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Evaluation of proline analogs as trypanocidal agents through the inhibition of a *Trypanosoma cruzi* proline transporter

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ABSTRACT

Background: *Trypanosoma cruzi*, the etiological agent of Chagas disease, uses proline as its main carbon source, essential for parasite growth and stage differentiation in epimastigotes and amastigotes. Since proline is involved in many essential biological processes in *T. cruzi*, its transport and metabolism are interesting drug targets.
Methods: Four synthetic proline analogues (ITP-1B/1C/1D/1G) were evaluated as inhibitors of proline transport mediated through the *T. cruzi* proline permease TcAAAP069. The trypanocidal activity of the compounds was also assessed.
Results: The compounds ITP-1B and ITP-1G inhibited proline transport mediated through TcAAAP069 permease in a dose-dependent manner. The analogues ITP-1B, -1D and -1G had trypanocidal effect on *T. cruzi* epimastigotes with IC_{50} values between 30 and 40 μM. However, only ITP-1G trypanocidal activity was related with its inhibitory effect on TcAAAP069 proline transporter. Furthermore, this analogue strongly inhibited the parasite stage differentiation from epimastigote to metacyclic trypomastigote. Finally, compounds ITP-1B and ITP-1G were also able to inhibit the transport mediated by other permeases from the same amino acid permeases family, TcAAAP.
Conclusions: It is possible to design synthetic amino acid analogues with trypanocidal activity. The compound ITP-1G is an interesting starting point for new trypanocidal drug design which is also an inhibitor of transport of amino acids and polyamines mediated by permeases from the TcAAAP family, such as proline transporter TcAAAP069 among others.
General significance: The *Trypanosoma cruzi* amino acid transporter family TcAAAP constitutes a multiple and promising therapeutic target for the development of new treatments against Chagas disease.

1. Introduction

Trypanosoma cruzi is the protozoan parasite that causes Chagas disease, an illness that affects approximately 6 million people in Latin America [1]. Nowadays there are only two drugs approved for Chagas treatment, the nitroimidazole benznidazole and the nitrofuran nifurtimox. Both drugs were discovered over 50 years ago, and despite their high efficacy during the acute phase of the disease, they have limited antiparasitic activity in the chronic phase. Moreover, both drugs have many side effects, such as anorexia, nauseas and dermopathies, highlighting the urgent need for develop new therapies and find new alternative drug targets [2]. Since proline is involved in many essential biological processes in *T. cruzi*, its transport and metabolism are interesting drug targets. Besides its role as carbon source, proline sustains cell invasion and differentiation from intracellular epimastigotes to trypomastigotes [3,4]. There is also evidence of its participation in resistance to nutritional and oxidative stress as well as drug resistance [5,6].

Proline can be obtained from glutamate or it can be acquired from the extracellular medium *via* membrane transporters. Two proline transport systems with different affinities have been biochemically characterized in *T. cruzi*, and one proline transporter has been recently identified [6,7]. The proline transporter, named TcAAAP069, belongs to the first multigenic family of amino acid transporters identified in *T. cruzi*, the TcAAAP family (*Trypanosoma cruzi* Amino Acid/Auxin Permeases) [8]. This protein family is absent in mammals and its members are responsible for *T. cruzi* ability for acquiring essential metabolites like amino acids and polyamines, thus the TcAAAP family constitutes not only an interesting drug target *per se* but also a novel way of entering drugs like toxic analogues or metabolites conjugated with

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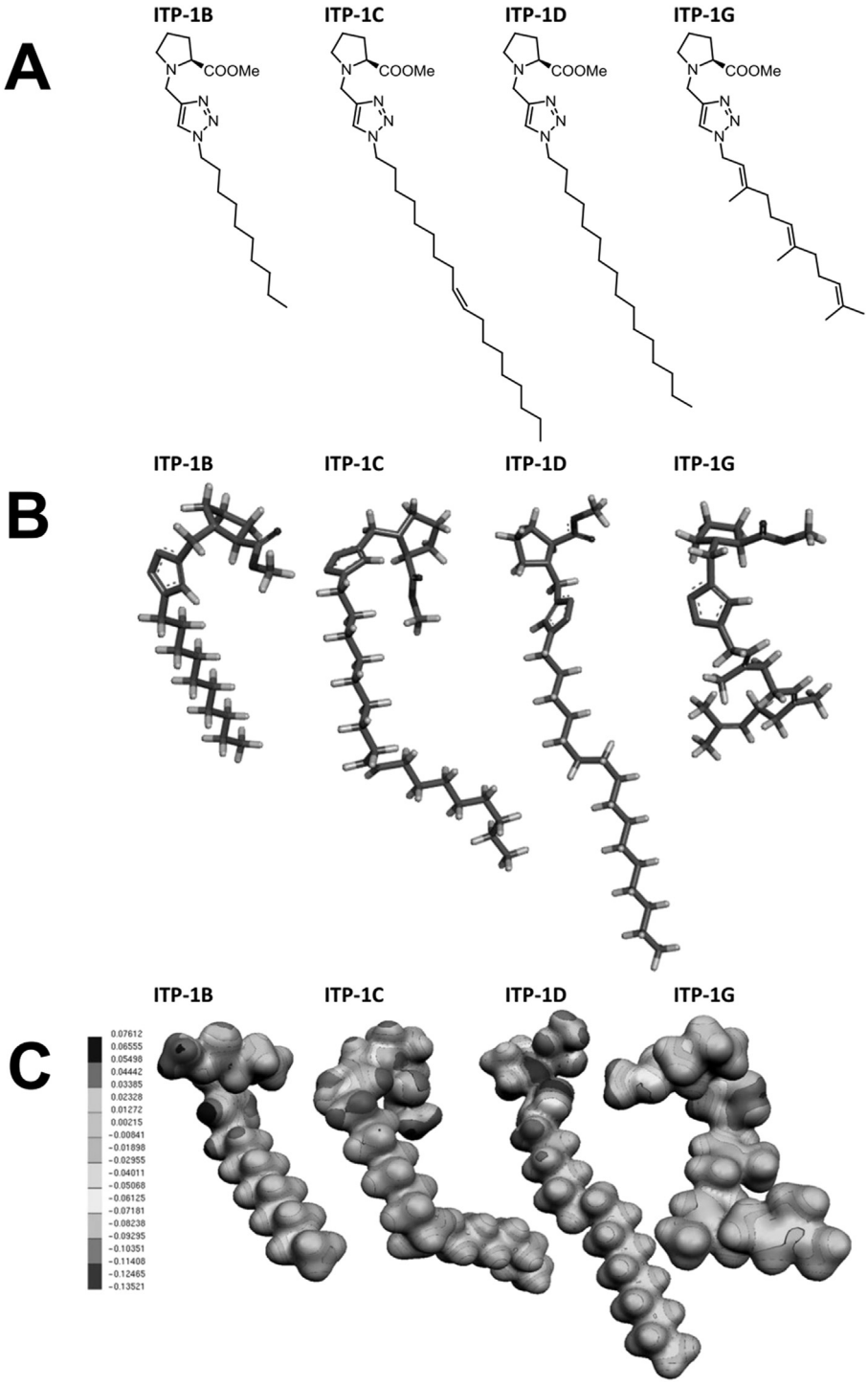


Fig. 1. Proline analogues used in this work. **A**) **Structure of the four synthetic analogues**. ITP-1B: (*S*)-methyl 2-(methyl((1-undecyl-1H-1,2,3-triazol-4-yl)methyl)amino) propanoate; ITP-1C: (S,Z)-methyl 2-(methyl((1-(nonadec-10-en-1-yl)-1H-1,2,3-triazol-4-yl)methyl)amino)propanoate; ITP-1D: (*S*)-methyl 2-(((1-heptadecyl-1H-1,2,3-triazol-4-yl) methyl)(methyl)amino)propanoate; ITP-1G: (*S*)-methyl 2-methyl((1-(3E,7E)-4,8,12-trimethyltrideca-3,7,11-trien-1-yl)-1H-1,2,3-triazol-4-yl)methyl)amino)propanoate. **B**) **Global minimum energy conformers of the four synthetic analogues**. The conformation of the compounds is highly dependent on the number of double bounds and substituents of the triazol chain, going from linear for ITP-1B to a twisted conformation for ITP-1G. **C**) **Molecular electronic potential maps**. Molecular electronic potential maps onto a van der Waals surface (isodensity 0.001 e/au^3) for compounds ITP-1B/1C/1D/1G. The grayscale-code comprises the range from − 0.1352 au to + 0.0761 au.

inhibitors into the parasite.

The use of metabolite analogues has been largely exploited, mainly as metabolic pathway inhibitors. One of the most known metabolic inhibitors used in therapy is the eflornithine (α-difluoromethylornithine or DFMO), an ornithine analogue which is one of the four drugs currently used as treatment for Human African Trypanosomiasis (HAT), a disease caused by *Trypanosoma brucei* [9]. The proline analogue L-thiazolidine-4-carboxylic acid (T4C) has been proved to diminish *T. cruzi* viability and also decreased the resistance to nutritional and oxidative stress [5]. Related to the use of chimeric molecules, some uracil amino acid conjugates have been tested as *T. cruzi* dUTPase inhibitors [10].

Several efforts have been also made to design site-directed drugs. The melamine moiety present in melarsoprol, another drug used to treat HAT, directs its entry into the parasite through the TbAT1 (P2) aminopurine transporter [11,12]. Many compounds have been synthesized using this moiety in combination with different trypanocidal agents, such as polyamine analogues, nitroheterocycles, fluoroquinolones, artesunate and eflornithine [13–16]. In addition, many quinone conjugates have been designed in combination with amino acids or polyamines and successfully tested for leishmanicidal activity [17,18].

The aim of this study was to evaluate new proline analogues that may target the *Trypanosoma cruzi* proline transporter TcAAAP069 and

also to test the trypanocidal effect and specificity of such synthetic compounds.

2. Materials and methods

2.1. Parasite culture and growth

Epimastigotes of the *Trypanosoma cruzi* Y strain were cultured at 28 °C in plastic flasks (25 cm^2), containing 5 mL of BHT medium (started with 5 × 10^6 parasites/mL) supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin [19]. Cells were counted using a hemocytometer or a colorimetric cell proliferation assay based on tetrazolium reagent (MTS).

2.2. Plasmid constructions and parasite transfection

TcAAAP069 (TriTrypDB ID: TcCLB.504069.120) was amplified using genomic *T. cruzi* DNA as template. Amplification product was subcloned into a modified pTREX expression plasmid called pTREXL [20,21]. A pTREXL containing the green fluorescent protein (GFP) was used to generate a transgenic control parasite culture. Constructions were transfected into *T. cruzi* epimastigotes as follows. 10^8 parasites grown at 28 °C in BHT medium were harvested by centrifugation, washed with PBS, and resuspended in 0.35 mL of electroporation buffer (PBS containing 0.5 mM $MgCl_2$ and 0.1 mM $CaCl_2$). This cell suspension was mixed with 50 μg of plasmid DNA in 0.2 cm gap cuvettes (Bio-Rad Laboratories, Hercules, CA). The parasites were electroporated using a single pulse of 400 V, 500 μF with a time constant of about 5 ms.

2.3. Yeast culture, plasmids constructions and yeast transformation

Saccharomyces cerevisiae cells from the Y01645 strain (MATα *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 put4::kanMX4*) were cultured at 30 °C in YPD medium (1% yeast extract, 2% peptone, 2% glucose). Yeasts were transformed with p416 vector containing the TcAAAP069 gene or with empty p416 vector as control. Transformation was performed according to Gietz and Woods [22]. Ura^+ transformants were selected on SC medium (2% glucose, 0.17% yeast nitrogen base -without amino acids-, 0.5% ammonium sulfate and 2% agar).

2.4. Proline analogues

The structures of the four synthetic compounds as well as their minimum energy conformations and molecular electronic potential maps are shown in Fig. 1. ITP-1B: (*S*)-methyl 2-(methyl((1-undecyl-1H-1,2,3-triazol-4-yl)methyl)amino)propanoate; ITP-1C: (S,Z)-methyl 2-(methyl((1-(nonadec-10-en-1-yl)-1H-1,2,3-triazol-4-yl)methyl)amino) propanoate; ITP-1D: (*S*)-methyl 2-(((1-heptadecyl-1H-1,2,3-triazol-4-yl)methyl)(methyl)amino)propanoate; ITP-1G: (*S*)-methyl 2-methyl (((1-(3E,7E)-4,8,12-trimethyltrideca-3,7,11-trien-1-yl)-1H-1,2,3-triazol-4-yl)methyl)amino)propanoate.

2.5. Metabolite transport assays

Aliquots of epimastigote cultures (10^7 parasites) or yeast cultures (1 OD) were centrifuged at 8000 × *g* for 30 s, and washed once with PBS. Cells were resuspended in 0.1 mL PBS and then added 0.1 mL of the transport mixture containing L-[^{3}H] proline, [^{3}H] thymidine, [^{3}H] amino acid mix (aspartate, glutamate, glutamine, glycine, leucine, lysine, phenylalanine, serine, tryptophan and valine), [^{3}H] putrescine, L-[^{3}H] glutamate or L-[^{3}H] lysine (PerkinElmer's NEN Radiochemicals; 0.4 mCi). Following incubation at 28 °C, reaction was stopped by adding 1 mL of ice-cold PBS. Cells were centrifuged as indicated above, and washed twice with ice-cold PBS. Cell pellets were resuspended in 0.2 mL of water and counted for radioactivity in UltimaGold XR liquid scintillation cocktail (Packard Instrument Co., Meridien CT, USA) [23]. Cell viability was assessed by direct microscopic examination. Non-specific uptake and carry over were assayed without incubation (T_0), or incubated at 4 °C.

2.6. Proline determination

Measurements of proline concentration were adapted from the ninhydrin colorimetric reaction [24]. Briefly, 10^7 parasites were washed with PBS, resuspended in 100 mM Tris-HCl pH 7.0 and then lised by sonication. The supernatant was treated with 5% trichloroacetic acid (TCA) for 1 h at 4 °C. The new supernatant was mixed with ninhydrin acidic solution (0.25 g ninhydrin dissolved in 6 mL acetic acid and 4 mL 6 M phosphoric acid) and then incubated for 1 h at 100 °C. The reaction was stopped in ice, followed by toluene addition. The organic phase was recovered in a new tube and measured at 520 nm using a spectrophotometer.

2.7. Trypanocidal activity assays

Epimastigotes of *T. cruzi* were cultured as described above, in 24-wells plate at a start density of 0.5 × 10^7 cells/mL in BHT medium. Parasites growth was evaluated at different concentrations of proline analogues and parasite proliferation was determined after 48 h unless otherwise indicated. Compound ITP-1B: 0, 1, 10, 25, 50, 100 and 200 μM. Compound ITP-1C: 0, 1, 10, 25, 50, 100, 200 and 400 μM. Compound ITP-1D: 0, 1, 10, 25, 50, 100, 200 and 400 μM. Compound ITP-1G: 0, 10, 25, 50, 100, 200 and 400 μM. Cells were counted with a Neubauer chamber using a blinded design or by viability assays using "Cell Titer 96® Aqueous One Solution Cell Proliferation Assay (MTS)" (Promega, Madison, WI, USA) according to the manufacturer instructions.

2.8. T. cruzi in vitro metacyclogenesis assay

The *in vitro* differentiation assay was performed as previously described [25]. Briefly, 7-day-old *T. cruzi* epimastigotes cultured in SDM-20 were harvested and incubated for 2 h at 37 °C in triatomine artificial urine (TAU) medium. Next, parasites were diluted in TAU3AAG medium (TAU supplemented with 10 mM L-proline, 50 mM L-sodium glutamate, 2 mM L- sodium aspartate and 10 mM D-glucose) with the addition of 0, 0.2 or 2 μM ITP-1G proline analog. Parasites were cultured at 28 °C 96 h. The epimastigotes and differentiated metacyclic trypomastigotes mix was harvested by centrifugation at 600 × *g* for 15 min and resuspended in 0.5 mL of fresh human serum, which selectively lyses epimastigotes [26]. Metacyclic trypomastigotes, easily seen by light microscopy, were quantified using a hemocytometer chamber.

2.9. Computational methods

The geometries of the proline products were optimized using density functional at the B3LYP together with 6–311 + + G(d,p) basis set. Frequency calculations were computed to verify the nature of the true minima. All calculations were performed with the Gaussian 09 package [27].

2.10. Molecular docking

Three dimensional structure of the proline transporter TcAAAP069 was obtained by homology modeling using as template the *Escherichia coli* amino acid antiporter (AdiC; PDB ID: 3LRB) (http://swissmodel.expasy.org/). The model was refined using the software Modeller v7 [28]. The obtained structure was evaluated by Ramachandran plot analysis using Chimera v1.8 [29,30]. Docking assays were performed with AutoDock Tools 1.5.6 using a grid covering the whole transporter molecule, without defining specific flexible residues and using the same

spacing and automatic centering. AutoDock 4.2.6 was used for calculation of optimal energy conformations for the ligands interacting with the putative substrate recognition site, running the Lamarckian Genetic Algorithm 100 times, with a population size of 300, and 2.7×10^4 as maximum number of generations.

2.11. Statistics and data analysis

All the experiments were carried out at least by triplicate in at least three independent experiments. Standard procedures were used to determine kinetic parameters. K_m and V_{max} values were obtained by nonlinear regression fit of the data to the Michaelis-Menten equation. IC_{50} values were obtained by non-linear regression of dose-response logistic functions. Statistics, curve fittings, K_m and V_{max} were calculated using GraphPad Prism 6.01 for Windows.

3. Results

3.1. Inhibition effect of proline analogs on TcAAAP069 activity

In order to investigate if the proline analogues ITP-1B, ITP-1C, ITP-1D and ITP-1G may inhibit TcAAAP069 activity, the effect on proline transport was measured in the presence of 40 μM of each compound. First, the ability of inhibit proline transport was evaluated on yeasts from *S. cerevisiae* Y01645 strain overexpressing the TcAAAP069 proline permease (Tc069 yeasts) and yeasts bearing an empty expression plasmid (Fig. S1). Only the compound ITP-1G was able to significantly inhibit proline transport on Tc069 yeasts while control yeasts have low-background proline transport (about 20-fold lower transport) and did not present any inhibition effect. Next, the proline transport inhibition was evaluated using transgenic parasites that overexpresses the TcAAAP069 proline permease (Tc069 parasites) or the green fluorescent protein (TcGFP parasites) as control. Due to the overexpression, the Tc069 parasites have about 9-fold increase in proline transport when compared to control culture (Fig. S2). So, if proline analogues affect TcAAAP069 activity the inhibition effect should be higher in these parasites. Initially the analogues were tested at 40 μM because this value represents approximately a 10-fold lower concentration than TcAAAP069 transporter K_m value for proline (K_m = 0.38 mM) [6].

The results showed that ITP-1B, ITP-1C and ITP-1D do not produce any effect on proline transport in control parasites. However, the ITP-1G analogue produced a significant inhibition of 33.1% (± 4.2, $p < 0.0001$), (Fig. 2, left panel).

The results obtained with the Tc069 parasites treated with the proline analogues were in accordance with those observed for the control parasites. Only the ITP-1G analogue produced a significant inhibition of 51.9% (± 11.2, $p < 0.001$), while the compounds ITP-1B, ITP-1C and ITP-1D did not produce any effect on proline transport (Fig. 2, right panel).

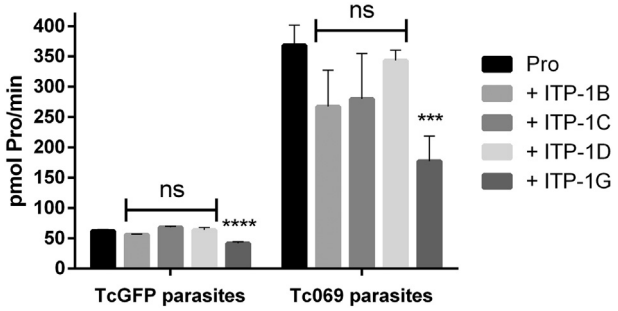


Fig. 2. Inhibition effect on proline transport on *T. cruzi* transgenic epimastigotes. The effect of the proline analogues was evaluated in TcGFP parasites (control) and Tc069 parasites. Both assays were performed incubating 10^7 parasites with or without 40 μM of each compound for 15 min. ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant. The assays were performed by triplicate.

These results suggest that the analogue ITP-1G inhibits the proline transport through the proline permease TcAAAP069, since its effect appears to be enhanced by the overexpression of the TcAAAP069 transporter.

T. cruzi transfections might lead to different overexpression levels even under the same experimental conditions. To discard that the observed inhibition could be due to the transporter expression levels, proline analogues were evaluated throughout the growth curve of Tc069 parasites when a dramatic change in the levels of TcAAAP069 expression were observed (Fig. S3). The proline transporter TcAAAP069 expression in these parasites presents a cell density-dependent expression and activity, with a maximum transport when culture densities are between 1.1 and 1.5 × 10^7 parasites/ml that gradually diminishes until it becomes undetectable when density approaches 10 × 10^7 parasites/ml [31]. The observed inhibition for compound ITP-1G corresponds with 45.3% (± 2.2), 53.4% (± 0.9) and 52.1% (± 5.7) for each culture density tested respectively. The results indicate that the compound ITP-1G has the same effect on proline transport regardless the TcAAAP069 permease expression.

3.2. Dose-dependent inhibition

Only the ITP-1G analogue showed inhibition effect both in control and Tc069 parasites at a 40 μM concentration. In order to test if the other compounds exert their effect on proline transport mediated through TcAAAP069 permease at higher concentrations, dose-response curves for all the analogues were performed in Tc069 epimastigote cultures.

The compounds ITP-1C and ITP-1D did not show inhibition at any concentration evaluated (data not shown). Both ITP-1B and ITP-1G analogues presented a dose-dependent inhibition (Fig. 3A), but this effect was greater in the presence of the ITP-1G compound. For example, at 25 μM the inhibitions observed were 19.28% (± 0.36) and 31.83% (± 2.15) for ITP-1B and ITP-1G analogues, respectively. At the maximum concentration assayed (100 μM) the inhibition achieved with ITP-1G was 62% higher than the obtained with ITP-1B (76.58% ± 7.21 and 47.03% ± 10.52 respectively). These results showed that the inhibition effect of these two analogues is dose-dependent and could explain why the effect of ITP-1B on proline transport was not detected using a concentration of 40 μM.

To evaluate the inhibition mechanism produced by the ITP-1B and ITP-1G analogues, the kinetic parameters, K_m and V_{max} were determined for proline transport in the presence or absence of these compounds (Fig. 3B). The V *vs* [S] curves were performed in the presence of 100 μM of compounds ITP-1B or ITP-1G. The parameters obtained for proline transport without proline analogues were K_m = 0.35 mM (± 0.09) and V_{max} = 373 pmol/min (± 24.7). The parameters obtained with the addition of ITP-1B were K_m = 0.43 mM (± 0.08) and V_{max} = 317 pmol/min (± 18.9), and with ITP-1G the values were K_m = 0.51 Mm (± 0.07) and V_{max} = 200 pmol/min (± 16.7). These results suggest that compound ITP-1B produced a non-competitive inhibition on proline transport whilst analogue ITP-1G seemed to act through a mixed inhibition mechanism.

3.3. Intracellular proline levels determination

The intracellular proline concentration was determined using a colorimetric method (Fig. 4). The Tc069 parasites were incubated for 1 h in the presence of 10 mM proline with 40 μM of each analogue and then the proline concentration was measured. The intracellular proline levels were also determined in Tc069 parasites without proline incubation as control. Calculated concentration, before incubation with 10 mM proline, was 5.90 mM (± 0.01), while after 1 h incubation was 8.32 mM (± 0.24). This represented a 41% increase in proline levels. Tc069 parasites treated with proline analogues ITP-1B, ITP-1C and ITP-

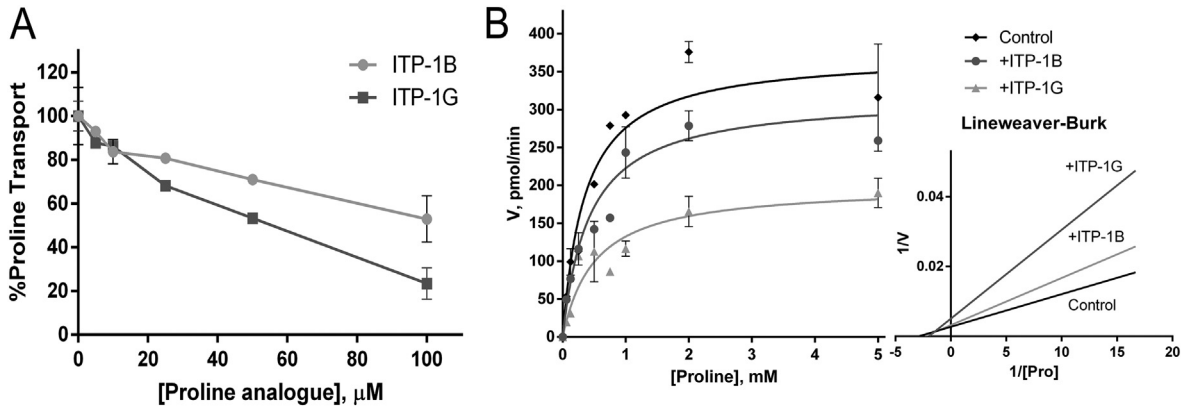


Fig. 3. A) Dose-dependent inhibition on proline transport. Compounds ITP-1B (• black circles) and ITP-1G (■ gray squares) were added at different concentrations between 0 and 100 μM and then proline uptake was measured. **B) Kinetic parameters analysis**. The curves V *vs* [S] were assessed in absence (◆ black diamonds, control) or presence of 100 μM of analogues ITP-1B (· light gray circles) and ITP-1G (▲ dark triangles). The assays were performed using 10^7 Tc069 parasites/ml and were carried out by triplicate.

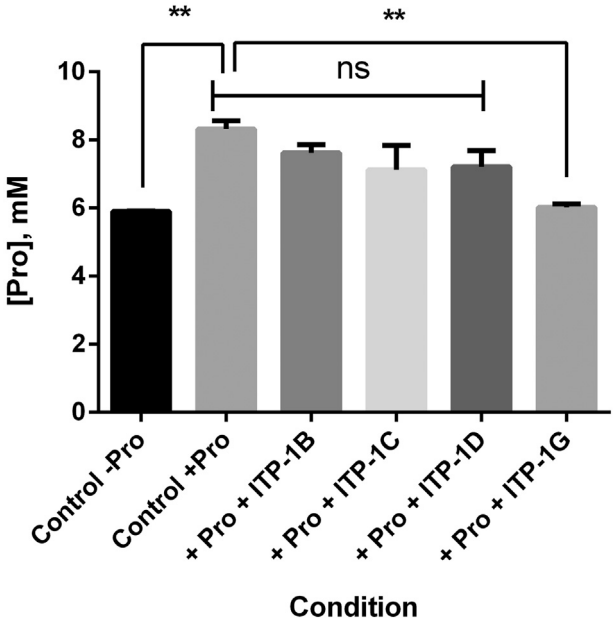


Fig. 4. Intracellular proline levels. Proline concentration was measured after 1 h incubation of 1×10^7 Tc069 parasites in PBS-proline 10 mM with or without (control + Pro) the proline analogues at 40 μM. Tc069 parasites without any treatment were used as baseline control (control -Pro). The assays were carried out by triplicate. **, $p < 0.01$; ns, not significant.

1D did not show significant differences in proline concentration when compared to the proline incubated control without treatment (7.62 mM ± 0.24, 7.12 mM ± 0.72 and 7.21 mM ± 0.48, respectively). On the other hand, parasites treated with the compound ITP-1G did not incorporate proline after 1 h incubation (5.90 mM ± 0.01 and 6.02 mM ± 0.10, before and after incubation, respectively).

The fact that ITP-1G was able to completely inhibit proline transport at a 250-fold lower concentration than proline suggests that this analogue probably acts on the TcAAAP069 proline permease through an irreversible inhibition mechanism.

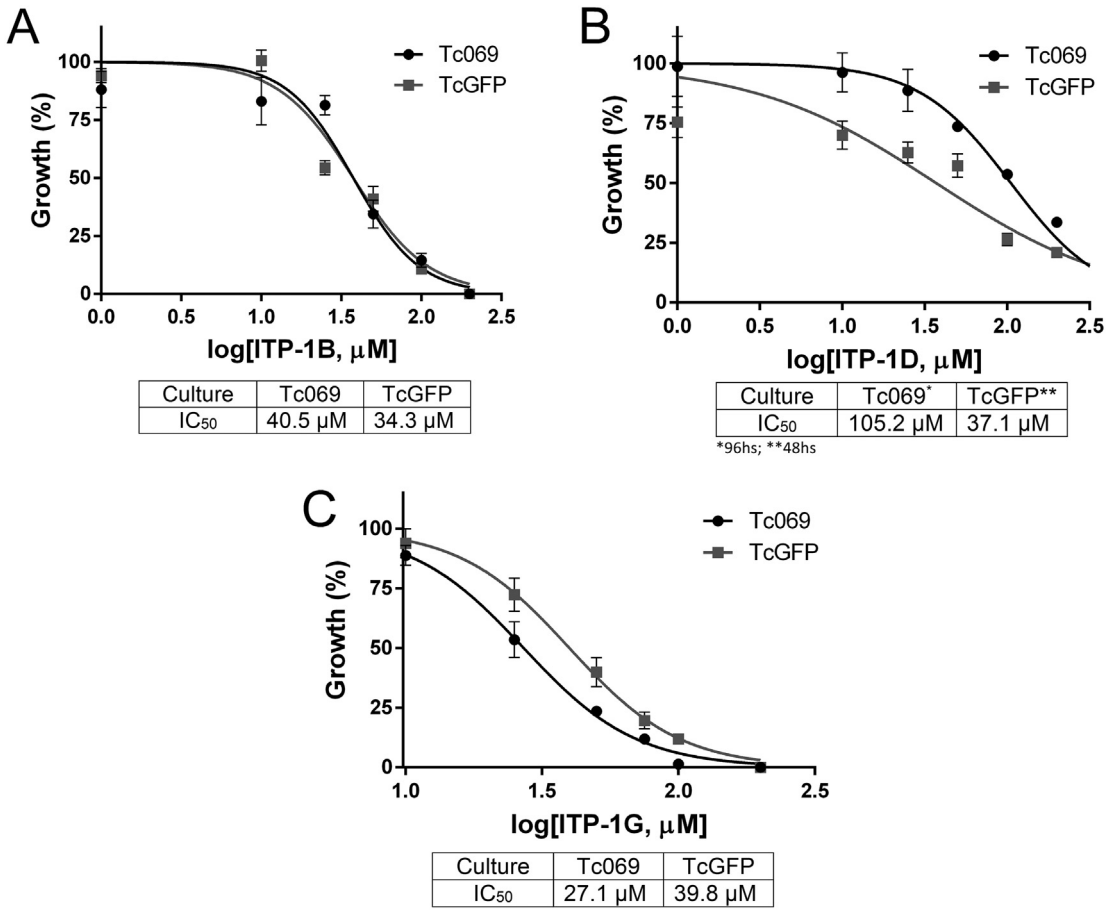
3.4. Trypanocidal effect

The trypanocidal activity was evaluated for all the proline analogues in Tc069 parasites and it was compared to control parasites (TcGFP). The IC_{50}s were calculated 48 h post treatment. The treatment with the compound ITP-1B did not present differences in the trypanocidal effect between the Tc069 and control parasites ($p = 0.23$) (Fig. 5A). The calculated IC_{50}s were 40.5 μM and 34.3 μM, respectively. These results indicate that the ITP-1B trypanocidal activity is not augmented by the overexpression of the TcAAAP069 proline transporter. Only compounds ITP-1D and ITP-1G produced significant differences in the growth curves when compared to control parasites. For the treatment with the ITP-1D analogue, the IC_{50} for control parasites was 37.8 μM, and for Tc069 parasites it was 105.2 μM ($p < 0.0001$). However, the latter was calculated 96 h post-treatment because the compound did not produce any effect during the first 72 h (Fig. 5B). The ITP-1G treatment also presented significantly different IC_{50}s between Tc069 and control parasites ($p < 0.0001$) (Fig. 5C). The IC_{50}s calculated were 27.1 μM and 39.8 μM, respectively. The compound ITP-1C did not show any trypanocidal activity on the assayed conditions. These results, together with those obtained for the proline transport inhibition, reinforced the hypothesis that the ITP-1G analogue acts through the TcAAAP069 transporter.

Also, the addition of the compound ITP-1G during the parasite stage differentiation from epimastigote to the metacyclic trypomastigote (metacyclogenesis) caused a significant decrease on trypomastigotes obtained from wild-type epimastigotes. Metacyclogenesis diminished 55.2% (± 3.1) when 0.2 μM ITP-1G was added, whilst the addition of 2 μM ITP-1G caused a 96.9% (± 0.9) decrease compared with non-treated parasites.

3.5. Effect on other transport processes

In order to evaluate if the proline analogues may be exerting their trypanocidal effect through another amino acid transporters from the TcAAAP family or even other unrelated permeases, the incorporation of other metabolites was determined in presence and absence of the ITP-1B/1C/1D/1G compounds. First, the incorporation of a mix of 10 amino acids was evaluated with and without the proline analogues (Fig. 6A). Only the compounds ITP-1B and ITP-1G were able to produce a significant inhibition on amino acids transport. However, when the activity of an unrelated transporter, such as the thymidine permease was evaluated, none of the proline analogues showed any inhibition effect (Fig. 6B). To further investigate the observed effect of the proline analogues ITP-1B and ITP-1G on other amino acid transporters, the inhibition was tested on single TcAAAP member activities. The selected amino acids were glutamate and lysine, which transports are mediated through TcAAAP649 and TcAAP7 permeases, and also the substrate of the polyamine transporter TcPAT12 was evaluated (Fig. 6C and D) [unpublished data, [32,33]]. While the analogue ITP-1G inhibited all tested transports, the compound ITP-1B only inhibited glutamate and lysine transports, but had no effect on putrescine transport. These results suggest that both compounds are able to act through several members of the TcAAAP family.



Culture	Tc069	TcGFP
IC_{50}	40.5 μM	34.3 μM

Culture	Tc069*	TcGFP**
IC_{50}	105.2 μM	37.1 μM

*96hs; **48hs

Culture	Tc069	TcGFP
IC_{50}	27.1 μM	39.8 μM

Fig. 5. Trypanocidal effect of proline analogues. A) Effect of ITP-1B. B) Effect of ITP-1C. C) Effect of ITP-1G. The trypanocidal activity was evaluated for Tc069 and TcGFP parasites as control. 0.5×10^7 exponential phase epimastigotes were treated with different concentrations of proline analogue between 0 and 400 μM during 48 h. The IC_{50}s were calculated at 48 h post-incubation, except for Tc069 parasites treated with ITP-1D analogue (96 h post-treatment). The assays were performed by triplicate.

3.6. Molecular docking

The three dimensional structure of proline transporter TcAAAP069 was modeled using as template the *Escherichia coli* amino acid antiporter (PDB ID: 3LRB). The obtained model was validated by assessment of the Ramachandran plot that predicted only 8 residues (2.8%) outside the allowed regions of the plot (Fig. S4A). Using this model the ability of ITP-1G to bind the TcAAAP069 proline binding residues was tested by a computer-assisted simulation method. AutoDock 4.0 software was used to calculate possible conformations of the ligand that binds to TcAAAP069 permease. To analyze the docking results ITP-1G free binding energy was compared with the obtained for the natural substrate proline under the same program parameters. Interestingly, according to docking model, both compounds shared all (13) putative binding residues (distance < 5 Å). These binding sites are within the hydrophobic channel of the transporter mainly close to the transmembrane spanners 1 and 6 (Fig. S4B). Proline and ITP-1G possess a ligand efficiency of -3.45 and -5.41 kcal mol^{-1} for the proline binding site of TcAAAP069, respectively. A total of 72/100 and 46/100 structures were found in the most populated clusters for proline and ITP-1G, respectively.

4. Discussion

Transport systems comprise an important feature of every living cell: they allow the entry of essential nutrients into the cell and regulate the intracellular concentrations of metabolites [34]. Since amino acids participate in a wide variety of metabolic routes, they are essential compounds for survival of *T. cruzi*. Taking into account these data and the metabolic differences between trypanosomatids and their hosts, transporters and enzymes related to their metabolism become interesting targets for drug design [35].

TcAAAP transporters are not present in the mammalian host, so, they can also be used to direct the entry of toxic compounds into parasite. In fact, the drug eflornithine, used to treat HAT disease, entries into *T. brucei* through the neutral amino acids permease TbAAT6, a TcAAAP069 homologue, and its loss causes resistance to the treatment with this drug [36]. In *Leishmania major*, the aquaporin LmAQP1 mediates the uptake of the drug sodium stibogluconate. Overexpression of this protein produces increased sensitivity to antimony- and arsenic-containing compounds, and the deletion of the LmAQP1 gene induces resistance to the drug [37]. Despite the fact that the LmAQP1 protein does not belong to the AAAP family, these examples show how the transport systems can be used to deliver drugs into parasites.

Previously, Magdaleno et al. [5] demonstrated that proline analogue L- thiazolidine-4-carboxylic acid (T4C) interferes with proline metabolism and reduced up to 56% trypomastigote bursting from mammalian cells. Also, this compound inhibits proline transport mediated through the transporter TcAAAP069 [6]. Despite these results, the analogue T4C presents high IC_{50} values (0.5–0.9 mM) for epimastigote cultures, making it an interesting inhibitor but not a suitable trypanocidal drug. Our results showed that it is possible to design proline analogues with IC_{50} values in therapeutic ranges (between 40 and 60 μM) similar to those obtained for benznidazole for many strains from different discrete typing units (DTU, IC_{50} values between 7 and 30 μM) [38].

Only two of the designed proline analogues produced a significant inhibition on the proline transporter TcAAAP069 activity. The

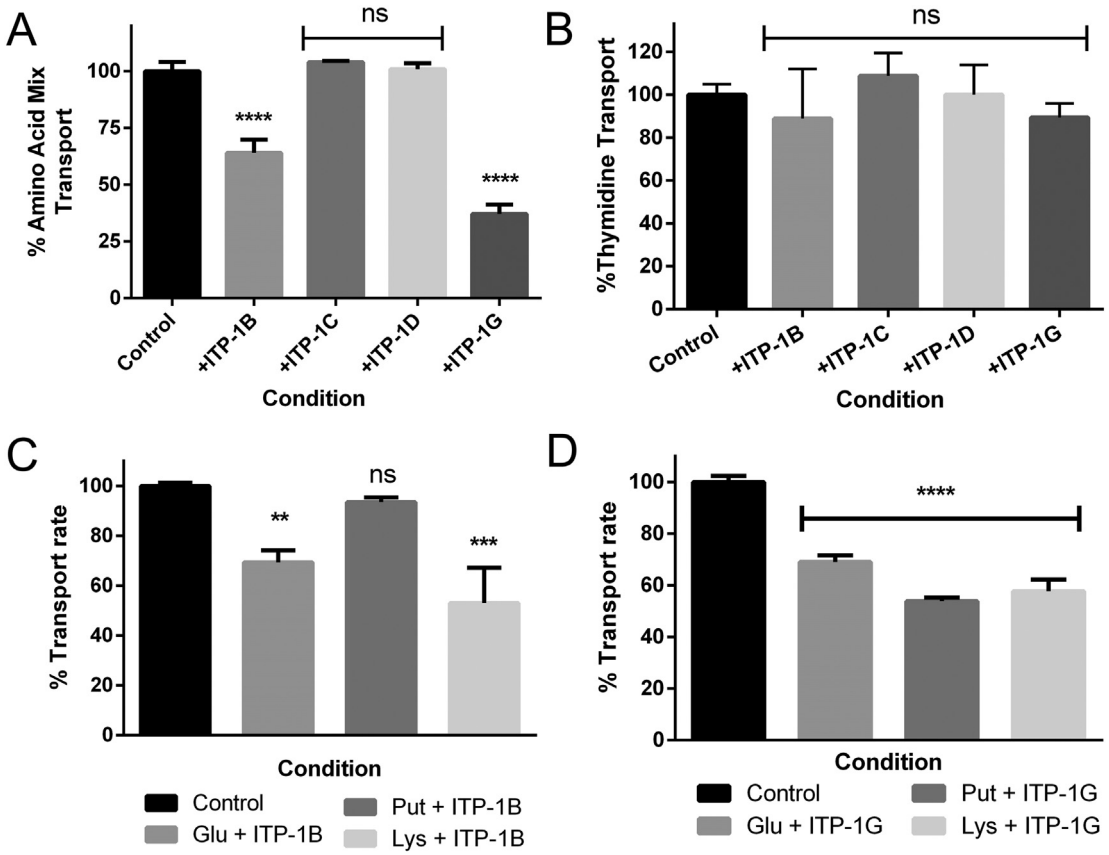


Fig. 6. Effect on other transport processes. Evaluation of the proline analogues on other metabolites transport. **A) Amino acids mix transport**. **B) Thymidine transport**. **C) Evaluation of ITP-1B effect on other TcAAAP members**. **D) Evaluation of ITP-1G effect on other TcAAAP members**. All the assays were performed incubating 10^7 Tc069 parasites with or without each proline analogue at 40 μM. The assays were performed by triplicate. *, $p < 0.1$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant.

compound ITP-1G seemed to act as an irreversible inhibitor of the TcAAAP069 permease since its addition in combination with 10 mM proline did not produce any increment in proline intracellular concentration and its presence at 40 μM inhibited up to 51% the proline transport. The ITP-1B analogue was able to inhibit proline transport too, but only in doses higher than its IC_{50} value, and this inhibition effect did not seem to be related with the trypanocidal action because the TcAAAP069 overexpression did not produce changes on the IC_{50} when compared to control parasites. However, both proline analogues affected the amino acid mix transport, showing that the inhibition activity of the compounds is not specifically limited to the proline transporter TcAAAP069. The inhibition assays with glutamate, lysine and putrescine confirmed this hypothesis, although only the ITP-1G analogue inhibited the putrescine transport mediated through the polyamine permease TcPAT12. Since all these transporters and also the proline permease TcAAAP069 belong to the TcAAAP amino acid transporters family, the multitarget action of these two analogues may be due to its interaction with the most conserved regions. In fact, one remarkable feature of the members of the TcAAAP family is the variability of the N-terminal domain (about 90 amino acids with only 5% of consensus positions), in contrast to the central and C-terminal domains, which have a very similar sequence [8]. The fact that ITP-1B analogue had no effect on putrescine transport is not surprising, because the permease TcPAT12 and the other putative polyamine transporters from the TcAAAP family are the most divergent members, presenting the lowest percentage of amino acid identity within the central and C-terminal regions. Interestingly, ITP-1G also produced a strong inhibition on parasites metacyclogenesis; more that 50% at 200 nM concentration suggesting that ITP-1G could be active, not only as trypanocidal agent but also inhibiting the parasite differentiation steps where proline is needed. Taken together all this information, the compound ITP-1G was the most promising drug candidate as proline transport inhibitor, since it presented the higher inhibition effect on proline transport, abolished completely the increment in intracellular proline levels and also proved to be an effective inhibitor on other amino acids and derivatives transport.

The trypanocidal action of compound ITP-1D seems to be unrelated to the proline transporter TcAAAP069 since its addition did not inhibit the transport activity neither alter the intracellular proline concentration. Strikingly, the treatment with the ITP-1D analogue was less effective against Tc069 parasites. The diminished efficacy against parasites overexpressing the transporter TcAAAP069 could be related to the mechanism of action of this compound rather than the transport itself (*e.g.* Tc069 parasites are more resistant to oxidative and nitrosative stress [6]), but further studies are required. Regarding the analogue ITP-1C, it did not show any effect, neither on proline transport nor on parasites´ growth.

The higher selectivity of the ITP-1G analogue towards the transporter TcAAAP069 could be rationalized based on the flexibility of the substitution of the 1,2,3-triazole. All the compounds contain an aliphatic carbon chain, with different degree of unsaturation. ITP-1B and -1D are saturated (decyl and cetyl, respectively), while ITP-1C has one double bond (oleyl) and ITP-1G has not only multiple double bonds but also it has methyl groups along the chain. Those differences produce a topological effect that can be observed on the minimum energy conformation of the compounds. As can be seen on Fig. 1A, compounds ITP-1B and ITP-1D have extended linear conformation on the olefinic carbon and a high degree of conformational freedom. The ITP-1C analogue has a twisted conformation produced by the C9– double bond that also restricts the rotation around the neighbor bonds. The compound ITP-1G shows a nested conformation that is due to the presence of three double bonds and the methyl group bound to them. The clear *E*-

farnesyl inhibition of the transporter could be attributed to the markedly different conformation of the analog due to the restricted rotation of the isoprenyl chain. Those restrictions should probably contribute to block the transporter once the proline region is recognized. That is not unusual in drug discovery and development strategies where the conformational restriction have been an effective tactic to improve selectivity and potency [39]. Molecular electrostatic potentials (MEPs) have been used for analyzing drug-receptor and enzyme-substrate interaction and other recognition processes. That is based on the assumption that those potentials described how different molecular regions will interact with another approaching chemical species. In order to complement the conformational analysis, a tridimensional molecular electrostatic potential map of the proline transport inhibitors at the van der Waals contact surface was prepared. Fig. 1C show the electrostatic potentials superimposed onto a surface of constant electron charge density (0.001 e/au^3). The electrostatic potential has been defined as the energy of interaction of a positively charge point with the nuclei and the electrons of a molecule. On Fig. 1C the gray scale area represents the electrostatic potential, V(r), providing a measurement of the molecular charge distribution. The calculated MEP maps for the four analogues varies from − 0.1352 au for the strongest attraction, to + 0.0761 au for the strongest repulsion. Negative regions V(r) are usually associated with the lone pair of electronegative atoms and the π electrons of the unsaturated hydrocarbons. In this case, the MEPs are very similar for the four compounds studied. The higher negative potentials are at the oxygen of the carbonyl group and the nitrogen of the proline and the N—N double bond of the 1,2,3-triazol. Those results show that the different proline transporter inhibition profile is mostly due to the conformational difference and not the electrostatic profile. In addition, calculated lowest free binding energy of ITP-1G to TcAAAP069 putative substrate recognition site was − 5.41 kcal mol^{-1}. This value is lower than the calculated for proline (− 3.45 kcal mol^{-1}) suggesting that the stability of the ITP-1G-transporter complex is higher than the stability of the complex formed with the natural substrate.

To our knowledge, this is the first report in *Trypanosoma cruzi* of synthetic amino acid analogues, with trypanocidal activity, targeting multiple amino acid transporters. However, *in vivo* studies must be carry out in order to evaluate its true potential as drug candidates for Chagas disease treatment.

4.1. Conclusions

The *Trypanosoma cruzi* amino acid transporter family TcAAAP constitutes a multiple and promising therapeutic target for the development of new treatments against Chagas disease. The compound ITP-1G is an interesting start point for design of new proline transport inhibitors and trypanocidal drugs.

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