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Short communication

Pyrimido[1,2-*a*]quinoxaline 6-oxide and phenazine 5,10-dioxide derivatives and related compounds as growth inhibitors of *Trypanosoma cruzi*

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Abstract

Two different families of *N*-oxide containing heterocycles were evaluated as *in vitro* growth inhibitors of *T. cruzi*. Both families of heterocycles were selected from our in-house library of compounds as analogues of active anti-*T. cruzi N*-oxide containing heterocycles. Derivatives from pyrimido[1,2-*a*]quinoxaline 6-oxide family were poorly active at the assayed doses. However, phenazine 5,10-dioxide derivatives displayed good to excellent anti-*T. cruzi* activities. The anti-*T. cruzi* activity of phenazine derivatives was related to substituent' electronic descriptors, σ_p^-. Derivatives **19**, **20** and **23** were the most cytotoxic compounds against the protozoan and became excellent hit for further structural modifications.
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Keywords: Chagas disease; Pyrimido[1,2-a]quinoxaline 6-oxide; Phenazine 5,10-dioxide

1. Introduction

Chagas' disease or American trypanosomiasis is an important health problem that affects around 20 million people in Central and South America [1]. Around 2–3 million individuals develop the typical symptoms of this disease that results in 50 000 yearly deaths [2]. The causative agent of this disease is the haemoflagellate protozoan *Trypanosoma cruzi* (*T. cruzi*) [3]. The parasite presents three main morphological forms in a complex life cycle. It replicates within the crop and midgut of Chagas' disease vectors as the epimastigote form, it is released with the insect excrements as the non-dividing highly infective metacyclic trypomastigotes that invade mammalian tissues via wounds provoked by blood sucking action. The parasite multiplies intracellularly as amastigote form which is released as the non-dividing bloodstream trypomastigote form that invades other tissues. The existence of the epimastigote form as an obligate mammalian intracellular stage has been revisited [4,5] and confirmed recently [6]. The sequencing of the *T. cruzi* genome has been recently completed [7] and large progress made in the study of *T. cruzi* biochemistry and physiology [3], in which several crucial enzymes for parasite survival absent in the host have been identified as potential targets for the design of new drugs [8–11]. In spite of these recent achievements, the chemotherapy to control this parasitic infection remains undeveloped. In fact, although Nifurtimox (Nfx) and Benznidazole (Bnz), the only two drugs currently in use for clinical treatment of this disease, are able to wipe out parasitemia and reduce serological titres, they are not specific enough to all *T. cruzi* strains to guarantee complete cure [12–14]. The research and development of new active anti-*T. cruzi* compounds have been based on different strategies, i.e. inhibition of specific parasite enzymes, actions on parasite DNA, and oxidative stress damage. In the first group a great number of enzymes have been employed as targets for new inhibitors, especially trypanothione

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reductase inhibitors belonging to phenothiazine derivatives (i.e. compound **1**, Fig. 1) are specific competitive inhibitors [15,16]. Agents based on DNA-damage include, between others, camptothecin derivatives (i.e. **2**, Fig. 1) [17,18] and acridine derivatives (i.e. **3**, Fig. 1) [19]. In reference to oxidative stress-producer agents the well known nitrofurans have been the most studied compounds [20–22]. In this sense, we identified some *N*-oxide containing heterocycles with ability to produce oxidative stress intraparasitically (i.e. **4**, Fig. 1) [23]. Recently, we have analyzed some quinoxaline dioxide derivatives (i.e. **5**, Fig. 1) as anti-*T. cruzi* agents identifying good inhibitors with a potential free radical production as mechanism of action [24].

Looking for new *N*-oxides with anti-*T. cruzi* activity we have selected, from our in-house chemical library of *N*-oxides, derivatives from pyrimido[1,2-*a*]quinoxaline 6-oxide and phenazine 5,10-dioxide systems with structural motives related to compounds **1**–**5** (Fig. 1). These heterocyclic systems include planar backbone with two or three heteroatoms, nitrogens, distributed like in derivatives **1**, **2** or **5**. In order to analyze structural requirements we have chosen different substituents (R^1–R^5, Fig. 1) with differential physicochemical behaviours. Consequently, we have synthesized and biologically evaluated some selected pyrimido[1,2-*a*]quinoxaline 6-oxide and phenazine 5,10-dioxide derivatives as anti-trypanosomal agents. The 25 derivatives described in the present paper were carefully selected from our near to 100 derivatives-library in order to cover a wide structural spectrum, synthesized and biologically evaluated. From the data generated here a new series of compounds will be selected.

2. Chemistry

The studied compounds, **6**–**30** (Fig. 2, Table 1), were prepared following synthetic procedures previously reported [25–29]. The pyrimido[1,2-*a*]quinoxaline 6-oxides **6**–**14** were obtained by the cyclization of the corresponding nitroaminoamide reactants in presence of ethyl polyphosphate (EPP). Compound **6** was then transformed into the deoxygenated derivative **15** by reaction with $NaBH_4$. Compounds **6**, **7**, and **9** were methylated with CH_3I to generate derivatives **16**–**18**. The phenazine 5,10-dioxide derivatives **19**–**28** were obtained from the corresponding benzofuroxan reactants as inseparable mixtures of 7- and 8-isomers which were evaluated without further separation. In Table 1 are shown the main isomers obtained in the synthetic procedures. Similar to pyrimidoquinoxaline 6-oxides deoxygenated derivatives, **29** and **30**, were included in this study in order to evaluate the relevance of the *N*-oxide moiety in the bioactivity.

All compounds were identified by IR, MS, ^{1}H NMR, ^{13}C NMR, HSQC and HMBC experiments, and their purity established by TLC and microanalysis.

3. Pharmacology

All the compounds were tested *in vitro* against *T. cruzi*, as previously described [30]. The compounds were incorporated into the media at 25 μM and its ability to inhibit the parasite growth was evaluated in comparison to the control (no drug added to the media). Nfx and Bnz were used as the reference trypanocidal drugs. The percentage of growth inhibition (PGI) at 25 μM is gathered in Table 1. The IC_{50} (50% inhibitory concentration) was assessed for compounds presenting higher trypanocidal activity (Table 2). In the case of the most active derivatives, phenazine 5,10-dioxides, three different *T. cruzi* strains were studied, Tulahuen 2, CL Brener and Y strains.

4. Discussion

We reported the biological activity of 25 pyrimido[1,2-*a*]quinoxaline 6-oxide and phenazine 5,10-dioxide derivatives and related compounds, against the epimastigote form of *T. cruzi*. The most active derivatives, belonging to phenazine dioxide family, were evaluated against three different strains of *T. cruzi* (Table 1). Derivatives **19**, **20**, and **23** were the most active compounds against the studied strains. Consequently, they were selected to determine its IC_{50} together with Nfx and Bnz against the three strains (Table 2). The selected derivatives have similar levels of bio-activities against *T. cruzi* as the reference drugs. Derivatives belonging to

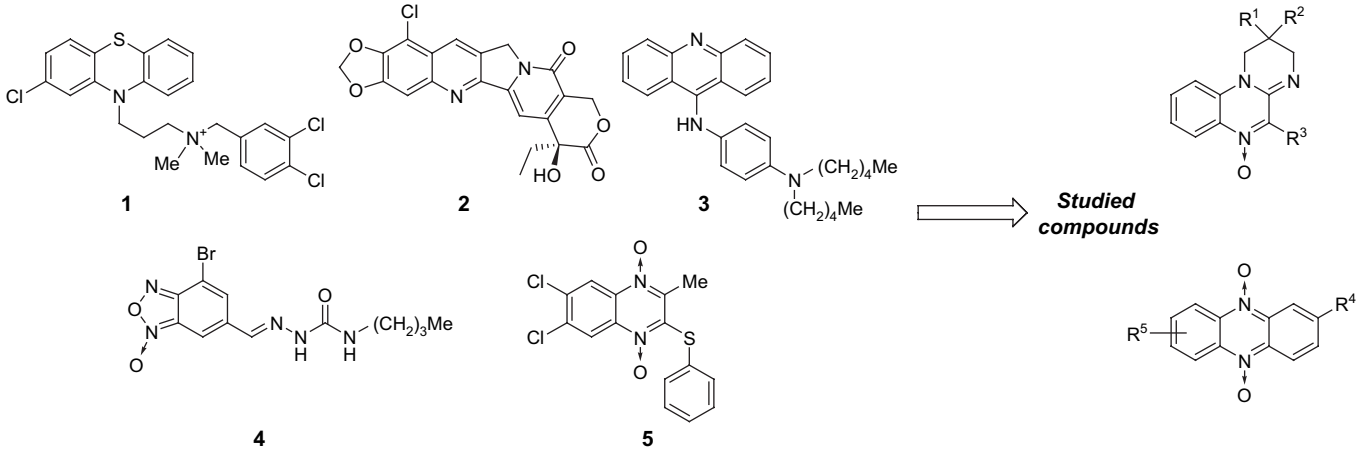


Fig. 1. Inhibitors of *T. cruzi* and structural related *N*-oxides studied for anti-*T. cruzi* activity.

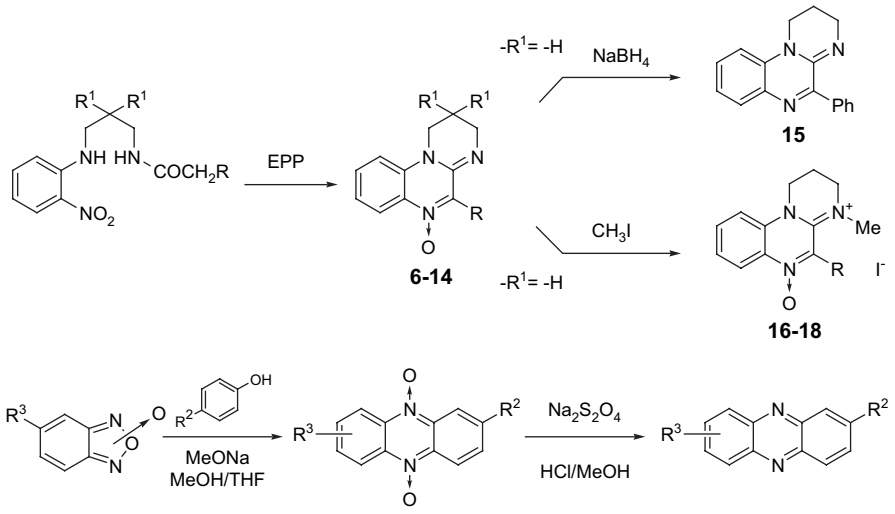
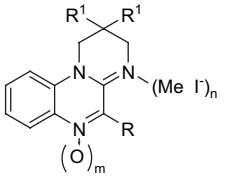


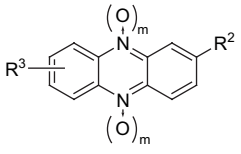
Fig. 2. Synthetic procedures used for the preparation of the studied compounds.

pyrimido[1,2-*a*]quinoxaline 6-oxide family were less active than phenazine 5,10-dioxides. It was not possible to establish any clear relationship between structure of pyrimido[1,2-*a*]quinoxaline 6-oxide derivatives and anti-*T. cruzi* activity. Apparently, the 5-phenyl substituent is relevant for activity (compare activity of derivative **6**–**13** and **14**'s activities). Furthermore, the absence of the *N*-oxide moiety (i.e. derivative **15**) or the quaternization of nitrogen in 4 position (i.e. derivative **16**) did not modify significantly the bioactivity. In contrast, comparing anti-*T. cruzi* activity of derivatives **7** and **12**, some steric requirements for the correct activity are necessary.

For the phenazine 5,10-dioxide derivatives relevant structural information was amassed. For example, the 2-hydroxy derivatives are, in general, less active than the 2-amino analogues. In the 2-amino series, derivatives **19**–**23**, a clear relationship between the $-R^3$ electronic characteristic and activity was observed (Fig. 3). Therefore, derivatives substituted by

Table 1
Structure and biological characterization of the studied compounds





Ref.	*n*	*m*	$-R$	$-R^1$	PGI[a] (%)[b,c] Tul 2[d]	Ref.	*m*	$-R^2$	$-R^3$	Ratio 7-isomer:8-isomer[e]	PGI[a] (%)[b,c] Tul 2[d]	CLB[f]	Y[g]
6	0	1	−Ph	−H	36	**19**	1	$-NH_2$	−Br	54:46	81	93	88
7	0	1	−(*p*-OMe)Ph	−H	36	**20**	1	$-NH_2$	−Cl	63:37	61	99	78
8	0	1	−(*p*-Cl)Ph	−H	14	**21**	1	$-NH_2$	−OMe	95:5	38	34	21
9	0	1	−(*p*-NO_2)Ph	−H	9	**22**	1	$-NH_2$	−Me	70:30	37	25	34
10	0	1	−(*o*-Cl)Ph	−H	14	**23**	1	$-NHCOCH_2Cl$	−(1,3-Dioxolan-2-yl)	55:45	79	95	94
11	0	1	−(*o*-NO_2)Ph	−H	12	**24**	1	−OH	−Br	64:36	0	40	8
12	0	1	−(*p*-OMe)Ph	−Me	13	**25**	1	−OH	−H	–	0	14	0
13	0	1	$-CH_2Ph$	−H	13	**26**	1	−OH	−OMe	100:0	0	9	0
14	0	1	−Me	−H	22	**27**	1	−OH	−Me	76:24	43	6	9
15	0	0	−Ph	−H	29	**28**	1	−OH	−(1,3-Dioxolan-2-yl)	35:65	0	23	17
16	1	1	−Ph	−H	20	**29**	0	−OH	−Br	70:30	0	23	8
17	1	1	−(*p*-OMe)Ph	−H	9	**30**	0	−OH	−Me	56:44	45	25	10
18	1	1	−(*p*-NO_2)Ph	−H	20								

[a] PGI: percentage of growth inhibition.
[b] Inhibition of epimastigotes growth, dose = 25 μM.
[c] The results are the means of three different experiments with an SD less than 10% in all cases.
[d] Tul 2: Tulahuen 2 strain.
[e] Determined by ^{1}H NMR from the reaction mixture [27–29]. The compounds were evaluated as mixture of isomers.
[f] CLB: CL Brener strain.
[g] Y: Y strain.

Table 2
IC_{50} (μM) values for the most active derivatives

Ref.	IC_{50} (μM)[a]		
	Tul 2[b]	CLB[c]	Y[d]
19	9.0	8.0	8.2
20	5.3	13.0	7.7
23	14.2	6.5	4.5
Nfx	7.7	8.5	6.5
Bnz	7.4	4.5	3.8

[a] The results are the means of three different experiments with an SD less than 10% in all cases.
[b] Tul 2: Tulahuen 2 strain.
[c] CLB: CL Brener strain.
[d] Y: Y strain.

electron withdrawing groups, like −Br and −Cl, show better bioactivity than derivatives substituted by electron donors, like −OMe, −Me, 1,3-dioxolan-2-yl moiety. In the case of activity against Y strain an excellent linear relationship ($r = 0.9994$) between R^3 electronic descriptor, Hammett σ_p^- parameters, and activity was observed (Fig. 3). This phenomenon has been already observed previously for quinoxaline dioxide analogues [24]. The correlation with R^3 electronic property could indicate that a reductive metabolism, throughout the *N*-oxide moiety, could be implicated in the mechanism of action. However, derivative **23**, with an electron donor moiety, displays high activity against the three studied strains (Table 2) maybe as consequence of the excellent electrophile centre, chloroacetamide moiety, in the 2-amino position. This structural motif could unselectively react with biological nucleophiles (amino and phosphate groups from DNA, mercapto, hydroxyl and amino groups from proteins). Methyl derivatives **27** and **30**, 2-hydroxy dioxide and 2-hydroxy deoxygenated derivatives, are medium active against Tulahuen 2 strain. Maybe another structural factor could play a role in the activity of these compounds.

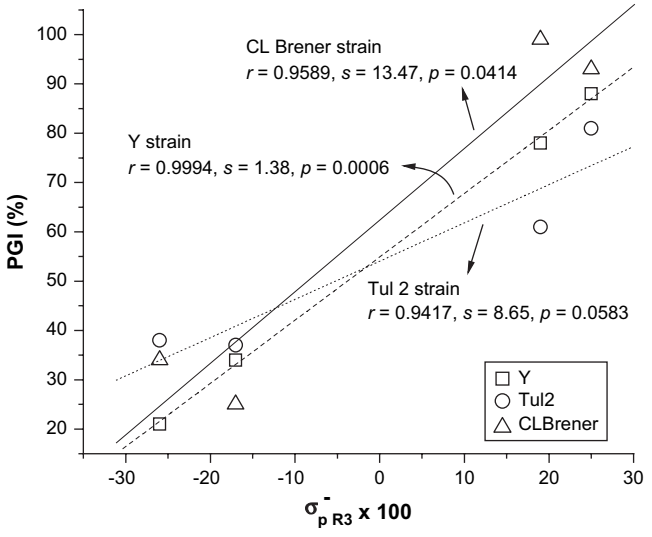


Fig. 3. Substituent electronic descriptors ($\sigma^-_{pR^3} \times 100$) *vs* percentages of trypanosome growth inhibition at 25 μM (PGI). The curves show the tendency.

Furthermore, Lipinski has described desired ranges for certain properties thought to be important for pharmacokinetics and drugs development. They are log $P < 5$, number of hydrogen bond donors ≤ 5, number of hydrogen bond acceptors ≤ 10 and molecular weight < 500 [31]. A compound that fulfils at least three out of the four criteria is said to adhere to Lipinski's 'rule of 5'. Table 3 lists the values of these properties for the best derivatives, **19**, **20** and **23**, and suggests that these compounds are reasonable starting points for a drug discovery effort. This information led us to re-design new structures with increased desired activity.

5. Conclusions

The results presented above indicate, according to the *in vitro* activity and adherence to Lipinski's rules, that new phenazine 5,10-dioxide derivatives **19**, **20** and **23** are excellent hits for further *in vivo* studies in an animal model of Chagas' disease.

6. Experimental

6.1. Chemistry

All starting materials were commercially available research-grade chemicals and used without further purification. Compounds **6**–**30** and nitroaminoamide and benzofuroxans reactants were prepared following literature procedures [25–29]. All solvents were dried and distilled prior to use. All the reactions were carried out in a nitrogen atmosphere. Melting points were determined using a Leitz Microscope Heating Stage Model 350 apparatus and are uncorrected. Elemental analyses were obtained from vacuum-dried samples (over phosphorous pentoxide at 3–4 mm Hg, 24 h at room temperature) and performed on a Fisons EA 1108 CHNS-O analyser. Infrared spectra were recorded on a Perkin Elmer 1310 apparatus, using potassium bromide tablets. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker DPX-400 (at 400 and 100 MHz) instrument, with tetramethylsilane as the internal reference. Mass spectra were recorded on a Shimadzu GC–MS QP 1100 EX instrument at 70 eV.

6.2. Biology

The Tulahuen 2, CL Brener and Y strains stocks of *T. cruzi* were used in this study. Handling of live *T. cruzi* was done

Table 3
Physicochemical properties and Lipinski's 'rule of 5'

Ref.	log *P* [a]	H-bond donors	H-bond acceptors	Molecular weight	'Rule of 5' criteria met
Rule	<5	≤5	≤10	<500	At least 3
19	6.8	2	5	306	3
20	6.6	2	5	262	3
23	6.1	1	8	376	3

[a] Calculated using Ghose–Crippen algorithm, implemented in Spartan'04 modeling package [32,33].

according to established guidelines [34]. The epimastigote form of the parasite was grown at 28 °C in an axenic medium (BHI-Tryptose), complemented with 5% foetal calf serum. Cells from a 5-day-old culture 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 daily measuring the absorbance of the culture at 600 nm for 11 days. Before inoculation, the media was supplemented with 25 μM solutions of compounds from a stock DMSO solution. The final DMSO concentration in the culture media never exceeded 0.4% (vol/vol) and had no effect by itself on the proliferation of the parasites (no effect on epimastigote growth was observed by the presence of up to 1% DMSO in the culture media). The compounds' ability to inhibit growth of the parasite was evaluated, in triplicate, in comparison to the control (no drug added to the media). The control was run in the presence of 0.4% DMSO and in the absence of any drug. The percentage of growth inhibition (PGI) was calculated as follows:

$$\mathrm{PGI}(\%) = \left\{1 - \left[\left(A_\mathrm{p} - A_{0\mathrm{p}}\right)/\left(A_\mathrm{c} - A_{0\mathrm{c}}\right)\right]\right\} \times 100,$$

where $A_\mathrm{p} = A_{600}$ of the culture containing the drug at day 5; $A_{0\mathrm{p}} = A_{600}$ of the culture containing the drug just after addition of the inocula (day 0); $A_\mathrm{c} = A_{600}$ of the culture in the absence of any drug (control) at day 5; and $A_{0\mathrm{c}} = A_{600}$ in the absence of the drug at day 0. The 50% effective concentrations (IC_{50}) were obtained assaying five different points, 1.0, 5.0, 10.0, 15.0 and 25.0 μM. Each point was analyzed in three different experiments with an SD less than 10%. Nifurtimox and Benznidazole were used as the reference trypanocidal drug.

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