In vitro evaluation of antitrypanosomal activity and molecular docking of benzoylthioureas

Patricia M.L. Pereira, Priscila G. Camargo, Bruna T. Gonçalves, Luiz A.P. Flores-Junior, Luiza R.S. Dias, Camilo H.S. Lima, Phileno Pinge-Filho, Lucy M.Y. Lioni, Sueli F. Yamada-Ogatta, Marcelle L.F. Bispo, Fernando Macedo

PII: S1383-5769(20)30175-6

DOI: https://doi.org/10.1016/j.parint.2020.102225

Reference: PARINT 102225

To appear in: *Parasitology International*

Received date: 30 June 2020

Accepted date: 12 October 2020

Please cite this article as: P.M.L. Pereira, P.G. Camargo, B.T. Gonçalves, et al., In vitro evaluation of antitrypanosomal activity and molecular docking of benzoylthioureas, *Parasitology International* (2019), https://doi.org/10.1016/j.parint.2020.102225

IN VITRO EVALUATION OF ANTITRYPANOSOMAL ACTIVITY AND MOLECULAR DOCKING OF BENZOYLTHIOUREAS

Patricia M.L. Pereira[a], Priscila G. Camargo[b], Bruna T. Gonçalves[a], Luiz A. P. Flores-Junior[c], Luiza R. S. Dias[c], Camilo H. S. Lima[c,d], Phileno Pinge-Filho[e], Lucy M. Y. Lioni[a], Sueli F. Yamada-Ogatta[a], Marcelle L. F. Bispo[b,*], Fernando Macedo Jr[b].

[a]Laboratório de Biologia Molecular de Microrganismos, Departamento de Microbiologia, Centro de Ciências Biológicas, Universidade Estadual de Londrina, Londrina, Paraná, Brazil.

[b]Departamento de Química, Centro de Ciências Exatas, Universidade Estadual de Londrina, Londrina, Paraná, Brazil.

[c]Laboratório de Química Medicinal, Faculdade de Farmácia, Universidade Federal Fluminense, Niterói, Rio de Janeiro, Brazil.

[d]Departamento de Química Orgânica, Instituto de Química, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil.

[e]Laboratório de Imunopatologia Experimental, Departamento de Ciências Patológicas, Centro de Ciências Biológicas, Universidade Estadual de Londrina.

Abstract

A series of sixteen benzoylthioureas derivatives were initially evaluated *in vitro* against the epimastigote form of *Trypanosoma cruzi*. All of the tested compounds inhibited the growth of this form of the parasite, and due to the promising anti-epimastigote activity from three of these compounds, they were also assayed against the trypomastigote and amastigote forms. ADMET-Tox *in silico* predictions and molecular docking studies with two main enzymatic targets (cruzain and CYP-51) were performed for the three compounds with the highest activity. The docking studies showed that these compounds can interact with the active site of cruzain by hydrogen bonds and can be coordinated with Fe-heme through the carbonyl oxygen atom of the CYP51. These findings can be considered an important starting point for the proposal of the benzoylthioureas as potent, selective, and multi-target antitrypanosomal agents.

Keywords: Chagas disease, CYP51, cruzain, *Trypanosoma cruzi*, molecular docking.

1. Introduction

American trypanosomiasis or Chagas disease is a neglected tropical illness caused by the protozoan parasite *Trypanosoma cruzi*. Although still endemic in Latin America, the presence of Chagas disease in other continents can be related to immigration and the globalization events, which have changed its epidemiological profile. According to the World Health Organization (WHO), about 8 million people are infected with this protozoan worldwide, and more than 10000 deaths occur each year [1,2].

The *T. cruzi* life cycle alternates between the insect vector and the host mammalian. The natural human infection results from the bites of infected hematophagous reduviid vectors which release the non-proliferative metacyclic trypomastigotes into their excreta (feces and urine) close to the injured skin or intact mucosal membranes [3,4]. In host cells, metacyclic trypomastigotes differentiate into replicative amastigote forms [5], which multiply by binary fission. The intracellular amastigote forms in high density transform into trypomastigotes, which can invade new cells or are released into the bloodstream, reaching other organs [6]. The blood-circulating parasites can serve to complete the life cycle when they are taken up by the insect vector during its blood meal. In this invertebrate host, bloodstream trypomastigotes differentiate into the proliferative epimastigotes, which multiply in the midgut, also by binary fission. Finally, epimastigotes migrate and adhere to the rectal epithelium and differentiate into metacyclic trypomastigotes [4]. *T. cruzi* can also be transmitted by contaminated food, blood transfusion, organ transplantation, and vertically from mother to newborn [7].

Overall, the clinical course of Chagas disease comprises acute and chronic phases. The acute phase is generally asymptomatic or presents non-specific symptoms [8]. This phase is characterized by high parasitemia and lasts 4-8 weeks; if untreated, patients remain chronically infected and around 30-40% can develop organ involvement such as cardiomyopathy and megaesophagus and/or megacolon after 10-30 years of infection [7]. Progression of heart failure accounts for the most of Chagas disease morbidity and related mortality [9]. The etiologic treatment for Chagas disease is restricted to two nitroheterocyclic drugs introduced in the 1960-1970s: Benznidazole and Nifurtimox [10]. Both are effective only in the acute or early chronic phases, require prolonged treatment durations, have mutagenic potential [11] and exhibit a wide

range of side effects, which lead to discontinuation of therapy by patients [12]. In addition, there is no vaccine for the prevention of Chagas disease [13] and efforts are focused on controlling transmission, which impulses to the search of new antitrypanosomal agents with low toxicity and greater efficiency [14].

Due to its great synthetic and biological versatility, thiourea plays an important role in drug discovery since thiourea-containing compounds have a wide range of biological activities, such as antitumoral [15], anti-diabetes [16], anti-adipogenesis[17], antibacterial [18], antifungal [19] and antiviral [20]. Furthermore, there are reports in the literature indicating that thiourea scaffold could represent a promising pharmacophore in the search for new therapies against protozoal diseases, such as malaria [21], leishmaniasis [22] and Chagas disease [23]. Considering this huge potential, our research group has been investigating the antimicrobial potential of a series of benzoylthiourea, which showed interesting results against *Mycobacterium tuberculosis* (minimal inhibitory concentration = 423.1 - 9.6 μM)[24] and against other species of bacteria and fungi (unpublished data). Owing to these exciting results and supported by the literature reports on antiprotozoal activity of thiourea derivatives, we decided to investigate the antitrypanosomal activity of this class of compounds. Therefore, in this work we described the *in vitro* evaluation of sixteen benzoylthiourea derivatives (**1a-p**, Figure **1**) against epimastigote forms of *T. cruzi* as well as the cytotoxic evaluation in mammalian cells. Thereafter, a preliminary structure-activity relationship (SAR) study was conducted to determine the important structural features of this series of compounds for the biological activity and also to find out the hit compounds. The most promising compounds were then selected to be assayed against the infective trypomastigote and the intracellular replicative amastigote forms of *T. cruzi*. Finally, molecular docking studies were carried out in two important molecular targets of *T. cruzi*, cruzipain (CRZ) and C14α-sterol demethylase (CYP51) with the aim to demonstrate possible molecular mechanisms of action of these compounds.

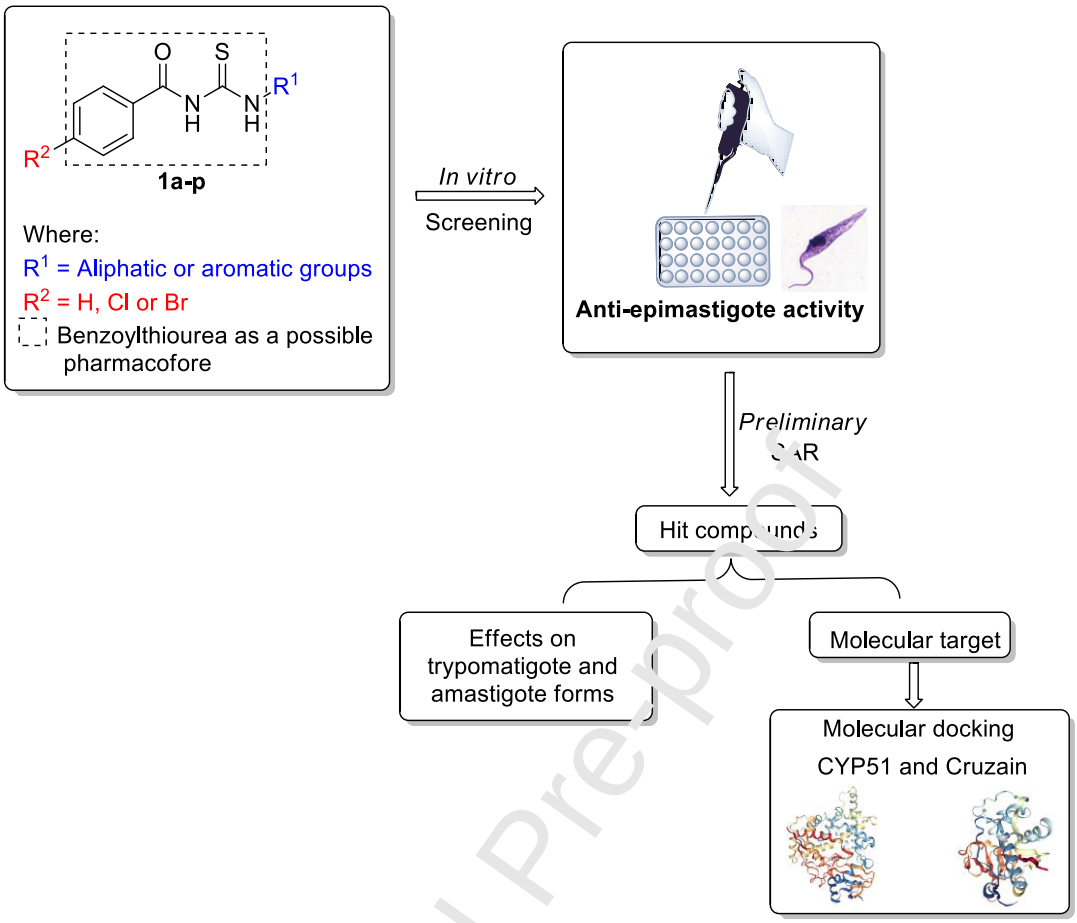


Figure 1. Experimental approach to evaluate the antitrypanosomal potential of benzoylthioureas (**1a-p**).

2. Experimental section

2.1 Cytotoxicity to mammalian cells

LLCMK2 (kidney epithelial cells of *Macaca mulatta*, CCL-7, ATCC, USA) cell line was maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 2 mM L-glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin, 1% tylosin and 5% CO_2 at 37°C. Peritoneal macrophages were obtained from male BALB/c mice (6 to 8 weeks old). 5% thioglycolate (1.0 mL) was injected intraperitoneally into the animals, and 4 days later, the macrophages were harvested by washing the peritoneal cavities with sterile and cold 0.1 M phosphate-buffered saline (PBS), pH 7.2. The cytotoxicity of all benzoylthioureas were evaluated on LLCMK2 cells; and the toxicity of lead compounds were also evaluated on peritoneal macrophages. For both mammalian cells, 2.5 x 10^5 cells/mL

were cultured into 96-well plate for 24 h [25]. Non-adherent cells were removed by washing with sterile PBS. The medium containing different concentrations of compounds (3.12 - 400 µg/mL) was added to each well and the plates were incubated for further 48 h. Cells cultured in growth medium alone were used as controls. Cell viability was determined by the reduction of the tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide - MTT (Merck) method according to the manufacturer's recommendation. The 50% cytotoxic concentration (CC_{50}) that corresponds to the concentration of compounds needed to inhibit the viable cells up to 50% was calculated by regression analysis. The selectivity index (SI) was calculated using the equation: SI = CC_{50}/IC_{50}, where IC_{50} corresponds the minimal concentration of the compounds that causes 50% of antitrypanosomal activity [25]. This study protocol was approved by the Ethics in Animal Utilization Committee (CEUA) of the Universidade Estadual de Londrina (UEL), Paraná, Brazil (CEUA/UEL number 15516.2018.20).

2.2 *Trypanosoma cruzi*

Trypanosoma cruzi Y strain was used in all assays. Epimatigotes were maintained by weekly transfers in liver infusion tryptose (LIT) medium [26] supplemented with 10% of heat inactivated FBS at 28°C; and cells obtained from 4-day incubations were used in all assays. Trypomastigotes were harvested by centrifugation (850 x *g* for 5 min) from the supernatant of infected LLCMK2 cells cultured in 25 cm^2 flasks at 37°C after 120 h post-infection.

2.3 Effect on growth of epimastigotes

Epimastigotes (1.0 x 10^6 cells/mL) in exponential growth phase were inoculated into 24-well plate containing LIT-FBS medium with different concentrations of compounds (6.25 - 100 µg/mL). The cultures were incubated at 28°C and cell growth was estimated by direct counting in a hemocytometer (Improved Double Neubauer) after 72 h. Wells containing growth medium alone and medium plus 1% DMSO were used as controls. The results were expressed as the minimal concentration of the compounds that causes 50% (IC_{50Epi}) of growth inhibition compared to the untreated control.

2.4 Effect on viability of trypomastigotes

Trypomastigotes (1.0 x 10^7 cells) were incubated in DMEM supplemented with 10% FBS containing different concentrations of compounds (6.25 – 100 µg/mL) in 24-well plate at 37°C and 5% CO_2 for 24 h. Viable trypomastigotes were estimated by motility and counted in a hemocytometer under a light microscope (Olympus CX31), according to the Brener method [27]. The results were expressed as the minimal concentration of the compounds that inhibits the viability of 50% ($IC_{50Trypo}$) of parasites compared to the untreated control.

2.5 Effect on intracellular amastigotes

Peritoneal macrophages (2.5 x 10^5 cells/mL) were inoculated into the 24-well plates containing round glass coverslips. At confluence, non-adherent cells were removed by gently washing with PBS. Trypomastigotes at a protozoan-cell-ratio of 5:1 was inoculated into the macrophages monolayer and incubated at 37°C in 5% CO_2 atmosphere for 2 h. Non-interiorized trypomastigotes were removed by gently washing with PBS. DMEM containing different concentration of benzoylthioureas (6.25 and 100 µg/mL) was added and the plates were incubated for 48 h. The coverslips were subjected to fixation with methanol, stained with Giemsa for 20 min and permanently prepared with Entellan (Merck). The number of infected cells and parasites per cell were determined by direct counting randomly 200 cells under a light microscope. The survival index (%) was determined by multiplying the percentage of infected cells by the average number of amastigotes per infected cell. The survival index observed in the control without treatment was considered as 100%. The results were expressed as the minimal concentration of the compounds that causes 50% (IC_{50Ama}) reduction in survival index compared to the untreated control [28].

2.6 *In silico* predictions of pharmacokinetic and toxicity parameters (ADME-Tox)

In silico ADME and the parameters related to oral bioavailability of the compounds **1c**, **1g,** and **1p** were assessed by rule-based filters from Linpinski [29] and Veber [30] using the SwissADME platform (http://www.swissadme.ch/) [31].

Prediction of the *in silico* toxicity of these compounds were performed using DataWarrior software (Version 5.2.1) [32].

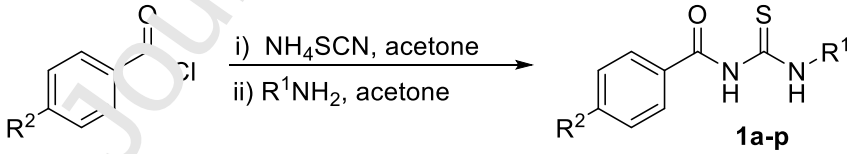
2.7 Molecular docking

Molecular docking studies were performed using Autodock 4.2 [33] and the X-ray crystal structures of CYP51 (PDB ID: 4C27, resolution: 1.95 Å) [34] and CRZ (PDB ID: 4KLB, resolution 2.62 Å) [35] available in the Protein Data Bank (PDB) server (rcsb.org) [36]. The protein preparation and the grid box size dimensions were prepared according to the protocol previously described by our research group [37]. The intermolecular interactions analyses and figures were generated by the PyMOL program (DeLano Scientific).

3. Results and Discussion

3.1. Anti-epimastigote activity and *in vitro* cytotoxicity of the Benzoylthioureas (1a-p): a preliminary SAR study

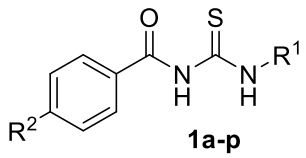
Benzoylthioureas (**1a-p**, Scheme 1) were previously synthesized by our research group by treatment of the corresponding benzoyl chloride with ammonium isothiocyanate and different nucleophilic amines. The structure of the products was fully characterized by assignments of IR, MS and ^{1}H and ^{13}C NMR spectra [24,38].



Scheme 1. Synthetic route for the preparation of Benzoylthioureas **1a-p**, previously described by our research group[24,38].

In the first step, the inhibitory effect of all benzoylthioureas was evaluated on growth of epimastigote forms of *T. cruzi* Y strain, and on viability of LLCMK2 cells. Except **1o** compound, all benzoylthioureas **(1a-n, 1p**) inhibited the growth of epimastigotes forms at non-toxic concentration to LLCMK2 cells *in vitro*. The IC_{50Epi} and CC_{50} values of the compounds ranged from 13.4 to 217.3 μM and 54.5 to 946.0 μM, respectively (Table 1).

Table 1. *In vitro* antitrypanosomal activity on epimastigotes forms (IC_{50Epi}) of *Trypanosoma cruzi*, toxicity (CC_{50}) to LLCMK22 cells and selectivity index (SI) of Benzoylthioureas **(1a-p)**.

N°	 1a-p		IC_{50Epi} (µM)	CC_{50} (µM)	SI
	R^1	R^2			
1a	n-Bu	H	98.4	303.2	3.20
1b	*n*-Hex	H	186.4	424.7	2.27
1c	*c*-Hex	H	26.4	261.3	10.6
1d	CH_2Bn	H	180.8	493.1	2.72
1e	4-OH-Ph	H	98.3	946.0	9.61
1f	4-OMe-Ph	H	141.2	457.1	3.23
1g	4-*t*-Bu-Ph	H	13.4	255.1	19.0
1h	4-Cl-Ph	H	82.6	459.4	5.56
1i	4-Br-Ph	H	64.0	392.0	6.12
1j	3-NO_2-Ph	H	87.3	910.3	10.4
1k	4-OMe-Ph	Cl	132.0	652.5	4.94
1l	4-Cl-Ph	Cl	217.3	495.6	2.28
1m	3-NO_2-Ph	Cl	29.8	152.1	5.10
1n	4-OMe-Ph	Br	193.7	282.6	1.46
1o	4-Cl-Ph	Br	62.3	54.5	0.87
1p	3-NO_2-Ph	Br	61.1	945.7	15.5

Based on the results presented in Table 1, a preliminary study of SAR was conducted with the aim to indicate important structural features to the anti-epimastigote activity of the benzoylthioureas series (**1a-p**). In general, considering only the R^1-

substituted derivatives (**1a-j**), the aromatic subset (**1e-j**) showed more relevant IC_{50} values than the aliphatic ones (**1a-d**) for the epimastigote forms. Among the aliphatic subset (**1a-d**), the chain length could be considered a critical feature for the anti-epimastigote effect since longer and bulky chains such as n-Hex (**1b**) and CH_2Bn (**1d**) have shown the lowest activities in comparison with *n*-Bu (**1a**) and *c*-Hex (**1c**). For the aromatic subset (**1e-j**), the presence of electron-withdrawing groups attached to the benzene ring, such as chlorine (**1h**), bromine (**1i**) and nitro (**1j**), were more effective against epimastigote forms than strong electron releasing groups, such as hydroxyl (**1e**) and methoxyl (**1f**). However, the best compound of this subset was **1g**, which has a *t*-butyl group attached at 4-position of the benzene ring, which is a weak electron releasing group suggesting steric, conformational, or lipophilic effects also could be important for the anti-epimastigote effect.

In regard to the subset of derivatives (**1k-n**), which have chloro or bromo at *para*-position of benzoyl group (R^2), the chlorinated derivatives (**1k-m**) were more active than brominated ones (**1n-o**) against epimastigote forms, except for **1o.** However, the replacement of the hydrogen by a halogen seems not to contribute for the improvement of IC_{50} values when 4-OMe-Ph (**1k** and **1n**) and 4-Cl-Phe (**1l** and **1o**) are attached to R^1 position. On the other hand, when derivatives have a 3-NO_2-Ph at R^1, the presence of chloro (**1m**) or bromo (**1p**) increases the activity when compared to the respective unsubstituted derivative (**1j**).

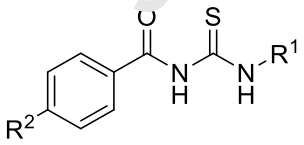
Generally, most benzoylthioureas (**1a-n, 1p**) were more relatively selective towards the epimastigote forms of *T. cruzi* than to the mammalian cells, which can be demonstrated by SI values that ranged from 1.46 to 19.0, except for **1o** (SI = 0.87). Noteworthy, the introduction of an electronegative atom at R^2 position leads to the rising of cytotoxicity, except for **1p** (SI = 15.5). Among all benzoylthioureas, the most promising compounds were **1c**, **1g**, **1j** and **1p** that showed SI values > 10 [39]. However, in combination with IC_{50} values, the derivatives **1c**, **1g** and **1p** were selected as the hit compounds of this series, since they also exhibited lower IC_{50} values, being 26.4, 13.4 µM and 61.1 µM, respectively. Therefore, these three derivatives were selected to be subjected to more specific assays, in order to also verify their effects against trypomastigote and amastigote forms of the parasite.

3.2. Anti-trypomastigote and anti-amastigote activity of the Benzoylthioureas 1c, 1g and 1p

The antitrypanosomal activity of lead compounds **1c**, **1g** and **1p** were also evaluated in clinically significant developmental forms *in vitro*, using benznidazole (BZD) as a reference drug. All derivatives selectively inhibited the viability of trypomastigote forms (Table 2). Compound **1g** was 3 times more active than the reference drug, which is also 12.2 and 6.7 times more active than **1c** and **1p**. The inhibitory effect on trypomastigotes is particularly of note, since in general these non-proliferative cells exhibit a higher inherent capacity to resist the action of antitrypanosomal compounds [40] including BZD [41].

During the acute phase of Chagas disease, macrophages play an important role in controlling *Trypanosoma cruzi* infection. Besides acting as effector cells in the initial immune defense response, these cells can serve as host for the parasite replication [42]. In this sense, we investigate the effect of the lead compounds on replication of amastigotes in murine peritoneal macrophages. The benzoylthiourea **1c**, **1g** and **1p** displayed relatively low toxicity to macrophage cells, exhibiting CC_{50} values of 261.3, 255.1 and 94.57 µg/mL, respectively. The derivative **1p** showed the most promising antiamastigote effect, exhibiting both the lowest IC_{50} and highest SI_{ama} values, indicating that this compound could be tolerable and safer.

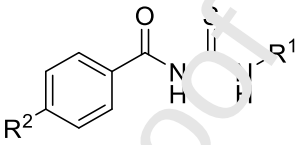
Table 2. *In vitro* antitrypanosomal activity in trypomastigote ($IC_{50Trypo}$) and amastigote (IC_{50Ama}) forms of *T. cruzi* and selective index (SI) of Benzoylthioureas **(1a-p)** and benznidazole (BZD).

N°			**$IC_{50Trypo}$ (µM)**	**IC_{50Ama} (µM)**	**CC_{50} (µM)**	**SI_{Trypo}**	**SI_{Ama}**
	R^1	**R^2**					
1c	*c*-Hex	H	54.9	24.8	261.3	4.76	10.5
1g	4-*t*-Bu-Ph	H	4.5	21.9	255.1	59.9	11.6
1p	3-NO_2-Ph	Br	30.3	16.8	945.7	31.2	56.2
BZD	-----	-----	13.5	5.28[43]	768.5	56.9	145.5

3.3 *In silico* pharmacokinetic and toxicity parameters (ADME-Tox) predictions

In order to predict the oral bioavailability, permeability profile and drug-likeness of the selected compounds (**1c**, **1g** and **1p**), we conducted an *in silico* study of their physicochemical properties (Table 3).

Table 3. *In silico* prediction of physicochemical and pharmacokinetic properties and toxicological risks of the lead compounds **1c**, **1g** and **1p**.

ADMET-Tox Properties			
	1c	**1g**	**1p**
	R^1 = *c*-Hex **R^2** = H	**R^1** = 4-*t*-Bu-Ph **R^2** = H	**R^1** = 3-NO_2-Ph **R^2** = Br
MW (g.mol^{-1})	262.3	312.4	365.2
cLogP	2.96	4.20	3.41
H-bond acceptor	2	1	2
H-bond donor	2	2	2
TPSA (Å²)	73.22	73.22	82.45
Rotatable bonds	5	6	6
Violations	0	0	0
GI absorption	High	High	High
BBB permeant	Yes	Yes	No
P-gp substrate	No	No	No
Mutagenic	None	None	None
Tumorigenic	None	None	None
Reproductive Effective	High	High	High
Irritant	None	None	None

MW: molecular weight; cLog P: partition coefficient log; TPSA: topological polar surface area; GI: gastrointestinal; BBB: blood-brain barrier; P-gp: P-glycoprotein-; Calculated on: SwissADME platform (http://www.swissadme.ch/). Toxicological risks are calculated using DataWarrior software (Version 5.2.1)

The three compounds (**1c, 1g**, and **1p**) do not present violations according to Lipinski's [29] and Veber's rules [30] (Table 3), following the requirements that indicate that these substances have drug-likeness features, they are likely to be membrane-permeable and have high bioavailability following oral administration.

The ability to permeate membranes can be reinforced by the parameters of gastrointestinal absorption and permeation of the blood-brain barrier. Except for **1p**, these compounds showed a high probability to exhibit absorption through the

gastrointestinal tract, although they may also access the central nervous system, which could lead to possible adverse effects [44]. Due to the P-glycoprotein affects the pharmacokinetics of several structurally and pharmacologically distinct drugs [45], inhibition or induction of this protein can result in toxicity or under treatment caused by decrease absorption at the intracellular level [46]. However, the *in silico* results indicate that these compounds are not P-glycoprotein substrates. The toxicity profile of **1c**, **1g,** and **1p** compounds was performed using DataWarrior software [32]. The results indicated that the compounds have no mutagenic, tumorigenic, and irritant effects, but showed reproductive effective risk. Moreover, it is important to be mentioned that experimental data should be obtained aiming to corroborate with these predictions.

3.2. Molecular docking

Acyl urea and thioureas can be considered lead scaffolds for the development of non-covalent inhibitors of cruzain (CRZ) [23], and thioureas derivatives could interact with fungal C14α-sterol demethylase (CYP51) [19,47], as reported in the literature. Since the inhibitory activity of benzoylthioureas derivatives could be associated with multi-target in the *T. cruzi* [48,49], we carried out molecular docking simulations of the highest activity compounds (**1c**, **1g**, and **1p**) in two of the main enzymatic targets of *T. cruzi*, CRZ [50,51] and CYP51 [52,53] to obtain more information about the antitrypanosomal activity.

The validation of the docking protocol was carried out through a re-docking simulation, considering the protocol described in the literature [37], from the co-crystallized structure of each enzyme deposited in Protein Data Bank (PDB): CRZ (PDB ID: 4KLB; resolution: 2.62 Å) [34] and CYP51 (PDB ID: 4C27; resolution: 1.95 Å) [35]. The protocol selected from the AutoDock program was able to reproduce the crystal conformation with showed a root mean square deviations (RMSD) values below 2 Å (1.01 Å and 0.68 Å for CYP51 and CRZ, respectively).

The docking complexes between the ligands (**1c**, **1g**, and **1p**) and CRZ showed that these compounds could interact with this enzyme by hydrogen bonds, but the simulation showed different orientations for these compounds (Figure 2). The compound **1c** can interact by a hydrogen bond with Leu160 in the S3 subsite (Figure 2-A). Although **1p** (R^1= 3-NO_2-Ph) can also interact in the S3 subsite, this would happen with the Ser64 residue (Figure 2-C), while the 4-bromophenyl group would be inserted

into the cavity formed by S1 and S2. However, the R^1 replacement by a 4-(*tert*-butyl)-phenyl group allows the interaction in Gly66 in the S2 subsite (**1g**, Figure 2-B), which is a residue already described for CRZ inhibition [34].

The molecular docking simulations of **1c**, **1g**, and **1p** as CYP51-ligands revealed that the three compounds can coordinate with Fe-heme through the carbonyl oxygen atom. Compound **1c** can also interact by hydrogen bonding with the NH group from Ala291 and by hydrophobic interactions with cyclohexane group and residues Phe291, Met460, and Val461 (Figure 2-D), while compound **1p** can interact by hydrogen bonding with –CONH- group and Tyr103 residue (Figure 2-F). On the other hand, the compound **1g** showed that the pose underwent rotation, leading to loss of the hydrogen bonding interaction and allowing the *tert*-butyl phenyl group to be conducted to a hydrophobic area near the Tyr103 and Tyr116 residues (Figure 2-E). The interactions by coordination with ferryl heme, hydrogen bonds, and/or hydrophobic interactions indicate that these compounds are capable of reducing the enzyme's reactivation time as described in the literature [35,54–56].

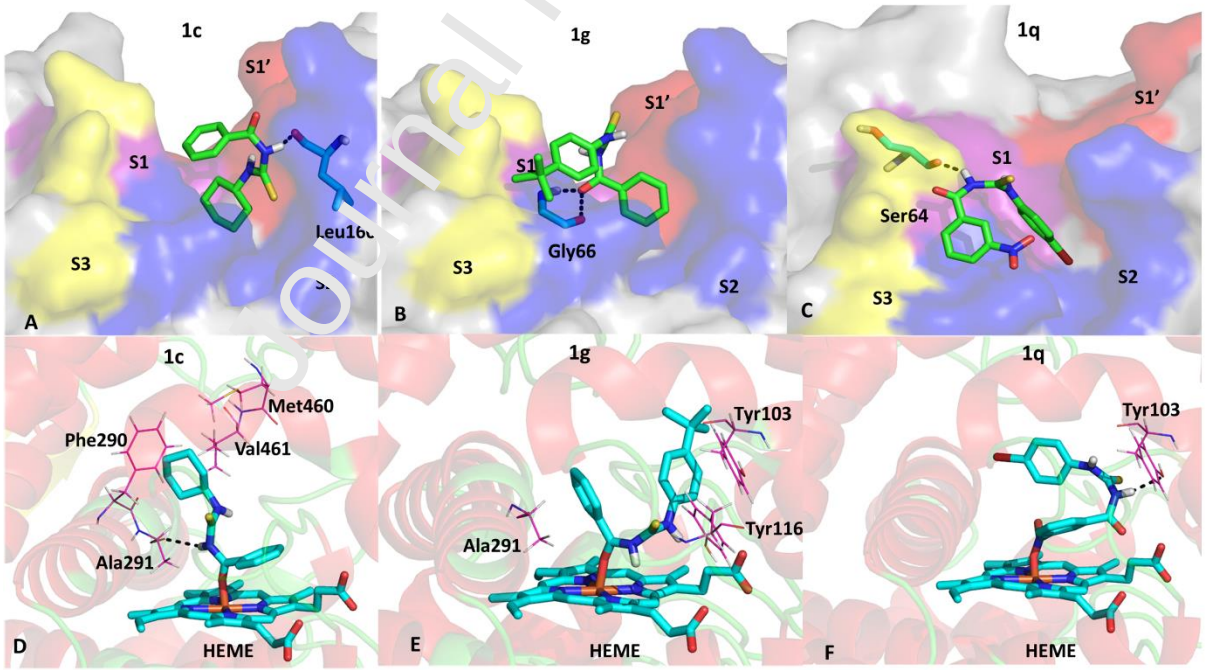


Figure 2. Main intermolecular interactions of the top docking pose of **1c** (A and D), **1g** (B and E) and **1p** (C and F) in the active site of CRZ (top) and CYP51 (down), respectively.

4. Conclusions

In summary, we observed that the series of benzoylthiourea derivatives inhibited *in vitro* the growth of epimastigote forms of *T. cruzi*. A preliminary SAR study indicated that aromatic substituents attached at N2 of the thiourea moiety improve the antiepimastigote activity, and among these aromatic derivatives, the steric effects are more prominent to biological activity than electronic effects. Moreover, it was possible to identify the three most promising compounds (**1c**, **1g**, and **1p**), due to their highest activity and SI > 10. These hit compounds also inhibited the viability and replication of trypomastigote and amastigote forms, respectively, in non-toxic concentrations for mammalian cells.

The results of the molecular docking study showed that these compounds may interact with the active site of CRZ and CYP51 enzymes indicating that the **1c**, **1g**, and **1p** compounds can inhibit these enzymes. Furthermore, ADME-Tox *in silico* predictions indicated that these compounds may have high bioavailability following oral administration, and no mutagenic, tumorogenic and irritant toxicological risks. Our results reinforce the highlight of the benzoylthioureas as a scaffold for trypanosomal activity.

Funding sources

This study was supported by Coordenadoria de Aperfeiçoamento Pessoal de Nível Superior (CAPES, Brazil).

Declarations of Competing Interest

The authors declare no conflict of interest.

References

[1] WORLD HEALTH ORGANIZATION (WHO), Chagas Disease (American Trypanosomiasis) - Epidemiology, (2020). https://www.who.int/chagas/epidemiology/en/ (accessed February 16, 2020).

[2] L.S. Lara, C.S. Moreira, C.M. Calvet, G.C. Lechuga, R.S. Souza, S.C. Bourguignon, V.F. Ferreira, D. Rocha, M.C.S. Pereira, Efficacy of 2-hydroxy-3-phenylsulfanylmethyl-[1,4]-naphthoquinone derivatives against different Trypanosoma cruzi discrete type units: Identification of a promising hit compound, Eur. J. Med. Chem. 144 (2018) 572–581. doi:10.1016/j.ejmech.2017.12.052.

[3] C. Lin, F. Hulpia, C.F. Da Silva, D.D.G.J. Batista, K. Van Hecke, L. Maes, G. Caljon, M.D.N.C. Soeiro, S. Van Calenbergh, Discovery of Pyrrolo[2,3-b]pyridine (1,7-Dideazapurine) Nucleoside Analogues as Anti- Trypanosoma cruzi Agents, J. Med. Chem. 62 (2019) 8847–8865. doi:10.1021/acs.jmedchem.9b01275.

[4] K.M. Tyler, D.M. Engman, The life cycle of Trypanosoma cruzi revisited, Int. J. Parasitol. 31 (2001) 472–481. doi:10.1016/S0020-7519(01)00153-9.

[5] M. Cholewiński, M. Derda, E. Hadaś, Parasitic diseases in humans transmitted by vectors, Ann. Parasitol. 61 (2015) 137–157. doi:10.17420/ap6103.01.

[6] J.A. Atwood, D.B. Weatherly, T.A. Minning, B. Bundy, C. Cavola, F.R. Opperdoes, R. Orlando, R.L. Tarleton, Microbiology: The Trypanosoma cruzi proteome, Science (80-.). 309 (2005) 473–476. doi:10.1126/science.1110289.

[7] L.E. Echeverria, C.A. Morillo, American Trypanosomiasis (Chagas Disease), Infect. Dis. Clin. North Am. 33 (2019) 119–134. doi:10.1016/j.idc.2018.10.015.

[8] K. Babanezhad Harikandei, P. Salehi, S.N. Ebrahimi, M. Bararjanian, M. Kaiser, A. Al-Harrasi, Synthesis, in-vitro antiprotozoal activity and molecular docking study of isothiocyanate derivatives, Bioorganic Med. Chem. 28 (2020). doi:10.1016/j.bmc.2019.115185.

[9] L. Capuani, A.L. Bierrenbach, A. Pereira Alencar, A. Mendrone, J.E. Ferreira, B. Custer, A.L. Antonio, E. Cerdeira Sabino, Mortality among blood donors seropositive and seronegative for Chagas disease (1996–2000) in São Paulo, Brazil: A death certificate linkage study, PLoS Negl. Trop. Dis. 11 (2017) 1–14. doi:10.1371/journal.pntd.0005542.

[10] A. González, N. Becerra, M. Kashif, M. González, H. Cerecetto, E. Aguilera, B. Nogueda-Torres, K.F. Chacón-Vargas, J. José Zarate-Ramos, U. Castillo-Velázquez, C.O. Salas, G. Rivera, K. Vázquez, In vitro and in silico evaluations of new aryloxy-1,4-naphthoquinones as anti-Trypanosoma cruzi agents, Med. Chem. Res. 29 (2020) 665–674. doi:10.1007/s00044-020-02512-9.

[11] M.E.B. Melo, L.C.S. Ferreira, Screening the mutagenic activities of commonly used antiparasite drugs by the simultest, a simplified Salmonella/microsome plate incorporation assay, Rev. Inst. Med. Trop. Sao Paulo. 32 (1990) 269–274. doi:10.1590/S0036-46651990000400006.

[12] W. Apt B, I. Heitmann G, M.I. Jercic L, L. Jotré M, P. Muñoz C. del V, I. Noemí

H, A.M. San Martin V, J. Sapunar P, M. Torres H, I. Zulantay A, Guías clínicas de la enfermedad de Chagas: Parte IV. Enfermedad de Chagas en pacientes inmunocomprometidos, Rev. Chil. Infectología. 25 (2008) 289–292. doi:10.4067/s0716-10182008000400008.

[13] A.E. Bivona, A.S. Alberti, N. Cerny, S.N. Trinitario, E.L. Malchiodi, Chagas disease vaccine design: the search for an efficient Trypanosoma cruzi immune-mediated control, Biochim. Biophys. Acta - Mol. Basis Dis. 1866 (2020). doi:10.1016/j.bbadis.2019.165658.

[14] C.M. Beaumier, P.M. Gillespie, U. Strych, T. Hayward, P.J. Hotez, M.E. Bottazzi, Status of vaccine research and development of vaccines for Chagas disease, Vaccine. 34 (2016) 2996–3000. doi:10.1016/j.vaccine.2016.03.074.

[15] A.R. McCarthy, M.C.C. Sachweh, M. Higgins, J. Campbell, C.J. Drummond, I.M.M. van Leeuwen, L. Pirrie, M.J.G.W. Ladds, N.J. Westwood, S. Lain, Tenovin-D3, a Novel Small-Molecule Inhibitor of Sirtuin SirT2, Increases p21 (CDKN1A) Expression in a p53-Independent Manner, Mol. Cancer Ther. 12 (2013) 352–360. doi:10.1158/1535-7163.MCT-12-0900.

[16] H.M. Faidallah, M.M. Al-Mohammadi, K.A. Alamry, K.A. Khan, Synthesis and biological evaluation of fluoropyrazolesulfonylurea and thiourea derivatives as possible antidiabetic agents, J. Enzyme Inhib. Med. Chem. 31 (2016) 157–163. doi:10.1080/14756366.2016.1180594.

[17] A. Khan, J. Hashim, N. Arshad, J. Khan, N. Siddiqui, A. Wadood, M. Ali, F. Arshad, K.M. Khan, M.I. Choudhary, Dihydropyrimidine based hydrazine dihydrochloride derivatives as potent urease inhibitors, Bioorg. Chem. 64 (2016) 85–96. doi:10.1016/j.bioorg.2015.12.007.

[18] N. Dolan, D.P. Gavin, A. Eshwika, K. Kavanagh, J. McGinley, J.C. Stephens, Synthesis, antibacterial and anti-MRSA activity, in vivo toxicity and a structure-activity relationship study of a quinoline thiourea, Bioorganic Med. Chem. Lett. 26 (2016) 630–635. doi:10.1016/j.bmcl.2015.11.058.

[19] L. Antypenko, E. Meyer, O. Kholodniak, Z. Sadykova, T. Jirásková, A. Troianova, V. Ruhaiova, S. Cao, S. Kovalenko, L.A. Garbe, K.G. Steffens, Novel acyl thiourea derivatives: Synthesis, antifungal activity, gene toxicity, drug-like and molecular docking screening, Arch. Pharm. (Weinheim). 352 (2019) 1–14. doi:10.1002/ardp.201800275.

[20] G.G. A. Bielenica, G. Sanna, S. Madeddu, M. Strugac, M. Jóźwiak, A. E. Koziol, A. Sawczenko, I. B. Materek, A. Serra, New thiourea and 1,3-thiazolidin-4-one derivatives effective on the HIV-1 virus, Chem. Biol. Drug Des. 90 (2017) 883–891. doi:10.1111/cbdd.13009.

[21] N. Sunduru, K. Srivastava, S. Rajakumar, S.K. Puri, J.K. Saxena, P.M.S. Chauhan, Synthesis of novel thiourea, thiazolidinedione and thioparabanic acid derivatives of 4-aminoquinoline as potent antimalarials, Bioorganic Med. Chem. Lett. 19 (2009) 2570–2573. doi:10.1016/j.bmcl.2009.03.026.

[22] G.M. Viana, D.C. Soares, M.V. Santana, L.H. Do Amaral, P.W. Meireles, R.P. Nunes, L.C.R.P. Da Silva, L.C. De Sequeira Aguiar, C.R. Rodrigues, V.P. De Sousa, H.C. Castro, P.A. Abreu, P.C. Sathler, E.M. Saraiva, L.M. Cabral,

Antileishmanial thioureas: Synthesis, biological activity and in Silico evaluations of new promising derivatives, Chem. Pharm. Bull. 65 (2017) 911–919. doi:10.1248/cpb.c17-00293.

[23] X. Du, E. Hansell, J.C. Engel, C.R. Caffrey, F.E. Cohen, J.H. McKerrow, Aryl ureas represent a new class of anti-trypanosomal agents, Chem. Biol. 7 (2000) 733–742. doi:10.1016/S1074-5521(00)00018-1.

[24] T.O. Brito, L.O. Abreu, K.M. Gomes, M.C.S. Lourenço, P.M.L. Pereira, S.F. Yamada-Ogatta, Â. de Fàtima, C.A. Tisher, F.M. Jr, M.L.F. Bispo, Benzoylthioureas: Design, Synthesis and Antimycobacterial Evaluation, Med. Chem. (Los. Angeles). 16 (2018) 93–103. doi:10.2174/1573406415666181208110753.

[25] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays., J. Immunol. Methods. 65 (1983) 55–63. doi:10.1016/0022-1759(83)90303-4.

[26] E.P. CAMARGO, Growth and differentiation in Trypanosoma cruzi. I. Origin of metacyclic trypanosomes in liquid media, Rev. Inst. Med. Trop. Sao Paulo. 6 (1964) 93—100. http://europepmc.org/abstract/MED/14177814.

[27] Z. BRENER, Therapeutic activity and criterion of cure on mice experimentally infected with Trypanosoma cruzi., Rev. Inst. Med. Trop. Sao Paulo. 4 (1962) 389–396.

[28] E. Izumi, L.G. Morello, T. Ueda-Nakamura, S.F. Yamada-Ogatta, B.P.D. Filho, D.A.G. Cortez, I.C.P. Ferreira, J.A. Morgado-Díaz, C.V. Nakamura, Trypanosoma cruzi: Antiprotozoal activity of parthenolide obtained from Tanacetum parthenium (L.) Schultz Bip. (Asteraceae, Compositae) against epimastigote and amastigote forms, Exp. Parasitol. 118 (2008) 324–330. doi:10.1016/j.exppara.2007.08.015.

[29] C.A. Lipinski, F. Lombardo, B.W. Dominy, P.J. Feeney, Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings, Adv. Drug Deliv. Rev. 64 (2012) 4–17. doi:10.1016/j.addr.2012.09.019.

[30] D.F. Veber, S.R. Johnson, H.Y. Cheng, B.R. Smith, K.W. Ward, K.D. Kopple, Molecular properties that influence the oral bioavailability of drug candidates, J. Med. Chem. 45 (2002) 2615–2623. doi:10.1021/jm020017n.

[31] A. Daina, O. Michielin, V. Zoete, SwissADME: A free web tool to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules, Sci. Rep. 7 (2017) 1–13. doi:10.1038/srep42717.

[32] T. Sander, J. Freyss, M. Von Korff, C. Rufener, DataWarrior: An open-source program for chemistry aware data visualization and analysis, J. Chem. Inf. Model. 55 (2015) 460–473. doi:10.1021/ci500588j.

[33] R. Huey, G.M. Morris, S. Forli, Using AutoDock 4 and AutoDock Vina with AutoDockTools: A Tutorial, Scripps Res. Inst. Mol. (2012) 32.

[34] H.J. Wiggers, J.R. Rocha, W.B. Fernandes, R. Sesti-Costa, Z.A. Carneiro, J. Cheleski, A.B.F. da Silva, L. Juliano, M.H.S. Cezari, J.S. Silva, J.H. McKerrow,

C.A. Montanari, Non-peptidic Cruzain Inhibitors with Trypanocidal Activity Discovered by Virtual Screening and In Vitro Assay, PLoS Negl. Trop. Dis. 7 (2013). doi:10.1371/journal.pntd.0002370.

[35] D.F. Vieira, J.Y. Choi, C.M. Calvet, J.L. Siqueira-Neto, J.B. Johnston, D. Kellar, J. Gut, M.D. Cameron, J.H. McKerrow, W.R. Roush, L.M. Podust, Binding Mode and Potency of N -Indolyloxopyridinyl-4-aminopropanyl-Based Inhibitors Targeting Trypanosoma cruzi CYP51, J. Med. Chem. 57 (2014) 10162–10175. doi:10.1021/jm501568b.

[36] H.M. Berman, T. Battistuz, T.N. Bhat, W.F. Bluhm, P.E. Bourne, K. Burkhardt, Z. Feng, G.L. Gilliland, L. Iype, S. Jain, P. Fagan, J. Marvin, D. Padilla, V. Ravichandran, B. Schneider, N. Thanki, H. Weissig, J.D. Westbrook, C. Zardecki, The protein data bank, Acta Crystallogr. Sect. D Biol. Crystallogr. 58 (2002) 899–907. doi:10.1107/S0907444902003451.

[37] C.H. da Silva Lima, J.C. de Araujo Vanelis Soares, J.L. de Sousa Ribeiro, E.M.F. Muri, S. de Albuquerque, L.R.S. Dias, Anti-Trypanosoma cruzi Activity and Molecular Docking Studies of 1Hpyrazolo[3, 4-b]pyridine Derivatives, Lett. Drug Des. Discov. 17 (2019) 184–191. doi:10.2174/1570180816666190305141733.

[38] T.O. Brito, A.X. Souza, Y.C.C. Mota, V.S.S. Morais, L.T. De Souza, Â. De Fátima, F. Macedo, L. V. Modolo, Design, syntheses and evaluation of benzoylthioureas as urease inhibitors of agricultural interest, RSC Adv. 5 (2015) 44507–44515. doi:10.1039/c5ra07c86e.

[39] E. Chatelain, Chagas Disease Drug Discovery : Toward a New Era, (2014). doi:10.1177/1087057114550585.

[40] L.M. MacLean, J. Thomas, M.D. Lewis, I. Cotillo, D.W. Gray, M. De Rycker, Development of Trypanosoma cruzi in vitro assays to identify compounds suitable for progression in Chagas' disease drug discovery, PLoS Negl. Trop. Dis. 12 (2018) 1–22. doi:10.1371/journal.pntd.0006612.

[41] S. Revollo, B. Oury, A. Vela, M. Tibayrenc, D. Sereno, In vitro benznidazole and nifurtimox susceptibility profile of trypanosoma cruzi strains belonging to discrete typing units tci, tcii, and tcv, Pathogens. 8 (2019). doi:10.3390/pathogens8040197.

[42] T.C. de Araujo-Jorge, The Biology of Trypanosoma cruzi - Macrophage Interaction, Meml. Inst. Oswaldo Cruz. 84 (1989) 441–462. doi:10.1590/S0074-02761989000400001.

[43] A.M.A. Velásquez, R.A. De Souza, T.G. Passalacqua, A.R. Ribeiro, M. Scontri, C.M. Chin, L. De Almeida, M.L.D. Cistia, J.A.D. Rosa, A.E. Mauro, M.A.S. Graminha, Antiprotozoal activity of the cyclopalladated complexes against leishmania amazonensis and trypanosoma cruzi, J. Braz. Chem. Soc. 27 (2016) 1032–1039. doi:10.5935/0103-5053.20150360.

[44] W. Shinoda, Permeability across lipid membranes, Biochim. Biophys. Acta - Biomembr. 1858 (2016) 2254–2265. doi:10.1016/j.bbamem.2016.03.032.

[45] J.H. Lin, M. Yamazaki, Role of P-Glycoprotein in Pharmacokinetics, Clin. Pharmacokinet. 42 (2003) 59–98. doi:10.2165/00003088-200342010-00003.

[46] W. Jäger, E. Gehring, B. Hagenauer, S. Aust, A. Senderowicz, T. Thalhammer, Biliary excretion of flavopiridol and its glucuronides in the isolated perfused rat liver: Role of multidrug resistance protein 2 (Mrp2), Life Sci. 73 (2003) 2841–2854. doi:10.1016/S0024-3205(03)00699-4.

[47] V. Bala, S. Jangir, D. Mandalapu, S. Gupta, Y.S. Chhonker, N. Lal, B. Kushwaha, H. Chandasana, S. Krishna, K. Rawat, J.P. Maikhuri, R.S. Bhatta, M.I. Siddiqi, R. Tripathi, G. Gupta, V.L. Sharma, Dithiocarbamate-thiourea hybrids useful as vaginal microbicides also show reverse transcriptase inhibition: design, synthesis, docking and pharmacokinetic studies., Bioorg. Med. Chem. Lett. 25 (2015) 881–886. doi:10.1016/j.bmcl.2014.12.062.

[48] G.R. Zimmermann, J. Lehár, C.T. Keith, Multi-target therapeutics: when the whole is greater than the sum of the parts., Drug Discov. Today. 12 (2007) 34–42. doi:10.1016/j.drudis.2006.11.008.

[49] M.T. Scotti, I. Castro-Gamboa, E.I. Ferreira, C.M. de S. Menezes, M.V.R. Velasco, V. da S. Bolzani, C. Cardoso, L. Scotti, P. Padrela, Modelagem molecular aplicada ao desenvolvimento de moléculas com atividade antioxidante visando ao uso cosmético, Rev. Bras. Ciências Farm. 43 (2007). doi:10.1590/s1516-93322007000200002.

[50] M. Sajid, S.A. Robertson, L.S. Brinen, J.H. McKerrow, Cruzain: The path from target validation to the clinic, Adv. Exp. Med. Biol. 712 (2011) 100–115. doi:10.1007/978-1-4419-8414-2_7.

[51] V. Duschak, A. Couto, Cruzipain, the Major Cysteine Protease of Trypanosoma cruzi: A Sulfated Glycoprotein Antigen as Relevant Candidate for Vaccine Development and Drug Target. A Review, Curr. Med. Chem. 16 (2009) 3174–3202. doi:10.2174/092986709788802971.

[52] M.L. Sykes, V.M. Avery, 3-Pyridyl inhibitors with novel activity against Trypanosoma cruzi reveal in vitro profiles can aid prediction of putative cytochrome P450 inhibition, Sci. Rep. 8 (2018) 1–12. doi:10.1038/s41598-018-22043-z.

[53] C.M. Calvet, D.F. Vieira, J.Y. Choi, D. Kellar, M.D. Cameron, J.L. Siqueira-Neto, J. Gut, J.B. Johnston, L. Lin, S. Khan, J.H. McKerrow, W.R. Roush, L.M. Podust, 4-Aminopyridyl-based CYP51 inhibitors as anti-Trypanosoma cruzi drug leads with improved pharmacokinetic profile and in vivo potency, J. Med. Chem. 57 (2014) 6989–7005. doi:10.1021/jm500448u.

[54] T.Y. Hargrove, Z. Wawrzak, P.W. Alexander, J.H. Chaplin, M. Keenan, S.A. Charman, C.J. Perez, M.R. Waterman, E. Chatelain, G.I. Lepesheva, Complexes of trypanosoma cruzi Sterol 14α-Demethylase (CYP51) with Two Pyridine-based Drug Candidates for Chagas Disease: Structural basis for pathogen selectivity, J. Biol. Chem. 288 (2013) 31602–31615. doi:10.1074/jbc.M113.497990.

[55] Y. C. Jun; R. R. William, Structure Based Design of CYP51 Inhibitors, Curr. Top. Med. Chem. 17 (2017) 30–39. doi:10.2174/1568026616666160719.

[56] L. Friggeri, T.Y. Hargrove, G. Rachakonda, A.D. Williams, Z. Wawrzak, R. Di Santo, D. De Vita, M.R. Waterman, S. Tortorella, F. Villalta, G.I. Lepesheva, Structural basis for rational design of inhibitors targeting Trypanosoma cruzi

Sterol 14α-demethylase: Two regions of the enzyme molecule potentiate its inhibition, J. Med. Chem. 57 (2014) 6704–6717. doi:10.1021/jm500739f.

Highlights

- Antitrypanosomal activity evaluation *in vitro* of benzoylthioureas.
- All tested compounds presented anti-epimastigote activity.
- Three promising compounds were evaluated against trypomastigote and amastigote forms of *T. cruzi*.
- ADME-Tox *in silico* predictions was performed.
- Docking studies with two main enzymatic targets (cruzain and CYP-51) were investigated.