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Trypanocidal activity of N-isopropyl oxamate on cultured epimastigotes and murine trypanosomiasis using different *Trypanosoma cruzi* strains

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Abstract

The trypanocidal activity of N-isopropyl oxamate (NIPOx) and the ethyl ester of N-isopropyl oxamate (Et-NIPOx) were tested on cultured epimastigotes (*in vitro)* and on murine trypanosomiasis (*in vivo)* using five different *T. cruzi* strains. When benznidazole and nifurtimox, used for comparison, were tested we found that only three of these *T. cruzi* strains were affected, whereas the other two strains, Miguz and Compostela, were resistant to the *in vitro* and the *in vivo* trypanocidal activity of these substances. In addition, when NIPOx was tested on cultured epimastigotes and on mice parasitaemia, trypanocidal activity was not obtained on either of these *T. cruzi* strains. Our experiments strongly suggest that NIPOx does not penetrate intact epimastigotes due to the polarity of its carboxylate whereas Et-NIPOx, acting as a prodrug, exhibited *in vitro* and *in vivo* trypanocidal activity in the five tested *T. cruzi* strains.

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

Introduction

Chagas' disease which is caused by *Trypanosoma cruzi*, is an endemic parasitic disease in Latin America, specially in the Southern part of Mexico [1], Central and South America countries, where vectorial transmission is effected by triatomine insects [2]. It is estimated that 16–18 million people are infected by *Trypanosoma cruzi* and that 100 million of the population are at risk of acquiring the infection [2,3]. There are two stages of the human disease: the acute stage which appears shortly after the infection and the chronic stage which may last several years and irreversibly affects internal organs such as the heart, esophagus, colon and the peripheral nervous system [4].

Chagas' disease remains essentially incurable. The pharmaceutical industry has had limited interest in developing new antichagasic drugs due principally to a lack of commercial incentive [5,6]. Benznidazole (Bz) and nifurtimox (Nfx) are the available drugs for the treatment of Chagas' disease; both drugs are toxic nitroheterocyclic derivatives used as long-term treatment [7]. These drugs cure only a very low percentage of chronic patients [6] and natural resistance of *T. cruzi* to nitro derivatives has been suggested as an important factor to explain the low rates of cure detected in chagasic patients [6]. Thus, a search for new agents that exhibit activity against *T. cruzi*, seems justifiable.

Since glycolysis is the only ATP-generating process in the bloodstream form of trypanosomes [8], it has been perceived as a promising drug target for the design of new anti-trypanosomatide drugs [9,10], following the argument that inhibitors which decrease significantly the glycolytic flux will kill the parasites. Thus, the work presented here focuses on glycolysis in

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trypanosomes as a target for the possible trypanocidal effect of some oxamates.

It has been demonstrated that *T. cruzi* possesses an enzyme similar to the LDH-x from mammalian spermatozoa [11]. The enzyme designated α-hydroxyacid dehydrogenase (HADH), exhibits two molecular forms (I and II), which have been purified and characterized [12]. It has been established that HADH-isozyme II is integrated in a shuttle system transferring reducing equivalents from the cytosol to the mitochondria [13]. In addition, HADH-isozyme II is actually integrated into metabolic pathways supplying energy for the motility of the flagellum and survival of the parasites [13] and therefore, it has been proposed that inhibitors of this isozyme could reduce the motility and survival of this parasite [14,15].

Previously, we were able to show that N-isopropyl oxamate was an effective and selective inhibitor of HADH-isozyme II due to the close chemical structure existing between N-isopropyl oxamic acid (NIPOx) and the best substrate for HADH-isozyme II, the α-ketoisocaproate [15]. We also found that the ethyl ester of N-isopropyl oxamate (Et-NIPOx) exhibited trypanocidal activity on intact epimastigotes [15]. Accordingly, in the present investigation we studied the possible trypanocidal activity of NIPOx and Et-NIPOx (Figure 1) on intact epimastigotes and on murine trypanosomiasis, using different *T. cruzi* strains.

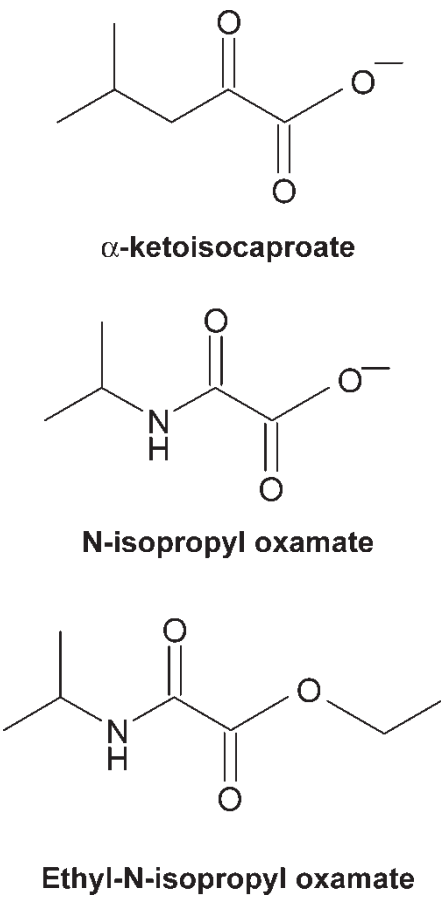

Figure 1. Structure of α-ketoisocaproate, the substrate of *T. cruzi* α-HADH-isozyme II and the inhibitors: N-isopropyl oxamate and the ethyl ester of N-isopropyl oxamate.

Materials and methods

Chemicals

NAD, NADH, pyruvate (2-oxopropanoate), α-ketoisocaproate (2-oxo-4-methyl-pentanoate) as sodium salts, were 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-furfurylidene)amine)-2-methyltetrahydro-1,4-thiazine-4,4-dioxide) was from Bayer-México. NIPOx and Et-NIPOx were synthesized according to methods reported elsewhere [15].

T. cruzi strains isolated from triatomines, chagasic patients and wild animals

T. cruzi stock strains were isolated through xenoculture [16] from chagasic patients, from triatomines and wild animals captured in the state of Nayarit, México. Miguz strain was isolated from a chronic chagasic patient. V2R strain from *Didelphis marsupialis*, Parra and Compostela strains from *Triatoma longipennis* and Nayarit strain from *Triatoma picturata*. The primary isolation was made by xenodiagnosis with bred triatoma infestans. Faeces of infected 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 [17].

Organisms

Epimastigotes of *T. cruzi* of the different strains were cultivated at 28°C in monophasic liquid medium (LIT medium). The parasites were collected after 7 days of culture. At this stage, the growth is exponential. The liquid phase was centrifuged at 3000 × g for 15 min and the pellet washed three times with 20 parts of 0.15 M phosphate buffer pH 7.2 containing 0.15 M NaCl. The pellet was dispersed in 4 parts of the same buffer to a final concentration of 1×10^3 or 1×10^6 organisms/mL. All operations were carried out at 4°C.

Enzyme preparation from T. cruzi homogenates

Isozymes I and II of HADH were purified as described by Coronel et al. [12]. 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 is active on a broad spectrum of linear and branched chain substrates, especially α-ketocaproate and α-ketoisocaproate [12].

T. cruzi homogenates

The pellet of parasites was washed three times with about 20 parts of 0.15 M, sodium phosphate buffer, 0.15 M NaCl, pH 7.2. The final pellet of washed parasites was resuspended in 4 parts of the same buffer (1:5), frozen in liquid nitrogen, and the suspension was thawed at room temperature. The freeze and thaw operations were repeated three times. The disruption of parasites was monitored by microscopic examination of the resulting suspension. The preparation was frozen at −20°C until experiments were performed (usually 24 h later). There was no loss of activity in the homogenates after storage at −20°C for up to 2 months. Immediately before study, the suspension was thawed and centrifuged at 20,000 × g for 20 min at 4°C. The supernatant was used for the analysis of HADH and esterases.

Enzyme assay

The α-hydroxyacid dehydrogenase isozyme II activity was determined by using α-ketoisocaproate as substrate [12]. The reagent mixture contained, in a final volume of 3 mL: 0.115 mM NADH, 100 mM sodium phosphate buffer pH 7.4, α-ketoisocaproate as neutral sodium salt (concentrations indicated in results), enzyme preparation, diluted with sodium phosphate buffer 0.1 M (pH 7.4) in order to obtain an absorbance change at 340 nm of 0.05–0.08 per min with a 5 mM concentration of substrate. Assays were incubated at 20–23°C. Change in absorbance at 340 nm was recorded during a 4 min period.

Determination of K_i values

The enzyme HADH-isozyme II was incubated with the buffer used in the assay, the inhibitor (NIPOx) and the coenzyme NADH for 10 min at room temperature before starting the reaction by adding the substrate. Values of K_i were calculated from those of K_m and V obtained with and without the inhibitor added to the assay mixture. Concentrations of substrates, coenzymes and inhibitors that were used are given in the results.

*Evaluation of drug activity on cultured epimastigotes (*in vitro*)*

To aliquots (980 μL in eppendorf tubes) of *T. cruzi* suspension in phosphate buffer (1×10^6 epimastigotes/mL, final concentration) the drugs dissolved in 20 μL of ethanol, up to 0.1 mM final concentrations were added. Two controls were used: either ethanol added in the same proportion utilized to dissolve the drugs (20 μL/mL), or a parasite suspension without any addition. All samples were incubated at 28°C. Observations and counts were made with a Neubauer hemocytometer after 10, 20, 30, 40, 50 and 60 min incubations, according to Brener's method [18]. 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 the epimastigotes was confirmed by the trypan blue dye exclusion method [19].

*Evaluation of drug activity on mice parasitaemia induced by different T. cruzi strains (*in vivo*)*

Male NIH albino mice, 18–20 g (ten mice per group), were inoculated intraperitoneally with 1×10^3 blood trypomastigotes. At the peak of maximum parasitaemia (40 days for Compostela and Miguz strains, 14 days for Nayarit strain, 28 days for V2R strain and 18 days for Parra strain) a single dose of 500 mg/kg of the drugs to be tested was given by oral route as proposed by Filardi and Brener [20]. The level of parasitaemia was checked by counting in a Neubauer chamber 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 Filardi and Brener [20] and Barr et al. [21], before and 2, 4 and 6 h after drug administration. Untreated mice similarly inoculated were used as controls, and the trypanocidal drugs Nfx and Bz were used as reference drugs. The percentage of reduction in parasitaemia was calculated by comparing the number of parasites obtained at each interval of time after drug administration with that found before treatment. The investigation was performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health [22].

Results

T. cruzi homogenates used in these experiments contain HADH-isozyme II and the carboxylesterases. The HADH-isozyme II was competitively inhibited by NIPOx with a K_i of 0.074 mM with respect to α-ketoisocaproate, the same as that obtained with the purified *T. cruzi* HADH-isozyme II 15 (Figure 2).

Effect of the inhibitors on the activity of purified HADH isozyme II from T. cruzi

Figure 3 shows the effect of the inhibitors, NIPOx and Et-NIPOx, on the activity of purified HADH isozyme II from *T. cruzi*. The ethyl ester is not an inhibitor of

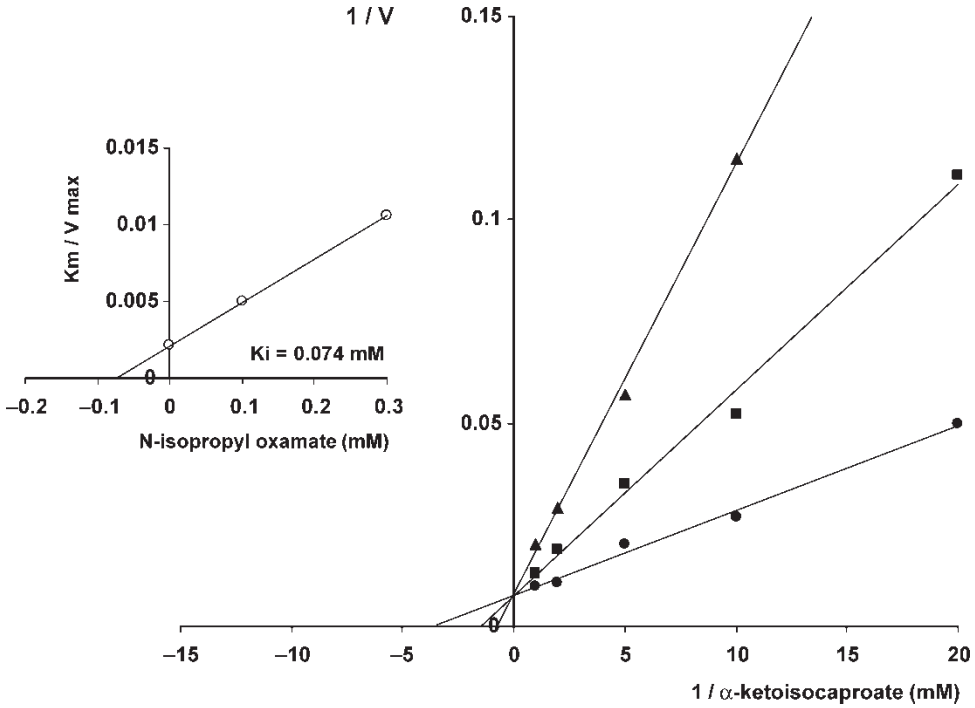

Figure 2. Effect of α-ketoisocaproate on the inhibitory activity of N-isopropyl oxamate on α-HADH-isozyme II from a *T. cruzi* strain homogenate. Reciprocal of V (expressed as ΔE_{340}/min) are plotted against reciprocal of α-ketoisocaproate concentration. The concentration of substrate used were 0.05, 0.1, 0.2, 0.5 and 1 mM; NADH concentration was kept constant at 0.12 mM. Assays without (•) and with (■) 0.1 mM or (▲) 0.3 mM N-isopropyl oxamate added to the reagent mixture. The K_m for α-ketoisocaproate was 0.3 mM. Upper left, determination of K_i from replot of slope values against inhibitor concentration.

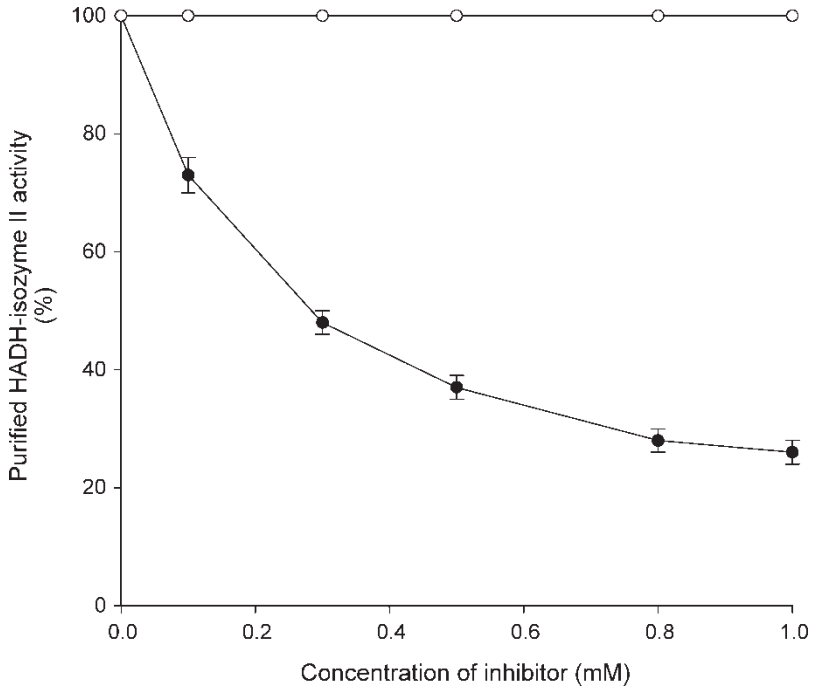

Figure 3. Effect of the inhibitors on the activity of purified HADH isozyme II from *T. cruzi*. Inhibitors: NIPOx (•), Et-NIPOx (○). α-Ketoisocaproate was used as substrate.

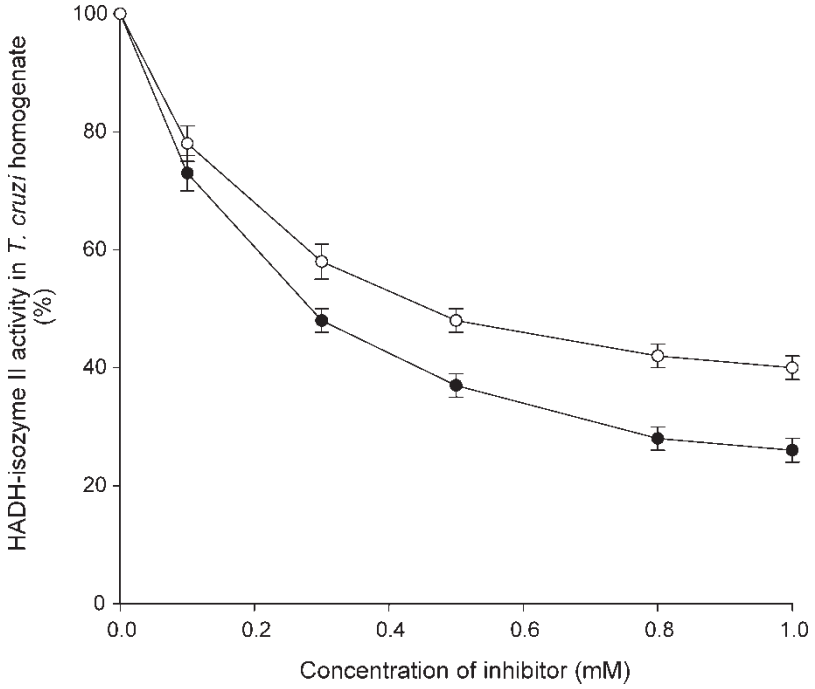

Figure 4. Effect of the inhibitors on *T. cruzi* homogenates containing carboxylesterases and HADH isozyme II. Inhibitors: NIPOx (•), Et-NIPOx (○). α-Ketoisocaproate was used as substrate.

the purified isozyme, whereas increasing concentrations of N-isopropyl oxamic acid produced increasing inactivation of the isozyme and at 1 mM produced 70% inhibition.

Effect of the inhibitors on T. cruzi homogenates containing carboxylesterases and HADH isozyme II

Figure 4 shows the effect of NIPOx and the Et-NIPOx on the activity of HADH from *T. cruzi* homogenate containing also the carboxylesterases. The Et-NIPOx was readily hydrolyzed by the *T. cruzi* extract and the generated NIPOx inhibited the HADH isozyme II present in the homogenate. The inhibition was comparable to that produced by NIPOx.

In vitro *evaluation of trypanocidal activity of the drugs*

Figure 5 shows the trypanocidal activity of Et-NIPOx, Bz and Nfx on intact epimastigotes of five *T. cruzi* strains. The trypanocidal activities of Bz and Nfx were evident on three of five *T. cruzi* strains, whereas

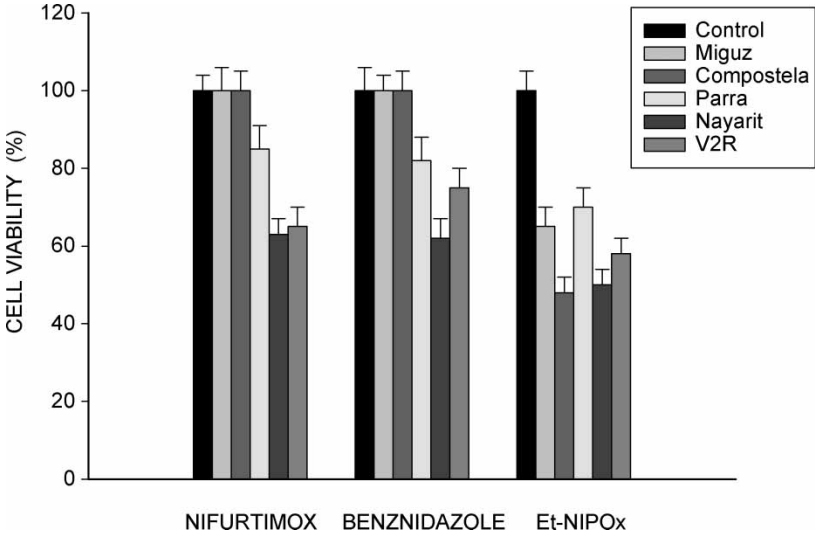

Figure 5. Trypanocidal effect of nifurtimox, benznidazole and the ethyl ester of N-isopropyl oxamate on intact epimastigotes of different *T. cruzi* strains. Concentration of drugs 0.1 mM. Final concentration of epimastigotes 1×10^6 /mL. Time of incubation one hour.

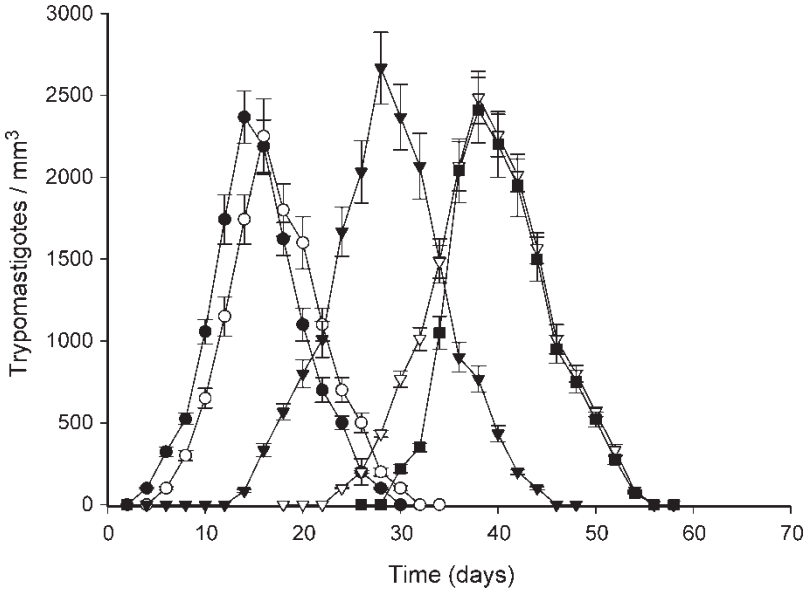

Figure 6. Parasitaemias induced in NIH mice by different *T. cruzi* strains. Parra (○), V2R (▼), Nayarit (•), Miguz (■) and Compostela (∇) *T. cruzi* strains. Groups of ten mice each were respectively inoculated with the different *T. cruzi* strains, using a single dose of 1×10^3 blood trypomastigotes.

the other two, Miguz and Compostela strains were resistant to these drugs. In addition, Et-NIPOx shows trypanocidal activity in all evaluated *T. cruzi* strains, including those *T. cruzi* strains that were resistant to Bz and Nfx. On the contrary, the acid (NIPOx) did not show trypanocidal activity on any of the tested *T. cruzi* strains (results not shown).

Parasitaemia induced in NIH mice by different T. cruzi strains

Figure 6 shows the parasitaemia induced in NIH mice by different *T. cruzi* strains. The peaks of parasitaemias were: 40 days for Compostela and Miguz strains, 14 days for Nayarit strain, 28 days for V2R strain and 18 days for Parra strain.

Effect of Nifurtimox on mice parasitaemia induced by different T. cruzi strains

Figure 7 shows the trypanocidal effect of Nfx on the parasitaemia induced by Parra, V2R and Nayarit *T. cruzi* strains. Whereas the Miguz and the Compostela *T. cruzi* strains were resistant to Nfx treatment.

Effect of Benznidazole on mice parasitaemia induced by different T. cruzi strains

Figure 8 shows the trypanocidal effect of Bz on the parasitaemia induced by Parra, V2R and Nayarit *T. cruzi* strains. Whereas the Miguz and the Compostela *T. cruzi* strains were resistant to Bz treatment.

Effect of the ethyl ester of N-isopropyl oxamic acid on mice parasitaemia induced by different T. cruzi strains

Figure 9 shows the trypanocidal effect of Et-NIPOx on the parasitaemia induced by blood trypomastigotes of Miguz, Parra, V2R, Nayarit and Compostela *T. cruzi* strains. This ester showed a trypanocidal effect in all the tested *T. cruzi* strains, including those that were resistant to Nfx and Bz. The most potent trypanocidal effect was observed with the Compostela strain, that it was resistant to Nfx and Bz.

Discussion

In a preceding investigation [15] we demonstrated that NIPOx was a selective inhibitor of HADH-isozyme II from *T. cruzi* and therefore we expected trypanocidal activity with this inhibitor. However, when we tested the effect of NIPOx on cultured epimastigotes of the V2R *T. cruzi* strain we did not detect any trypanocidal activity with this substance. Whereas, with Bz and Nfx used for comparison, the trypanocidal activity was evident on these *T. cruzi* epimastigotes. Nevertheless, when we tested the corresponding ethyl ester of N-isopropyl oxamate (Et-NIPOx) on these cultured epimastigotes we obtained the expected trypanocidal activity which was even better than that with Nfx and Bz [15]. Since carboxylesterases activity corresponding to aliphatic or non-specific esterases has been demonstrated in *T. cruzi* epimastigotes [23,24], we assumed that the increased effectiveness of the Et-NIPOx to reduce the motility and to kill these cultured *T. cruzi* epimastigotes most probably resulted from their better

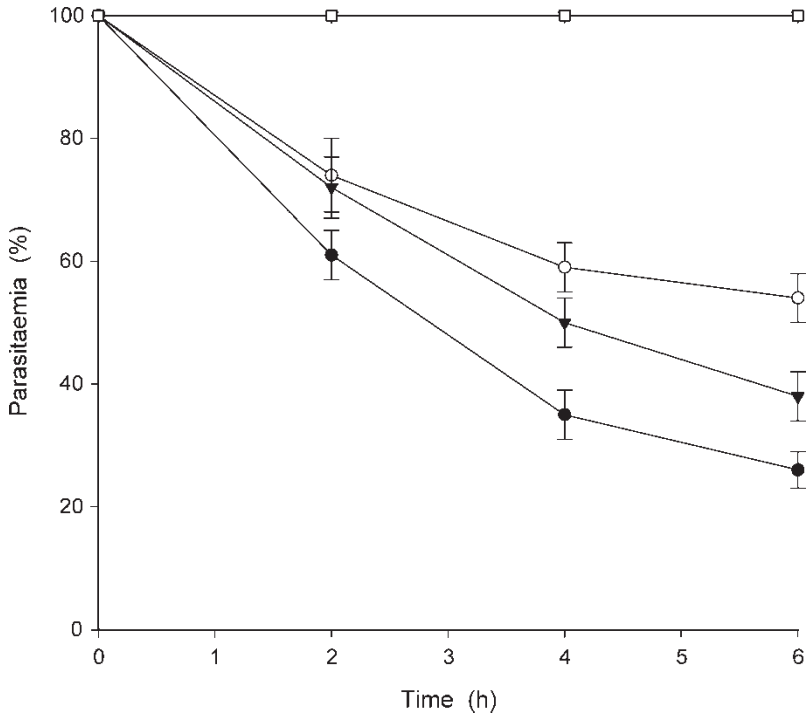

Figure 7. Effect of nifurtimox on mice parasitaemias induced by different *T. cruzi* strains. Parra (○), V2R (▼), Nayarit (•), Miguz (■) and Compostela (∇) *T. cruzi* strains, control (□) without treatment. Miguz and Compostela strains were resistant to the treatment of Nfx. At the peak of maximum parasitaemia, a single dose of 500 mg/kg of Nfx was given by the oral route [20].

absorption by this parasite and its efficient hydrolysis *in situ* into the active HADH inhibitor [15]. In the present investigation, using the purified HADH isozyme II from *T. cruzi* and a *T. cruzi* homogenate containing the carboxyl esterases and the HADH isozyme II we were able to confirm that this was the case since Et-NIPOx was not an inhibitor for the purified *T. cruzi* HADH isozyme II, whereas NIPOx

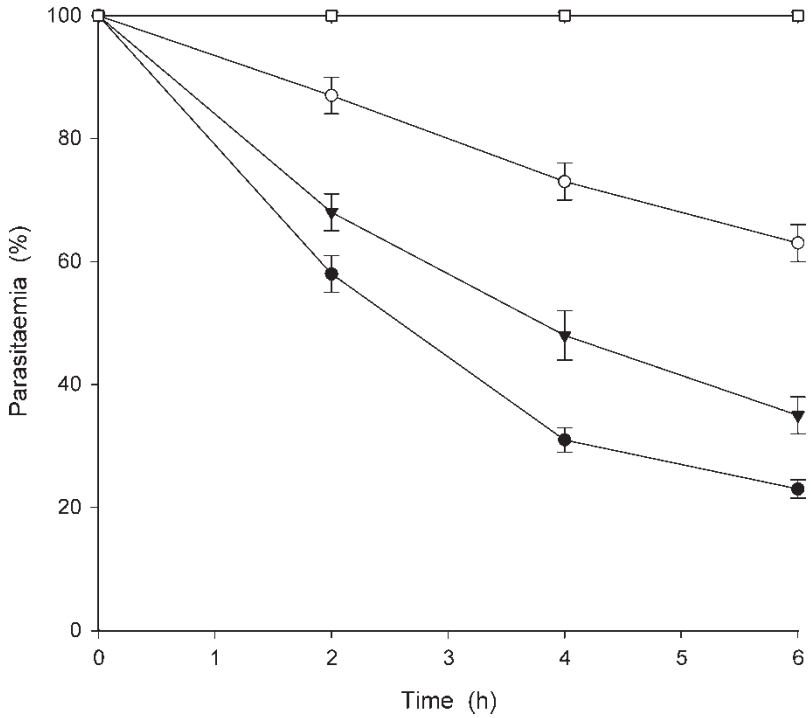

Figure 8. Effect of benznidazole on mice parasitaemias induced by different *T. cruzi* strains. Parra (○), V2R (▼), Nayarit (•), Miguz (■) and Compostela (∇) *T. cruzi* strains, control (□) without treatment. Miguz and Compostela strains were resistant to the treatment of Bz. At the peak of maximum parasitaemia, a single dose of 500 mg/kg of Bz was given by the oral route [20].

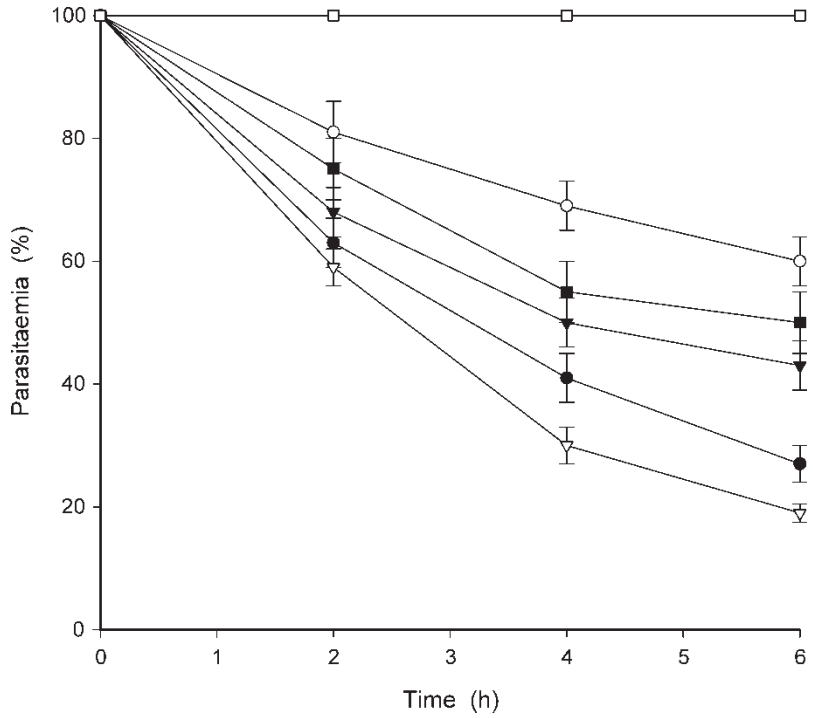

Figure 9. Effect of the ethyl ester of N-isopropyl oxamic acid on mice parasitaemias induced by different *T. cruzi* strains. Parra (○), V2R (▼), Nayarit (•), Miguz (■) and Compostela (∇) *T. cruzi* strains, control (□) without treatment. At the peak of maximum parasitaemia, a single dose of 500 mg/kg of Et-NIPOx was given by the oral route [20].

really inhibited this isozyme. On the contrary, when we used the above mentioned *T. cruzi* homogenate, the ester (Et-NIPOx) was hydrolyzed by the esterases and the *in situ* generated acid (NIPOx) readily inhibits the HADH isozyme II of the *T. cruzi* extract. These experiments strongly suggest that NIPOx does not penetrate intact epimastigotes, due to the polarity of the carboxylate, whereas the non polar ethyl ester of N-isopropyl oxamate, with affinity for membranes, really penetrates inside the parasite where it is hydrolyzed and exerts its pharmacological effect.

Since the available drugs for treatment of Chagas' disease are the nitroderivatives, Bz and Nfx, these drugs were used to compare the possible, *in vitro* and *in vivo*, trypanocidal activity of NIPOx and its corresponding ethyl ester (Et-NIPOx) using five different *T. cruzi* strains.

When Bz and Nfx were tested on cultured epimastigotes (*in vitro*) or blood trypomastigotes (*in vivo*) of these *T. cruzi* strains, it was found that only three of these *T. cruzi* strains were affected. Whereas, the other two strains, Miguz and Compostela, were resistant to the *in vitro* and the *in vivo* trypanocidal activity of these substances. Natural resistance of *T. cruzi* to nitroderivatives was suggested as an important factor to explain the low rate of cure detected in chagasic patients [18]. Since the trypanocidal action of Bz and Nfx derives from their ability to generate free radicals inside *T. cruzi* [25], the lack of trypanocidal activity of these substances can be explained on the basis of poor generation of free radicals inside these *T. cruzi* strains, or probably, because these substances do not penetrate inside these *T. cruzi* strains. In addition, when we tested NIPOx in the five *T. cruzi* strains on cultured epimastigotes and on mice parasitaemia induced by blood trypomastigotes, we did not obtain any trypanocidal activity on any of these *T. cruzi* strains. It is well known that membranes behave like hydrophobic barriers against polar substances such as NIPOx, with a negative charge on the carboxylate at physiological pH, which explains why this oxamate derivative do not show trypanocidal activity on these *T. cruzi* strains. Whereas, when EtNIPOx, a non-polar substance, with affinity for membranes was evaluated, the trypanocidal activity was evident.

When the Et-NIPOx was tested on intact epimastigotes and on mice parasitaemia, this substance acting as a prodrug, exhibited *in vitro* and *in vivo* trypanocidal activity in the five tested *T. cruzi* strains, including those that were resistant to Nfx and Bz. The trypanocidal effect was more evident on the mice parasitaemia. The better trypanocidal activity observed with Et-NIPOx, in comparison with Bz and Nfx, most probably resulted from the better absorption of this compound by the parasite and probably to the accumulation or trapping of the generated acid (NIPOx) inside *T. cruzi*, due to the polarity of the carboxylate.

Our experiments clearly indicate that HADH-isozyme II was correctly selected as a target enzyme

for the inhibition of the energetic metabolism of *T. cruzi*. A close relationship was shown between the inhibition of HADH-isozyme II and the trypanocidal effect on cultured epimastigotes and on mice blood trypomastigostes.

It is evident that the parasitaemia induced by different *T. cruzi* strains were affected to different extents by the treatment with Et-NIPOx. These differences do not correlate with K_i values because K_i for HADH-isozyme II, remains constant in the homogenate of every one of the five tested *T. cruzi* strains (results not shown). So, the different trypanocidal activity of Et-NIPOx observed in these *T. cruzi* strains, must be attributed to a different rate of absorption of Et-NIPOx by each of the five tested *T. cruzi* strains, in the *in vitro* and *in vivo* experiments.

The *in vivo* experiments showing the trypanocidal activity of Et-NIPOx in mice, also suggest that Et-NIPOx was resistant to the hydrolytic action of the carboxyl esterases also presents in mice tissues.

Further investigations are needed to ascertain whether the intracellular amastigotes (stage found in tissues of the mammalian host) are also susceptible to Et-NIPOx. Additional studies on the action of this compound on experimental infections of mammalian cell cultures and of laboratory animals are necessary to evaluate its activity as a possible therapeutic agent in trypanosomiasis.

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