

In vitro activity of thienyl-2-nitropropene compounds against *Trypanosoma cruzi*

Cristina Herrera^{1/+}, Gabriel A Vallejos², Randall Loaiza³, Rodrigo Zeledón³, Andrea Urbina³, Silvia Sepúlveda-Boza⁴

¹Instituto de Investigaciones Farmacéuticas, Facultad de Farmacia, Universidad de Costa Rica, San José, Costa Rica ²Instituto de Química, Facultad de Ciencias, Universidad Austral de Chile, Valdivia, Chile ³Laboratorio de Zoonosis, Escuela de Medicina Veterinaria, Universidad Nacional, Heredia, Costa Rica ⁴Laboratorio Emory Black, Escuela de Medicina, Facultad de Ciencias Médicas, Universidad de Santiago de Chile, Santiago, Chile

The in vitro activity of four 2-nitropropene derivatives, 1-(3-benzothienyl)-2-nitropropene (N1), 1-(3-thienyl)-2-nitropropene (N2), 1-(5-bromo-2-thienyl)-2-nitropropene (N3) and 1-(4-bromo-2-thienyl)-2-nitropropene (N4), were tested against cultures of the parasite Trypanosoma cruzi. Cytotoxicity studies were performed using Vero cells. The blood trypomastigotes, amastigotes and epimastigotes showed differential degrees of sensitivity towards the four tested compounds; the highest activity against the epimastigotes and blood trypomastigotes was exhibited by N1, followed by N3, N4 and finally N2. In contrast, whereas the compounds N1, N3 and N4 exerted similar magnitudes of activity against amastigotes, N2 was found to be a much less potent compound. According to our results, the compound N1 had the highest level of activity (IC₅₀: 0.6 μM) against epimastigotes.

Key words: *Trypanosoma cruzi* - thienyl-2-nitropropene compounds - Chagas disease - antitrypanosomal activity

Chagas disease, caused by *Trypanosoma cruzi*, is responsible for significant economic and public health burdens in Latin America (Dias et al. 2002, Urbina 2002, Moncayo 2003, Cerecetto & González 2008). The drugs currently in use for the treatment of Chagas disease are nifurtimox (4[(5-nitrofurfuryl-idene)amino]-3-methylthiomorpholine-1,1-dioxide), which is derived from nitro-furan (Lampit[®], Bayer), and benznidazole (*N*-benzyl-2-nitroimidazole-1-acetamide) (Rochagan[®], Roche). Both of these drugs are trypanocidal for all forms of the parasite (Mady et al. 2008, Mecca et al. 2008) and act via the production of free radicals by a metabolic pathway. As enzymes that scavenge free radicals are either absent or have very low levels of activity in the parasite, *T. cruzi* is highly susceptible to the damage that is induced by these metabolites (Urbina 1999, 2002 Docampo 2001). The two drugs listed above are more effective during the acute phase of the disease. During the chronic phase, their activity is limited. Additionally, nifurtimox and benznidazole can also cause systemic toxicity and adverse effects that include anorexia, nausea, vomiting, headache, central nervous system depression, maniacal symptoms, seizures, vertigo, paraesthesias, peripheral polyneuropathies and dermatitis (Coura & De Castro 2002, Urbina & Docampo 2003, Kirchhoff 2005, Rodríguez 2005, Urbina 2005).

Both the high prevalence of trypanosomiasis and the limitations to the treatments that are currently available indicate the need for new drugs that are ef-

fective, selective and well tolerated. The mechanism of action of natural and synthetic drugs against *T. cruzi* and their interactions with the mammalian host were reviewed by Maya et al. (2007).

Several imidazole and nitro-furan analogues have been tested against *T. cruzi* and these analogues have been found to exert similar activities against the epimastigote and the trypomastigote forms (Pozas et al. 2005). Although megalol, a 5-nitroimidazole derivative, has been used to treat *Trypanosoma brucei* infection (Darsaud et al. 2004) and at least one study highlights its therapeutic efficacy against *T. cruzi* infection (Maya et al. 2003), its utility has been called into question as a result of reports that it can cause chromosomal alterations and DNA damage (Enanga et al. 2003, Nesslany et al. 2004).

In contrast, González et al. (2007) synthesized a series of bis-2,5-(4-guanidinophenyl) thiophenes that had antichagasic properties, but lacked cytotoxic effects. The most active of these compounds had an IC₅₀ of 1.7 μM. Another thiophen derivative, 2-(5-nitrothiophen-2-ylmethylene) malononitrile, which is less structurally complex than the previous compounds, exerted a degree of anti-amastigote activity that was similar to nifurtimox and benznidazole; however, this compound was also a lot more cytotoxic than the reference drugs (Muelas-Serrano et al. 2002). The cytotoxic and antitumor effects of phenyl-2-nitropropenes were demonstrated two decades ago (Cassels et al. 1982).

Based on the cytotoxic characteristics of the phenyl-2-nitropropenes and the potential pharmacophoric effect of the thiophene group, we decided to investigate the efficacy of thienyl-2-nitropropene compounds as *T. cruzi* inhibitors. Furthermore, we tested the effect of removing the nitro group of the aromatic system and placing it on a lateral chain that was conjugated with the π system of the propene group.

+ Corresponding author: cristina.herrera@ucr.ac.cr

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In this paper, the activities of four synthetic compounds related to thienyl-2-nitropropenes were tested in vitro against the amastigotes, epimastigotes and blood trypomastigotes of three strains of *T. cruzi*. Cytotoxicity tests were performed on Vero cells.

MATERIALS AND METHODS

Compounds - 1-(3-benzothieryl)-2-nitropropene (N1), 1-(3-thienyl)-2-nitropropene (N2), 1-(5-bromo-2-thienyl)-2-nitropropene (N3) and 1-(4-bromo-2-thienyl)-2-nitropropene (N4) (Fig. 1) were assayed as possible agents against *T. cruzi*. We have previously reported the synthesis and NMR characterization of all of the nitropropenes used (Vallejos et al. 2005).

Biological assays - For the biological assays, the test substances were dissolved in 0.1% or less dimethyl sulphoxide (DMSO), according to the literature (De Souza et al. 2002, Kamnaing et al. 2003).

Parasites - Three strains of *T. cruzi* were used: the Jennifer strain, which was isolated in 2003 from an acute case of Chagas disease in a Costa Rican child (6 years old), the GA strain, which was isolated in 1998 from an adult (48 years old) from Costa Rica and the CL transgenic strain (kindly donated by the Universidad Complutense, Madrid), as defined by Le-Senne et al. (2002). Epimastigotes were grown on LIT medium supplemented with 10% bovine foetal serum (bfs). Blood trypomastigotes were harvested in Locke solution and the intracellular forms were obtained from Vero cells, as described below.

Cell cultures - Vero cells were cultivated on Dulbecco's Minimal Essential Medium (Sigma-Aldrich) supplemented with 10% bfs, 20 μ M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and antibiotics (benicillillin 200 μ g/mL - streptomycin 200 U/mL).

Cytotoxicity test - 40,000 Vero cells/well were incubated overnight on 96-well plates at 37°C with CO₂ (5%). The cells were exposed to different concentrations of the

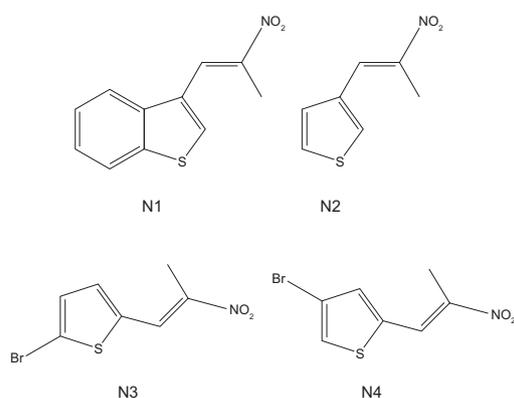


Fig. 1: chemical structures of thienyl-2-nitropropene [N1: 1-(3-benzothieryl)-2-nitropropene; N2: 1-(3-thienyl)-2-nitropropene; N3: 1-(5-bromo-2-thienyl)-2-nitropropene; N4: 1-(4-bromo-2-thienyl)-2-nitropropene].

compounds (100-3 μ M) for a period of 24 h (Sánchez et al. 2002). Cell quantification was performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolide bromide (MTT) reduction method that was described by Mosman (1983), with the following slight modification: to increase sensitivity and reduce the speed of transformation of MTT to formazan, 5 mg/mL of MTT were supplemented with 2 mM phenazine methosulphate (PMS). After incubation for 45 min, the supernatants were extracted, the cells were washed twice with PBS (pH 7.2) and formazan crystals were dissolved in 100 μ L DMSO. The absorbance was quantified on a Termo Labsystems[®] microplate reader at 540 nm.

Activity against epimastigotes expressed as IC₅₀ - A total of 1 x 10⁶ epimastigotes/mL were incubated with different concentrations of the compounds (100-0.1 μ M) for periods of 72 h at 28°C. The Jennifer strain and the CL strain epimastigotes were quantified by the reduction of MTT/PMS, as described previously (Mosman 1983).

Activity against amastigotes expressed as IC₅₀ - A total of 4,000 Vero cells/well were incubated overnight in 96-well plates. Cells were then infected for 2 h with either 20 parasites/cell of the Jennifer strain or 10 parasites/cell of the CL strain. Non-infective parasites were removed with PBS and the cells were incubated with different concentrations of the compounds for six days. The number of intracellular parasites was determined indirectly by counting the numbers of extracellular parasites in a Neubauer chamber (Hyde & Dvorak 1973).

Activity against trypomastigotes - Trypomastigotes of the GA strain were isolated from the heart blood of Balb/c infected mice, diluted in Asever's solution and centrifuged at 206 g to eliminate the red cells. The supernatant was collected and then centrifuged at 1,288 g and the pellet was resuspended to a concentration of 1 x 10⁶ trypomastigotes/mL. A 25 μ L volume of the suspension (25,000 parasites/well) was exposed to different concentrations of the compounds (100-6.25 μ M) in 25 μ L of Locke solution, for a period of 4 h. DMSO (0.5%) was used as a positive control and Locke solution was used as a negative control. The numbers of parasites and their motility were determined using an inverted light microscope and 400X amplification. The experiments were performed twice for each concentration of the drugs; these assays are considered to be preliminary tests.

Statistical analysis - The cytotoxicity percentage (CP) against Vero cells and the activity percentages (AP) against epimastigotes and amastigotes were calculated using the following formula: AP (or CP) = (1-Mt/Mc)*100 (Muelas et al. 2002, Sánchez et al. 2002, Petray et al. 2004), where Mt represents the measurement taken after the treatment and Mc is the measurement of the negative control. Each concentration of the compounds was assayed in triplicate on the same day and the assays were repeated at least three times on different days. The IC₅₀ value for each compound was calculated by plotting the AP (or CP) against the logarithm of the com-

pound concentration for each assay (Petray et al. 2004). Comparisons of the mean IC_{50} values were made using the one-tailed ANOVA with the Tukey test as a *post-hoc* test. Differences with a p-value equal to or less than 0.05 were considered to be significant.

RESULTS

The IC_{50} values of compounds N1, N2, N3 and N4 for cytotoxicity against Vero cells, epimastigotes and amastigotes are shown in Table I and Fig. 2. Statistical analysis of the IC_{50} values revealed that N2 exerted significantly less cytotoxicity against Vero cells than the other three compounds N1 ($p = 0.014$), N3 ($p = 0.001$) and N4 ($p = 0.002$).

In contrast, N1 was the most active compound against epimastigotes of the Jennifer strain. The effect of N1 was significantly larger than that produced by either N2 ($p = 0.001$) or N4 ($p = 0.037$); however, there was no significant difference between the effects produced by N1 and those of N3 ($p = 0.390$). Compared to N2, there were significant differences in the IC_{50} values of N3 ($p = 0.002$) and N4 ($p = 0.017$) against epimastigotes of the Jennifer strain. Similarly, N1 was also the most active compound against epimastigotes of the CL strain; the effects resulting from the use of N1 were significantly different from those produced by N2 ($p = 0.001$) and N4 ($p = 0.008$), but they were not different to N3 ($p = 0.970$). Compared to N2, the IC_{50} values of N3 ($p = 0.001$) and N4 ($p = 0.007$) against epimastigotes of the CL strain were significantly different. The activity of each compound against epimastigotes was similar for both the Jennifer and CL strains.

N1, N3 and N4 exhibited comparable activities against amastigotes of the Jennifer strain. The IC_{50} value of N2 was significantly different to the IC_{50} values of each of the other three compounds: N1 ($p = 0.001$), N3 ($p = 0.001$) and N4 ($p = 0.001$). A similar result was obtained against amastigotes of the CL strain. N2 was found to have lower efficacy than each of the other three compounds: N1 ($p = 0.001$), N3 ($p = 0.001$) and N4 ($p = 0.001$). There were no significant differences in the activities of any of the four compounds between amastigotes of the Jennifer strain and those of the CL strain.

When tested against trypanomastigotes of the GA strain, all compounds produced decreases in parasite motility and small reductions in the amount of parasites. N1 ex-

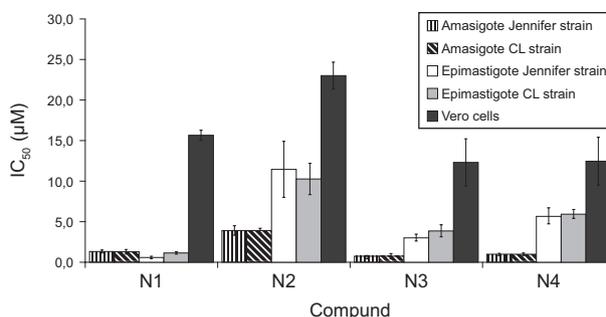


Fig. 2: IC_{50} values against Vero cells, epimastigotes and amastigotes of 1-(3-benzothienyl)-2-nitropropene (N1), 1-(3-thienyl)-2-nitropropene (N2), 1-(5-bromo-2-thienyl)-2-nitropropene (N3) and 1-(4-bromo-2-thienyl)-2-nitropropene (N4) compounds.

erted the highest activity, followed by N3 and N4 (which had similar levels of activity). N2 exhibited the lowest activity (Table II). At a concentration of 12.5 µM, N1 and N4 decreased the numbers of parasites after 4 h, whereas N2 and N3 did not. At a concentration of 6.25 µM, all of the compounds decreased the motility of parasites after 4 h. A greater suppression of activity was observed when 12.5 µM concentrations of N1 and N4 were used.

DISCUSSION

The four compounds under investigation in our study were found to exert differential toxic effects against Vero cells and all parasitic forms at different concentrations. These results suggest that, in contrast to mammalian cells, the four compounds exhibit a degree of specificity for parasites. The most pronounced differences were observed with compound N1, which was found to be the most active against all parasite forms tested.

As N1 had the greatest effect against both epimastigotes and trypanomastigotes, followed by N3, N4 and finally N2, it might be suggested that these compounds act on a similar target in the flagellar forms of parasites and that their effects are independent of the parasite strain used.

In contrast, the activities of the compounds against amastigotes showed a different pattern. Whereas compounds N1, N3 and N4 exerted a similarly high inhibitory effect against amastigotes, N2 produced only modest activity.

TABLE I

IC_{50} (µM) values against Vero cells, epimastigotes and amastigotes of Jennifer and CL strain

| Compound | Vero cells | Epimastigotes | | Amastigotes | |
|----------|------------|---------------|------------|-------------|-----------|
| | | Jennifer | CL | Jennifer | CL |
| N1 | 15.6 ± 0.6 | 0.6 ± 0.2 | 1.1 ± 0.3 | 1.3 ± 0.2 | 1.3 ± 0.2 |
| N2 | 23.1 ± 1.7 | 11.5 ± 3.4 | 10.3 ± 1.9 | 3.8 ± 0.6 | 3.9 ± 0.3 |
| N3 | 12.4 ± 2.9 | 3.0 ± 0.5 | 3.9 ± 0.7 | 0.6 ± 0.6 | 0.7 ± 0.2 |
| N4 | 12.5 ± 2.8 | 5.6 ± 1.2 | 6.0 ± 0.6 | 1.1 ± 0.1 | 1.0 ± 0.1 |

TABLE II
Activity of each compound against trypomastigotes of GA strain

| Compound | Concentration (μ M) | Amount of parasites ^a and time (min) | | | | | Motility of parasites ^b and time (min) | | | | |
|----------------|-----------------------------|-------------------------------------------------|------|------|------|------|---------------------------------------------------|------|------|------|------|
| | | 30 | 60 | 120 | 180 | 240 | 30 | 60 | 120 | 180 | 240 |
| N1 | 100 | ++++ | +++ | ++ | + | + | ++ | + | + | + | + |
| | 50 | ++++ | ++++ | +++ | ++ | + | +++ | ++ | + | + | + |
| | 25 | ++++ | ++++ | +++ | ++ | ++ | +++ | ++ | ++ | ++ | + |
| | 12.5 | ++++ | ++++ | ++++ | ++++ | +++ | ++++ | +++ | +++ | ++ | ++ |
| | 6.25 | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | +++ | +++ | +++ | +++ |
| N2 | 100 | ++++ | ++++ | ++++ | ++++ | +++ | +++ | +++ | ++ | ++ | + |
| | 50 | ++++ | ++++ | ++++ | ++++ | +++ | +++ | +++ | +++ | ++ | ++ |
| | 25 | ++++ | ++++ | ++++ | ++++ | ++++ | +++ | +++ | +++ | ++ | ++ |
| | 12.5 | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | +++ | +++ | +++ | +++ |
| | 6.25 | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | +++ | +++ | +++ | +++ |
| N3 | 100 | ++++ | ++++ | +++ | ++ | + | ++ | + | + | + | + |
| | 50 | ++++ | ++++ | ++++ | +++ | ++ | +++ | ++ | ++ | ++ | + |
| | 25 | ++++ | ++++ | ++++ | ++++ | +++ | ++++ | +++ | +++ | +++ | + |
| | 12.5 | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | +++ | +++ | +++ | +++ |
| | 6.25 | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | +++ | +++ | +++ | +++ |
| N4 | 100 | ++++ | +++ | +++ | + | + | ++ | + | + | + | + |
| | 50 | ++++ | ++++ | ++++ | +++ | ++ | +++ | ++ | ++ | + | + |
| | 25 | ++++ | ++++ | ++++ | ++++ | +++ | +++ | +++ | +++ | ++ | ++ |
| | 12.5 | ++++ | ++++ | ++++ | ++++ | +++ | ++++ | +++ | +++ | +++ | ++ |
| | 6.25 | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | +++ | +++ | +++ | +++ |
| Locke solution | | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ |
| DMSO 0.5% | | ++++ | ++++ | +++ | +++ | ++ | +++ | +++ | +++ | +++ | ++ |

a: amount of parasites (++++: parasites with normal motility; +++: parasites with slow and normal motility; ++: parasites with slow motility; +: parasites with slow motility and without motility); *b*: motility (++++: more than 20 parasites; +++: 11-20 parasites; ++: 6-10 parasites; +: 1-5 parasites); DMSO: dimethyl sulphoxide; N1: 1-(3-benzothienyl)-2-nitropropene; N2: 1-(3-thienyl)-2-nitropropene; N3: 1-(5-bromo-2-thienyl)-2-nitropropene; N4: 1-(4-bromo-2-thienyl)-2-nitropropene.

Compared to the epimastigote forms, an activity-switch was observed for compounds N2, N3 and N4 against amastigotes; however, this behaviour was not observed for N1. Although no definitive conclusions can be drawn, it might be hypothesized that metabolic differences between the distinct forms of the parasite could account for the varying effects of N1. However, although the incubation periods for the blood forms lasted for less than 72 h, the experiments against the amastigotes were conducted over a period of six days; therefore, compared to the other compounds, faster inactivation of N1 in the latter case might partially explain these results.

The four compounds, N1, N2, N3 and N4, share the same thienyl-2-nitropropene structure; therefore, the differences in their activity are related to the presence of benzene in N1, but bromide in N3 and N4. If N1, N2, N3 and N4 interacted with a protein, they might fit into a hydrophobic pocket. Under this assumption, because it is the most bulky side substituent, the benzene in N1 would fit better into the hydrophobic pocket; this would be followed by the bromide groups in N3 and N4. In the case of N2, the absence of a hydrophobic substituent, leads to a decrease in its activity. As one may expect a general

increase in activity against *T. cruzi* with the presence of hydrophobic and bulky substitutions at positions 2 and 3 of the thiophen group, this could be very important. Therefore, to increase its activity, it is possible that compound N1 might be chemically modified.

To compare the effect of the nitropropene group with that of the aminopropane group, we proposed the synthesis of the following thienyl-2-aminopropanes compounds: 1-(3-benzothienyl)-2-aminopropane (A1), 1-(3-Thienyl)-2-aminopropane (A2) and 1-(5-bromo-2-thienyl)-2-aminopropane (A3) from thienyl-2-nitropropene compounds N1, N2 and N3 (in which the nitro groups were reduced to amino groups), respectively. The compounds A1, A2 and A3 did not show activity against both epimastigotes and trypomastigotes. These data suggest that the nitropropene group is important for the activity of the compounds (unpublished observations).

In summary, two properties of the compounds that we investigated appear to be responsible for their activity against the *T. cruzi* parasite: (i) the thiophene group, which is present in each of the four compounds, resembles the imidazole and tiazole groups that bind iron from the haem groups of the cytochrome P450 enzymes

and thereby inhibit its function (De Souza et al. 2002) and (ii) the nitro group, which might produce oxidative stress by interacting with macromolecules through the production of nitrous derivatives (Henderson et al. 1988, Stoppani 1999, Cerecetto et al. 2000, Muelas et al. 2002, Enanga et al. 2003, De Oliveira et al. 2003, 2006, Maya et al. 2003, Olea-Azar et al. 2003, Stewart et al. 2004, Pozas et al. 2005, Quesada et al. 2006).

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