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In vitro and *in vivo* trypanocidal activity of the ethyl esters of N-allyl and N-propyl oxamates using different *Trypanosoma cruzi* strains

CHARMINA AGUIRRE-ALVARADO[1], FABIOLA ZARAGOZA-MARTÍNEZ[2], LORENA RODRÍGUEZ-PÁEZ[1], BENJAMÍN NOGUEDA[2], ISABEL BAEZA[1], & CARLOS WONG[1]

[1]*Departamento de Bioquímica y, and* [2]*Departamento de Parasitología, Escuela Nacional de Ciencias Biológicas- Instituto Politécnico Nacional, Apartado Postal 4-129, Admon. 4, México City 06401, México*

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Abstract

The trypanocidal activity of N-allyl (NAOx) and N-propyl (NPOx) oxamates and that of the ethyl esters of N-allyl (Et-NAOx) and N-propyl (Et-NPOx) oxamates were tested on cultured epimastigotes (*in vitro*) and murine trypanosomiasis (*in vivo*) using five different *T. cruzi* strains. NAOx and NPOx did not penetrate intact epimastigotes and therefore we were not able to detect any trypanocidal effect with these oxamates. Whereas the ethyl esters (Et-NAOx and Et-NPOx), acting as prodrugs, exhibited *in vitro* and *in vivo* trypanocidal activity on the five tested *T. cruzi* strains. On the contrary, when Nifurtimox and Benznidazole used as reference drugs were tested, we found that only three of the five tested *T. cruzi* strains were affected, whereas the other two strains, Miguz and Compostela, were resistant to the *in vitro* and *in vivo* trypanocidal activity of these compounds.

Keywords: Trypanosoma cruzi, *(α-hydroxyacid dehydrogenase-isozyme II inhibition, ethyl N-allyl oxamate, ethyl N-propyl oxamate, (α-hydroxyacid dehydrogenase (HADH)*

Introduction

Chagas' disease exists only on the American continent. It is caused by a flagellate protozoan parasite, *Trypanosoma cruzi*, transmitted to humans in two ways, either by the vector, a blood-sucking reduviid bug or directly by transfusion of infected blood and organ donations. Humans, wild and domestic animals are the natural reservoirs of *T. cruzi*. The geographical distribution of the human *T. cruzi* infections extends from Mexico to the south of Argentina. The disease affects 16–18 million people, killing around 20,000 people annually and some 100 million, i.e. about 25% of the population of Latin America, is at risk of acquiring Chagas' disease [1]. There are two stages of the human disease: the acute stage which appears shortly after the infection and the chronic stage which appears after a silent asymptomatic period that may last several years. The lesions of the chronic phase irreversibly affect internal organs such as the heart, oesophagus and colon, and the peripheral nervous system [2]. Patients with severe chronic disease become progressively ill and ultimately die, usually from heart failure. There is at present no effective treatment for such cases [1]. Chronic Chagas' disease is the major health problem in many Latin America countries [1] and remains practically incurable, due principally to the limited interest in developing new antichagasic drugs and to the fact that the current clinically available drugs for the treatment of Chagas' disease, Benznidazole (Bz) and Nifurtimox (Nx), cure only a very low percentage of chronic patients [3]. Natural resistance of *T. cruzi* to these drugs has been

Correspondence: C. Wong, Departamento de Bioquímica, Escuela Nacional de Ciencias Biológicas- Instituto Politécnico Nacional, Apartado Postal 4-129, Admon. 4, México City, 06401, México. Tel/fax: 52 55 57296000. Ext. 62321. E-mail: cwong@encb.ipn.mx

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suggested as an important factor to explain the low rate of cure detected in chagasic patients [4].

Since there is presently no satisfactory drug for the treatment of Chagas' disease, the search for new antichagasic drugs is amply justified.

In previous investigations we designed and synthesized N-allyl oxamate (NAOx) and N-propyl oxamate (NPOx) as possible inhibitors of the *T. cruzi* α-hydroxyacid dehydrogenase (HADH)-isozyme II and we found that these oxamates were indeed competitive and selective inhibitors of this isozyme [5].

Since HADH-isozyme II participates in the energetic metabolism of *T. cruzi*, a trypanocidal effect can be expected with these inhibitors [6,7].

This paper examines the trypanocidal effect of N-allyl (NAOx) and N-propyl (NPOx) oxamates and the corresponding ethyl esters, Et-NAOx and Et-NPOx on cultured epimastigotes (*in vitro*) and on mice parasitaemia (*in vivo*) using five different *T. cruzi* strains.

Materials and methods

Chemicals

Trypan blue (tetrasodium salt) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Other chemicals used were of the highest purity available. Benznidazole (*N*-benzyl-2-nitro-1-imidazol-1-acetamide) was from Roche-México and Nifurtimox (tetrahydro-3-methyl-4-[(5-nitro-furfurilidene) amine]-2-methyl-tetrahydro-1,4-thiazine-4,4′-dioxide was from Bayer-México. NAOx and NPOx and the corresponding esters, Et-NAOx and Et-NPOx, were synthesized according to the methods reported elsewhere [5].

Isolation of T. cruzi *strains*

Five *T. cruzi* strains from different endemic areas of México were used in this investigation. All the *T. cruzi* stock strains were isolated by xenoculture according to Bronfen et al. [8]. *V2R* strain was isolated from *Didelphis marsupialis*. *Miguz* strain from a chronic chagasic patient, *Parra* and *Compostela* strains from *Triatoma longipenis* and *Nayarit* strain from *Triatoma pincturata*.

Following the method described by Chiari et al. [9], faeces of infected bugs were inoculated intraperitoneally into laboratory mice and cardiac blood was cultured subsequently on the monophasic liquid medium, liver infusion tryptone broth (LIT medium), supplemented with 10% heat-inactivated fetal calf serum.

Epimastigotes of T. cruzi *strains*

Epimastigotes of the five *T. cruzi* strains were cultivated at 28°C in the monophasic medium (LIT medium). The parasites were collected from the liquid phase after 7 days of culture. At this stage, the growth is exponential. The liquid phase was centrifuged at 3000 *g* for 15 min to obtain the parasites pellet. This was washed 3 times with about 20 parts of 0.15 M sodium phosphate and 0.15 M sodium chloride buffer pH 7.4. The final pellet of washed parasites was resuspended in 4 parts of the same buffer (1:5) to a final concentration of 1×10^3 or 1×10^6 epimastigotes/mL. All operations were carried out at 4°C.

T. cruzi *homogenates*

One mL of the above mentioned *T. cruzi* suspension, with a concentration of 1×10^6 epimastigotes/mL was frozen in liquid nitrogen, and thawed at 37°C. The freezing and the thawing operations were repeated 3 times. The disruption of parasites was monitored by microscopic examination. The homogenates attained were frozen at −20°C until experiments were performed (usually 24 h later). There was no loss of activity of HADH-isozyme II in the homogenates after storage at −20°C for up to two months. Before the enzymatic assays, the suspension was thawed at room temperature and centrifuged at 1200 *g* for 20 min at 4°C. The supernatant (0.1 mL properly diluted to obtain a change in absorbance of 0.05–0.08/min at 340 nm) was used for the analysis of HADH-isozyme II.

Enzyme assay

HADH-isozyme II activity was determined using α-ketoisocaproate as substrate [10]. The reagent mixture contained in a final volume of 3 mL: 0.12 mM NADH, 0.1 M sodium phosphate buffer pH 7.4, α-ketoisocaproate as neutral sodium salt, enzyme preparation properly diluted with 0.1 M sodium phosphate buffer pH 7.4 in order to obtain an absorbance change at 340 nm of 0.05–0.08 per min with 5 mM concentration of substrate. Changes in absorption at 340 nm were recorded during a 4 min period at room temperature.

Trypanocidal effect of drugs on cultivated epimastigotes of different T. cruzi *strains*

Drug tests were carried out in eppendorf tubes. A suspension of epimastigotes with a final concentration of 1×10^6 parasites/ mL was prepared. The drugs (Bz, Nx, NPOx, NAOx, Et-NPOx and Et-NAOx), dissolved in 20 μL of ethanol in a final concentration up to 0.1 mM, were added to aliquots (980 μL/mL) of this suspension. Two controls were used: one had ethanol added in the same proportion used to dissolve the drugs (20μL/mL), the other was a parasite suspension without any addition. All samples were incubated at 28°C. Observations and counts were

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made with a Neubauer haemocytometer after 10, 20, 30, 40, 50 and 60 min incubations, according to Barr et al. [11]. These experiments were carried out in comparison with Nx and Bz. The addition of ethanol (20 μL/mL) to the parasite suspension did not modify motility. All experiments were run twice in triplicate, and the results are given as mean ± standard deviation. Death of epimastigotes was confirmed by the trypan blue dye exclusion method [12].

Trypanocidal effect of drugs on mice parasitaemia induced by different T. cruzi *strains*

In previous experiments, the parasitaemia induced by different *T. cruzi* strains was followed with time and we found that the peaks of maximum parasitaemia were: 40 days for *Miguz* and *Compostela* strains, 28 days for *V2R* strain, 18 days for *Parra* strain and 14 days for *Nayarit* strain [13]. To test the trypanocidal activity of drugs (Bz, Nx, NPOx, NAOx, Et-NPOx and Et-NAOx), at the peak of maximum parasitaemia, a single dose of 500 mg/kg of a drug was given by the oral route and the parasitaemia was determined, before, 2, 4, and 6 h after drug administration, according to the method proposed by Filardy and Brener [14]. To induce parasitaemia, male NIH albino mice, 18–20 g (ten mice per group) were inoculated intraperitoneally with 1×10^3 blood trypomastigotes. The level of parasitaemia was checked by counting, in a Neubauer haemocytometer, the number of parasites in 5 μL of blood drawn from the tail of the mice and diluted 1:10 in ammonium chloride, according to Barr et al. [11]. Untreated mice similarly inoculated were used as control. The trypanocidal drugs Bz and Nx were used as reference drugs. The investigation was performed according to the Guide for Care and Use of Laboratory Animals published by the US National Institute of Health [15].

Results

Effect of N-propyl and N-allyl oxamates and the corresponding ethyl esters (Et-NPOx and Et-NAOx) on the activity of HADH from a T. cruzi *homogenate also containing carboxyl esterases*

Figure 1 shows that Et-NAOx and Et-NPOx (esters) were hydrolyzed by *T. cruzi V2R* strain homogenate and that the generated derivatives (NPOx and NPOx) inhibited the HADH-isozyme II also present in the homogenate. The inhibition was comparable to that produced by the synthesized NPOx and NAOx. Similar results were obtained with homogenates of the five tested *T. cruzi* strains (results not shown). Previously, we had demonstrated that the esters (Et-NAOx and Et-NPOx) were not inhibitors for the purified HADH-isozyme II [5].

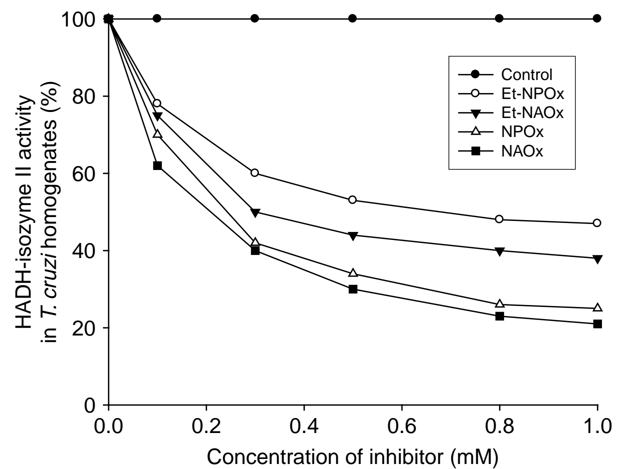

Figure 1. Effect of the inhibitors N-allyl and N-propyl oxamates (NAOx and NPOx) and the corresponding ethyl esters (Et-NAOx and Et-NPOx) on the activity of HADH-isozyme II in a *T. cruzi* V2R strain homogenate, containing also carboxyl esterases and using α-ketoisocaproate as a substrate. Similar results were obtained in the five tested *T. cruzi* strains. Control without inhibitor.

Trypanocidal effect of Benznidazole and Nifurtimox on cultured epimastigotes of different T. cruzi *strains*

Figure 2 shows the effects of Bz and Nx on five different *T. cruzi* strains. The trypanocidal effects of these drugs were evident in only three of the five tested *T. cruzi* strains, whereas the other two, *Migus* and *Compostela*, were resistant to these drugs. The *in vitro* trypanocidal effects of Bz and Nx on *Parra*, *V2R* and *Nayarit T. cruzi* strains, were relatively low in comparison with the trypanocidal effects obtained with Et-NAOx and Et-NPOx under the same conditions.

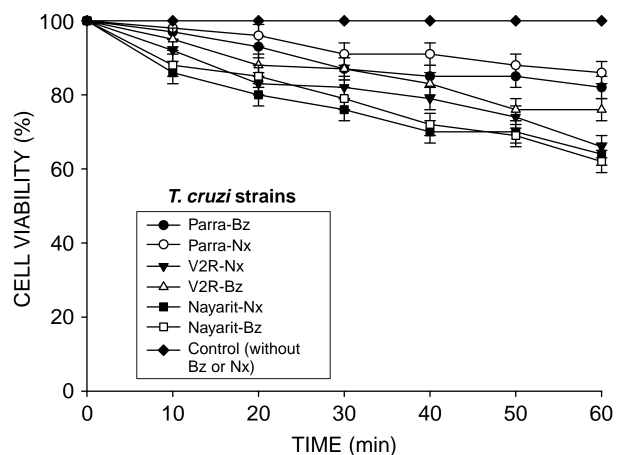

Figure 2. Trypanocidal effect of Benznidazole (Bz) and Nifurtimox (Nx) on cultured epimastigotes of different *T. cruzi* strains. Control (without Bz or Nx). Drug concentration was 0.1 mM. Final concentration of epimastigotes was 1×10^6/ mL. Cell viability was determined every 10 min during 1 h, according to Barr et al. [11]. *Compostela* and *Miguz* strains were resistant to the treatment with Nx and Bz.

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Trypanocidal effect of the ethyl esters of N-allyl and N-propyl oxamates on cultured epimastigotes of different T. cruzi *strains*

Figure 3 shows the trypanocidal effect of Et-NAOx and Et-NPOx on epimastigotes from five different *T. cruzi* strains, using NAOx and NPOx as controls. The Et-NAOx and Et-NPOx produced a trypanocidal effect in all the evaluated *T. cruzi* strains, including those *T. cruzi* strains that were resistant to Bz and Nx, whereas NAOx and NPOx did not show a trypanocidal effect. The trypanocidal effect of Et-NAOx and Et-NPOx were higher than those obtained with Bz and Nx under the same conditions.

Trypanocidal effect of Benznidazole and Nifurtimox on mice parasitaemia induced by different T. cruzi *strains*

Figure 4 shows the effect of Bz and Nx on mice parasitaemia induced by trypomastigotes of five *T. cruzi* strains. Nx and Bz produced a trypanocidal effect in only three of the five tested *T. cruzi* strains, whereas the other two, *Compostela* and *Miguz* strains, were resistant to these drugs.

Trypanocidal effect of the ethyl esters of N-allyl and N-propyl oxamates on mice parasitaemia induced by different T. cruzi *strains*

Figure 5 shows the effect of Et-NAOx and Et-NPOx on mice parasitaemia induced by trypomastigotes of five *T. cruzi* strains. Et-NAOx and Et-NPOx produced a trypanocidal effect in all the evaluated *T. cruzi* strains, including those that were resistant to Bz and Nx, whereas NAOx and NPOx did not show a trypanocidal effect.

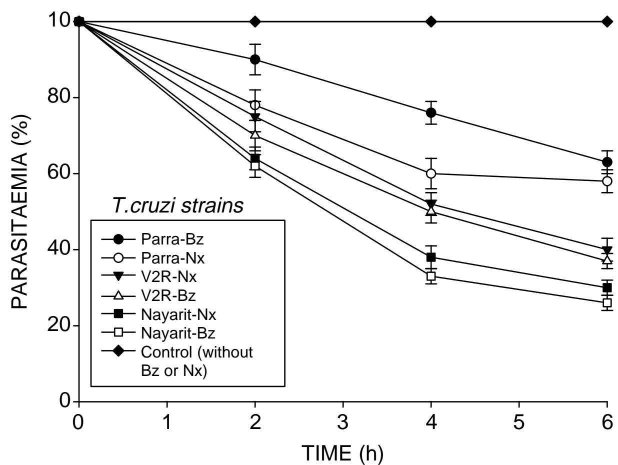

Figure 4. Trypanocidal effect of Benznidazole (Bz) and Nifurtimox (Nx) on mice parasitaemia induced by different *T. cruzi* strains. Control (without Bz or Nx). At the peak of maximum parasitaemia, a single dose of the drug (500 mg/kg) was given by the oral route and the parasitaemia was determined before and 2,4 and 6 h after treatment, according to Filardi and Brener [14]. *Compostela* and *Miguz* strains were resistant to the treatment with Nx and Bz.

Comparative trypanocidal effect of Benznidazole, Nifurtimox, the ethyl ester of N-propyl oxamate and the ethyl ester of N-allyl oxamate on cultured epimastigotes and on parasitaemia using different T. cruzi *strains*

Figure 6 shows: (A) the effect of Bz, Nx, Et-NPOx and Et-NAOx on cultured epimastigotes, and (B) on mice

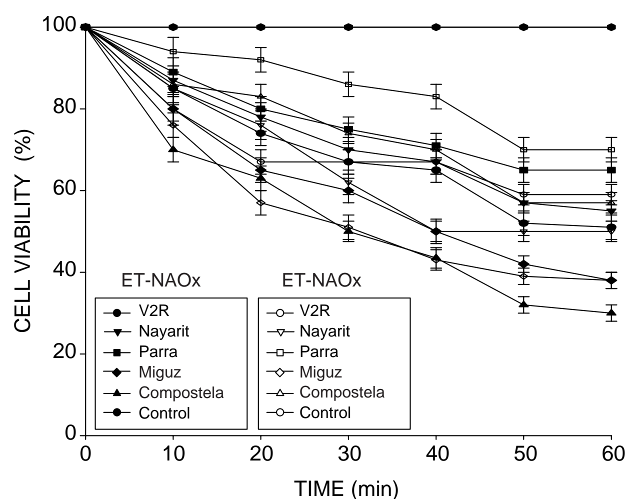

Figure 3. Trypanocidal effect of the ethyl esters of N-allyl oxamate (Et-NAOx) and N-propyl oxamate (Et-NPOx) on cultured epimastigotes of different *T. cruzi* strains using N-allyl oxamic acid (NAOx) or N-propyl oxamic acid (NPOx) as a control. Drug concentration was 0.1 mM. Final concentration of epimastigotes was 1×10^6/ mL. Cell viability was determined every 10 min during 1 h, according to Barr et al. [11].

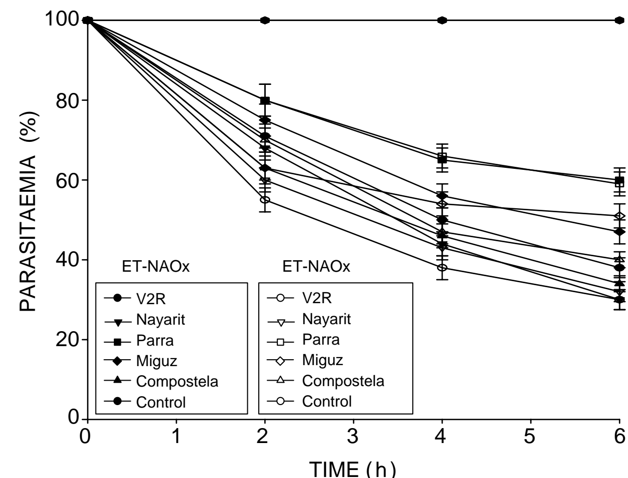

Figure 5. Trypanocidal effect of the ethyl esters of N-allyl oxamate (Et-NAOx) and N-propyl oxamate (Et-NPOx) on mice parasitaemia induced by different *T. cruzi* strains using N-allyl oxamic acid (NAOx) or N-propyl oxamic acid (NPOx) as a control. At the peak of maximum parasitaemia, a single dose of the drug (500 mg/kg) was given by the oral route and the parasitaemia was determined before and 2, 4 and 6 h after the treatment, according to Filardi and Brener [14].

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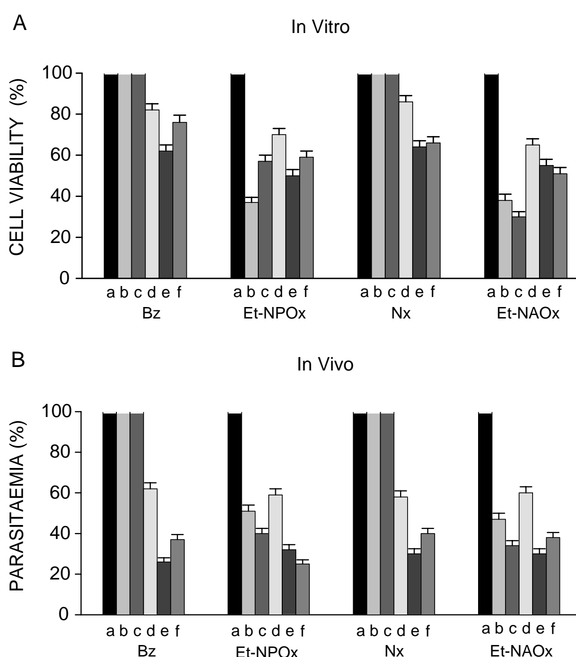

Figure 6. (A) Comparative trypanocidal effect of Benznidazole (Bz), the ethyl ester of N-propyl oxamate (Et-NPOx), Nifurtimox (Nx) and the ethyl ester of N-allyl oxamate (Et-NAOx) on cultured epimastigotes, and (B) On mice parasitaemia using five different *T. cruzi* strains. *In vitro*, the drug concentration was 0.1 mM. Time of incubation was 1 h. Data were taken from Figures 2 and 3. Whereas, *in vivo*, at the peak of maximum parasitaemia of each strain, a single dose of the drug (500 mg/kg) was given by the oral route and the parasitaemia was determined 6 h after drug administration. Data were taken from Figures 4 and 5. The employed *T. cruzi* strains were: a) Control, b) *Miguz*, c) *Compostela*, d) *Parra*, e) *Nayarit*, f) *V2R*.

parasitaemia using five different *T. cruzi* strains. This figure clearly shows that Nx and Bz produced a trypanocidal effect in only three of the five tested *T. cruzi* strains, whereas the other two, Miguz and Compostela, were resistant to these drugs. On the contrary, Et-NPOx and Et-NAOx produced trypanocidal effects in all tested *T. cruzi* strains, including those *T. cruzi* strains that were resistant to Bz and Nx.

Discussion

Since glycolysis provides virtually all the energy for the bloodstream form of trypanosomatides [6], enzymes that participate in glycolysis or in its regulation have been suggested as a target for antitrypanosomatid drug design [7,16]. Thus, the work presented here focuses on glycolysis in trypanosomes as a target for the possible trypanocidal effect of some oxamates.

Trypanosoma cruzi, possesses a NAD-linked oxidoreductase designated (α-hydroxyacid dehydrogenase (HADH). This enzyme exhibits two molecular forms (I and II), which have been purified and characterized [17,18]. Isozyme I is responsible for the weak lactate dehydrogenase activity found in *T. cruzi* extracts while isozyme II does not show activity against pyruvate and it is active on a broad range of linear and branched chain substrates especially α-ketocaproate and α-ketoisocaproate [17]. It has been established that HADH-isozyme II is actually integrated into metabolic pathways supplying energy for the motility of flagellum and survival of the parasites [10], because this isozyme participates in the reoxidation of NADH during glycolysis [17], and in a shuttle system transferring reducing equivalents (NADH) from cytosol into the mitochondria [10]. Consequently, an inhibitor of HADH-isozyme II will not only inhibit glycolysis but will also inhibit the shuttle system, and therefore, it has been proposed that inhibitors of this isozyme could reduce the motility and survival of this parasite [5,19,20,21].

Since HADH-isozyme II showed maximum activity with the substrates α-ketocaproate and α-ketoisocaproate [17], we assumed that N-allyl oxamate (NAOx) and N-propyl oxamate (NPOx) would fulfil the structural and steric requirements for inhibitors of *T. cruzi* HADH-isozyme II due to the close chemical structure existing between NAOx and NPOx and the substrates α-ketocaproate and α-ketoisocaproate. Thus, we synthesized NAOx and NPOx and found that these oxamates were indeed competitive and selective inhibitors of this isozyme, rather than as general inhibitors of dehydrogenases [5].

In the present investigation, using five different *T. cruzi* strains, we evaluated the trypanocidal effect of the acids NAOx and NPOx and the corresponding ethyl esters, Et-NAOx and Et-NPOx, on cultured epimastigotes (*in vitro*) and on mice parasitaemia (*in vivo*) in comparison with Bz and Nx, the current clinically available drugs used for the treatment of Chagas' disease.

In previous investigations we demonstrated that NAOx and NPOx were selective inhibitors of HADH-isozyme II [5]. Since this isozyme participates in the energetic metabolism of *T. cruzi*, a trypanocidal effect can be expected with these inhibitors [6,7]. However, in the present investigation, we were not able to detect any trypanocidal activity with these oxamates in the five tested *T. cruzi* strains. On the contrary, when the trypanocidal effect of Et-NAOx and Et-NPOx was tested on cultured epimastigotes of five different *T. cruzi* strains, we found that these oxamates produced a trypanocidal effect in all the tested *T. cruzi* strains, including those *T. cruzi* strains that were resistant to Bz and Nx. The increased effectiveness of the Et-NAOx and Et-NPOx to reduce the motility and to kill cultured *T. cruzi* epimastigotes of this strain resulted from their better absorption by this parasite and their efficient hydrolysis inside *T. cruzi* by the carboxyl esterases also present in this parasite [22,23] so generating *in situ* the active HADH inhibitors, NAOx and NPOx. The trypanocidal effect of Et-NAOx and Et-NPOx cannot be attributed to a direct inhibition of HADH-isozyme

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II by these esters since Et-NAOx and Et-NPOx were not inhibitors for the purified HADH-isozyme II from *T. cruzi* [5]. Whereas when we used the *T. cruzi* homogenates of the five tested *T. cruzi* strains, containing also carboxyl esterases, the esters were rapidly hydrolyzed and the generated acids really inhibited the HADH-isozyme of these extracts; those inhibitions were similar to those produced by the synthesized NPOx and NAOx.

Similar results were obtained in the *in vivo* experiments. NAOx and NPOx were inactive, whereas the ethyl esters, Et-NAOx and Et-NPOx, showed trypanocidal activity on mice parasitaemia in all the evaluated *T. cruzi* strains, including those *T. cruzi* strains that were resistant to Bz and Nx. These experiments also demonstrated that the acids, NAOx and NPOx, did not penetrate intact trypanosomatids, *in vitro* and *in vivo*, due to the polarity of their carboxylates. Since biological membranes behave like hydrophobic barriers against polar substances, the negative charge of the carboxylate present in these oxamates at physiological pH was responsible for the lack of trypanocidal activity of these substances, whereas the highly hydrophobic Et-NPOx and Et-NAOx with high affinity for biomembranes really penetrated inside this parasite where they were hydrolyzed and produced their pharmacological effect.

It is evident that in cultured epimastigotes as well as in blood trypomastigotes, the five different *T. cruzi* strains were affected to different extents by the treatment with Et-NAOx and Et-NPOx. These differences must be attributed to a different rate of absorption of Et-NAOx and Et-NPOx by each of the five tested *T. cruzi* strains, and to a different rate of hydrolysis inside *T. cruzi* by the carboxyl esterases.

When Bz and Nx were tested on cultured epimastigotes, as well as in circulating trypomastigotes of different *T. cruzi* strains, we found that these substances showed trypanocidal activity in only three of the five tested *T. cruzi* strains, whereas the other two, *Compostela* and *Miguz* strains, were resistant to Bz and Nx. Natural resistance of *T. cruzi* to Bz and Nx has been suggested as an important factor to explain the low rate of cure detected in chagasic patients [4]. Some aspects such as variations in absorption and metabolism of drugs are implicated in the resistance of some *T. cruzi* strains to the chemotherapeutic effect of these drugs, and according to Brener, the resistance of *T. cruzi* to these drugs varies from 0%–100%, and the success of the treatment of chagasic patients with Bz and Nx depends mainly on the strain of *T. cruzi* [24,25].

In the present investigation, Bz and Nx showed trypanocidal activity *in vitro* and *in vivo* in only three of the five tested *T. cruzi* strains, whereas Et-NAOx and Et-NPOx showed trypanocidal activity, *in vitro* and *in vivo*, in the five tested *T. cruzi* strains. These experiments strongly suggest that these oxamates are potentially promising drugs for the treatment of Chagas' disease. Nevertheless, additional studies on the action of Et-NAOx and Et-NPOx on the experimental infections of mammalian cell cultures and of animals are necessary to test their possible use as therapeutic agents in trypanosomiasis.

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