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3-Trifluoromethylquinoxaline *N*,*N*′-Dioxides as Anti-Trypanosomatid Agents. Identification of Optimal Anti-*T. cruzi* Agents and Mechanism of Action Studies

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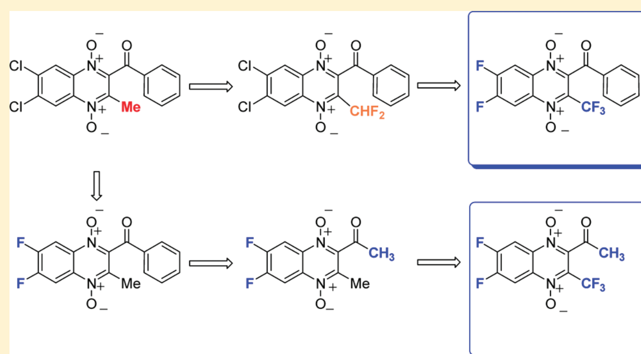
ABSTRACT: For a fourth approach of quinoxaline *N*,*N*′-dioxides as anti-trypanosomatid agents against *T. cruzi* and *Leishmania*, we found extremely active derivatives. The present study allows us to state the correct requirements for obtaining optimal in vitro anti-*T. cruzi* activity. Derivatives possessing electron-withdrawing substituents in the 2-, 3-, 6-, and 7-positions were the most active compounds. With regard to these features and taking into 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 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.



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 characteristics regarding drug treatment, include Chagas' disease (*Trypanosoma cruzi*) and leishmaniasis (*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) and benznidazole (Bnz), introduced empirically over 3 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 leishmaniasis are sodium stibogluconate, meglumine antimoniate, 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 because 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 deoxygenated quinoxaline analogues were studied to establish the importance of the *N*-oxide moiety. 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

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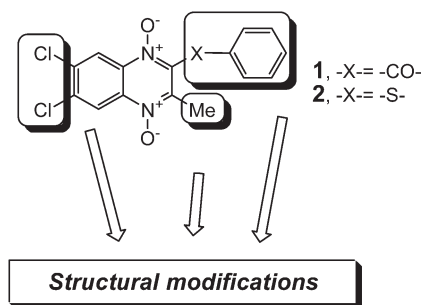


Figure 1. Quinoxaline *N,N′*-dioxide derivatives previously described as *T. cruzi* growth inhibitors.[5a]

effects on the mitochondrion dehydrogenase activity promoted by quinoxaline *N,N′*-dioxide derivatives were studied. The most promising quinoxalines were studied for their mutagenicities and in vivo activity on an acute murine model of Chagas disease.

METHODS AND RESULTS

Selected Compounds and Synthesis. Ten families of quinoxaline *N,N′*-dioxides were selected in order to analyze their in vitro anti-*T. cruzi* activity (Table 1). Derivatives **3−8**, 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, electronic behaviors, and polarities. In the second family, made up of derivatives **9−15**, 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.

The third group of derivatives (**16−19**) 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 the $-CF_3$ moiety, yielding derivative **20**, and when 6- and 7-substituents were concomitantly modified, the resulting derivatives were **21−26**. Other modifications involved the inclusion of a 3-Ph group as voluminous moiety in this position, a nonvoluminous electrophilic moiety in position 2, $-CO_2Et$ group, and different electronic behavior substitutions in the 6- and/or 7-benzo cycle (derivatives **27−33**). In the sixth and seventh families of compounds we modified derivatives **3−8** in the benzo substitutions, yielding 6,7-dimethyl derivatives (**34−39**) and benzo-nonsubstituted analogues (**40−43**). The $-CF_3$ substitution in the 3-position, i.e., derivatives **20−26**, resulted in very active compounds. From here we designed the eighth group of studied compounds (**44−64**), maintaining the $-CF_3$ at the 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**). Finally, the last group made up of quinoxalines **71** and **72**, 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. Only compounds with analytical results for C, H, and N within ±0.4 of the theoretical values were considered pure enough.

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**, 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).

Additionally, quinoxaline derivatives **21** and **44** were tested in vitro against the bloodstream trypomastigote form of *T. cruzi*, CL Brener clone. The compounds were incorporated into the biological media at 250 μg/mL and their ability to inhibit growth of the parasite was evaluated, by microscopy, in comparison to that of the control (no drug added to the media) after 24 h of incubation. Gentian violet (GV) was used as the trypanosomicidal reference drug (Table 4).

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 the 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.

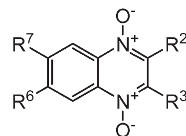
In Vitro Toxicity Studies. To explore the potential of these quinoxaline *N,N′*-dioxides as drugs, we performed two different studies. First, we evaluated their in vitro unspecific mammal cytotoxicity, using J-774 mouse macrophages. Second, we studied the mutagenicity capacity using the Ames test.

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 5. The selectivity indexes, SI, were expressed as the ratio of ID_{50} in macrophages to 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 derivatives **21** and **44** and its 3-methyl analogue, **9**, 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 6). 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 the toxic effect on *S. typhimurium*.

Studying the Mechanism of Action. It is well-known that some quinoxaline *N,N′*-dioxides are species that suffer a bioreductive process in hypoxic conditions to produce •OH and quinoxaline.[14] This led us to

Table 1. *T. cruzi* Antiproliferative Activity of Quinoxaline *N*,*N*′-Dioxide and Related Derivatives



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

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

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,c,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, a procedure described for *Leishmania* parasite.[17] We compared the changes of the mitochondrial dehydrogenase activities of four active agents, parent compound **2** and the three 3-trifluoromethyl derivatives **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.

Scheme 1. Synthetic Procedures Used To Prepare the New Derivatives

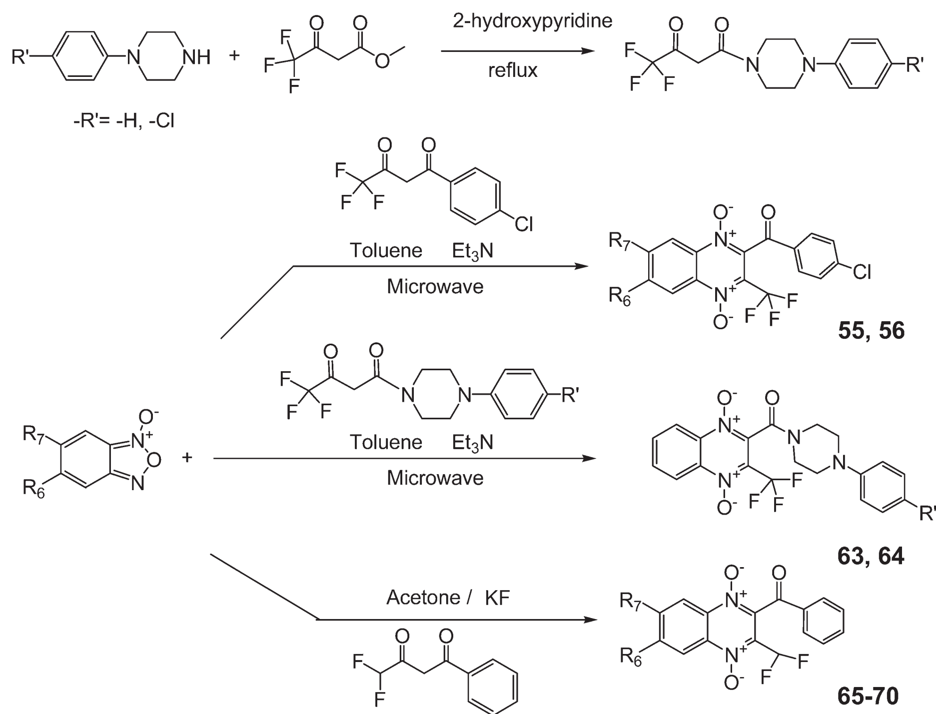


Table 2. ID_{50} **for Relevant Quinoxaline** ***N,N′*****-Dioxides and for Parent Compounds in Tulahuen 2 Strain**

compd	ID_{50} (μM)[a]	compd	ID_{50} (μM)[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	390	**65**	4700
45	500	**66**	6400
46	780	**67**	6300
47	1400	**68**	10000
Nfx	7700	**69**	25000

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

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 the 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 amino acids 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

Table 3. Percentage of Cytotoxicity and ID_{50} **for Relevant Quinoxaline** ***N,N′*****-Dioxides and for Parent Compound 2 against Different** ***T. cruzi*** **Strains and** ***L. brazilensis*** **LTB300 Strain**

	PCyt (%)[a,b]/ID_{50} (μM)				ID_{50} (μM)[b,d]
compd	CL Brener clone	Y strain	Colombiana strain	compd	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[c]	90.0/4.9	80.0/9.7	87.0/3.4	Mtf	9.0

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

Table 4. In Vitro Trypanosomicidal Activity (against Trypomastigote Form of CL Brener Clone)

compd	% lysis[a]
21	84 (63)[b]
44	80
GV	100

[a] % lysis = percentage of parasite lysis at 250 μg/mL compound. Results are the mean of three different experiments with a SD less than 10% in all cases. [b] Value in parentheses corresponds to the inhibition using 100 μg/mL compound.

effects on the mitochondrial dehydrogenase since the beginning of the incubation, i.e., **2** and **21**.

Preliminary in Vivo Anti-*T. cruzi* Studies. We evaluated derivatives **21** and **44** in vivo in murine models of acute Chagas' disease. Bnz was used as the in vivo active reference drug. In one of the preliminary studies (experiment 1), male Swiss albino mice were infected with CL Brener trypomastigotes and treatment began 10 days after infection with oral administration of each compound (10 or 50 (mg/kg bw)/day for **21** and 50 (mg/kg bw)/day for Bnz) during 10 days.[20] In the second experiment (experiment 2), the only differences were that the animals were infected with Y-trypomastigotes, compound **21** was administered at 10 or 30 (mg/kg bw)/day, and derivative **44** was included in the study at 10 (mg/kg bw)/day. In the third experiment (experiment 3), male BALB/c mice infected with CL Brener clone were used and derivatives **21** (10 (mg/kg bw)/day) and **44** (10 (mg/kg bw)/day) or Bnz (50 (mg/kg bw)/day) was administered orally. Three different parameters were used to evaluate the in vivo activity, weekly parasitemia (Figure 3), animal survival percentages (Figure 4a), and anti-*T. cruzi* antibody levels at 30 and 60 or 90 days after infection (Figure 4b).

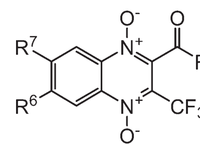
DISCUSSION

We reported the in vitro biological activity of nearly 70 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 bioresponses similar to or higher than those of the reference drug, Nfx, and parent compound **2** (Table 3).

Parent compound **2** and the new 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, derivatives **21** and **44** displayed significant activity against the circulating form of *T. cruzi*, trypomastigote form (Table 4).

Optimal anti-*T. cruzi* quinoxaline *N*,*N*′-dioxides were identified from a structural point of view (Figure 5). 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

Table 5. Biological Characterization of Quinoxaline *N*,*N*′-Dioxides against Mammal Macrophages



compd	-R	-R6	-R7	ID50 (nM)[a]	SI[b]
21	-Ph	-F	-F	8000	11.4
44	$-CH_3$	-F	-F	10600	27.2
49	-*i*-Pr	-F	-F	11450	<0.5
50	-*t*-Bu	-F	-F	16250	<0.6
51	-*t*-Bu	-H	-H	8700	<0.3
52	$-CH_3$	-H	-H	8550	2.8
53	-1-naphthyl	-H	-H	7920	5.5
56	-Ph-*p*-Cl	-H	-H	8500	5.3
57	-2-furyl	-H	-H	7130	3.0
62	-2-thienyl	-H	-H	4690	4.3
63	-4-Ph-1-piperazinyl	-H	-H	9510	12.5
64	-4-(Ph-*p*-Cl)-1-piperazinyl	-H	-H	8250	10.4
Nfx				316000	41.0

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

electron-withdrawing substituent in position 2, were completely inactive at the assayed doses (Table 1), showing the significance of this exigency at position 2 (compare activities of parent compound **1** and derivative **11** to activities of derivatives **16**, and **17**, respectively; Table 1). However, better electron-withdrawing substituents in positions 6 and 7 (i.e., 6,7-difluoro in derivative **10**, Figure 5), increased the activity. Clearly, in some cases the electronic character of 6- and 7-substituents also improve the activities of the quinoxaline *N*,*N*′-dioxides (i.e., 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 (compare **65** vs **1**, **21** vs **65**, or **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*-Pr, −CO-*t*-Bu, −CO-1-naphthyl, −COPh-*p*-Cl, −CO-2-furyl, −CO-2-thienyl, and −CONRR′ (derivatives **5**, **9**, **44**−**48**, **50**, and **52**−**68**), also produced good active derivatives.

The optimal activity was found in derivative **44** (Figure 5), 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 positions 3, 6, and 7.

Along with these findings, derivatives **21** and **44** were selective against the parasite (Table 5), with the methylketone **44** having the best selectivity index (macrophage/*T. cruzi*). Additionally, these compounds were not mutagenic in the Ames test (Table 6), making them excellent leads to further studies. Surprisingly, the 3-methyl analogue of compound **44**, derivative **9**, was mutagenic in this test, showing the significance of the 3-trifluoromethyl substituent in this property.

Table 6. Number of Revertants of Derivatives 21, 44, 9, and Nfx on TA98 *S. typhimurium* Strain

	21			44			9			Nfx		
	D[a]	NR[b,c]	M[d]	D[a]	NR[b,c]	M[d]	D[a]	NR[b,c]	M[d]	D[a]	NR[b,c]	M[d]
−S9	0.0	14 ± 6		0.0	12 ± 1		0.0	13 ± 3		0.0	21 ± 4	
−S9	0.005	10 ± 6		0.05	9 ± 1		0.2	29 ± 7		0.5	29 ± 6	
−S9	0.05	22 ± 8		0.15	9 ± 1		0.6	97 ± 10		1.0	43 ± 17	
−S9	0.5	22 ± 1		0.44	14 ± 3		1.7	245 ± 10	(+)[e]	3.0	62 ± 2	
−S9	1.5	20 ± 4		1.3	9 ± 1		5.0	348 ± 10		10.0	144 ± 11	(+)[e]
−S9	5.0	27 ± 4		4.0	18 ± 5		15.0	270 ± 4		30.0	117 ± 17	
−S9	15.0	35 ± 14										
+S9	0.0	19 ± 4		0.0	9 ± 2		0.0	12 ± 1		0.0	31 ± 10	
+S9	0.005	18 ± 1		0.05	12 ± 3		0.2	21 ± 3		0.5	37 ± 5	
+S9	0.05	20 ± 4		0.15	14 ± 1		0.6	34 ± 1		1.0	39 ± 18	
+S9	0.5	15 ± 1		0.44	14 ± 1		1.7	97 ± 2	(+)[e]	3.0	53 ± 9	
+S9	1.5	20 ± 2		1.3	13 ± 3		5.0	178 ± 5		10.0	64 ± 6	
+S9	5.0	20 ± 4		4.0	15 ± 3		15.0	236 ± 14		30.0	139 ± 11	(+)[e]
+S9	15.0	21 ± 4										

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

[a] D: dose in μg/plate. [b] NR: number of revertants. [c] The results are the mean of two independent experiments. [d] M: mutagenicity, according to ref 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.

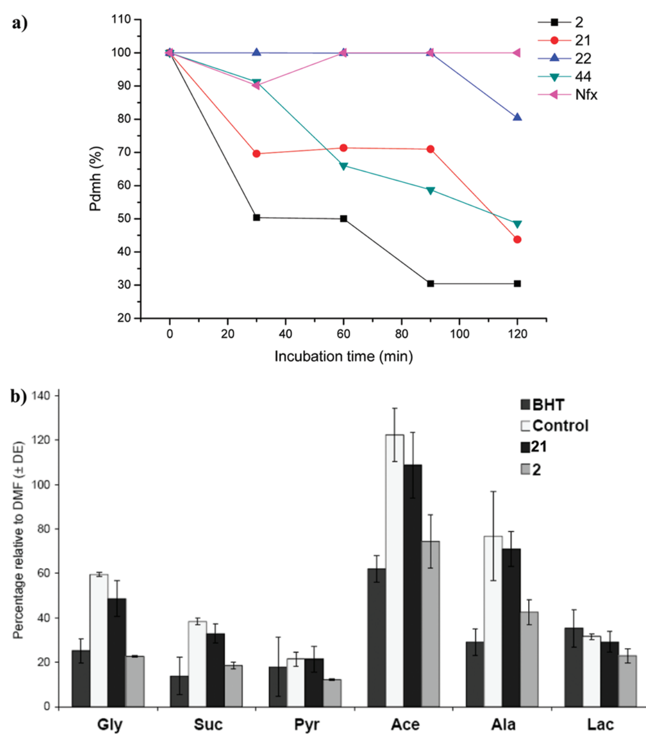


Figure 2. (a) Variation of the percentage of mitochondrial dehydrogenase activities (Pmdh), produced by the compounds with respect to time compared to the untreated control of *T. cruzi* epimastigote, Y strain (for details see Experimental Section). (b) Percentage of the end-products excreted to the medium in the different treatment, expressed respect to DMF,[19] by *T. cruzi* epimastigote, Y strain (for details see Experimental Section).

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 (Figure 2a). In general, parent compound **2** promoted greater diminishing of the studied end-metabolite concentrations than 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 dehydrogenase inhibitor (Figure 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* form 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 dehydrogenase inhibition does not correlate with the potency of the studied compounds (compare activity of the less potent parent compound **1** to activities of the most potent derivatives **21**, **22**, and **44**; Table 2), showing that other phenomena are also involved in the mode of action of this family of compound.

The in vivo analyzed 3-trifluoromethylquinoxaline dioxide derivatives **21** and **44** displayed relevant behavior. In the three experiments, the animals treated with derivative **21** showed the best survival rates at the end of the experiment. For CL Brener experiments, the blood-study findings showed that compound **21**, at a dose 5 times lower than that used for Bnz, exhibited a particular biological profile, shifting the parasitemia maximum 10 days with respect to the control and lacking the second

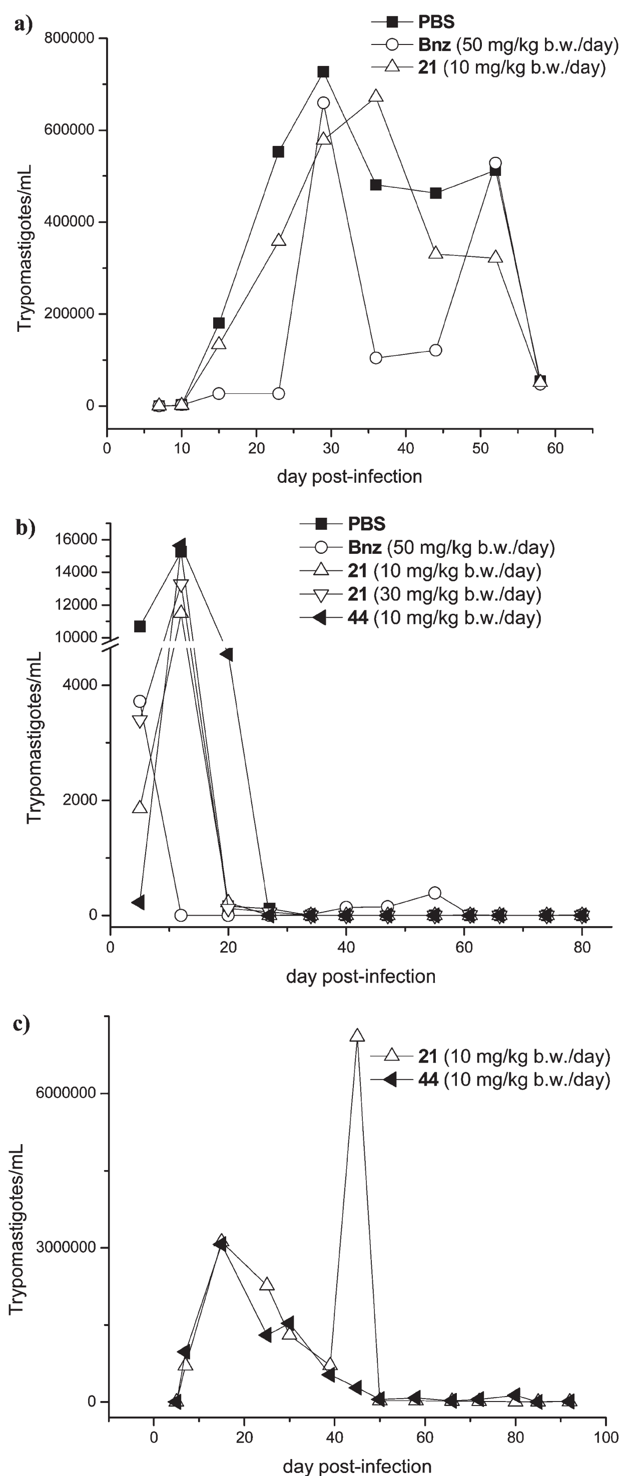


Figure 3. Parasitemia of mice treated with 50 (mg/kg bw)/day of Bnz (○), group treated with 10 (mg/kg bw)/day of **21** (△), group treated with 30 (mg/kg bw)/day of **21** (▽), group treated with 10 (mg/kg bw)/day of 44 (left-pointing triangle), and control group (■): (a) experiment 1 (CL-Brener clone); (b) experiment 2 (Y strain); (c) experiment 3 (CL-Brener clone).

parasitaemia maximum, evidenced in untreated and Bnz-treated animals at day 53 of the assay (experiment 1, Figure 3a). On the analysis days, the number of trypomastigotes on **21**-treated animals was lower than the parasite number in untreated mice. According to experiment 3, treatment with derivative **44** exhibited a better parasitemia profile than derivative **21** (Figure 3c). These results were in agreement with the values of anti-*T. cruzi* antibodies where derivative **44** showed lower findings than **21**, at 30 and 90 days after infection (Figure 4b). For Y experiments (experiment 2, Figure 3b), the blood-study findings showed that the number of trypomastigotes in **21**-treated animals was lower than the parasite number in untreated mice for both doses, in a nondose dependence, while **44**-treated animals did not improve the parasitemia profile. These results were completely in agreement with the animals' survival percentages in the Y-infected mice (Figure 4a). On the other hand, both derivatives **21** and **44** avoided the second parasitemia maximum, evidenced in Bnz-treated animals at day 55 of the assay. No significant differences were observed regarding the antibody levels, at day 90 after infection, between the four studied groups (quinoxalines and Bnz); however, the antibody levels for **21**, at both doses, and Bnz were lower than the values for infected animals (upper line in Figure 4b).

These findings promote us further thorough studies, modifying doses, administration routes, and combinations with other drugs.

■ CONCLUSIONS

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. Because of the lack of mutagenic properties of derivatives **21** and **44**, the previously described lower acute systemic toxicity in Wistar rats for **21**,[22] and the preliminary in vivo anti-trypanosoma results, these compounds could be proposed as drug candidates. Additional and more thorough in vivo studies are currently being performed on animal models of Chagas disease and leishmaniasis.

■ EXPERIMENTAL SECTION

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 ^{1}H 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, WI, U.S.) in KBr pellets. To determine the purity of the compounds, elemental microanalysis results obtained on a CHN-900 elemental analyzer (Leco, Tres Cantos, Spain) from vacuum-dried samples were used to confirm ≥95% purity. 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.

a)

Experiment	Treatment with	Doses (mg/kg b.w./day)	Percentage of survival (%)[a]
1	PBS	-	100
	Bnz	50	100
	21	10	100
		50	100
2	PBS	-	75
	Bnz	50	87.5
	21	10	100
		30	87.5
	44	10	62.5
3	PBS	-	100
	Bnz	50	75
	21	10	100
	44	10	100

[a] At the end of the studies (day 90).

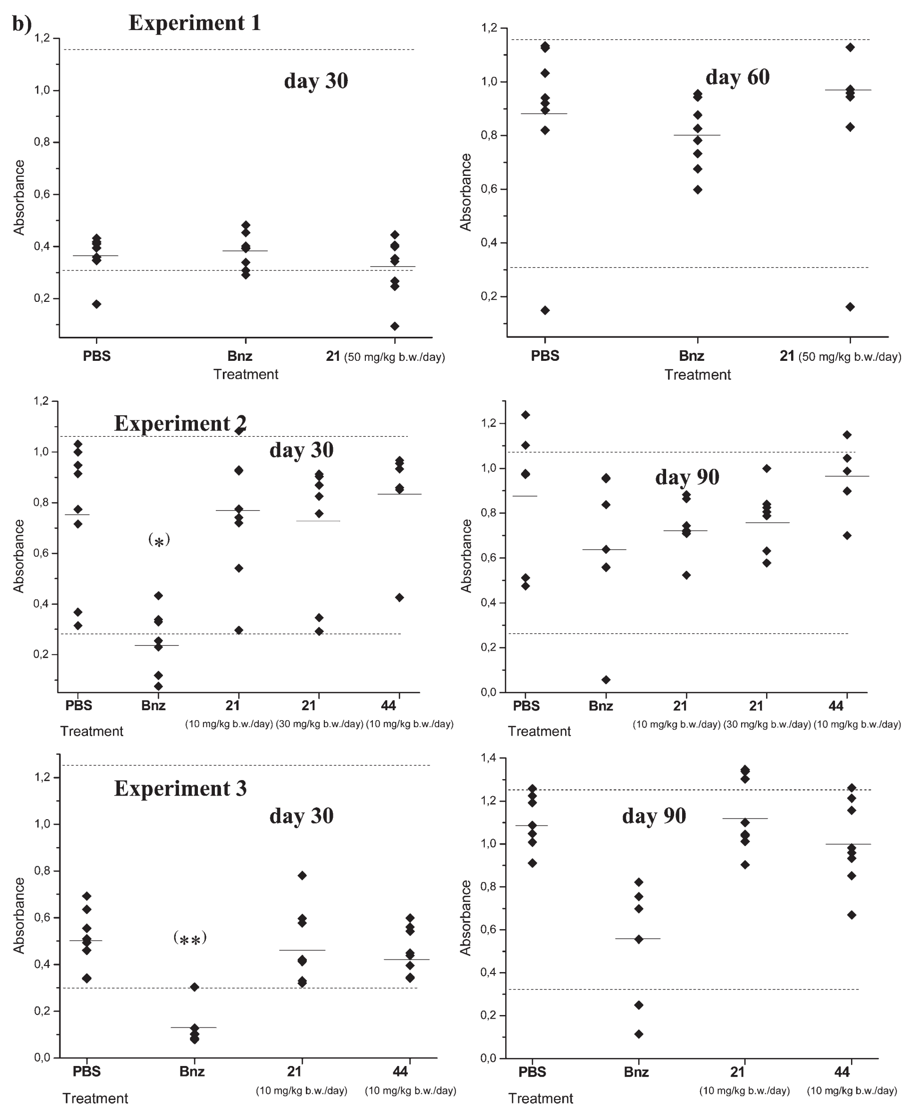


Figure 4. (a) Animal survival percentages in the different treatment-schedules. (b) Examples of anti-*T. cruzi* antibody levels in the different treatment-schedules. Upper lines represent the antibody levels for infected animals, and lower lines represent the antibody levels for healthy animals: (*) $p < 0.05$; (**) $p < 0.01$.

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 min, and then it was put in the

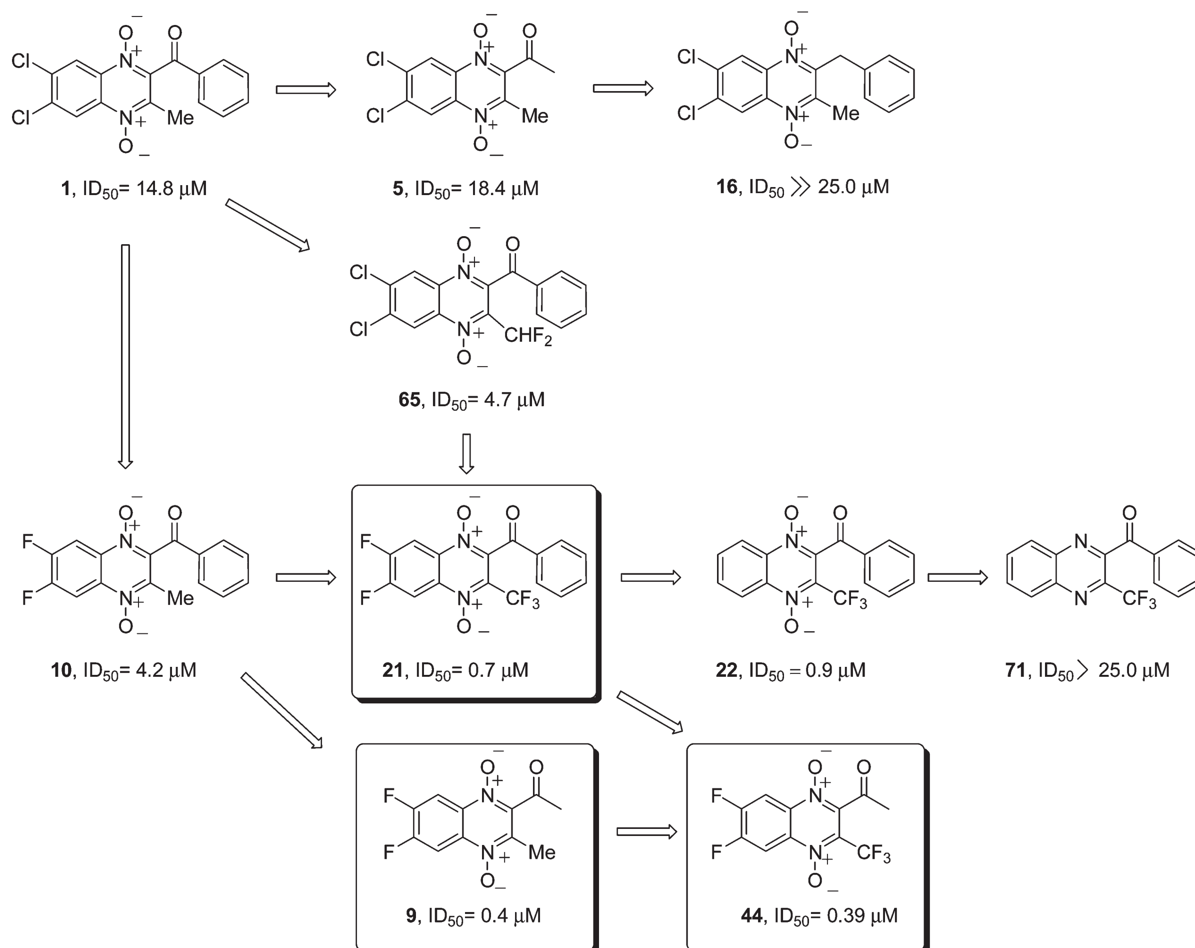


Figure 5. Structural-anti-*T. cruzi* activity for the studied quinoxalines. The ID_{50} refers to the Tulahuen 2 strain.

microwave reactor. The mixture was then subjected to microwave irradiation at 70 W for 45 min, keeping the temperature at 70−80 °C. 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 the 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) δ ppm: 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) δ ppm: 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 a similar compound *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 169 °C 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 piperazinylamide 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 min, and then it was put in the microwave reactor. The mixture was then subjected to microwave irradiation at 50 W for 25−35 min, keeping the temperature at 90 °C. 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 evaporated to dryness in vacuo, and the 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) δ ppm: 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, $H_{3'}$ + $H_{5'}$, $J_{3'\text{-}4'}$ = $J_{5'\text{-}4'}$ = 7.1 Hz, $J_{3'\text{-}2'}$ = $J_{5'\text{-}6'}$ = 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 amide), 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) δ ppm: 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, νN-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 the 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 h. 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) δ ppm: 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, νN-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) δ ppm: 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, νN-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) δ ppm: 8.73 (dd, 1H, H$_5$, $J_{5\text{-FC7}}$ = 4.9 Hz, J_{5-6} = 9.52 Hz); 8.26 (dd, 1H, H$_8$, J_{8-6} = 2.6 Hz, $J_{8\text{-FC7}}$ = 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, $J_{6\text{-FC7}}$ = 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, νN-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) δ ppm: 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$_3$O). IR (KBr): 3111 (w, νC−H Ar), 1685 (s, νC=O), 1343 (s, νN-oxide), 1042(m, νC−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) δ 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$_{3\text{-C7}}$). IR (KBr): 3064 (w, νC−H Ar), 1683 (s, νC=O), 1340 (s, νN-oxide), 1044 (m, νC−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) δ 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, νC−H Ar), 1638 (s, νC=O), 1340 (s, νN-oxide), 1044 (m, νC−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. Antitrypanosomatid in Vitro Evaluation. *Anti-T. cruzi in Vitro Test Using Tulahuen 2 Strain. Trypanosoma cruzi* epimastigotes (Tulahuen 2 strain) were grown at 28 °C 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 medium was supplemented with the indicated quantity of the drug from a stock solution in DMSO. The final concentration of DMSO in the culture medium 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 because of the presence of up to 1% DMSO in the culture medium. 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 on 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) on day 5, and A0c = A_{600} in the absence of the drug on day 0. In order to determine ID$_{50}$, 50% inhibitory concentration, parasite growth was followed in the absence (control) and presence of increasing concentrations of the corresponding drug. On day 5, the absorbance of the culture was measured and related to the control. The ID$_{50}$ 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 °C 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. Afterward, the cells were washed twice with PBS−glucose (5.5 mM) and resuspended in 200 μL of PBS−glucose. Then an amount of 100 μL of suspension was incubated (28 °C) with 0.4 mg/mL MTT (Sigma) for 4 h. Then the lysis of cells was carried out with SDS−isopropanol (1:5), and optical densities were measured at 610 nm. 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 (Lampit) and Mtf (Impavido) were used as reference drugs for *T. cruzi* and *L. braziliensis*, respectively. For this propose, Mtf was dissolved in ethanol. The ID_{50} was taken as the concentration of drug needed to reduce the absorbance ratio to 50%.

Trypanosomicidal (Trypomastigotes) in Vitro Test[20,24]. Products were evaluated in vitro on the trypomastigote form of *T. cruzi* (CL Brener clone). BALB/c mice infected with *T. cruzi* were used 7 days after infection. Blood was obtained by cardiac puncture using 3.8% sodium citrate as anticoagulant in a 7:3 blood/anticoagulant ratio. The parasitemia in infected mice ranged from 1×10^5 to 5×10^5 parasites/mL. The products were dissolved in a minimal quantity of DMSO and added to PBS to give a final concentration of 100 and 250 μg/mL. Aliquots (10 μL) of each solution in triplicate were mixed in microtiter plates with 100 μL of infected blood containing parasites at a concentration near 10^6 parasites/mL. Infected blood and infected blood containing GV at 250 μg/mL were used as control. The plates were shaken for 10 min at room temperature and kept at 4 °C for 24 h. Each solution was examined microscopically (Olympus BH2) for parasite counting. The activity (% of parasites reduction) was compared with that of the standard drug GV.

Mutagenicity Assay. The method of direct incubation in plate was performed. A 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 *S. typhimurium* TA98 strain was assayed. DMSO solutions of **9**, **21**, **44**, and Nfx at different doses (starting at the highest doses without toxic effects, 15.0 and 30.0 μg/plate, respectively) 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 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, U.S.). 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 of 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 °C. 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. An amount of 50 μL of a solution containing 5 mg/mL of MTT in PBS was 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 of HCl 10 N in 100 mL of isopropanol). The absorbance was measured at 570 nm. Under our conditions, compounds did not interfere with the reaction mixture. The percentage of mitochondrial dehydrogenase activity (Pmdh (%)) was determined using untreated parasite activity as 100%.

^{1}H NMR Study of the Excreted Metabolites. For the spectroscopic studies, an amount of 5 mL of a 2-day-treated *T. cruzi* (Y strain) was centrifuged with each studied compound (5 μM) at 1500*g* for 10 min at 4 °C. The pellet was discarded, and the parasite-free supernatant was stored at −20 °C until use. Before measurement, 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 and 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 or Swiss albino 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 or 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 parasitemia was carried out 5 days after infection (week 1), and the treatment was begun 7 days later. Compounds were administered orally, as aqueous solution in PBS, at 10, 30, or 50 (mg/kg bw)/day for quinoxaline derivative **21**, 10 (mg/kg bw)/day for quinoxaline derivative **44**, and 50 (mg/kg bw)/day for Bnz, during 10 days. Parasitemia 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 or 90 days after 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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ABBREVIATIONS USED

Nfx, nifurtimox; Bnz, benznidazole; Mtf, miltefosine; PGI, percentage of growth inhibition; PCyt, percentage of cytotoxicity; GV, gentian violet; SI, selectivity index; 4-NPD, 4-nitro-*o*-phenylendiamine; AF, 2-aminofluorene; NR, number of revertants; Pmdh, percentage of mitochondrial dehydrogenase activity; Lac, lactate; Ace, acetate; Pyr, pyruvate; Suc, succinate; Ala, alanine; Gly, glycine

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