Identification and Preliminary Structure-Activity Relationship Studies of Novel Pyridyl Sulfonamides as Potential Chagas Disease Therapeutic Agents

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Abstract: Chagas disease is a neglected pathology responsible for about 12,000 deaths every year across Latin America. Although six million people are infected by the *Trypanosoma cruzi*, current therapeutic options are limited, highlighting the need for new drugs. Here we report the preliminary structure activity relationships of a small library of 17 novel pyridyl sulfonamide derivatives. Analogues **4** and **15** displayed significant potency against intracellular amastigotes with EC_{50} of 5.4 µM and 8.6 µM. In cytotoxicity assays using mice fibroblast L929 cell lines, both compounds indicated low toxicity with decent selectivity indices (SI) >36 and >23 respectively. Hence these compounds represent good starting points for further lead optimization.

Chagas disease (CD) is a parasitic infection caused by the protozoan *Trypanosoma cruzi* (*T. cruzi*). This neglected disease is endemic to 21 countries across Latin America with more than six million people infected worldwide. A further 70 million people are at risk of infection and approximately 12,000 deaths annually are associated with CD.[1] CD has become an important public health concern in other non-endemic countries across North America, Europe and Asia because of globalization mainly due to the migration of infected individuals to these areas.[2-3] CD treatment is based on two nitroheterocyclic drugs: nifurtimox (Nf) and benznidazole (Bz), both introduced in clinics more than 50 years ago.[4] These nitroderivatives require long administration periods and produce adverse side effects, which may result in the discontinuation of the treatment by 20-30% of the patients. Furthermore, there are several naturally resistant strains and very low cure rates (≤ 20 %) once the disease reaches chronic phase.[5-6]

Two inhibitors of the ergosterol biosynthetic route Posaconazole and E1224 (a pro-drug of ravuconazole) were recently evaluated in clinical trials on chronic chagasic patients. Unfortunately, both failed to sustain high therapeutic cure rates after one year of follow up despite promising results in experimental murine and canine models of *T. cruzi* infection.[7-8] Also, the recently concluded clinical trial BENEFIT, revealed that Bz is not able to impair or reduce the progression of the chagasic cardiomyopathy when administered to chronic patients, despite the remarkable reduction of the parasite load assessed through qPCR.[9] Those findings emphasize the importance of pre-clinical studies to investigate the biological activity of novel molecules against *T. cruzi*, aiming to find new alternative treatments that are more tolerable, potent, orally adequate, with broader efficacy, lower costs and reduced administration periods.[10]

Presently, we have identified a 3-pyridyl sulfonamide derivative (compound **1**) active against *T. cruzi* from a high throughput screening of a small set of 100 diverse compounds based on our previous study.[11] Compound **1** showed an EC_{50} (minimal concentration able to reduce the infection index by 50%) of 5.5 µM against intracellular forms of *T. cruzi* (Silvio X10/7 strain), with minimal toxicity to the mammalian hosts (Vero cell line), and comparable activity to nifurtimox, with EC_{50} of 0.9 µM (Figure 1).

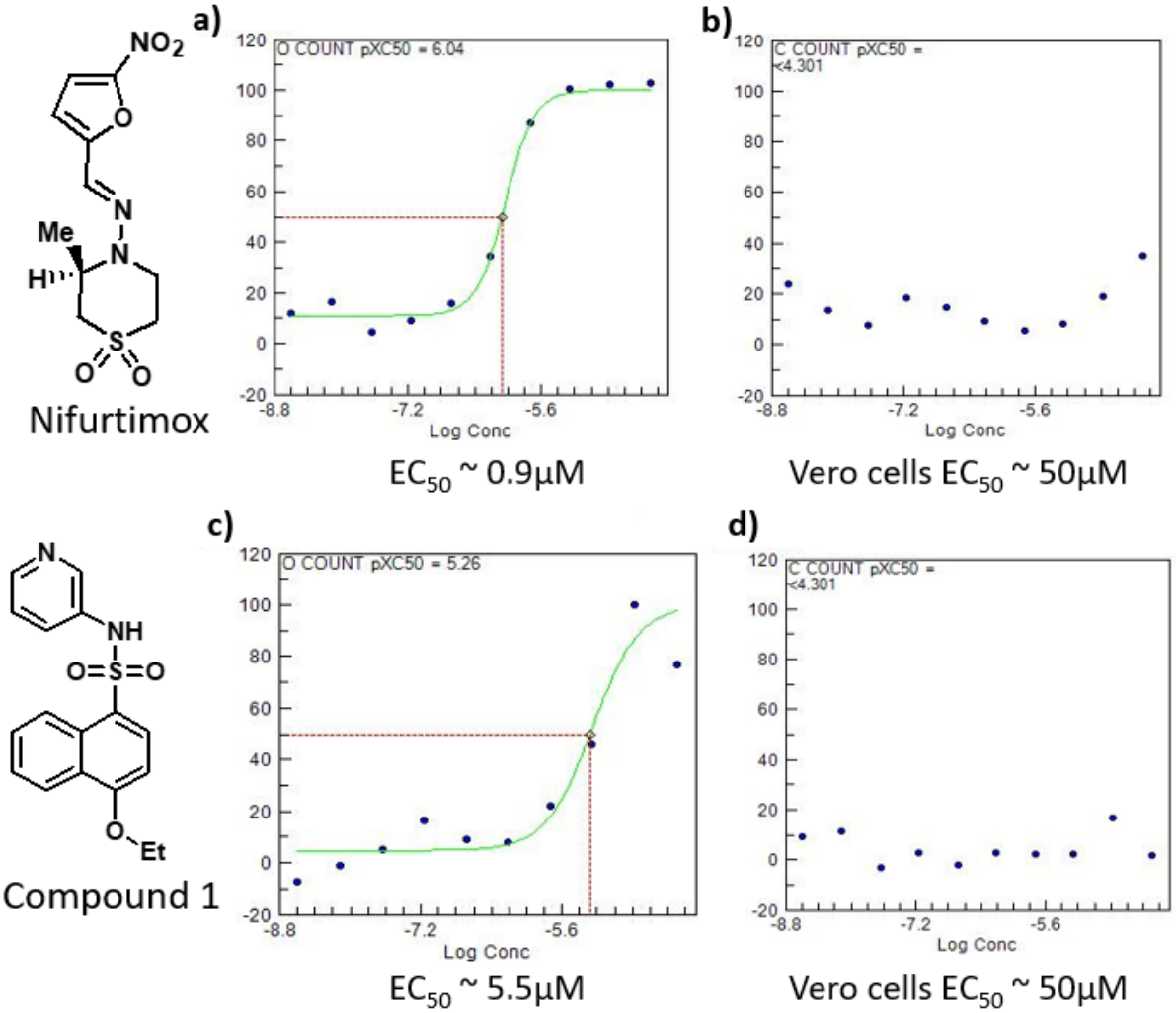


Figure 1. *Silvio strain of T. cruzi*; *Ten-point dose response curves and Vero cells toxicity of Nifurtimox (**a** and **b**) and compound **1** (**c** and **d**) with calculated potency values. The X-axis shows log of compound molar concentrations (M) and Y-axis shows the normalized activity based on the measurement of number of amastigotes per host cell.*

As a part of the project related to Chagas disease drug discovery that recommends testing against different parasite strains and forms relevant for mammalian infection (intracellular and bloodstream forms), compound **1** was selected as a lead compound due to its low toxic profile, drug likeness (Lipinski's rule of five) and synthetic accessibility. A small set of 16 analogues (**2-17**) were purchased (Table 1) to establish the initial structure activity relationships (SARs). As a part of SAR development, compounds (Supporting information) were selected based on various modifications that differ in steric properties (naphthyl *Vs* phenyl), location of nitrogen on the pyridyl ring (2^{nd}, 3^{rd} and 4^{th} position), alkyl chain length (methoxy, ethoxy and propoxy substituents) and extent of branching (methyl, ethyl, isopropyl and tertiary butyl). Compounds with methylene linker were also selected and parameters such as the EC_{50} values against the intracellular parasites were taken into consideration while establishing the SARs.

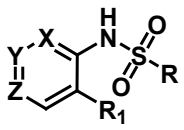
Next, regarding the phenotypic biological analysis, the novel analogs were initially assayed using a fixed concentration (10 µM) that corresponds to the EC_{90} of Bz. The non-infected cells (controls) were treated with DMSO. Analogues that reached similar or higher activity than Bz were further evaluated in assays with increasing concentrations (serially diluted) for the determination of the EC_{50}. In both assays, the cultures were maintained at 37 ºC for 96 h.[12]

When compound **1** was further screened against intracellular forms of *T. cruzi* but now against Tulahuen strain using Bz as reference drug, the lead compound with 3-pyridyl moiety and 4-ethoxy naphthyl ring **A** displayed lower activity than Bz (Table 1), but sustained a low toxicity profile against other cell line (L929 cells reaching $LC_{50} > 400$). When a small set of analogues (**2-4**) was screened to investigate the contribution of pyridyl moiety towards the anti-*T.cruzi* activity we found that the replacement of 3-pyridyl moiety from compound **1** (EC_{50} >25 µM)

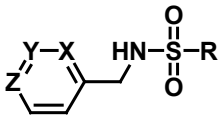
either with 4-pyridyl (**2**) or 2-substituted pyridyl (**3**) moieties did not improve the potency of the compounds towards the intracellular forms (see Table 1 regarding the % of reduction levels using a fixed concentration of 10 μM). These results suggest that the position of the nitrogen on the pyridyl ring is not relevant for the anti-parasitic activity.

Replacement of 4-ethoxy naphthyl ring **A** in compound **1** with 6-methoxy naphthyl ring **C** (**4** in Table 1) resulted in over 5-fold increase in potency (EC_{50} of ~5.44 μM). This suggests that the substitution pattern of the naphthyl ring is important for activity.

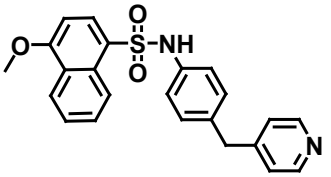
Table 1. *Modifications of pyridyl sulfonamide derivatives and their in vitro activity profiles. Activity (% reduction of the L929 culture infection) was determined using a fixed concentration at 10 µM. Activity (EC_{50} - Mean ± SD - 96h), toxicity (L929 – 96 h) and selectivity were determined against intracellular forms of T. cruzi (Tulahuen B-Galactosidase transfected strain).*



TYPE I



TYPE II
(Methylene spacer)



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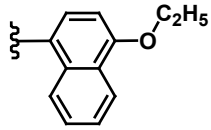
'R' Substitutions



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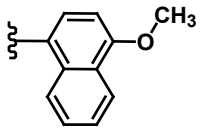

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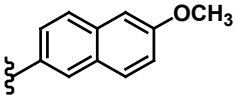

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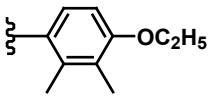

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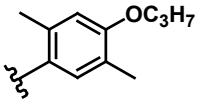

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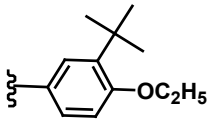

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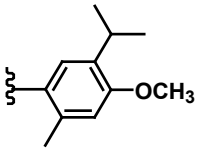
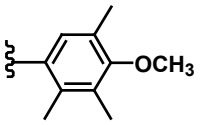


I



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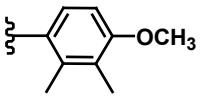

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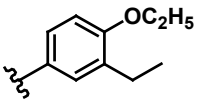

F

ID	Type	*X*	*Y*	*Z*	R_1	R	% reduction on the infection level*	EC_{50} µM	LC_{50} µM	SI (LC_{50}/ EC_{50})
Bz		-	-	-	-	-	89.3 ± 2.8	3.51 ± 0.78	>400	>114
1	I	C	N	C	H	**A**	ND	>25	>400	ND
2	I	C	C	N	H	**A**	0.1 ± 0.1	-	>200	-
3	I	N	C	C	CH_3	**A**	0	-	>200	-
4	I	C	N	C	H	**C**	41.1 ± 11.5	5.44 ± 2.76	>200	>36
5	I	C	N	C	H	**D**	62 ± 5.6	14.79 ± 3.55	>200	>13.5
6	I	C	C	N	H	**D**	3.6 ± 7.1	-	>200	-
7	I	C	N	C	H	**E**	78 ± 6.1	13.21 ± 0.02	>200	>15
8	I	C	N	C	H	**F**	3 ± 0.5	-	>200	-
9	I	C	N	C	H	**H**	2.1 ± 1.8	-	>200	-
10	I	C	N	C	H	**I**	26.70 ± 17.51	-	>200	-
11	I	C	N	C	H	**J**	1.9 ± 3.5	-	>200	-
12	-	-	-	-	-	**-**	0	-	>200	
13	II	C	N	C	-	**A**	1.1 ± 1.9	-	>200	-
14	II	C	N	C	-	**B**	28.0 ± 24.9	-	>200	-
15	II	C	C	N	-	**D**	65 ± 13.9	8.62 ± 8.64	>200	>23
16	II	N	C	C	-	**G**	7.2 ± 5.9	-	>200	-
17	II	N	C	C	-	**D**	3.6 ± 0	-	>200	-

ND: not determined

Then, we have examined the effect of combination of 3-pyridyl moiety with substituted phenyl rings (**D** to **J**) towards the activity. 3-pyridyl moiety in combination with 2,3-dimethyl-4-ethoxy substituted phenyl ring **D** (**5**) and 2,5-dimethyl-4-propoxy substituted phenyl ring **E** (**7**) displayed better trypanocidal activity than the lead compound **1**, with EC_{50} of ~14 µM and ~13 µM, respectively. The compound **6** with 4-pyridyl moiety resulted in complete loss of activity.

Interestingly combination of 3-pyridyl moiety with bulky ethyl (compound **8** with **F**), trimethyl substitutions (compound **9** with **H**), branched isopropyl (i-Pr; compound **10** with **I**) and tertiary butyl (t-butyl; compound **11** with **J**) moieties were not tolerated suggesting that dimethyl substitutions (**D** and **E**) along with ethoxy or propoxy or methoxy groups on the phenyl ring are optimal. The substitution pattern on the aromatic ring attached to the sulfonyl group also had a large impact on activity as the methyl substituted phenyl derivatives **5** and **7** showed activity with an EC_{50} of ~14 µM and ~13 µM compared to inactive branched i-Pr and t-butyl analogues **10** and **11**. Extension of the aromatic amine with methylene-4-pyridyl ring (**12**) did not result in anti-parasitic effect suggesting that aromatic amine ring extension has no impact on the potency of the compounds (Table 1).

Introduction of methylene spacer between 3-pyridyl ring and amine group of the compound **1** resulted in compound **13** (type II) that showed no anti-*T.cruzi* activity. Compound **14** (type II) with 3-pyridyl ring was also inactive similarly to compounds **16**, **17** (type II) with 2-pyridyl moiety and 2,3-dimethyl-4-methoxy **G** and 2,3-dimethyl-4-ethoxy phenyl substitutions **D** that did not display anti-parasitic effect. Interestingly replacement of 2-pyridyl moiety in compound **17** with 4-pyridyl moiety (**15)** improved the activity with an EC_{50} of ~8.62 µM. This

indicates that 4-pyridyl nitrogen could be involved in hydrogen bonding interaction with the parasite target.

Sulfonamide derivatives **1**-**17** (Table 1) were further evaluated for their toxicity on mouse fibroblast cell lines NTCL clone 929 (L929). The cell cultures were incubated with increasing concentrations of the compounds **1**-**17** and host cell viability assessed through colorimetric assays using AlamarBlue for determining the LC_{50} that corresponds to the compound concentration reducing viability by 50%.[12] The ratio of LC_{50} and EC_{50} values is reported as Selectivity Index (SI) in Table 1. In general, all these compounds showed an excellent *in vitro* safety profile, with no toxicity noticed until 200 µM. Although benznidazole showed the higher selectivity index (SI) value (> 114), among the sulfonamide analogs, compound **4** with 6-methoxy naphthyl ring substitution displayed better selectivity with SI value >36. Compounds **15**, **7** and **5** displayed moderate selectivity with SI values >23, >15, and >13.5 respectively (Table 1).

Overall four analogues (**4**, **5**, **7** and **15**) of compound **1** showed activity against intracellular forms of *T.cruzi*. Compound **4** is the most potent with EC_{50} of ~ 5.44 µM. These compounds were further analysed using bloodstream trypomastigotes (BT - Y strain, DTU II) obtained by cardiac puncture from Swiss Webster mice during the parasitemia peak.[13] All animal procedures carried out in accordance with the guidelines established by the FIOCRUZ Committee of Ethics for the Use of Animals (CEUA LW16/14). After 24 h incubation at 37 °C, the indexes of parasite death were determined by quantifying the motile and live parasites in a Neubauer chamber using light microscope for the determination of EC_{50} values. The results are shown in the following Table 2.

Table 2. *In vitro effect (EC_{50} values in µM) of the selected compounds (3 assays using triplicates) against bloodstream trypomastigotes of T. cruzi (Y strain) and cardiac cell culture toxicity (LC_{50} values in µM) after treatment for 24 h at 37 °C, and their corresponding selectivity index (SI)*

Cpd ID	EC_{50} µM	LC_{50} µM	SI (LC_{50}/ EC_{50})
Bz	11.20 ± 2.4	>1000	>89
4	>50	>100	ND
5	>50	>100	ND
7	>50	>100	ND
15	>50	>100	ND

ND: not determined

As the heart is an important target organ for *T.cruzi* infection and inflammation, the toxicity upon cardiac cells was evaluated using heart samples of Swiss Webster mice embryos as reported by Meirelles *et al.*[13] Uninfected cardiac cell cultures were incubated for 24 h with different concentrations of these compounds and then cell viability assessed with PrestoBlue for determination of the LC_{50} values.[14] All four compounds did not induce loss of cellular viability after incubation for 24 h with concentrations up to 100 µM, whereas reference compound (Bz) showed LC_{50} > 1000 µM. After 24 h of incubation at 37 °C, all compounds did not show trypanocidal effect against BT up to 50 µM, while Bz potency was 11 µM (Table 2).

Study of overall pharmacokinetic compliance of the active compounds. *In silico* studies were carried out to predict the pharmacokinetic (PK) characteristics. Computational assessment of PK properties for the novel drug candidates at early stages of drug discovery programmes has gained importance as experimental determination of these properties using *in vivo* models is costly and time consuming. This information is widely used to refine the PK properties at the early stage of lead identification or lead optimization.[15] *In silico* prediction of the drug likeness and pharmacokinetic properties of five active sulfonamide compounds (Table 3) was carried out using the QikProp module of Schrodinger and admetSAR.[16]

Table 3. *Assessment of Drug likeness and pharmacokinetic properties of the active compounds.*

		Recommended compliance score (range for 95% of drugs)	Compound ID				
			1	**4**	**5**	**7**	**15**
Rule of five	M.Wt	≤ 500	328.38	314.36	306.38	320.41	320.40
	HBD	≤ 5	1	1	1	1	1
	HBA	≤ 10	5	5	5	5	5
	cLogP	≤5	3.57	2.98	3.35	3.93	2.95
Rule of three	QPlogS	–6.5 to 0.5	-3.91	-3.6	-3.64	-3.98	-2.44
	QPCaco	<25 poor, >500 great	1205	1050	1209	1276	960
	Primary metabolites	<7	4	4	6	6	6
% Human oral absorption	-	>80% is high, <25% is low	100	93	96	100	93
QPPMDCK	-	>500 great, <25 poor	609	525	611	645	508
QPlogKhsa	-	–1.5 to 1.5	-0.12	-0.22	-0.21	-0.08	-0.25
QPlog HERG	-	concern below –5	-6.15 (wi)	-5.99 (wi)	-5.39 (wi)	-5.43 (wi)	-3.64 (ni)
AMES Toxicity	-	Non-AMES toxic					
Carcinogenicity	-	Non-carcinogens					

M.Wt: Molecular Weight; HBD: hydrogen bond donor; HBA: hydrogen bond acceptor; cLogP: calculated logarithm of partition coefficient; QPlogS: the logarithm of aqueous solubility; QPCaco: Caco-2 cell permeability in nm/sec, model for the gut-blood barrier; QPPMDCK: Madin-Darby canine kidney (MDCK) cell permeability in nm/sec, model for the blood-brain barrier; QPlogKhsa: the logarithm of predicted binding constant to human serum albumin; QPlog HERG: Predicted IC_{50} *value for blockage of HERG K+ channels*; wi: week inhibitor; ni: not inhibitor.

Recommended compliance scores for the PK properties[17] are used to assess the drug likeliness of these compounds. All showed compliance to the "Rule of five"[18] and "Rule of three".[19-20] Hence these compounds could show good aqueous solubility (within the recommended range), and excellent permeability (greater than 500) properties. Good solubility and permeability are crucial parameters for absorption. Excellent absorption properties of these sulfonamide derivatives (average > 90 %) from the gastrointestinal tract could lead to good oral bioavailability. Possible metabolic sites of these compounds have also been predicted to understand their ability to reach the parasitic target site. The recommended possible metabolic sites for small molecules are 7. Possible metabolic sites for

all the active compounds are between 4 and 6. These computed properties clearly indicate the drug-likeness of these sulfonamide derivatives.

QPPMDCK values are the prediction of MDCK cell permeability (nm/s) of the compounds. Greater values of QPPMDCK indicates higher cell permeability.[21] All active compounds displayed good QPPMDCK values and are thus likely to show good blood brain permeation. QPlogKhsa values are the prediction of ability of the compounds to bind to human serum albumin. All the active compounds displayed compliance to the recommended values, indicating that these compounds would have lower binding to serum albumin and the unbound fraction could have access to the putative receptor drug target. In recent years hERG screening has been introduced earlier in drug discovery program.[22] Human ether-a-go-go related gene (HERG) encodes a potassium ion (K+) channel that is implicated in the fatal arrhythmia. If a drug molecule blocks the potassium ion (K+) channel then it develops the risk of heart failure. Hence HERG potassium channel blockers are potentially toxic.[23] The recommended compliance score for blockage of HERG K+ channels is > −5. Compound **15** showed an acceptable level (log HERG -3.64) of HERG inhibition. Compounds **1, 4, 7** and **5** displayed marginal levels of HERG inhibition. Furthermore, all compounds are predicted to be non-mutagenic and non-carcinogenic (Table 3).

Literature data reported the promising trypanocidal effect of sulfonamides in *in vitro* studies suggesting the importance of this chemical group as a scaffold for the development of new anti-*T.cruzi* agents.[24] Also, aromatic/heterocyclic sulfonamides incorporating halogeno/methoxyphenacetamido tails were able to inhibit the α-carbonic anhydrase of *T.cruzi*, being less effective against the human off target isoforms, but not active against *in vivo* infection in animal models.[25] The use of Nano emulsion oil formulation increased the

effects upon *T. cruzi* probably due to an enhancement of the compounds permeation, suggesting that this type of formulation may lead to novel therapeutic approaches against this neglected disease.[26] It has also been reported that benzenesulfonylhydrazone (BS-H) and N-propionylbenzene sulfonylhydrazone (BS-NAH) derivatives displayed anti-trypanosomal activity against the NINOA and INC-5 strains of T.cruzi which are endemic in Mexico. This suggests that aryl sulfonyl derivatives has potential trypanocidal activity against different strains of T.cruzi.[27] As a part of our anti-trypanosomal drug development strategy, we have previously reported a study involving parallel evaluation of arylimidamide derivatives for their efficacy and ADMET properties[28]. This strategy has resulted in compounds with submicromolar trypanocidal activity and provided a better understanding of their drug likeness and pharmacokinetic properties at the early stage of our lead optimization process. We are adopting a similar strategy in our sulfonamide lead optimisation studies. Our present phenotypic data, especially upon intracellular forms, demonstrate that 3-pyridyl sulfonamide compound (**4**) and pyridin-4-yl-methylsulfonamide (**15**) derivative are potential lead compounds for the development of novel anti-*Trypanosoma cruzi* drugs. The reported results should be useful in guiding future efforts to develop compounds with increased potency and maintaining the lower toxicity profile against mammalian hosts. Currently synthesis programme is underway in our laboratory to design and develop pyridyl sulfonamides as potential drug like candidates targeting *T. cruzi*.

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Supplementary data

This include HRMS data, ^{1}HNMR spectroscopic data of the sulfonamide derivatives.

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