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**RESEARCH ARTICLE**

# Trypanocidal activity of the ethyl esters of *N*-propyl and *N*-isopropyl oxamates on intracellular amastigotes of *Trypanosoma cruzi* acute infected mice

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**Abstract**

In this investigation we studied the trypanocidal activity of the ethyl esters of N-propyl (Et-NPOX) and N-isopropyl (Et-NIPOX) oxamates on bloodstream trypomastigotes and on the clinically relevant intracellular amastigotes of Trypanosoma cruzi acute infected mice. In the infected and treated mice, the levels of parasitemia were drastically reduced between days 15 and 20 of treatment and almost to zero between days 35 and 40. We also found that Et-NPOX completely eliminated amastigote nests in the myocardium of mice infected with INC-5 or NINOA T. cruzi strain, and in skeletal muscle the reduction in the number of amastigote nests was between 60 and 80% in both strains. Also, Et-NIPOX reduced by 60–80% the number of amastigote nests in the myocardium and skeletal muscle of mice infected with these T. cruzi strains. In contrast, nifurtimox, used for comparison, produced a reduction of amastigote nests of only 20–40% in the studied tissues in both strains.

**Keywords:** Trypanosoma cruzi*; prodrugs; ethyl* N*-isopropyl oxamate; ethyl* N*-propyl oxamate; amastigotes*



## Introduction

Chagas' disease is an endemic parasitic disease in Latin America, and it is caused by *Trypanosoma cruzi*[1]. The vectorial transmission is effected by triatomine insects[2]; humans and wild and domestic animals are the natural reservoirs of *T. cruzi*. It is estimated that 16–18 million people are infected by *T. cruzi* and that some 100 million are at risk of acquiring Chagas' disease[2,3]. There are three stages of the human disease: the acute stage which appears shortly after infection, characterized by a large increase of bloodstream trypomastigotes, followed by a silent or asymptomatic stage, and the chronic stage, characterized by a large increase of intracellular amastigotes, which may last several years and irreversibly affects internal organs such as the heart, esophagus, colon, and peripheral nervous system[4].

Chagas' disease remains practically incurable due to the limited interest in developing new antichagasic drugs and the fact that the clinically available drugs for the treatment of Chagas' disease, benznidazole and nifurtimox (Nx), markedly reduce the parasitemia in the acute stage but they are ineffective in the chronic stage[5]. Natural resistance to these drugs has been suggested as an important factor to explain the low rate of cure detected in chagasic patients[6]. Thus, a search for new agents that exhibit trypanocidal activity against intracellular *T. cruzi* amastigotes seems justifiable.

In previous investigations we designed and synthesized *N*-propyl oxamate (NPOX) and *N*-isopropyl oxamate (NIPOX) as possible inhibitors of *T. cruzi* α-hydroxyacid dehydrogenase (HADH)-isozyme II, and we found that these oxamates were indeed competitive and selective inhibitors of this isozyme (Figure 1)[7,8]. Since HADH-isozyme II participates in the energetic metabolism of *T. cruzi*[9–11], a trypanocidal effect can be expected with these inhibitors[12,13]. However, when we tested the trypanocidal activity of NPOX and NIPOX, we were not able to detect any trypanocidal effect with these oxamates. In contrast, the corresponding

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ethyl esters (Et-NPOX and Et-NIPOX), acting as prodrugs, exhibited trypanocidal activity on cultured epimastigotes (*in vitro*) and murine trypanosomiasis (*in vivo*) in all the tested *T. cruzi* strains[8,14,15]. The increased effectiveness of Et-NPOX and Et-NIPOX resulted from their better absorption by this parasite and their efficient hydrolysis inside *T. cruzi* by its carboxylesterases[16,17], generating the active HADH-isozyme II inhibitors and NIPOX *in situ*[14,15].

Accordingly, in the present investigation we studied the possible trypanocidal activity of the ethyl esters of *N*-propyl and *N*-isopropyl oxamates on bloodstream trypomastigotes and intracellular amastigotes of *Trypanosoma cruzi* acute infected mice.

## Materials and methods

### *Chemicals*

Trypan blue (tetrasodium salt), hematoxylin, eosin, and crystal violet were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Other chemicals used were of the highest purity available. Nifurtimox (tetrahydro-3-methyl-4-[5-nitro-furfurilidene)amine]-2-methyl-tetrahydro-1,4-thiazine-4,4-dioxide was from Bayer, Mexico. NPOX, NIPOX, and the corresponding esters, Et-NPOX and Et-NIPOX, were synthesized according to methods reported elsewhere[7,14].

### **T. cruzi** *strains*

Two *T. cruzi* strains, NINOA and INC-5, isolated from chronic chagasic patients of two different endemic areas of Mexico, were used in this investigation. The *T. cruzi* stock strains were isolated by xenoculture according to Bronfen *et al.*[18]. Following the method described by Chiari *et al.*[19], feces of infected triatomine insects were inoculated intraperitoneally into laboratory mice and cardiac blood was cultured subsequently at 28°C on either enriched biphasic blood agar medium or the monophasic liquid medium, liver infusion tryptone broth (LIT), supplemented with 10% heat-inactivated fetal calf serum.

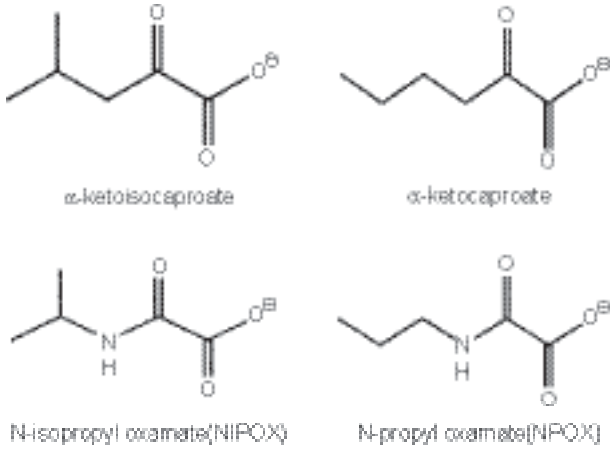


**Figure 1.** Structures of α-ketocaproate and α-ketoisocaproate, the best substrates of *T. cruzi* α-HADH-isozyme II, and the inhibitors *N*-propyl oxamate and *N*-isopropyl oxamate.

### *Trypanocidal assay*

The level of parasitemia was determined by counting in a Neubauer chamber the number of parasites in 5 μL of blood collected from the mouse tail and diluted 1:10 in ammonium chloride, according to Filardi and Brener[20] and Barr *et al.*[21]. The reduction in parasitemia was evaluated by comparing the number of parasites obtained at each time point after drug administration with the number of parasites obtained at the same time points in infected, non-treated mice.

### *Evaluation of drug activity on intracellular amastigotes of* **T. cruzi** *acute infected mice*

Eighty male National Insitutes of Health (NIH) albino mice (18–20 g per mouse, 10 mice per group) were inoculated intraperitoneally with $1 \times 10^3$ bloodstream trypomastigotes. Four groups were infected with *T. cruzi* NINOA strain and the other four groups were infected with *T. cruzi* INC-5 strain. The trypanocidal prodrugs Et-NIPOX and Et-NPOX, and the drug Nx, were administered orally, dissolved in a 5% gum Arabic solution, at a dose of 10 mg/kg per day during 60 days. The first dose was given 24 h after the infection.

Mice were divided into the following groups: (1) infected with *T. cruzi* (NINOA) as a control; (2) infected with *T. cruzi* (NINOA) and treated with Et-NPOX; (3) infected with *T. cruzi* (NINOA) and treated with Et-NIPOX; (4) infected with *T. cruzi* (NINOA) and treated with Nx; (5) infected with *T. cruzi* (INC-5) as a control; (6) infected with *T. cruzi* (INC-5) and treated with Et-NPOX; (7) infected with *T. cruzi* (INC-5) and treated with Et-NIPOX; (8) infected with *T. cruzi* (INC-5) and treated with Nx. The experimental procedure was carried out in accordance with the "Guide for the care and use of laboratory animals" published by the US NIH, publication number[22]. Levels of parasitemia were determined in a Neubauer hemocytometer beginning 24 h after infection.

Histopathological studies of mouse hearts and left legs were done 60 days post-infection. Thin (3 μm) sections of heart tissue from *T. cruzi* infected mice, treated or not treated with NPOX, NIPOX, or Nx ( 10 mg/kg per day), were formaldehyde-fixed, dehydrated, and embedded in paraffin. Sections were stained by hematoxylin–eosin (H&E) and analyzed by light microscopy (×40). Fifty randomly selected microscopic fields were examined to quantify the number of amastigote nests. Histopathological studies of the hearts and legs of each group were done in triplicate. The mean number of amastigote nests in the tissue slices of the infected group not submitted to drug treatment was taken as 100%. The results were evaluated statistically, using Student's *t* test for parasitemia and the $\chi^2$ test for histopathology; the significance level was set at $p < 0.05$.

## Results

### *Effect of Et-NIPOX, Et-NPOX, and Nx on acute parasitemia of mice infected with INC-5* **T. cruzi** *strain*

Figure 2 shows that treatment of the infected mice with Et-NIPOX, Et-NPOX, and Nx, at a dose of 10 mg/kg during 60

days, markedly decreased the parasitemia induced by INC-5 *T. cruzi* strain. This figure also shows that after 20 days of treatment, parasitemia sharply decreased, and after 40 days of treatment the parasitemia was reduced to zero in the groups treated with Et-NPOX and Nx. In the group treated with Et-NIPOX, the parasitema was no longer evident after 55 days of treatment. In contrast, in the infected and non-treated group the parasitemia remained very high between days 20 and 40 after infection.

***Effect of Et-NIPOX, Et-NPOX, and Nx on acute parasitemia of mice infected with NINOA* T. cruzi *strain***

Figure 3 shows that treatment of the infected mice with Et-NIPOX, Et-NPOX, and Nx, at a dose of 10 mg/kg during 60 days, markedly decreased the parasitemia induced by NINOA *T. cruzi* strain. This figure also shows that after 15 days of treatment, the parasitemia sharply decreased, and after 35 days of treatment the parasitemia was reduced almost to zero in all the treated groups. In contrast, in the infected and non-treated group the parasitemia remained very high between days 15 and 40 after infection.

***Effect of Et-NIPOX, Et-NOPX, and Nx on amastigote nests in myocardium of mice infected with INC-5 or NINOA T. cruzi strain***

Figure 4 shows that Et-NIPOX, administered at a dose of 10 mg/kg during 60 days, reduced by 60–80% the number of amastigote nests in cardiac muscle of mice infected with INC-5 or NINOA *T. cruzi* strain. Also, Et-NPOX completely eliminated amastigote nests in cardiac muscle of mice infected with INC-5 or NINOA *T. cruzi* strain. In contrast, nifurtimox, the drug used for comparison, produced a reduction of amastigote nests of only 20–40%. These experiments are in agreement with previous reports describing the low trypanocidal effect of nifurtimox in the chronic stage of Chagas' disease[7].

***Effect of Et-NPOX, Et-NIPOX, and Nx on amastigote nests in skeletal muscle of mice infected with INC-5 or NINOA* T. cruzi *strain***

Figure 5 shows that Et-NPOX and Et-NIPOX, administered at a dose of 10 mg/kg during 60 days, produced in skeletal muscle a reduction in the number of amastigote nests of 60–80%. In contrast, nifurtimox, the drug used for comparison, produced a reduction of amastigote nests of only 20–40% in skeletal muscle of mice infected with INC-5 or NINOA T. cruzi strain. These experiments are in agreement with previous reports describing the low trypanocidal effect of this compound in the chronic stage of Chagas' disease7.

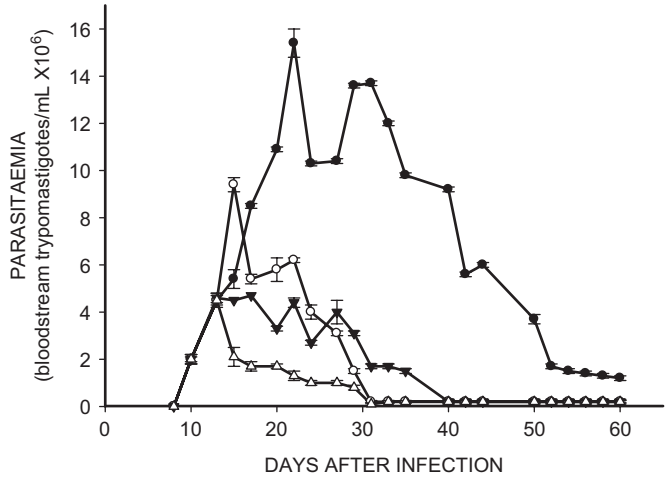


**Figure 3.** Effect of ethyl ester of *N*-propyl oxamate (Et-NPOX) (△), ethyl ester of *N*-isopropyl oxamate (Et-NIPOX) (▼), and nifurtimox (○) on acute parasitemia of mice infected with NINOA *T. cruzi* strain, using as a control (●) infected and non-treated mice. The drugs were administered orally 10 mg/kg per day during 60 days.

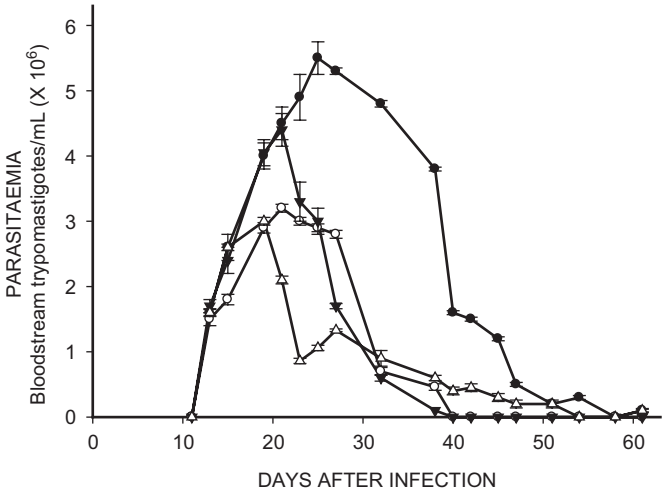


**Figure 2.** Effect of ethyl ester of *N*-propyl oxamate (Et-NPOX) (△), ethyl ester of *N*-isopropyl oxamate (Et-NIPOX) (▼), and nifurtimox (○) on acute parasitemia of mice infected with INC-5 *T. cruzi* strain, using as a control (●) infected and non-treated mice. The drugs were administered orally 10 mg/kg per day during 60 days.

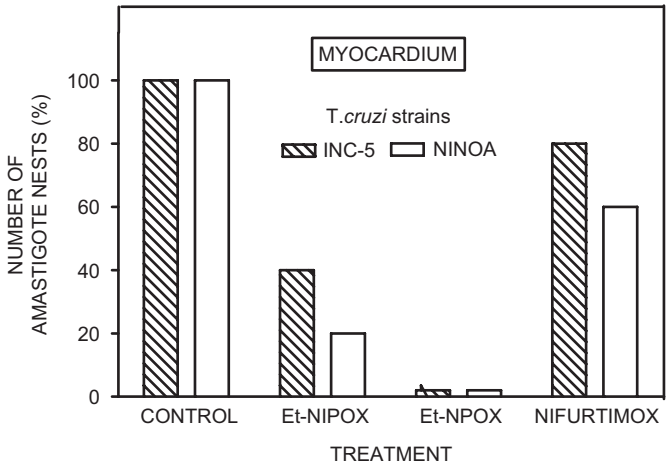


**Figure 4.** Effect of ethyl ester of *N*-isopropyl oxamate (Et-NIPOX), ethyl ester of *N*-propyl oxamate (Et-NPOX), and nifurtimox on *T. cruzi* amastigote nests in myocardium of mice infected with INC-5 (hatched columns) or NINOA (open columns) *T. cruzi* strain. The drugs were administered orally 10 mg/kg per day during 60 days.

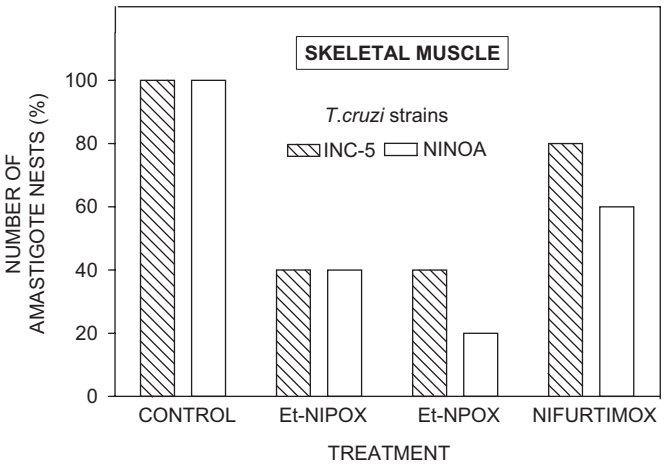


**Figure 5.** Effect of ethyl ester of *N*-isopropyl oxamate (Et-NIPOX), ethyl ester of *N*-propyl oxamate (Et-NPOX), and nifurtimox on *T. cruzi* amastigote nests in skeletal muscle of mice infected with INC-5 (hatched columns) or NINOA (open columns) *T. cruzi* strain. The drugs were administered orally 10 mg/kg per day during 60 days.

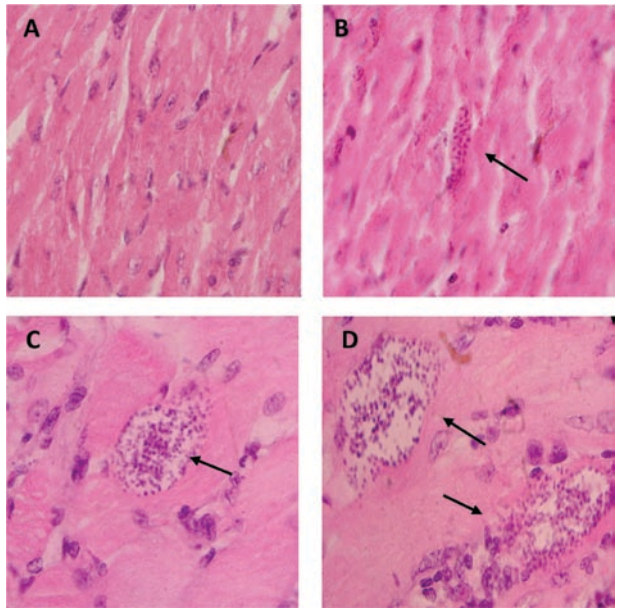


**Figure 6.** Histopathological studies of myocardium and skeletal muscle of mice infected with NINOA or INC-5 *T. cruzi* strain. (A) Myocardium from (NINOA) *T. cruzi* infected mice treated with the ethyl ester of *N*-propyl oxamate (Et-NPOX). (B) Myocardium from (NINOA) *T. cruzi* infected, non-treated mice. (C) Skeletal muscle from (NINOA) *T. cruzi* infected mice treated with Et-NPOX. (D) Skeletal muscle from (NINOA) *T. cruzi* infected, non-treated mice. Similar results were obtained in myocardium and skeletal muscle from (INC-5) *T. cruzi* infected mice, non-treated and treated with the ethyl ester of *N*-isopropyl oxamate (Et-NIPOX). The drugs were administered orally 10 mg/kg per day during 60 days. Tissue slices were stained with hematoxylin–eosin and were analyzed with a ×40 objective. The arrows indicate the location of amastigote nests.

### *Histopathological studies of myocardium and skeletal muscle of mice infected with NINOA or INC-5* T. cruzi *strain and treated with Et-NPOX*

Figure 6 shows some of the histopathological studies of myocardium and skeletal muscle of mice infected with NINOA or INC-5 *T. cruzi* strain and treated with Et-NPOX, 10 mg/kg per day during 60 days. This figure also shows that Et-NPOX completely eliminated amastigote nests in the myocardium of mice infected with NINOA or INC-5 *T. cruzi* strain, whereas in skeletal muscle the reduction of amastigote nests by Et-NPOX was around 60–80%.

## Discussion

Since the clinically available drugs for the treatment of Chagas' disease reduce only the severity of the acute disease, but they are ineffective in chronic stages of the infection[5], in this investigation we studied the possible trypanocidal activity of the ethyl esters of *N*-propyl and *N*-isopropyl oxamates on bloodstream trypomastigotes and on the intracellular amastigotes of *Trypanosoma cruzi* acute infected mice.

*T. cruzi* presents a complex life cycle involving several morphological and functionally different stages that adapt to a variety of conditions imposed by the insect vector and mammalian host environments. The trypomastigotes, the infective form of *T. cruzi*, live in the blood, and they are dependent upon their own vigorous cell motility for extravasation and dissemination within the host[23]. It is evident that cell motility plays an important role in the pathogenesis of Chagas' disease. Taking advantage of this cell motility, the bloodstream trypomastigotes invade mammalian cells where they undergo differentiation into amastigotes, the replicative form of *T. cruzi*. The amastigotes then undergo many cycles of multiplication by binary fission, and transform again into mobile bloodstream trypomastigotes, leading to the rupture of colonized cells during the chronic stage of the infection. These tissue lesions can be detected as amastigote nests in histopathological studies. So, to evaluate the possible trypanocidal effect of Et-NPOX and Et-NIPOX on intracellular amastigotes, we used a murine model of acute Chagas' disease. Male NIH albino mice were inoculated with bloodstream trypomastigotes of INC-5 or NINOA *T. cruzi* strain. The prodrugs Et-NPOX and Et-NIPOX and the drug Nx used for comparison were administered at a dose of 10 mg/kg per day during 60 days, and at the end of the treatment, the trypanocidal effect of these drugs on intracellular amasatigotes was evaluated by detecting and counting the amastigote nests in myocardium and skeletal muscle of infected, treated and non-treated mice. Additionally, the level of parasitemia was followed over time in the treated and non-treated mice.

In the infected and non-treated mice we obtained classic bell shaped curves of parasitemia, with a maximum peak of $5.5 \times 10^6$ bloodstream trypomastigotes/mL with the INC-5 *T. cruzi* strain and of $15 \times 10^6$ trypomastigotes/mL with the NINOA *T. cruzi* strain. In the infected and treated mice the parasitemia was drastically reduced between days 15 and 20, and was reduced almost to zero between days 35 and 40. In contrast, in the infected and non-treated group the parasitemia remained very high between days 15 and 40 after infection.

The trypanocidal experiments on intracellular *T. cruzi* amastigotes showed that Et-NPOX completely eliminated

amastigote nests in the cardiac muscle of mice infected with INC-5 or NINOA *T. cruzi* strain, and in skeletal muscle the reduction in the number of amastogote nests was between 60 and 80% in both strains. Also, Et-NIPOX reduced by 60–80% the number of amastigote nests in both cardiac and skeletal muscles of mice infected with these *T. cruzi* strains. In contrast, Nx, the drug used for comparison, produced a reduction of amastigote nests of only 20–40% in cardiac and skeletal muscles of mice infected with these *T. cruzi* strains. These findings are in agreement with previous reports describing the low trypanocidal effect of this drug in the chronic stage of Chagas' disease[7].

These pharmacological studies show that the prodrugs Et-NPOX and Et-NIPOX have an *in vivo* trypanocidal effect not only on bloodstream trypomastigotes, but also on the clinically relevant intracellular proliferative form of the parasite. The reduction in the number of both circulating and intracellular parasites produced by Et-NPOX and Et-NIPOX was clearly demonstrated. In contrast, Nx showed a poor trypanocidal effect on intracellular amastigotes in this experimental model of the acute stage of Chagas' disease.

In conclusion, the results we have presented above show that Et-NPOX and Et-NIPOX have trypanocidal activity against bloodstream trypomastigotes and intracellular amastigotes of INC-5 and NINOA *T. cruzi* strains in a murine model of acute Chagas' disease.

At the dose and the treatment duration used in this work, Et-NPOX and Et-NIPOX given by the oral route were very well tolerated by mice. No deleterious effects on weight gain and general physical condition of the treated animals, or in the histopathological studies of tissues such as the myocardium and the skeletal muscle, were observed.

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***Declaration of interest*:** The authors report no conflicts of interest.

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