Analysis of the effect of methyl 2-acetamide-3-methylquinoxaline-7-carboxylate 1,4-di-*N*-oxide on the relative expression of the trypanothione reductase gene in *Trypanosoma cruzi* epimastigotes

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Abstract: In recent decades, some quinoxaline 1,4-di-*N*-oxide derivatives have been shown to have better trypanocidal activity than the reference drugs; however, their mechanism of action is not yet clear, although it is suggested that they mainly produce reactive oxygen species that cause oxidative stress and parasite death. *Trypanosoma cruzi* relies on the enzyme trypanothione reductase, among others, to defend itself against oxidative stress. With the aim of contributing to the elucidation of the mechanism of action of quinoxaline 1,4-di-*N*-oxide derivatives on *Trypanosoma cruzi*, this study was carried out to evaluate the effect of methyl 2-amide-3-methylquinoxaline-7-carboxylate 1,4-di-*N*-oxide (compound M-8) on the expression of the trypanothione reductase gene in an *in vitro* model on *Trypanosoma cruzi* epimastigotes of the CL-Brener strain. The results show that compound M-8 does not cause a significant effect on the trypanothione reductase gene, suggesting a mechanism of action not related to oxidative stress.

Keywords: Gene expression, quinoxaline, 1,4-di-*N*-oxide, trypanothione reductase, *Trypanosoma cruzi*, Chagas disease

INTRODUCTION

Trypanosoma cruzi (*T. cruzi*) is an intracellular parasite characterized by the presence of a single flagellum and a single mitochondrion. Its genome is ordered in a complex and compact region called a kinetoplast. *T. cruzi* is the etiological agent of Chagas disease, which is also known as American trypanosomiasis (Carrada-Bravo, 2004; Bombaca *et al*., 2019). Initially, this disease only appeared in Latin America; however, its presence has now been reported in other continents. In Latin America, it is estimated that between 6 and 7 million people are infected (Hotez *et al.,* 2013; Tarleton *et al.,* 2014; Lara-Ramirez *et al*., 2017). To treat the disease, two nitroheterocyclic compounds are available: a nitrofuran (nifurtimox, Nfx) and a nitroimidazole derivative (benznidazole, Bzn), which generate oxidative stress on the parasite. Oxidative stress is defined as the imbalance between the production of reactive oxygen species and the antioxidant capacity of cells. It has been proposed that the nitro group of Bzn and Nfx is reduced to an amino group by nitroreductases of the parasite with the formation of an intermediate free radical and electrophilic metabolites (D 'Silva *et al.,* 2002; Irigoín *et al.,* 2008; Rivera *et al*., 2014; Kashif *et al*., 2018). Since the reactive oxygen species generated by these drugs do not discriminate between parasite and host molecules, both compounds produce severe side effects which include in Bzn: allergic dermatitis, peripheral neuropathy, weight loss and insomnia, while Nfx produces weight loss, polyneuropathy, nausea, vomiting, headache and vertigo (Rivera *et al*., 2014; Vázquez-Jiménez *et al*., 2019). Based on the aforementioned, the search for new therapeutic alternatives for Chagas disease is a priority.

Quinoxaline 1,4-di-*N*-oxide derivatives have been shown to have multiple biological properties (Abid and Azam, 2005, Deepika *et al.,* 2011, Estevez *et al.,* 2011; Quiliano *et al*., 2017; Chacón-Vargas *et al*., 2018; Palos *et al*., 2018); among these we can highlight their trypanocidal properties. However, their mechanism of action has not been clearly established, although it has been suggested that quinoxaline 1,4-di-*N*-oxide derivatives generate oxidative stress through the production of reactive oxygen species (Maya *et al.,* 2007; Chacón-Vargas *et al*., 2018). Oxidative stress in *T. cruzi* is mainly regulated by the enzyme trypanothione reductase (TR), which is a flavoprotein disulfide reductase dependent on NADPH that maintains the oxidation-reduction balance inside the parasite (Algranati *et al.,* 2006; Vázquez *et al*., 2017; Bombaca *et al*., 2019). *T. cruzi* does not produce catalase or glutathione reductase as in mammals.

In 2014, our research group performed the *in vitro* evaluation of 33 compounds derived from methyl and ethyl quinoxaline-7-carboxylate 1,4-di-*N*-oxide on the strains NINOA and INC-5 in the trypomastigote phase of *T. cruzi.* In this study, three compounds (M7, M8 and E4, fig. 1) showed a greater anti-*T. cruzi* activity than the reference drugs (Nfx= 32.7% and 48.3%, Bnz= 30.8%

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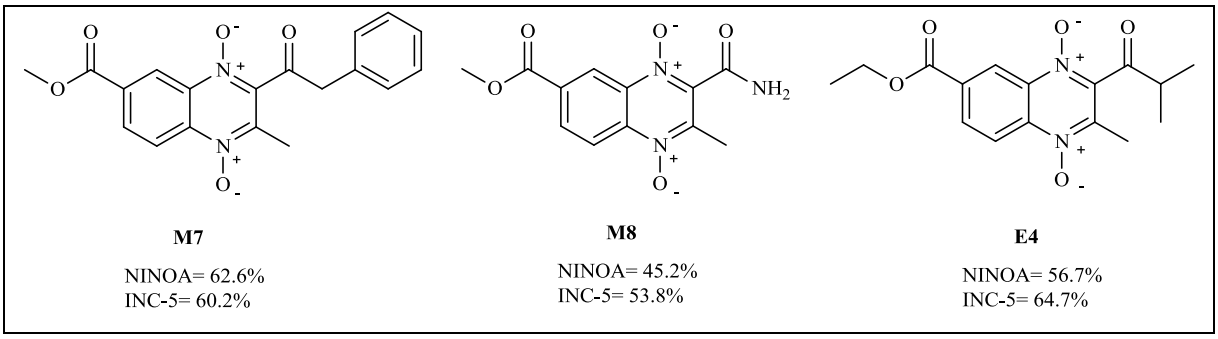

Fig. 1: Structure and trypanocidal activity of methyl and ethyl quinoxaline-7-carboxylate 1,4-di-*N*-oxide derivatives at 10 µg/mL

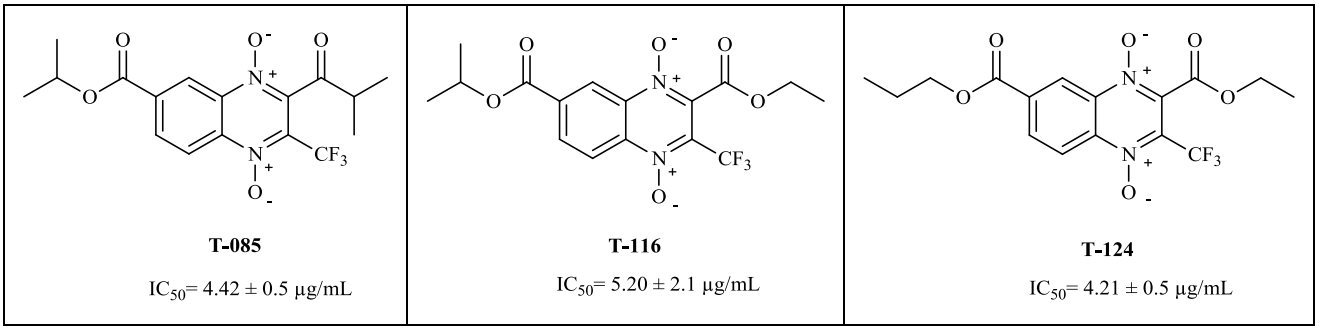

Fig. 2: Structure and trypanocidal activity of n-propyl and isopropyl quinoxaline-7-carboxylate 1,4-di-*N*-oxide derivatives in epimastigotes of the INC-5 strain of *T. cruzi*

and 38.4%, respectively, for NINOA and INC-5 strains). In addition, through a study of molecular coupling, it was possible to determine that this type of compounds could be *T. cruzi* trypanothione reductase inhibitors (Villalobos-Rocha *et al.*, 2014).

Subsequently, in 2017, 26 new n-propyl and isopropyl quinoxaline-7-carboxylate 1,4-di-*N*-oxide derivatives were tested against the INC-5 strain of *T. cruzi* in the epimastigote and trypomastigote phases. Of these derivatives, three compounds presented trypanocidal activity in epimastigotes of strain INC-5 with an IC_{50} less to 10 µM (fig. 2). Molecular coupling studies and enzyme assays indicated that compound T-085 acts as a trypanothione reductase inhibitor (Chacón-Vargas *et al*. 2018).

Based on the foregoing, in order to contribute to the elucidation of the mechanism of action of quinoxaline 1,4-di-*N*-oxide derivatives on *T. cruzi*, in this work, compound M-8 was selected (fig. 1). This compound was previously reported by Villalobos-Rocha *et al.* in 2014 as a trypanocidal agent; therefore, in this study its effect on the expression level of the TR gene was determined. This would indirectly indicate an effect on trypanothione reductase through two possible mechanisms: inhibition of trypanothione reductase or overproduction of trypanothione reductase to regulate the oxidative stress produced by the presence of reactive oxygen species. In order to contribute to the development of more effective quinoxaline 1,4-di-*N*-oxide derivatives for the treatment of Chagas disease, this study was done.

MATERIALS AND METHODS

Strain cultures

The epimastigotes of the CL-Brener strain of *T. cruzi* obtained from the National School of Biological Sciences of the National Polytechnic Institute in Mexico City were cultured. The parasites were preserved in the middle of heart brain infusion (BHI) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 500 µg/mL at 28°C. Epimastigotes grew exponentially by passes every 7 days.

Trypanocidal activity

The evaluation of the trypanocidal activity of compound M-8 on epimastigotes of *T. cruzi* was carried out with the procedure described by Benítez *et al.* in 2011 with some modifications. Initially, BHI medium supplemented with 10% FBS and 1% penicillin/streptomycin 500 µg/mL was prepared. Afterwards, parasites were assessed in the exponential phase. In a 96-well plate, 90 µL of the parasites ($1x10^6$) and 10 µL of compound M-8 were evaluated at seven different concentrations (400, 200, 100, 50, 25, 12.5 and 6.25 µg/mL) prepared with

Table 1: Primers and reaction conditions used for the TR and HGPRT gene

Gene	Foward	Reverse	Fragment size	Amplification conditions
TR	CTCTACAAGAAGCGGGTTGC	CTGAGAGTGGTGCGATCAAA	191 bp	95ºC for 5min; 39 cycles at 95°C for 15 s, 60°C for 20 s and 72°C for 15 s
HGPRT	CTACAAGGGAAAGGGTCTGC	ACCGTAGCCAATCACAAAGG	412 bp	

dimethylsulfoxide (DMSO) at a concentration of 2%, using parasites without treatment as negative controls and Nfx and Bzn-treated parasites as positive controls. Subsequently, the plate was incubated at 28°C for 24 h, then 10 µL of diphenyltetrazolium bromide (MTT) and 2.5 mg/mL in 1X phosphate buffered saline (PBS) were added and the plate was incubated for 4 h at 37°C; 100 µL of sodium dodecyl sulfate-hydrochloric acid (SDS-HCl) (10% -0.01 N) was added and the plate was again incubated at 28 °C for 24 h. Finally, the optical density was determined at 595 nm in a spectrophotometer (Biorad iMark™). The mortality percentages were obtained using the following formula: Percentage of mortality= 100-(DO(t)/DO(c)x100), where DO(t) is the optical density of the culture after exposure to the concentration of the substance being evaluated and DO(c) is the optical density of the control culture. Trypanocidal activity was expressed as the percentage of reduction of cell viability with respect to the controls and was carried out in triplicate. The results of the trypanocidal activity obtained were subjected to a one-way ANOVA analysis. The comparison of means was carried out using the Tukey test. A $p < 0.05$ was considered significant. The statistical analysis was carried out using SPSS v.22.0 statistical software (IBM Corp., Armonk, NY).

T. cruzi RNA extraction

BHI medium supplemented with 10% FBS and 1% penicillin-streptomycin 500 µg/mL was prepared and 90 µL of epimastigotes [$1x10^6$] and 10 µL of compound M-8 at three concentrations (25, 100 and 200 µg/mL) were added to a 96-well plate. Samples were taken at different times: 0, 2, 4, 8, 12 and 24 h (in triplicate), and the plate was kept in incubation at 28°C for 24 h. Total RNA extraction was carried out using the protocol of the SV Total RNA Isolation System commercial extraction kit (Promega®). Quantitation of total RNA was performed on a Thermo Scientific® Nanodrop 2000 device.

T. cruzi cDNA synthesis

Initially, 2 ng of total RNA was incubated at 70°C for 10 min, and then centrifuged for 30 s and kept on ice. The synthesis of first-strand cDNA was carried out using the protocol of the commercial transcription case A3500 of Promega®. Reverse transcription reactions containing 25 mM $MgCl_2$, 10X reverse transcription buffer, 10 mM dNTP's, 1 U ribonuclease inhibitor/µL, 25 U/µL reverse transcriptase, oligonucleotide $(dT)_{15}$ 0.5 µg/µL were prepared. Nuclease-free water was used to obtain a volume of 20 µL. Finally, the reactions were incubated in a SimpliAmp Thermal Cycler® thermocycler at 42 °C for 15 min, then heated at 95 °C for 5 min and incubated at 5°C for 5 min.

Analysis of the relative expression of the trypanothione reductase gene

The expression levels of the TR gene in the epimastigotes exposed to compound M8 at concentrations of 25, 100 and 200 µg/mL at 0, 2, 4, 8, 12 and 24 h were compared with time 0 in all cases. The reaction conditions and the primers used for the TR gene (Mejía-Jaramillo *et al*., 2011), as well as for the reference gene (hypoxanthineguanine phosphoribosyltransferase, HGPRT) (Murta *et al*., 2006), are shown in table 1. Reactions were established in a total volume of 10 µL using sterile MiliQ water, 1 µL of cDNA, 5 µL Green Supermix and 1 µL of each specific primer in the CFX96 Real-Time System, BIO RAD®. For the relative quantification, a standard deviation of ± 1 and a $p < 0.05$ were used. This was evaluated using BIO RAD CFX Manager Version 3.1 software (Vázquez-Jiménez *et al*., 2019).

RESULTS

Trypanocidal activity

The trypanocidal activity (percentage of mortality) of compound M-8 and the reference drugs at six concentrations on epimastigotes of the CL-Brener strain of *T. cruzi* is shown in fig. 3.

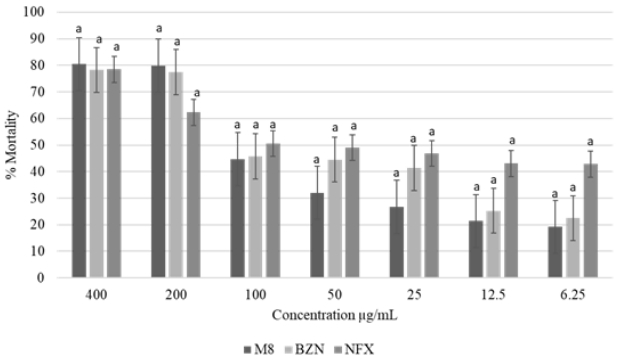

Fig. 3: Mortality percentage of the compound M8 and the reference compounds benznidazole (Bzn) and nifurtimox (Nfx) on the CL-Brener strain. The means that do not share a letter are significantly different according to the Tukey Test ($P<0.05$). The values are presented as the mean ± SD of the experiment in triplicate.

Analysis of the relative expression of the TR gene

The results of the effect of compound M-8 on the expression of the TR gene of the CL-Brener strain of *T. cruzi* is shown in fig. 4. The determination of the constitutive gene *HGPRT* allowed a comparison of the expression of the TR gene by the effect of compound M-8, observing that at no time was there a significant effect of compound M-8 on the expression level of the TR gene, obtaining an average of the mean cycle threshold (Ct) of 33.29 ± 0.250 (mean ± SE) for the TR gene and of 33.90 ± 0.532 (mean ± SE) for the constitutive gene. The average value of Ct that was obtained for the samples was 33.59 cycles.

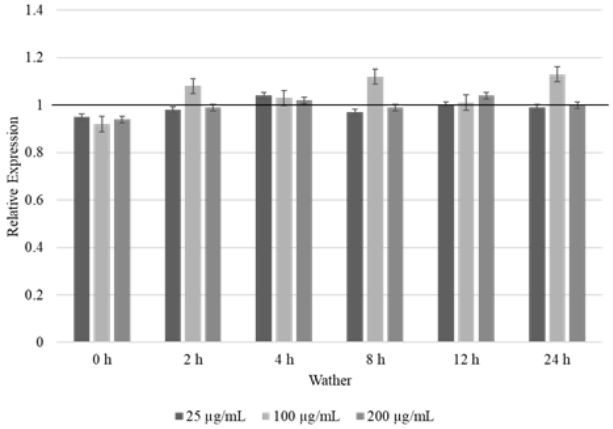

Fig. 4: Relative expression levels of the trypanothion reductase gene of the CL-Brener strain of *T. cruzi*. The graph shows the data of the average Ct value of each gene evaluated in duplicate in three independent experiments; the bars in gray represent expression of the TR gene; the black line represents expression of the *HGPRT* gene. The bar above represents the SE of three independent experiments performed in duplicate.

DISCUSSION

Trypanocidal activity

Compound M-8 caused a mortality percentage of 80.45% at the highest concentration used (400 µg/mL) and a mortality percentage lower than 20% at the lowest concentration used (6.25 µg/mL). The trypanocidal effect observed in the latter concentration contrasts with that reported by Villalobos-Rocha *et al.* in 2014 on trypomastigotes (NINOA strain 45.2%; strain INC-5 53.8% at 5 µg/mL). This may be due to a difference between the parasitic phases analyzed in each study. Considering the reference drugs at the concentration of 400 µg/mL both had a trypanocidal effect (Nfx 78.47% and Bzn 78.27%) equal to that of compound M8. The same trypanocidal activity between Bzn and compound M8 was maintained in all the concentrations analyzed.

Analysis of the relative expression of the TR gene

Different mechanisms of action have been described in the literature for quinoxaline-1,4-di-*N*-oxide derivatives. These include the direct action of the nitro-anion radical on macromolecules of great importance such as DNA; generation of oxygen free radicals; production of nitrous oxide and hydroxylamine derivatives that possess high cytotoxicity; and finally, inhibition of the TR enzyme (Ihsan *et al.*, 2011; Viñas *et al.*, 2012; García *et al.*, 2013; Chacon-Vargas *et al*., 2017). However, it remains unclear whether all quinoxaline 1,4-di-*N*-oxide derivatives act in the same way or are specific protein inhibitors.

The real-time PCR analysis of the effect of compound M-8 on the TR gene of the CL-Brener strain of *T. cruzi* allowed us to determine the expression of this gene at the evaluated times (0, 2, 4, 8, 12 and 24 h) and at three concentrations (25, 100 and 200 µg/mL). The results show that compound M-8 did not alter the expression levels of the *T. cruzi* TR gene at any time at any evaluated concentration. This may be due to several situations: a) a mechanism of resistance of the CL-Brener strain to reactive oxygen species produced by compound M-8 that does not affect production of the TR enzyme; b) a normal production of the TR enzyme that efficiently eliminates reactive oxygen species produced by compound M-8; c) compound M-8 does not show any inhibitory effect on the TR enzyme that alters gene expression as a compensatory effect. This confirms what was reported by Chacón-Vargas in 2017. However, it should be mentioned that compound M-8 had a trypanocidal effect on the CL-Brener strain, with which it can be suggested that this activity is due to the involvement of another or other mechanisms of action (Villalobos-Rocha *et al.*, 2014; Chacón-Vargas *et al.*, 2017).

CONCLUSION

The analysis of the effect of compound M-8 (Methyl 2-acetamide-3-methylquinoxaline-7-carboxylate 1,4-di-*N*-oxide) on the expression levels of the TR gene in *T. cruzi* epimastigotes by real-time PCR show a level of stable TR gene expression at all times and concentrations analyzed; however, compound M-8 showed trypanocidal activity suggesting that its biological effect is due to another type of mechanism of action that does not involve the trypanothione reductase enzyme.

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