 ± 0.06	55.3 ± 0.5***	nt	52.5 ± 0.51/87
5e		>10	46.5 ± 6***	nt	—
5f		—	—	34.8 ± 4***	—
5g		—	—	98.4 ± 1***	—
5h		—	—	97.1 ± 3***	—
5i		>10	—	nt	—
5j		>10	—	nt	—
5k		>10	—	nt	—
5l		>10	40.7 ± 1**	nt	—
5m		>10	21.2 ± 5**	nt	—
5n		>10	—	nt	—
5o		>10	—	nt	—
Pentamidine		4.4 ± 0.9	67.3 ± 2***	nt	—

For footnotes, see table 1.

Table 3. Antileishmanial activity and cytotoxicity of disubstituted AGH derivatives against *L. chagasi* amastigotes.

Code	R ₁	R ₂	IC ₅₀ (μM)	Maximal effect (%)	% Cytotoxicity

6a	Ph	Ph	–	–	75.3 ± 2***
6b			>10	43.4 ± 3**	nt
6c			>10	–	18 ± 3**
6d			>10	24.0 ± 6.6**	nt
6e			>10	33.3 ± 5**	nt
6f			>10	31.8 ± 3**	nt
PTD			4.4 ± 0.9	67.3 ± 2***	nt

For footnotes, see table 1.

Table 4. Antileishmanial activity and cytotoxicity of *N*-substituted AGH derivatives against *L. chagasi* amastigotes.

Code	R ₁	R ₃	IC ₅₀ (μM)	Maximal effect (%)	% Cytotoxicity

7a	<i>m,p</i> -Cl ₂	Et	>10	35.8 ± 4.9**	nt
7b	<i>m,p</i> -Cl ₂	Ph	–	–	85.5 ± 0.4***
7c	<i>m,p</i> -Cl ₂	Bn	–	–	97.1 ± 3***
7d	<i>m,p</i> -Cl ₂	(CH ₂) ₂ Ph	–	–	94.1 ± 0.6***
7e	<i>m,p</i> -Cl ₂	(CH ₂) ₃ Ph	–	–	97.1 ± 3***
7f	<i>m,p</i> -(OMe) ₂	Et	>10	35.8 ± 5**	nt
7g	<i>m,p</i> -(OMe) ₂	Bn	–	–	75.9 ± 3***
7h	<i>m,p</i> -(OMe) ₂	(CH ₂) ₂ Ph	–	–	56.1 ± 3***
Pentamidine			4.4 ± 0.9	67.3 ± 2***	nt

For footnotes, see table 1.

Replacement of the guanidine moiety in **3a** with its isosteric thiourea yielded **9a**, which was not toxic, but showed less activity (E_{max} of 40.49%), when compared with the corresponding AGH (**3a**). A set of substituted TSC's **9a-9w** was tested, as reported in Table 5. A first remark emphasizes TSC derivatives with a lack of cytotoxicity (except derivative **9u**, which showed moderate cytotoxicity; 30.6 % of *J774.A1* killed) and more actives when compared with AGH's. Many non-toxic TSC's presented less activity against the parasite when compared with **3a** (**9b**, **9f**, **9m-n**,

9q-t and **9x**; $E_{max} < 40.49\%$) or were completely inactive toward the parasite (**9c-e**, **9g-h**, **9j**, **9l**, **9p**, **9v**, and **9w**). However, some activity could be recovered in the substituted TSC-series through beneficial effects of aromatic substitutions, and this beneficial effect is not the same for the two series AGH's and TSC's. In particular, the introduction in the *para* position of a methoxyl group (**9o**), a trifluoromethyl group (**9k**), or *m,p*-dichlorine (**9i**) led to active compounds with IC₅₀ values ranged from 1.6 to 3.2 μM, satisfactory SI values ranging from 25 to 44, and E_{max}

between 60.2 and 66.38%. Interestingly, the most active TSC's all possess a substituent in *para* position (**9i**, **9k**, **9o**), whereas some of the most active AGH's present a substituent in the *ortho* position (**3i**, **4a**).

Furthermore, as observed previously, the *N*-substituted TSCs led to inactive compounds **10a** and **10b**. Finally, with

respect to the other oxygen isosters (S→O), the corresponding semicarbazones presented moderate cytotoxicity (**11a-b**; 50% < *J774.A1* killed > 30%), and were too discarded from the first screening.

Table 5. Antileishmanial activity and cytotoxicity of substituted TSC derivatives against *L. chagasi* amastigotes.

Code	R ₁	X	R ₃	IC ₅₀ (μM)	Maximal effect (%)	% Cytotoxicity	CC ₅₀ /SI

9a	H	S	H	>10	40.49 ± 0.9***	nt	—
9b	<i>p</i> -Cl	S	H	>10	28.37 ± 2.6**	nt	—
9c	<i>m</i> -Cl	S	H	>10	—	nt	—
9d	<i>o</i> -Cl	S	H	>10	—	nt	—
9e	<i>m,m'</i> -Cl ₂	S	H	>10	—	nt	—
9f	<i>p</i> -Br	S	H	8.73 ± 0.2	56.91 ± 0.6***	nt	69.66 ± 0.85/8
9g	<i>o,m</i> -Cl ₂	S	H	>10	—	nt	—
9h	<i>o,o'</i> -Cl ₂	S	H	>10	—	nt	—
9i	<i>m,p</i> -Cl ₂	S	H	2.2 ± 1.8	66.38 ± 4***	nt	69.01 ± 0.4/31
9j	<i>o</i> -CF ₃	S	H	>10	—	nt	—
9k	<i>p</i> -CF ₃	S	H	3.2 ± 1	60.4 ± 1.6***	nt	82.08 ± 1.2/25
9l	<i>p</i> -CN	S	H	>10	—	nt	—
9m	<i>p</i> -CO ₂ Me	S	H	>10	30.37 ± 4.4**	nt	—
9n	<i>p</i> -CO ₂ H	S	H	>10	26.6 ± 0.8**	nt	—
9o	<i>p</i> -OMe	S	H	1.6 ± 1.1	60.2 ± 1.8***	nt	70.3 ± 0.97/44
9p	<i>m</i> -OMe	S	H	>10	—	nt	—
9q	<i>p</i> -OH	S	H	>10	38.02 ± 0.2**	nt	—
9r	<i>p</i> -OH, <i>m</i> -OMe	S	H	>10	26.42 ± 1**	nt	—
9s	<i>p</i> -OMe, <i>m</i> -OH	S	H	>10	40.25 ± 2.5***	nt	—
9t	<i>m,p</i> -(OMe) ₂	S	H	>10	35.56 ± 0.2**	nt	—
9u	<i>p</i> -Ph	S	H	—	—	30.6 ± 6**	—
9v	<i>p</i> -F	S	H	>10	—	nt	—
9w	<i>p</i> -OH, <i>m,m'</i> -(<i>t</i> -Bu) ₂	S	H	>10	—	nt	—
9x	<i>m,p</i> -(OCH ₂ O)	S	H	>10	27.65 ± 3.7*	nt	—
10a	<i>m,p</i> -Cl ₂	S	Et	>10	—	nt	—
10b	<i>m,p</i> -Cl ₂	S	Bn	>10	—	nt	—
11a	<i>m,p</i> -Cl ₂	O	H	—	—	40.4 ± 2**	—
11b	<i>m,p</i> -(OMe) ₂	O	H	—	—	31 ± 2**	—
Pentamidine				4.4 ± 0.9	67.3 ± 2***	nt	—

For footnotes, see table 1.

Rigidification for both AGH and TSC moieties involving terminal nitrogens (or nitrogen and sulfur in TSC, mode *a*) led to the corresponding dihydroimidazolines **15a-c** (X = NH, *n* = 1), tetrahydropyrimidines **16a-c** (X = NH, *n* = 2), thiazolidines **12a-c** (X = S, *n* = 2) or dihydrothiazines **13a-c** (X = S, *n* = 2) (Table 6). As observed with highly cytotoxic *N*-substituted AGH (R₁ ≠ H), all the cyclized AGH **15** and **16** showed a relative cytotoxic effect, except the tetrahydropyrimidine **15a** (*m*-Cl-Ph), which was not toxic, but inactive against the parasite. All the cyclized TSC **12** and **13** were devoted to antileishmanial properties, but were inactive or presented poor activity against the parasite. Finally, the mode *a* of cyclization of both AGH and TSC was disappointing and was not more explored.

AGH and their isosteres present a specific chemotype (aminoguanidine) surrounded by different substituents (aryl, R₁, R₂ ≠ H). Efforts in heterocyclic chemistry led to scaffolds with the efficient incorporation of the aminoguanidine into a rigid frame. Thus, the dihydropyrimidines (**18**), azines (**19** and **20**), and benzotriazepines (**23-25**) were designed as putative mimetics (Chart 4, modes *b* and *c*) synthesized and tested as antileishmanial agents. However, most of the resulting constrained AGH (**18**, **19**, **25**) and TSC (**20**, **24**) were found cytotoxic or presented poor antileishmanial activity (Table 7).

Table 6. Antileishmanial activity and cytotoxicity of cyclized AGH (dihydroimidazolines **15**, n= 1 and tetrahydropyrimidines **16**, n= 2) and TSC isosteres (thiazolidines **12** and dihydrothiazines **13**) against *L. chagasi* amastigotes.

Code	R ₁	X	n	IC ₅₀ (μM)	Maximal effect (%)	% Cytotoxicity

15a	<i>m</i> -Cl	NH	1	>10	—	nt
16a	<i>m</i> -Cl	NH	2	—	—	47.4 ± 1.2***
15b	<i>m,p</i> -Cl ₂	NH	1	—	—	46.8 ± 2.5***
16b	<i>m,p</i> -Cl ₂	NH	2	—	—	42.05 ± 2***
15c	<i>m,p</i> -(OMe) ₂	NH	1	—	—	27.4 ± 0.3***
16c	<i>m,p</i> -(OMe) ₂	NH	2	—	—	27.2 ± 1.8***
12a	<i>m</i> -Cl	S	1	>10	—	nt
13a	<i>m</i> -Cl	S	2	>10	—	nt
12b	<i>m,p</i> -Cl ₂	S	1	>10	—	nt
13b	<i>m,p</i> -Cl ₂	S	2	>10	—	nt
12c	<i>m,p</i> -(OMe) ₂	S	1	>10	27.6 ± 2**	nt
13c	<i>m,p</i> -(OMe) ₂	S	2	>10	—	nt
Pentamidine				4.4 ± 0.9	67.3 ± 2***	nt

For footnotes, see table 1.

Table 7. Antileishmanial activity and cytotoxicity of constrained AGH and TSC derivatives against *L. chagasi* amastigotes.

Code	Structures	R ₁	IC ₅₀ (μM)	Maximal effect (%)	% Cytotoxicity
18a		<i>m,p</i> -Cl ₂	>10	—	nt
18b		<i>m</i> -Cl	>10	44.25 ± 3.8***	nt
18c		<i>m,p</i> -(OMe) ₂	>10	28.7 ± 6.9*	nt
19a		<i>m,p</i> -Cl ₂	>10	—	nt
19b		<i>m</i> -Cl	—	—	35.4 ± 1.7**
19c		<i>m,p</i> -(OMe) ₂	—	—	34.6 ± 4.3**
20a		<i>m,p</i> -Cl ₂	—	—	45.7 ± 7.3***
20b		<i>m</i> -Cl	—	—	54 ± 2***
20c		<i>m,p</i> -(OMe) ₂	>10	—	nt
23a		<i>m,p</i> -Cl ₂	>10	—	nt
23b		<i>m</i> -Cl	—	—	36.5 ± 2.6***
24a		<i>m,p</i> -Cl ₂	—	—	46.7 ± 6.4***
24b		<i>m</i> -Cl	—	—	45.7 ± 0.7***
25a		<i>m,p</i> -Cl ₂	—	—	63.5 ± 3.2***
25b		<i>m</i> -Cl	—	—	63 ± 1.2***
Pentamidine			4.4 ± 0.9	67.3 ± 2***	nt

For footnotes, see table 1.

After the obtainment of all information derived from the SAR's analysis, it was possible to rationalize a chemical structure representative for all chemical derivatives from

AGH's and TSC's containing the most significant structural modifications, in terms of medicinal chemistry. Finally, these important points are summarized in Figure 4.

Fig. 4. The most significant structure modifications rationalized from the SAR analysis.

The exact mechanism of antileishmanial activity of the actives TSC and AGH derivatives studied in this work is not known. Although, the hydrazones have been previously reported as potential inhibitors of TryR from *Leishmania* [23], an enzyme that participates in the crucial antioxidant system of the parasite [56]. The TryR has a negatively charged active site that requires a positively charged amino chain in the substrate or inhibitor. Likewise, a structure-property relationship analysis of 4-phthalazinylhydrazones, realized by Romero and López [57], showed that the chloro on the aryl group plays an important role in the ligand-TryR binding. Deeming these data and in order to provide some insights about a potential mechanism of action, *in silico* studies involving molecular docking analyses were also performed in this work.

3.3. Molecular Docking Analyses upon Trypanothione Reductase (TryR) from *Leishmania*

Considering the docking simulations performed on the TryR enzyme from *L. infantum*, it was verified that compounds **3i**, **4a**, **5d**, **9i**, **9k**, and **9o** interact with this target with FitScore values of 53.63, 63.33, 66.76, 49.71, 54.11, and 49.75, respectively. These values suggest that there is no a FitScore-IC₅₀ correlation. Therefore, the visual inspection revealed important molecular features. Initially, it is possible to verify that the active compounds are placed into the same binding site (Figure 5A). Interestingly, AGH **3i** and **4a** are overlapped in the binding site, and they present the highest IC₅₀ values, 7.27 ± 0.3 and 4.97 ± 0.8 μ M, respectively. Similarly, TSC **9k** and **9o** analogs are also overlapped, and they demonstrated similar IC₅₀ values, 3.2 ± 1 and 1.6 ± 1.1 μ M, respectively. The TSC **9i** derivative presents a hybrid binding mode between **3i** and **9o** analogs, which probably results in an IC₅₀ value of 2.2 ± 1.8 μ M. Lastly, the AGH **5d** (the highest FitScore value) showed a different binding mode than other hit compounds,

suggesting which it could be essential for a low an IC₅₀ value, being equal to 0.6 ± 0.06 μ M.

TSC derivatives (**9i**, **9k**, and **9o**) present interactions with 4-6 amino acids. Regarding this, **9i** hydrophobically interacts with Ser¹⁴, Thr⁵¹, Lys⁶⁰, Ser¹⁷⁸, and Ile¹⁹⁹, while shows two hydrogen-bonding interactions with Thr³³⁵ residue. Interestingly, **9k** and **9o** interact with Tyr¹⁹⁸ (via H-bond for **9o**), Met³³³ (via two H-bonds for **9k** and **9o**), and Ala³³⁸ residues. Additionally, **9k** interacts with Leu³³⁴, while the best TSC analog (**9o**) presents hydrophobic interaction with Ser¹⁴. In contrast, AGH compounds, **3i**, **4a**, and **5d**, interact with the Ser¹⁷⁸, Tyr¹⁹⁸ (via H-bond for **3i**), Ile¹⁹⁹, Phe²⁰³, and Met³³³ (via H-bond for **3i**) (See supplementary material). Surprisingly, the best AGH analog (**5d**) presents four hydrogen bonding interactions with the TryR enzyme. However, only **5d** interacts with Ala³⁶⁵ and His⁴⁶¹ amino acids (Figure 5B). It was observed that **5d** performs two hydrogen bonding interactions with Ala³⁶⁵ at distances of 1.8 and 2.13 Å, respectively. Also, it has hydrogen bonding interactions with His⁴⁶¹ residue at distances of 2.1 and 2.0 Å. Then, it is possible to suggest that both of these residues are critical for the antileishmanial activity. Also, Romero *et al.* [58,59] have developed some studies involving 4-phthalazinyl-hydrazones targeting enzyme related to oxidative stress mechanisms from *Leishmania*. In one of these studies has been revealed that the most active inhibitor interacts with the Ala³⁶⁵ residue from *Leishmania* TryR enzyme. Then, the authors suggested that this inhibitor could act by an oxidative stress mechanism, in which the parasite would be unprotected against oxidative oxygen-derived species. Moreover, Verma *et al.* [60] performed a study aiming to demonstrate the anti-TryR activity of 2H-chromene-2-thiones. So, it was observed that the most active analog synthesized was able to interact with the His⁴⁶¹ residue. In both of these studies, the most active inhibitors are bioisosteres of the quinoline nucleus present in the compound **5d**, corroborating with the molecular docking

results presented in this section and thus, suggesting that it could disrupt the redox mechanism from the *L. chagasi* parasite. Finally, it was verified that all AGH (**3i**, **4a**, and **5d**) and TSC (**9i**, **9k**, and **9o**) analogs are surrounded by residues

belonging from the TryR active site and thus, it could be concluded that the interaction of the ligands with these residues might be responsible for the antileishmanial activity observed in this study.

Fig. 5. The cluster of the hit AGH and TSC derivatives (A) and the interactions of AGH **5d** (B) in the active site of trypanothione reductase (TryR) from *Leishmania infantum* (PDB ID: 2JK6). In (A) - red: **3i**; blue: **4a**; green: **5d**; magenta: **9i**; yellow: **9k**; and cyan: **9o**. In (B) – yellow dots represent hydrogen bond interactions.

CONCLUSION

Based on the impact of the *L. chagasi* parasite worldwide, no vaccines, and undesired side-effects from first-line chemotherapy (pentavalent antimonials) for treatment of this infection, the search for effectiveness compounds (with low IC₅₀ values and less cytotoxic effects) is extremely important. Besides, it contributes to advances in medicinal chemistry of neglected diseases, as well to the development of new antileishmanial alternatives. In this work, we have performed a screening of a series of AGH derivatives, isosteres, and semi-rigid structurally-related compounds for the rational search of novel antileishmanial agents. Amastigotes of *L. chagasi* was selected as the most valuable evolutionary forms, and after the exclusion of cytotoxic compounds, the final selection includes three AGH derivatives **3i**, **4a**, and **5d** and three TSC derivatives **9i**, **9k** and **9o**. These compounds presented antileishmanial activity compared with PTD, showing IC₅₀ values ranged from 0.6 to 7.27 μM, maximal effects up to 55.3%, and satisfactory SI values (ranged from 11 to 87). Based on this study, it is interesting to notice that TSC class is more promising than its isosteric AGH analogs, and the beneficial aromatic substituent effects are not similar in both AGH (*ortho*-substitutions) and TSC series (*para*-substitutions), suggesting different modes of action in both very close sub-series.

Furthermore, *in silico* studies involving molecular docking have demonstrated that the most active analog, compound **5d**, interacts with a different orientation in the active site of trypanothione reductase enzyme from *L. infantum*, and it performs hydrogen bonding interactions with Ala³⁶⁵ and His⁴⁶¹ residues, which have been reported in other studies. From *in silico* analyses, it is possible

suggesting that the hit compounds act by interfering in the redox system from the *L. chagasi* amastigotes. Finally, the next step of further pharmacological assays (profiling of selected hits *versus* other leishmania strains) will help to choose the best compounds for *in vivo* experiments.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

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CONFLICT OF INTEREST

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article.

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