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In vitro activity and mechanism of cell death induction of cyanomethyl vinyl ethers derivatives against *Trypanosoma cruzi*

Carlos J. Bethencourt-Estrella [a,b], Samuel Delgado-Hernández [c,d], Atteneri López-Arencibia [a,b,e], Desirée San Nicolás-Hernández [a,b], David Tejedor [c,**], Fernando García-Tellado [c,***], Jacob Lorenzo-Morales [a,b,e,*], José E. Piñero [a,b,e,****]

[a] Instituto Universitario de Enfermedades Tropicales y Salud Pública de Canarias, Universidad de La Laguna, Avda. Astrofísico Fco. Sánchez, S/N, 38203, La Laguna, Tenerife, Islas Canarias, Spain
[b] Departamento de Obstetricia y Ginecología, Pediatría, Medicina Preventiva y Salud Pública, Toxicología, Medicina Legal y Forense y Parasitología, Universidad de La Laguna, Tenerife, Islas Canarias, Spain
[c] Instituto de Productos Naturales y Agrobiología, Consejo Superior de Investigaciones Científicas, Avda. Fco. Sánchez 3, 38206, La Laguna, Tenerife, Islas Canarias, Spain
[d] Departamento de Química, Unidad Departamental de Química Analítica, Universidad de La Laguna (ULL), Tenerife, 38206, Spain
[e] Centro de Investigación Biomédica en Red de Enfermedades Infecciosas (CIBERINFEC), Instituto de Salud Carlos III, 28220, Madrid, Spain

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ABSTRACT

Chagas disease causes a problematic pathology that can lead to megacolon and heart disease, and can even cause the death of the patient. Current therapies for this disease are the same as they were 50 years ago, are not fully effective and have strong side effects. The lack of a safe and effective therapy makes it necessary to search for new, less toxic and totally effective compounds against this parasite. In this work, the antichagasic activity of 46 novel cyanomethyl vinyl ether derivatives was studied. In addition, to elucidate the type of cell death that these compounds produce in parasites, several events related to programmed cell death were studied. The results highlight four more selective compounds, E63, E64, E74 and E83, which also appear to trigger programmed cell death, and are therefore postulated as good candidates to use in future therapeutics for Chagas disease.

1. Introduction

Chagas disease or American trypanosomiasis is one of the neglected tropical diseases due to its characteristics and its relationship with areas of poverty. Endemic in 21 Latin American countries, including South America, Central America and Mexico, it affects between 6 and 8 million people in these areas. In recent decades, due to globalization and immigration, the number of cases outside endemic areas has been increasing, with an estimated 300,000 infected people in the United States (Bern y Montgomery, 2009; Organization, 2018; Stanaway y Roth, 2015).

Discovered by Carlos Chagas in 1909 and caused by the protozoan parasite *Trypanosoma cruzi*, this disease is mainly transmitted by the triatomine, although other less common routes of transmission also occur, which are the cause of cases outside endemic areas, such as congenital transmission, transfusions, or transplants (Chagas, 1909; Guarner, 2019; Rassi et al., 2010).

Chagas disease presents two phases, the initial phase, known as the acute stage, and the late phase, known as the chronic stage, separated by the indeterminate phase (Tanowitz et al., 1992). The acute phase is

* Corresponding author. Instituto Universitario de Enfermedades Tropicales y Salud Pública de Canarias, Universidad de La Laguna, Avda. Astrofísico Fco. Sánchez, S/N, 38203, La Laguna, Tenerife, Islas Canarias, Spain.
** Corresponding author.
*** Corresponding author.
**** Corresponding author. Instituto Universitario de Enfermedades Tropicales y Salud Pública de Canarias, Universidad de La Laguna, Avda. Astrofísico Fco. Sánchez, S/N, 38203, La Laguna, Tenerife, Islas Canarias, Spain.
E-mail addresses: cbethene@ull.edu.es (C.J. Bethencourt-Estrella), sdelgadh@ull.es (S. Delgado-Hernández), atlopez@ull.edu.es (A. López-Arencibia), dsannico@ull.edu.es (D. San Nicolás-Hernández), dtejedor@ipna.csic.es (D. Tejedor), fgarcia@ipna.csic.es (F. García-Tellado), jmlorenz@ull.edu.es (J. Lorenzo-Morales), jpinero@ull.edu.es (J.E. Piñero).

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characterized by the absence of symptoms, in few cases there is indeterminate symptomatology (fever, sweating, muscle aches, general malaise, heart condition, among others), which is easily confused with other pathologies, although sometimes the most characteristic sign of the disease occurs, inflammation in the bite area (inoculation chagoma or Romaña sign) (Coura, 2007). Following this initial acute phase, which can last approximately 2–8 weeks, the patient enters the indeterminate phase, characterized by the absence of symptoms, but with positive serology (Linhares-Lacerda et al., 2018). Most patients usually settle at this indeterminate stage until death. But about 20–30% of these patients develop the chronic phase, characterized by the presence of cardiac and digestive problems (megacolon and megaesophagus). This phase can appear years, even decades after the acute phase (Echavarría et al., 2021)(Echavarría et al., 2021). It is important to mention that the symptomatology may vary according to genetics and geographic area, which makes its clinical diagnosis and treatment even more complicated (Campbell et al., 2004; Miles et al., 1981; Urbina, 2010).

As the third most common parasitic disease worldwide, it is important to emphasize its prevention. This prevention should be carried out in three main approaches. Firstly, by avoiding transmission (vector control, transfusions, transplants), secondly, by improving the detection of cases, thus allowing early treatment (periodic analysis, antiparasitic treatment), and lastly, by improving the patient's quality of life once the disease has developed (treatment of symptoms, health monitoring) (Abad-Franch et al., 2010; Rassi et al., 2009).

Current treatments for Chagas disease are the same as in the 1960s, benznidazole and nifurtimox, whose activity varies according to the geographical area. Treatments with these drugs are prolonged and, in addition, they are toxic, producing many side effects. These side effects mainly include gastrointestinal and central nervous system problems and, in the case of benznidazole, the appearance of allergic dermatitis. All these effects often make it necessary to stop the treatment (Pérez-Molina y Molina, 2018; Rassi et al., 2012). These side effects, in addition to their variability in efficacy, make it necessary to develop new, more effective, and less toxic therapies for this pathology.

Despite being a disease discovered more than 100 years ago by Carlos Chagas, there is still a lack of research on the progression of the disease and its mode of action, which makes the search for new drugs very complicated for researchers.

Over the past 50 years, efforts have been made to develop new treatments for both the acute and chronic phases of Chagas disease. Although no new drugs currently used in the treatment of Chagas disease have been developed, many promising candidates for further study of trypanosomiasis have been developed. These candidates can be divided into two main groups, a first group comprising compounds of synthetic and semi-synthetic origin, and a second group containing products of natural origin. Compounds of synthetic or semi-synthetic origin include, among others, sterol biosynthesis inhibitors, such as ravuconazole (Urbina et al., 2003) or fenarimol analogues (Keenan et al., 2013), antimicrobial peptides, such as AS-48 bacteriocin (Martín-Escolano et al., 2020), cruzipain inhibitors, such as K777 (Barr et al., 2005), nitro-heterocyclic compounds, such as fexinidazole (Raether y Seidenath, 1983) or nitrotriazoles (Papadopoulou et al., 2016), or quinolones (Nefertiti et al., 2018). Compounds of natural origin include flavonoids from Delphinium staphisagria (Marín et al., 2011) or Arrabidaea brachypoda (da Rocha et al., 2014), artemisinins from Artemisia annua (Mishina et al., 2007), lignans from Piper jericoense (García-Huertas et al., 2018) or Zanthoxylum naranjillo (Bastos et al., 1999), celastroloids from Maytenus chiapensis (Núñez et al., 2021) or indolocarbazoles from Streptomyces sanyensis (Cartuche et al., 2020).

The use of compounds based on nitrile or cyano groups has been developed for decades against *Trypanosoma* spp. (Bethencourt-Estrella et al., 2021; Quilles et al., 2020). In addition, these compounds have been linked to the inhibition of cystein proteases, more specifically against cruzain (Beaulieu et al., 2010; Burtoloso et al., 2017; Fonseca Lameiro et al., 2021).

In this study the activity and cytotoxicity of 46 novel cyanomethyl vinyl ethers derivatives were tested. In addition, the mechanism of action was studied to determine the type of cell death that these compounds produce in the parasites.

2. Materials and methods

2.1. Compounds

The 46 tested novel compounds, included in Table 1, were synthetized as described by Delgado-Hernández et al., (2021) (Delgado-Hernández et al., 2021). These 46 cyanomethyl vinyl ethers were dissolved in dimethyl sulfoxide (DMSO, Merck, Darmstadt, Germany) and stored at −20 °C in the dark. When adding the compounds to the parasites, care must be taken not to exceed 1% DMSO, as higher concentrations could be toxic to the parasites.

2.2. Cultures

The trypanocidal analysis were developed using epimastigotes of *Trypanosoma cruzi* (Y strain) cultured in Liver Infusion Tryptose medium (LIT) supplemented with 10% of fetal bovine serum (FBS) at 26 °C. The cytotoxicity assays were performed using murine macrophages J774A.1 (ATCC #TIB-67) cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS at 37 °C and 5% CO_2 atmosphere.

2.3. Activity against epimastigote stage

The studies of activity against the epimastigote stage were performed using a 96 wells plate, serial dilutions of the cyanomethyl vinyl ethers were added with a quantity of 10^5 epimastigotes of *T. cruzi,* in a final volume of 200 µl of LIT per well. To see the fluorescence reaction a 10% of alamarBlue Cell Viability Reagent® (Thermo Fisher Scientific, Madrid, Spain) was added. After 3 days of incubation, which is the time when the parasite culture is in its exponential growth phase, i.e. maximum division and minimum elimination, the fluorescence was determined using the EnSpire Multimode Plate Reader® (PerkinElmer, ThermoFisher Scientific, Madrid, Spain). After corroborating at microscope that the parasites responsible of the colorimetric change are in process of death, the inhibitory concentration 50 (IC_{50}) was calculated using a nonlinear regression analysis (Núñez et al., 2021).

2.4. Studies of cytotoxicity on murine cells

To determine de cytotoxicity concentration 50 (CC_{50}) the same colorimetric assay based on the alamarBlue reagent was done. In this case, 10^4 cells per well were added previously until full cell adhesion is achieved to the 96 well plate, after that, serial dilutions of the compounds were added in a final volume of 100 µl of RPMI 1640 medium (Gibco, Waltham, MA, USA). Medium should be changed from DMEM as it may interfere with the fluorescence readings with alamarBlue, due to its colour intensity. After 24 h at 37 °C with 5% CO_2, which is sufficient time for the macrophage culture to reach its exponential growth phase, fluorescence was measured using the EnSpire Multimode Plate Reader® (PerkinElmer, ThermoFisher Scientific, Madrid, Spain). Finally, with the CC_{50} and the IC_{50} the selectivity index was calculated (CC_{50}/IC_{50}) (Cartuche et al., 2020).

2.5. Activity against amastigote stage

To determine the activity of the compounds against the amastigote stage of *T. cruzi,* the same colorimetric method based on the reduction of alamarBlue was used. A culture of epimastigotes was incubated for 4 days in LIT medium. In a 96 wells plate, 10^4 murine macrophages were added per well at a final volume of 50 µl in DMEM medium. After a minimum of 2 h of incubation at 37 °C with 5% CO_2, 5×10^4 parasites

Table 1
Molecular structure of the cyanomethyl vinyl ethers derivatives. BENZ: benznidazole.

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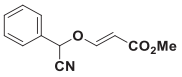
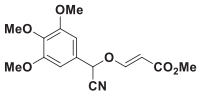
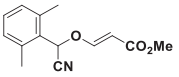
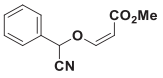
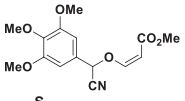
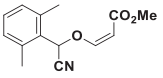
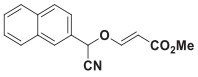
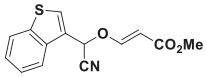
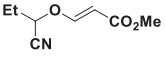
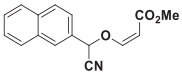
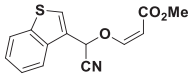
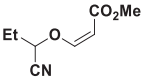
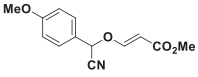
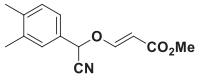
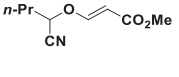
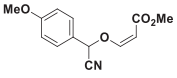
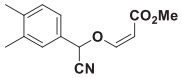
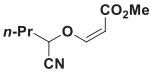
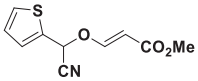
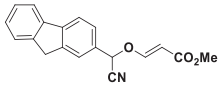
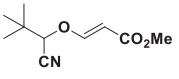
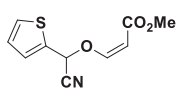
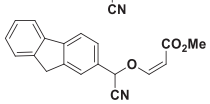
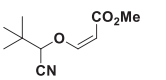
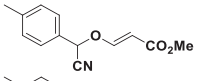
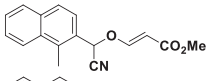
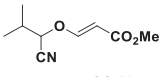
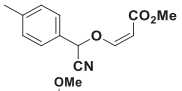
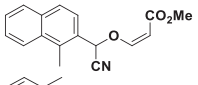
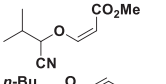
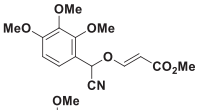
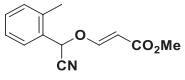
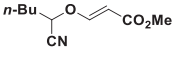
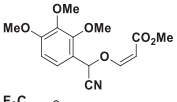
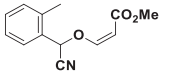
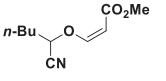
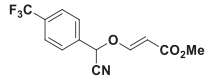
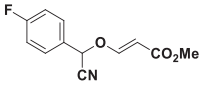
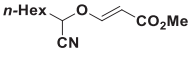
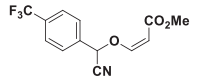
E51		E67		E83	
E52		E68		E84	
E53		E69		E85	
E54		E70		E86	
E55		E71		E87	
E56		E72		E88	
E57		E73		E89	
E58		E74		E90	
E59		E75		E91	
E60		E76		E92	
E61		E77		E93	
E62		E78		E94	
E63		E79		E95	
E64		E80		E96	
E65		E81		BENZ	
E66		E82			

were added to each well (ratio 5:1 parasite:macrophage) and incubated for 24 h at 37 °C with 5% CO_2. Following this incubation, wells were washed 3 times with fresh medium to eliminate the non-internalized parasites, and serial dilutions of the compounds were added at a final volume of 100 µl of DMEM. After an incubation of 24 h at 37 °C with 5% CO_2, the DMEM was removed and 30 µl of SDS at 0.05% in LIT was added for 30 s to induce the lysis of macrophages, following this, 170 µl of fresh LIT was quickly added to reach a final volume of 200 µl. Finally, 10% of alamarBlue was added, and after 72 h of incubation at 26 °C, the fluorescence was measured using the EnSpire Multimode Plate Reader® (PerkinElmer, ThermoFisher Scientific, Madrid, Spain) and the IC_{50} of the compounds was calculated using a nonlinear regression analysis.

2.6. Chromatin condensation assay

To analyze the presence of chromatin condensation the Vybrant® Apoptosis Assay Kit n◦5, Hoechst 33342/Propidium Iodide (ThermoFisher Scientific, Madrid, Spain) were used. Epimastigotes were incubated with the inhibitory concentration 90 (IC_{90}) of the compounds. After 24 h the solution was centrifuged (825 g, 10 min, 4 °C), resuspended in 50 µl of buffer with Hoechst (5 µg/mL) and propidium iodide (PI) (1 µg/mL) and incubated for 20 min at 26 °C. To see the results EVOS® FL Cell Imaging System (ThermoFisher Scientific, Madrid, Spain) was used. To determine the cell concentration of DAPI light cube and RFP light cube were processed with FIJI ImageJ 2.0 software, in

triplicate using 40x images. A negative control (without any treatment) and a reference treatment (benznidazole) were added (Bethencourt-Estrella et al., 2022).

2.7. Plasmatic membrane permeability assay

To determine the alterations in the plasmatic membrane permeability the SYTOX® Green nucleic acid stain fluorescent dye (ThermoFisher Scientific, MA, USA) was used. This probe is normally impermeable to intact plasma membranes, but is able to penetrate cells with increased permeability, reaching the nucleus and binding to nucleic acids, resulting in a strong increase in its fluorescence. The epimastigotes were incubated with the IC_{90} of the compounds for 24 h at 26 °C. Then, the solution was centrifuged (825 g, 10 min, 4 °C) and resuspended in 50 µl of buffer with 1 µM concentration of the kit. The measure of green cells concentration was developed using the Countess II FL (ThermoFisher Scientific, Madrid, Spain) and the images were done using GFP light cube in the EVOS® FL Cell Imaging System (ThermoFisher Scientific, Madrid, Spain). A positive control (Triton 0.5% for 30 min), a reference treatment (benznidazole) and a negative control (without any treatment) were added (López-Arencibia et al., 2019b).

2.8. ATP alterations assay

The alterations in the ATP levels were determined using CellTiter-Glo® Luminescent Cell Viability Assay (Promega, WI, USA). After an incubation of 24 h of epimastigotes with the IC_{90} of the compounds, the solution was centrifuged (825 g, 10 min, 4 °C) and resuspended in 25 µl of buffer and 25 µl of the kit. After 10 min of incubation, the luminescence was measured using the EnSpire Multimode Plate Reader® (PerkinElmer, ThermoFisher Scientific, Madrid, Spain) in a white plate. A positive control (sodium azide 20 mM for 3 h), a reference treatment (benznidazole) and a negative control (without any treatment) were added (López-Arencibia et al., 2019a).

2.9. Mitochondrial membrane potential alterations assay

To determine the alterations in the mitochondrial membrane potential the JC-1 Mitochondrial Membrane Potential Assay Kit® (Cayman Chemical, MI, USA) was used. The epimastigotes were incubated with the IC_{90} of the compounds for 24 h at 26 °C. After centrifuge (825 g, 10 min, 4 °C) and resuspend in 50 µl, 5 µl of the kit were added. Then, the fluorescence was measured using the EnSpire Multimode Plate Reader® (PerkinElmer, ThermoFisher Scientific, Madrid, Spain) in a black plate. A positive control (Carbonyl Cyanide Chlorophenylhydrazone, CCCP 100 µM for 3 h), a reference treatment (benznidazole) and a negative control (without any treatment) were added (Bethencourt-Estrella et al., 2022).

2.10. Presence of reactive oxygen species assay

The presence of reactive oxygen species (ROS) was measured using the CellROX® Deep Red Reagent (Thermo Fisher Scientific, Madrid, Spain). After incubating the epimastigotes with the IC_{90} of the compounds at 26 °C for 24 h, centrifuging (825 g, 10 min, 4 °C) and resuspending in 50 µl of buffer, the kit was added at 5 µM concentration for 30 min at 26 °C. The measure of red cells concentration was carried out using the Countess II FL (ThermoFisher Scientific, Madrid, Spain) and the images were done using Cy5 light cube in the EVOS® FL Cell Imaging System (ThermoFisher Scientific, Madrid, Spain) (Bethencourt-Estrella et al., 2021).

2.11. Phosphatidylserine externalization assay

The externalization of phosphatidylserine from the inner to the outer part of the plasma membrane is another characteristic event of apoptosis. This was determined using the Tali™ Apoptosis Kit- Annexin V Alexa Fluor® 488 (ThermoFisher Scientific, Madrid, Spain). This kit containing Annexin and PI divides cells into 3 populations: live (unstained), dead (stained with PI) or apoptotic (stained with annexin). The cells were quantified using the cytometer Tali® (ThermoFisher Scientific, Madrid, Spain) (López-Arencibia et al., 2021).

2.12. Statistical methods

The IC_{50} and CC_{50} were calculated using a non-linear regression analysis with 95% confidence, with the statistical software GraphPad Prism 9.0.0. All experiments were done in duplicate in three different days and expressed as mean ± standard desviation. After verifying the normal distribution of the data by Shapiro-Wilk test, a Tukey's test was used, considering significant values of $p < 0.05$. The results obtained from the mechanism of cell death induced were processed performing a one-way ANOVA, the GraphPad Prism 9.0.0 was also used.

3. Results

3.1. Activity against epimastigote stage

The results of activity against the epimastigote stage of *T. cruzi* are presented in Table 2, the inhibitory concentration 50 were expressed in µM as mean ± standard desviation.

3.2. Studies of cytotoxicity

The results of cytotoxicity against murine macrophages are presented in Table 3, the cytotoxicity concentration 50 were expressed in µM as mean ± standard desviation.

3.3. Selectivity index against epimastigote stage

The selectivity indexes of the epimastigote stage were calculated as the ratio CC_{50}/IC_{50}, these values were included in Table 4. The most selective cyanomethyl vinyl ethers were selected to do the different analysis to elucidate the type of cell death produced in the parasite.

3.4. Activity against amastigote stage

The results of activity against the amastigote stage of *T. cruzi* of the most selective compounds against amastigote stage were presented in Table 5, the inhibitory concentration 50 were expressed in µM as mean ± standard desviation.

Table 2
Activity of the cyanomethyl vinyl ethers derivatives against epimastigote stage of Trypanosoma cruzi, represented as inhibitory concentration 50 (IC_{50} in µM). *BENZ: benznidazole.*

ID	IC_{50} (µM)	ID	IC_{50} (µM)	ID	IC_{50} (µM)
E51	39.96 ± 3.73	E67	>150	E83	11.25 ± 1.47
E52	50.09 ± 0.28	E68	>150	E84	67.6 ± 1.35
E53	8.84 ± 0.37	E69	>150	E85	>150
E54	13.4 ± 1.24	E70	>150	E86	>150
E55	36.24 ± 0.65	E71	>150	E87	>150
E56	49.1 ± 2.79	E72	>150	E88	>150
E57	>150	E73	38.48 ± 3.51	E89	>150
E58	>150	E74	8.88 ± 0.95	E90	>150
E59	>150	E75	10.1 ± 1.74	E91	>150
E60	>150	E76	10.27 ± 1.64	E92	>150
E61	>150	E77	>150	E93	>150
E62	>150	E78	>150	E94	>150
E63	5.43 ± 0.14	E79	>150	E95	>150
E64	8.91 ± 1.75	E80	>150	E96	>150
E65	47.77 ± 1.3	E81	>150		
E66	46.62 ± 5.21	E82	>150	BENZ	6.92 ± 0.77

Table 3
Cytotoxicity of the cyanomethyl vinyl ethers derivatives against murine macrophages, represented as cytotoxic concentration 50 (CC_{50} in μM). ND: not determined. BENZ: benznidazole.

ID	CC_{50} (μM)	ID	CC_{50} (μM)	ID	CC_{50} (μM)
E51	709.44 ± 20.48	E67	ND	E83	339.82 ± 6.36
E52	>700	E68	ND	E84	>300
E53	47.47 ± 52.24	E69	ND	E85	ND
E54	200.46 ± 6.04	E70	ND	E86	ND
E55	>700	E71	ND	E87	ND
E56	>700	E72	ND	E88	ND
E57	ND	E73	>300	E89	ND
E58	ND	E74	>1300	E90	ND
E59	ND	E75	96.76 ± 23.36	E91	ND
E60	ND	E76	157.98 ± 18.99	E92	ND
E61	ND	E77	ND	E93	ND
E62	ND	E78	ND	E94	ND
E63	167.13 ± 4.13	E79	ND	E95	ND
E64	>300	E80	ND	E96	ND
E65	>700	E81	ND		
E66	>700	E82	ND	BENZ	399.91 ± 1.4 μM

Table 4
Selectivity index (SI) of the cyanomethyl vinyl ethers derivatives against epimastigote stage of Trypanosoma cruzi. ND: not determined. BENZ: benznidazole.

ID	SI	ID	SI	ID	SI
E51	11.52	E67	ND	E83	30.2
E52	>13.97	E68	ND	E84	4.4
E53	5.37	E69	ND	E85	ND
E54	15.0	E70	ND	E86	ND
E55	>19.3	E71	ND	E87	ND
E56	>14.3	E72	ND	E88	ND
E57	ND	E73	7.8	E89	ND
E58	ND	E74	146.4	E90	ND
E59	ND	E75	9.6	E91	ND
E60	ND	E76	15.4	E92	ND
E61	ND	E77	ND	E93	ND
E62	ND	E78	ND	E94	ND
E63	30.8	E79	ND	E95	ND
E64	>33.7	E80	ND	E96	ND
E65	>14.7	E81	ND		
E66	>15.0	E82	ND	BENZ	57.8

Table 5
Activity of the cyanomethyl vinyl ethers derivatives against amastigote stage of Trypanosoma cruzi, represented as inhibitory concentration 50 (IC_{50} in μM). BENZ: benznidazole.

ID	IC_{50} (μM)	ID	IC_{50} (μM)	ID	IC_{50} (μM)
E63	16.11 ± 0.70	E74	38.62 ± 8.88		
E64	9.94 ± 0.80	E83	23.27 ± 2.93	BENZ	2.67 ± 0.39

3.5. Selectivity index against amastigote stage

The selectivity indexes against the amastigote stage of the most selective cyanomethyl vinyl ethers were presented in Table 6.

3.6. Chromatin condensation analysis

The presence of chromatin condensation and dead cells was expressed in mean fluorescence intensity in Fig. 1. The images of DAPI and RFP light cubes taken EVOS® FL Cell Imaging System were included in figure S1 and S.2.

Table 6
Selectivity index of the cyanomethyl vinyl ethers derivatives against amastigote stage of Trypanosoma cruzi. BENZ: benznidazole.

ID	SI	ID	SI	ID	SI
E63	10.4	E74	33.7		
E64	30.2	E83	14.6	BENZ	149.8

3.7. Plasmatic membrane permeability analysis

The presence of plasmatic membrane permeability was expressed in percentage relative to the negative control. These results were included in Fig. 2. The images of GFP light channel obtained with the EVOS® FL Cell Imaging System were included in figure S3.

3.8. ATP alterations analysis

The levels of ATP were expressed in percentage relative to the negative control and included in Fig. 3.

3.9. Mitochondrial membrane potential alterations analysis

The results of alterations in the mitochondrial membrane potential were expressed in percentage relative to the negative control and included in Fig. 4.

3.10. Presence of reactive oxygen species analysis

The presence of reactive oxygen species was presented in Fig. 5, as a percentage relative to the negative control. The images obtained with the Cy5 light cube in the EVOS® FL Cell Imaging System were included in figure S4.

3.11. Phosphatidylserine externalization analysis

The externalization of phosphatidylserine was expressed as percentage of live (black), dead (red) and apoptotic (green) cells. The percentage of stained cells obtained with the Tali® was presented in Fig. 6.

4. Discussion

From the 46 cyanomethyl vinyl ethers studied 16 presented activity against epimastigote stage of *T. cruzi* at the range between 5.43 and 67.6 μM. These most active compounds were selected to study the cytotoxicity and to determine the activity against amastigote stage. Out of these compounds, four of them, E63, E64, E74 and E83, showed a good selectivity index against epimastigote stage, close to the reference treatment, benznidazole. Furthermore, E74 showed a selectivity index almost three times higher than benznidazole. These four compounds showed activity against the amastigote stage with selectivity indexes between 10.4 and 33.7.

Comparing the results of activity against the different forms of the parasite, cyanomethylvinyl ethers show better activity against the epimastigote forms of the parasite. This is corroborated by the idea that, because they contain cyano groups, they act on cruzipain, which is expressed to a greater extent in the epimastigote forms than in the amastigote forms (Beaulieu et al., 2010; Burtoloso et al., 2017).

These four most selective compounds were selected to study the type of mechanism they perform for the elimination of the parasite. For this purpose, different events characteristic of apoptotic-like death were studied. Events studied include chromatin condensation, decreased cellular ATP level, altered plasma membrane permeability, decreased mitochondrial membrane potential, exposure to phosphatidylserine and accumulation of reactive oxygen species. All of these are characteristic of apoptotic cell death (Basmaciyan et al., 2019; Basmaciyan y Casanova, 2019; Bethencourt-Estrella et al., 2022; López-Arencibia et al., 2019a; Menna-Barreto, 2019).

In this step, to demonstrate that these four most selective compounds produced apoptotic cell death, the results shown that these four

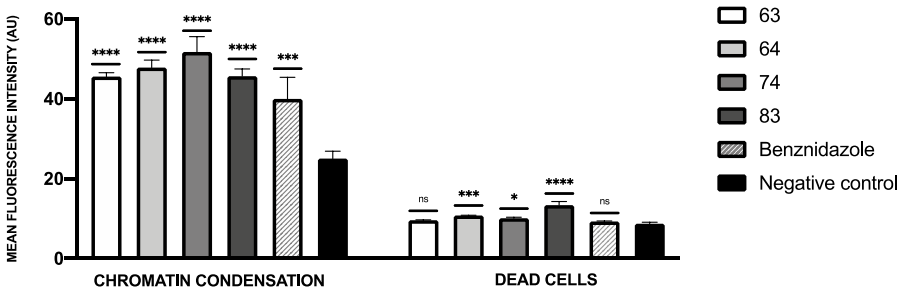


Fig. 1. Results of the chromatin condensation and the number of dead cells represented as mean fluorescence intensity, benznidazole was used as the reference treatment. A Tukey test with the GraphPad.PRISM® 9.0 software was done to test the statistical differences between means. (non-significant [ns]; $p < 0.05$ [*]; $p < 0.001$ [***]; $p < 0.0001$ [****]).

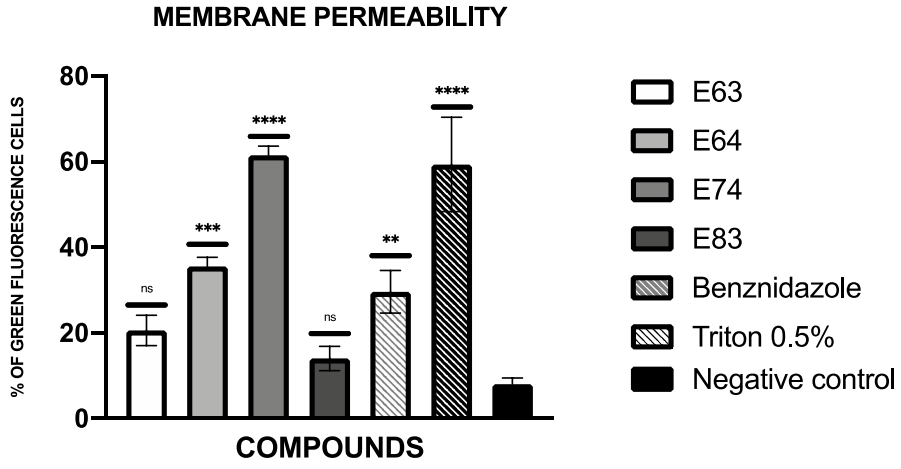


Fig. 2. Results of the plasma membrane permeability represented as the percentage of green cells, benznidazole was used as the reference treatment, and Triton at 0.5% was used as positive control (C+). A Tukey test with the GraphPad.PRISM® 9.0 software was done to test the statistical differences between means. (non-significant [ns]; $p < 0.01$ [**]; $p < 0.001$ [***]; $p < 0.0001$ [****]).

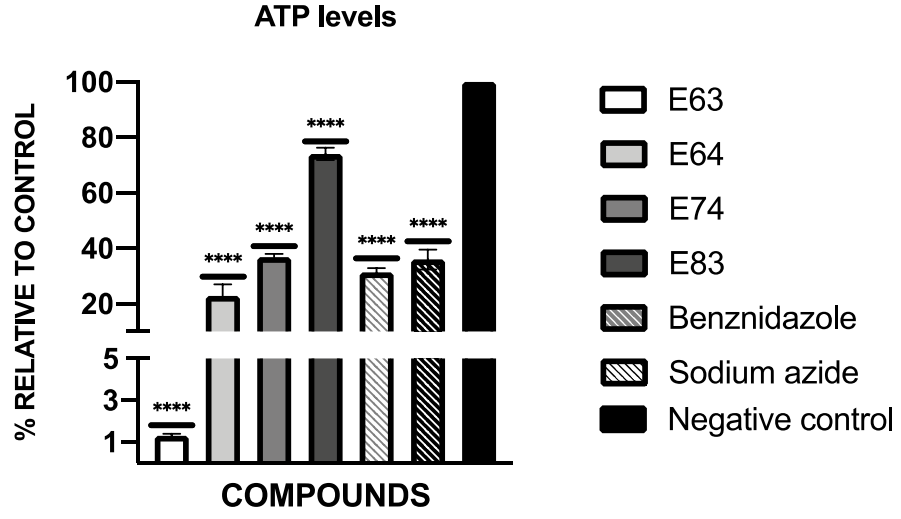


Fig. 3. Results of the decrease in the ATP levels represented as the percentage relative to negative control (C-), benznidazole was used as the reference treatment, and sodium azide was used as positive control (C+). A Tukey test with the GraphPad.PRISM® 9.0 software was done to test the statistical differences between means. ($p < 0.0001$ [****]).

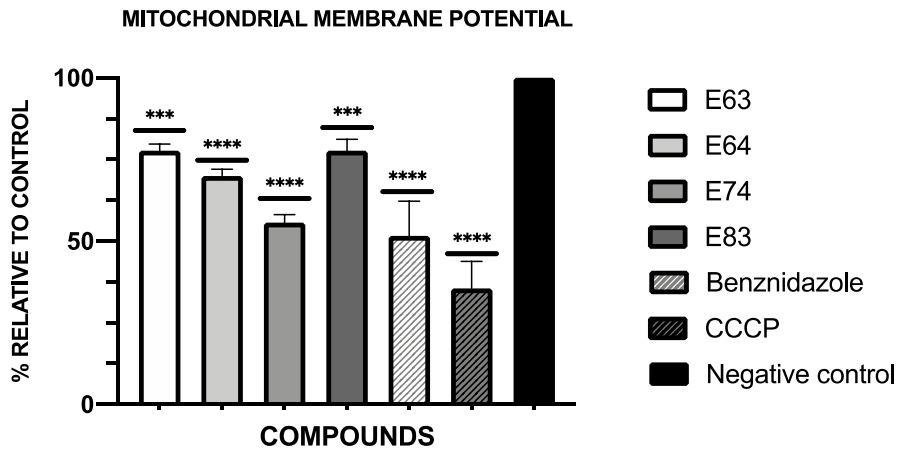


Fig. 4. Results of mitochondrial membrane potential alterations (△Ψm) represented as the percentage relative to negative control (C-), benznidazole was used as the reference treatment, and CCCP was used as positive control (C+). A Tukey test with the GraphPad.PRISM® 9.0 software was done to test the statistical differences between means. ($p < 0.001$ [***]; $p < 0.0001$ [****]).

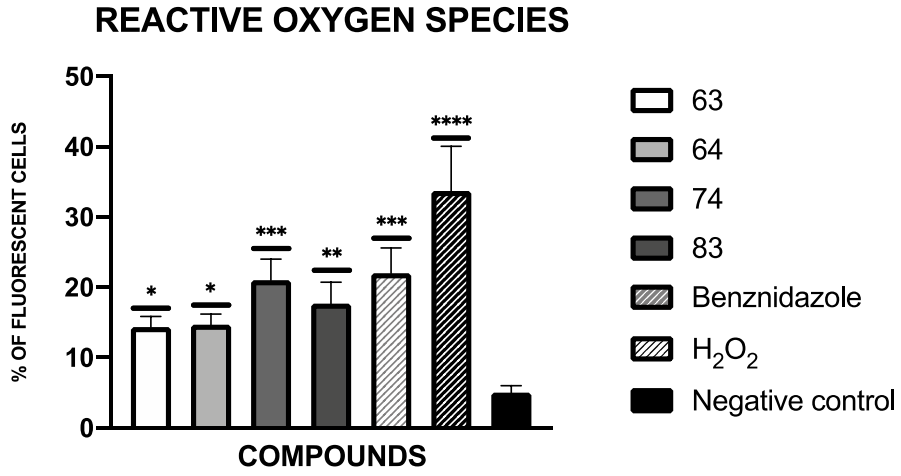


Fig. 5. Results of reactive oxygen species represented as percentage of fluorescent cells, benznidazole was used as the reference treatment, and H_2O_2 was used as positive control (C+). A Tukey test with the GraphPad.PRISM® 9.0 software was done to test the statistical differences between means. ($p < 0.05$ [*]; $p < 0.01$ [**]; $p < 0.001$ [***]; $p < 0.0001$ [****]).

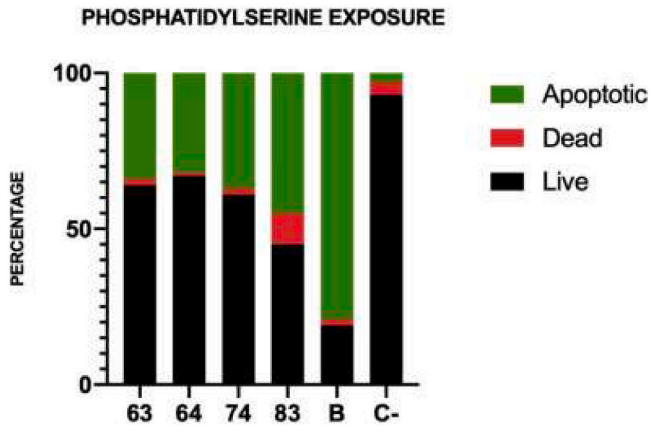


Fig. 6. Results of phosphatidylserine exposure represented as percentage of apoptotic (green), dead (red) and live (black) cells, benznidazole was used as the reference treatment. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

compounds produce the above-mentioned events characteristic of this type of programmed cell death. It can therefore be concluded that they appear to be good trypanocides, as they will not produce the problems caused by necrotic cell death.

Regarding the IC_{50} values, the configuration of the double bond does not seem to play an important role because the activity of each pair of isomers is very similar in most of the cases. In fact, there are no cases where one of the isomers lacks activity (>150) while its counterpart has a significant activity (<100).

The most significant differences between pairs of isomers are 73–74, where the Z-isomer is over four times more active than the E-isomer, and 83–84, where the E-isomer is this time six times more active than the Z-isomer. There is no evident explanation for the enhanced activity for one of the two isomers in each case.

The difference in the IC_{50} values of these cyanomethyl vinyl ethers is therefore mainly due to the substituent present at the sp3 carbon other than the cyano group. While aliphatic (85–96) or heteroaromatic (57–58, 69–70) have no significant activity, some aromatic substituents present IC_{50} in the 1–70 μM range. Among all the aromatic substituents, again, there is no clear explanation on how the electronic nature of the aromatic ring affects the biological activity but there are numerous

examples in which the IC_{50} values are in the 5–15 μM range.

Paying attention to the structures of these compounds, it seems that part of their activity comes from the cyano group, but clearly the other substituents modify the activity of these compounds. This is corroborated with previous literature, where antikinetoplastid activity from compounds with cyano groups has already been reported. One example is the study by Gerpe et al. where the best results of IC_{50} against epimastigotes of two different strains of *Trypanosoma cruzi* were obtained as values of 7 and 16 μM, and another example of work is that of Ancizu et al. where the lowest IC_{50} values were around 10 and 19 μM (Ancizu et al., 2009; Gerpe et al., 2006). All this, when compared to the present work, highlights the better activity of the ethers, which presents up to 8 compounds with values in these ranges, where compound E63 stands out for its activity with an IC50 value of 5.43 μM. Therefore, this study confirms the literature, and puts compounds with cyano groups as a line to continue the search for possible trypanocidal compounds (Barea et al., 2012; Chao et al., 2019; Farahat et al., 2018; Torrence et al., 2006).

5. Conclusions

In conclusion, compounds E63, E64, E74 and E83 show *in vitro* activity against *Trypanosoma cruzi*, as well as apoptotic cell death, and are therefore promising antichagasic compounds with which to continue studies.

Author contributions

Conceptualization, J.E.P. and J.L.-M.; methodology, C.J.B.-E. and A. L.-A.; software, C.J.B.-E.; validation, A.L.-A.; J.E.P. and J.L.-M.; formal analysis, C.J.B.-E., D.S.N.-H. and A.L.-A.; investigation, C.J.B.-E.; resources, S.D.-H.; data curation, C.J.B.-E.; writing—original draft preparation, C.J.B.-E.; writing—review and editing, J.L.-M. and J.E.P; visualization, F.G.-T. and D.T.; supervision, J.E.P.; project administration, J.L.-M.; funding acquisition, J.L.-M. and J.E.P. All authors have read and agreed to the published version of the manuscript.

Note

Supplementary data associated with this article.

Declaration of competing interest

The authors declare no conflicto of interest.

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Appendix A. Supplementary data

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