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Research paper

Targeting polyamine transport in *Trypanosoma cruzi*

Chantal Reigada [a, b], Otto Phanstiel IV [c], Mariana R. Miranda [a, b], Claudio A. Pereira [a, b, *]

[a] Universidad de Buenos Aires, Facultad de Medicina, Instituto de Investigaciones Médicas A. Lanari, Buenos Aires, Argentina
[b] Consejo Nacional de Investigaciones Científicas y Técnicas, Universidad de Buenos Aires, Instituto de Investigaciones Médicas (IDIM), Laboratorio de Parasitología Molecular, Buenos Aires, Argentina
[c] University of Central Florida, Department of Medical Education, 12722 Research Parkway, Orlando, FL, 32826-3227, USA

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ABSTRACT

Polyamines play critical roles as regulators of cell growth and differentiation. In contrast with other protozoa, the human parasite *Trypanosoma cruzi*, the etiological agent of Chagas disease, is auxotrophic for polyamines. Therefore, their intracellular availability depends exclusively on polyamine transport and inhibition of these uptake processes can alter the viability of the parasite. The polyamine analogues used in this work were successfully tested as antiproliferative agents in cancer cells, bacteria, fungi and also showed a potent antiplasmodial effect. We evaluated the activity of these compounds on polyamine transport in *T. cruzi* and assessed the effects on parasite viability. Three polyamine derivatives, AMXT1501, Ant4 and Ant44, inhibited the putrescine transport in epimastigotes (the insect stage *of T. cruzi*) with calculated IC_{50} values of 2.43, 5.02 and 3.98 μM, respectively. In addition, only Ant4 and Ant44 inhibited spermidine transport with IC_{50} of 8.78 μM and 13.34 μM, respectively. The Ant4 analogue showed a high trypanocidal effect on trypomastigotes (the bloodstream stage of *T. cruzi*) with an IC_{50} of 460 nM, (SI = 12.7) while in epimastigotes the IC_{50} was significantly higher (16.97 μM). In addition, we studied the effect of the combination of benznidazole, a drug used in treating Chagas disease, with Ant4 on the viability of epimastigotes. The combined treatment produced a significant increase on the inhibition of parasites growth compared with individual treatments. In summary, these results suggest that Ant4, a putrescine conjugate, is a promising compound for the treatment of Chagas disease because it showed a potent trypanocidal effect via its inhibition of polyamine import.

1. Introduction

Chagas disease, or American trypanosomiasis, is a neglected tropical infection caused by the hemoflagellate protozoan parasite *Trypanosoma cruzi* [1]. According to the World Health Organization, around 6–7 million people worldwide are estimated to be infected with the parasite, mostly in Latin America, and over 10,000 deaths per year are caused by the disease. Moreover, Chagas disease is a global health problem largely due to population movements from endemic countries to the rest of the world [2,3]. Benznidazole (BZN) and nifurtimox (NFX) are the only drugs approved for the treatment of Chagas disease. These drugs often have serious side-effects and very limited efficacy [4]. In addition, the "Benznidazole Evaluation for Interrupting Trypanosomiasis" (BENEFIT) in patients with chronic Chagas' cardiomyopathy did not significantly reduce cardiac clinical progression through 5 years of follow-up [5]. Thus, the development of new therapeutic alternatives and the identification of novel drug targets in *T. cruzi* are necessary.

Polyamines (putrescine, spermidine and spermine; Fig. 1, compounds **1**–**3**) are polycationic compounds essential for the growth and function of cells [6]. In trypanosomes, they are involved in crucial cellular processes including the synthesis of trypanothione (Fig. 1, compound **4**), a bis-glutathionyl conjugate of spermidine, which is necessary for protection against oxidative damage [7]. Most organisms synthesize their own polyamines from ornithine and in some cases from arginine by the action of ornithine decarboxylase (ODC) and arginine decarboxylase (ADC) [8,9]. *T. cruzi* cannot synthesize putrescine *de novo* due to the lack of both enzymes and, therefore, the intracellular availability of these metabolites relies exclusively on transport processes. Thus, the inhibition of this important uptake process can alter the viability of the parasite. This is the case of the trypanocidal drug, pentamidine (Fig. 1, compound **5**), that blocks polyamine transport in *T. cruzi*

* Corresponding author. IDIM, Av. Combatientes de Malvinas 3150, 1427, Buenos Aires, Argentina.
E-mail address: cpereira@retina.ar (C.A. Pereira).

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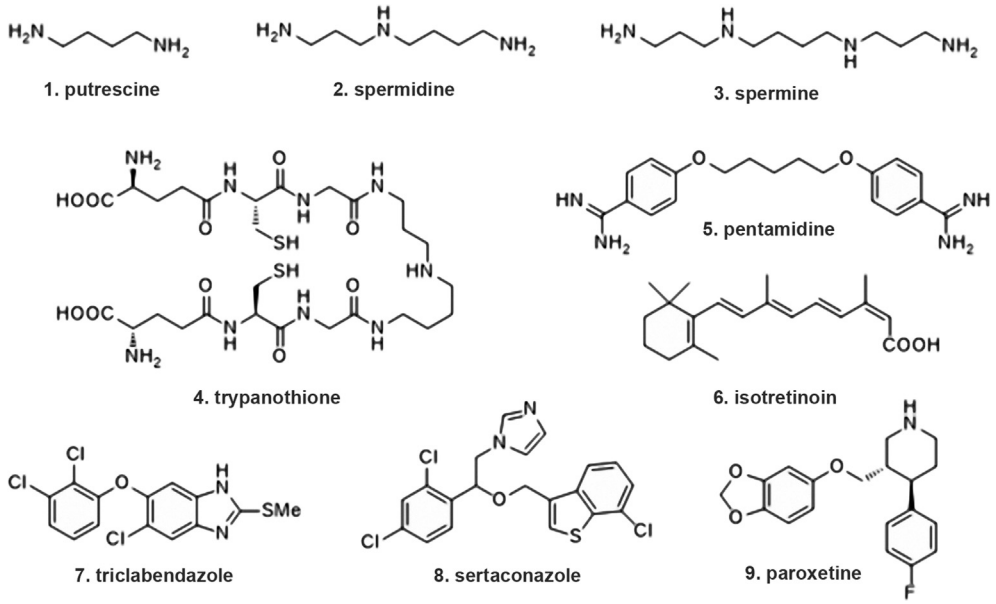

Fig. 1. Chemical structure of polyamines, related metabolites and transport inhibitors. Structures of the native polyamines putrescine, spermidine and spermine (**1**–**3**), the reduced form of trypanothione (**4**) and the previously reported polyamine transport inhibitors pentamidine, isotretinoin, triclabendazole, sertaconazole and paroxetine (**5**–**9**).

[10,11]. In addition, recent studies have shown that isotretinoin (Fig. 1, compound **6**), a retinol derivative drug, has a potent trypanocidal effect by inhibiting polyamine and amino acid uptake in this parasite [12]. Moreover, the polyamine analogues triclabendazole, sertaconazole and paroxetine (Fig. 1, compounds **7**–**9**), discovered by computational simulation, had inhibitory effects on the proliferation of the parasite and also inhibited putrescine transport [13]. Furthermore, different polyamine-based analogs, e.g., diamine, triamine and tetramine derivatives, also had antikinetoplastid activity [14].

Polyamine analogues tested in this work (Fig. 2) have been successfully used as inhibitors of intraerythrocytic *Plasmodium falciparum* proliferation, as polyamine transport inhibitors for cancer treatment, and for bacterial (*Proteus mirabilis*) infections. For example, the anthracene-putrescine conjugate **15** (Ant4) and the anthracene-homospermidine conjugate **16** (Ant44) inhibited the uptake of putrescine and spermidine in the trophozoite stage of *P. falciparum* parasites and showed a potent antiplasmodial effect [15]. The combination of α-difluoromethylornithine (DFMO), an inhibitor of ODC, with polyamine transport inhibitors results in intracellular polyamine depletion and cell death, providing a method to target cancers with high polyamine requirements [16].

Muth et al. have shown that the polyamine derivatives **10** (Trimer44) and **11** (Trimer44NMe) blocked spermidine transport in DFMO-treated human pancreatic cancer cells [17]. An additional study has reported that the polyamine transport inhibitor **17** (AMXT1501) in combination with DFMO also blocked tumor growth by targeting tumor polyamines [18]. The putrescine analogue **12** (Triamide44) has been tested on *Proteus mirabilis*, a bacterium that causes urinary tract infections in humans. This compound inhibited putrescine transport and consequently decreased putrescine-stimulated swarming and urothelial cell invasion in bacteria. In addition, other polyamine derivatives were tested against the fungus *Pneumocystis carinii* that causes pneumonia in immunocompromised hosts. The compounds reduced organism burden, decreased lung inflammation and prolonged the survival of treated *Pneumocystis* pneumonia (PCP) rats [19,20].

Considering the effects of these polyamine-transport-targeting compounds in protozoan organisms, cancer cells and in bacterial and fungi systems, in this work, we evaluated these polyamine analogues as transport inhibitors and trypanocidal drugs in *T. cruzi* via several *in vitro* assays.

2. Results

2.1. Inhibition of putrescine and spermidine transport

The eight polyamine derivatives (**10**–**17**) were tested for polyamine transport inhibition. *Trypanosoma cruzi* epimastigotes were used for putrescine and spermidine transport assays (5 μM and 15 μM, respectively), in the presence of the polyamine derivatives over a wide concentration range (0–30 μM). Those compounds that showed transport inhibition activity were re-evaluated adjusting the concentration range according to the inhibition values obtained in the first assay. The calculated IC_{50} value denotes the concentration of the compound needed to inhibit 50% of uptake of radiolabeled putrescine or spermidine. Only compounds **15**–**17** presented significant putrescine transport inhibition activity with calculated IC_{50} values of 5.02 μM (±0.39), 3.98 μM (±0.24) and 2.43 μM (±0.15), respectively (Table 1 and Supplementary Material, Fig. S1A). In the case of spermidine transport only **15** (Ant4) and **16** (Ant44), but not **17** (AMXT 1501), produced a significant inhibition. IC_{50} values for spermidine transport inhibition were of 8.78 μM (±1.04) and 13.34 μM (±0.94) for **15** and **16**, respectively (Table 1 and Supplementary Material, Fig. S1B).

2.2. Trypanocidal effect of polyamine conjugates

All compounds that showed a significant inhibition of polyamine transport (**15**–**17**) were also examined for trypanocidal activity. First, the effect was evaluated on the epimastigote stage. Cells were incubated with different concentrations of each compound, between 0 and 100 μM, over a 48 h incubation period. The IC_{50} values were then determined, where the IC_{50} value is the concentration of compound which gave 50% relative viability of parasites compared to the untreated control. Among the three compounds analyzed, only conjugate **15** presented a detectable trypanocidal activity with a calculated IC_{50} of 16.97 μM (±1.16) (Table 1 and

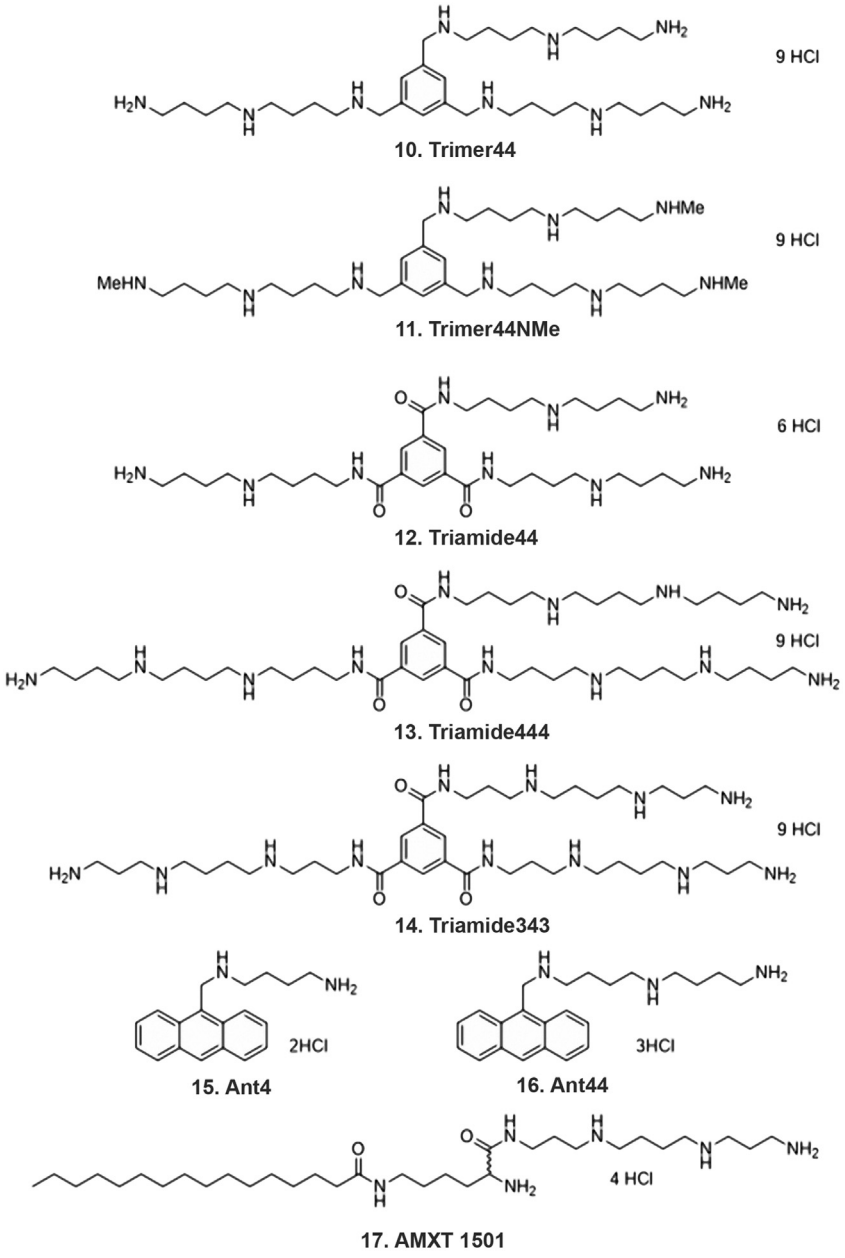

Fig. 2. Chemical structures of the putative TcPAT12 inhibitors. Structure of all the compounds tested as polyamine transport inhibitors: Trimer44, Trimer44Nme, Triamide44, Triamide444, Triamide343, Ant4, Ant44, and AMXT1501 (**10**–**17**).

Supplementary Material, Fig. S2A). Then, culture-derived trypomastigotes were subjected to the treatment with the conjugate **15** in a concentration range of 0–2.5 μM during 24 h. Interestingly, the trypomastigote stage was 37-fold more sensitive to **15** than the epimastigote stage, with a calculated IC_{50} of 460 nM (±25.2) (Table 1 and Supplementary Material, Fig. S2B). Finally, using a model of *in vitro* infection in VERO cells, trypomastigote infected cells were exposed to **15** for five days in the concentration range of 0–2.5 μM. At very low concentrations, the compound **15** inhibited the trypomastigotes burst after six days of infection, with a calculated IC_{50} similar to that obtained using culture derived trypomastigotes (520 nM ± 24) (Table 1 and Supplementary Material, Fig. S2C). Additionally, VERO cells exposed to compound **15** for 24 h in a concentration range from 0 to 50 μM, showed an IC_{50} of 5.86 μM. The calculated selectivity index of conjugate **15** (IC_{50} VERO cells/IC_{50} trypomastigotes) was 12.74. This cytotoxicity value is in agreement with others previously published in other cell lines; for example CHO cells (Chinese hamster ovary, 7.7 μM) and HL60 cells (Human promyelocytic leukemia cells, 20 μM) [21,22].

2.3. Inhibition of polyamine transport by Ant4 (15) in T. cruzi trypomastigotes

In order to further correlate the trypanocidal activity of the conjugate **15** with the polyamine transport activity, this compound was also tested for transport inhibition in the trypomastigote stage of *T. cruzi*. As occurred with the epimastigote stage, the conjugate **15** presented a significant putrescine and spermidine transport inhibition activity in trypomastigotes with calculated IC_{50} values of 4.94 μM (±0.62) and 4.86 μM (±0.51), respectively. However, the

Table 1
Effect of polyamine conjugates on polyamine transport and parasites viability.[a]

	Polyamine conjugate		
	Ant4	Ant44	AMXT 1501
Inhibition of polyamine transport IC_{50} (μM ± SE)			
Putrescine	5.02 ± 0.39	3.98 ± 0.24	2.43 ± 0.15
Spermidine	8.78 ± 1.04	13.34 ± 0.94	–
Trypanocidal activity IC_{50} (μM ± SE)			
Epimastigote	16.97 ± 1.16	–	–
Trypomastigote	0.46 ± 0.02	–	–
Trypomastigotes burst (infected cells)	0.52 ± 0.02	–	–
VERO cells	5.86 ± 0.26		
Selectivity index (SI)			
VERO/*T. cruzi*	12.74	–	–

[a] IC_{50}s for the inhibition of polyamines transport of epimastigote stage of *T. cruzi* parasites and IC_{50}s for the inhibition of growth of epimastigote and trypomastigote stages of *T. cruzi*, infected and wild type VERO cells by the compounds Ant4, Ant44 and AMXT 1501. IC_{50}s are expressed at μM concentrations ± standard errors (SE). SI (selectivity index) = (ANT4 IC_{50} of VERO cells)/(ANT IC_{50} in *T. cruzi* trypomastigotes).

measured transport rate in trypomastigotes was about 3-fold lower (0.96 pmol min^{-1} per 10^7 cells) than in *T. cruzi* epimastigotes.

2.4. Influence of Ant4 (15) on the effect of benznidazole

Epimastigote cells were treated during 96 h with 30 μM of BZN, the drug used for the treatment of Chagas disease, which has IC_{50} values on epimastigotes around this value according to previous bibliography reports [23]. Treatments were performed using BZN alone or combined with 20 μM **15**. Results showed that the combined treatment with BZN + **15** produced a significant increase in the potency of BZN of 1.5-fold at 24 h, 1.9-fold at 48 h, 6.2-fold at 72 h and 11.2-fold, at 96 h.

3. Discussion

In *Trypanosoma brucei*, the ornithine analogue eflornithine (DFMO) has been used to treat the second stage of the African trypanosomiasis or sleeping sickness. Eflornithine is a "suicide inhibitor," irreversibly binding to ODC producing a strong decrease on parasites putrescine intracellular concentration [24,25]. The depletion of polyamines in *T. cruzi* is also lethal for parasites, but eflornithine is not effective since *T. cruzi* lacks ODC and ADC, therefore the only way to achieve this effect is to target the polyamine transport from the extracellular medium [8,9]. Taking advantage of this *T. cruzi* "Achilles heel", in this work we tested eight synthetic polyamine transport inhibitors **10**–**17** that were previously tested in different organisms and diseases [14–20]. Among the compounds assayed, compound **15**, the only putrescine analog, produced a strong trypanocidal effect over trypomastigotes cells, one of the clinically relevant stages of the *T. cruzi* life cycle. The calculated IC_{50} for the trypanocidal action of the conjugate **15** was 460 nM, which is promising since BZN, used to treat Chagas disease, has IC_{50} values about 10 to 100-fold higher, depending the parasite strain. Since compound **15** has an anthracene moiety, a DNA-intercalating agent, other targets, apart from the polyamine transporter, are likely to be critical in determining trypanocidal activity. Once compound **15** is transported inside the cell, it may interact at the level of the parasites DNA and induces DNA damage as has been described previously for human cancer cells [22]. Furthermore, studies showed that anthracene also reacts with other targets like polynucleotides, nucleotides and nucleosides and in particular with purines [26].

To our knowledge, there are no previous data on the transport of polyamines in trypomastigotes. In this work it was reported that compound **15** is able to inhibit the transport of polyamines with a similar IC_{50} (≈5 μM) in epimastigotes and trypomastigotes, although in the latter stage the transport rate is lower (about 3-fold). This stage of *T. cruzi*, present in the mammalian host, lives in a very stable environment in terms of extracellular conditions including polyamine availability, while the epimastigote is adapted to a very fluctuating environment within the intestinal tract of the insect vector. These differences could explain that trypomastigote has a low but constant level of polyamine transport unlike the epimastigote that is adapted to variable extracellular concentration of polyamines, therefore, trypomastigotes are more sensitive to the inhibition of this process.

Several polyamine analogues were tested as PTIs in trypanosomatids resulting in limited effectiveness. For example, the compound 1,4-diamino-2-butanone (DAB), which was tested in *T. cruzi* epimastigotes and *Leishmania amazonensis*, showed a limited putrescine transport inhibition at 10 mM DAB and a slight toxic effect with an IC_{50} of 144 μM [27,28]. These data reinforce the need to explore more exhaustively the effect of synthetic polyamine analogues on *T. cruzi*. On the other hand, as previously mentioned, new polyamine transport inhibitors which are not polyamine analogs, were identified using different techniques, mostly by virtual screening [12,13]. Related to the inhibition of polyamine transport, three of the eight compounds assayed in this work have IC_{50} values of ≤5 μM. These compounds may act via inhibition of the previously identified *T. cruzi* polyamine transporter, TcPAT12, also called TcPOT [29,30]. The strong effect of polyamine transport inhibitors over *T. cruzi* viability could be attributed to the fact that TcPAT12 is likely the only polyamine transporter of *T. cruzi* and its inhibition cannot be compensated by other similar transporters. The unique metabolic characteristics of *T. cruzi* together with the results presented here suggest that polyamine transport inhibitors are promising compounds for the treatment of Chagas disease.

Lastly, It is interesting to note that in the series of PTIs evaluated here, only the lipophilic PTI designs **15**–**17** showed activity in inhibiting putrescine import and only **15** and **16** showed activity in inhibiting the import of spermidine in *T. cruzi*. In contrast the three-armed designs **10**–**14**, which are more hydrophilic, do not present a lipophilic "tail" to the putative receptor and were not effective in these assays. This suggests that membrane affinity likely plays a role in contributing to the effectiveness of these agents *in vitro*. Our observation that the trypomastigote stage was approximately 4-fold more sensitive to PTI compound **15** (Ant4) than the epimastigote stage, suggests that putrescine import plays a critical role in the escape of trypomastigotes from host cells. Finally, conjugate **15** dramatically increased the trypanocidal effect of BZN by more than an order of magnitude, suggesting that the use of a combined treatment would reduce the effective dose of BZN and potentially limit its severe side effects.

4. Conclusion

Considering that the human parasite *Trypanosoma cruzi*, the etiological agent of Chagas disease is auxotrophic for polyamines and their intracellular availability depends exclusively on polyamine transport, the inhibition of these uptake processes dramatically alter the viability of the parasite. Here we demonstrate that Ant4 not only inhibits the polyamine uptake but also has a high trypanocidal effect on trypomastigotes, the bloodstream stage of *T. cruzi*. Another interesting feature of Ant4 is that it can enhance the effect of benznidazole, the drug used in treating Chagas disease, in more than 10-fold.

Taking into account the urgent need for the development of new drugs for Chagas disease, we can conclude that polyamine transport inhibitors could be a therapeutic alternative either alone or in combination with the current drugs used in therapy.

5. Experimental section

5.1. Synthetic polyamines

The syntheses of the polyamine derivatives have been previously described in detail for compounds **10** [31], **11**–**14** [17], **15** [32], **16** [33], and **17** [34,35]. All compounds were tested at >95% purity as confirmed by elemental analysis.

5.2. Parasites

Epimastigote stage of *T. cruzi* from the Y strain (5×10^6 cells per milliliter) was grown in Brain-Heart infusion-Tryptose (BHT) media (pH 7) supplemented with 10% fetal calf serum, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and 20 μg mL^{-1} hemin at 28 C [36]. VERO cells (African green monkey Kidney) were cultured in MEM medium supplemented with 10% heat inactivated Fetal Calf Serum (FCS), 0.15% (w/v) $NaHCO_3$, 100 U/mL penicillin and 100 U/mL streptomycin at 37 °C in 5% CO_2. Trypomastigotes were obtained from infected VERO cells as previously described [37]. *T. cruzi* parasites of the Y strain used in this work were obtained from our laboratory stock.

5.3. Polyamine transport assays

Aliquots of epimastigote or trypomastigote cells were centrifuged at 8000 × *g* for 30 s, and washed twice with phosphate-buffered saline (PBS). Parasites (1×10^7) in 0.1 mL PBS supplemented with 2% (w/v) glucose (PBS-G) were added to an equal volume of PBS-G containing the corresponding radiolabeled substrate: [^{3}H]-putrescine or [^{3}H]-spermidine (PerkinElmer's NEN® Radiochemicals; 0.4 μCi). It was incubated during 10 min with the corresponding polyamine analogue at the indicated concentrations at 28 °C or 37 °C. At the selected time, the reaction was stopped by the addition of 1 mL of ice-cold PBS-G, the cells were centrifuged as indicated above, and washed twice with ice-cold PBS-G. The pellets were then resuspended in 0.2 mL of water and counted for radioactivity in UltimaGold XR liquid scintillation cocktail (Packard Instrument Co., Meridien CT, USA) [38,39]. Non-specific transport and carry over were measured in transport mixtures containing 100-fold molar excess of the corresponding substrate. Cell viability was assessed by direct microscopic examination.

5.4. Trypanocidal activity assays

Epimastigotes from *T. cruzi* Y strain were cultured as described above, in 24-well plates by inoculation with 10^7 cells/well in BHT medium. Parasite growth was evaluated at different concentrations of the corresponding compounds (polyamine analogues or BZN) at the indicated concentrations. The plates were incubated at 28 C for 48 h and parasite proliferation was determined. Trypanocidal activity in trypomastigotes was performed using 10^6 cells.mL^{-1} in 24-well plates and incubated at 37 C for 24 h in the presence of the indicated drug or polyamine compound. In the controls, the parasites were incubated in the absence of the compounds. The plates were assessed visually under a microscope to check for motility of parasites and the activity was determined by counting in a Neubauer chamber or by viability assays using "Cell Titer 96® Aqueous One Solution Cell Proliferation Assay (MTS)" (Promega, Madison, WI, USA) according to the manufacturer's instructions.

5.5. Cell viability assay

The toxicity of synthetic polyamines on VERO cells was determined by the crystal violet staining assay. The cells (1×10^4 cells/well) were incubated in 96-well plates with the indicated compound or PBS only as negative control and maintained at 37 C for 24 h. At the end of treatment, cells were fixed for 15 min, and stained with 0.5% crystal violet. After washing with water and drying, the absorbance of stained cells was measured at 570 nm. **Statistical analysis**. All the experiments were made at least in triplicate and results presented here are representative of three independent assays. IC_{50} values were obtained by non-linear regression of dose-response logistic functions, using GraphPad Prism 6.01 for Windows.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ejmech.2018.01.083.

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