Esters of quinoxaline-7-carboxylate-1,4-di-*N*-oxide as *Trichomonas vaginalis* triosephosphate isomerase inhibitors

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Trichomoniasis is a public health problem world-
wide. Trichomoniasis treatment consists of the use
of nitroimidazole derivatives; however, therapeutic
ineffectiveness occurs in 5 to 20 % of the cases.
Therefore, it is essential to propose new pharma-
cological agents against this disease. In this work,
esters of quinoxaline-7-carboxylate-1,4-di-*N*-oxide
(EQX-NO) were evaluated in *in vitro* assays as novel
trichomonocidal agents. Additionally, an *in vitro*
enzyme assay and molecular docking analysis
against triosephosphate isomerase of *Trichomonas*
vaginalis to confirm their mechanism of action
were performed. Ethyl (compound 12) and *n*-pro-
pyl (compound 37) esters of quinoxaline-7-carboxy-
late-1,4-di-*N*-oxide derivatives showed tricho-
monocidal activity comparable to nitazoxanide,
whereas five methyl (compounds 5, 15, 19, 20 and
22), four isopropyl (compounds 28, 29, 30 and 34),
three ethyl (compounds 4, 13 and 23) and one *n*-
propyl (compound 35) ester derivatives displayed
activity comparable to albendazole. Compounds 6
and 20 decreased 100 % of the enzyme activity of
recombinant protein triosephosphate isomerase.

Keywords: quinoxaline 1,4-di-*N*-oxide, trichomo-
niasis, triosephosphate isomerase inhibitor

Trichomonas vaginalis (*T. vaginalis*) is a protozoon that poses an important public health
challenge (1–3). It colonizes the human urogenital tract by adhering to the epithelium.
T. vaginalis infection is often asymptomatic but can produce inflammation of the urinary

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tract. Trichomoniasis is a non-viral sexually diffused infection that affects approximately 276 million people per year (4, 5).

The drug of choice for trichomoniasis is metronidazole, a 5-nitroimidazole derivative (5); however, it causes severe adverse effects since it is carcinogenic and teratogenic (6). These effects have been associated with prolonged treatment or high doses (7, 8). Additionally, the expansion of resistant *T. vaginalis* strains makes new antitrichomoniasis agents necessary.

Quinoxaline (QX) is a privileged structure for the design and development of new antibacterial (9), anticancer (10), and antiparasitic (11) agents with some derivatives in the clinical phase studies (12, 13). Interestingly, quinoxaline 1,4-di-*N*-oxide (QX-NO) derivatives show biological effects against diverse protozoa (14–17); however, QX-NO has not been evaluated against *T. vaginalis*. Therefore, in this work, the esters of quinoxaline-7-carboxylate-1,4-di-*N*-oxide (EQX-NO) derivatives were evaluated as novel antitrichomoniasis agents. Furthermore, *in silico* and *in vitro* analyses were done against *T. vaginalis* triosephosphate isomerase to elucidate their mechanism of action.

EXPERIMENTAL

Syntheses

All EQX-NO derivatives were synthesized using the method reported by Gomez-Caro *et al.* (18). All molecules were identified by IR spectroscopy, ¹H NMR and elemental analysis of C, H and N (see Table SI, Supplementary material), as in previous research (14, 15, 17, 18).

In vitro evaluation against *T. vaginalis*

T. vaginalis strain GT3 (a wild strain isolated in Centro Medico Nacional Siglo XXI, Instituto Mexicano del Seguro Social, Mexico) was used. EQX-NO derivatives, albendazole (ABZ), metronidazole (MTZ), and nitazoxanide (NTZ) were tested against *T. vaginalis* strain GT3 *in vitro* using the previously reported method (19). Dimethylsulfoxide (DMSO) was used as a negative control. The half-maximal inhibitory concentration (*IC*₅₀) was calculated using the Probit analysis.

In silico procedures

Protein structure. – The atomic coordinates of triosephosphate isomerase (TIM) from *T. vaginalis* (TvTIM) from the Protein Data Bank (PDB, code 3QSR) were used with Molecular Operating Environment (MOE) software and CHARMM27 force field (20, 21). This structure was used to predict binding free energy (ΔG_{bind}) values between TvTIM and EQX-NO derivatives. Site Finder implemented in the MOE was used to determine potential binding sites (22, 23).

Molecular docking. – For molecular docking, the receptor (TIM, 3QSR) was kept inflexible, while examining twenty conformations for each of the ligands. All crystallographic water molecules were deleted from the initial structures. Molecular docking was done

with the MOE software using the previously reported procedure (24, 25). The molecules were analyzed on twenty-three potential binding sites from TvTIM (Table SII, Supplementary material) to propose a potential interaction site that explains the effect on enzyme activity.

To describe the interaction of the ligands, the binding affinity of each complex was estimated with the generalized Born/volume integral ratio (GB/VI) using parameters in MOE (26). The generalized Born or non-bonded interaction energies comprised van der Waals, Coulomb electrostatic interactions, and implied solvent interaction energies.

In vitro assays against TvTIM

Stock solutions of EQX-NO derivatives were prepared in DMSO (1 %) and further diluted with media to obtain 50 and 100 $\mu\text{g } \mu\text{L}^{-1}$ of each compound tested. Recombinant TvTIM was obtained as previously described (27, 28). The conversion of glyceraldehyde 3-phosphate (GAP) into dihydroxyacetone phosphate was followed to determine enzyme activity following a previous procedure (29–35).

RESULTS AND DISCUSSION

Methyl (*m*-), ethyl (*e*-), isopropyl (*i*-), and *n*-propyl (*np*-) EQX-NO derivatives were obtained (see Supplementary material) (18) and evaluated *in vitro* against *T. vaginalis*. Table I shows their trichomonocidal activity.

Trichomonocidal activity

Among *m*- and *e*-EQX-NO derivatives, twelve compounds had an IC_{50} between 1 and 10 $\mu\text{mol L}^{-1}$, and seven compounds had a higher IC_{50} value than ABZ (Table I). However, none of the *m*- and *e*-EQX-NO derivatives had trichomonocidal activity greater than MTZ and NTZ. Only compound **12**, an ethyl methyl-3-methyl-quinoxaline-2,7-dicarboxylate-1,4-di-*N*-oxide, had IC_{50} of 0.97 $\mu\text{mol L}^{-1}$, a value comparable or equal to both reference drugs (MTZ and NTZ). In general, the structure-activity relationship (SAR) analysis showed that aliphatic, ethoxy and methoxy groups at R1-position on the QX ring enhance biological effects. Additionally, the steric effect at R1-position is a key factor in the activity. A trifluoromethyl and a methyl group at R2- and R3-position, resp., also showed trichomonocidal activity.

None of the five *np*-EQX-NO derivatives showed trichomonocidal activity, but two compounds had IC_{50} values from 5.0 to 6.2 $\mu\text{mol L}^{-1}$, whereas two more had an IC_{50} value $< 3 \mu\text{mol L}^{-1}$ (Table I). Compounds **35** (*n*-propyl methyl-3-methylquinoxaline-2,7-dicarboxylate-1,4-di-*N*-oxide) and **37** (*n*-propyl ethyl-3-methylquinoxaline-2,7-dicarboxylate-1,4-di-*N*-oxide) showed better trichomonocidal activity than ABZ. Both compounds have an ester and methyl group at R1- and R2-position, resp., on the QX-NO ring. Additionally, compound **37** showed activity comparable to NTZ.

From *i*-EQX-NO derivatives, nine compounds showed no trichomonocidal activity ($IC_{50} > 10 \mu\text{mol L}^{-1}$) and eight compounds had an IC_{50} value from 2.9 to 6.4 $\mu\text{mol L}^{-1}$.

Table I. The half-maximal inhibitory concentration (IC_{50}) of EQX-NO derivatives against *T. vaginalis*

Compd.	R ₁	R ₂	R ₃	IC_{50} ($\mu\text{mol L}^{-1}$) ^a
1	C ₆ H ₅	CH ₃	CH ₃	> 10
2	C ₆ H ₅	CH ₃	CH ₃ CH ₂	> 10
3	CH ₃	CF ₃	CH ₃ CH ₂	> 10
4	C ₁₀ H ₇	CF ₃	CH ₃ CH ₂	3.52 ± 0.07
5	C ₄ H ₃ S	CF ₃	CH ₃	2.63 ± 0.01
6	C ₄ H ₃ S	CF ₃	CH ₃ CH ₂	> 10
7	NHC ₆ H ₅	CH ₃	CH ₃	> 10
8	OCH ₂ CH ₃	CH ₃	CH ₃	> 10
9	OCH ₂ CH ₃	CH ₃	CH ₃ CH ₂	5.93 ± 0.23
10	C ₆ H ₅	CF ₃	CH ₃	5.32 ± 0.12
11	OCH ₃	CH ₃	CH ₃	6.19 ± 0.32
12	OCH ₃	CH ₃	CH ₃ CH ₂	0.97 ± 0.04
13	CH ₂ CH ₃	CF ₃	CH ₃ CH ₂	2.51 ± 0.09
14	C ₆ H ₅ Cl	CF ₃	CH ₃ CH ₂	> 10
15	C ₁₀ H ₇	CF ₃	CH ₃	4.29 ± 0.12
16	C ₄ H ₃ O	CF ₃	CH ₃	> 10
17	CH ₃	CH ₃	CH ₃	> 10
18	OCH ₂ CH ₃	CH ₂ COOCH ₂ CH ₃	CH ₃	> 10
19	CH ₂ CH ₃	CF ₃	CH ₃	3.25 ± 0.05
20	CH(CH ₃) ₂	CF ₃	CH ₃	4.01 ± 0.11
21	OCH ₂ C ₆ H ₅	CH ₃	CH ₃	> 10
22	OCH ₂ CH ₃	C ₆ H ₅	CH ₃	2.55 ± 0.03
23	OCH ₂ CH ₃	C ₆ H ₅	CH ₃ CH ₂	2.92 ± 0.01
24	NH ₂	CH ₃	CH ₃ CH ₂	6.00 ± 0.04
25	OCH ₃	CH ₃	(CH ₃) ₂ CH	> 10
26	OCH ₂ CH ₃	CH ₃	(CH ₃) ₂ CH	> 10
27	OC(CH ₃) ₃	CH ₃	(CH ₃) ₂ CH	> 10
28	OCH ₂ CH ₃	CH ₂ COOCH ₂ CH ₃	(CH ₃) ₂ CH	3.50 ± 0.12
29	C ₄ H ₃ S	CF ₃	(CH ₃) ₂ CH	3.68 ± 0.04

Compd.	R ₁	R ₂	R ₃	IC ₅₀ (μmol L ⁻¹) ^a
30	CH ₃	CF ₃	(CH ₃) ₂ CH	3.77 ± 0.02
31	C ₆ H ₅	CF ₃	(CH ₃) ₂ CH	5.25 ± 0.01
32	C ₁₀ H ₇	CF ₃	(CH ₃) ₂ CH	5.41 ± 0.01
33	C ₄ H ₃ O	CF ₃	(CH ₃) ₂ CH	> 10
34	CH(CH ₃) ₂	CF ₃	(CH ₃) ₂ CH	3.21 ± 0.07
35	OCH ₃	CH ₃	CH ₃ CH ₂ CH ₂	2.56 ± 0.11
36	C ₄ H ₃ S	CF ₃	CH ₃ CH ₂ CH ₂	5.04 ± 0.12
37	OCH ₂ CH ₃	CH ₃	CH ₃ CH ₂ CH ₂	1.46 ± 0.01
38	C(CH ₃) ₃	CH ₃	CH ₃ CH ₂ CH ₂	> 10
39	NHC ₆ H ₅	CH ₃	(CH ₃) ₂ CH	> 10
40	C ₆ H ₅	CH ₃	(CH ₃) ₂ CH	6.46 ± 0.08
41	C(CH ₃) ₃	C(CH ₃) ₃	(CH ₃) ₂ CH	> 10
42	NH ₂	CH ₃	(CH ₃) ₂ CH	> 10
43	C ₄ H ₃ O	CF ₃	CH ₃ CH ₂ CH ₂	> 10
44	OCH ₂ CH ₃	CF ₃	(CH ₃) ₂ CH	2.98 ± 0.07
45	C(CH ₃) ₃	CF ₂ CF ₂ CF ₃	(CH ₃) ₂ CH	> 10
46	CF ₂ CF ₃	CF ₃	(CH ₃) ₂ CH	> 10
47	OCH ₂ CH ₃	CF ₃	CH ₃ CH ₂ CH ₂	> 10
48	NHC ₆ H ₅	CH ₃	CH ₃ CH ₂ CH ₂	> 10
49	CH ₃	CH ₃	CH ₃ CH ₂ CH ₂	6.24 ± 0.41
50	NH ₂	CH ₃	CH ₃ CH ₂ CH ₂	> 10
51 ^b	OCH ₃	CH ₃	CH ₂ CH ₃	> 10
52 ^b	C ₆ H ₅	CF ₃	CH ₃	4.94 ± 0.12
53 ^b	CH(CH ₃) ₂	CF ₃	CH ₃	> 10
ABZ				4.08 ± 0.21
MTZ				0.69 ± 0.01
NTZ				1.00 ± 0.05

ABZ – albendazole, MTZ – metronidazole, NTZ – nitazoxanide

^a Mean ± SD of triplicate determinations.

^b Quinoxaline derivatives without *N*-oxide groups.

Although five compounds had lower IC₅₀ values (< 4.0 μmol L⁻¹) than ABZ, none showed better trichomonocidal activity than MTZ and NTZ (Table I).

Finally, the *N*-oxide groups on the QX ring from some EQX-NO derivatives were eliminated (by reduction with sodium dithionite) to determine their effect on trichomonocidal activity (Table I). EQX-NO derivatives **10**, **12**, and **20** with trichomonocidal activity below

6.0 $\mu\text{mol L}^{-1}$ were selected to obtain QX derivatives **51**, **52**, and **53** (see Supplementary material), resp. These new compounds showed no or low biological activity, except compound **52** (methyl 2-benzoyl-3-trifluoromethyl quinoxaline-7-carboxylate), which had an antiprotozoal effect against *T. vaginalis*. These results show that the *N*-oxide groups are the key factor for the trichomonocidal activity and suggest that compound **52** has a different mechanism of action than EQX-NO derivatives. Still, the mechanism of action of QX-NO is currently not clear, as reported by Cheng *et al.* (12).

Enzymatic activity of TvTIM

Previously, Guzman *et al.* (32), reported that QX-NO derivatives affect the enzymatic activity of *Trypanosoma cruzi* TIM. Therefore, in this work, TvTIM was considered a potential drug target. All EQX-NO derivatives were evaluated in enzyme activity assays, but only four compounds (**6**, **10**, **20** and **42**) decreased TvTIM activity to some degree. The effect of compounds **6** (ethyl 2-(thiophene-2-carbonyl)-3-trifluoromethylquinoxaline-7-carboxylate-1,4-di-*N*-oxide), **10** (methyl 2-benzoyl-3-trifluoromethylquinoxaline-7-carboxyl-

Table II. Effect of EQX-NO derivatives against the enzymatic activity of TvTIM, predictive binding free energy value and main residues in the interaction on the active site

Compd.	TvTIM enzymatic activity (%) ^a		ΔG_{bind} (kcal mol ⁻¹)	Main amino acids in the interactions
	50 g L ⁻¹	100 g L ⁻¹		
6	49.6 ± 4.1	0	−0.6	Glu166
10	69.0 ± 4.5	38.3 ± 3.2	−7.3	Lys11, Gly172, and Ser212
20	49.6 ± 2.5	0	−3.2	Lys11, Ile171, and Gly172
42	51.6 ± 4.1	30.3 ± 4.0	−4.7	Lys11, Ala170, Ile171, and Gly172

ΔG_{bind} – binding free energy

^a Mean ± SD of triplicate determinations.

Fig. 1. The main interaction site on TvTIM for the compounds **6**, **10**, **20**, and **42**. Residues Lys11, His94, and Glu166 (red color) and seven conformers of each compound (pink color).

ate-1,4-di-*N*-oxide), **20** (ethyl methyl-3-methyl-quinoxaline-2,7-dicarboxylate-1,4-di-*N*-oxide), and **42** (isopropyl 2-amide-3-methyl-quinoxaline-7-carboxylate-1,4-di-*N*-oxide) on the activity of TvTIM was determined in two concentrations (50 and 100 $\mu\text{g mL}^{-1}$). The results are displayed in Table II. Three of the abovementioned EQX-NO derivatives (**6**, **20**, and **42**) showed similar enzyme activity inhibition (50 % approx.) at 50 $\mu\text{g mL}^{-1}$, and only compounds **6** and **20** showed 100 % enzyme inhibition at 100 $\mu\text{g mL}^{-1}$. These compounds caused a decrease in the function of TvTIM at higher concentrations than the other reported compounds (34–36). Nevertheless, this is the first report that shows that EQX-NO derivatives have an inhibitory effect against TvTIM. Previously, Cheng *et al.* (12) mentioned two sulfonamide QX-NO derivatives with a better trichomonocidal activity than metronidazole, however, the mechanism of action was not explored. Therefore, QX-NO derivatives are a new option to develop new TvTIM inhibitors.

Fig. 2. Interaction site between EQX-NO derivatives and the potential active site of TvTIM. a) compound **6**, b) compound **10**, c) compound **20**, and d) compound **42**.

Molecular docking analysis on TvTIM

Molecular docking analysis was done to describe the molecular interaction of the EQX-NO derivatives on 23 potential TvTIM binding sites (Table SII, Supplementary material).

The compounds that affected the enzymatic activity (**6**, **10**, **20**, and **42**) showed the best interactions with Lys11, His94, and Glu166, among other residues, near the active site (Fig. 1). The best interactions for the four compounds were determined in the region of residues near Lys11, His94, Glu166, Ala170, Ile171, Gly172, and Ser212 (Fig. 2). Additionally, predictive ΔG_{bind} was determined (Table II). Compound **10** showed the lowest ΔG_{bind} value ($-7.3 \text{ kcal mol}^{-1}$); however, this compound showed the lowest enzyme inhibition (31 %) at $50 \mu\text{g } \mu\text{L}^{-1}$. The other three compounds (**6**, **20**, and **42**) showed very different ΔG_{bind} values but comparable enzyme inhibition. These results suggest no correlation between *in vitro* and *in silico* analysis. Therefore, compounds **6**, **10**, **20**, and **42** could be interacting on another binding site of TvTIM, causing an effect on the enzyme activity assays. The molecular docking results from the other QX-NO derivatives showed a probable interaction in the interface and core of TvTIM, which can be related to not affecting the enzymatic activity of TvTIM.

CONCLUSIONS

Four different types of EQX-NO derivatives were tested as novel agents against *T. vaginalis*. e-EQX-NO derivatives showed a better trichomonocidal effect than BZN. In particular, compound **12** (ethyl methyl-3-methyl-quinoxaline-2,7-dicarboxylate-1,4-di-*N*-oxide) *in vitro* assays show a better trichomonocidal activity than ABZ and NTZ. Additionally, four compounds **6** (ethyl 2-(thiophene-2-carbonyl)-3-trifluoromethylquinoxaline-7-carboxylate-1,4-di-*N*-oxide), **10** (methyl 2-benzoyl-3-trifluoromethylquinoxaline-7-carboxylate-1,4-di-*N*-oxide), **20** (ethyl methyl-3-methyl-quinoxaline-2,7-dicarboxylate-1,4-di-*N*-oxide), and **42** (isopropyl 2-amide-3-methyl-quinoxaline-7-carboxylate-1,4-di-*N*-oxide) reduced the enzymatic activity of recombinant protein TvTIM. SAR analysis showed that methyl and ethyl groups at R3-position and *N*-oxide groups on the QX ring are the key moieties for the biological activity. Molecular docking analysis revealed that the main interaction site of these compounds is near to the active site where they interact with Lys11, Glu166, Ala170, Ile171, and Gly172. Therefore, these types of compounds could help in the development of new and more effective TvTIM inhibitors to combat the *T. vaginalis* resistance.

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