Synthesis and biological evaluation of quinoxaline di-*N*-oxide derivatives with *in vitro* trypanocidal activity

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Abstract: We report the synthesis and *in vitro* activity against *T. cruzi* epimastigotes of 15 novel quinoxaline derivatives. Ten of the derivatives presented IC_{50} values lower than the reference drugs Nfx and Bzn; four of them standed out with IC_{50} values lower than 1.5 µM. Moreover, unspecific cytotoxicity and genotoxicity studies are also reported. Compound **14** showed a SI higher than 24, whereas compound **10** was the only one that was negative in the genotoxicity screening.

Keywords: Chagas disease, *Trypanosoma cruzi*, Quinoxaline *N*-oxide, cytotoxicity, genotoxicity.

Chagas disease (CD) is classified as one of the 17 Neglected Tropical Diseases (NTD) as defined by WHO.[1] The 17 NTD account for a disease burden of at least 26 million disability-adjusted life years (DALYs) according to the Third WHO Report on Neglected Tropical Diseases[2] and are under the Sustainable Development Goals SDG3. However, only the 0.6% of the new therapeutic products registered from 2000 to 2011 were indicated for NTD and none of them were a NCE.[3] These facts highlight the urgent need of new effective and safe drugs for fighting NTDs.

CD, also known as American trypanosomiasis, is caused by the protozoan parasite *Trypanosoma cruzi*. It used to be considered as a zoonotic disease affecting rural areas in low-middle income countries in Latin America. Nevertheless, human migration has spread out the disease worldwide and, according to WHO, 8 million people are currently infected.[4] It is estimated that over 10000 people die every year from CD, and more than 25 million people are at risk of infection.

CD is curable if treatment is initiated soon after infection; therefore, access to diagnosis is essential. If not treated, 30% of the affected people develop heart damage and 10% suffer from digestive and/or neurological alterations. Nifurtimox (Lampit®) and Benznidazol (Rochagan®) are the only available drugs for CD and they were developed more than 40 years ago. Neither of them is approved by the FDA.[5] The major limitation of currently available drugs is their lower antiparasitic activity in the established chronic form of the disease, which is the most prevalent presentation. On the other hand, both drugs have undesired side effects that can lead to treatment discontinuation, which for Nfx include anorexia, nausea and vomiting causing severe weight loss, insomnia and irritability, while for Bnz the most common adverse effect is urticarial dermatitis.[6, 7]

Three clinical trials have recently been conducted. A phase II proof-of-activity study of oral posaconazole in the treatment of asymptomatic chronic CD was completed in January 2015 and no conclusions have been reported.[8] The phase II clinical trial for the treatment of chronic CD with posaconazole and benznidazole (CHAGASAZOL) and a higher treatment failure was observed in patients in the posaconazole groups than in the Bnz group.[9, 10] Finally, a proof-of-concept study of the promising NCE E1224 was recently completed but the development of E1224 as monotherapy has been stopped and it will be considered for new combinatory regimens.[5, 11, 12]

This background justifies the urgent need for novel and better drugs to treat both acute and chronic phases. Quinoxaline derivatives are a chemical scaffold that has showed a wide spectrum of biological activities.[13] Our group has vast experience in the synthesis

and biological evaluation of quinoxaline derivatives with anti-cancer, anti-mycobacterium and anti-inflammatory activities among others.[14-26] One of the projects has been focused on the study of novel quinoxaline derivatives as anti *T. cruzi* agents. In this field, over 200 derivatives have been prepared and evaluated as anti-trypanosomatid agents and some structural requirements have been established for their anti-chagasic *in vitro* activity. [20, 25, 27, 28] Therefore, it can be considered that the main structural requirements for the trypanocidal activity of quinoxaline derivatives are: the presence of the *N*-oxide moiety and the insertion of electro withdrawing substituents on the quinoxaline ring (e.g. fluoro, chloro, trifluoromethyl…) Despite the general opinion about the toxicity associated with *N*-oxides, it has been reported that quinoxaline derivatives mutagenicity seems to be associated with the substituents on the heterocycle.[29] With this background and with the aim of identifying a new lead with higher potency and selectivity and a better drug target profile a series of 15 novel quinoxaline derivatives were designed considering the structural requirements previously established by our group and the insertion of alicyclic amines of biological interest. Piperazine derivatives have been explored for their interest as anti-trypanosomatid agents and their activity as inhibitors of different targets of interest to fight against CD has been reported.[30-32] Structural similarity can be observed between the proposed compounds and fluoroquinolones (**Figure 1**), a family of well-known antibacterial agents that have shown interesting activity data against trypanosomatids.[33-35]
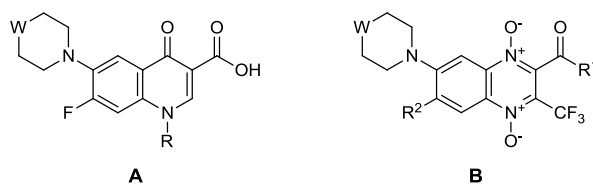

Figure 1. Structural similarity between fluoroquinolones (A) and proposed quinoxaline derivatives (B).

The design, synthesis and *in vitro* evaluation of new quinoxaline derivatives (**1-15**) as antitrypanosomal agents are described and SARs are discussed. The non-specific toxicity against mammalian cells was studied in order to evaluate the quinoxaline selectivity to the parasites and the SOS/umu test was included as a preliminary genotoxicity screening assay.

The designed compounds (**1-15**) were synthesized according to the sequence of reactions outlined in **Scheme 1**.
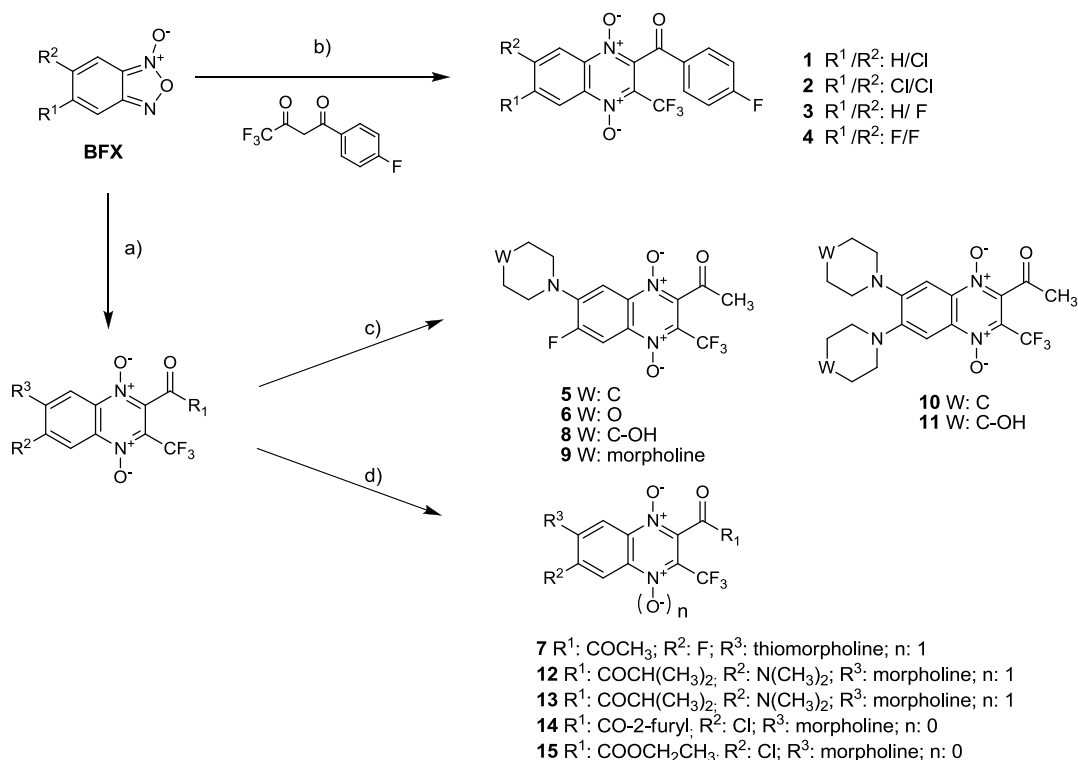
Scheme 1. Synthesis of quinoxaline derivatives **1-15**.

a) Previously reported in [27, 36]; b) toluene, triethylamine, MW; c) acetonitrile, triethylamine, r.t.; d) *N,N*-DMF, reflux.

4 new quinoxaline 1,4-di-*N*-oxide derivatives (**1-4**) were prepared using a variation of the Beirut reaction using microwave irradiation in which the corresponding BFX reacted with the 1-(4-fluorophenyl)-4,4,4-trifluoro-1,3-butanedione using toluene as solvent and triethylamine as base. The use of microwave assisted organic synthesis reduced the reaction times and simplifies the purification of quinoxaline derivatives. This fact led to an increase in the yield mainly when quinoxalines are substituted by halogens in positions 6 and/or 7 of the ring.

Compounds substituted by a cyclic amine on positions 6 and/or 7 of the quinoxaline ring were prepared by nucleophilic aromatic substitution of the corresponding quinoxaline. All the quinoxalines presented a chloro or fluoro substituted on positions 6 and/or 7 of the ring as leaving group and different cyclic amines were used as nucleophiles. As expected, the fluoro acts as a better leaving group decreasing the reaction times in comparison with the chloro. The longer reaction times needed when a chloro was substituted on positions 6 and 7 of the heterocycle led to the generation of the mono-reduced quinoxaline (**14** and **15**) complicating the purification of the desired compounds.[37, 38] Different synthetic methodologies were used with the aim of preparing the desired compounds. When the reaction was carried out in *N,N*-DMF, the fluoro

could be replaced not only by the cyclic amine but also by the solvent (compound **12**) making more difficult the isolation of the desired compound. Moreover, the use of reflux would facilitate the generation of 1-*N*-oxide quinoxaline derivatives (**14** and **15**). *N,N*-DMF was substituted by acetonitrile that could be used as aprotic polar solvent that would avoid the aforementioned difficulties.[39-41] Finally, triethylamine was used as base and 6 compounds were prepared using these conditions (**5, 6, 8-11**). All of the compounds were fully characterized by infrared spectroscopy, nuclear magnetic resonance and elemental microanalyses (See Supplementary material).

Compounds were assayed against the epimastigote form of *T. cruzi*, Tulahuen 2 strain in order to determine the percentage of growth inhibition (PGI) at 25 μM and the IC_{50}. Compounds showing IC_{50} lower than Nifurtimox (IC_{50}=7.7 μM) are considered active and would be considered for further testing.[42] Five compounds were selected and their unspecific cytotoxicity and genotoxicity were studied before moving on to advanced studies. Cytotoxicity was assayed in Vero cells and the IC_{50} was determined from the dose-response curve. The Selectivity Index (SI) is expressed as the ratio of IC_{50} in mammalian cells to IC_{50} in epimastigote. The mutagenic capacity of the selected compounds was studied using the SOS/umu screening test.

The primary screening data for all compounds at 25 μM are shown in **Table 1**. The results demonstrate that 13 out of the 15 evaluated compounds presented PGI higher than 80%. Moreover, 10 of the derivatives showed IC_{50} values lower than the reference drugs Nfx and Bzn, presenting 4 of them IC_{50} values lower than 1.5 μM.
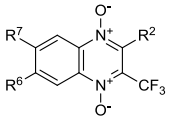
Analysis of the activity data presented in **Table 1** reveals that substitution of a halogen atom, on position 7 of the quinoxaline, by a cyclic amine led to a decrease of the *in vitro* anti-*T. cruzi* activity. This behavior can be observed when comparing compounds **5-9** with their parent compound **A** or, in general terms, comparing with compounds **1-4**.

If considering mono or di-substitution by cyclic amines no conclusion can be achieved. Di-substituted derivatives **11** and **13** showed lower PGI and higher IC_{50} than their mono-substituted analogues **8** and **12**. Meanwhile, derivative **10** presented very interesting data, even better than its mono-substituted analogue **5**.

Regarding the type of amine substituted on position 6 and/or 7, derivatives that present a piperidine on these positions (compounds **5** and **10**) stand out with IC_{50} (2.6 and 2.3 μM, respectively) much better than the reference drugs. When piperidine is substituted by its bioisosteres morpholine or tiomorpholine or a hydroxyl moiety is substituted on

position 6, the *in vitro* activity of these derivatives decreases (compound **5** vs. **6-8**). It can be confirmed that the most decisive structural requirement, in terms of *in vitro* biological activity, is the presence of electron withdrawing substituents on positions 6 and/or 7. On the other hand, substitution on position 2 of the quinoxaline ring seems not to play such an important role on biological activity as previously observed.[27, 36] This behavior can also be observed in compounds **1-4** that present the best activity data.

Table 1. *In vitro* trypanosomicidal activity of quinoxaline derivatives.

Comp.	R^2	R^6	R^7	PGI (%)[b]	IC$_{50}$ (μM)[c]
A[a]	COCH$_3$	F	F	100	0.39
1	COPh-*p*-F	H	Cl	86.1	1.0
2	COPh-*p*-F	Cl	Cl	89.3	1.3
3	COPh-*p*-F	H	F	95.6	1.1
4	COPh-*p*-F	F	F	91.8	0.6
5	COCH$_3$	F	–N(piperidine)	96.9	2.6
6	COCH$_3$	F	–N(morpholine)	100	6.4
7	COCH$_3$	F	–N(thiomorpholine)	83.4	2.0
8	COCH$_3$	F	–N(piperidine)-OH	84.9	6.3
9	COCH$_3$	F	–N(piperazine)-N(morpholine)	93.4	5.3
10	COCH$_3$	–N(piperidine)	–N(piperidine)	100	2.3
11	COCH$_3$	–N(piperidine)-OH	–N(piperidine)-OH	15.7	>25
12	COCH(CH$_3$)$_2$	N(CH$_3$)$_2$	–N(morpholine)	86.0	10.7
13	COCH(CH$_3$)$_2$	–N(morpholine)	–N(morpholine)	66.3	17.0
14[d]	CO-2-furyl	Cl	–N(morpholine)	91.7	12.1
15[d]	COOCH$_2$CH$_3$	Cl	–N(morpholine)	54.6	>25
Nfx				100	7.7
Bnz				100	8.0

[a] Previously reported in [27]. [b] Percentage of growth inhibition of epimastigote growth of Tulahen 2 strain, dose = 25 μM. [c] Concentration (in μM) that inhibits 50% of epimastigote form of *T. cruzi* growth. The results are the mean of three independent experiments with an SD less than 10% in all cases. [d] quinoxaline-1-*N*-oxide

Throughout the activity optimization process we were mindful of the importance of a good toxicological profile that would guarantee not only a potent compound but also a safe drug. Therefore and owing to the interesting *in vitro* trypanosomicidal activity

showed by the quinoxaline derivatives, 5 compounds (**3-5**, **10** and **14**) were selected considering their IC_{50} value (compounds **3, 4** and **5**) and from a structural point of view compound **10** was included due to its significant activity and in order to study the influence of the substitution of an halogen by an amine. Compound **14** was selected to evaluate the influence of the *N*-oxide on the toxicological profile of these derivatives. The *in vitro* cytotoxicity was studied using the tetrazolium-based colorimetric assay (MTT assay) in Vero cells. The IC_{50} values after 48 h of incubation are shown in **Table 2**. The selective indexes (SI) were calculated as the ratio of IC_{50} in Vero cells to IC_{50} in *T. cruzi* Tulahuen 2 strain.

Moreover, the compounds were tested in the SOS/umu screening test for their genotoxicity capacity. The results obtained from the SOS/umu screening test for the selected compounds (**3-5**, **10, 14**) with and without metabolic activation are shown in **Table 2**. The evaluated doses of each compound were determined in previous toxicity studies on the test system.

Table 2. *In vitro* toxicological profile of quinoxaline derivatives.

Comp.	*T. cruzi* epimastigote Tulahuen 2		MTT Vero Cells		SOS/umu	
	PGI (%)	IC_{50} (μM)	IC_{50} (μM)	SI	-S9	+S9
3	95.6	1.1	4.4	4.0	+	+
4	91.8	0.6	3.0	5.0	+	+
5	96.9	2.6	15.2	5.8	+	+
10	100	2.3	4.5	1.7	-	-
14	91.7	12.1	454.7	37.6	+	+

Regarding the unspecific cytotoxicity assay, 4 compounds (**3**-**5** and **10**) presented SI lower than 10. Compound **14** was the only one that presented a SI value higher than 25 and could be considered moderately selective. This increase in the SI is mainly due to the low non specific cytotoxicity presented by this compound which does not present a *N*-oxide moiety, generally related with toxic effects, in one of the nitrogens of the quinoxaline heterocycle.

The SOS/umu screening test revealed that 4 compounds (**3-5** and **14**) were genotoxic with and without metabolic activation and compound **10** was not genotoxic in any of the tested conditions. Interestingly, the absence of a fluoro substituent in the quinoxaline system was the only condition to loss the mutagenic capacity. From the genotoxicity point of view, this could point out the quinoxaline **10** for further studies.

According to the SI values showed in **Table 2** compound **14** could be considered for further testing so that its activity against the trypomastigote and amastigote forms of *T. cruzi* should be explored. However, compound **14** was genotoxic in both tested conditions, with and without metabolic activation. Therefore, none of the tested compounds will continue for further development.

In conclusion, thirteen new 1,4-di-*N*-oxide and two 1-*N*-oxide quinoxaline derivatives were synthesized and evaluated against *T. cruzi* identifying new derivatives with potent *in vitro anti-T. cruzi* activity. Ten out of the fifteen evaluated compounds resulted more active than the reference drugs Nfx and Bnz. Four of them stand out with IC_{50} lower than 1.5 μM. Due to the toxicity issues usually associated to this family of compounds; unspecific cytotoxicity assay and a genotoxicity screening test were included in the very early stages of the project. Four out of the five tested compounds were mutagenic in both tested conditions. Compound **10** was not mutagenic; however, its low SI value (SI=1.7) led us to discontinue the biological studies of this family of compounds against *T. cruzi*. An appropriate toxicological profile study should consider not only unspecific cytotoxicity as usual but also mutagenicity issues. Including toxicological studies in the very early phases of biological evaluation would allow reducing costs and time in the overall drug discovery process.

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