3-Trifluoromethylquinoxaline *N,N'*-Dioxides as Anti-trypanosomatid Agents. Identification of Optimal Anti-*T. cruzi* Derivatives and Mechanism of Action Studies

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Abstract

As a fourth approach of quinoxaline *N,N'*-dioxides as anti-trypanosomatid agents against *T. cruzi* and *Leishmania*, we found extremely active derivatives. The present study allows us to state the correct requirements for obtaining optimal *in vitro* anti-*T. cruzi* activity. Derivatives possessing electron-withdrawing substituent in the 2-, 3-, 6-, and 7-positions rendered the most active compounds. With regard to these features, and taking in account their mammal-cytotoxicity, some trifluoromethylquinoxaline *N,N'*-dioxides have been proposed as candidates for further clinical studies. Consequently, mutagenicity and *in vivo* analyses were performed with one of the most promising derivatives. In addition, with regard to the mechanism of action studies, it was demonstrated that mitochondrial dehydrogenases are involved in the anti-*T. cruzi* activity of the most active derivatives.

Running Title. Quinoxaline *N,N´*-Dioxide as Anti-trypanosomatid agents.

Key Words: Quinoxaline *N,N´*-Dioxide, *T. cruzi*, *Leishmania*

Introduction

Parasitic diseases affect hundreds of millions of people throughout the world, mainly in developing countries. Since parasitic protozoa are eukaryotic, they share many common features with their mammalian host, making the development of effective and selective drugs a hard task. Diseases caused by *Trypanosomatidae*, which share similar characterisitics regarding drug treatment, include Chagas' disease (*Trypanosoma cruzi*) and leishmaniosis (*Leishmania spp*.).[1] These trypanosomatids alone are responsible for an infected population of nearly 30 million, and more than 400 million persons are at risk. The drugs currently used in the treatment of Chagas' disease are two nitroaromatic heterocyles, Nifurtimox (Nfx, Lampit®, recently discontinued by Bayer) and Benznidazole (Rochagan®, Roche), introduced empirically over three decades ago.[2] Both drugs are active in the acute phase of the disease but efficacy is very low in the established chronic phase. In addition, differences in drug susceptibility among different *T. cruzi* strains lead to a variety of parasitological cure rates depending upon the geographical area.

The drugs of choice for the treatment of leishmaniosis are sodium stibogluconate (Pentostam®), meglumine antimoniate (Glucantime®), pentamidine and liposomal amphotericin B, but they sometimes meet with failure.[3] "WHO/TDR is currently developing a research program with Miltefosine (Mtf), a very promising leishmanicidal drug. However, new therapeutic alternatives should be found due to the fact that this drug is creating serious problems of resistance.[3b-d]

The capability of the quinoxaline *N,N'*-dioxide system to act as anti-infective agents toward a great number of microorganisms[4] led us to evaluate some selected derivatives as anti-*T. cruzi* agents from our quinoxaline-library.[5] This study allowed us to identify excellent *in vitro* anti-*T. cruzi* agents against Tulahuen 2 strain and CL Brener clone (derivatives **1** and **2**, Figure 1). New quinoxaline *N,N'*-dioxide derivatives were selected to analyze some structural changes in parent compounds **1** and **2** and they were biologically analyzed against different *T. cruzi* strains and against *Leishmania* protozoa. In particular, we selected quinoxaline *N,N'*-dioxides in which

substituents in positions 2, 3, 6, 7 of parent compounds **1** and **2** were then modified (Figure 1). In addition, some reduced quinoxaline analogues were studied. The unspecific toxicity against mammalian cells was studied in order to evaluate the quinoxaline selectivity to the parasites. In addition, in order to better understand the anti-*T. cruzi*-mechanism of action, the changes in the parasite-excreted metabolites and the effects on the mitochondrion dehydrogenase activity promoted by quinoxaline *N,N'*-dioxide derivatives was studied. One of the most promising quinoxalines was studied for its mutagenicitiy and *in vivo* activity on an acute murine model of Chagas disease.

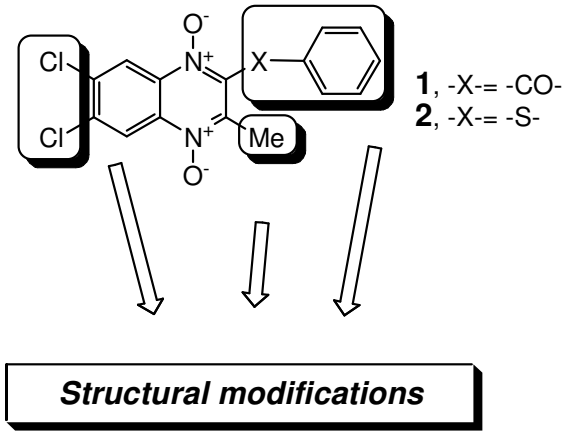

Figure 1. Quinoxaline *N,N'*-dioxide derivatives previously described as *T. cruzi* growth inhibitors.[5a]

Methods and Results

2.1. Selected compounds and synthesis

Ten families of quinoxaline *N,N*'-dioxides were selected in order to analyze their *in vitro* anti-*T. cruzi* activity. Derivatives **3-8** (Table 1), belonging to the first family, were included as derivatives in which modifications in position 2 of the parent compounds **1** and **2**, were then carried out. In this case, we included moieties with different volumes, electrophilic behaviors and polarities. In the second family, made up of derivatives **9-15** (Table 1), we maintained

substitutions in positions 2 and 3 of parent compound **1** or active derivative **5**, modifying the 6- and/or 7-*benzo*-substitutions of quinoxaline ring.

Table 1. *T. cruzi* Antiproliferative Activity of Quinoxaline *N,N'*-Dioxide and Related Derivatives.

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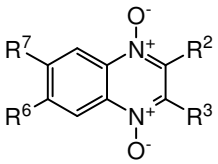


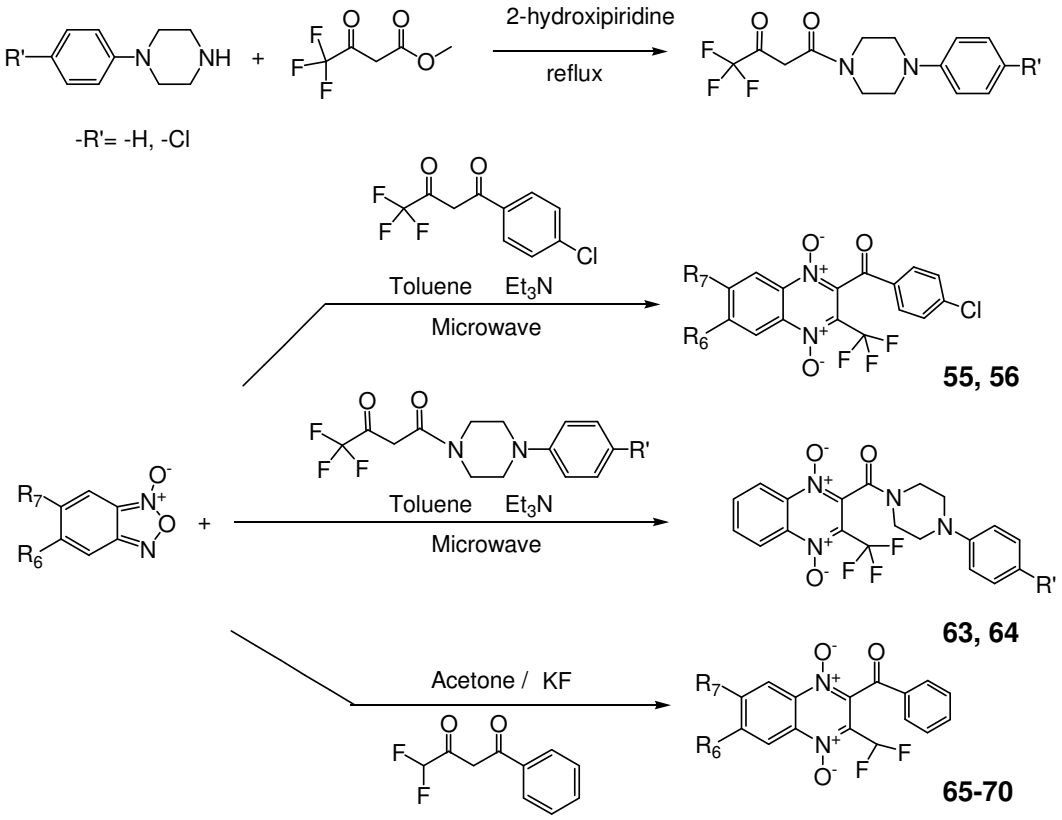
Cpd.	-R^2	-R^3	-R^6	-R^7	PGI^a(%)	Cpd.	-R^2	-R^3	-R^6	-R^7	PGI^a(%)
3	-CO_2CH_3				66.0	**40**	-CO_2CH_3				24.0
4	-CONHPh				61.0	**41**	-CONHPh	-CH_3	-H	-H	10.0
5	-$COCH_3$	-CH_3	-Cl	-Cl	60.0	**42**	-$COCH_3$				29.0
6	-CONHPh-*o*-CH_3				49.0	**43**	-CONHPh-*o*-CH_3				21.0
7	-$CO_2CH_2CH_3$				32.0	**44**	-$COCH_3$				100.0
8	-CONH-*t*-Bu				28.0	**45**	-$COCH_2CH_3$		-F	-F	100.0
9	-$COCH_3$		-F	-F	100.0	**46**					100.0
10					92.0	**47**			-Cl	-Cl	100.0
11			-Cl		33.0	**48**	-CO-*i*-Prop		-H	-H	100.0
12	-COPh	-CH_3	-H	-H	19.0	**49**					23.6
13			-OCH_3		9.0	**50**	-CO-*t*-Bu		-F	-F	100.0
14			-CH_3		8.0	**51**					26.0
15			-CH_3	-CH_3	3.0	**52**	-$COCH_3$		-H	-H	100.0
16			-Cl	-Cl	24.0	**53**	-CO-1-naphthyl				100.0
17	-CH_2Ph	-CH_3	-Cl	-H	0.0	**54**		-CF_3	-F	-F	100.0
18			-H	-H	0.0	**55**	-COPh-*p*-Cl				100.0
19			-CH_3	-CH_3	0.0	**56**			-H	-H	100.0
20			-Cl	-Cl	100.0	**57**	-CO-2-furyl				100.0
21			-F	-F	100.0	**58**			-F	-F	100.0
22			-H	-H	100.0	**59**			-CF_3	-H	100.0
23	-COPh	-CF_3	-CH_3	-H	99.0	**60**			-H	-CF_3	100.0
24			-Cl	-H	90.0	**61**	-CO-2-thienyl		-F	-F	100.0
25			-OCH_3	-H	98.0	**62**					100.0
26			-CH_3	-CH_3	72.0	**63**	-CO(4-Ph-Pi)^b		-H	-H	100.0
27			-F	-F	100.0	**64**	-CO[4-(Ph-*p*-Cl)-Pi]				100.0
28			-Cl	-Cl	99.0	**65**			-Cl	-Cl	100.0
29			-Cl	-H	88.0	**66**			-Cl	-H	100.0
30	-$CO_2CH_2CH_3$	-Ph	-H	-H	29.0	**67**	-COPh	-CHF_2	-F	-H	100.0
31			-CH_3	-H	13.0	**68**			-OCH_3	-H	100.0
32			-OCH_3	-H	0.0	**69**			-CH_3	-H	50.8
33			-CH_3	-CH_3	0.0	**70**			-H	-H	35.8
34	-CO_2CH_3				10.0	**71**^{c}	-COPh	-CF_3	-H	-H	49.0
35	-CONHPh				16.0	**72**^{c}	-CO-*t*-Bu-				43.0
36	-$COCH_3$	-CH_3	-CH_3	-CH_3	2.0	**1**	-SPh	-CH_3	-Cl	-Cl	93.0
37	-CONHPh-*o*-CH_3				12.0	**2**	-COPh				91.0
38	-$CO_2CH_2CH_3$				1.0	**Nfx**	-	-	-	-	100.0
39	-CONH-*t*-Bu				0.0						

[a] Percentage of growth inhibition. Inhibition of epimastigote growth of Tulahuen 2 strain, doses = 25 µM. The results are the means of three independent experiments with a SD less than 10% in all cases. [b] Pi= 1-piperazinyl. [c] Deoxygenated derivatives.

The third group of derivatives (derivatives **16-19**, Table 1) involved modifications in positions 6 and 7 together with -CO- or -S- substitution (-**X-** in parent compounds **1** and **2**, Figure 1) by a -CH_2- group. In the fourth family, the 3-CH_3 substituent, in parent compound **1**, was changed by -CF_3 moiety yielding derivative **20** and when 6- and 7-substituents were concomitantly modified, the resulting derivatives were **21-26** (Table 1). Other modifications involved the inclusion of a 3-Ph group, as voluminous moiety in this position, a non-voluminous electrophilic moiety in position 2, –CO_2Et group, and different electronic-behavior-substitutions in the 6- and/or 7-benzo-cycle (derivatives **27-33**, Table 1). In the sixth and seventh families of compounds we modified derivatives **3-8** (Table 1) in the *benzo*-substitutions, yielding 6,7-dimethyl derivatives (**34-39**, Table 1) and benzo-non-substituted analogues (**40-43**, Table 1). The –CF_3 substitution in 3-position, i.e. derivatives **20-26**, resulted in very active compounds (Table 1). From here we designed the eighth group of studied compounds (**44-64**, Table 1), maintaining the–CF_3 in 3-position, with modifications at 2-, 6- and/or 7-positions. In the same way, the ninth group included derivatives with –CHF_2 substitution in position 3 (derivatives **65-70**, Table 1). Finally, the last group made up of quinoxalines **71** and **72** (Table 1), deoxygenated analogues of **22** and **51**, respectively, was included in our study to investigate the relevance of the *N*-oxide moieties in the anti-*T. cruzi* activities.

Most of the studied compounds were prepared following previously reported[5,6] synthetic procedures, and the new compounds **55**, **56**, and **63-70** were prepared using the Beirut expansion process as shown in Scheme 1. Derivatives asymmetrically substituted in the benzo-cycle were obtained as a mixture of inseparable 6- and 7-isomers which were evaluated without further separation. For simplicity, Table 1 only shows one isomer. All the compounds were characterized by ^{1}H-NMR and IR. The purity was established by TLC and microanalysis.

OJO: En el gráfico, el término hay que escribir como: 2-hydroxypiridine (sustiuir “i” con”y”).



Scheme 1. Synthetic procedures used to prepare the new derivatives.

2.2. Biological characterization

***In vitro* anti-*T. cruzi* activity.** The new quinoxaline derivatives were initially tested *in vitro* against the epimastigote form of *T. cruzi*, Tulahuen 2 strain. The existence of the epimastigote form of *T. cruzi* as an obligate mammalian intracellular stage has been reviewed and confirmed.[7] The compounds were incorporated into the biological media at 25 μM and their ability to inhibit growth of the parasite was evaluated in comparison to that of the control (no drug added to the media) on day 5. Nfx was used as the trypanosomicidal reference drug. The percentage of growth inhibition (PGI) was calculated as indicated in the Experimental Section (Table 1).

In addition, the ID_{50} concentrations (50% inhibitory dose) were assessed for the most active

derivatives and Nfx (Table 2). The parent compound **2**, and derivatives **10**, **21**, **22**, and **28**, with a high anti-*T. cruzi* activity against the Tulahuen 2 strain and the less active derivatives **40**, and **41** were selected to study against the CL Brener clone and the *in vivo*-Nfx- and Bnz-partially resistant strains, Y and Colombiana (Table 3).[8] In these assays, viability of *T. cruzi* was colorimetrically assessed using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide).[9] For each derivative, the percentage of cytotoxicity (PCyt) was initially determined at 25 μM, as indicated in the Experimental Section, and then the ID_{50} concentration was calculated in a dose-response assay, between 1.0 and 50.0 μM, (Table 3).

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Table 2. ID_{50} Values for Relevant Quinoxaline *N,N'*-Dioxides and for Parent Compounds in Tulahuen 2 Strain.

Cpd.	ID_{50} (nM) [a]	Cpd.	ID_{50} (nM) [a]
1	11800 [b]	**48**	> 2500
2	6500 [b]	**50**	4900
5	18400	**52**	3000
9	400	**53**	1400
10	4200	**54**	3900
20	2400	**55**	2500
21	700	**56**	1600
22	900	**57**	2400
23	1300	**58**	2500
24	3000	**59**	5000
25	3500	**60**	4800
26	1400	**61**	2800
27	3300	**62**	1100
28	11300	**63**	760
29	12500	**64**	790
44	32	**65**	4700
45	500	**66**	6400
46	780	**67**	6300
47	1400	**68**	10000
Nfx	7700	**69**	25000

[a] The results are the means of three independent experiments with a SD less than 10% in all cases. [b] From reference [5a].

***In vitro* leishmanicidal activity.** We also selected derivatives **2**, **10**, **21**, **22**, **28**, **40**, and **41** to assess the leishmanicidal activity. They were tested *in vitro* against promastigote form of

Leishmania braziliensis (MHOM/BR/00/LTB300) strain. Viability of parasite was assessed colorimetrically using MTT assay.[10] For each derivative, the percentage of cytotoxicity was initially determined at 25 μM, as indicated in the Experimental Section, and then the ID_{50} concentration was calculated in a dose-response assay, between 1.0 and 50.0 μM, (Table 3). Mtf was used as the leishmanicidal reference drug.

Table 3. Percentage of Cytotoxicity and ID_{50} Values for Relevant Quinoxaline *N,N'*-Dioxides and for Parent Compound **2** Against Different *T. cruzi* Strains and *L. brazilensis* LTB300 Strain.

Cpd.	PCyt (%)[a,b,c] / ID_{50} (μM)			Cpd.	ID_{50} (μM)[c,e]
	CL Brener clone	Y strain	Colombiana strain		LTB300 Strain
2	98.0 / < 1.0	100.0 / 2.9	100.0 / 1.7	**2**	1.4
10	92.0 / 16.2	89.0 / 18.3	98.0 / 4.5	**10**	20.4
21	96.0 / 1.1	100.0 / 1.8	100.0	**21**	1.3
22	97.0 / 3.1	100.0 / 4.0	100.0	**22**	1.0
28	94.0 / 16.4	100.0 / 7.1	100.0	**28**	3.0
40	59.0	0.0	60.0	**40**	> 50.0
41	32.0	0.0	24.0	**41**	> 50.0
Nfx[d]	90.0 / 4.9	80.0 / 9.7	87.0 / 3.4	**Mtf**	9.0

[a] Percentage of cytotoxicity. [b] Doses = 25 μM. [c] The results are the means of three independent experiments with a SD less than 10% in all cases. [d] At 10 μM. [e] The results are the means of three independent experiments with a SD less than 10% in all cases.

In vitro toxicity studies

To explore the potential of these quinoxaline *N,N'*-dioxides as drugs we performed two different studies. Firstly, we evaluated their *in vitro* unspecific mammal cytotoxicity, using J-774 mouse macrophages. Secondly, we studied the mutagenicity capacity using the Ames test.

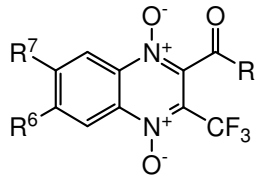
In vitro unspecific mammal cytotoxicity

The 3-trifluoromethylquinoxaline *N,N'*-dioxides were selected according to their anti-*T. cruzi* and leishmanicidal activity and trying to cover a wide range of structural characteristics. The ID_{50} values for the studied compounds are shown in Table 4. The selectivity indexes, SI, were expressed as the ratio between ID_{50} in macrophages and ID_{50} in *T. cruzi* (Tulahuen 2 strain,

Table 2).[11]

Mutagenicity assay. The method of direct incubation in plate[12] using culture of *Salmonella typhimurium* TA98 strain was performed on derivative **21** and Nfx. The influence of metabolic activation was tested by adding S9 fraction of mouse liver. Positive controls of 4-nitro-*o*-phenylendiamine and 2-aminofluorene were run in parallel. The revertant number was manually counted and compared to the natural revertant (Table 5). The compound is considered mutagenic when the number of revertant colonies is at least 2-fold of the spontaneous revertant frequencies for at least two consecutive dose levels.[13] The maximum assayed doses were determined according to toxic effect on *S. typhimurium.*

Table 4. Biological Characterization of Quinoxaline *N,N'*-Dioxides Against Mammal Macrophages.



Cpd.	-R	-R^6 -R^7	ID$_{50}$ (nM)[a]	SI[b]
21	-Ph	-F -F	8000	11.4
44	-CH_3		10600	331.2
49	-*i*-Prop		11450	<0.5
50	-*t*-Bu	———	16250	<0.6
51			8700	<0.3
52	-CH_3	-H -H	8550	2.8
53	-1-naphthyl		7920	5.5
56	-Ph-*p*-Cl		8500	5.3
57	-2-furyl		7130	3.0
62	-2-thienyl		4690	4.3
63	-4-Ph-1-piperazinyl		9510	12.5
64	-4-(Ph-*p*-Cl)-1-piperazinyl		8250	10.4

[a] The results are the means of three independent experiments with a SD less than 10% in all cases. [b] SI: selectivity index, $ID_{50,macrophage}/ID_{50,T.\ cruzi}$ (Tulahuen 2)

Table 5. Number of Revertants of Derivative **21** and Nfx on TA98 *S. typhimurium* Strain.

	21			Nfx		
	D[a]	**NR[b,c]**	**M[d]**	**D[a]**	**NR[b,c]**	**M[d]**
	0.0	14±6		0.0	21±4	
	0.005	10±6		0.5	29±6	
	0.05	22±8		1.0	43±17	
-S9	0.5	22±1		3.0	62±2	
	1.5	20±4		10.0	144±11	(+)[e]
	5.0	27±4		30.0	117±17	
	15.0	35±14				
	0.0	19±4		0.0	31± 10	
	0.005	18±1		0.5	37± 5	
	0.05	20±4		1.0	39± 18	
+S9	0.5	15±1		3.0	53± 9	
	1.5	20±2		10.0	64± 6	
	5.0	20±4		30.0	139±11	(+)
	15.0	21±4				

	4-NPD [f]			AF [g]	
	D[c]	**NR[d,e]**		**D[c]**	**NR[d,e]**
-S9	20.0	1223±237	**+S9**	10.0	801±82

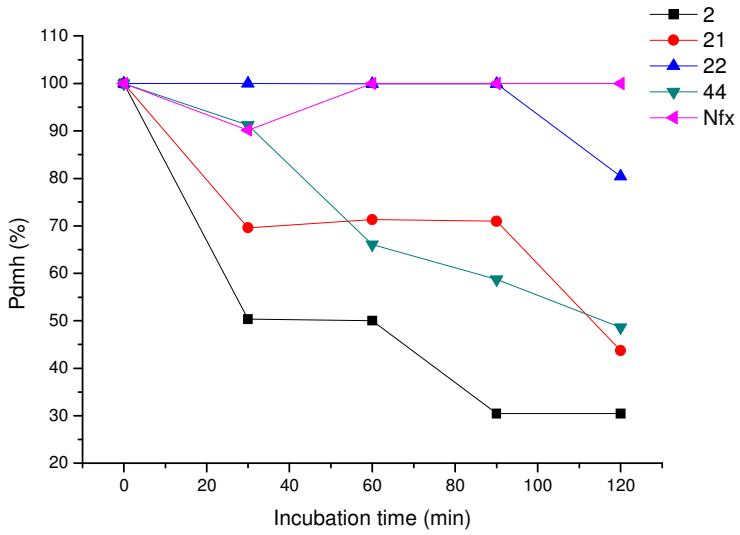
[a] D: doses in μg/plate. [b] NR: number of revertants. [e] The results are the means of two independent experiments. [d] M: mutagenicity, according to reference [13] (see text). [e] (+): Response is considered positive because it is the second dose in which the revertant levels are at least twice the spontaneous frequencies. [f] 4-NPD: 4-nitro-*o*-phenylendiamine. [g] AF: 2-aminofluorene

2.3. Studying the mechanism of action

It is well known that some quinoxaline *N*,*N*'-dioxides are species which suffer a bioreductive process in hypoxic conditions to produce ·OH and quinoxaline.[14] This led us to believe that these quinoxalines could produce parasitic damage through the production of radical species affecting the redox metabolism. Previously, we have studied a possible mechanism of action of *N*-oxide containing heterocycles from a theoretical point of view. We found that a bioreductive process could be involved.[5a,5c,15] In an attempt to investigate the mode in which these quinoxaline *N*,*N*'-dioxides act on parasites, we studied their effect on the mitochondrial dehydrogenase activities. We recently demonstrated that these enzymes could be involved in the mechanism of action of *N*-oxides containing heterocycles, such as furoxans and benzofuroxans.[16] The percentage of mitochondrial dehydrogenase activities (Pmdh) with respect

to untreated control was assessed using the colorimetric MTT assay performed at very short times, no more than 240 min of incubation, procedure described for *Leishmania* parasite.[17] We compared the changes of the mitochondrial dehydrogenase activities of four active agents, parent compound **2**, and three 3-trifluoromethyl derivatives, called **21**, **22**, and **44**, together with the reference drug Nfx (Figure 2a). Nfx does not affect the mitochondrial dehydrogenase activities while the quinoxalines produce decrease in a time-dependent manner.

a)



b)

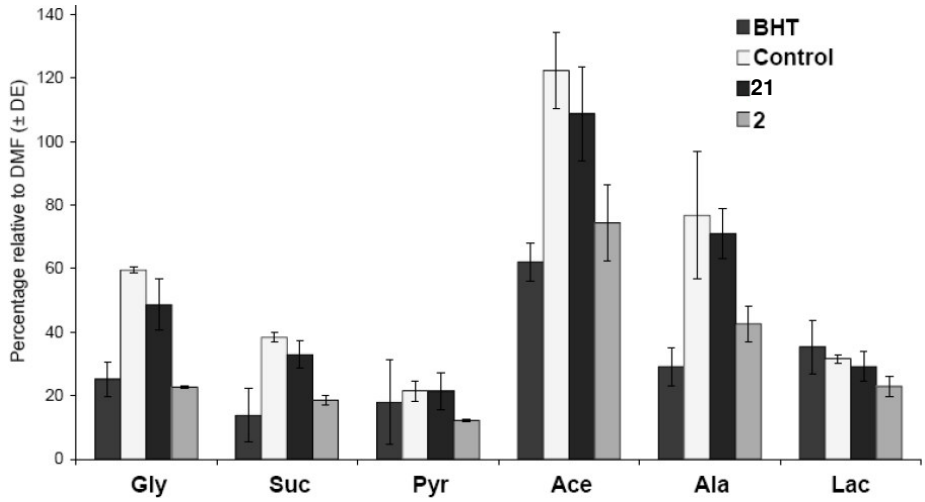


Figure 2. a) Variation of the percentage of mitochondrial dehydrogenase activities (Pmdh), produced by the compounds with respect to time compared to the untreated control of *T. cruzi* epimastigote, Y strain (for details see Experimental Section). **b)** Percentage of the end-products

excreted to the medium in the different treatment, expressed respect to DMF[19], by *T. cruzi* epimastigote, Y strain (for details see Experimental Section

Finally, in order to study the changes in the biochemical pathways promoted by two of our active quinoxalines, we have studied the modifications in the excreted metabolites by ^{1}H NMR spectroscopy. This type of studies has been proven to be a useful tool in mechanism of action elucidation.[16a,18] We compared the spectra of the cell-free medium of quinoxaline-treated parasites with those of the untreated *T. cruzi*-free medium as control. We have mainly focused on the changes of the excreted salts of the carboxylic acids, lactate (Lac), acetate (Ace), pyruvate (Pyr), and succinate (Suc) and the aminoacids, alanine (Ala) and glycine (Gly), being the most relevant modified metabolites. Figure 2b shows the changes in the excreted end-products without or after treatment with the studied compounds, quinoxalines with marked effects on the mitochondrial dehydrogenase since the beginning of the incubation, i.e. **2**, and **21**.

2.4. Preliminary *in vivo* anti-*T. cruzi* studies

We evaluated derivative **21** *in vivo* in a murine model of acute Chagas' disease. Bnz was used as the *in vivo* active reference drug. In one of the preliminary studies, male mice were infected with CL Brener-trypomastigotes and treatment began 10 days post-infection with oral administration of each compound (10 mg/kg b.w./day for **21** and 50 mg/kg b.w./day for Bnz) during 10 days.[20] In the second experiment, the only difference was that the animals were infected with Y-trypomastigotes. Three different parameters were used to evaluate the *in vivo* activity, weekly parasitaemia (Figure 3), weekly animal survival percentages, and anti-*T. cruzi* antibody levels at 30 and 60 days post infection. In both experiments, all the animals treated with derivative **21** survived at the end of the experiment. For CL Brener-experiments, the blood-study findings showed that compound **21**, at a dose five times lower than that used for Bnz, exhibited a particular biological profile, shifting the parasitaemia maximum 10 days with respect to the

control, and lacking the second parasitaemia maximum, evidenced in un- and Bnz-treated animals at day 53 of the assay. On the analysis days, the number of trypomastigotes on **21**-treated animals resulted lower than the parasites-number in untreated mice. No differences were observed in the antibody levels, at day 30 and 60 post infection, between the three studied groups. For Y-experiments, the blood-study findings showed that the number of trypomastigotes in **21**-treated animals was lower than the parasites-number in untreated mice for both doses, in a non-doses dependence. On the other hand, both doses of **21** avoided the second parasitaemia maximum, evidenced in Bnz-treated animals at day 55 of the assay. No differences were observed regarding the antibody levels, at day 30 and 60 post infection, between the three studied groups.

a) **b)**

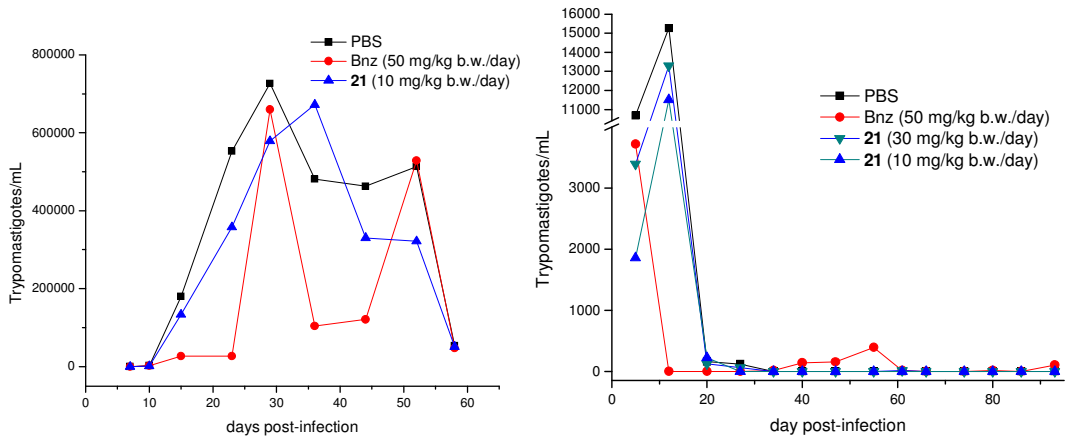


Figure 3. Parasitemia of mice treated with 50 mg/kg b.w./day of Bnz (•), group treated with 10 mg/kg b.w./day of **21** (▯),group treated with 30 mg/kg b.w./day of **21** (▯), and control group (▯). Animals infected with **a)** CL Brener, or **b)** Y strain.

Discussion

We reported the biological activity of nearly seventy quinoxaline derivatives against the epimastigote form of three different strains and one clone of *T. cruzi* and the promastigote form of one strain of *L. braziliensis*. Derivatives **9**, **10**, **20-25**, **27-29**, **44-48**, **50**, and **52-68** were the

most active ones against Tulahuen 2 strain (Table 1) with **9**, **21**, **22**, **44-46**, and **62-64** being at least 10 times more active than the reference drug and the parent compounds, Nfx and **1** and **2**, respectively (Table 2). The activity profiles against the other studied clone and strains, CL Brener, Y and Colombiana, were similar to Tulahuen 2 activities, identifying derivatives **21** and **22** with bio-responses similar or higher than the reference drug, Nfx, and parent compound **2** (Table 3). Parent compound **2**, and the recently studied quinoxalines **21**, **22**, and **28** showed excellent *in vitro* activities against Nfx- and Bnz-partially resistant strains, Y and Colombiana strains. The same profiles were observed in the study with *L. brasiliensis*, LTB300 strain, with compounds **2**, **21**, **22**, and **28** being at least 3 times more active than the reference drug, Mtf (Table 3). Concomitantly, derivative **21** was not mutagenic in the Ames test, making it an excellent lead to further studies.

Optimal anti-*T. cruzi* quinoxaline *N,N'*-dioxides were identified from a structural point of view (Figure 4). Electrophilic character changes in position 2 of parent compound **1** (Figure 1), producing compounds with unmodifiable or decreasing activity (derivatives **5** and **16**). Derivatives **16-19**, without an electrophilic substituent in position 2, were completely inactive at the assayed doses (Table 1), showing the significance of an electron-withdrawing center in position 2. However, better electron-withdrawing substituents in position 6 and 7 (i.e. 6,7-difluoro in derivative **10)**, increased the activity. Clearly, the electronic character of 6- and 7-substituents modify the activities of the quinoxaline *N,N'*-dioxides (compare activity of parent compound **1** to activities of derivatives **10-15**, Table 1). When a new electron-withdrawing substituent was included, the biological behavior was better (Compound **21** vs. **10**) . Finally, in the 3-CF_3-substituted derivatives, **20-26**, no relevant effects of the electronic character of 6- and 7-substituents were observed. However, the lack of *N*-oxide moieties, i.e. derivative **71**, produced less active derivatives. Other electrophilic centers different from –COPh, i.e. –$COCH_3$, -$COCH_2CH_3$, –CO-*i*-Prop, –CO-*t*-But, –CO-1-naphthyl, –COPh-*p*-Cl, –CO-2-furyl, –

CO-2-thienyl, or –CONRR' (derivatives **5**, **9**, **44-48**, **50**, or **52-68**) also produced excellent active derivatives. The optimal activity was found in derivative **44** (Fig. 4) which contained all the structural exigencies observed for the rest of the derivatives. Compound **44**, the best anti-Tulahuen 2 of the quinoxaline *N,N'*-dioxides studied, has a good electrophilic center in position 2 and three excellent electron-withdrawing moieties in position 3, 6 and 7.

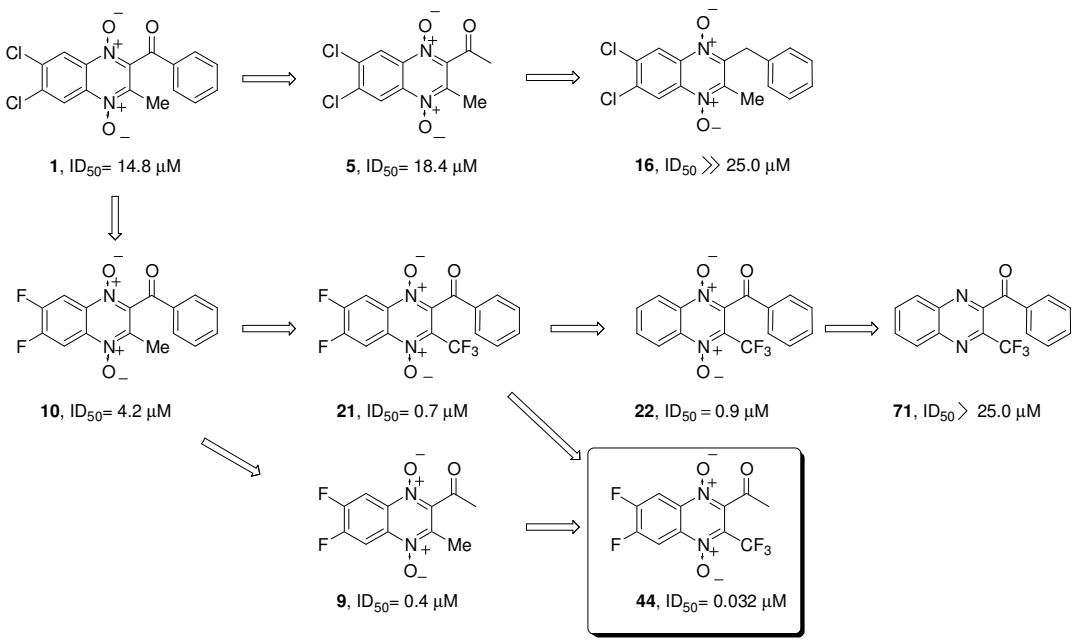


Figure 4. Structural-anti-*T. cruzi* activity for the studied quinoxalines. The ID_{50} refer to the Tulahuen 2 strain.

With regard to the mechanism of action studies, the studied quinoxalines, parent compounds **2** and **21**, **22**, **44**, decreased mitochondrial dehydrogenases activity, unlike what occurs with the untreated or Nfx-treated parasite (Fig. 2a). In general, parent compound **2** promoted greater diminishing of the studied end-metabolite concentrations than those for quinoxaline **21**-treated parasites. The lower levels of excreted Ace and Suc in the case of parasites treated with compound **2** compared to the excreted levels obtained with compound **21** could be due to the fact that **2** is a better mitochondrial dehydrogenases inhibitor (Fig. 2a). The following three facts

prove this statement[21]: 1) Mitochondrial Suc-dehydrogenase and fumarate reductase are very homologous enzyme complexes so most inhibitors of Suc-dehydrogenase could also act on fumarate reductase; 2) The *T. cruzi* stages with an active Krebs cycle, such as epimastigotes, produce Suc, implying that fumarate reduction has to occur at the same time as Suc oxidation; 3) Ace is the end-product of Pyr oxidative decarboxylation by mitochondrial Pyr-dehydrogenase.

The *in vivo*-analyzed 3-trifluoromethylquinoxaline dioxide derivative, **21**, displayed relevant behavior that should be thoroughly studied, modifying doses, administration routes, and combinations with other drugs.

Conclusions

In conclusion, we have identified quinoxaline *N,N'*-dioxides as excellent anti-trypanosomatid agents. In addition, we were able to establish that mitochondria are affected when these derivatives are used. Observing the lack of mutagenic properties of derivative **21**, the previously described lower acute systemic toxicity in Wistar rats,[22] and the preliminary *in vivo* anti-trypanosoma results, this compound could be proposed as a drug candidate. Additional and more thorough *in vivo* studies are currently being performed on animal models of Chagas disease and Leishmaniosis.

Experimental

Chemistry

All starting materials were purchased from Panreac Química S.A. (Barcelona, Spain), Sigma-Aldrich Química, S.A. (Alcobendas, Spain), Acros Organics (Janssen Pharmaceutical, Geel, Belgium) and Lancaster (Bischheim-Strasbourg, France). All solvents were dried and distilled prior to use. All the reactions were carried out in a nitrogen atmosphere. All of the synthesized compounds were chemically characterized by thin layer chromatography (TLC), infrared (IR), proton nuclear magnetic resonance (^{1}H-NMR) and elemental microanalyses (CHN). Alugram

SIL G/UV254 (Layer: 0.2 mm) (Macherey-Nagel GmbH & Co. KG., Düren, Germany) was used for TLC and Silica gel 60 (0.040-0.063 mm, Merck) was used for Flash Column Chromatography. The 1H-NMR spectra were recorded on a Bruker 400 Ultrashield instrument (400 MHz), using TMS as the internal standard and with $CDCl_3$-d_6 as the solvent; the chemical shifts are reported in ppm (δ) and coupling constants (J) values are given in Hertz (Hz). Signal multiplicities are represented by: s (singlet), d (doublet), dd (double doblet), t (triplet), tt (triple triplet) and m (multiplet). The IR spectra were recorded on a Nicolet Nexus FTIR (Thermo, Madison, USA) in KBr pellets. To determine the purity of the compounds, elemental microanalyses obtained on a CHN-900 Elemental Analyzer (Leco, Tres Cantos, Spain) from vacuum-dried samples were used. The analytical results for C, H and N, were within ± 0.4 of the theoretical values. Compounds **3-54, 57-62, 71** and **72** were prepared following synthetic procedures previously reported.[4,6.] Compounds **55**, **56** and **63-70**, were prepared as described below.

General procedure for the synthesis of compounds 55 and 56

1-(4-Chlorophenyl)-4,4,4-trifluoromethyl-1,3-butanedione (4.35 mmol) was added to a solution of the appropriate benzofuroxan (2.90 mmol) in toluene (20 mL) in a microwave vessel. The mixture was cooled and triethylamine was added dropwise (1.5 mL). The solution was stirred at room temperature for 30 minutes and then it was put in the microwave reactor. The mixture was then subjected to microwave irradiation at 70 W for 45 minutes, keeping temperature at 70-80 ºC. After an important conversion as indicated by TLC, the reaction mixture was cooled and the solvent was eliminated *in vacuo*. Brown oil was obtained and purified by column chromatography on silica gel, eluting with dichloromethane. The corresponding fractions were evaporated to dryness *in vacuo* and yellow oil obtained was precipitated by adding diethyl ether and filtered off.

2-(4-Chlorobenzoyl)-6,7-difluoro-3-(trifluoromethyl)quinoxaline 1,4-dioxide, ***55***. Yield: 8.6%. ^{1}H NMR (400 MHz, $CDCl_3$-d_6) δppm: 8.51 (dd, 1H, H_8, $J_{8\text{-FC6}}$ = 7.2 Hz, $J_{8\text{-FC7}}$ = 9.1 Hz), 8.39 (dd, 1H, H_5, $J_{5\text{-FC7}}$ = 7.1 Hz, $J_{5\text{-FC6}}$ = 9.0 Hz); 7.83 (d, 2H, $H_{2'}$+$H_{6'}$, $J_{2'\text{-}3'}$ = $J_{6'\text{-}5'}$= 8.6 Hz); 7.55 (d, 2H, $H_{3'}$+$H_{5'}$, $J_{3'\text{-}2'}$ = $J_{5'\text{-}6'}$= 8.6 Hz). IR (KBr): 3065 (w, νC-H Ar), 1698 (s, νC=O), 1353 (s, ν*N*-oxide), 1176 (s, νC-F), 910 (w, νAr-Cl) cm^{-1}. Calculated analysis for $C_{16}H_6ClF_5N_2O_3$: C: 47.46; H: 1.48; N: 6.92. Found: C: 47.50; H:1.71; N: 6.72.

2-(4-Chlorobenzoyl)-3-(trifluoromethyl)quinoxaline 1,4-dioxide, ***56***. Yield: 6%. ^{1}H NMR (400 MHz, $CDCl_3$-d_6) δppm: 8.73-8.70 (m, 1H, H_8); 8.61-8.58 (m, 1 H, H_5); 8.04-8.02 (m, 2H, H_6+H_7); 7.86 (d, 2H, $H_{2'}$+$H_{6'}$, $J_{2'\text{-}3'}$ = $J_{6'\text{-}5'}$= 8.7 Hz); 7.54 (d, 2H, $H_{3'}$+$H_{5'}$, $J_{3'\text{-}2'}$ = $J_{5'\text{-}6'}$ = 8.7 Hz). IR (KBr): 3100 (w, νC-H Ar), 1689 (s, νC=O), 1351 (s, ν*N-oxide*), 1162 (s, νC-F), 909 (w, νAr-Cl) cm^{-1}. Calculated analysis for $C_{16}H_8ClF_3N_2O_3$: C: 52.10; H: 2.17; N: 7.60. Found: C: 51.78; H:2.08; N: 7.39.

General procedure for the synthesis of compounds 63 and 64

The intermediates were prepared according to the synthetic procedure for similar compounds *N*-(3-oxobutyryl)piperazine[4d]. The trifluoromethyl acetoacetate was heated with the corresponding piperazine in the presence of 2-hydroxypyridine as catalyst in a bath at 169ºC for 5 h, under nitrogen atmosphere. The partly cooled mixture was then stirred into hot water. The resulting suspension was extracted with dichloromethane. The organic solvent was eliminated under pressure and a solid was obtained. The corresponding piperazinyl-amide without purification (5.7 mmol) was added to a solution of the appropriate benzofuroxan (2.8 mmol) in toluene (20 mL) in a microwave vessel. The mixture was cooled and triethylamine was added dropwise (1.5 mL). The solution was stirred at room temperature for 10 minutes and then it was put in the microwave reactor. The mixture was then subjected to microwave irradiation at 50 W for 25-35 minutes keeping temperature at 90ºC. After an important conversion as indicated by TLC, the reaction mixture was cooled and the solvent was eliminated *in vacuo*. A brown oil was obtained

and it was purified by column chromatography on silica gel, eluting with toluene:dioxane (9:1). The corresponding fractions evaporating to dryness *in vacuo* and yellow oil obtained was precipitated by adding diethyl ether and filtered off.

2-(4-Phenylpiperazine-1-carbonyl)-3-(trifluoromethyl)quinoxaline 1,4-dioxide, ***63***. Yield: 7%. ^{1}H NMR (400 MHz, $CDCl_3$-d_6) δppm: 8.68-8.66 (m, 1H, H_8); 8.64-8.62 (m, 1H, H_5); 8.04-7.96 (m, 2H, H_6+H_7), 7.32 (dd, 2H, $H_{3'}$+$H_{5'}$, $J_{3'-4'} = J_{5'-4'} = 7.1$ Hz, $J_{3'-2'} = J_{5'-6'} = 8.9$ Hz); 6.98-6.95 (m, 3H, $H_{2'}$+$H_{4'}$+$H_{6'}$); 4.04-4.00 (m, 2H, $CH_{2\text{-piperazine}}$); 3.60-3.15 (m, 6H, $3CH_{2\text{-piperazine}}$). IR (KBr): 3453 (w, νC-H Ar), 1655 (s, νC=O), 1444 (m, νC-N amida), 1356 (s, ν*N*-oxide), 1236 (m, νC-N Ar-amine), 1153 (m, νC-F) cm^{-1}. Calculated analysis for $C_{20}H_{17}F_3N_4O_3$: C: 57.41; H: 4.06; N: 13.39. Found: C: 57.46; H:4.11; N: 13.36.

2-[4-(4-Chlorophenyl)piperazine-1-carbonyl]-3-(trifluoromethyl)quinoxaline 1,4-dioxide, ***64***. Yield: 6%. ^{1}H NMR (400 MHz, $CDCl_3$-d_6) δppm: 8.63 (dd, 1H, H_8, $J_{8-6} = 1.7$ Hz, $J_{8-7} = 8.1$ Hz); 8.68 (dd, 1H, H_5, $J_{5-7} = 1.4$ Hz, $J_{5-6} = 8.1$Hz); 8.04-7.97 (m, 2H, H_6+H_7); 7.26 (d, 2H, $H_{2'}$+$H_{6'}$, $J_{2'-3'} = J_{6'-5'} = 8.6$ Hz); 6.88 (d, 2H, $H_{3'}$+$H_{5'}$, $J_{3'-2'} = J_{5'-6'} = 8.6$ Hz); 4.09-3.12 (m, 8H, 4 $CH_{2\text{-piperazine}}$). IR (KBr): 3440 (w, νC-H Ar), 1654 (s, νC=O), 1498 (m, νC-N amida), 1357 (s, ν*N*-oxide), 1236 (m, νC-N Ar-amine), 1157(m, νC-F) cm^{-1}. Calculated analysis for $C_{20}H_{16}ClF_3N_4O_3$: C: 53.05; H: 3.53; N: 12.37. Found: C: 52.90; H:3.4; N: 12.22.

General procedure for the synthesis of compounds 65-70

1-Phenyl-4,4-difluoro-1,3-butanedione (5.05 mmol) was added to a solution of appropriate benzofuroxan (5.05 mmol) and KF (in alumina 40% supported) in acetone (40 mL). The mixture was stirred at room temperature in darkness for 14-24 hours. The mixture was filtered to eliminate the KF in alumina. The organic phase was extracted with dichloromethane/water. The organic phase was dried with anhydrous Na_2SO_4 and filtered. The solvent was removed *in vacuo* and the resulting oil was precipitated by adding *n*-hexane (20 mL) and methanol (5 mL).The obtained yellow precipitate was washed with diethyl ether.

2-Benzoyl-3-(difluoromethyl)-6,7-dichloroquinoxaline 1,4-dioxide, **65**. Yield: 52%. ^{1}H NMR (400 MHz, $CDCl_3$-d_6) δppm: 8.78 (s, 1H, H_8); 8.70 (s, 1H, H_5); 7.89 (dd, 2H, $H_{2'}$+$H_{6'}$, $J_{2'-4'} = J_{6'-4'}$ = 1.2 Hz, $J_{2'-3'} = J_{6'-5'}$ = 8.3 Hz); 7.70 (dt, 1H, $H_{4'}$, $J_{4'-2'} = J_{4'-6'}$ = 1.2 Hz, $J_{4'-3'} = J_{4'-5'}$ = 7.2 Hz); 7.55 (dt, 2H, H_3+$H_{5'}$, $J_{3'-5'}$ = 1.6 Hz, $J_{3'-2'} = J_{5'-6'}$ = 8.1 Hz); 7.30 (t, 1H, CHF_2, J_{H-F} = 52.2 Hz). IR (KBr): 3090 (w, νC-H Ar), 1681 (s, νC=O), 1324 (s, ν*N*-oxide), 1051(m, νC-F) cm^{-1}. Calculated analysis for $C_{16}H_8Cl_2F_2N_2O_3$: C: 49.87; H: 2.08; N: 7.27. Found: C: 49.94; H:2.13; N: 6.98.

2-Benzoyl-3-(difluoromethyl)-7-chloroquinoxaline 1,4-dioxide, **66**. Yield: 44%. ^{1}H NMR (400 MHz, $CDCl_3$-d_6) δppm: 8.64 (d, 1H, H_5, J_{5-6} = 9.2 Hz); 8.60 (d, 1H, H_8, J_{6-8} = 2.1 Hz); 7.93-7.90 (m, 2H, $H_{2'}$+$H_{6'}$); 7.91 (dd, 1H, H_6, J_{6-8} = 2.1 Hz, J_{6-5} = 9.1 Hz); 7.70 (dt, 1H, $H_{4'}$, $J_{4'-2'}$ = 1.2 Hz, $J_{4'-3'} = J_{4'-5'}$ = 7.2 Hz); 7.55 (dt, 2H, H_3+$H_{5'}$, $J_{3'-5'}$ = 0.5 Hz, $J_{3'-4} = J_{5'-4'}$ = 7.7 Hz, $J_{3'-2'}$ = $J_{5'-6'}$ = 7.9 Hz); 7.32 (t, 1H, CHF_2, J_{H-F} = 52.2 Hz). IR (KBr): 3083 (w, νC-H Ar), 1679 (s, νC=O), 1333 (s, ν*N*-oxide), 1045(m, νC-F) cm^{-1}. Calculated analysis for $C_{16}H_9ClF_2N_2O_3$: C, 54.78; H, 2.57; N, 7.99. Found: C, 54.54; H, 2.83; N, 7.96.

2-Benzoyl-3-(difluoromethyl)-7-fluoroquinoxaline 1,4-dioxide, **67**. Yield: 8%. ^{1}H NMR (400 MHz, $CDCl_3$-d_6) δppm: 8.73 (dd, 1H, H_5, J_{5-FC7} = 4.9 Hz, J_{5-6} = 9.52 Hz); 8.26 (dd, 1H, H_8, J_{8-6} = 2.6 Hz, J_{8-FC7} = 8.1 Hz); 7.91 (dd, 2H, $H_{2'}$+$H_{6'}$, $J_{2'-4'} = J_{6'-4'}$ = 1.1 Hz, $J_{2'-3'} = J_{6'-5'}$ = 8.2 Hz); 7.73 (ddd, 1H, H_6, J_{6-8} = 2.8 Hz, J_{6-FC7} = 7.3 Hz, J_{6-5} = 9.9 Hz); 7.70 (dt, 1H, $H_{4'}$, $J_{4'-2'} = J_{4'-6'}$ = 0.8 Hz, $J_{4'-3'} = J_{4'-5'}$ = 7.3 Hz); 7.55 (dt, 2H, H_3+$H_{5'}$, $J_{3'-5'}$ = 1.0 Hz, $J_{3'-2'} = J_{5'-6'}$ = 7.7 Hz, $J_{3'-4'} = J_{5'-4'}$ =7.5 Hz); 7.33 (t, 1H, CHF_2, J_{H-F} = 52.2 Hz). IR (KBr): 3077 (w, νC-H Ar), 1675 (s, νC=O), 1338 (s, ν*N*-oxide), 1046(m, νC-F) cm^{-1}. Calculated analysis for $C_{16}H_9F_3N_2O_3$: C:57.48; H: 2.69; N:8.38. Found: C: 57.82; H:2.89; N: 8.18.

2-Benzoyl-3-(difluoromethyl)-7-methoxyquinoxaline 1,4-dioxide, **68**. Yield: 5%. ^{1}H NMR (400 MHz, $CDCl_3$-d_6) δppm: 8.58 (d, 1H, H_5, J_{5-6} = 9.5 Hz); 7.92 (dd, 2H, $H_{2'}$+$H_{6'}$, $J_{2'-4'} = J_{6'-4'}$ = 1.2 Hz, $J_{2'-3'} = J_{6'-5'}$ = 7.9 Hz), 7.89 (d, 1H, H_8, J_{8-6} = 2.6 Hz); 7.68 (dt, 1H, $H_{4'}$, $J_{4'-2'} = J_{4'-6'}$ = 1.2 Hz, $J_{4'-3'} = J_{4'-5'}$ = 7.0 Hz); 7.55 (dd, 1H, H_6, J_{6-8} = 2.2 Hz, J_{6-5} = 9.1 Hz); 7.53 (dd, 2H, $H_{3'}$+$H_{5'}$, $J_{3'-5'}$ =

0.6 Hz, $J_{3'-2'} = J_{5'-6'} = 7.2$ Hz, $J_{3'-4'} = J_{5'-4'} = 6.6$ Hz); 7.35 (t, 1H, CHF_2, J_{H-F} =52.4 Hz); 4.03 (s, 3H, CH_3O). IR (KBr): 3111 (w, C-H Ar), 1685 (s, C=O), 1343 (s, *N*-oxide), 1042(m, C-F) cm^{-1}. Calculated analysis for $C_{17}H_{12}F_2N_2O_4$: C: 58.95; H: 3.46; N: 8.09. Found: C: 58.78; H:3.50; N:7.96.

2-Benzoyl-3-(difluoromethyl)-7-methylquinoxaline 1,4-dioxide, **69**. Yield: 3%. ^{1}H NMR (400 MHz, $CDCl_3$-d_6) ppm: 8.58 (d, 1H, H_5, J_{5-6} = 8.8 Hz); 8.39 (d, 1H, H_8, J_{8-6} = 1.0 Hz); 7.91 (dd, 2H, $H_{2'}$+$H_{6'}$, $J_{2'-4'} = J_{6'-4'} = 1.2$ Hz, $J_{2'-3'} = J_{6'-5'} = 8.3$ Hz); 7.81 (dd, 1H, H_6, J_{6-8} = 1.46 Hz, J_{6-5} = 8.8 Hz); 7.68 (dt, 1H, $H_{4'}$, $J_{4'-2'} = J_{4'-6'} = 1.2$ Hz, $J_{4'-3'} = J_{4'-5'} = 7.2$ Hz); 7.54 (t, 2H, $H_{3'}$+$H_{5'}$, $J_{3'-2'} = J_{5'-6'} = 7.8$ Hz, $J_{3'-4'} = 7.4$ Hz); 7.35 (t, 1H, CHF_2, J_{H-F} = 52.4 Hz); 2.67 (s, 3H, CH_{3-C7}). IR (KBr): 3064 (w, C-H Ar), 1683 (s, C=O), 1340 (s, *N*-oxide), 1044(m, C-F) cm^{-1}. Calculated analysis for $C_{17}H_{12}F_2N_2O_3$: C:61.81; H: 3.63; N:8.48. Found: C: 61.65; H:4.00; N: 8.08.

2-Benzoyl-3-(difluoromethyl)quinoxaline 1,4-dioxide, **70**. Yield: 9%. ^{1}H NMR (400 MHz, $CDCl_3$-d_6) ppm: 8.70 (m, 1H, H_8); 8.62 (m, 1H, H_5); 8.00 (m, 2H, H_6+H_7); 7.92 (dd, 2H, $H_{2'}$+$H_{6'}$, $J_{2'-4'} = J_{6'-4'} = 1.2$ Hz, $J_{2'-3'} = J_{6'-5'} = 8.4$ Hz); 7.69 (dt, 1H, $H_{4'}$, $J_{4'-2'} = 1.2$ Hz, $J_{4'-3} = J_{4'-5'} = 7.2$ Hz); 7.54 (dt, 2H, $H_{3'}$+$H_{5'}$, $J_{3'-5'} = 1.5$ Hz, $J_{3'-4'} = J_{5'-4'} = 7.8$ Hz, $J_{3'-2'} = J_{5'-6'} = 7.7$ Hz); 7.36 (t, 1H, CHF_2, J_{H-F} = 52.3 Hz). IR (KBr): 3199 (w, C-H Ar), 1638 (s, C=O), 1340 (s, *N*-oxide), 1044 (m, C-F) cm^{-1}. Calculated analysis for $C_{16}H_{10}F_2N_2O_3$: C, 60.75; H, 3.16; N, 8.86. Found: C,60.75; H, 3.60; N, 8.52.

Biology

Anti-trypanosomatid *in vitro* evaluation

Anti-*T. cruzi in vitro* test using Tulahuen 2 strain. *Trypanosoma cruzi* epimastigotes (Tulahuen 2 strain) were grown at 28 ºC in an axenic medium (BHI-Tryptose) as previously described,[5,23] supplemented with 5% fetal bovine serum (FBS). Cells from a 10-day-old culture (stationary phase) were inoculated into 50 mL of fresh culture medium to give an initial

concentration of 1×10^6 cells/mL. Cell growth was followed by measuring the absorbance of the culture at 600 nm every day. Before inoculation, the media was supplemented with the indicated quantity of the drug from a stock solution in DMSO. The final concentration of DMSO in the culture media never exceeded 0.4%, and the control was run in the presence of 0.4% DMSO and in the absence of drugs. No effect on epimastigote growth was observed due to the presence of up to1% DMSO in the culture media. The percentage of inhibition (PGI) was calculated as follows: PGI (%) = {1 - [(Ap - A0p)/(Ac - A0c)]} × 100, where Ap = A_{600} of the culture containing the drug at day 5; A0p = A_{600} of the culture containing the drug just after addition of the inocula (day 0);Ac = A_{600} of the culture in the absence of drugs (control) at day 5; A0c = A_{600} in the absence of the drug at day 0. In order to determine ID_{50} values, 50% inhibitory concentrations, parasite growth was followed in the absence (control) and presence of increasing concentrations of the corresponding drug. At day 5, the absorbance of the culture was measured and related to the control. The ID_{50} value was taken as the concentration of drug needed to reduce the absorbance ratio to 50%.

Viability of CL Brener, Y or Colombiana strains of *T. cruzi* and LTB300 strain of *L. braziliensis*. *Trypanosoma cruzi* epimastigotes (CL Brener, Y or Colombiana strains) were grown as previously indicated for Tulahuen 2 strain. *Leishmania braziliensis* promatigotes (MHOM/BR/00/LTB300 strain) were grown at 28 ºC in an axenic-RPMI medium supplemented with 5% FBS as previously described.[23] Cell-culture plates consisting of 24 wells were filled at 1 mL/well with the corresponding strain of the corresponding parasite culture during its exponential growth in the corresponding medium. BHI-Tryptose medium was supplemented with 5% fetal bovine serum (FBS). Different doses of studied compounds dissolved in DMSO were added and maintained for 2 days. Afterwards, the cells were washed twice with PBS-glucose (5.5 mM) and resuspended in 200 µL of PBS-glucose. Then 100 µL of suspension were

incubated (28 ºC) with 0.4 mg/mL MTT (Sigma) for 4 h. Then, the lysis of cells was carried out with SDS-isoprapanol (1:5), and optical densities were measured at 610nm. Each concentration was assayed three times, and six growth controls were used in each test. Cytotoxicity percentages (PCyt (%)) were determined as follows: PCyt = [100 - (ODd - ODdm)/(ODc - ODcm)] × 100, where ODd is the mean of OD595 of wells with parasites and different concentrations of the compounds, ODdm is the mean of OD595 of wells with different compound concentrations in the medium, ODc is the growth control, and ODcm is the mean of OD595 of wells with medium only. Nfx and Mtf were used as reference drugs for *T. cruzi* and *L. braziliensis*, respectively. For this propose, Mtf. was dissolved in ethanol. The ID_{50} value was taken as the concentration of drug needed to reduce the absorbance ratio to 50%.

Mutagenicity assay. The method of direct incubation in plate was performed. Culture of *S. typhimurium* TA98 strain in the agar minimum glucose medium (AMG)—agar solution, Vogel Bonner E(VB) 50×, and 40% glucose solution—was used. First, the direct toxicity of the compounds under study against the bacteria was assayed. DMSO solutions of **22** and Nfx at different doses (starting at the highest doses without toxic effects, 15.0 and 30.0 μg/plate, respectively) were assayed (¿hay una palabra major que "used"? 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 of DMSO were run in parallel. The influence of metabolic activation was tested by adding 500 μL of 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.

Studying the mechanism of action

Mitochondrial dehydrogenase activities. Mitochondrial dehydrogenase activities were measured in 24-well plates. One million of *T. cruzi* epimastigotes (Y strain) in 500 µL medium were seeded in each well and 20 µM of studied compounds were added. Two wells with untreated parasites were maintained as controls corresponding to the given time of treatment. The cultures were incubated at 28 ºC. At the different incubation times, the epimastigotes were counted and the colorimetric MTT dye-reduction assay was performed, with the tetrazolium salt being converted into purple formazan by living mitochondria. Fifty µL of a solution containing 5 mg/mL of MTT in PBS were added to each well and plates were incubated for an additional 4 h. The reaction was stopped by the addition of 500 µL of acidic isopropanol (0.4 mL HCl 10 *N* in 100 mL isopropanol). The absorbance was measured at 570 nm. Under our conditions, compounds did not interfere with the reaction mixture. Percentage of mitochondrial dehydrogenase activities (Pmdh (%)) were determined using untreated parasites-activities as 100 %.

^{1}H NMR study of the excreted metabolites. For the spectroscopic studies, 5 mL of a 2-day-treated *T. cruzi* (Y strain) were centrifuged with each studied compound (5 µM) at 1500g for 10 min at 4 ºC. The pellet was discarded, and the parasite-free supernatant was stored at -20 ºC until use. Before measuring, approximately 0.1 mL of DMF (10 mM) as internal standard and 0.1 mL of D_2O, were added to 0.3 mL of the supernatant. The spectra were registered with water suppression in 5 mm NMR sample tubes. The chemical displacements used to identify the respective metabolites were confirmed by adding each analyzed metabolite to the studied supernatant as well as by the study of a control solution with 4 µg/mL of each metabolite in buffer phosphate, pH = 7.4.

In Vivo Anti-*T. cruzi* Activity (Acute Model)

BALB/c male mice (30 days old, 25-30 g) bred under specific pathogen-free (SPF) conditions, were infected by intraperitoneal injection of 10^3 blood trypomastigotes (CL Brener and Y). One group of 10 animals was used as control (PBS), and three groups of eight animals were treated with the two compounds and Bnz, respectively. First parasitaemia was carried out 5 days post-infection (week 1) and the treatment was begun 7 days later. Compounds were administered orally, as aqueous solution in PBS, at 10 mg/kg/day for quinoxaline derivative **21** and 50 mg/kg/day for Bnz, during 10 days. Parasitaemia in the control and treated mice was determined once a week after the first administration, for 60 days, in tail-vein blood; the mortality rate was recorded. All the sera obtained after centrifugation of the blood that was extracted from infected mice were tested twice by ELISA (enzyme linked immuno assay) at 30 and 60 days post-infection. A locally produced ELISA kit (Chagas test, IICS, Asunción, Paraguay) was used following the procedure recommended by the manufacturer (IICS Production Department, Asunción-Paraguay).[24] The optical density values were obtained in an ELISA plate reader (Titerek Unistan I). Wilconxon test was used in order to compare the levels of anti-*T. cruzi* antibodies between experimental groups. The experimental protocols with animals were evaluated and supervised by the local Ethics Committee and the research adhered to the Principles of Laboratory Animal Care.[25]

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