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In vitro activity of steroidal dendrimers on *Trypanosoma cruzi* epimastigote form with PAMAM dendrons modified by "click" chemistry

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ABSTRACT

The increasing use of dendrimers shows promise for the treatment of inflammatory diseases, Chagas disease and other conditions such as cancer. In this study, the activity of 1st and 2nd generation dendrimers over *T. cruzi* in the epimastigote stage was tested. Dendrimers were derived from α-ethynylestradiol (EE) modified with PAMAM-type dendrons through a triazole ring. The activity of each compound was evaluated in five doses (from 1.3 to 20 μmol/mL) by flow cytometry, including benznidazole (Bz) as positive control. The findings show that an equivalent concentration of 14.8 μmol/mL of 2nd generation (G) dendrimer is 8 times more effective than Bz at 24 h, and it maintains its superiority at 48 h with an $IC_{50} = 1.25 \pm 0.19$ μmol/mL. A TUNEL assay showed that dendrimers induce cell death in *T. cruzi* epimastigotes mostly via apoptosis, unlike Bz, which induces death via necrosis in more than 50% of cells.

1. Introduction

Chagas disease, a previously neglected infection, is now spreading to countries worldwide and found, not just, in developing countries [1]. Six to seven million people are estimated to be infected with *Trypanosoma cruzi* [2], and 40 million are considered at risk. Current chemotherapy treatment involves two drugs, benznidazole (Bz) and nifurtimox (Nfx) [3]. However, these drugs may have many side effects, such as anorexia, vomiting, dermopathy and peripheral polyneuropathy [1,4], which can cause patients to abandon treatment. Moreover, some strains of the parasite have developed resistance to these drugs [5].

A key point in the treatment of Chagas disease is the use of inhibitors of the ergosterol biosynthesis pathway. These inhibitors are used for the treatment of infections caused by fungi, yeasts and protozoa [6]. The Trypanosomatidae family produces ergosterol and other 24-methyl sterols which are required for parasitic growth and viability [7]. Therefore, these organisms are highly susceptible to ergosterol biosynthesis [8], creating a potential new target for the treatment of Chagas disease [9,10]. The suppression of ergosterol biosynthesis induces cell lysis in *T. cruzi*, causing an irreversible alteration in the plasma membrane and Kinetoplasto-Mitocondrion complex [11]. For this reason, the sterol biosynthesis pathway is considered one of the most attractive alternatives for the treatment of Chagas disease, specifically [12]. The enzyme, lanosterol-14α-demethylase (CYP51), is found in this biosynthesis, and can be inhibited with antifungal drugs known as azoles (imidazole and/or triazole) [7].

For more than 20 years azoles have been used to fight Chagas disease. Azoles, such as ketoconazole, inhibit the growth of *T. cruzi in vitro* [13]. However, studies in murine models and in humans, show their ineffectiveness at the chronic stage of the disease. For this reason, different azoles have been tested, i.e. fluconazole [14,15], itraconazole [16,17] and pozaconazole [18]. These drugs can induce a parasitological cure in murine models, and are used commercially in allopathic medicine for fungal diseases. However, they have not been

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effective in eliminating the parasite completely. In 1996, Urbina et al. reported the synthesis and evaluation of D0870, a triazole derivative capable of inducing a parasitological cure in 70–90% of murine models in both phases of the disease [19]. Unfortunately, the development of the compound was discontinued due to the resistance of a strain of *T. cruzi* [20].

Another alternative in the treatment of Chagas disease could be the introduction of dendrimers. These well-defined nanostructured macromolecules tend to be monodisperse when compared to traditional polymers, despite their high molecular weight. At a molecular level, dendritic branching gives rise to globular or semiglobular structures, mostly, with a high density of end groups on the surface along with small molecular volume [21]. Due to their multivalence, one of the major potential applications of dendrimers is as drug carriers [22]. Dendrimers, as drug carriers, improve the pharmacokinetic properties of drugs, increase water solubility, extend blood circulation, improve transit through the biological barriers and delay breakdown of the drug [23]. The cytotoxicity and permeability of dendrimers are related to concentration, generation and surface load [21]. These, in turn, instigate a cooperative action referred to as the "dendritic effect" in which a greater number of surface groups results in greater activity [24]. i.e., the higher the generation, the greater the dendrimeric effect.

Regarding the use of dendrimers in parasitic treatment, few results have been published. Giarolla et al. [25] showed the feasibility of dendrimers theoretically acting as drug carriers. These dendrimers were constructed with myo-inositol (core and directing group), D-mannose (directing group), L-malic acid (spacer and dendron) and hydroxymethylnitrofurazone (NFOH) as a bioactive agent. Fernández et al. [26] evaluated the use of a 1st G dendrimer as a candidate for intravenous administration of an anti-*T. cruzi* compound (2′-(benzo[1,2-c] 1,2,5-oxadiazol-5(6)-yl (N-1-oxide) methylidene]-1-methoxy methane hydrazide). They found that it could be an appropriate carrier for the antichagasic drug. Other research was developed by Matthews and Holan [27], who described the synthesis of prodrugs derived from PAMAM dendrimers with diverse terminal groups, 3,6,8 trisulfonaphthylthiourea, 3,5 dicarboxyphenylthiourea, 4 phosphonooxyphenylthiourea or 3,6-disulfonapthylthiourea. These prodrugs showed activity against *Plasmodium fulsiparum*, inhibiting its invasion, growth and replication. Additionally, when they were incubated with red blood cells, the cells did not suffer damage.

Due to the anti-chagasic activity of triazoles and the positive effect of the dendrimers as drug carriers and as prodrugs, the present work aimed to evaluate their role in the anti-*T. cruzi* activity of two different dendrimeric structures and the precursor dendron. A dendrimeric structure (Fig. 2) consists of a hydrophobic core derived from 1,3,5-tribromomethyl-benzene (tBrMeB), from the 1st G, and tri(3,5-bis ((hydroxymethyl)phenoxy)benzene from the 2nd G. The dendrons are derived from 17α-ethynylestradiol modified with PAMAM type fragments through "click" reaction to produce a triazolic ring. PAMAM fragments used were of 0.5 and 1.5 generations, with azide as focal point and carboxylic acid as end groups. Their synthesis has previously been described [28].

It is assumed that the triazole ring improves activity against *T. cruzi* due to dendritic effect, and that the carboxylic acid end groups diminish the cytotoxic effect over normal cells, as mentioned by Jevprasesphant [29].

2. Materials and methods

2.1. General

All reagents and solvents from the synthesis were purchased from Sigma Aldrich. Uncorrected melting points were determined by electrothermal 9100 Melting Point Apparatus, Fisher. NMR spectra were recorded on a JEOL ECA+500 instrument and FT-IR spectra, on a Varian 600-IR series spectrometer. Mass spectra were recorded with Agilent Technologies MS TOF using the ESI(+) technique. Flow cytometry was performed using the MACSQuant Analyzer (Miltenyi Biotec, Germany) at 615–620 nm for propidium iodide. Fluorescence intensity was analysed by 10,000 events using FlowJo X, version 10.0.7r2, and expressed as percentages of cells in each population.

A statistical analysis and the design of graphics was carried out with the GraphPad Prism program, version 5.02. The effect of each concentration group was compared, showing a mean significance level of $p < 0.05$ and a confidence interval of 95%. The IC_{50} was determined using the statistical program RStudio, version 1.1.383 [https://www.rstudio.com/products/rstudio/download/].

2.2. Synthesis of compounds

Compounds **2**, **5** and **6** were obtained as previously reported [28].

2.2.1. Compound *3*

Sodium ascorbate (0.329 g, 1.64 mmol), benzoic acid (1.024 g, 8.38 mmol) and $CuSO_4{\cdot}5\ H_2O$ (0.139 g, 0.87 mmol) were dissolved in 10 mL *tert*-butyl alcohol:H_2O (2:1 *v/v*) in a water bath at 40 °C. Next, EE **1** (1.155 g, 3.89 mmol) and azide **2** (1.528 g, 4.28 mmol) were added to the reaction mixture and stirred for 12 h at room temperature. During this process the colour changed from orange-yellow to light green. The reaction was monitored by thin-layer chromatography (TLC). After 12 h, 10 mL H_2O and 20 mL of CH_2Cl_2 was added to the reaction mixture. The organic layer was then washed with a saturated solution of $NaHCO_3$ and water (3 × 15 mL), dried over Na_2SO_4 and concentrated under vacuum. The raw product was dissolved in a minimal amount of CH_2Cl_2 and precipitated with hexane. This process was repeated until no remaining azide was detected by TLC. The substance obtained was dried under high vacuum to give 2.34 g (92%) of white solid. pf.: 70.9–72.0 °C. FT-IR (cm^{-1}):3361 (OH); 2974, 2929, 2866, 2824 (C—H aliph.); 1723 (C=O). ^{1}H NMR (500 MHz, CDCl3) δ (ppm): 7.63 (s, 1H, H-20), 7.00 (d, 1H, J = 8.4 Hz, H-1), 6.58 (dd, 1H, J = 8.4, 2.2 Hz, H-2), 6.55 (d, 1H, J = 2.2 Hz, H-4), 4.38 (t, 2H, J = 6.8 Hz, H-21), 2.38 (t, 2H, J = 5.8 Hz, H-23), 2.32 (t, 2H, J = 6.8 Hz, H-25), 1.43 (s, 18H, H-28), 1.02 (s, 3H, H-18). ^{13}C NMR (126 MHz, CDCl3) δ (ppm): 14.4 (C18), 23.4 (C22), 26.4 (C15), 27.4 (11) 28.2 (C7, C28), 29.8 (C6), 33.1 (C12), 33.6 (C25), 37.9 (C16), 39.5 (C8), 43.5 (C9), 47.4 (C13), 47.8 (C21), 48.6 (C14), 49.2 (C24), 50.2 (C23), 80.7 (C27), 82.4 (C17), 112.8 (C2), 115.4 (C4), 122.1 (C20), 126.5 (C1), 132.4 (C10), 138.2 (C5), 153.5 (C19), 153.7 (C3), 172.2 (C26). HR-ESI-TOF (*m/z*): calcd for $C_{37}H_{56}N_4O_6+H^+$, 653.42781, found 653.42677.

2.2.2. Compound *4*

Compound **3** (400 mg, 0.61 mmol) was dissolved in trifluoroacetic acid (TFA, 400 mg/3 mL) with 3 drops of water and stirred for 3 h. Next, TFA was evaporated under reduced pressure to give a viscous light brown oil, which was re-dissolved in CH_2Cl_2 (minimum quantity) and precipitated with hexane. The liquid was decanted several times until no further TFA was present, although hexane was present even after several hours under high vacuum. To obtain the product free of solvents, the compound was precipitated with acetone, then the product was dried under high vacuum. Finally, 0.312 g (0.56 mmol, 91%) of solid was obtained. This was decomposed without melting at 227 °C. FT-IR (cm^{-1}): 3600–2220 (COOH broad and weak), 2928, 2868 (C—H aliph.), 1675 (C=O). ^{1}H NMR (500 MHz, $DMSO_6$) δ (ppm): 7.82 (s, 1H, H-20), 6.91 (d, 1H, J = 8.4 Hz, H-1), 6.42 (d, 1H, J = 8.4 Hz, H-2), 6.37 (s, 1H, H-4), 4.31 (t, 2H, J = 6.4 Hz, H-21), 0.88 (s, 3H, H-18). ^{13}C NMR (126 MHz, $DMSO_6$) δ (ppm): 14.9 (C18), 24.1 (C22), 26.6 (C15), 27.1 (11) 27.7 (C7), 29.8 (C6), 31.3 (C12), 33.2 (C25), 37.7 (C16), 43.7 (C9), 47.2 (C13), 47.5 (C21), 48.0 (C14), 49.0 (C24), 50.2 (C23), 81.6 (C17), 113.2 (C2), 115.4 (C4), 123.2 (C20), 126.5 (C1), 130.9 (C10), 137.7 (C5), 154.7 (C19), 155.4 (C3), 173.6 (C26). HR-ESI-TOF (*m/z*): calcd for $C_{29}H_{40}N_4O_6+H^+$, 541.30206, found 541.302205.

Table 1
Concentrations for cytotoxic assay over epimastigotes.

Compounds	Dendron **4**	Dendrimer **5**	Dendrimer **6**	Bz
Concentration μmol/mL	20.00	6.67	3.33	20.00
	14.80	4.91	2.46	14.80
	10.00	3.30	1.65	10.00
	5.00	1.69	0.84	5.00
	1.30	0.42	0.21	1.30

2.3. In vitro biological activity

2.3.1. Antiproliferative effect over epimastigotes

The epimastigotes were grown in a Triptosa Liver Infusion (LIT) medium. The isolate strain, Queretaro (QRO), was used for this procedure. It was obtained from *Triatoma Barberi*, taken from a cave in Querétaro state (International Identification ITRI/MX/86/qro) and preserved in the Parasite Biology Laboratory at Faculty of Medicine at the Universidad Nacional Autónoma de México. The culture was maintained in the medium LIT and supplemented with 5% or 10% fetal bovine with 200 U/mL penicillin and 100 μg/mL streptomycin to prevent contamination.

Epimastigotes in the exponential phase i.e., after 7 days of growth (incubated at 28 °C), were used to evaluate the activity of compounds **4**, **5**, **6** and N-benzyl-2-nitro-1H-imidazole-1-acetamide (Bz, Sigma Aldrich) as positive control. Epimastigotes were observed under a microscope to evaluate the viability of the population and were counted in a Neubauer chamber [30]. Next, for the cytotoxicity assay, aliquots in Eppendorf tubes were prepared with 50 μL medium and 2×10^5 parasites, and were incubated twice with the compounds at 28 °C, for 24 and 48 h. The concentrations of each compound are those in Table 1, but for a better comparison in the results these are expressed as equivalents of dendron **4**, i.e., 1.3, 5.0, 10.0, 14.8, and 20.0 μmol/mL (hydrated with 2% DMSO (Sigma Aldrich) and afforded with PBS) in all compounds.

After incubation, the epimastigotes were washed with 1 mL sterile PBS, pH: 7.2, to remove the substances mentioned above. Then, they were centrifuged (Eppendorf Centrifuge 5415C) at 2500 rpm for 15 min, the cell button was maintained in 100 μL sterile PBS, pH 7.2 and transferred to other tubes for flow cytometry. Next, 1.5 μL diluted (1:10) propidium iodide was added (Miltenyi Biotec). Finally, the samples were placed in a flow cytometer MACSQuant Analyzer (Miltenyi biotec, Germany) and the results were analysed by FlowJo X, version 10.0.7r2. IC_{50} was determined using the statistical RStudio program, version 1.1.383.

2.3.2. Cytotoxic activity in lymphocytes

A cytotoxicity assay was conducted with 14.8 μmol/mL of each compound. 15 mL of peripheral blood was drawn with a sterile syringe and poured into a tube with 150 μL EDTA. The procedure recommended by Histopaque gradient density ®-1007 (Sigma-Aldrich catalog 10771, USA) was followed in this sample to obtain mononuclear cells. These were collected carefully with a Pasteur pipette and placed in a Falcon test tube of 15 mL. 5 mL PBS 1× was added, then the cells were centrifuged at 2000 rpm for 5 min at 17 °C. The supernatant was decanted, and the resulting pellet was suspended in 5 mL PBS 1×. Centrifugation in the aforementioned conditions, resulted in a suspended cellular package of 1 mL PBS 1×. The cells were counted in the Neubauer chamber (2 μL trypan blue per 8 μL sample) in an optical microscope. 50 μL aliquots of 2×10^5 cells were incubated with each compound for 2 h. The cells were washed to remove all residues, incubated with the Annexin V-FITC kit (Milteyi Biotec catalog 130-092-052, USA), and, finally, the samples were placed in a flow cytometer 10 MACSQuant Analyzer. The results were analysed by FlowJo X, version 10.0.7r2.

2.3.3. Test of DNA fragmentation

This test was conducted according to the protocol for the kit (Cat. Nos. 630107 ApoAlert™ DNA fragmentation Assay Kit User Manual) in the section relating to flow cytometry.

3. Results

3.1. Synthesis

The synthesis of steroidal dendron **4** (Fig. 1) was carried out by a "click" reaction between the alkyne in 17α-ethynylestradiol and the azide group in N-alkylated 3-azidopropanamine with *tert*-butyl acrylate [28], giving rise to compound **3**. Following this, *tert*-butyl terminals were hydrolysed in TFA to obtain compound **4**.

In the first step, the structure of compound **3** was corroborated by NMR and FTIR spectroscopy. The most characteristic signals in the ^{1}H NMR spectrum is the singlet at 7.63 ppm, associated with H-20 from the triazole ring, together with the corresponding signals from the dendrons at 4.38, 2.38, 2.32 and 1.43 ppm assigned to H-21, H-23, H-25 and H-28, respectively. The formation of a triazole ring is clear in ^{13}C NMR due to the displacement of signals C-19 and C-20. These signals correspond to the alkyne in EE, which shifts from 87.5 to 153.5 ppm and from 74.2 to 122.0 ppm due to the new chemical environment in the ring. An *m/z* value of 653.4278 for HR-ESI-TOF confirmed the expected structure, in accordance with the theoretical value of 653.4268, calculated for C37H56N4O6 H.

Hydrolysis of the *tert*-butyl group in compound **3** to produce compound **4** was confirmed by FTIR. The carbonyl stretching band changed from 1723 to 1675 cm^{-1}, i.e., from an ester to an acid group. In addition, hydrolysis was evident from the band of low intensity, ranging from 3600 to 2220 cm^{-1} because of the H-bond interactions.

Evidence of hydrolysis was seen in the absence of a signal at 1.43 ppm in ^{1}H NMR, assigned to the *tert*-butyl group, and the shift from 7.63 to 7.86 ppm in H-20. In the ^{13}C NMR spectrum, the quaternary carbon and methyl groups of the *tert*-butyl were absent, that is, the signals at 28.2 and 80.7 ppm, respectively. In addition, the ion at *m/z* of 541.3022, obtained by HRMS confirmed product formation.

Dendrimers **5** and **6** were synthesized as previously described. Their structures are shown in Fig. 2 [28].

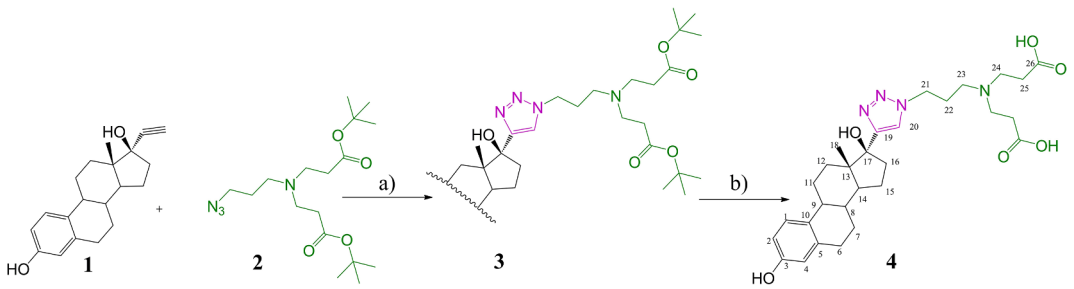

Fig. 1. Route of synthesis of dendron **4**.

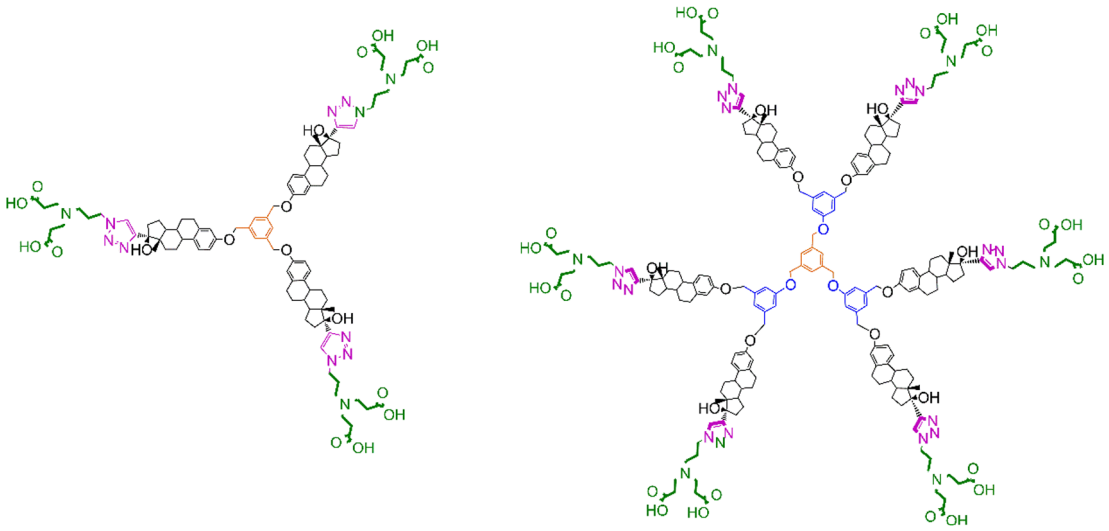

Fig. 2. Structures of dendrimers **5** (left) and **6** (right) [28].

3.2. Biological activity

3.2.1. In vitro antiproliferative activity

In vitro antiproliferative activity was determined by flow cytometry as a percentage of cell viability, using propidium iodide as a marker of dead or dying cells. Results at 24 h, with five concentrations for compounds **4**, **5**, **6** and Bz as a positive control are shown in Table 2.

Table 3 shows the results of *in vitro* activity in compounds incubated for 48 h against *T. cruzi* epimastigotes.

3.2.2. Cytotoxic activity in lymphocytes

The evaluation of the cytotoxicity upon lymphocytes by the target compounds was made at a concentration equivalent to dendron **4** of 14.8 µmol/mL. The results are shown in Fig. 3.

3.2.3. DNA fragmentation

Studies of DNA fragmentation in the epimastigotes were made by TUNNEL assay [31], the results for dendrimer **6** and the Bz are shown in Figs. 4 and 5, respectively.

4. Discussion

According to current research, triazole rings present a good alternative in the treatment of fungal, bacterial, and parasitic diseases [32]. However, their incorporation into dendrimeric structures presents an interesting challenge. For these purposes, steroidal dendron **4** (Fig. 1) and 1st and 2nd G dendrimers, compounds **5** and **6** respectively, (see Fig. 2) were evaluated as antiproliferative agents against *T. cruzi* epimastigote form.

Table 2
In vitro activity of compounds **4**, **5**, **6** and *Bz* against *T. cruzi epimastigotes* (24 h).

Concentration[#] (µmol/mL)	Values (Mean ± DS)			
	Bz	Dendron **4**	Dendrimer **5**	Dendrimer **6**
1.3	13.82 ± 4.92	2.87 ± 1.70	0.63 ± 0.27	0.86 ± 0.17
5.0	12.62 ± 3.90	1.18 ± 0.36	1.12 ± 0.43	1.68 ± 0.48
10.0	6.46 ± 1.54	1.16 ± 0.77	1.26 ± 0.41	25.98 ± 6.17
14.8	10.27 ± 3.70	1.01 ± 0.12	2.70 ± 0.58	79.06 ± 0.94[*]
20.0	12.52 ± 3.65	1.26 ± 0.07	2.16 ± 0.86	76.90 ± 6.32

The results are expressed as percentages of cell death and as means ± SD obtained in triplicate. A significant difference of [*] $P < 0.05$ was found using the Kruskal-Wallis test. The basal cytotoxity of epimastigotes was 0.73 ± 0.44.

[#] Concentration of dendrimers **5** and **6** is expressed as equivalents of dendron **4**, that is, with 3 and 6 unities, respectively.

The potential cytotoxic activity of dendron **4** and 1st G **5** and 2nd G **6** dendrimers was initially evaluated by incubation times at 2, 24, 48 and 72 h. The results at 2 and 72 h were discarded immediately because at 2 h, neither the control nor the compounds showed activity against *T. cruzi*. At 72 h, the environment in which the parasites were found, showed a loss of nutrients, therefore, the results obtained do not only correspond to the presence of the compounds. Therefore, the repetition and analysis of data focused on anti-*T. cruzi* epimastigote stage activity at 24 and 48 h.

Due to the high variability of the IC_{50} reported in Bz [30,33] the range of concentrations for Bz were varied from 1.3 to 20 µmol/mL. However, at 24 h, an IC_{50} was not reached, despite a positive report for this strain by Moreno-Rodriguez, et al. [30]. It was only at 48 h when 50% of *T. cruzi* epimastigote death was achieved, regardless of the Bz concentration (Table 2). The greatest activity found by other researchers could be due to a sensitization of the parasite by the excipients in commercial Bz formulation. Other reports indicate the use of a commercial brand of Bz, while we used a chemical grade Bz reagent to maintain the same conditions as those of the dendrimers.

The statistical analysis of cell death in percentages (Table 2) shows no significant differences with Bz, dendron **4** or dendrimer **5**, regardless of the concentration. Cell death percentages achieved for *T. cruzi* epimastigotes were very low for Bz, and compounds **4** and **5**. Just dendrimer **6** showed a significant difference at 2.46 and 3.33 µmol/mL, which expressed as equivalents of **4** correspond to 14.8 and 20 µmol/mL (for equivalences see Table 1), respectively, with almost 80% of parasite death, and with an IC_{50} calculated at 2.22 ± 0.14 µmol/mL at 24 h.

After 24 h incubation at 14.8 µmol/mL, the activity of dendrimer **6** was almost 8 times greater than that of Bz. Therefore, dendrimer **6**, could be a good candidate as anti *T. cruzi* after *in vivo* evaluation of their activity. Even at 10 µmol/mL, dendrimer **6** showed activity to be four times greater than with Bz (Fig. 6).

A clear dendrimeric effect [34–36] is noted in the screening conducted at 24 h, seen in the large increase in cell death in Table 2. At 14.8 µmol/mL cell death due to dendron **4** increased significantly when incorporated with 1st G dendrimer **5** and is enhanced almost 80 times when incorporated in a 2nd G structure **6** (Fig. 1). These results clearly show the effect of Fréchet core-type dendrimers over compound activity.

At 48 h, dendron **4** and dendrimer **5** show dose-dependent responses. The higher the concentration, the greater the activity. However, even at 20.0 µmol/mL, activity is below that displayed in Bz

Table 3
In vitro activity of compounds **4**, **5**, **6** and *Bz* against *T. cruzi* epimastigotes (48 h).

Concentration[#] (μmol/ml)	Values (Mean ± DS)			
	Bz	Dendron **4**	Dendrimer **5**	Dendrimer **6**
1.3	61.53 ± 13.44	11.24 ± 0.48	13.19 ± 1.41	17.13 ± 2.12
5.0	65.87 ± 3.00	20.18 ± 4.85	16.56 ± 6.39	36.61 ± 4.89
10.0	64.23 ± 2.48	25.00 ± 2.06	16.27 ± 3.86	78.03 ± 9.17
14.8	69.03 ± 2.07	35.26 ± 6.87*	31.38 ± 2.12	84.33 ± 0.71*
20.0	66.87 ± 3.02	32.93 ± 1.18*	45.61 ± 6.22*	75.23 ± 2.21

The results are expressed as percentages of cell death and as means ± SD obtained in triplicate. A significant difference of * $P < 0.05$ was found using the Kruskal-Wallis test. The concentration of the control (Bz) showed 0.73 ± 0.44 μmol/mL anti *T cruzi* activity.

[#] Concentration of dendrimers **5** and **6** is expressed as equivalent to dendron **4**, with 3 and 6 unities, respectively (see Table 1).

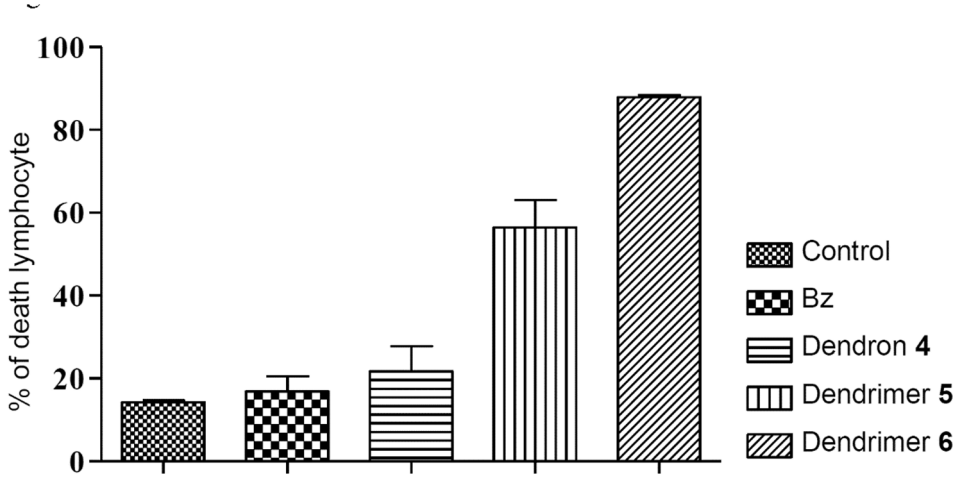

Fig. 3. Activity of Bz, **4**, **5** and **6** at 14.8 μmol/mL over lymphocytes after 2 h of incubation (14.20 ± 0.93 was the percentage of basal death).

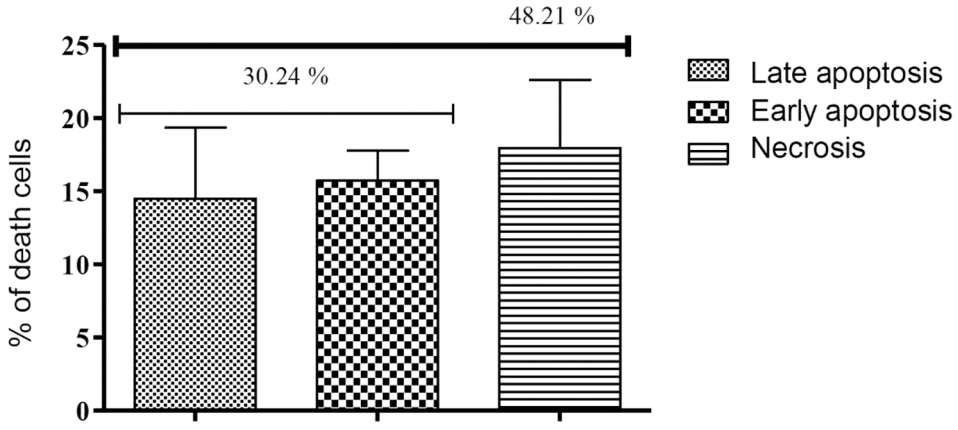

Fig. 4. Fragmentation of the DNA due to dendrimer **6** at 2.46 μmol/mL (equivalent 14.8 μmol of **4**/mL).

(60–70%). Although there is no significant increase in activity between 24 and 48 h, dendrimer **6** could have potential in the treatment of Chagas disease based on the activity displayed. It shows a greater effect at 1.69 μmol/mL (corresponding to 10.0 μmol of **4**/mL), with almost 80% of activity, reaching a mortality of 84% at 2.40 μmol/mL (corresponding to 14.8 μmol of **4**/mL), with an IC_{50} calculated at 1.25 ± 0.19 μmol/mL.

It is well known that dendrimers have longer circulation times than conventional drugs due to the polyanionic surfaces, which can avoid glomerular filtration. In theory, the reduced affinity of anionic dendrimers dictates that these molecules are relatively non-toxic [37]. This makes it possible to envision a scenario with lower doses added over time.

Keeping in mind the use of dendrimers against *T. cruzi*, it is important to evaluate the effect of these compounds over lymphocytes, because these are important cell effectors in Chagas disease. The effect was evaluated at 2.46 μmol/mL, concentration equivalent to 14.8 μmol/mL, which is shown to be the most effective against *T. cruzi* epimastigotes. The same trend produced by dendrimers over *T. cruzi* parasites was observed in the case of lymphocytes. Dendron **4** and dendrimer **5** showed the lowest percentages of lymphocyte death, 21.68 ± 10.57 and 56.45 ± 11.42, respectively, while dendrimer **6** showed the greatest mortality 87.83 ± 0.89. However, as reported by other authors [29], dendrimer cytotoxicity depends, largely, on the cell lines tested, and lymphocytes are the most sensitive. Although cytotoxicity in lymphocytes was high, *in vivo* behaviour cannot be extrapolated, although it is expected to be negligible due to the anionic charge on its surface.

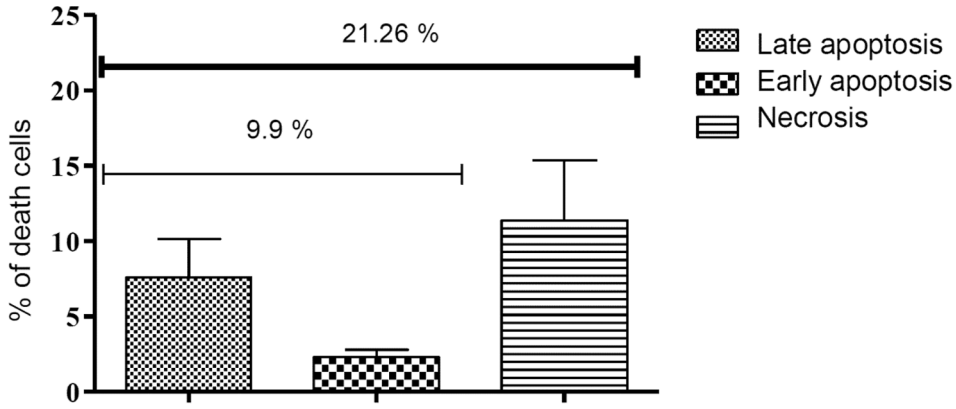

Fig. 5. Fragmentation of DNA due to Bz at 14.8 µmol/mL.

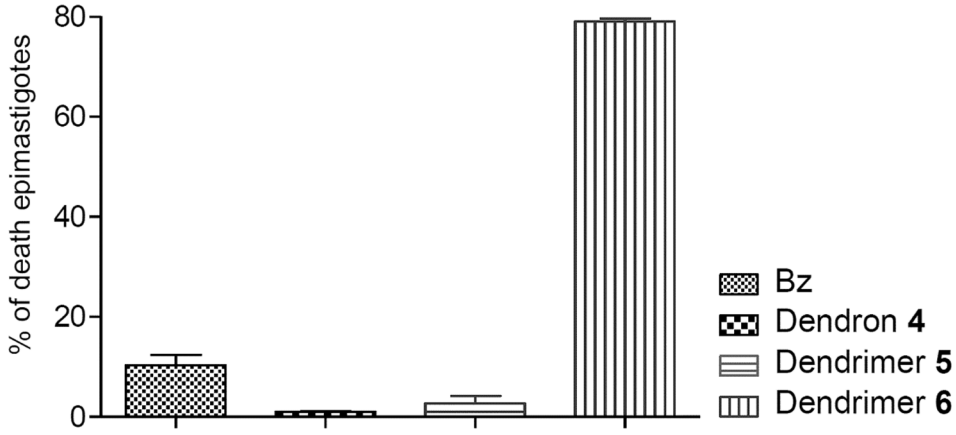

Fig. 6. Activity of Bz, **4**, **5** and **6** at 14.8 µmol/mL over epimastigotes after 24 h of incubation.

Additionally, the type of death induced by dendrimer **6** and Bz was determined by DNA fragmentation assay, which showed that the induction of death, mostly by apoptosis in dendrimer **6** (30.2%) (Fig. 4), differs from Bz where it occurs, mainly by necrosis (Fig. 5). Since necrosis is a violent process, causing rupture of the membrane and, release of cytoplasmic and organelle content, this mechanism is not the preferred one. The opposite of necrosis, apoptosis is a process in which no inflammation occurs, and the cellular membrane is not destroyed. This characteristic, together with the percentage of epimastigote death, and the short time needed for activation, endorses the use of this compound as a possible anti-chagasic drug.

In conclusion, the present work shows the antiproliferative activity of dendrimer **6**. This dendrimer showed higher activity against *T. cruzi* epimastigotes *in vitro* compared to Bz at both 24 and 48 h. Additionally, dendrimer **6** induces cell death mainly via apoptosis, as was determined by TUNNEL assay, unlike Bz. Therefore, this dendrimer is a good candidate for continued *in vivo* analysis to stablish new compounds for treating Chagas disease.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.01.056.

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