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Research Article

Synthesis, Cytotoxicity and *In Vitro* Antileishmanial Activity of Naphthothiazoles

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The leishmaniasis is a spectral disease caused by the protozoan *Leishmania* spp., which threatens millions of people worldwide. Current treatments exhibit high toxicity, and there is no vaccine available. The need for new lead compounds with leishmanicidal activity is urgent. Considering that many lead leishmanicidal compounds contain a quinoidal scaffold and the thiazole heterocyclic ring is found in a number of antimicrobial drugs, we proposed a hybridization approach to generate a diverse set of semi-synthetic heterocycles with antileishmanial activity. We found that almost all synthesized compounds demonstrated potent activity against promastigotes of *Leishmania* (*Viannia*) *braziliensis* and reduced the survival index of *Leishmania* amastigotes in mammalian macrophages. Furthermore, the compounds were not cytotoxic to macrophages at fivefold higher concentrations than the EC_{50} for promastigotes. All molecules fulfilled Lipinski's Rule of Five, which predicts efficient orally absorption and permeation through biological membranes, the *in silico* pharmacokinetic profile confirmed these characteristics. The potent and selective activity of semi-synthetic naphthothiazoles against promastigotes and amastigotes reveals that the 2-amino-naphthothiazole ring may represent a scaffold for the design of compounds with leishmanicidal properties and encourage the development of drug formulation and new compounds for further studies *in vivo*.

Key words: fragment embedment, *Leishmania braziliensis*, leishmaniasis, naphthothiazoles, neglected tropical disease

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Leishmaniasis is caused by protozoan parasites from the *Leishmania* genus and comprises two major diseases: visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL). While VL is fatal if untreated, CL may heal spontaneously or leave disfiguring scars. Leishmaniasis is one of the most neglected tropical diseases and is associated with high morbidity levels. Moreover, the mortality rate of VL is approximately 60 000 per year, which is the second highest rate among parasitic diseases, only surpassed by malaria (1). The expansion of the geographical distribution of leishmaniasis in developing and developed countries in Europe (e.g. France, Italy, Spain and Greece) and North America is an alert to health authorities worldwide (2,3). This scenario makes evident that leishmaniasis is no longer a poverty-associated disease (2), since climate change (4–6), human migration (7,8) and additional socio-environmental factors (9) have become important variables affecting the incidence of this parasitic infection. Although the disease is treatable, its control is difficult for the absence of an effective vaccine, the adaptation of the vector and reservoirs to human environments, and the emergence of resistant lines. The relative high toxicity of available chemotherapy and the lack of more effective, safer and orally available therapies for all clinical manifestations is another relevant problem that substantially makes leishmaniasis a high-risk disease (10). The long-lasting lack of commercial incentives to support the R&D for drugs against tropical diseases has been overcome by organized networks between public–private partnerships (PPPs) (11,12).

The characterization of physico-chemical factors could drive the design and innovation of potential leishmanicidal hits. Specifically, the Rule of Five, which is useful for prediction of good permeability and drug-likeness for compounds, is more likely when H-bond donors (nOHNH) are ≤ 5, H-bond acceptors (nON) ≤ 10, molecular weight (MW) ≤ 500 and calculated lipophilicity (xlog P is the calculated logP using the specific logarithm of the software) ≤ 5 (13,14). In addition, oral bioavailability has also been correlated with the total polar surface area (TPSA) parameter, which represents the sum of surfaces of polar

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atoms in a molecule (≤ 140 Å^2) including N, O, P and S (TPSA_NOPS), the number of rotatable bonds (rotatable bonds count – rbc ≤ 10) and water solubility (logS) (15). To facilitate the identification of effective antileishmanial drug candidates, Nwaka *et al.* proposed a set of criteria based on biological activity, physico-chemical characteristics and pharmacokinetics. According to the authors, a leishmanicidal hit should fit the following criteria: EC_{50} for the amastigote (in macrophages) about 1–2 μg/mL, selectivity over parasites >20 (selectivity index, SI), confirmed and elucidate structure, established synthetic route, good drug-likeness scores (DLS), no violation of Lipinski's Rule of Five and chemically exploitable (16).

Heteroaromatic rings found in small bioactive molecules have been successfully exploited for different medicinal purposes, including bioavailability, due to their shape, hydrophobicity, hydrogen bond potential, polarizability, and established diversified reactivity for manipulation of functional groups and structures (17). The thiazole ring system is a useful structural element in medicinal chemistry. This structure can be found in numerous biologically active molecules used for the treatment of allergies, hypertension, inflammation, fungal and bacterial infections and has found broad application in drug development (18,19). In particular, diverse classes of therapeutic drugs, including antimicrobials, contain a 2-aminothiazole ring. Several synthetic and natural naphthoquinones are also known as antiparasitic lead compounds, acting by different sort of mechanisms of action against trypanosomatids (20–23). The aim of this study was to make the fragment embedment of the thiazole ring into naphthoquinoidal structures to generate a small library of naphthothiazoles, including a hybrid thiazoloquinone, aiming compounds with different pattern of substitution to search for improved druggability. We adopted chemical and biological criteria to design and select hit compounds anti-Leishmania. The compounds containing a 2-aminothiazole core within a naphthalene skeleton were synthesized, the structure was correlated with drug-likeness index and drug-relevant properties, the activity against promastigote and amastigote forms was tested, and the cytotoxicity was evaluated.

We have provided a new rationale for the development of novel lead compounds with leishmanicidal activity based on thiazole ring properties. We have shown a potent and selective activity of semi-synthetic naphthothiazoles against promastigotes and amastigotes revealing that the 2-amino-naphthothiazole ring may represent a scaffold for the design of molecules with anti-Leishmania activities.

Methods and Materials

Chemical synthesis, drug-relevant properties and pharmacokinetics

All chemicals and reagents were purchased from Sigma-Aldrich Brasil Ltda (Sao Paulo, Brazil), and the solvents were obtained from Vetec Química Fina Ltda (Duque de Caxias, RJ, Brazil) and Synth (Labsynth, São Paulo, Brazil). Iodoxybenzoic acid was freshly prepared as previously described (24). All reactions were monitored by thin layer chromatography using Merck Silica gel 60F254 plates, and synthesized compounds were purified by flash column chromatography on silica gel 60 (200–430 mesh; Merck Brasil, Sao Paulo, Brazil) or by recrystallization. High-resolution mass spectra were obtained using an electrospray ionization time-of-flight mass spectrometer. The NMR data were recorded with a Bruker DPX-500 instrument using DMSO-d6, $CDCl_3$ or CD_3OD as a solvent and tetramethylsilane as an internal standard. IR spectra were measured with a Perkin-Elmer Spectrum RX IFTIR System.

Drug-relevant properties and DLS were calculated with the Vortex data analysis and visualization software from Dotmatics (http://www.dotmatics.co.uk/) and with the web-based molecular property calculator from OSIRIS Property Explorer (http://www.organic-chemistry.org/prog/peo/). OSIRIS was used to compare the DLS (drug similarity based on the occurrence of substructure fragments – 80% of drugs have DLS higher than −4.0) of the naphthothiazoles and antileishmanial drugs on the market. Vortex was used to compare MW, xlogP, rbc, TPSA, nOHNH and nON and violations to Lipinski's Rule of Five. The probable pharmacokinetic behaviour of the naphthothiazoles was predicted using the web tool 'PK/DB Database for Pharmacokinetics Properties' (25) which expressed the results as: human intestinal absorption (HIA); human oral bioavailability (F); plasma protein binding (PPB).

Leishmania *culture*

The parasites Leishmania (V.) braziliensis, strain H3227 (MHOM/BR/94/H-3227), were maintained *in vitro* in M199 medium (GIBCO, Grand Island, NY, USA) at 26 °C supplemented with 10% heat-inactivated foetal calf serum, 2% human urine, 20 mM HEPES, 4 mM $NaHCO_3$, 10 U/mL of penicillin and 100 μg/mL of streptomycin (GIBCO).

Antipromastigote assay

The antipromastigote assay was carried out according to the method of Dutta *et al.* (26). The naphthothiazole compounds were serially diluted from 0.02 to 10 μM in Schneider's medium (supplemented with 10% FBS and 2% human urine) containing 2×10^5 parasites/mL. A total of 4×10^4 parasites were seeded per well in 96-well microplates incubated at 26 °C until the end of the log phase. After, 100 μg of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) dissolved in 10 μL of PBS was added per well, and the plates were incubated at 37 °C for 4 h. Following incubation, the plates were centrifuged at 3000 × *g* for 5 min, the supernatant was removed and precipitated formazan was dissolved in 100 μL of dimethyl sulfoxide (DMSO). The absorbance was measured at 492 nm, and the data for two independent assays

performed in triplicate were analysed using GRAPH PAD PRISM 5.0 software (GraphPad Software Inc., La Jolla, CA, USA). The EC_{50} was defined as the effective drug concentration that inhibits parasite proliferation by 50% compared with a non-treated culture. Geneticin (G418) was used as a positive control.

Determination of cytotoxicity against mammalian cells

Cytotoxicity was determined using the MTT assay. Briefly, BALB/c mice were intraperitoneally injected with 1.5 mL of 5% thioglycolate medium. After 72 h, murine peritoneal cells were harvested in RPMI 1640 medium (GIBCO Invitrogen Corporation, New York, NY, USA), pH 7.6 supplemented with 10% heat-inactivated foetal calf serum (GIBCO). A total of 1×10^5 cells were plated in 96-well microtiter plates and allowed to adhere for 12 h at 37 °C in an atmosphere of 5% CO_2. The naphthothiazole compounds were serially diluted from 0.024 to 12.5 μM in RPMI medium, and macrophages were incubated in the presence of these compounds for 48 h at 37 °C in 5% CO_2. Control cells were incubated in DMSO without drugs. Analysis of the mean of two assays performed in triplicate was carried out with GRAPH PAD PRISM 5.0 software.

Anti-amastigote assay

A suspension of 5×10^5 macrophages/mL in RPMI 1640 medium was dispersed into polypropylene tubes and infected with *L. (V.) braziliensis* (10 parasites per cell) for 3 h. Non-internalized parasites were removed by centrifugation at 50 × *g*, three washing cycles with RPMI medium. The infected macrophages were plated on glass coverslips (13-mm diameter) in 24-well plates and incubated for 1 h at 33 °C. The naphthothiazole compounds which show activity against promastigotes were diluted in RPMI medium, and infected macrophages were incubated in the presence of these compounds for 48 h at 33 °C in 5% CO_2. The coverslips were washed with PBS, stained with Panotic stain (Panótico Rápido LB kit; Laborclin, Paraná, Brazil), dried, mounted on glass slides with Tissue-Tek® mounting medium (Sakura Finetek Europe B.V., Alphen aan den Rijn, Holland) and examined microscopically. Two hundred cells were evaluated per assay. The results were expressed as infection index (percentage of infected macrophages multiplied by the average number of amastigotes per macrophage). Analysis of the mean of two assays performed in duplicate was carried out with GRAPH PAD PRISM 5.0 software.

Results

Synthesis of naphthothiazoles

Despite all the efforts on the R&D to discover new lead compounds active against *Leishmania* parasites, the chemotherapy currently available to treat leishmaniasis is not suitable (10,27). This work reports a molecular hybridization strategy for the design and synthesis of six semi-synthetic naphthothiazoles.

The naphthothiazoles were designed based on a hybridization strategy, in which a 2-amino-thiazole ring is embedded into a naphthoquinoidal skeleton (Figure 1). The naphthothiazoles compounds **4**, **5** and **6** were obtained in a single step by the addition of thiourea to the respective naphthoquinones (1, 4-naphthoquinone [1], lawsone [2] and menadione [3]), in an ethanolic/HCl solution, as previously described (28,29) (Scheme 1). The compound **6** was subjected to double acetylation in presence of acetic anhydride and triethylamine to produce compound **8**, and methylated in the presence of dimethyl sulphate and sodium carbonate in ethanol to produce the compound **7** (Scheme 2). The compound **4** was oxidized in presence of 2-iodoxybenzoic acid and acetonitrile: water (2:1) to produce the compound **9** (Scheme 3). All reactions gave good-to-average yields of respective compounds. The crystal and molecular structure of compound **7** were determined by single crystal X-ray diffraction (Supporting information), and it was deposited at 'The Cambridge Crystallographic Data Centre' (www.ccdc.cam.ac.uk; deposit number: 854691). Details of all synthetic procedures, structure characterization, spectroscopic and spectrometric data, and crystallographic results can be found in the Supporting information.

Biological activity

The anti-Leishmania activity of semi-synthetic naphthothiazoles against *Leishmania* promastigotes (extracellular stage) was accessed in biological screening by MTT reduction, and activity against amastigotes (intracellular stage) was determined by reduction in infection index.

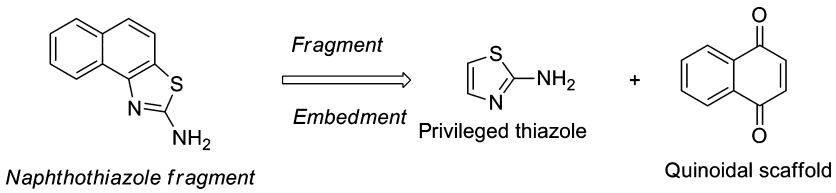
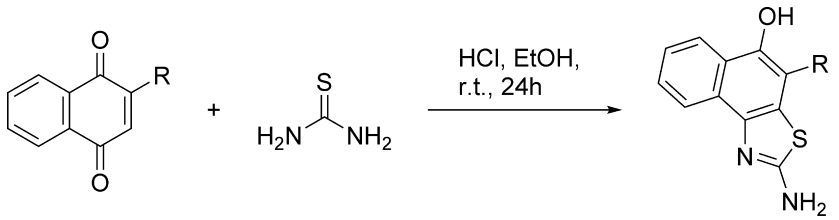
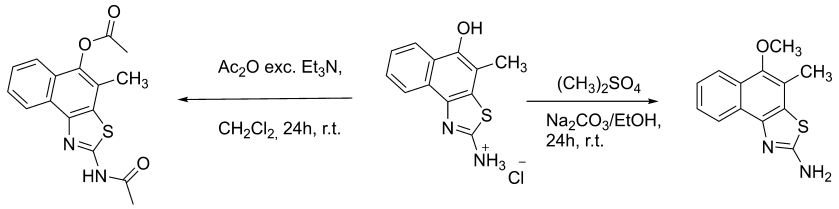


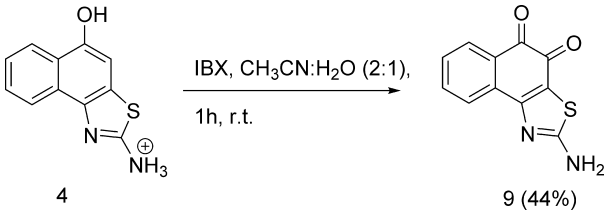
Figure 1: General strategy for the development of new heterocyclic derivatives from quinones.



Scheme 1: Schematic representation of the synthesis of aminothiazoles from napthoquinones.



Scheme 2: Methylation and diacetylation of naphthothiazole 6.



Scheme 3: Oxidation of hydroxyl-thiazole 4 to quinone 9.

Table 1: *In vitro* antipromastigote activity of 2-amino-naphthothiazoles

Compound	Promastigote EC_{50} [μM (μg/mL)]
1,4-naphthoquinone	0.11 (0.01)
Lawsone	Inactive
Menadione	1.78 (0.30)
4	1.44 (0.31)
5	1.21 (0.28)
6	4.6 (1.081)
7	0.97 (0.304)
8	1.18 (0.27)
9	Inactive
Control (G418)	2.42 (1.2)

The compounds **4**, **5** and **6** possess a free basic amine, and the water-soluble aminothiazole hydrochloride form was used for biological studies. All compounds tested, except for the compound **9**, showed potent activity against promastigotes (Table 1). According to EC_{50} determined for promastigotes, the cytotoxicity was tested on mammalian cells. None of the compounds were cytotoxic,

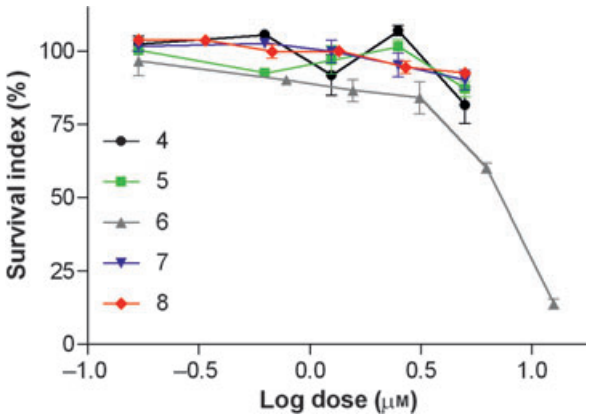


Figure 2: Cytotoxicity on macrophages. Naphthothiazoles effect on peritoneal macrophage from BALB/c mice. Cell viability determined by MTT method with different concentrations of compounds **4**, **5**, **6**, **7** and **8**. Results expressed in % of means ± standard deviations from tree independent experiments.

excluding the compound **6**, which displayed some cytotoxicity at 6–12 μM concentration (Figure 2). Based on the EC_{50} determined for promastigotes, and given the lack of cytotoxicity to macrophages, the naphthothiazole compounds (except compounds **6** and **9**) were tested at a concentration of 3 and 6 μM. All derived tested compounds (**4**, **5**, **7** and **8**) were able to reduce number of macrophage's parasite loads (Figure 3). Compounds **4** and **5** reduced the survival index of intracellular amastigote *Leishmania* by 75% at concentrations of 1.29 μg/mL (6 μM) and 0.69 μg/mL (3 μM), respectively (Table 1). The hybrid compounds **4**, **5**, **7** and **8** fit the biological criteria in terms of efficacy and toxicity (Table 1).

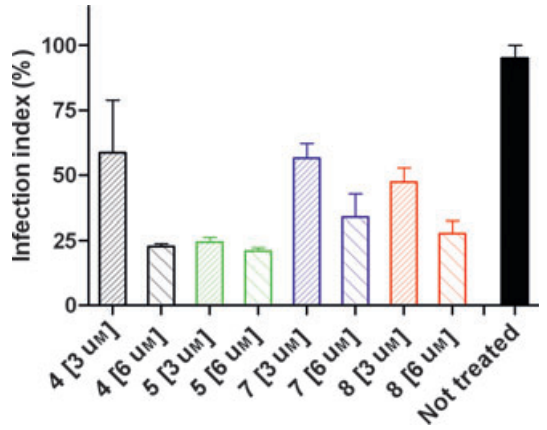


Figure 3: *In vitro* anti-amastigote activity of naphthothiazoles. Reduction in infection index of peritoneal macrophage infected by *Leishmania* and treated with different concentrations of compounds **4**, **5**, **7** and **8**. Macrophages were infected with *Leishmania* (*Viannia*) *braziliensis* promastigotes and treated with naphthothiazoles compounds (3 or 6 μg/mL). The number of intracellular amastigotes per 200 macrophages was determined under optical microscope analysis. Values represent the averages of three independent experiments with the error bars indicating the range of values; *$p < 0.05$.

Drug-like properties and pharmacokinetics prediction

There is ample evidence that the characterization of physicochemical properties could optimize the drug discovery process. We have used cheminformatic tools to examine and compare the drug-like properties, DLS and pharmacokinetics of the naphthothiazoles herein described with the ones commonly used in chemotherapy against leishmaniasis.

To evaluate and compare the potential oral bioavailability and drug-likeness of naphthothiazoles and leishmanicidal drugs, we used the Lipinski's Rule of Five, rbc, TPSA and DLS (14). All compounds synthesized adhered to Lipinski's Rule of five. The most active compounds (**4**, **5**, **7** and **8**) display values of xlogP (value ranges from 3.75 to 4.88) lower than miltefosine, which has xlogP-value of 5.59, violating the Lipinski's statement (Table 2). All synthesized compounds show a TPSA lower than 140 Å^2 and also lower rbc (<3), especially when compared to the oral miltefosine (rbc = 20). The active naphthothiazoles have DLS higher than −1.9 and comparable to almost all leishmanicidal drugs, except for miltefosine and pentamidine, which have very low DLS (Table 2). In addition, according to the pharmacokinetic prediction, the naphthothiazoles display high values of HIA (>80% for active compounds) and high values of oral bioavailability, especially the most active compounds **4** and **7**, with F of 66% (Table 2). Furthermore, the compounds are approximately 50% free in plasma. The probable human HIA, F and PPB of the tested compounds indicated that naphthothiazoles have a pharmacokinetic profile which would allow oral administration (Table 3).

Discussion

Leishmaniasis are among the sixth most threaten infectious diseases worldwide (http://www.who.int/leishmaniasis/en/index.html). Nowadays, some PPPs are stimulating R&D of new therapies for tropical and neglected diseases, including leishmaniasis (10,30). As a result, some non-antimony-based oral therapies have become available over the past decade, including miltefosine and liposomal amphotericin (31). However, none of these new chemotherapeutics are suitable to treat all clinical manifestations of leishmaniasis (27).

In this study, we employed a molecular hybridization strategy for the design and synthesis of six semi-synthetic

Table 2: Predicted molecular properties of synthesized compounds and leishmanicidal drugs

Compound	MW	xlogP	HBA	HBD	LIPINSKI	LC	TPSA_NOPS (Å^2)	RBC	DLS
1	158.15	1.64	2	0	Pass	0	34.1	0	−2.44
2	174.15	0.89	3	1	Pass	0	54.4	0	−1.89
3	172.18	2.07	2	0	Pass	0	34.1	0	−4.08
4	217.27	4.12	3	2	Pass	0	89	0	−1.79
5	233.27	3.75	4	3	Pass	0	109.2	0	−1.89
6	231.29	4.4	3	2	Pass	0	89	0	−1.90
7	244.31	4.24	3	1	Pass	0	76.4	1	−1.73
8	314.36	4.88	5	1	Pass	0	96.5	3	0.28
9	230.24	2	4	1	Pass	0	101.3	0	−5.72
Amphotericin B	924.12	0.21	17	11	Fail	3	299.4	3	−0.14
Meglumine	195.21	−3.71	6	6	Pass	1	113.2	6	1.76
Miltefosine	407.57	5.59	5	0	Pass	1	68.4	20	−54.74
Paromomycin	615.63	−10.36	19	13	Fail	3	347.3	9	2.49
Pentamidine	340.42	3.01	6	4	Pass	0	118.2	10	−5.35
Stibogluconate	676.77	−6.08	17	5	Fail	2	284.8	8	1.02

MW, molecular weight; HBA, H-bond acceptor; HBD, H-bond donor; LC, Lipinski count violations; TPSA_NOPS, total polar surface area; RBC, rotatable bond count; DLS, drug-likeness score; All molecular properties were calculated using Vortex data analysis and visualization software from Dotmatics, except DLS, which was calculated with the web-based molecular property calculator from OSIRIS Property Explorer.

Table 3: Predicted pharmacokinetic behaviour of synthesized naphthothiazoles

Compound	HIA (%)	F (%)	PPB (%)
4	83.82	66.71	57.29
5	56.41	55.78	51.61
6	82.59	58.66	54.28
7	85.39	66.03	55.08
8	83.28	43.36	59.15
9	72.50	73.89	67.31

HIA, human intestinal absorption; F, human oral bioavailability; PPB, plasma protein binding. The predicted pharmacokinetic parameters of the compounds were obtained from PK/DB Database for Pharmacokinetics Properties (http://miro.ifsc.usp.br/pkdb).

naphthothiazoles that significantly reduced the proliferation of promastigotes and intracellular amastigotes *in vitro*. Moreover, none of the tested compounds shown cytotoxicity to mammalian macrophages up to 12.5 μM.

The potent activity against promastigotes of water-soluble compounds **4** and **5** (0.31, 0.28 μg/mL), and moderate activity of 6 (1.08 μg/mL), suggests that the naphthothiazole ring, oxygenated at C-5, is important for antiparasitic activity and become a potential fragment for further structure–activity relationship exploration, as indicated by the biological data herein presented; free hydroxyl, O-alkylation or O-acylation did not affect the antiparasitic activity. On the other hand, the substitution on C-4 is clearly a potential site for functional group diversification, because, as the biological activity revealed, comparing to compound **4**, alkylation or hydroxylation of C-4 does not appear to affect the biological activity of the naphthothiazoles. The compound **5** (the most active against both forms of the parasite) contains 2 hydroxyl groups at C-4 and C-5, but the oxidation of this catechol to an *o*-quinoidal structure (Compound **9**) lead to loss of activity. As for the hydroxyl group, compounds with free amines or *N*-acyl substitution (e.g. compounds **4** and **8**, respectively) showed activity in both promastigote and amastigote. It is noticeable that free amine (compounds **4**, **5**, **6** and **7**) could be an important structural feature because this functional group is protonated in acidic microenvironment. Considering that the parasitophorous vacuoles containing *Leishmania* parasites maintain an acidic pH (pH 4.5–6.0) (32,33), this physicochemical free amine characteristic could lead to the accumulation of these compounds within the phagolysosomal compartment increasing the leishmanicidal effect. Molecular modification of compound **6** by either alkylation (compound **7**; EC_{50} 0.97 μg/mL against promastigotes) or acylation (Compound **8**; EC_{50} 1.18 μg/mL against promastigotes) of the hydroxyl group at position C-5 improved the antiparasitic activity, which could be related to higher lipophilicity. These preliminary structural requirements could be explored in a hit-to-lead evolution towards leishmanicidal compounds.

We have applied a preliminary analysis of the physicochemical properties and predicted the absorption and bioavailability for synthesized compounds. We have found that the semi-synthetic naphthothiazoles (compounds **4**–**9**) show no violations of Lipinski's Rule of Five. Among leishmanicidal drugs available on the market, only miltefosine does not violate Lipinski's Rule of Five; all other drugs have, at least, 1 violation. Moreover, the xlogP and TPSA value ranges from 2.0 to 4.88 and 76.4 to 109.2, respectively, for naphthothiazoles, suggesting that these compounds are potentially able to cross cell membranes in a permeation process, which could explain the ability to reach the amastigote inside of phagolysosome. According to our theoretical study, the TPSA of most leishmanicidal drugs currently on the market is higher than 140 $Å^2$ which reflects on poor absorption and bioavailability. The good drug-like properties of the naphthothiazoles are comparable to the ones for miltefosine suggesting a good oral bioavailability, because only miltefosine has an oral route of administration, while the other drugs are parenterally administered. Compared with miltefosine, the compounds **4**, **5**, **7** and **8** display higher DLS, which indicates that these compounds are more similar to commercial drugs, in terms of molecular properties, as those described in this work, and appear to have potential for oral administration (Table 2). In addition, the pharmacokinetic behaviour predicted to naphthothiazoles show a better profile for oral administration than miltefosine, which is almost 100% bound to plasma protein (34). There is no available data for humans about F and HIA, although some experiments in mice suggest good intestine absorption (35).

Thus, as the compounds described in this study demonstrated leishmanicidal activity, met the biological and chemical parameters, and potentially overcome the limitations of low oral bioavailability and absorption (36), they may represent oral drug candidates for hit-to-lead progression. Moreover, further structural modification of the bioactive compounds should be driven by these parameters to obtain higher drug score indexes and improved leishmanicidal activity.

Conclusions and Future Directions

The identification of the lead compounds or chemical starting point is essential in drug discovery pipeline. This study revealed four potential candidates for *in vivo* studies that drastically inhibited promastigote proliferation and the macrophages amastigote loads without affecting host cell viability, with high selectivity index and very good probable oral bioavailability. The relatively simple laboratory synthesis and the possibility to modify naphthothiazoles chemical structure constitute important advantages for development of new antileishmanial therapy. The results of this study provide a new rationale for the development of novel, semi-synthetic naphthothiazole lead compounds with leishmanicidal activity, which are

derived from natural sources of naphthoquinones. The potent and selective activity of semi-synthetic naphthothiazoles against amastigotes infection *in vitro* encourage the development of pharmaceutical drugs for future studies *in vivo*.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Complementary data from spectroscopy, crystallography, synthesis and leishmanicidal activity of naphthothiazoles.