Effect of 4-amino-3-nitrobenzoic acid on the expression level of the *trans*-sialidase gene in *Trypanosoma cruzi* epimastigotes

Lenci K Vazquez-Jimenez[1], Alma D Paz-Gonzalez[1], Muhammad Kashif[1], Karina J Juarez-Rendon[1], Benjamín Nogueda-Torres[2], Virgilio Bocanegra-Garcia[1] and Gildardo Rivera[1]*

[1]Laboratorio de Biotecnología Farmacéutica, Centro de Biotecnología Genómica, Instituto Politécnico Nacional, Reynosa, México
[2]Departamento de Parasitología, Escuela Nacional de Ciencias Biologicas Instituto Politécnico Nacional, Ciudad de México, México

Abstract: *Trans*-sialidase of *Trypanosoma cruzi* (TcTS) is a key enzyme in the infection process from parasite to host; therefore, it has been considered an important target for developing new anti-Chagas drugs. Different compounds with trypanocidal activity and/or inhibition of TcTS have been reported; however, some benzoic acid derivatives have shown high enzymatic inhibition but low trypanocidal activity and viceversa. These results show that each compound may possess a different mechanism of action. Based on the above, the compound 4-amino-3-nitrobenzoic acid (16), a potent TcTS inhibitor (77% inhibition in enzymatic assays) was selected to evaluate its effects on the expression level of the TS gene in *T. cruzi* epimastigotes and determine its involvement in the mechanism of action. Results showed an increase in the expression level of the TcTS gene, which confirmed that compound 16, has a direct effect on TcTS.

Keywords: Benzoic acid, *trans*-sialidase, *Trypanosoma cruzi*, Chagas disease.

INTRODUCTION

Trypanosoma cruzi (*T. cruzi*) is the causative agent of Chagas disease or American trypanosomiasis, which was first described more than one hundred years ago and still challenges clinicians worldwide. *T. cruzi* is a flagellated protozoan parasite transmitted to humans via insects such as *Triatoma infestans*, *Rhodnius prolixus* and *Triatoma dimidiata*. Transmission can also happen congenitally, by blood transfusion, during organ transplantation, and by the ingestion of contaminated food or drink (Rassi *et al.*, 2012; Pérez-Molina *et al.*, 2015; Kashif *et al.*, 2017a).

Chagas disease is divided into two phases: acute and chronic. The acute phase is usually asymptomatic; patients only show signs of infection (Romaña´s sign and Chagoma). In the chronic phase, patients present alterations in the esophagus, colon and heart. Only two drugs are currently available for treatment of the disease, nifurtimox (Nfx) and benznidazole (Bzn). These have been in use from the last four decades with an efficacy of approximately 80% and 40-60% in the acute and chronic phase, respectively. In addition, both drugs cause severe adverse effects, such as nausea, vomiting, anorexia, headache, depression of the central nervous system, seizures, paresthesias, vertigo, dermatitis and peripheral polyneuropathies, among others, which often cause treatment to stop (Rivera *et al.*, 2014; Bern, 2015; Arce-Fonseca *et al.*, 2018).

One of the most important pharmacological targets in *T. cruzi* (Tc) is the enzyme *trans*-sialidase (TS), a glycosylphosphatidylinositol (GPI) protein that is associated with the parasite membrane and catalyzes the process of the transfer of sialic acid from the host cell to its own surface mucin as a glycoprotein. Sialic acid is vital for the growth of *T. cruzi* since the parasite is unable to synthesize it de novo (Neres *et al.*, 2008; Rivera *et al.*, 2014; Kashif *et al.*, 2017a; Arce-Fonseca *et al.*, 2018). Epimastigotes and trypomastigotes express active TcTS as a shed acute-phase antigen (SAPA). These stages have identical activities but differ in the SAPA domain (Chaves *et al.*, 1993; Briones *et al.*, 1995; Jäger *et al.*, 2008). In epimastigotes, SAPA is a transmembrane protein, while in trypomastigotes, TcTS is associated with the membrane via a GPI anchor (Agusti *et al.*, 1997). On the other hand, mucins and other sialylated molecules on the surface of *T. cruzi* participate directly in the recognition and invasion of infected host cells. As a consequence, cell lines deficient in the synthesis of sialic acid are very poorly infected by the parasite (Vandekerckhove *et al.*, 1992; Scudder *et al.*, 1993); therefore, TS constitutes a novel metabolic pathway exclusive of *T. cruzi*.

In the looking for potent non-sugar inhibitor of TcTS, Neres *et al.* (2007) reported the benzoic acid/pyridine derivatives, 4-acetylamino-3-hydroxymethylbenzoic acid (fig. 1, compound 1) with an IC_{50} of 0.54 mM and 5-acetylamino-6-aminopyridine-2-carboxylic acid (fig. 1, compound 2) with an IC_{50} of 0.44 mM. After, through a virtual screening of a natural product library, Arioka *et al.* (2010) showed that flavonoid and anthraquinone derivatives (fig. 1, compounds 3 and 4) have potent TcTS inhibition with IC_{50}= 78μM and IC_{50}= 0.58μM, respectively.

Recently, Kashif *et al.* (2017b), evaluated the benzoic acid derivatives, 4-amino-benzoic acid and 4-amino-3-

**Corresponding author:* e-mail: gildardors@hotmail.com

nitrobenzoic acid (fig. 1, compounds 10 and 16, respectively) to determine trypanocidal activity and TcTS inhibition. Compound 10 showed a high trypanocidal activity with IC_{50} values of 0.52 µM and 1.24 µM for NINOA and INC-5, respectively, but a low TcTS inhibition (30%). Compound 16 also showed a high trypanocidal activity with an IC_{50} of 1.37 µM for NINOA and IC_{50}= 0.63µM for INC-5 strains and a high TcTS inhibition (77%). These results showed that benzoic acid derivatives could have a different trypanocidal mechanism of action. Therefore, in this work, the main aim was to determine the effect of compound 16 on the expression level of the TS gene in epimastigotes of *T. cruzi* to confirm its mechanism of action.

MATERIALS AND METHODS

Chemical synthesis

All chemicals were purchased from Sigma-Aldrich, Mexico and were used without further purification. The melting points were determined on a Mel-Temp capillary apparatus and are uncorrected. Infrared spectra were recorded using a Bruker Alpha FTIR spectrometer. ^{1}H-NMR spectra were obtained in $CDCl_3$ or DMSO-d_6 with Me_4Si as an internal standard on a Bruker Avance-300 Spectrometer operating at 400 MHz for ^{1}H-NMR. The reactions were monitored at 254-265 nm by thin-layer chromatography (TLC) performed on silica gel plates prepared with silica gel 60 (PF-245 with gypsum, Merck Japan).

4-acetamido-3-nitrobenzoic acid (compound 16) was synthesised by adopting the procedure described in the literature (Kashif *et al.,* 2017b). *Para*-amino benzoic acid (2 g, 14.5 mmol), was added to a mixture of 20 mL of acetic acid (CH_3COOH) and 20 mL of acetic anhydride ($(CH_3CO)_2O$) in a flask of 100 mL with stirred and refluxed for 30 min. After, the mixture was stirred at room temperature for 10 min and subsequently poured onto 100 g of ice. The solid was isolated by filtration in a Büchner funnel and washed 3 times with distilled water (100 mL), obtaining a white solid (4-acetylamino benzoic acid). Afterwards, in a ball flask of 100 mL, 4-acetylamino benzoic acid (2.5 g, 14 mmol) was added slowly in a mixture of 30 mL of concentrated sulfuric acid (H_2SO_4) and 30 mL of nitric acid (HNO_3) and stirred for 10 min at 0 °C. Subsequently, the mixture was stirring at room temperature for 30 min. After, the mixture was poured onto 100 g of ice, filtered in a Büchner funnel and washed 3 times with distilled water (100 mL), obtaining a yellow solid (4-acetylamino-3-nitro benzoic acid). Afterward, 4-acetylamino-3-nitro benzoic acid (2 g, 8 mmol) was added in a ball flask of 100 mL and 30 mL of H_2SO_4 was added dropwise under stirring for 15 min, and bringing the reaction to 100 °C for 15 min more. The mixture was poured onto 100 g of ice. The solid obtained was filtered in a Buchner funnel and washed 3 times with distilled water (100 mL). The crude product was recrystallized in EtOH, yielding the compound 4-amino-3-nitrobenzoic acid, 16 (90%). The FTIR and ^{1}H-NMR data showed agreement with the reported data (Kashif *et al*., 2017b).

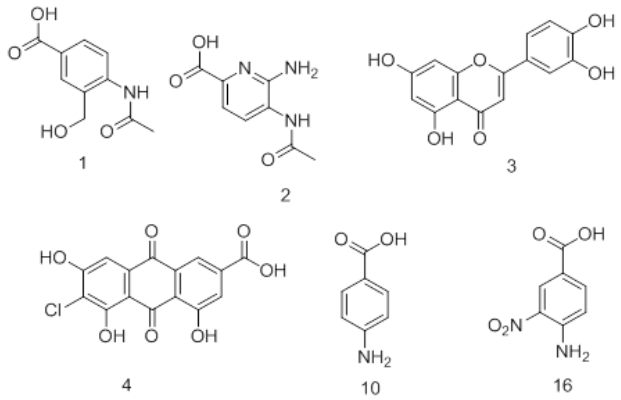

Fig. 1: Structure of small molecules as TcTS inhibitors

Culture of NINOA strain

The epimastogotes of NINOA strain were obtained from the Escuela Nacional de Ciencias Biológicas (ENCB) del Instituto Politécnico Nacional (IPN), Mexico City, and preserved in the middle of heart brain infusion (BHI) supplemented with 10% fetal bovine serum (SFB) and 1% penicillin/streptomycin at 500 µg/mL at 28 °C. The strain remained in exponential growth, and every 7 days was transferred to a new medium.

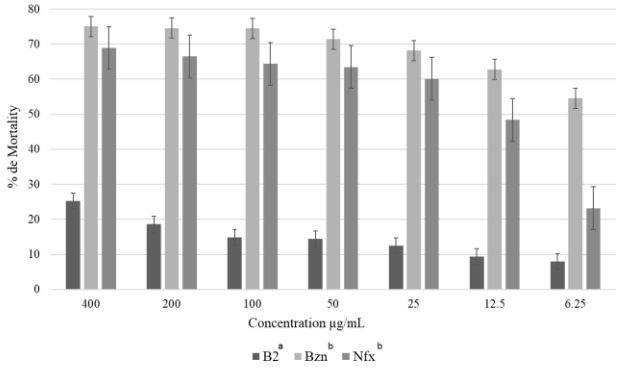

Fig. 2: Mortality percentage of compound 16 and the reference drugs Bzn and Nfx on the NINOA strain. The values are presented as the mean ± SE of the experiment in triplicate. The means that do not share a letter are significantly different, according to the Tukey Test ($p < 0.05$).

Trypanocidal activity

The epimastigotes from culture were taken and the parasite concentration was adjusted 1 x 10^6/mL. Compound 16, Nfx and Bzn were dissolved in dimethyl sulfoxide (DMSO) and seven concentrations were prepared (400, 200, 100, 50, 25, 12.5 and 6.25 µg/mL). The concentration of DMSO was maintained less than 2% in the epimastigote culture. 90 µL of epimastigotes (1 x

10^6 /mL) and 10 µL of each test compound concentration were placed in a 96-well plate, and a solution (90 µL) with epimastigotes (1 x 10^6/mL) without treatment was used as a negative control and a solution (90 µL) with parasites (1 x 10^6/mL) treated with (Nfx and Bzn at seven concentrations) was used as a positive control. Subsequently, the plate was incubated at 28 °C for 24 h, then 10 µL (2.5 mg/mL) of diphenyltetrazolium bromide (MTT) in 1X phosphate buffered saline (PBS) were added and incubated for 4 h at 37°C. After, 100 µL of sodium dodecyl sulfate-hydrochloric acid (SDS-HCl) (10% -0.01 N) was added to dissolve the MTT crystals and this was reincubated at 28 °C for 24 h. Finally, absorbance was measured at (OD) 595 nm using a (Biorad iMark ™) spectrophotometer and the final result (percentage of mortality) was calculated by the formula:

$$\text{Percentage of mortality} = \frac{100 - (DO(t)}{OD(c)} \times 100$$

Where:
OD(t) is the optical density of the culture after exposure to a concentration of the substance being evaluated.
OD(c) is the optical density of the control culture. Each test was performed in triplicate.

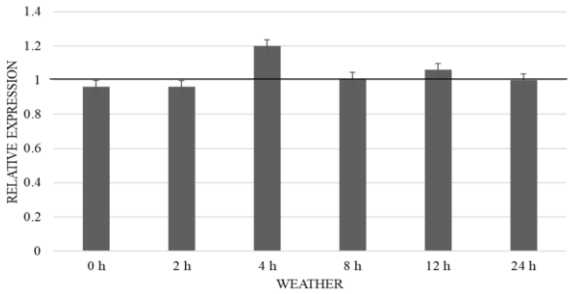

Fig. 3: Relative expression levels of the *trans*-sialidase gene. The graph shows the data of the mean TC value of each gene evaluated in duplicate in three independent experiments; the black line represents the expression of the *HGPRT* gene; the bars in gray represent the expression of the TcTS gene. The bar above represents the EE of three independent experiments performed in duplicate.

The statistical analysis was carried out using the software SPSS v.22.0 (IBM Corp., Armonk, NY). The percentage of mortality results were analyzed by a one-factor ANOVA and the comparison of means was made by the Tukey test. A $p<0.05$ was considered significant

Preparation of T. cruzi RNA
A total of 90 µL of epimastigotes (1 x 10^6 /mL) and 400 µg/mL of test compound 16 were mixed and incubated at 28 °C for 24 h. The sample for the isolation of RNA was taken at different time intervals: 0, 2, 4, 8, 12 and 24 h.

Total RNA was isolated by following the protocol of the SV Total RNA Isolation System commercial extraction kit (Promega®). The concentration and purity of the nucleic acid were determined by the Thermo Scientific® NanoDrop 2000 spectrophotometer.

cDNA synthesis
Prior to cDNA synthesis, 2 ng of total RNA was incubated at 70 °C for 10 min; it was then centrifuged for 30 seconds and kept on ice. cDNA synthesis was carried out with the protocol of the commercial transcription case A3500 of Promega®. A reaction mixture was prepared with 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 and nuclease-free water to obtain a volume of 20 µL. The reaction was incubated at 42 °C for 15 min, heated to 95 °C for 5 min and incubated at 5 °C for 5 min.

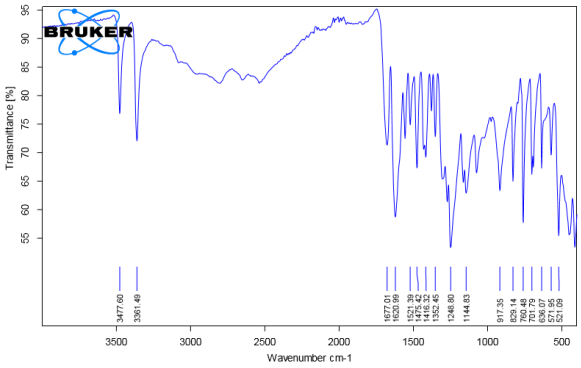

Fig. 4: FTIR spectrum of compound 16.

Relative expression analysis by real-time PCR
Expression levels of the TcTS gene in the epimastigotes after treatment with compound 16 at 400 µg/mL in different times (2, 4, 8, 12 and 24 h) were analyzed by comparing with 0 time, and expression of the normalizing gene hypoxanthine-guanine phosphoribosyltransferase (*HGPRT*) was used as a reference (Murta *et al.*, 2006). The amplification conditions and primers used for the normalizing gene as well as for the TcTS gene (designed in the Primer Select program of DNASTAR® software) are shown in table 1. The 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®. The relative quantification was determined using the BIO-RAD CFX Manager software version 3.1, using a standard deviation of ± 1 and a $p<0.05$.

RESULTS

Chemical synthesis
From *para*-aminobenzoic acid, through a process of protection, nitration and deprotection, *4*-amino *3*-nitro benzoic acid (compound 16) was obtained with a yield of 90% and a single melting point of 250 °C. The infrared

Table 1: Primers and reaction conditions for the HGPRT and TcTS gene

Gen	Forward	Reverse	Fragment size(base pair, bp)	Amplification conditions
HGPRT	CTACAAGGGAAAGGGTCTGC	ACCGTAGCCAATCACAAAG	412 bp	95°C for 5 min; 39 cycles of 95 °C for 15 s, 60°C for 20 s and 72°C for 15 s
TcTS	TTAATGTGGACGGGGTGATGGTTG	GACGATGCACGACTGTTCTTGATG	151 bp	

spectroscopy analysis showed the characteristic bands of the N-H bonds at 3477.60 and 3361.49 cm^{-1} and C=O at 1677.01 cm^{-1} (fig. 4) present in compound 16.

Trypanocidal activity

Fig. 2 shows the trypanocidal activity of compound 16 and the reference drugs Bzn and Nfx on NINOA strain. The compound 16 to the seven concentration evaluated (400, 200, 100, 50, 25, 12.5 and 6.25 µg/mL) showed a trypanocidal activity from 25.30 to 7.98% on epimastigotes of *T. cruzi*, while the reference drugs Bzn and Nfx showed activity from 75.02 to 54.55% and from 68.89 to 23.20%, respectively.

Analysis of relative expression by real-time PCR

The relative expression of the TcTS gene in *Trypanosoma cruzi* epimastigotes produced by the compound 16 at 400 µg/mL at different times is shown in fig. 3.

DISCUSSION

Trypanocidal activity

Bzn and Nfx, showed a same trypanocidal effect in five concentrations (400, 200, 100, 50 and 25 µg/mL). However, at 12.5 and 6.25 µg/mL Bzn, showed a better trypanocidal activity that Nfx. These results shown that epimastigotes are more sensitive to the reference drug Bzn compared to the second reference drug Nfx. Kashif *et al.* (2017b), also demonstrated that Bzn shown a higher mortality on trypomastigotes. Additionally, these results also exhibit that the reference drugs Bzn and Nfx have three times more potent trypanocidal activity than compound 16. However, Kashif *et al*. reported that compound 16 had better trypanocidal effect than Bzn and Nfx on trypomastigotes . The contrast in results has been justified by Chacón-Vargas *et al.* (2017) who reported that no correlation can be established between the trypanocidal activity of compounds on epimastigotes and trypomastigotes, they suggest that this may due to different morphological forms of the parasite, and different conditions of culture and evaluation.

Analysis of relative expression by real-time PCR

An increase in the expression level of the TcTS gene was observed at 4 and 12 h. The mean of the TC (Threshold cycle) for all the samples was found at 28.83 cycles, while the TcTS gene showed a TC of 28.36 ± 0.292 (mean ± SE) and 29.30 ± 0.295 (mean ± SE) for the normalizing gene (*HGPRT*).

Compound 16 showed the same basal expression level in the first two times (0 and 2 h) of TcTS that the normalizing *HGPRT* gene. However, the unit increase of 0.22 times was observed at 4 h, possibly due to the inhibitory effect of compound 16 on TcTS. After 8 h, the TcTS gene returned to its basal state and showed that the increase in expression level was temporary. These results could correlation with a low trypanocidal activity (25.30 %) of compound 16. Therefore, we suggest that a comprehensive and continuous study of the TcTS gene can be helpful to understand the mechanism of action of benzoic acid derivatives on TcTS.

CONCLUSION

Compound 16 showed a low trypanocidal activity on epimastigotes of NINOA strain of *T. cruzi* compared to reference drugs Bzn and Nfx. However, a unit increase of 0.22 times at 4 h, in the expression of the TcTS gene was observed, suggesting that this effect could be due to TcTS inhibition, confirming the mechanism of action of compound 16 on *T. cruzi.*

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