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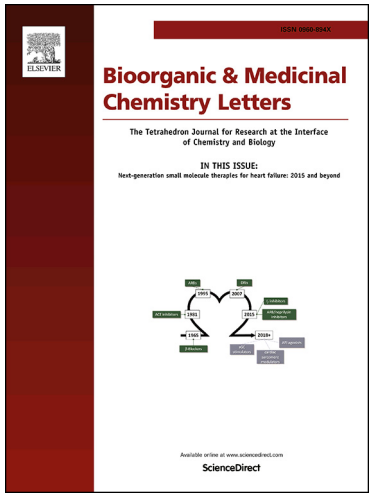

Synthesis and biological evaluation of isoxazolyl-sulfonamides: a non-cytotoxic scaffold active against *Trypanosoma cruzi*, *Leishmania amazonensis* and *Herpes Simplex Virus*

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Synthesis and biological evaluation of isoxazolyl-sulfonamides: a non-cytotoxic scaffold active against *Trypanosoma cruzi*, *Leishmania amazonensis* and *Herpes Simplex Virus*.

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Highlights

- Synthesis and characterization of 20 isoxazolyl-sulfonamides are reported.
- The compounds were active against *T. cruzi*, *L. amazonensis*, and HSV-1.
- Druglikeness evaluation showed potential for further pre-clinical development.

Abstract

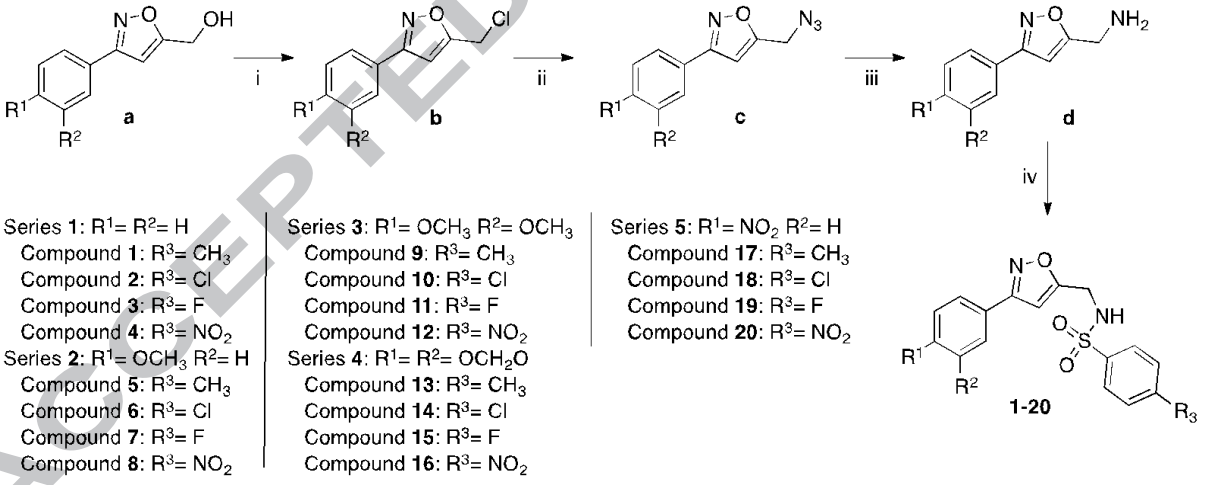
In this study we report the synthesis, characterization, biological evaluation, and druglikeness assessment of a series of 20 novel isoxazolyl-sulfonamides, obtained by a four-step synthetic route. The compounds had their activity against *Trypanosoma cruzi*, *Leishmania amazonensis*, *Herpes Simplex Virus type 1* and cytotoxicity evaluated in phenotypic assays. All compounds have drug-like properties, showed low cytotoxicity and were promising regarding all other biological activities reported herein, especially the inhibitory activity against *T. cruzi*. The compounds **8** and **16** showed significant potency and selectivity against *T. cruzi* (GI_{50}=14.3 µM, SI>34.8 and GI_{50}=11.6 µM, SI=29.1, respectively). These values, close to the values of the reference drug benznidazole (GI_{50}=10.2 µM), suggest that compounds **8** and **16** represent promising candidates for further pre-clinical development targeting Chagas disease.

Keywords: isoxazole; sulfonamide; *Trypanosoma cruzi*; *Leishmania amazonensis*; *Herpes Simplex Virus*

Since the discovery of Prontosil (4-[(2,4-diaminophenyl)diazenyl]benzenesulfonamide) as an antibacterial agent in 1932, sulfa drugs have a prominent place in therapeutics[1]. Soon after, sulfonamide-containing antidiabetics[2] and carbonic anhydrase inhibitor drugs[3,4] reached the market and remain relevant until current days. In recent decades, research on these classes continues active[5–9] although it also has moved to distinct applications. Sulfa-bearing compounds displaying anti-inflammatory (COX-2 inhibitors)[10], anti-proliferative[11–14], anti-parasitary[15–18], anti-A® fibrillogenisis[19,20], among other properties, have been reported and showcase the versatility of their biological potential.

Our research group has been working on the synthesis of heterocyclic compounds based on natural scaffolds to obtain new trypanocidal and leishmanicidal agents. In a previous work, a series of 3,5-disubstituted isoxazole derivatives was reported active against *T. cruzi,* and in an effort to expand the understanding of the structure-activity relationship, isoxazole-triazole bis-heterocyclic derivatives were later synthesized and assayed against the parasite[21,22]. Considering that sulfonamide moieties are frequent in numerous molecules of medicinal interest, we planned derivatives containing this functional group between the aromatic rings present in the previously reported molecules. The biological potential of these compounds was assessed by their cytotoxic, trypanocidal, leishmanicidal and antiherpes activities together with a druglikeness evaluation assisted by *in silico* tools.

Synthetically, the derivatives were obtained in four steps from 5-hydroxymethyl-3-phenyl-isoxazole derivatives (Compound **a**, Scheme 1)[23].



Scheme 1. Synthesis of isoxazolyl-sulfonamide compounds. i) $SOCl_2$, CH_2Cl_2, N_2, r.t. ii) NaN_3, DMF, r.t. iii) TPP, THF, r.t. iv) sulfonyl chloride, pyridine, CH_2Cl_2, r.t.

In the first step, the hydroxyl group was converted into a chlorine (Compound **b**), which is a suitable leaving group for the S_N2 reaction with sodium azide in the second step (Compound **c**). The azide derivative was reduced to an amine through the Staudinger reaction using triphenylphosphine in THF (Compound **d**) and, finally, the amine derivatives were coupled with sulfonyl chlorides leading to the isoxazolyl-sulfonamide derivatives **1**-**20** in satisfactory yields (from 17% to 62%). The final

compounds were characterized by ^{1}H and ^{13}C NMR spectroscopy and HRMS. The spectral details are described in Supplementary Data.

The biological evaluation of derivatives **1**-**20** started by measuring the effects of compounds on cell viability (VERO, THP-1, A549 and HCT-8 cell lines), using either MTT or sulforhodamine B assays. The compounds were tested at the maximum concentration of 500 µM, since a low cytotoxicity is desired because it would contribute to high selectivity indexes (SI) in the other activities.

Most of the compounds had no effects on cell viability even at the highest concentration tested. Compounds **5**, **11**, **13**, and **16** showed, in general, low cytotoxicity, which should not impact their biological application. Their concentration values responsible for reducing cell viability by 50% (CC_{50} µM) can be found in Table 1.

Table 1. Cytotoxicity of compounds **5**, **11**, **13** and **16**.

ID	A549[a]	HCT-8[a]	VERO[a]	THP-1[b]
5	>500	>500	>331.1 (130.2 – 842.2)	>500
11	327.3 (232.2 – 461.3)	373.8 (219.7 – 536.2)	397.2 (191.5 – 524.0)	299.8 (283.0 – 317.0)
13	357.8 (246.3 – 519.7)	436.9 (279.3 – 683.3)	>500	>500
16	283.5 (192.7 – 417.2)	>500	387.0 (188.6 – 793.9)	339.1 (321.0 – 357.0)

[a,b]Data represent the mean (95% confidence interval) of two or three independent experiments, respectively, and are expressed as CC_{50} values.

Next, we aimed to investigate compounds **1**-**20** as potential *hits* that could be developed to treat Chagas disease - a life-threatening condition caused by the protozoan parasite *Trypanosoma cruzi*. Although the disease is highly prevalent in Latin America countries, benznidazole (Rochagan®) and nifurtimox (Lampit®) are the only drugs currently available for pharmacological treatment and both show limited efficacy[24–26]. Having in mind the urgency of identifying new chemical entities which are active against the parasite, we used intracellular amastigotes of *T. cruzi* (Tulahuen strain expressing ß-galactosidase) cultivated in THP-1 cells to evaluate the trypanocidal activity of the compounds using a colorimetric assay. Phenotypic approaches are highly recommended in drug discovery for neglected tropical diseases once they allow identifying cell-permeating compounds that might affect parasites by reaching different molecular targets[27–29].

Compounds **1-20** were screened at 50 µM and, then, if the growth inhibition (%GI) was higher than 50%, they were serially diluted to determine the exact concentration responsible for inhibiting 50% of the parasite growth (GI_{50}). Table 2 shows that 14 out of 20 compounds inhibited the parasite growth to some extent and four of them presented GI_{50} values lower than 50 µM.

Table 2. Trypanocidal screening of isoxazolyl-sulfonamides **1**-**20**.

ID	%GI (50 µM)	GI_{50} (µM)	SI

1	5.8±1.1	>50	-
2	16.6±4.0	>50	-
3	NA	NA	-
4	34.0±1.2	>50	-
5	NA	NA	-
6	NA	NA	-
7	44.5±4.9	>50	-
8	85.8±1.0	14.3±0.9	>34.8
9	25.6±14.8	>50	-
10	27.4±2.5	>50	-
11	72.8±1.9	42.4±0.9	7.1
12	53.9±6.7	>50	-
13	NA	NA	-
14	14.2±0.7	>50	-
15	NA	NA	-
16	86.3±0.5	11.6±1.0	29.1
17	9.8±1.0	>50	-
18	NA	NA	-
19	7.8±1.0	>50	-
20	76.2±1.2	38.6±1.3	>12.9
BZN	96.6±0.2[a]	10.2±0.3	>49.1

Data represent the mean ± SD of three independent experiments. [a]Growth inhibition at 20 µM; SI: selectivity index = CC_{50} THP-1/GI_{50}; NA: not active at the tested concentration; BZN: benznidazole.

The obtained results suggest that a nitro substituent at the sulfonamidic aromatic ring plays an important role in the trypanocidal activity, once compounds **8**, **12**, **16**, and **20** are among the most potent. However, it is important to highlight that compound **4** does not show a relevant trypanocidal activity in spite of having this substituent. The main difference between compound **4** and the other compounds is the presence of substituents in the other phenyl ring, such as in compounds **8**, **12**, **16**, and **20**, suggesting that this might be a requirement for a higher trypanocidal activity due to pharmacodynamic factors. Considering the other unsubstituted compounds **1**-**3**, this hypothesis strengthens, once their activity is among the weakest in the entire series.

As an electron withdrawing group, the nitro substituent decreases the pKa of the sulfonamide acidic hydrogen assisting the hydrosolubility of the molecules. The highest aqueous solubility of the active compounds could indicate that pharmacokinetic factors also might be fundamental for this activity (Figure 1).

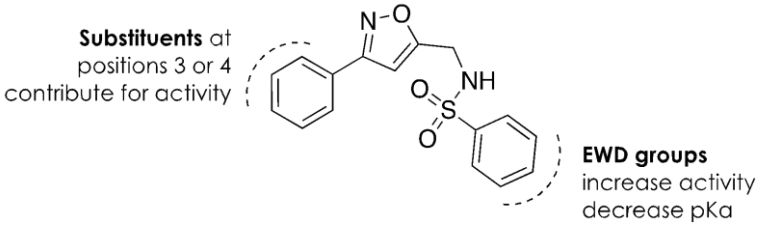


Figure 1. Suggested SAR for the trypanocidal activity of compounds **1**-**20**.

Interestingly, the fluoro-substituted compound **11** also showed a good trypanocidal activity, which might be related to the high electronegativity of fluorine. On the other hand, the fluoro-substituted molecules **3**, **7**, **15**, and **19** showed different behaviors; implying that the presence of methoxy groups in the other aromatic ring could have a determining role in their biological application.

Overall, compound **16** was the most active of the series showing a GI_{50} value comparable with that of the reference drug benznidazole (11.6 µM and 10.2 µM, respectively), and displays a high selectivity index (SI= 29.1). The potency in combination with selectivity demonstrates that compound **16** has the potential to be further investigated regarding its activity against *T. cruzi*.

Altogether with Chagas disease, leishmaniasis, which is caused by *Leishmania* parasites, is also a neglected kinetoplastid disease that has a great impact on socioeconomic indicators in developing countries[30,31]. Considering the promising results obtained from the trypanocidal screening, the activity against intracellular amastigotes of *L. amazonensis* (LTB0016 strain expressing ß-galactosidase) was also assessed.

Table 3. Leishmanicidal screening of isoxazolyl-sulfonamides **1**-**20**.

ID	%GI (50 µM)
1	10.9±2.6
2	17.2±0.7
3	13.2±1.0
4	18.4±0.5
5	2.0±0.5
6	6.4±2.0
7	14.0±1.4
8	48.7±0.4
9	4.1±1.0
10	NA
11	6.6±2.4
12	10.2±4.0
13	11.4±3.8
14	30.7±8.6
15	12.8±2.2
16	43.4±2.0

17	9.3±0.9
18	2.0±0.5
19	15.7±1.7
20	25.2±3.0
AB	92.1±0.9[a]

Data represent the mean ± SD of three independent experiments. [a]Growth inhibition at 2 µM; NA: not active at the tested concentration; AB: amphotericin B.

Except for compound **10**, all other compounds slightly affected the parasite growth (%GI > 0) even though their GI_{50} values are higher than 50 µM in all cases (Table 3). And, similarly to the results of the trypanocidal screening, compounds **8** and **16** were also the most active against *L. amazonensis*.

In order to guide us in exploring other biological activities of compounds **1**-**20**, a publicly available virtual prediction tool (SEA – Similarity Ensemble Approach, http://sea.bkslab.org) was used to locate molecular targets in which these compounds could bind[33]. Among different proteins, SEA suggested us to target the inhibition of the catalytic subunit of the DNA polymerase of *Human herpesvirus 6A* (HHV-6A). The Herpesviridae family comprises eight viruses highly prevalent in the population, including the causative agent of herpes labialis (*Herpes Simplex Virus* type 1, HSV-1). It is known that the catalytic subunit of HHV-6A's DNA polymerase displays around 62% of similarity and 37% of identity with the homologous enzyme of HSV-1. Therefore, we initially screened compounds **1**-**20** for their ability to inhibit HSV-1 replication (Table 4)[34,35]. The antiherpes screening was performed using a plaque number reduction assay[36]. Vero cells were inoculated with HSV-1 (KOS strain) and treated with compounds **1**-**20** at 100 µM to determine the percentages of replication inhibition (%RI 100 µM). Then, the most active compounds were assayed at different concentrations to determine their RI_{50} values (concentration that inhibits 50% of viral replication).

Table 4. Anti-HSV-1 screening of isoxazolyl-sulfonamides **1**-**20**.

ID	CC_{50} (µM) Vero cells	%RI (100 µM)	RI_{50} (µM)	SI
1	>500	25.3±12.4	>100	-
2	>500	17.8±2.3	>100	-
3	>500	40.2±8.7	>100	-
4	>500	10.4±3.4	>100	-
5	331.1 (130.2 – 842.2)	65.0±7.5	95.9±6.3	>3.4
6	>500	32.0±4.1	>100	-
7	>500	100±8.8	87.4±9.7	>5.7
8	>500	60.0±7.9	137.4±10.3	>3.6
9	>500	NA	NA	-
10	>500	2.0±0,9	>100	-

11	397.2 (191.5 – 524.0)	22.8±5.8	>100	-
12	>500	NA	NA	-
13	>500	19.1±7.1	>100	-
14	>500	NA	NA	-
15	>500	70.1±11.4	173.2±12.5	>2.9
16	387.0 (188.6 – 793.9)	25.3±5.7	>100	-
17	>500	NA	NA	-
18	>500	NA	NA	-
19	>500	NA	NA	-
20	>500	NA	NA	-
ACV	>2000	-	1.29±0.38	>1500

Data represent the mean ± SD or mean (95% confidence interval) of two independent experiments. SI: selectivity index = CC_{50} Vero/RI_{50}; NA: not active at the tested concentration; ACV: Acyclovir.

Within the group of 13 active molecules, compounds **5**, **7**, **8**, and **15** showed a significant anti-herpes activity inhibiting ≥ 60% of the HSV-1 replication at the tested concentration. Furthermore, the compounds **5** and **7** presented the lowest RI_{50} values among the evaluated compounds (RI_{50}= 95.9 and 87.4, respectively), and their SI values demonstrated that they are acting selectively against HSV-1 replication. Although the inhibition of DNA polymerase was not directly assessed in this assay, we could identify promising compounds to upcoming investigations regarding their mode of action.

In addition to potency, different factors are crucial in the decision of moving new chemical entities through the preclinical drug development pipeline. Physicochemical and structural descriptors are often associated to ADME properties, which are a frequent cause of failure in drug development process[37]. Toxicological concerns also impact how the pace of development is dictated and might restrict the application of a bioactive hit[38].

Aided by the software DataWarrior 4.7.2 (http://www.openmolecules.org), we calculated molecular weight (MW), Clog*P*, number of hydrogen bond acceptors (HBA), number of hydrogen bond donors (HBD), number of rotatable bonds (nROTB) and topological polar surface area (tPSA) of compounds **1**-**20** (Supplementary Data, p. S32). These properties are associated to Lipinski's Rule of Five and Veber rules, used as predictors of oral bioavailability, a desired aspect for bioactive molecules aiming to proceed in development[39,40]. The results have shown that most compounds fit all criteria of these rules, with the exception of compounds **12**, **16**, and **20** that have more than 140 Å² of polar surface area due to a combination of polar aromatic substituents.

Regarding toxicity, Hughes (2008) demonstrated that physicochemical properties, specifically Clog*P* and tPSA, could be related to *in vivo* adverse outcomes. In a set of preclinical molecules, those with Clog*P*> 3 and tPSA< 75 Å² showed 2.5 more likelihood of being toxic, possibly because of the increased probability of an off-target binding[41]. Moreover, DataWarrior 4.7.2 also provides a toxicity risk assessment by means of a fragment-based approach, being possible to predict whether

tumorigenic, mutagenic, and irritative functions are present in chemical structures. The data obtained for compounds **1**-**20** suggest that they are not prone to present toxic effects, thereby, positively influencing their druglikeness.

In conclusion, twenty non-cytotoxic isoxazolyl-sulfonamides were obtained through a four-step synthetic route from 5-hydroxymethyl-3-phenyl-isoxazole derivatives, previously described by our research group. To the best of our knowledge, all final compounds are not yet described in literature[42] and were phenotypically assayed against *Herpes Simplex Virus* type 1, and intracellular amastigotes of *T. cruzi* and *L. amazonensis*.

Our results have shown that the isoxazolyl-sulfonamide scaffold is promising regarding all biological activities studied herein, especially the inhibitory activity against *T. cruzi*. Compounds **8** and **16** were the most trypanocidal compounds, presenting high selectivity and potency near to those of the reference drug benznidazole. Furthermore, compound **7** was the most active in the anti-HSV-1 screening giving us an opportunity to continue improving this scaffold to obtain more potent antiherpetic derivatives. Also, all compounds could be ranked as highly *drug-like*, once they possess features that are related to adequate permeation, solubility, and low toxicity.

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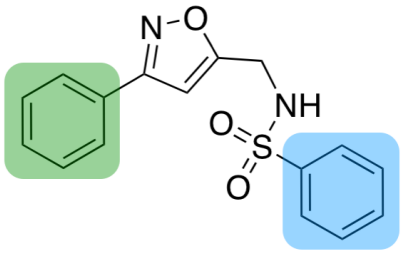
Highlights (3 to 5 of 85 characters each)

- Synthesis and characterization of 20 isoxazolyl-sulfonamides are reported.
- The compounds were active against *T. cruzi*, *L. amazonensis*, and HSV-1.
- Druglikeness evaluation showed potential for further pre-clinical development.

20 isoxazolyl-sulfonamide derivatives

Four-step synthetic route



Different aromatic substituents

-4-H
-4-OCH_3
-3,4-OCH_3
-3,4-OCH_2O
-4-NO_2

-4-CH_3
-4-Cl
-4-F
-4-NO_2

Biological investigation

Cytotoxicity
Anti-*Trypanosoma cruzi*
Anti-*Leishmania amazonensis*
Anti-*Herpes Simplex Virus*