Accepted Manuscript

Novel Imidazo[4,5-*c*][1,2,6]thiadiazine 2,2-dioxides as antiproliferative *trypanosoma cruzi* drugs: Computational screening from neural network, synthesis and *in vivo* biological properties

Angela Guerra, Pedro Gonzalez-Naranjo, Nuria E. Campillo, Javier Varela, María L. Lavaggi, Alicia Merlino, Hugo Cerecetto, Mercedes González, Alicia Gomez-Barrio, José A. Escario, Cristina Fonseca-Berzal, Gloria Yaluf, Jorge Paniagua, Juan A. Páez

PII: S0223-5234(17)30357-4

DOI: 10.1016/j.ejmech.2017.04.075

Reference: EJMECH 9426

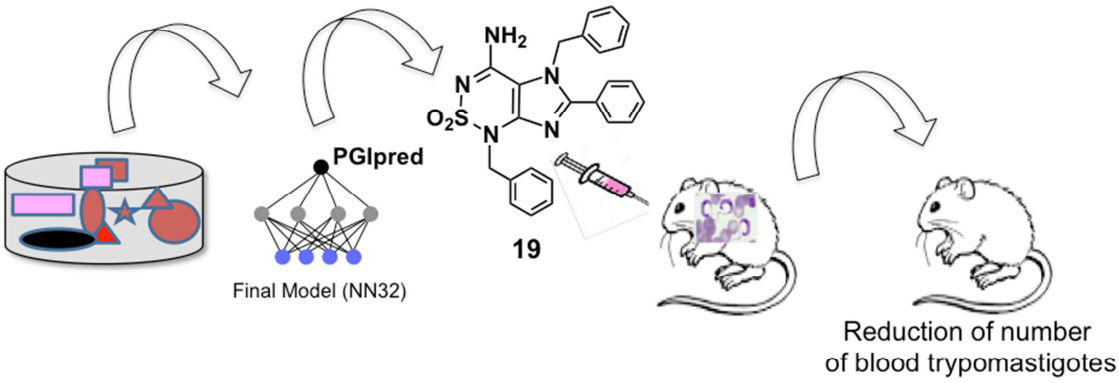
To appear in: *European Journal of Medicinal Chemistry*

Received Date: 1 February 2017

Revised Date: 25 April 2017

Accepted Date: 29 April 2017

Please cite this article as: A. Guerra, P. Gonzalez-Naranjo, N.E. Campillo, J. Varela, Marí.L. Lavaggi, A. Merlino, H. Cerecetto, M. González, A. Gomez-Barrio, José.A. Escario, C. Fonseca-Berzal, G. Yaluf, J. Paniagua, J.A. Páez, Novel Imidazo[4,5-*c*][1,2,6]thiadiazine 2,2-dioxides as antiproliferative *trypanosoma cruzi* drugs: Computational screening from neural network, synthesis and *in vivo* biological properties, *European Journal of Medicinal Chemistry* (2017), doi: 10.1016/j.ejmech.2017.04.075.



Novel Imidazo[4,5-*c*][1,2,6]thiadiazine 2,2-Dioxides as Antiproliferative *Trypanosoma Cruzi* Drugs: Computational Screening from Neural Network, Synthesis and *in vivo* Biological Properties

Angela Guerra[a], Pedro Gonzalez-Naranjo[a], Nuria E. Campillo[b], Javier Varela[c], María L. Lavaggi[c], Alicia Merlino[c], Hugo Cerecetto[c], Mercedes González[c], Alicia Gomez-Barrio[d], José A. Escario[d], Cristina Fonseca-Berzal[d], Gloria Yaluf[e], Jorge Paniagua[f], and Juan A. Páez[a]*

[a] Instituto de Química Médica. Consejo Superior de Investigaciones Científicas (CSIC), Juan de la Cierva 3, 28006 Madrid, Spain.

[b] Centro de Investigaciones Biológicas. Consejo Superior de Investigaciones Científicas (CSIC). Ramiro de Maeztu 9, 28040 Madrid, Spain.

[c] Grupo de Química Medicinal, Laboratorio de Química Orgánica, Facultad de Ciencias-Facultad de Química, Universidad de la República, Iguá 4225, 11400 Montevideo, Uruguay.

[d] Departamento de Parasitología. Facultad de Farmacia. Universidad Complutense de Madrid, Pza. Ramón y Cajal s/n, 28040, Madrid, Spain.

[e]Instituto de Investigaciones en Ciencias de la Salud (iics), Universidad Nacional de Asunción, Asunción-Paraguay

[f] Laboratorios Silanes IDF, S.L. *Calle Santiago Grisolia, Nº 2- PTM 148 Parque Tecnologico de Madrid 28760-Tres Cantos-Madrid.*

*Corresponding author. Dr. Juan A. Páez. Instituto de Química Médica (CSIC). Juan de la Cierva, 3, 28006-Madrid. Spain. Phone: +34 915622900; Fax: +34 915644853; E-mail: jpaez@iqm.csic.es

Abstract

A new family of imidazo[4,5-*c*][1,2,6]thiadiazine 2,2-dioxide with antiproliferative *Trypanosoma cruzi* properties was identified from a neural network model published by our group. The synthesis and evaluation of this new class of trypanocidal agents are described. These compounds inhibit the growth of *Trypanosoma cruzi*, comparable with benznidazole or nifurtimox. *In vitro* assays were performed to study their effects on the growth of the epimastigote form of the Tulahuen 2 strain, as well as the epimastigote and amastigote forms of CL clone B5 of *Trypanosoma cruzi*. To verify selectivity towards parasite cells, the non-specific cytotoxicity of the most relevant compounds was studied in mammalian cells, i.e. J774 murine macrophages and NCTC clone 929 fibroblasts. Furthermore, these compounds were assayed regarding the inhibition of cruzipain. *In vivo* studies revealed that one of the compounds, **19**, showed interesting trypanocidal activity, and could be a very promising candidate for the treatment of Chagas disease.

Keywords: CODES®, Chagas disease, imidazo[4,5-*c*][1,2,6]thiadiazine, neural network, *trypanocidal, Trypanosoma cruzi*

1. INTRODUCTION

Chagas disease, also known as American trypanosomiasis, is the result of infection with the protozoan parasite *Trypanosoma cruzi*. This zoonosis can be transmitted to humans by insect vectors that are found only in the American continent, mainly in rural areas with unhealthy housing conditions where poverty is a general concern. The World Health Organization (WHO) estimates that 6-7 million people are currently infected with *Trypanosoma cruzi* in the 21 endemic countries of the American continent [1]. In recent years, migration and travel have extended the distribution of Chagas disease to non-endemic areas of other continents, including Europe [2,3] parts of the Western Pacific [4] and North America [5, 6]. Most people with *Trypanosoma cruzi* acquire their infections in endemic countries [5].

Despite the efforts and initiatives taken by the WHO in terms of transmission control of Chagas disease, it is estimated that over 25 million people are at risk of infection. The economic cost of Chagas disease was estimated in 2013 at more than US $7 billion per year, with more than 7000 deaths per year caused by this disease [7].

Currently, only two drugs are used for the treatment of Chagas disease. These are two nitro heterocyclic compounds, i.e. a nitrofuran (nifurtimox) and a nitroimidazole (benznidazole) whose anti-*Trypanosoma cruzi* activities were discovered empirically over four decades ago [8]. These drugs are not approved by the FDA and are available only from the Centers for Disease Control and Prevention (CDC) under investigational drug protocols [9]. The production of nifurtimox has been discontinued; thus, benznidazole is currently the only commercially available treatment in most endemic countries. Two alternative drugs for Chagas disease treatment are allopurinol and itraconazole, which have been used in some selected cases and in certain patients [10,11] (Figure 1). Several clinical trials for the treatment of chronic Chagas disease with posaconazole [12, 13] and E1224 [14, 15] have been completed in the last year. Unfortunately, these trials did not show significant advantages in relation to benznidazole [16-18].

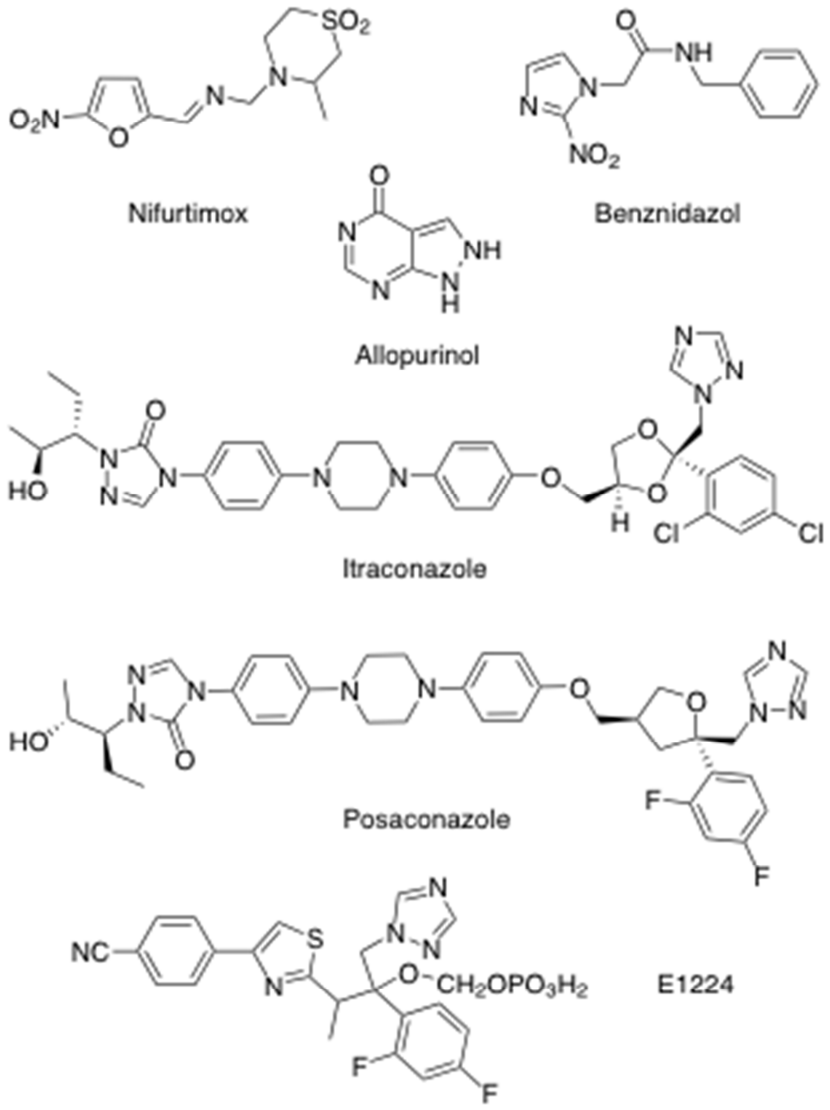


Figure 1. Chemical structures of drugs introduced to the market and in clinical trials for Chagas disease.

The current treatments are unsatisfactory because of their low effectiveness in the chronic phase and their adverse side effects, in addition to the fact that they do not address the serious consequences of this illness [8]. The side effects of these drugs include dermatological reactions [19] agranulocytosis and polyneuropathy [20]. Benznidazole and nifurtimox should not be taken by pregnant women [21] or by people with kidney or liver failure [21- 23]. It has been found that benznidazole can provoke lymphoma in a rabbit model [24]. However, in humans and at the doses used for Chagas treatment, lymphoma has not been detected. In addition, a mechanism for cross-resistance to nifurtimox and benznidazole has been described [25].

It is, therefore, necessary and urgent to develop new and effective trypanocidal drugs for the suitable treatment of

this disease. Motivated by our interest in the development of effective drugs for Chagas disease, a virtual screening approach was applied to discover new hits against *Trypanosoma cruzi*. In this study, we report the identification, synthesis and pharmacological assessment of a new family of heterocyclic compounds derived from imidazothiadiazines with interesting trypanocidal properties.

2. RESULTS AND DISCUSSION

2.1 Rational Design

One of the key steps in drug design is the hit identification process. In line with our ongoing efforts on the development of trypanocidal drugs, we developed a neural network model with a three-layered 4-4-1 architecture to predict anti-*T. cruzi* activity against the epimastigote form of the Tulahuen strain with 78% accuracy in the external prediction [26]. The codification of the structures was achieved with the CODES® program [27]. In this context, we employed the model to predict the potential activity of a diverse set of compounds developed in our laboratory. Among these compounds, we identified several imidazo[4,5-*c*][1,2,6]thiadiazine 2,2-dioxide derivatives showing interesting anti-*T. cruzi* activity.

Based on these hits, we proposed a virtual library of derivatives, taking into account that the structure of the imidazo[4,5-*c*][1,2,6]thiadiazine 2,2-dioxide system can have variations on the substituents at three different positions. Thus, at position 6, a variety of groups such as alkyl, cycloalkyl, benzyl, aryl or heteroaryl were tested. *N1*-substituted groups included hydrogen, methyl, benzyl, or 4-chlorobenzyl. At position 5 of the heterocycle, only hydrogen and benzyl were proposed (Figure 2). Finally, we predicted the values of the percentage of growth inhibition (PGIe) against epimastigotes of *T. cruzi* at a fixed dose for a set of 20 imidazo[4,5-*c*][1,2,6]thiadiazine 2,2-dioxides that include *N*-1*H*, *N*-1 substituted and *N*-1 and *N*-5 substituted imidazothiadiazines (Figure 2).

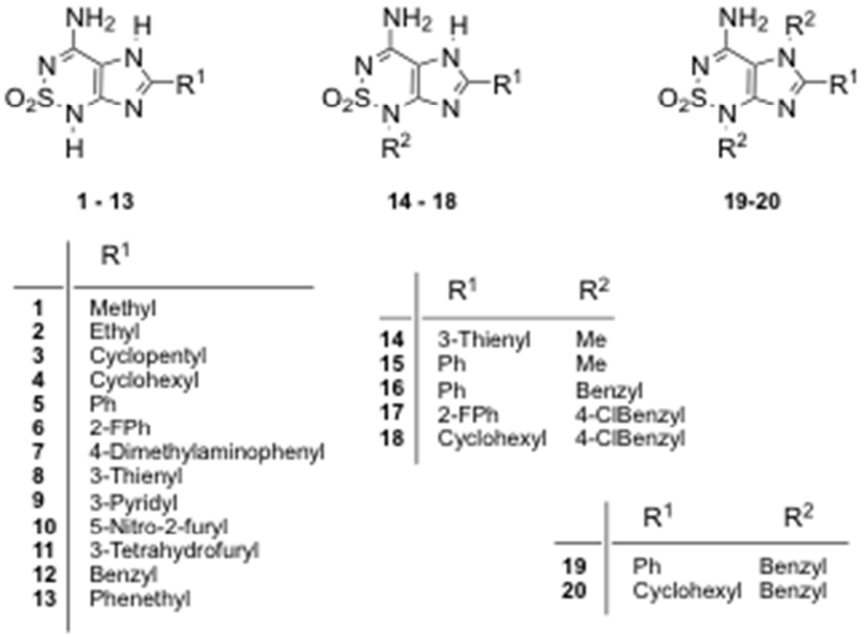


Figure 2. Chemical structures of the imidazo[4,5-*c*][1,2,6]thiadiazine derivative family.

Thus, using the methodology developed previously, we achieved the codification of the molecules from a non-supervised neural network using the CODES® program [27]. This program codifies each molecule into a set of numerical parameters, exclusively taking into account the information on its chemical structure. A subsequent dimension reduction procedure by means of supervised back-propagation neural network (NN) provided a set of four parameters for each structure [28, 29] (Figure 3). Thus, the biological activity expressed as PGIe was calculated and is presented in Table 1.

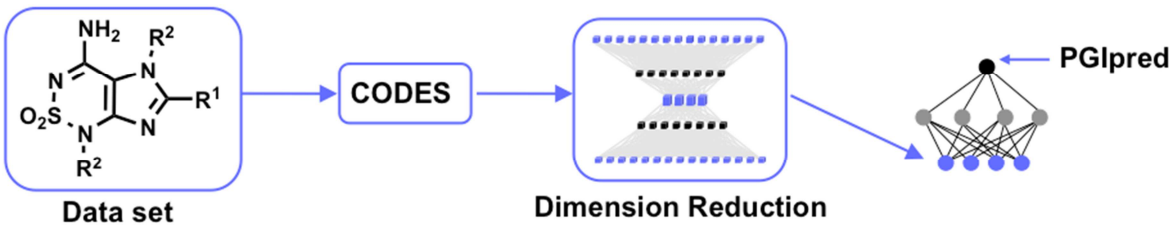
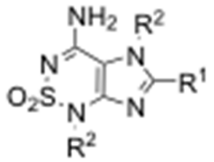


Figure 3. Protocol used to develop the neural network model.

The calculated values indicate an interesting predicted activity (> 98% at 25 μM) for four derivatives that include the *N*-1*H* **4**, *N*-1 substituted **15**, **17** and *N*-1,*N*-5 substituted **19** imidazo[4,5-*c*][1,2,6]thiadiazine 2,2-dioxides. On the basis of these findings, all compounds were tested *in vitro* as trypanomicidal agents against the Tulahuen 2 strain. The growth inhibition of the epimastigote form of *T. cruzi* by imidazothiadiazine derivatives **1-20** was determined and the results are shown in Table 1.

Table 1. Predicted and experimental values of the PGIe and IC_{50} of imidazothiadiazine derivatives **1-20**.



Compd	R^2	R^3	R^1	PGIe (calcd)[a]	PGIe (exp)[b]	IC_{50} (μM)
Nifurtimox						7.7 ± 0.5
1	H	H	CH_3	40	38.7± 5	> 25
2	H	H	CH_2-CH_3	13	43.0± 2	28.5 ± 0.8
3	H	H	cyclopentyl	57	42.2± 3	28.9 ± 0.4
4	H	H	cyclohexyl	100	72.3± 1	12.7± 0.6
5	H	H	Ph	53	39.6± 4	> 25
6	H	H	2-fluorophenyl	28	57.8 ± 2	20.2 ± 0.6
7	H	H	4-dimethylaminophenyl	6	42.8± 1	28.5 ± 0.5
8	H	H	3-thienyl	59	51.3 ± 5	22.2 ± 0.7
9	H	H	3-pyridinyl	59	37.5 ± 5	> 25
10	H	H	5-nitro-2-furyl	6	31.3± 3	> 25
11	H	H	3-tetrahydrofuryl	59	56.4± 4	20.3 ± 0.4
12	H	H	CH_2-Ph	59	59.7± 2	16.2± 0.2
13	H	H	CH_2-CH_2-Ph	7	73.6± 1	11.5± 0.5

14	CH_3	H	3-thienyl	51	46.3± 3	26.2 ± 0.8
15	CH_3	H	Ph	99	58.0± 1	16.7± 0.4
16	CH_2-Ph	H	Ph	5	41.6± 1	29.5 ± 0.5
17	CH_2-4-ClPh	H	2-fluorophenyl	100	86.3± 2	7.0 ± 0.5
18	CH_2-4-ClPh	H	cyclohexyl	51	0± 5	> 25
19	CH_2-Ph	CH_2-Ph	Ph	99	100.0± 1	4.5 ± 0.3
20	CH_2-Ph	CH_2-Ph	cyclohexyl	5	45.7± 3	28.7 ± 0.3

[a]PGIe(calcd): Predicted value of the % of growth inhibition (PI) of the parasite by our NN model;

[b]PGIe(exp): Experimental value of the % of growth inhibition (PI) of the parasite at 25 μM.

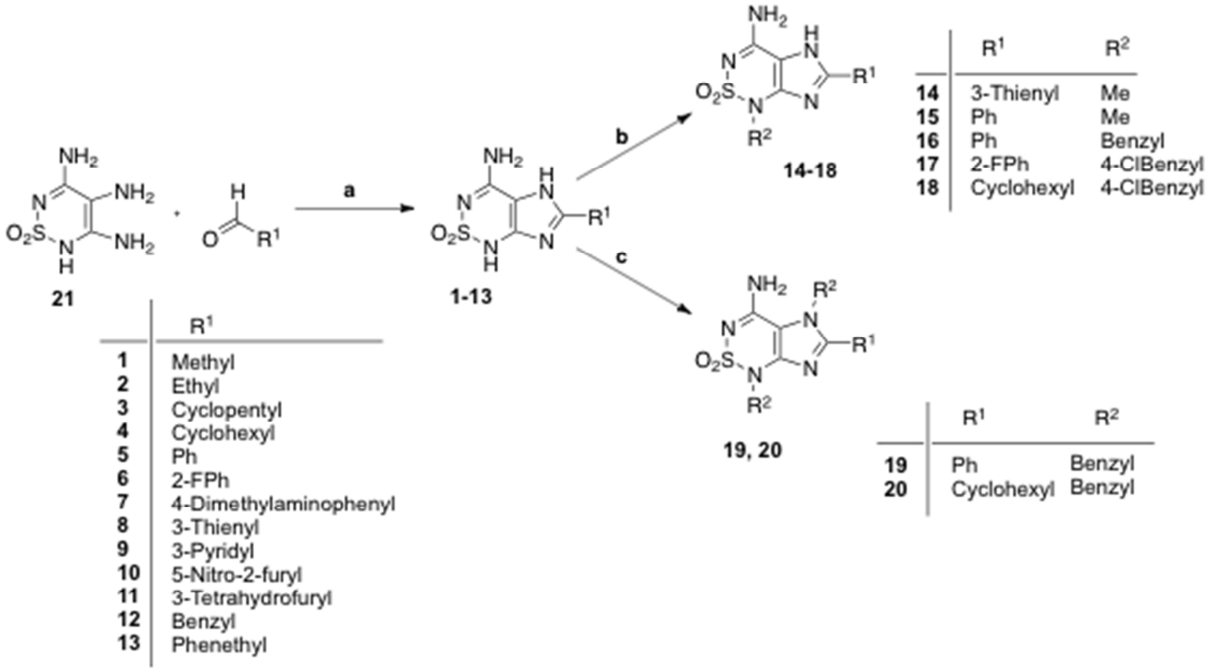
2.2 Chemistry

The structures of the imidazo[4,5-*c*][1,2,6]thiadiazine 2,2-dioxide system show variations on the substituents at three different positions. At position 6, a variety of groups such as alkyl, cycloalkyl, benzyl, aryl and heteroaryl were considered. *N1*-substituted groups such as hydrogen, methyl, benzyl, or 4-chlorobenzyl were considered. At position 5 of the heterocycle, only hydrogen and benzyl were assessed.

The compounds prepared for this study are presented in Figure 2. Their general synthetic route (outlined in Scheme I) involves formation of the parent 4-amino-1*H*,5*H*-imidazo[4,5-*c*][1,2,6]thiadiazine 2,2-dioxide [30] bearing different substituents at C-5, and subsequent introduction of the substituents at *N*-1 or at *N*-1 and *N*-5.

The first step, preparation of the imidazo[4,5-*c*][1,2,6]thiadiazine derivatives, consists of the reaction of the 3,4,5-triamino-2*H*-1,2,6-thiadiazine 1,1-dioxide **21** [31] with suitably functionalized aldehydes. Compound **21** was prepared, according to a process described in three reaction steps, from sulfamide and sulfuryl chloride [31, 32]. So, the reaction of **21** with aliphatic aldehydes such as acetaldehyde, propionaldehyde, cyclopentanecarbaldehyde, or cyclohexanecarbaldehyde afforded the corresponding 5-methyl, 5-ethyl, 5-cyclopentyl or 5-cyclohexyl compounds **1-4**, respectively (Figure 2). The preparation of 5-phenyl [30], 5-(2-fluorophenyl), and 5-(4-dimethylaminophenyl)-1*H*,5*H*-imidazo[4,5-*c*][1,2,6]thiadiazine 2,2-dioxide **5-7** were carried

out starting from **21** and the corresponding aromatic aldehydes. In a similar way, the reaction of **21** with heterocyclic aldehydes such as 3-thienyl, 3-pyridyl, 5-nitro-2-furyl, or 3-tetrahydrofuryl afforded the corresponding imidazo[4,5-*c*][1,2,6]thiadiazine 2,2-dioxides **8-11**, respectively. The 5-benzyl and 5-phenethyl-1*H*-[4,5-*c*][1,2,6]thiadiazine 2,2-dioxides **12** and **13** were prepared from **21** and benzyl and phenethyl aldehydes, respectively.



Scheme 1. General synthetic route for the preparation of imidazothiadiazine derivatives. Reagents and conditions: a) H_2O/AcOH, rt; b) MeI/K_2CO_3, acetone, reflux for **14,** Me_2SO_4 /K_2CO_3/H_2O for **15**; R^2-Br, Et_3N, acetone, reflux for **16-18** ; c) benzyl bromide, sodium hydroxide, H_2O, reflux for **19**; benzyl bromide, K_2CO_3, acetone, reflux for **20**.

The second step of the route shown in Scheme 1 is the functionalization of the *N*-1 and *N*-5 positions. Different alkylation procedures were tested to selectively obtain the final *N*-1-substituted derivatives. Substitution at position 1 of the corresponding *N*-1*H* imidazo[4,5-*c*][1,2,6]thiadiazine 2,2-dioxides was performed with alkyl halides in acetone using either potassium carbonate or triethylamine as the base. When the alkylation reaction was carried out with dimethyl sulfate or methyl iodide in potassium carbonate, it was possible to obtain only the *N*-1 methyl derivatives **14** and **15**, respectively (Scheme 1). The benzylation of 6-phenylimidazothiadiazine **5** in potassium carbonate afforded a mixture of the monobenzyl derivative **16** and the dibenzyl derivative **19** at the *N*-1

and *N*-5 position. The benzyl *N*-1 selectively mono-substituted derivatives were obtained by the reaction of the corresponding halide in the presence of triethylamine. Thus, the reaction of *N*-1*H*-imidazo[4,5-*c*][1,2,6]thiadiazine derivatives **4-6** with the corresponding bromides afforded the corresponding benzyl **16** and 4-chlorobenzyl derivatives **17** and **18**.

Finally, when the imidazo[4,5-*c*][1,2,6]thiadiazine had the same substituent at both the *N*-1 and *N*-5 positions, the reaction was carried out in acetone and potassium carbonate or in an aqueous solution of sodium hydroxide. Thus, the preparation of the substituted 1,5-dibenzylimidazothiadiazine 2,2-dioxides **19** and **20** were carried out starting from imidazothiadiazine **5** or **4** and benzyl bromide, respectively (Scheme 1).

2.3. Pharmacology

2.3.1. Trypanocidal activity against the epimastigote form of *T. cruzi* strain Tulahuen 2

First, the percentage of growth inhibition of the epimastigote form of the Tulahuen 2 strain was determined at 25 μM of all compounds **1-20** using nifurtimox as the positive control. The compounds that showed a PGIe cut-off higher than 70% (Table 1) were considered as good anti-*T. cruzi* agents [26, 33]. In general, as can be seen in Table 1, the experimental PGIe values for derivatives **1-20** are in good agreement with those predicted by our neural network model, showing an prediction accuracy greater than 80%.

According to PGIe(exp) values (Table 1), three N1-H derivatives **4, 12, 13**, two *N*-1 substituted derivatives **15, 17** and *N*-1, *N*-5-substituted compound **19** showed interesting anti-*T. cruzi* properties in the epimastigote form of the Tulahuen 2 strain, of all imidazo[4,5-*c*][1,2,6]thiadiazine 2,2-dioxide derivatives considered. The IC_{50} values of these imidazo[4,5-*c*][1,2,6]thiadiazine 2,2-dioxide derivatives were calculated and the values are presented in Table 2. The results show that derivatives **4, 12, 13, 15, 17** and **19** have excellent trypanocidal activity with potency, in cases such as **17** and **19**, equal to or greater than nifurtimox, used as the reference drug.

Table 2. Trypanocidal activities against the epimastigote form of strain Tulahuen 2 presented as IC_{50} and non-specific cytotoxicities (IC_{50}) in murine macrophages (J774) of selected imidazothiadiazine derivatives.

Compd	IC_{50} (μM)[a] Tulahuen 2 strain	IC_{50} (μM)[a] J774	SI[b]
Nifurtimox	7.7 ± 0.5	316 ± 5	41
4	16.2 ± 0.2	>400	> 25
12	12.7 ± 0.6	>400	>31
13	11.5 ± 0.5	>400	>34.8
17	7.0 ± 0.5	<100	<14.2
15	16.7 ± 0.4	128 ± 2	7.7
19	4.5 ± 0.3	100 ± 5	22

[a] IC_{50} : Concentration that inhibit the 50% of parasite growth; [b.] SI: Selectivity index defined as $IC_{50(J774)}/IC_{50(Tulahuen2)}$.

2.3.2. Evaluation of non-specific cytotoxicity in mammalian cell lines.

The non-specific cytotoxicity against J774 murine macrophages was assayed using the imidazo[4,5-*c*][1,2,6]thiadiazine derivatives that showed an IC_{50} < 20 μM for the epimastigote form of *T. cruzi* strain Tulahuen 2. The results are presented in Table 2, including nifurtimox as the reference drug.

It was clearly observed that derivatives **4**, **12**, **13**, **15**, **17**, and **19** showed selectivity for parasite cells; derivatives **4, 12,** and **13** showed similar or better selectivity compared to the reference compound.

In view of these results, all derivatives were taken into account for additional studies. Thus, the cytotoxicity and trypanocidal activity against another strain were evaluated.

2.3.3. Trypanocidal activity against the epimastigote form of *T. cruzi* strain CL-clone B5

The activity of the imidazo[4,5-*c*][1,2,6]thiadiazine 2,2-dioxide derivatives **4, 12, 13**, **15, 17,** and **19**, selected to test against the epimastigote forms of *T. cruzi* strain CL-clone B5, is expressed as the PGIe. The IC_{50} values are presented in Table 3, including nifurtimox and benznidazole as the reference drugs.

Regarding the trypanocidal activity against *T. cruzi* strain CL-clone B5, the results indicate that all derivatives showed higher IC_{50} values in relation to the Tulahuen strain, except for derivative **4** that showed the highest anti-T. cruzi activity. Of note, this level of trypanocidal activity for compound **4** is ten times higher that of benznidazole, the only available treatment for Chagas disease.

Table 3. Trypanocidal activity of imidazothiadiazine derivatives against the epimastigote form of the CL-clone B5 strain (PGIe, IC_{50}e) and non-specific cytotoxicity (IC_{50}) in murine fibroblasts (NCTC clone 929).

Compd	Anti-epimastigote activity		IC_{50} (µM) NCTC-929	SI
	PGIe (25 µM)	**IC_{50}e (µM)**		
Nifurtimox	82.2 ± 2.8	3.6	> 256	> 71.1
Benznidazol	49.0 ± 1.2	27.1	> 256	> 9.4
4	54.8 ± 3.3	2.5	> 256	> 102.4
12	17.7 ± 5.1	> 25	ND[a]	ND
13	51.1 ± 0.5	17.5	> 256	> 14.6
15	11.3 ± 2.8	> 25	ND	ND
17	42.1 ± 7.4	> 25	ND	ND
19	60.8 ± 2.1	3.6	< 64	< 17.8

[a] ND: Not determined

2.3.4. Evaluation of non-specific cytotoxicity in murine fibroblasts (NCTC clone 929)

The non-specific cytotoxicity in murine fibroblasts was determined for the most interesting derivatives **4, 12, 13**, and **19** that showed trypanocidal activities against the Tulahuen 2 and CL-clone B5 strains. Selectivity indexes were calculated in order to confirm the specific action of these drugs against the parasite (Table 3), showing that derivatives **4, 12, 13,** and **19** exhibited selectivity indexes on the same order or higher than that of benznidazole.

2.3.5. Trypanocidal activity against intracellular amastigotes of *T. cruzi* (CL-clone B5 strain)

According to these preliminary results, the most interesting compounds, **4** and **19,** were chosen to study their activities against *T. cruzi* intracellular forms. The percentage of growth inhibition of the amastigote stage (PGIa) are shown in Table 4, together with their IC_{50} values (IC_{50a}). Nifurtimox and benznidazole were also tested as reference drugs.

The results obtained from this study show that the *N*-1H derivative **4** did not have anti-amastigote activity at 25 µM. However, derivative **19** show interesting trypanocidal activity according to its capacity to inhibit the amastigote form of T. *cruzi*.

Table 4. Trypanocidal activities of **19** and **4** against the intracellular amastigote form of the CL-clone B5 strain (PGIa, IC_{50a}).

Compd	Anti-amastigote activity	
	PGIa (25 µM)	IC_{50a} (µM)
Nifurtimox	100 ± 2.6	< 0.5
Benznidazol	100 ± 0.0	0.8
19	90.2 ± 13.8	16.9
4	0.0 ± 9.0	ND [a]

[a] ND: Not determined

2.3.6. Cruzipain inhibitory activity

Cruzipain, the most abundant endoproteinase of *T. cruzi,* has been described as one of the most promising candidate targets for Chagas disease drug development [34,35]. Its biological relevance has been demonstrated using different cruzipain inhibitors that block parasite development *in vitro,* regardless of the chemical structure [14, 36]. Most cruzipain inhibitors described up to now belong to the peptidomimetic class, particularly 6-((3,5-difluorophenyl)amino)-9-ethyl-9*H*-purine-2-carbonitrile**,** an imidazopyrimidine [37]. Taking into account that this

compound shares structural motifs with our imidazothiadiazine family, the capacity to inhibit the cruzipain enzyme was assessed in order to study the mechanism of action of the most active compounds **4, 12, 13, 15, 17,** and **19** (Table 5). These six imidazothiadiazine derivatives presented variable degrees of cruzipain inhibition. The results indicate that some imidazo[4,5-*c*][1,2,6]thiadiazine 2,2-dioxides showed significant cruzipain inhibition, particularly the *N*-1*H* derivatives **4** and **12.** Therefore, trypanocidal activity may be mediated partly through cruzipain inhibition. However, it is clearly that other mechanism of action should be involved since the most interesting compound shows the highest value of IC_{50}.

Table 5. Cruzipain inhibitory activity.

Compd	IC_{50} (μM)
4	12 ± 2
12	23 ± 3
13	39.0 ± 0.5[a]
15	27.4 ± 0.5[a]
17	86 ± 4
19	81 ± 4
mbth [b]	0.10 ± 0.01

[a] Percentage of inhibition of cruzipain at 100 μM. [b] (*m*-bromophenyl)ethylketone thiosemicarbazone (reference compound)

2.3.7. Mutagenicity study (Ames test)

An important issue in drug development is to establish the mutagenicity of the molecule. Regarding nifurtimox and benznidazole, besides the side effects described above, these compounds show mutagenicity [38, 39]. Therefore, driven by our interest in determining the druggability of our compounds, we assessed the mutagenic capacity of a representative set of derivatives including *N*-1*H* (**12**), *N*-1 substituted (**15**), and *N*-1, *N*-5-substituted (**19**) compounds in the Ames test.

For this, *Salmonella typhimurium* strain TA98 in the absence and presence of S9 was used in order to simulate the metabolic process [40] (Table 6). Unlike the anti-Chagas drug nifurtimox, none of the studied compounds were mutagenic according to the criterion that a compound is mutagenic when the number of revertant colonies is at least two-fold higher than the spontaneous revertant frequencies for at least two consecutive dose levels [41]. This allowed us to confirm the druggability of these compounds.

Table 6. Ames test on selected imidazotiadiazine derivatives.

	19			**15**			**12**			**Nifurtimox**[a]		
	D[b]	**NR**[c,d]	**M**[e]	**D**[b]	**NR**[c,d]	**M**[e]	**D**[b]	**NR**[c,d]	**M**[e]	**D**[b]	**NR**[c,d]	**M**[e]
-S9	500	18±5		500	10±2		500	12±3		10.0	144±11	
	167	15±3		167	11±2		167	14±4		3.0	62±2	
	56	10±1		56	10±1		56	12±2		1.0	43±17	(+)
	19	9±3		19	10±1		19	9±2		0.5	29±6	
	6	7±3		6	5±3		6	9±2		0.0	21±4	
	0	10±3		0	10±3		0	10±3				
+S9	500	13±3		500	8±1		500	10±0		0.0	31± 10	
	167	15±1		167	9±1		167	26±9		0.5	37± 5	
	56	5±2		56	7±2		56	8±1		1.0	39± 18	
	19	6±1		19	14±5		19	4±2		3.0	53± 9	
	6	9±2		6	15±8		6	9±6		10.0	64± 6	
	0	11±1		0	11±1		0	11±1				

	4-NPD[f]			**AF**[g]	
	D[b]	**NR**[c,d]		**D**[b]	**NR**[c,d]
-S9	20.0	1460±228	**+S9**	10.0	856±55

[a] The data for Nifurtimox were taken from reference [33]. [b] D: Doses in μg/plate. [c] NR: Number of revertants. [d] The results are the mean of two independent experiments ±SD. [e] M: Mutagenicity, according to reference [41]. [f]NPD: 4-nitro-*o*-phenylendiamine. [g] AF: 2-aminofluorene.

2.3.8. In vivo trypanocidal assay

The compounds designated **19** and **4** were also chosen to evaluate their efficacy as trypanocidal agents in a murine model of acute infection caused by *T. cruzi* strain Y. Benznidazole was also included as a reference drug, and was administered to the animals in the same therapeutic scheme (i.e. 100 mg/kg/day p.o. for five consecutive days). As shown in Figure 4, **19** reduced the number of blood trypomastigotes from the fourth day of treatment. However, mice treated with **4** maintained high levels of parasitemia throughout the experiment. Regarding survival, only one death was registered in the experimental group treated with **19**, providing a survival fraction of 87.5% (Figure 5).

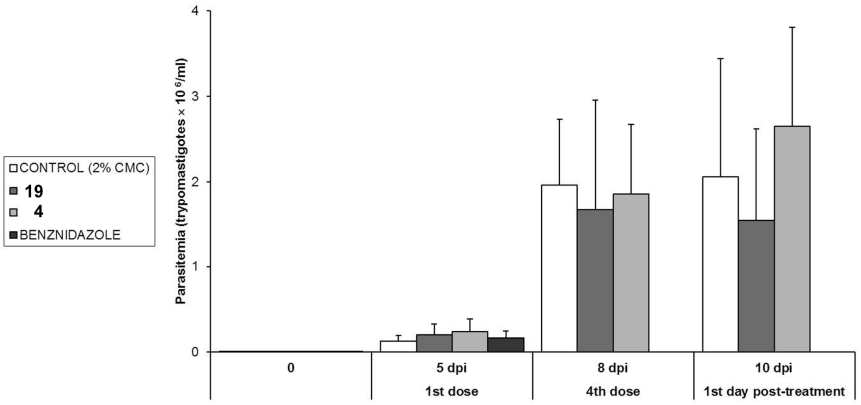


Figure 4. Parasitemia levels in mice experimentally infected with *T. cruzi* (Y strain) and treated orally for five consecutive days with 100 mg/kg/day of **19**, **4** and benznidazole.

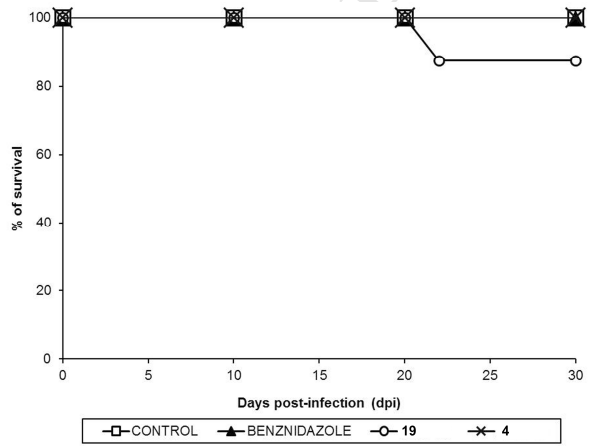


Figure 5. Survival rates of **19** and **4** treated mice expressed as the percentage of living animals during the acute phase of the infection (30 dpi).

3. CONCLUSIONS

This new family of imidazo[4,5-*c*][1,2,6]thiadiazine 2,2-dioxide with anti-*T. cruzi* properties has been patented by us in different countries [42-44] and in the USA [45], where the patent has been recently approved.

On the basis of this investigation, artificial neural network techniques (ANN) were used effectively to find new trypanocidal agents with an innovative heterocyclic system containing an imidazothiadiazine 2,2-dioxide structure. Six derivatives (**4, 12, 13, 15, 17,** and **19**) of the evaluated compounds showed the capacity to inhibit the epimastigote form of the Tulahuen 2 strain. The cruzipain studies provided insight into a possible mode of action of these compounds.

In summary, the anti-epimastigote and anti-amastigote effects in the Tulahuen 2 and CL-clone B5 strains of *Trypanosoma cruzi*, along with the absence of mutagenicity, suggest that these compounds can be regarded as trypanocidal agents. Among them, the 1,5 substituted imidazothiadiazine **19** showed *in vivo* trypanocidal activity and thus can be considered a potential agent for the treatment of Chagas disease.

4. EXPERIMENTAL

4.1. Chemistry

Melting points were determined using a Reichert-Jung Kofler apparatus. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on Varian INNOVA-300 (300 MHz), Varian MERCURY-400 (400 MHz) and Varian UNITY-500 (500 MHz) spectrometers. Mass spectrometry (ES^+) was performed using a Hewlett-Packard 1100SD apparatus. Flash chromatography was performed using silica gel 60 (230-400 mesh) from Merck. High performance liquid chromatography (HPLC) was performed using a Waters 2695 apparatus with a diode array UV/Vis detector and coupled to a micromass ZQ using silica based columns X-Bridge™ C18 (2.1x100 mm, 3.5 µm). The sample injection volume was set to 5 µL of a solution of 1 mg/mL methanol and experiments were carried out with a flow rate of 0.25 µL and a different gradient elution specified in each case. Both solvents contained a 0.1% v/v formic acid. Elemental analyses were performed on a Heraeus CHN-O Rapid Analysis apparatus. The purity of all compounds was >95% prior to biological testing (Supporting Information).

4.1.1. General procedure for the preparation of 4-amino-1*H*,5*H*-imidazo[4,5-*c*][1,2,6]thiadiazine 2,2-dioxides 1-13. To a suspension of **21** in water and acetic acid, the aldehyde was added in small fractions and stirred at room temperature until the complete elimination of **21**. The resulting precipitate was separated by vacuum filtration and recrystallized in each case. Amounts of reagents, time reaction, conditions and specific procedures are particularly specified in each case.

4.1.2. 4-Amino-6-methyl-1*H*,5*H*-imidazo[4,5-*c*][1,2,6]thiadiazine 2,2-dioxide (1). From **21** (300 mg, 1.69 mmol), acetaldehyde (0.29 mL, 5.13 mmol), 12 mL water and 2 mL glacial acetic acid. Reaction time 24 h. Yield **1** (213 mg, 62%). Mp 303-5 °C. ^{1}H-NMR (400 MHz, DMSO-d_6): δ = 11.91, 11.05 (bs, 2H, NH); 7.38 (bs, 2H, NH_2); 2.30 (s, 3H, CH_3). ^{13}C-NMR (100 MHz, DMSO-d_6): δ = 153.1 (C-4); 150.3 (C-7a); 148.0 (C-6); 101.8 (C-4a); 14.3 (CH_3). HPLC-MS (ES^+): CH_3CN/H_2O 5:95-100:0, t_G: 18.00 min, t_R: 1.70 min, $[M+H]^+$ = 202.

4.1.3. 4-Amino-6-ethyl-1*H,5H*-imidazo[4,5-*c*][1,2,6]thiadiazine 2,2-dioxide (2). From **21** (1.5 g, 8.50 mmol), propionaldehyde (0.90 mL, 12.47 mmol) in 40 mL water and 4 mL glacial acetic acid. Reaction time 24 h. Yield **2** (1.4 g, 78%). Mp 304-7 °C. ^{1}H-NMR (400 MHz, DMSO-d_6): δ = 11.93, 11.02 (bs, 2H, NH); 7.58 (bs, 2H, NH_2); 2.68 (c, 2H, $\underline{CH_2}CH_3$); 1.20 (t, 3H, $CH_2\underline{CH_3}$, *J* = 7.5 Hz). ^{13}C-NMR (125 MHz, DMSO-d_6): δ = 153.7 (C-4); 152.0 (C-7a); 148.8 (C-6); 103.2 (C-4a); 21 .8 ($\underline{CH_2}CH_3$); 12.0 ($CH_2\underline{CH_3}$). HPLC-MS (ES$^+$): CH_3CN/H_2O 5:95-100:0, t_G: 18.00 min, t_R: 1.53 min, $[M+H]^+$ = 216.

4.1.4. 4-Amino-6-cyclopentyl-1*H,5H*-imidazo[4,5-*c*][1,2,6]thiadiazine 2,2-dioxide (3). From **21** (300 mg, 1.69 mmol) and cyclopentanecarboxaldehyde (0.28 mL, 2.61 mmol) in 30 mL water and 4 mL glacial acetic acid. Reaction time 96 h. Yield 3 (195 mg, 45%). Mp dec. ^{1}H-NMR (400 MHz, DMSO-d_6): δ = 11.86, 11.04 (bs, 2H, NH); 7.43 (bs, 2H, NH_2); 3.11 (bs, 1H, 61-H); 2.01 – 1.62 (m, 8H, 62-H / 63-H). ^{13}C-NMR (100 MHz, DMSO-d_6): δ = 155.7 (C-4); 152.8 (C-7a); 150.5 (C-6); 101.4 (C-4a); 38.7 (C-61); 31.6 (2C, C-62); 25.0 (2C, C-63). HPLC-MS (ES$^+$): CH_3CN/H_2O 5:95-100:0, t_G: 18.00 min, t_R: 3.93 min, $[M+H]^+$ = 256.

4.1.5. 4-Amino-6-cyclohexyl-1*H,5H*-imidazo[4,5-*c*][1,2,6]thiadiazine 2,2-dioxide (4). From 21 (1.5 g, 8.50 mmol), cyclohexanecarboxaldehyde (3.2 mL, 25.5 mmol) in 30 mL water and 4 mL glacial acetic acid. Reaction time 24 h. Yield 4 (1.0 g, 46%). Mp 263-5°C (recrystallized from EtOH:H_2O (1:1)). ^{1}H-NMR (400 MHz, DMSO-d_6): δ = 11.82, 11.00 (bs, 2H, NH); 7.42 (bs, 2H, NH_2); 2.67 (bs, 1H, 61-H); 1.94 – 1.18 (m, 10H, 62-H / 63-H / 64-H). ^{13}C-NMR (100 MHz, DMSO-d_6): δ = 155.8 (C-4); 152.9 (C-7a); 150.4 (C-6); 101.2 (C-4a); 37.2 (C-61); 30.9 (2C, C-62); 25.4, 25.2 (3C, C-63 / C-64). HPLC-MS (ES$^+$): CH_3CN/H_2O 5:95-100:0, t_G: 18.00 min, t_R: 5.80 min, $[M+H]^+$ = 270.

4.1.6. 4-Amino-6-phenyl-1*H,5H*-imidazo[4,5-*c*][1,2,6]thiadiazine 2,2-dioxide (5). From **21** (300 mg, 1.69 mmol), benzaldehyde (0.26 mL, 2.54 mmol) in 12 mL water and 2 mL glacial acetic acid. Reaction time 24 h. Yield **5** (329 mg, 74%). Mp 296-8°C. ^{1}H-NMR (500 MHz, DMSO-d_6): δ = 12.72, 11.22 (bs, 2H, NH); 7.92 - 7.47 (bs, 2H, NH_2); 7.91 (d, 2H, 62-H, *J* = 7.2 Hz); 7.55 - 7.47 (m, 3H, 63-H / 64-H). ^{13}C-NMR (125 MHz, DMSO-d_6): δ

= 152.9 (C-4); 150.9 (C-7a); 147.7 (C-6); 130.1 (C-64); 129.1 (2C, C-63); 128.6 (C-61); 125.6 (2C, C-62); 103.1 (C-4a). HPLC-MS (ES^+): CH_3CN/H_2O 5:95-100:0, t_G: 18.00 min, t_R: 6.03 min, $[M+H]^+$ = 264.

4.1.7. 4-Amino-6-(2-fluorophenyl)-1*H,5H*-imidazo[4,5-*c*][1,2,6]thiadiazine 2,2-dioxide (6). From **21** (300 mg, 1.69 mmol) and 2-fluorobenzaldehyde (0.28 mL, 2.54 mmol) in 12 mL water and 2 mL glacial acetic acid. Reaction time 24 h. Yield **6** (253 mg, 53%). Mp 274-8°C (recrystallized from water). ^{1}H-NMR (300 MHz;d_6): δ = 12.57, 11.30 (bs, 2H, NH); 7.97, 7.79 (bs, 2H, NH_2); 8.09 (t, 66-H, J = 7.5 Hz); 7.58 – 7.37 (m, 3H, 64-H, 65-H, 63-H). ^{13}C-NMR (100 MHz;d_6): δ = 159.8 (d, C-62, J = 247.1 Hz); 152.7 (C-4); 150.3 (C-7a); 142.8 (C-6); 133.2 (d, C-64, J = 5.9 Hz); 130.2 (d, C-66, J = 1.5 Hz); 126.1 (d, C-65, J = 4.1 Hz); 117.2 (d, C-63, J = 21 .2 Hz); 116.9 (d, C-61, J = 13.7 Hz); 103.3 (C-4a). HPLC-MS (ES^+): CH_3CN/H_2O 5:95-100:0, t_G: 18.00 min, t_R: 6.87 min, $[M+H]^+$ = 282.

4.1.8. 4-Amino-6-(4-dimethylaminephenyl)-1*H,5H*-imidazo[4,5-*c*][1,2,6]thiadiazine 2,2-dioxide (7). From **21** (300 mg, 1.69 mmol), 4-dimethylaminebenzaldehyde (379 mg, 2.54 mmol) in 12 mL water and 2 mL glacial acetic acid. Reaction time 26 h. Yield **7** (219 mg, 42%). Mp > 330 ºC. ^{1}H-NMR (400 MHz, DMSO-d_6): δ = 12.24, 11.12 (bs, 2H, NH); 7.50 (bs, 2H, NH_2); 7.71 (d, 2H, 62-H, J = 8.6 Hz); 6.80 (d, 2H, 63-H, J = 8.6 Hz); 2.96 (s, 6H, N($\underline{CH_3}$)$_2$). ^{13}C-NMR (75 MHz, DMSO-d_6): δ = 153.0 (C-4); 151.8 (C-64); 150.8 (C-7a); 148.9 (C-6); 127.3 (C-62); 115.6 (C-61); 112.2 (C-63) 102.8 (C-4a); 40.0 (2C, N($\underline{CH_3}$)$_2$). HPLC-MS (ES^+): CH_3CN/H_2O 5:95-100:0, t_G: 18.00 min, t_R: 7.20 min, $[M+H]^+$ = 307.

4.1.9. 4-Amino-6-(3-thienyl)-1*H,5H*-imidazo[4,5-*c*][1,2,6]thiadiazine 2,2-dioxide (8). From **21** (300 mg, 1.69 mmol) and thiophen-3-carboxaldehyde (0.23 mL, 2.54 mmol) in 12 mL water and 2 mL glacial acetic acid. Reaction time 24 h. Yield **8** (240 mg, 53%). Mp 314-6°C (recrystallized from water). ^{1}H-NMR (400 MHz;d_6): δ = 12.70, 11.14 (bs, 2H, NH); 8.00 – 7.20 (bs, 2H, NH_2); 8.04 (dd, 62-H, $J_{62\text{-H, }65\text{-H}}$ = 1.2 Hz / $J_{62\text{-H, }64\text{-H}}$ = 2.9 Hz); 7.70 (dd, 64-H, $J_{62\text{-H, }64\text{-H}}$ = 2.9 Hz / $J_{64\text{-H, }65\text{-H}}$ = 5.0 Hz); 7.55 (dd, 65-H, $J_{62\text{-H, }65\text{-H}}$ = 1.2 Hz / $J_{64\text{-H, }65\text{-H}}$ = 5.0 Hz). ^{13}C-NMR (100 MHz;d_6): δ = 153.7 (C-4); 149.4 (C-7a); 143.9 (C-6); 131.0 (C-61); 128.6 (C-64); 125.7 (C-65); 125.5 (C-62); 104.2 (C-4a). HPLC-MS (ES^+): CH_3CN/H_2O 5:95-100:0, t_G: 18.00 min, t_R: 4.98 min, $[M+H]^+$ = 270.

4.1.10. 4-Amino-6-(3-pyridyl)-1*H,5H*-imidazo[4,5-*c*][1,2,6]thiadiazine 2,2-dioxide (9). From **21** (300 mg, 1.69 mmol) and 3-pyridincarboxaldehyde (0.25 mL, 2.54 mmol) in 12 mL water and 2 mL glacial acetic acid. Reaction time 24 h. Yield **9** (154 mg, 35%). Mp 309-11°C (recrystallized from water). ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 13.29, 12.90, 11.32 (bs, 2H, NH); 7.92, 7.15 (bs, 2H, NH_2); 9.05 (s, 62-H); 8.60 (d, 64-H, J = 10.6 Hz); 8.22 (d, 66-H, J = 4.4 Hz); 7.55 (dd, 65-H, $J_{64\text{-H, }65\text{-H}}$ = 10.6 Hz / $J_{65\text{-H, }66\text{-H}}$ = 4.4 Hz). ^{13}C-NMR (75 MHz, DMSO-d_6): δ = 153.0 (C-4); 150.7 (C-64); 150.5 (C-7a); 146.8 (C-62); 145.9 (C-6); 133.1 (C-66); 125.0 (C-61); 124.1 (C-65); 103.7 (C-4a). HPLC-MS (ES$^+$): CH_3CN/H_2O 5:95-100:0, t_G: 18.00 min, t_R: 1.77 min, $[M+H]^+$ = 265.

4.1.11. 4-Amino-6-(5-nitro-2-furyl)-1*H,5H*-imidazo[4,5-*c*][1,2,6]thiadiazine 2,2-dioxide (10). From **21** (200 mg, 1.12 mmol) and 5-nitro-2-furaldehyde (240 mg, 1.72 mmol) in 12 mL water and 2 mL glacial acetic acid. Reaction time 48 h. Yield **10** (153 mg, 46%). Mp dec. ^{1}H-NMR (500 MHz, DMSO-d_6): δ = 12.16, 11.29 (bs, 2H, NH); 8.15 (bs, 2H, NH_2); 7.89 (d, 2H, 64-H, J = 3.9 Hz); 7.38 (d, 2H, 65-H, J = 3.9 Hz). ^{13}C-NMR (125 MHz, DMSO-d_6): δ = 153.9 (C-4); 148.3 (C-7a); 135.6 (C-6); 151.6 (C-63); 146.4 (C-62);115.3 (C-65); 112.6 (C-64); 107.0 (C-4a). HPLC-MS (ES$^+$): CH_3CN/H_2O 5:95-100:0, t_G: 18.00 min, t_R: 6.90 min, $[M+H]^+$ = 264.

4.1.12. 4-amino-6-(3-tetrahydrofuryl)-1*H,5H*-imidazo[4,5-*c*][1,2,6]thiadiazine 2,2-dioxide (11). From **21** (300 mg, 1.69 mmol) and tetrahydrofuryl-3-carboxaldehyde (0.49 mL, 2.54 mmol) in 30 mL water and 2 mL glacial acetic acid. Reaction time 48 h. Yield **11** (181 mg, 41%). Mp 238–40°C (recrystallized from ethanol). ^{1}H-NMR (500 MHz, DMSO-d_6): δ = 11.97, 11.00 (bs, 2H, NH); 7.63 – 7.22 (bs, 2H, NH_2); 3.98 – 3.74 (m, 4H, 62-H / 64-H); 3.54 – 3.45 (bs, 1H, 61-H); 2.30 – 2.23 (m, 1H, 65_a-H); 2.12 – 2.05 (m, 1H, 65_b-H). ^{13}C-NMR (125 MHz, DMSO-d_6): δ = 152.9 (2C, C-4 / C-7a); 150.3 (C-6); 101.9 (C-4a); 71.2 (C-62); 67.4 (C-64); 38.3 (C-61); 31.3 (C-65). HPLC-MS (ES$^+$): CH_3CN/H_2O 5:95-100:0, t_G: 18.00 min, t_R: 2.42 min, $[M+H]^+$ = 258.

4.1.13. 4-Amino-6-benzyl-1*H,5H*-imidazo[4,5-*c*][1,2,6]thiadiazine 2,2-dioxide (12). From **21** (300 mg, 1.69 mmol), phenylacetaldehyde (475 mg, 3.75 mmol) in 2 mL acetone, 30 mL water and 2 mL glacial acetic acid. Reaction time 75 h. Yield **12** (190 mg, 40%). Mp 210-2°C (recrystallized from acetone). ^{1}H-NMR (400 MHz;d_6): δ

= 12.9 – 10.5 (bs, 2H, NH); 7.45 (bs, 2H, NH_2); 7.37 – 7.27 (m, 5H, Ar); 4.04 (s, 2H, 61-H). ^{13}C-NMR (100 MHz;d_6): δ = 153.8 (C-4); 149.2 (C-7a, C-6); 136.9 (C-62); 128.8 (2C, C-64); 128.7 (2C, C-63); 126.8 (C-65); 104.0 (C-4a); 34.5 (C-61). HPLC-MS (ES^+): CH_3CN/H_2O 5:95-100:0, t_G: 18.00 min, t_R: 5.88 min, $[M+H]^+$ = 278.

4.1.14. 4-Amino-6-phenethyl-1*H*,5*H*-imidazo[4,5-*c*][1,2,6]thiadiazine 2,2-dioxide (13). From **21** (300 mg, 1.69 mmol) and 3-phenylpropionaldehyde (0.34 mL, 2.54 mmol), 12 mL water and 2 mL glacial acetic acid. Reaction time 24 h. Yied **13** (369 mg, 75%). Mp 190-2°C (recrystallized from EtOH:H_2O (1:1)). ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 12.09, 11.96, 11.03 (bs, 2H, NH); 7.81, 7.66 (bs, 2H, NH_2); 7.27 – 7.17 (m, 5H, Ar); 2.99 – 2.96 (m, 4H, 61-H / 62-H). ^{13}C-NMR (125 MHz, DMSO-d_6): δ = 154.2 (C-7a); 152.7 (C-4); 150.5 (C-6); 140.5 (C-63); 128.5 (2C, C-65); 128.3 (2C, C-64); 126.2 (C-66); 101.5 (C-4a); 32.9 (C-61); 29.9 (C-62). HPLC-MS (ES^+): CH_3CN/H_2O 5:95-100:0, t_G: 18.00 min, t_R: 6.72 min, $[M+H]^+$ = 292.

4.1.15. General procedure for *N*-1 substituted 5*H*-imidazo[4,5-*c*][1,2,6]thiadiazine 2,2-dioxides (compounds 14, 16-18). A mixture of 4-amino-5*H*-imidazo[4,5-*c*][1,2,6]thiadiazine 2,2-dioxide derivative and the appropriate base in acetone was boiled and stirred. Once reflux was achieved, the corresponding halide and a catalytic amount of potassium iodide were added and the mixture was allowed to react until the complete elimination of the N(*H*) derivative. The remaining solvent was eliminated under vacuum. The resulting crude product was processed in each case. Work-up, amounts, the time of reaction, conditions, and specific procedures are specified in each case.

4.1.16. 4-Amino-1-methyl-6-(3-thienyl)-*5H*-imidazo[4,5-*c*][1,2,6]thiadiazine 2,2-dioxide (14). From **8** (300 mg, 1.11 mmol), K_2CO_3 (150 mg, 1.11 mmol), methyl iodide (150 mg, 11.1 mmol) in 60 mL acetone. Reaction time 4 days. To the residue aqueous HCl was added and the precipitate was purified by flash chromatography (CH_2Cl_2:MeOH, 100:1) to afford **14** (197 mg, 63%). Mp 305-8°C. ^{1}H-NMR (400 MHz;d_6): δ = 13.04 (bs, 1H, NH); 8.00 – 7.20 (bs, 2H, NH_2); 8.04 (dd, 62-H, $J_{62\text{-}H,\ 65\text{-}H}$ = 1.2 / $J_{62\text{-}H,\ 64\text{-}H}$ = 2.9); 7.70 (dd, 64-H, $J_{62\text{-}H,\ 64\text{-}H}$ = 2.9 / $J_{64\text{-}H,\ 65\text{-}H}$ = 5.0); 7.55 (dd, 65-H, $J_{62\text{-}H,\ 65\text{-}H}$ = 1.2 / $J_{64\text{-}H,\ 65\text{-}H}$ = 5.0); 3.29 (CH_3). ^{13}C-NMR (100 MHz;d_6) δ: 153.7 (C-4); 149.4 (C-7a); 143.9 (C-6); 131.0 (C-61); 128.6 (C-64); 125.7 (C-65); 125.5 (C-62); 104.2 (C-4a): 34.4 (CH_3). HPLC-MS (ES^+): CH_3CN/H_2O 5:95-100:0, t_G: 18.00 min, t_R: 9.42 min, $[M+H]^+$ = 284.

4.1.17. 4-Amino-1-benzyl-6-phenyl-5*H*-imidazo[4,5-*c*][1,2,6]thiadiazine 2,2-dioxide (16). From **5** (300 mg, 1.15 mmol), Et_3N (0.64 mL, 4.60 mmol), benzyl bromide (1.12 mL, 5.82 mmol) in 70 mL acetone. Reaction time 3 days. To the residue aqueous HCl was added and the precipitate was recrystallized from EtOH:H_2O (1:1) to yield **16** (167 mg, 36%). Mp 304-6°C. ^{1}H-NMR (400 MHz, DMSO-d_6): δ = 13.01 (bs, 1H, NH); 8.12 (bs, 2H, NH_2); 7.92 (d, 2H, 62-H, *J* = 7.0 Hz); 7.57 – 7.48 (m, 3H, 63-H / 64-H); 7.43 (d, 2H, 13-H, *J* = 7.3 Hz); 7.34 – 7.22 (m, 3H, 14-H / 15-H); 5.03 (s, 2H, 11-H). ^{13}C-NMR (125 MHz, DMSO-d_6): δ = 152.4 (C-4); 151.4 (C-7a); 148.2 (C-6); 137.3 (C-12); 130.1 (C-61); 128.9 (2C, C-63); 128.4 (C-64); 128.0 (2C, C-14); 127.7 (C-15); 127.0 (2C, C-13); 125.7 (2C, C-62); 103.3 (C-4a); 47.2 (C-11).). HPLC-MS (ES$^+$): CH_3CN/H_2O 5:95-100:0, t_G: 18.00 min, t_R: 12.95, $[M+H]^+$ = 354.

4.1.18. 4-Amino-1-(4-chlorobenzyl)-6-(2-fluorophenyl)-5*H*-imidazo[4,5-*c*][1,2,6]thiadiazine 2,2-dioxide (17). From **6** (500 mg, 1.78 mmol), Et_3N (1.00 mL, 7.12 mmol), 4-chlorobenzyl bromide (740 mg, 3.56 mmol) in 100 mL acetone. Reaction time 2 days. To the residue aqueous HCl was added and the precipitate was recrystallized from acetone:H_2O (1:2) affording **17** (158 mg, 39%). Mp 304-5°C. ^{1}H-NMR (500 MHz, DMSO-d_6): δ = 12.47 (bs, 1H, NH); 8.21 , 7.96 (bs, 2H, NH_2); 8.10 (t, 1H, 66-H, *J* = 7.0 Hz); 7.58 – 7.37 (m, 7H, Ar); 5.02 (s, 2H, 11-H). ^{13}C-NMR (125 MHz, DMSO-d_6): δ = 159.1 (d, C-62, *J* = 250.0 Hz); 152.4 (C-4); 150.7 (C-7a); 143.4 (C-6); 136.5 (C-12); 132.4 (d, C-64, *J* = 8.2 Hz); 132.0 (C-15); 129.8 (2C, C-13 or C-14) 129.8 (d, C-66, *J* = 6.4 Hz); 128.2 (2C, C-13 or C-14); 125.4 (C-65); 116.5 (d, C-63, *J* = 22.0 Hz); 116.2 (d, C-61, *J* = 11.9 Hz); 103.7 (C-4a); 47.0 (1C, C-11). HPLC-MS (ES$^+$): CH_3CN/H_2O 5:95-100:0, t_G: 18.00 min, t_R: 13.90, $[M+H]^+$ = 406.

4.1.19. 4-Amino-6-cyclohexyl-1-(4-chlorobenzyl)-5*H*-imidazo[4,5-*c*][1,2,6]thiadiazine 2,2-dioxide (18). From **4** (300 mg, 1.11 mmol), Et_3N (1.00 mL, 7.12 mmol), chlorobenzyl bromide (465 mg, 2.27 mmol) in 40 mL acetone. Reaction time 3 days. To the residue aqueous HCl was added and the precipitate was purified by flash chromatography starting from 200:1 to 10:1 (CH_2Cl_2:MeOH) to afford **18** (125 mg, 29%). Mp 220-2°C. ^{1}H-NMR (500 MHz, DMSO-d_6): δ = 11.97 (bs, 1H, NH); 7.89 (bs, 2H, NH_2); 7.37 (d, 4H, Ar); 4.89 (s, 2H, CH_2); 2.70 (m, 1H, 61-H); 1.95 – 1.20 (m, 10H, 62-H / 63-H / 64-H). ^{13}C-NMR (125 MHz, DMSO-d_6): δ = 157.2 (C-4); 153.0 (C-

7a); 151.4 (C-6); 137.1 (C-12); 132.3 (C-15); 130.2 (2C, C-13 or C-14); 128.6 (2C, C-13 or C-14); 102.1 (C-4a); 46.6 (C, C-11); 37.7 (C-61); 31.2 (2C, C-62); 25.8, 25.5 (3C, C-63 / C-64). HPLC-MS (ES^+): CH_3CN/H_2O 5:95-100:0, t_G: 18.00 min, t_R: 11.67, $[M+H]^+$ = 394.

4.1.20. 4-Amino-1-methyl-6-phenyl-*5H*-imidazo[4,5-*c*][1,2,6]thiadiazine 2,2-dioxide (15). To a suspension of **3** (300 mg, 1.11 mmol), K_2CO_3 (2,685 g, 19.10 mmol) in 30 mLof water, dimethyl sulphate (1.12 mL, 5.82 mmol) was added. After 2 days, the reaction mixture was acidulated with aqueus HCl and the precipitate is filtered and purified by flash chromatography (CH_2Cl_2:MeOH, 100:1) to afford **15** (111 mg, 36%). Mp 278-80°C. ^{1}H-NMR (400 MHz, DMSO-d_6): δ = 11.61 (bs, H, NH); 7.92 - 7.32 (bs, 2H, NH_2); 7.92 (d, 2H, 62-H, J = 7.5 Hz); 7.51 - 7.43 (m, 3H, 63-H / 64-H); 3.28 (s, 3H, CH_3). ^{13}C-NMR (100 MHz, DMSO-d_6): δ = 153.4 (C-4); 151.5 (C-7a); 148.4 (C-6); 129.7 (C-64); 129.0 (2C, C-63); 128.4 (C-61); 125.8 (2C, C-62); 105.3 (C-4a); 34.4 (CH_3). HPLC-MS (ES^+): CH_3CN/H_2O 5:95-100:0, t_G: 18.00 min, t_R: 9.23, $[M+H]^+$ = 292.

4.1.21. 4-Amino-1,5-dibenzyl-6-phenylimidazo[4,5-c][1,2,6]thiadiazine 2,2-dioxide (19). To a suspension of **6** (533 mg, 2.01 mmol), NaOH (200 mg, 5 mmol) in 100 mL water, benzylbromide was added dropwise (0.7 mL, 8.0 mmol) at reflux temperature. After 24 h, the reaction mixture was cooled, filtered and the residue was purified by flash chromatography (CH_2Cl_2:MeOH, 100:1) to afford **19** (260 mg, 29%). Mp 238-40°C. ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 8.0 – 7.0 (bs, 2H, NH_2); 7.63 – 7.60 (m, 2H, Ph); 7.54 – 7.50 (m, 3H, Ar); 7.39 – 7.20 (m, 10H, Ar); 5.53 (s, 2H, 51-H); 5.02 (s, 2H, 11-H). ^{13}C-NMR (75 MHz, DMSO- $CDCl_3$): δ = 153.7 (C-4); 152.8 (C-7a); 152.1 (C-6); 137.4 (C-12 or C-52); 136.2 (C-12 or C-52); 130.9 (C-61); 129.6, 129.2, 129.1, 128.5, 128.4, 128.2, 127.9, 127.5 (11C, Ar); 125.9 (C-62); 105.0 (C-4a); 49.2 (C51); 46.9 (C11). HPLC-MS (ES^+): CH_3CN/H_2O 5:95-100:0, t_G: 18.00 min, t_R: 12.63, $[M+H]^+$ = 444.

4.1.22. 4-Amino-1,5-dibenzyl-6-cyclohexylimidazo[4,5-*c*][1,2,6]thiadiazine 2,2-dioxide (20). To a suspension of **14** (300 mg, 1.11 mmol) and K_2CO_3 (310 mg, 2.24 mmol) in 40 mL acetone, benzylbromide was added (0.27 mL, 2.27 mmol) at reflux temperature. After 3 days, the rection mixture was cooled, filtered and the residue was purified by flash chromatography starting from 200:1 to 10:1 (CH_2Cl_2:MeOH) to afford **20** (59 mg, 12%). Mp 202-

3°C. ^{1}H-NMR (500 MHz, DMSO- $CDCl_3$): δ = 7.05 – 7.04 (d, 2H, NH_2); 7.62 – 7.60 (m, 2H, , Ar); 7.45 – 7.37 (m, 4H, , Ar); 7.33 – 7.24 (m, 4H, , Ar); 5.15 (s, 2H, 51-H); 4.80 (s, 2H, 11-H); 2.61- 2.56 (m, 1H, 61-H); 1.86 – 1.25 (m, 10H, 62-H / 63-H / 64-H). ^{13}C-NMR (125 MHz, DMSO-d_6): δ = 159.6 (C-4); 153.2 (C-7a); 152.0 (C-6); 136.8 (1C, C-12 or C-52); 134.5 (1C, C-12 or C-52); 129.2 (1C, C-15 or C-55); 127.6 (1C, 15 or C-55); 130.0; 129.3, 128.2, 125.3 (8C, C13 / C14 / C53 / C54); 103.7 (C-4a); 47.7 (C51); 47.6 (C11); 36.0 (C-61); 31.5 (2C, C-62); 25.8, 25.4 (3C, C-63 / C-64). HPLC-MS (ES^+): CH_3CN/H_2O 5:95-100:0, t_G: 18.00 min, t_R: 14.08, $[M+H]^+$ = 450.

4.2. Pharmacology

***4.2.1. In vitro* Tulahuen 2 antiepimastigote form activity.** The parasites were cultured at 28ºC in axenic medium (BHI-tryptose), supplemented with 5% fetal bovine serum. Cells cultured for 5-7 days (exponential phase) were inoculated with 50 mL of fresh culture medium, giving an initial concentration of 1 x 10^6 cells/mL. The growth of the parasite was followed for 11 days by culture absorbance measurements at 600 nm, proportional to the number of cells present.

Before inoculation, a pre-established quantity of each test compound was incorporated into the medium, dissolved in DMSO (dimethylsulfoxide). The final concentration of DMSO in the culture medium never exceeded 0.4%, using a blank (absence of product) with 0.4% DMSO. The compounds were incorporated into the culture medium at a final concentration of 25 µM and, for those that were most active, the dose was progressively decreased to 1 nM. The percentage of inhibition of growth (PGIe) of the parasite was evaluated in comparison with the blank, using nifurtimox as the trypanosomicide reference drug. The PGIe was calculated as follows: **PGIe=**{1-[(Ap-A0p)/(Ac-A0c)]}x100, where Ap=A_{600nm} of the culture containing the product on day 5; A_{0p}=A_{600nm} of the culture containing the product after addition of the culture (day 0); Ac=A_{600nm} of the blank on day 5; A0c=A_{600nm} of the blank on day 0. The A_{600nm} taken on day 5 corresponds to the late exponential phase in the culture growth curve.

To determine IC_{50} values, parasite growth was followed in the absence (control) and presence of increasing concentrations of the corresponding compound. The IC_{50} value was determined as the compound concentration required to reduce the absorbance by half compared to that of the untreated controls.

***4.2.2. In vitro* CL antiepimastigote assay.** The trypanocidal activity was evaluated by modifying a previously described colorimetric method [46] against *T. cruzi* (CL strain, clone B5) parasites stably transfected with *Escherichia coli* ß-galactosidase gene *(lacZ)* [47]. Epimastigote forms were axenically cultured at 28ºC in liver infusion tryptose medium (LIT), supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (100 µg/mL streptomicyn and 100 U/mL penicillin). Stock solutions of the compounds were prepared in dimethylsulfoxide (DMSO) and added to the cultures in a final DMSO concentration lower than 0.2%. Epimastigotes in the exponential phase of growth were seeded in 96-well plates at an initial concentration of 2.5 $\times$ 10^5 parasites/mL and incubated with the compounds for 72 h at 28ºC. The activity of the tested compounds was evaluated by measuring absorbance at 595 nm after the addition to each well of 50 µL of a chlorophenol red ß -D-galactopyranoside (CPRG) solution in 0.9% Triton X-100 (200 µM, pH 7.4), followed by incubation for 3 h at 37ºC. Nifurtimox and benznidazole were included in these assays as reference drugs. Antiepimastigote activity was expressed as the percentage of growth inhibition (PGIe), as follows: PGIe=100-{[(APc-APm)/(Ac-Am)]*100}, where APc represents the absorbance of treated cultures, APm the absorbance of the compound in medium, Ac the absorbance of cultures without compound (growth control) and Am the absorbance of the medium (blank). The PGIe obtained were used to calculate the IC_{50} value for epimastigotes (IC_{50e}) of each compound.

***4.2.3. In vitro* antiamastigote assay.** This *in vitro* assay was performed in 48-well tissue culture plates and the activity of the selected compounds was evaluated in the presence of the chromogenic substrate CPRG. NCTC-929 fibroblasts were seeded at a density of 10,000 cells/well with 120 µL of MEM medium. The cells were allowed to attach for 2-3 hours at 37ºC with 5% CO_2 and, afterwards, NCTC-929 derived trypomastigotes (TCT) of the CL-B5 *lac*Z strain were added in a 1:6 ratio (cells:TCT) and incubated for 24 h at a 33ºC with 5% CO_2.

Afterwards, infected cells were washed with PBS to remove non-penetrated trypomastigotes and then drugs in DMSO were diluted in fresh medium (2 µL/mL) to give a final volume of 450 µL/well. The plates were incubated for 7 days at 33ºC with 5% CO_2, then 50 µL of a CPRG solution in 3% Triton X-100 (400 µM, pH 7.4) was added. Finally, the plates were incubated at 37ºC for 3 h and the absorbance was read at 595 nm. Antiamastigote activity was expressed as the percentage of growth inhibition (PGIa), calculated as: PGIa=100-{[(APc-APm)/(Aci-Am)]*100}, were APc represents the absorbance of the treated group, APm the absorbance of the compound in medium, Aci the absorbance of infected cells without compound (control of infection) and Am the absorbance of the medium (blank). the absorbance of non-infected wells (only NCTC-929 cells) was subtracted from both groups (experimental and control of infection) as the background. The PGIa values were used to calculate the IC_{50} value of each compound against amastigotes (IC_{50a}).

***4.2.4. In vivo* trypanocidal assay.** Four groups of NMRI mice with an average weight of 20 g were intraperitoneally infected by the injection of 10^4 blood trypomastigotes of the *T. cruzi* Y strain, previously obtained by intracardiac puncture from anesthetized donor mice. The groups were distributed as follows: animals treated only with the solvent (control of infection), animals treated with benznidazole (reference drug), animals treated with **9** (experimental group 1) and animals treated with **4** (experimental group 2). This assay was carried out in order to assess the efficacy of these compounds to reduce parasitemia levels in acute disease, following previously established guidelines [48]. Accordingly, 100 mg/kg/day doses of the tested drugs were administered orally for five consecutive days, with the initiated on the treatment the fifth day post-infection (5 dpi). A solution of 2% carboxymethylcellulose sodium salt (CMC) was used as the drug vehicle. Only animals with positive parasitemia were included in the experiment. Parasitemia levels were estimated by counting trypomastigotes from tail blood samples as previously described [49] at 5, 8, and 10 dpi. Survival of the animals was assessed during acute infection (30 dpi).

All experiments were carried out according to directive 86/609/CEE and controlled in Spain by the then-current Royal Decree 1201/2005 of 10 October on the protection of animals used for research and other scientific purposes.

4.2.5. Cytotoxicity assay in murine macrophages. The non-specific cytotoxicity against J774 murine macrophages was assayed for the products that presented a percentage of growth inhibition of the epimastigote form of T. *cruzi* of the Tulahuen 2 strain greater than 50% at 25 μM on day 5 of the study. For this, murine macrophages were cultured in an atmosphere of 5% CO_2 and 95% air at 37ºC for 48 h with the products dissolved in DMSO at three concentrations: 100, 200 and 400 μM. Cell viability was determined based on the conservation of mitochondrial activity by the MTT/formazan method. This assay included nifurtimox, as an anti-*T. cruzi* reference drug.

4.2.6. Cytotoxicity assay in murine fibroblasts. NCTC-929 fibroblasts were seeded at a density of 15,000 cells/well in 96-well plates with 100 μL of MEM medium, and maintained for 3 h at 37ºC with 5% CO_2. Following cell attachment, the culture medium was replaced with 200 μL of compounds diluted in fresh medium and the plates were incubated for 48 h at 37ºC with 5% CO_2. Growth and medium controls were included in each plate. Benznidazole was also assayed as the reference drug. Cell viability was evaluated by applying the resazurin method [50], 20 μL of this substrate in PBS solution (2 mM, pH 7.0) was added before incubating the plates for another 3 h (37ºC with 5% CO_2). Finally, fluorescence intensity, with excitation at 535 nm and emission at 590 nm, was measured to assess cytotoxicity.

4.2.7. Cruzipain inhibitory activity [51]. The capacity to inhibit the cruzipain enzyme was assessed according to the following protocol: cruzipain (10 μL) was incubated with a reaction mixture containing a final concentration of 50 mM of Tris-HCl buffer solution, pH 7.6, 10 mM of mercaptoethanol and 25, 50 or 100 μM of the inhibitor for 10 min at ambient temperature. Later, the chromogenic substrate Bz-Pro-Phe-Arg-p-NA was aggregated at a final concentration of 150 μM, and the increase in absorbance at 410 nm was followed for 5 min at ambient

temperature in a Beckman DU 650 spectrophotometer. The inhibitors were dissolved in DMSO and the controls (100% enzymatic activity) contained the same concentration of the solvent.

4.2.8. Ames test. The method of direct incubation in a plate was performed. A culture of *S. typhimurium* strain TA98 was preprared in agar minimum glucose medium (AMG), containing agar solution, Vogel Bonner E(VB) 50×, and 40% glucose. First, the direct toxicity of the compounds under study against *S. typhimurium* strain TA98 was assayed. DMSO solutions of the imidazothiadiazines (starting at the highest dose without toxic effects, 500 μg/plate) were assayed in triplicate. Positive controls of 4-nitro-*o*-phenylendiamine (20.0 μg/plate, in the run without S9 activation) and 2-aminofluorene (10.0 μg/plate, in the cases of S9 activation) and negative control (DMSO) were run in parallel. The influence of metabolic activation was tested by adding 500 μL of the S9 fraction of mouse liver treated with Aroclor, obtained from Moltox, Inc. (Annapolis, MD, USA). The revertant number was counted manually. The sample was considered mutagenic when the number of revertant colonies was at least double that of the negative control for at least two consecutive dose levels.

4.3. Calculation of the anti-*T. cruzi* activity of imidazo[4,5-*c*][1,2,6]thiadiazine using the neural network model

4.3.1. Descriptor of the data set (CODES®). The numerical definition of the structures was achieved by using the CODES® program. CODES®, created by Prof. Manfred Stud at Instituto de Química Médica, CSIC, was initially designed for Mac computers with a graphical interface where molecules were drawn. A new version for Windows has been developed in collaboration with Advanced Software Production Line, S.L where molecules are read through their SMILES code.

Regarding the graphical molecular representation, aromatic bonds were set in the ChemDraw “bond type” option only for benzene and heteroaryl rings when drawing the structures to obtain the SMILES code using

CHEMDRAW 8.0 software. Once the SMILES code of the structural drawing was obtained (see Supplementary Material Table S4) it was included in the CODES® program.

4.3.2. Reduction of dimensions (RD). The philosophy of this process consists of reducing the complexity of any system without any loss of the intrinsic characteristics or information about the chemical nature. Several techniques, such as principal component analysis (PCA) or artificial neural networks (ANN), can be used for this purpose. In this work, the process was carried out by a back-propagation neural network (ReNDer type) with an architecture (IxA)-c-y-c-(IxA) where *(IxA)* represents the CODES® matrix, *c* is the number of neurons in the codification layer and *y* is the number of hidden neurons. Thus, it is possible to compress the dynamic matrix data into a set of four numeric codes for each molecule (hidden neurons: a_1, a_2, a_3 and a_4; see Supporting Information, Tables S1 and S2). The RD process was carried out using TSAR© software (v3.0) which applies a Monte Carlo algorithm [52]. Convergence parameters were 6000 iterations/cycle, 500 cycles past best (the number of cycles that are completed without improving on the best root mean square (RMS) fit before the training is terminated) and convergence of 0.001 RMS. The neural network is considered trained when the convergence plot shows consistent behavior.

4.3.3. Calculation of anti-*T. cruzi* activity. From our best predictive model obtained with a three-layered 4-4-1 architecture, the anti-*T. cruzi* activity of all the imidazothiadiazine derivatives was calculated using TSAR software (v3.0).

Supporting Information

Tables S1 and S2 show the SMILES codes and molecule descriptors (a1, a2, a3, and a4) of the data set, respectively.

Acknowledgments

We are grateful to the MINECO project (CTQ2015-66313), the MICINN project (TRA2009_0085, 2010-2011), the CSIC project (2009UY0003), CYTED net (http://ridimedchag.fq.edu.uy; RIDIMEDCHAG) and Emilia Bayo for her help with the development of this work. We are also very grateful for the financial support from LABORATORIOS SILANES IDF, S.L. *and* CSIC (Intramural project 200480E453).

References

[1] Chagas disease (American trypanosomiasis). *World Health Organization* **2016** (Updated March 2016)**,** *http://www.who.int/mediacentre/factsheets/fs340/en/* (*accessed October 31, 2016).*

[2] M. Navarro, B. Navaza, A. Guionnet, R. Lopez-Velez, Chagas disease in Spain: need for further public health measures. *PLoS Negl. Trop. Dis. 6(12)* (**2012***)* e1962.

[3] J. Strasen, T. Williams, G. Ertl, T. Zoller, A. Stich, O. Ritter, Epidemiology of Chagas disease in Europe: many calculations, little knowledge. *Clin. Res. Cardiol.* 103 (2014) 1-10.

[4] Y. Jackson, A. Pinto, S. Pett, Chagas disease in Australia and New Zealand: risks and needs for public health interventions. *Trop. Med. Int. Health* 19 (2014) 212-218.

[5] J. Milei, R.A. Guerri-Guttenberg, D.R. Grana, R. Storino, Prognostic impact of Chagas disease in the United States. *Am. Heart J.* 157 (2009) 22-29.

[6] S.P. Montgomery, M.C. Starr, P.T. Cantey, M.S. Edwards, S.K. Meymandi, Neglected parasitic infections in the United States: Chagas disease. *Am. J. Trop. Med. Hyg.* 90 (2014) 814-818.

[7] *Investing to overcome the global impact of neglected tropical diseases, WHO,* **2015**, http://www.who.int/neglected_diseases/9789241564861/en/, (*accessed October 31, 2016).*

[8] F. Sanchez-Sancho, N.E. Campillo, J.A. Páez, Chagas Disease: Progress and New Perspectives. *Curr. Med. Chem.*, 17 (2010) 523-552.

[9] Center for Disease Control and Prevention. Division of Parasitic Diseases (DPD), Antiparasitic treatment of Chagas disease Available at. *(Center for Disease Control and Prevention). http://www.cdc.gov/parasites/chagas/*, (*accessed October 31, 2016).*

[10] W. Apt, A. Arribada, I. Zulantay, A. Solari, G. Sanchez, K. Mundaca, X. Coronado, J. Rodriguez, L.C. Gil, A. Osuna,Itraconazole or allopurinol in the treatment of chronic American trypanosomiasis: the results of clinical and parasitological examinations 11 years post-treatment. *Ann. Trop. Med. Parasitol.*, 99 (2005) 733-741.

[11] W. Apt, I. Heitmann, I. Jercic, L. Jofré, P. Muñoz, I. Noemí, A.M. San Martín, J. Sapunar, M. Torres, I. Zulantay, [Part VI. Antiparasitic treatment for Chagas disease]. *Rev. Chilena Infectol.*, 25 (2008) 384-389, discussion 387.

[12] I. Molina, F. Salvador, A. Sanchez-Montalva, The use of posaconazole against Chagas disease. *Curr. Opin. Infect. Dis.,* 28 (2015) 397-407.

[13] A Study of the Use of Oral Posaconazole (POS) in the Treatment of Asymptomatic Chronic Chagas Disease (P05267) (STOP CHAGAS). *https://clinicaltrials.gov/ct2/show/NCT01377480?term=NCT01377480&rank=1. (accessed October 31, 2016). ClinicalTrials.gov Identifier NCT01377480.* **2015 (Last updated)**.

[14] F.S. Buckner, J.A. Urbina, Recent Developments in Sterol 14-demethylase Inhibitors for Chagas Disease. *Int. J. Parasitol: Drugs Drug Resist. 2* (2012) 236-242.

[15] Proof of Concept Study of E1224 To Treat Adults Patients with Chagas Disease. https://clinicaltrials.gov/ct2/show/NCT01489228?term=chagas&rank=23.(*accessed October 31, 2016)*. ClinicalTrials.gov Identifier NCT01489228.

[16] I. Molina, J. Gomez i Prat, F. Salvador, B. Trevino, E. Sulleiro, N. Serre, D. Pou, S. Roure, J. Cabezos, L. Valerio, A. Blanco-Grau, A. Sanchez-Montalva, X. Vidal, A. Pahissa, Randomized trial of posaconazole and benznidazole for chronic Chagas' disease. *N. Engl. J. Med.,* 370 (2014) 1899-1908.

[17] E. Chatelain, Chagas disease drug discovery: toward a new era. *J. Biomol. Screen,* 20 (2015) 22-35.

[18] L. Gaspar, C.B. Moraes, L.H. Freitas-Junior, S. Ferrari, L. Costantino, M.P. Costi, R.P. Coron, T.K. Smith, J.L. Siqueira-Neto, J.H. McKerrow, A. Cordeiro-da-Silva, Current and Future Chemotherapy for Chagas Disease. *Curr. Med. Chem.,* 22 (2015) 4293-4312.

[19] B. Carrilero, L. Murcia, L. Martinez-Lage, M. Segovia, Side effects of benznidazole treatment in a cohort of patients with Chagas disease in non-endemic country. *Rev. Esp. Quimioter.,* 24 (2011) 123-126.

[20] S.R. Wilkinson, C. Bot, J.M. Kelly, B.S. Hall, Trypanocidal activity of nitroaromatic prodrugs: current treatments and future perspectives. *Curr. Top. Med. Chem.,* 11 (2011) 2072-2084.

[21] J.A. Castro, M.M. de Mecca, L.C. Bartel, L. C. Toxic side effects of drugs used to treat Chagas' disease (American trypanosomiasis). *Hum. Exp. Toxicol.,* 25 (2006) 471-479.

[22] R. Viotti, C. Vigliano, B. Lococo, M.G. Alvarez, M. Petti, G. Bertocchi, A. Armenti, Side effects of benznidazole as treatment in chronic Chagas disease: fears and realities. *Expert Rev. Anti Infect. Ther.,* 7 (2009) 157-163.

[23] P.M. Guedes, G.K. Silva, F.R. Gutierrez, J.S. Silva, Current status of Chagas disease chemotherapy. *Expert Rev. Anti Infect. Ther.,* 9 (2009) 609-620.

[24] R. Viotti, B. Alarcon de Noya, T. Araujo-Jorge, M.J. Grijalva, F. Guhl, M.C. Lopez, J.M. Ramsey, I. Ribeiro, A.G. Schijman, S. Sosa-Estani, F. Torrico, J. Gascon, Towards a paradigm shift in the treatment of chronic Chagas disease. *Antimicrob. Agents Chemother.,* 58 (2014) 635-639.

[25] S.R. Wilkinson, M.C. Taylor, D. Horn, J.M. Kelly, I. Cheeseman, A mechanism for cross-resistance to nifurtimox and benznidazole in trypanosomes. *Proc. Natl. Acad. Sc.i USA*, 105 (2008) 5022-5027.

[26] A. Guerra, P. Gonzalez-Naranjo, N.E. Campillo, H. Cerecetto, M. Gonzalez, J.A. Páez, Artificial neural networks based on CODES descriptors in pharmacology: identification of novel trypanocidal drugs against Chagas disease. *Curr. Comput. Aided Drug Des.,* 9 (2013) 130-140.

[27] *M. Stud CODES©; v1.0 (revision 3) ed.*

[28] A. Guerra, N.E. Campillo, J.A. Páez, Neural computational prediction of oral drug absorption based on CODES 2D descriptors. *Eur. J. Med. Chem.,* 45 (2010) 930-940.

[29] A. Guerra, J.A. Páez, N.E. Campillo, Artificial neural networks in ADMET modeling: Prediction of blood-brain barrier permeation. *Qsar & Comb. Sci.,* 27 (2008) 586-594.

[30] A. Herrero, C. Ochoa, J.A. Páez, A new rote for the synthesis of 6-substituted imidazo[4,5-c]-1,2,6-thiadiazine 2,2-dioxides: NMR study and crystal structure of a complex with dimethylformamida. *Heterocycles* , 26 (1987) 3123-3133.

[31] G. Garcia-Muñoz, R. Madroñero, C. Ochoa, M. Stud, Synthesis of purine-like ring systems derived from 1,2,6-thiadiazine 1,1-dioxide. *J. Heterocyclic Chem.,* 13 (1976) 793-796.

[32] I. Alkorta, V.J. Aran, A.G. Bielsa, M. Stud, Reactivity of malononitrile towards sulphamide and N-substituted sulphamides: synthesis and hydrolysis reactions of 3,5-diamino-1,2,6-thiadiazine 1,1-dioxides. *Chem. Soc., Perkin Trans.,* (1988) 1271-1275.

[33] D. Benitez, M. Cabrera, P. Hernandez, L. Boiani, M.L. Lavaggi, R. Di Maio, G. Yaluff, E. Serna, S. Torres, M.E. Ferreira, N. Vera de Bilbao, E. Torres, S. Perez-Silanes, B. Solano, E. Moreno, I. Aldana, A. Lopez de Cerain, H. Cerecetto, M. Gonzalez, A. Monge, 3-Trifluoromethylquinoxaline N,N'-dioxides as anti-

trypanosomatid agents. Identification of optimal anti-T. cruzi agents and mechanism of action studies. *J. Med. Chem.,* 54 (2011) 3624-3636.

[34] J.J. Cazzulo,Proteinases of Trypanosoma cruzi: patential targets for the chemotherapy of Chagas disease. *Curr. Top. Med. Chem.,* 2 (2002) 1261-1271.

[35] V.G. Duschak, A.S. 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.

[36] J.C. Engel, P.S. Doyle, J. Palmer, I. Hsieh, D.F. Bainton, J.H. McKerrow, Cysteine protease inhibitors alter Golgi complex ultrastructure and function in Trypanosoma cruzi. *J. Cell Sci.,* 111 (Pt 5) (1998) 597-606.

[37] B.T. Mott, R.S. Ferreira, A. Simeonov, A. Jadhav, K.K. Ang, W. Leister, M. Shen, J.T. Silveira, P.S. Doyle, M.R. Arkin, J.H. McKerrow, J. Inglese, C.P. Austin, C.J. Thomas, B.K. Shoichet, D.J. Maloney, Identification and optimization of inhibitors of Trypanosomal cysteine proteases: cruzain, rhodesain, and TbCatB. *J. Med. Chem.,* 53 (2010) 52-60.

[38] S. Patterson, S. Wyllie, Nitro drugs for the treatment of trypanosomatid diseases: past, present, and future prospects. *Trends Parasitol.,* 30 (2014) 289-298.

[39] A. Buschini, L. Ferrarini, S. Franzoni, S. Galati, M. Lazzaretti, F. Mussi, C. Northfleet de Albuquerque, T. Maria Araujo Domingues Zucchi, P. Poli, Genotoxicity revaluation of three commercial nitroheterocyclic drugs: nifurtimox, benznidazole, and metronidazole. *J. Parasitol. Res.,* 2009 (2009) 463575.

[40] D.M. Maron, B.N. Ames, Revised methods for the Salmonella mutagenicity test. *Mutat Res* , 113 (1983) 173-215.

[41] K.C. Chu, K.M. Patel, A.H. Lin, R.E. Tarone, M.S. Linhart, V.C. Dunkel, Evaluating statistical analyses and reproducibility of microbial mutagenicity assays. *Mutat. Res.,* 85 (1981) 119-132.

[42] J.A. Paéz, N.E. Campillo, A. Guerra, M. Gonzalez, H. Cerecetto, Una nueva familia de antichagásicos derivados de 2,2-dióxido de imidazo[4,5-c][1,2,6]tiadiazina. *Patente ESP 200900225; PCT1641-296.* January 26, 2010.

[43] J.A. Páez, N.E. Campillo, A. Guerra, M.M. Gonzalez, H. Cerecetto, Una nueva familia de antichagásicos derivados de 2,2-dióxido de imidazo[4,5-c][1,2,6]-tiadiazina. Argentina, Uruguay, Paraguay, Bolivia, Venezuela 2010.

[44] J.A. Páez, N.E. Campillo, A. Guerra, M.M. Gonzalez, H. Cerecetto, New family of antichagasics derived from imidazo[4,5-c][1,2,6]thiadiazine 2,2-dioxide. EP1641 296, 2012.

[45] J.A. Páez, N.E. Campillo, A. Guerra, M.M. Gonzalez, H. Cerecetto, New family of antichagasics derived from imidazo[4,5-c][1,2,6]thiadiazine 2,2-dioxide. US 8,815,846 B2, August 26, 2014.

[46] C. Vega, M. Rolon, A.R. Martinez-Fernandez, J.A. Escario, A. Gomez-Barrio, A new pharmacological screening assay with Trypanosoma cruzi epimastigotes expressing beta-galactosidase. *Parasitol. Res.,* 95 (2005) 296-298.

[47] F.S. Buckner, C.L. Verlinde, A.C. La Flamme, W.C. Van Voorhis, Efficient technique for screening drugs for activity against Trypanosoma cruzi using parasites expressing beta-galactosidase. *Antimicrob. Agents Chemother.,* 40 (1996) 2592-2597.

[48] A.J. Romanha, S.L. Castro, N. Soeiro Mde, J. Lannes-Vieira, I. Ribeiro, A. Talvani, B. Bourdin, B. Blum, B. Olivieri, C. Zani, C. Spadafora, E. Chiari, E. Chatelain, G. Chaves, J.E. Calzada, J.M. Bustamante, L.H. Freitas-Junior, L.I. Romero, M.T. Bahia, M. Lotrowska, M. Soares, S.G. Andrade, T. Armstrong, W. Degrave, A. Andrade Zde, In vitro and in vivo experimental models for drug screening and development for Chagas disease. *Mem. Inst. Oswaldo Cruz* , 105 (2010) 233-238.

[49] 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.

[50] M. Rolon, C. Vega, J.A. Escario, A. Gomez-Barrio, Development of resazurin microtiter assay for drug sensibility testing of Trypanosoma cruzi epimastigotes. *Parasitol. Res.,* 99 (2006) 103-107.

[51] A. Merlino, P. Benítez, N.E. Campillo, J.A. Páez, L.W. Tinoco, M. Gonzalez, H. Cerecetto, Amidines bearing benzofuroxan or benzimidazole 1,3-dioxide core scaffolds as Trypanosoma cruzi-inhibitors: Structural basis for their interactions with cruzipain. *Med. Chem. Commun.,* 1 (2010) 216-228.

[52] *TSAR® v3.3. San Diego: Accelrys Software Inc., 2000*.

Figure Captions

Figure 1. Chemical structures of drugs introduced to the market and in clinical trials for Chagas disease.

Figure 2. Chemical structures of the imidazo[4,5-*c*][1,2,6]thiadiazine derivative family.

Figure 3. Protocol used to develop the neural network model.

Figure 4. Parasitemia levels in mice experimentally infected with *T. cruzi* (Y strain) and treated orally for five consecutive days with 100 mg/kg/day of **19**, **4** and benznidazole.

Figure 5. Survival rates of **19** and **4** treated mice expressed as the percentage of living animals during the acute phase of the infection (30 dpi).

Scheme 1. General synthetic route for the preparation of imidazothiadiazine derivatives. Reagents and conditions: a) H_2O/AcOH, rt; b) MeI/K_2CO_3, acetone, reflux for **14,** Me_2SO_4 /K_2CO_3/H_2O for **15**; R^2-Br, Et_3N, acetone, reflux for **16-18** ; c) benzyl bromide, sodium hydroxide, H_2O, reflux for **19**; benzyl bromide, K_2CO_3, acetone, reflux for **20**.

Highlights

- Artificial neural networks approach to identify a new class of anti-T.cruzi agents

- Development of a new family of imidazo[4,5-*c*][1,2,6]thiadiazines with anti-epimastigote and anti-amastigote effects

- Novel heterocyclic derivative **19** with *in vivo* trypanocidal activity as potential agent for the treatment of Chagas disease

Suplementary Information

Novel Imidazo[4,5-*c*][1,2,6]thiadiazine 2,2-Dioxides as Antiproliferative *Trypanosoma Cruzi* Drugs: Computational Screening from Neural Network, Synthesis and *in vivo* Biological Properties

Angela Guerra[a], Pedro Gonzalez-Naranjo[a], Nuria E. Campillo[b], Javier Varela[c], María L. Lavaggi[c], Alicia Merlino[c], Hugo Cerecetto[c], Mercedes González[c], Alicia Gomez-Barrio[d], José A. Escario[d], Cristina Fonseca-Berzal[d], Gloria Yaluf[e], Jorge Paniagua[f], and Juan A. Páez[a]*

[a] Instituto de Química Médica. Consejo Superior de Investigaciones Científicas (CSIC), Juan de la Cierva 3, 28006 Madrid, Spain.
[b] Centro de Investigaciones Biológicas. Consejo Superior de Investigaciones Científicas (CSIC). Ramiro de Maeztu, 9
Madrid, Spain
[c] Grupo de Química Medicinal, Laboratorio de Química Orgánica, Facultad de Ciencias-Facultad de Química, Universidad de la República, Iguá 4225, 11400 Montevideo, Uruguay.
[d] Departamento de Parasitología. Facultad de Farmacia. Universidad Complutense de Madrid, Pza. Ramón y Cajal s/n, 28040, Madrid, Spain.
[e] Instituto de Investigaciones en Ciencias de la Salud (iics), Universidad Nacional de Asunción, Asunción-Paraguay
[f] Laboratorios Silanes IDF, S.L. *Calle Santiago Grisolia, Nº 2- PTM 148 Parque Tecnologico de Madrid 28760-Tres Cantos-Madrid.*

Table of Contents:

Table S1. SMILES codes of the data set

Compd	Smile codes
1	NC1=NS(NC2=C1NC(C)=N2)(=O)=O
2	NC1=NS(NC2=C1NC(CC)=N2)(=O)=O
3	NC1=NS(NC2=C1NC(C3CCCC3)=N2)(=O)=O
4	NC1=NS(NC2=C1NC(C3CCCCC3)=N2)(=O)=O
5	NC1=NS(NC2=C1NC(c3ccccc3)=N2)(=O)=O
6	NC1=NS(NC2=C1NC(c3ccccc3F)=N2)(=O)=O
7	NC1=NS(NC2=C1NC(c3ccc(N(C)C)cc3)=N2)(=O)=O
8	NC1=NS(NC2=C1NC(C3=CSC=C3)=N2)(=O)=O
9	NC1=NS(NC2=C1NC(c3cccnc3)=N2)(=O)=O
10	NC1=NS(NC2=C1NC(C3=CC=C([N+]([O-])=O)O3)=N2)(=O)=O
11	NC1=NS(NC2=C1NC(C3COCC3)=N2)(=O)=O
12	NC1=NS(NC2=C1NC(Cc3ccccc3)=N2)(=O)=O
13	NC1=NS(NC2=C1NC(CCc3ccccc3)=N2)(=O)=O
14	NC1=NS(N(C)C2=C1NC(C3=CSC=C3)=N2)(=O)=O
15	NC1=NS(N(C)C2=C1NC(c3ccccc3)=N2)(=O)=O
16	NC1=NS(N(Cc4ccccc4)C2=C1NC(c3ccccc3)=N2)(=O)=O
17	NC1=NS(N(Cc4ccc(Cl)cc4)C2=C1NC(c3ccccc3F)=N2)(=O)=O
18	NC1=NS(N(Cc2ccc(Cl)cc2)C3=C1NC(C4CCCCC4)=N3)(=O)=O
19	NC1=NS(N(Cc4ccccc4)C2=C1N(Cc5ccccc5)C(c3ccccc3)=N2)(=O)=O
20	NC1=NS(N(Cc2ccccc2)C3=C1N(Cc4ccccc4)C(C5CCCCC5)=N3)(=O)=O

Table S2. Molecule descriptors (a_1, a_2 , a_3 and a_4) of the data set

Compd	a_1	a_2	a_3	a_4
1	0.932982	0.963147	0.940952	0.969182
2	0.986657	0.968009	0.763236	0.982532
3	0.276861	0.334238	0.561609	0.521102
4	0.905326	0.016024	0.03183	0.919626
5	0.163054	0.299929	0.930569	0.378914
6	0.224737	0.992553	0.204261	0.468652
7	0.986065	0.56182	0.992083	0.704709
8	0.610626	0.632634	0.017324	0.999687
9	0.675676	0.009432	0.743043	0.83699
10	0.721326	0.799836	0.997788	0.986109
11	0.800578	0.425448	0.096816	0.840056
12	0.696575	0.708217	0.918472	0.122085
13	0.879363	0.380809	0.971733	0.09105
14	0.916315	0.841631	0.306352	0.005446
15	0.972819	0.134136	0.145266	0.612621
16	0.003762	0.008269	0.454581	0.04898
17	0.887942	0.052361	0.048125	0.034586
18	0.915978	0.024077	0.034503	0.014056
19	0.030904	0.882515	0.022557	0.023453
20	0.932736	0.015817	0.015983	0.017912