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Inhibition of *Trypanosoma cruzi* α-Hydroxyacid Dehydrogenase-isozyme II by N-Isopropyl Oxamate and its Effect on Intact Epimastigotes

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The effect of N-isopropyl oxamate on the activity of α-hydroxyacid dehydrogenase-isozyme II (HADH-isozyme II) from *Trypanosoma cruzi* was investigated. The kinetic studies showed that this substance was a competitive inhibitor of this isozyme. The attachment of the nonpolar isopropylic branched chain to the nitrogen of oxamate increased 12-fold the affinity of N-isopropyl oxamate for the active site of *T. cruzi* HADH-isozyme II. N-isopropyl oxamate was a selective inhibitor of HADH-isozyme II, since other *T. cruzi* dehydrogenases were not inhibited by this substance. Since HADH-isozyme II participates in the energy metabolism of *T. cruzi*, a trypanocidal effect can be expected with inhibitors of this isozyme. However, although it was not possible to detect any trypanocidal activity with N-isopropyl oxamate when the ethyl ester was tested as a possible trypanocidal prodrug, the expected trypanocidal effect was obtained, comparable to that obtained with nifurtimox and benznidazole.

Keywords: *T. cruzi* α-hydroxyacid dehydrogenase inhibition; *T. cruzi* α-hydroxyacid dehydrogenase-isozyme II inhibition; N-isopropyl oxamate; Ethyl N-isopropyl oxamate; Trypanocidal activity

INTRODUCTION

Trypanosoma cruzi, the flagellate parasite that causes Chagas' disease, possesses an enzyme similar to the lactate dehydrogenase isozyme-x (LDH-C4) from mammalian spermatozoa.[1] The enzyme designated as HADH is a NAD-linked oxidoreductase whose catalytic properties resemble those of mouse[2] and rat[3] LDH-C4. This enzyme has two molecular forms (HADH-isozyme I and HADH-isozyme II), which have been purified and characterized in accordance with their substrate specificity and kinetic constants.[4] Isozyme I is responsible for the weak lactate dehydrogenase activity found in *T. cruzi* extracts,[5] while isozyme II does not show activity against pyruvate[6] and is active on a broad spectrum of linear and branched chain substrates, specially α-ketoisocaproate.[6]

Since *T. cruzi* HADH-isozyme II shows a substrate specificity similar to that of LDH-C4,[6,7] it is probably that HADH-isozyme II may accomplish similar functions in *T. cruzi*[8] to those ascribed to LDH-C4 in mammalian spermatozoa. On account of the similarities between HADH-isozyme II and LDH-C4, it has been proposed that these enzymes must be integrated in metabolic pathways supplying energy for the motility of flagellum and survival of the parasite,[6,8] or the spermatozoa respectively.[9] HADH-isozyme II is also located in the cytosol and in the matrix of mitochondria[8] as described for LDH-C4.[9] On the basis of this dual subcellular distribution, it was proposed that this dehydrogenase could be also integrated in a shuttle system, transferring reducing equivalents from cytosol to mitochondria using branched-chain 2-hydroxyacids/2-oxo acids as the redox couple.[8,9]

Taking into account the close chemical structure existing between N-isopropyl oxamate and the substrate α-ketoisocaproate and that HADH-isozyme II showed maximum activity and lowest K_m with α-ketoisocaproate,[6] we assumed that

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N-isopropyl oxamate would fulfill the structural and steric requirements for a competitive and selective inhibitor of *T. cruzi* HADH-isozyme II. Thus, we investigated the effect of N-isopropyl oxamate on the activity of HADH-isozyme II from *T. cruzi* and also its effect on the activity of several oxidoreductases from *T. cruzi*. We also tested the possible trypanocidal effect of N-isopropyl oxamate and that of the ethyl ester of N-isopropyl oxamate, in epimastigotes of the V2R strain.

MATERIALS AND METHODS

Chemicals

NAD, NADP, NADH, NADPH, D-glucose-6-phosphate, pyruvate, oxamate, α-ketoisocaproate, L-malate and L-glutamate all as sodium salts and succinate as the free acid were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Benznidazole (N-benzyl-2-nitro-1-imidazolacetamide) was from Roche-México and Nifurtimox (tetrahydro-3-methyl-4-[(5-nitro-furfurylidene)amine]-2-methyl-tetrahydro-1,4-thiazine-4,4-dioxide) was from Bayer-México. Other chemicals used were of the highest purity available.

Synthesis of Ethyl N-isopropyl Oxamate

Isopropylamine (5.9 g, 0.1 mol) in ether (50 mL) was added dropwise to an ice cold solution of diethyl oxalate (14.6 g, 0.1 mol) dissolved in ether (100 mL). After being stirred for 2 h the reaction mixture was allowed to warm to room temperature and left overnight. A crystalline product separated which was filtered off and the filtrate evaporated under slightly reduced pressure. The residue was then fractionated in vacuum. The ethyl N-isopropyl oxamate distilled at 75°C at 2 mm, as a colorless oily product. The overall yield was 13.6 g (85.5%), ^{1}H-NMR ($CDCl_3$) δ 1.17 (t, $J = 7.0$ Hz, 6H), 1.31 (t, $J = 7.0$ Hz, 3H), 4.07 (qd, $J = 7.0$ Hz, 1H), 4.28 (q, $J = 7.0$ Hz, 2H), 7.25 (broad s, 1H).

Synthesis of N-isopropyl Oxamate

Ethyl N-isopropyl oxamate (8.05 g, 0.05 mol) was shaken with 50 mL of 1 N NaOH for 30 min and extracted with ether. The aqueous phase was separated and acidified with 2 N HCl. Ether extraction and evaporation gave a crude product which, on mixing with light petroleum, soon becomes crystalline. The crystals were purified by recrystallization from chloroform (5.3 g, 81%): mp 113–114°C, ^{1}H-NMR ($CDCl_3$) δ 1.25 (d, $J = 6.5$ Hz, 6H), 4.07(m, 1H), 7.25 (broad s, 1H), 9.4 (broad s, 1H), IR (KBr) 3294, 2980, 1770, 1677, 1558, 1360 cm^{-1}.

Epimastigotes of *T. cruzi*

T. cruzi V2R stock strain was isolated from *Didelphis marsupialis* captured in the State of Nayarit, México. The primary isolation was made by xenodiagnosis with laboratory bred *Triatoma infestans*. Faeces of infected bugs were inoculated, intraperitoneally, into laboratory mice and cardiac blood was cultured subsequently 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.[10] Epimastigotes of *T. cruzi* V2R strain were cultivated in biphasic or liquid medium. The parasites were collected from the liquid phase after 7 days of culture at 28°C. At this stage, the growth is exponential. The liquid phase was centrifuged at 3000 × g for 15 min to obtain the parasite pellet.

T. cruzi Homogenates

The parasite pellet 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) to a final concentration of 1×10^6 organisms/mL. The suspension was frozen in liquid nitrogen, and thawed at 37°C. The freeze and thaw procedure was repeated three times. The disruption of parasites was monitored by microscopic examination of the resulting suspension. The homogenates obtained were 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 at room temperature and centrifuged at 20,000 × g for 20 min at 4°C. The supernatant was used for the analysis of HADH isozymes, malic enzyme, malate, glucose-6-phosphate, succinate and glutamate dehydrogenases.

Enzyme Preparations

The isozymes I and II of HADH were purified as described by Coronel *et al.*[6] Malate dehydrogenase (EC 1.1.1.37, L-malate:NAD oxidoreductase), glutamate dehydrogenase (EC 1.4.1.4, L-glutamate: NADP oxidoreductase (deaminating)), malic enzyme (EC 1.1.1.40, L-malate:NADP oxidoreductase (oxaloacetate-decarboxylating)), glucose-6-phosphate dehydrogenase (1.1.1.49, D-glucose-6-phosphate:NADP 1-oxidoreductase) and succinate dehydrogenase (EC 1.3.99.1, succinate:(acceptor) oxidoreductase) were assayed in crude extracts of *T. cruzi*.

Enzyme Assays

HADH-isozyme II activity was determined using α-ketoisocaproate as substrate.[7] 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 (at the concentrations indicated in results), enzyme preparation, diluted with 0.1 M sodium phosphate buffer pH 7.4 in order to obtain an absorbance change at 340 nm of 0.03–0.08 per min with a 5 mM concentration of substrate. Enzyme assays were incubated at 37°C and changes in absorption at 340 nm were recorded during a 4 min period. HADH-isozyme I was determined by the same method using pyruvate as substrate.[7]

The succinate dehydrogenase was assayed by the method of DerVartanian and Zeylemaker,[11] glucose-6-phosphate dehydrogenase by the method of Deutsch,[12] malate dehydrogenase by the technique described by Yoshida,[13] glutamate dehydrogenase by the method used by Juan *et al.*[14] and malic enzyme by the procedure used by Cannata *et al.*[15] All the enzyme assays were performed at 37°C.

Determination of Ki Values

HADH-isozyme II was incubated with the buffer used in the assay, the inhibitor oxamate or N-isopropyl oxamate and the coenzyme NADH were incubated for 10 min at 37°C before starting the reaction by adding the substrate. Values of Ki were calculated from those of Km 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 results.

In Vitro Evaluation of Trypanocidal Activity of the Drugs

To aliquots of *T. cruzi* suspension in liquid medium (1×10^6 trypomastigotes/mL) were added the test compounds in ethanol, up to 0.1 and 0.2 mM final concentrations. Two controls were used: one had ethanol added in the same proportion used to dissolve the test compounds (14 μL/mL), the other was a parasite suspension without any addition. All samples were incubated at 30°C. The observations and counts were made with a Neubauer hemocytometer after 10, 20, 30, 40, 50 and 60 min incubation. It was assumed that immobilized organisms had died. These experiments were also conducted using nifurtimox and benznidazole for comparison.

All experiments were run at least in triplicate and the results are given as mean ± standard deviation.

RESULTS

α-hydroxyacid Dehydrogenase Isozyme II Inhibition

A double-reciprocal plot of initial velocities of HADH-isozyme II at different substrate concentrations and the effect of N-isopropyl oxamate are shown in Figure 1. The inhibition was competitive and the Ki value with respect to α-ketoisocaproate was 74 μM. The K_m value (0.3 mM) was similar to

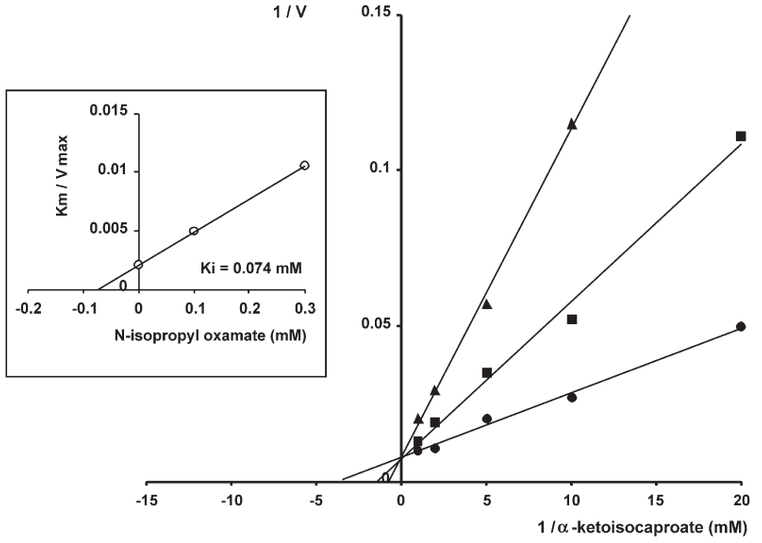

FIGURE 1 Effect of N-isopropyl oxamate on α-HADH-isozyme II from *T. cruzi* with α-ketoisocaproate as substrate. Reciprocal of V (expressed as ΔE_{340}/min) are plotted against reciprocal of α-ketoisocaproate concentration. The concentration of substrate used were 0.05, 0.1, 0.2, 0.5 and 1 mM; NADH concentration 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 Ki from replot of slope values against inhibitor concentration.

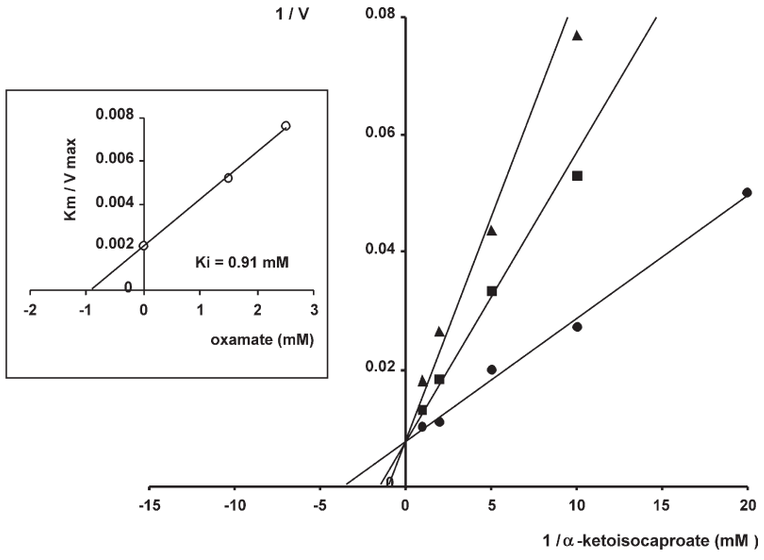

FIGURE 2 Effect of oxamate on α-HADH-isozyme II from *T. cruzi* with α-ketoisocaproate as substrate. Reciprocal of V (expressed as ΔE_{340}/min) are plotted against reciprocal of α-ketoisocaproate concentration. The concentration of substrate used were 0.05, 0.1, 0.2, 0.5 and 1 mM; NADH concentration was kept constant at 0.12 mM. Assays without (●) and with (■) 1.5 mM or (▲) 2.5 mM oxamate added to the reagent mixture. The K_m for α-ketoisocaproate was 0.3 mM. Upper left, determination of Ki from replot of slope values against inhibitor concentration.

those reported by others.[8] Results with crude extracts of HADH isozymes were the same as those presented for the purified isozymes, using α-ketoisocaproate for isozyme II and pyruvate for isozyme I.

Double-reciprocal plot of initial velocities of the activity of HADH-isozyme II at different substrate concentrations and the effect of the oxamate are shown in Figure 2. The inhibition was competitive and the Ki value with respect to α-ketoisocaproate was 0.91 mM.

Activity of Other Dehydrogenases

The effect of N-isopropyl oxamate was also tested on the activity of other dehydrogenases present in a crude extract of *T. cruzi* V2R strain. At very high concentrations of N-isopropyl oxamate (10 mM), glucose-6-phosphate dehydrogenase and HADH-isozyme I were only slightly inhibited, while malic enzyme, glutamate, malate and succinate dehydrogenases were unaffected. On the contrary, crude HADH-isozyme II was inhibited by 72% using 0.5 mM of N-isopropyl oxamate. Table 1 summarizes the results of the inhibitory action of N-isopropyl oxamate on the seven studied enzymes, using a crude extract of the *T. cruzi* V2R strain.

In Vitro Trypanocidal Activity

Figure 3 shows the *in vitro* trypanocidal activity of N-isopropyl oxamate, nifurtimox, benznidazole and the ethyl N-isopropyl oxamate against epimastigotes of the V2R strain. It is observed that a concentration of 0.1 mM N-isopropyl oxamate acid did not show trypanocidal activity, whereas the corresponding ethyl ester, the ethyl N-isopropyl oxamate, showed even better trypanocidal activity than nifurtimox and benznidazole. At a higher concentration, 0.2 mM, the ethyl ester of N-isopropyl oxamate showed a higher trypanocidal effect.

TABLE I Effect of N-Isopropyl oxamate on the activity of dehydrogenases from *Trypanosoma cruzi*

Enzyme	Substrates (mM)	N-isopropyl oxamate (mM)	Inhibition (%)*
HADH-isozyme II	α-ketoisocaproate (5) + NADH (0.12)	0.5	72.0
HADH-isozyme I	Pyruvate (5)+ NADH (0.12)	10	17.0
Glutamate dehydrogenase	α-ketoglutarate (2.5) + NADPH (0.12)	10	0.0
Succinate dehydrogenase	Succinate (20)	10	0.0
Glucose-6-phosphate dehydrogenase	Glucose-6-phosphate (3.3) + NADP (0.38)	10	23.8
Malate dehydrogenase	L-malate (8.5) + NAD (2.5)	10	0.0
Malic enzyme	L-malate (20) + NADP (0.30) + $MnCl_2$ (1)	10	0.0

*Initial velocities were determined in the presence and absence of N-isopropyl oxamate. Maximum enzymatic activity was attained at the millimolar concentrations of substrates and coenzymes indicated for each dehydrogenase, using a crude extract of the *T. cruzi* V2R strain.

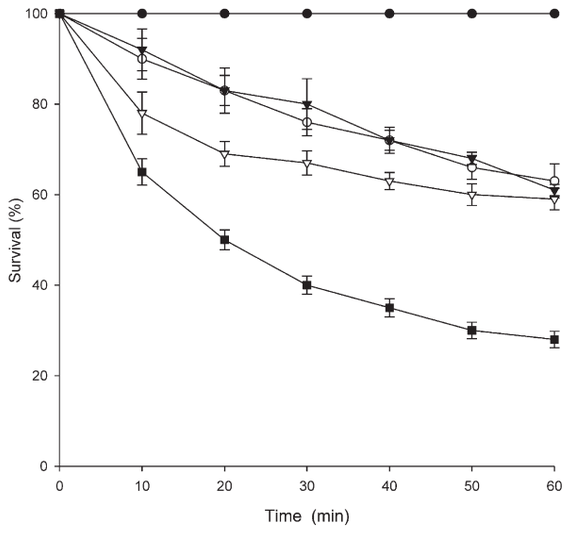

FIGURE 3 *In vitro* trypanocidal activity of several compounds on *T. cruzi* epimastigotes of V2R strain. The compounds were evaluated as described in Materials and Methods, at the indicated final concentrations. (●) 0.1 mM N-isopropyl oxamate, (○) 0.1 mM Nifurtimox, (▼) 0.1 mM Benznidazole, (▽) 0.1 mM Ethyl N-isopropyl oxamate, (■) 0.2 mM Ethyl N-isopropyl oxamate.

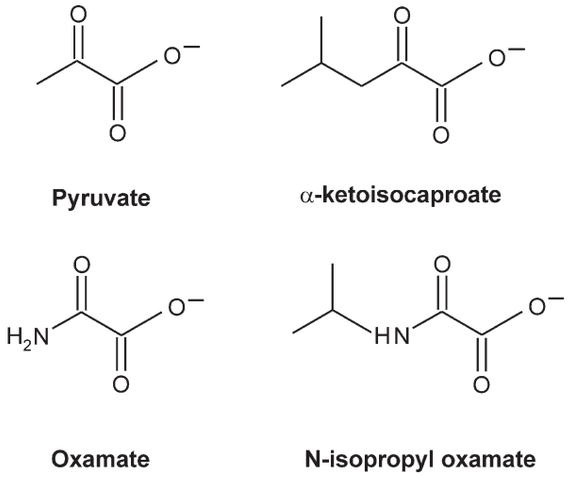

FIGURE 4 Chemical and structural relationships between the α-hydroxyacid dehydrogenase substrates pyruvate and α-ketoisocaproate, and the inhibitors oxamate and N-isopropyl oxamate.

DISCUSSION

In this work, the effect of N-isopropyl oxamate on the activity of *T. cruzi* HADH-isozyme II was studied because this isozyme has been associated with metabolic pathways supplying energy for the motility and survival of this parasite.[6,8] Furthermore, since glycolysis provides virtually all the energy for the bloodstream form of trypanosomatids, enzymes that participate in glycolysis or in its regulation have been suggested as a target for antitrypanosomatid drugs design.[16–18]

HADH-isozyme II, the main component of *T. cruzi* HADH, does not show activity against pyruvate and is active on a broad spectrum of α-keto and α-hydroxyacids from 4–6 nonpolar linear or branched (5–7) carbon chain substrates. Apparently α-keto-isocaproate is the best substrate for HADH-isozyme II because this isozyme showed the maximum activity and lowest K_m with this substrate.[6] α-Ketoisocaproate is a physiological metabolite derived from leucine by transamination, that participates in a shuttle system transferring reducing equivalents from cytosol to mitochondria.[8] In addition, HADH-isozyme II is the isozyme that it is actually integrated in metabolic pathways supplying energy for motility and survival of the parasite,[6,8] because this isozyme participates in the reoxidation of NADH during glycolysis.[6] Consequently, an inhibitor of HADH-isozyme II will not only inhibit glycolysis but will also inhibit the shuttle system transferring reducing equivalents from cytosol to mitochondria, and therefore, it has been proposed that inhibitors of this isozyme could reduce the motility and survival of this parasite.[19–21]

On account of the close chemical structure of N-isopropyl oxamate to that of the substrate α-ketoisocaproate (see Figure 4) we assumed that N-isopropyl oxamate would fulfill the structural and steric requirements to be a competitive and selective inhibitor of *T. cruzi* HADH-isozyme II. The kinetic studies showed that this compound was really an effective inhibitor of this isozyme and that the inhibition was competitive with respect to the substrate α-ketoisocaproate. The kinetic studies also showed that the introduction of the nonpolar isopropyl branched chain on the nitrogen of the oxamate molecule significantly increased the affinity of N-isopropyl oxamate for HADH-isozyme II. Consistent with this was the 12-fold better binding of N-isopropyl oxamate to the active site of *T. cruzi* HADH-isozyme II, as indicated by the Ki values (with α-ketoisocaproate as substrate) for oxamate ($Ki = 0.91\,mM$) and N-isopropyl oxamate ($Ki = 74\,\mu M$), respectively. It seems that oxamate, a well known competitive inhibitor of HADH,[22] directs the N-isopropyl oxamate molecule into the active site of *T. cruzi* HADH-isozyme II and the attachment of the nonpolar isopropylic branched chain to the nitrogen of oxamate provides N-isopropyl oxamate with more affinity and selectivity for the active site of HADH-isozyme II due to its possible hydrophobic interaction with a nonpolar region in or near the substrate binding site of the isozyme. These results are in accordance with those of Baker,[22] who has pointed out that the increase in the effectiveness of substrates and inhibitors induced by nonpolar substituents can be due only to

hydrophobic binding in an enzyme-inhibitor or enzyme-substrate complex.[22]

The slightly inhibitory effect of very high concentrations of N-isopropyl oxamate (10 mM) on glucose-6-phosphate dehydrogenase and HADH-isozyme I, and the lack of any inhibitory activity of N-isopropyl oxamate on malic enzyme, glutamate, malate and succinate dehydrogenases, supports a specific interaction with HADH-isozyme II, rather than a general inhibition of dehydrogenases. These experiments clearly indicated that N-isopropyl oxamate was a selective inhibitor of *T. cruzi* HADH-isozyme II. Our results also suggest the presence of an hydrophobic pocket, similar to that proposed for LDH-C4,[23] that can accommodate the nonpolar isopropylic group of N-isopropyl oxamate, near or in the active site of *T. cruzi* HADH-isozyme II. This hydrophobic pocket might explain the selective inhibition of this isozyme in comparison with the other studied dehydrogenases and the increasing affinity for α-keto and α-hydroxyacid substrates containing nonpolar side carbon chains.[6]

Investigations carried out in our laboratory, demonstrated that N-isopropyl oxamate was also an inhibitor of the mouse sperm-specific lactate dehydrogenase isozyme-C4.[23] Thus, besides the well known similarities in substrate specificity between *T. cruzi* HADH-isozyme II and isozyme-x of lactate dehydrogenase from spermatozoa (LDH-C4)[2,6] other biochemical homologies between these two isozymes can now be added. Apparently both isozymes are inhibited by N-isopropyl oxamate because the two isozymes use α-ketoisocaproate as a substrate.

Since in the present investigation we demonstrated that N-isopropyl oxamate was a selective inhibitor of HADH-isozyme II from *T. cruzi*, we then investigated if this inhibitor also showed trypanocidal activity on this parasite. To establish whether inhibition of HADH-isozyme II could affect the motility of whole parasites, we conducted studies with cultured epimastigotes diluted with the liquid medium (LIT) to a concentration of 1×10^6 trypanosomes per milliliter. N-isopropyl oxamate was then added to the suspension at a final concentration of 0.1 mM and surprisingly we were not able to detect any trypanocidal activity whereas with benznidazole and nifurtimox, used for comparison, trypanocidal activity was evident in *T. cruzi* epimastigotes of the V2R strain.

In view of the fact that biological membranes behave like hydrophobic barriers against polar substances, we supposed that N-isopropyl oxamate did not penetrate to the interior of the parasite. Assuming that the negative charge of the carboxylate present in N-isopropyl oxamate at physiological pH, was responsible for the lack of trypanocidal activity of this substance the ethyl ester of N-isopropyl oxamate was synthesized to transform N-isopropyl oxamate, into a more nonpolar or hydrophobic compound. This ester, gave the expected trypanocidal activity, even a little more potent than nifurtimox and benznidazole in this *T. cruzi* V2R strain. At a higher concentration, 0.2 mM, the ethyl N-isopropyl oxamate showed a higher trypanocidal effect.

Since carboxylesterase activity corresponding to type A, aromatic esterases, and type B, aliphatic or non-specific esterases, has been demonstrated in *T. cruzi* epimastigotes,[24] the increased effectiveness of the ethyl ester of N-isopropyl oxamate to reduce the motility and to kill *T. cruzi*, most probably results from its better absorption by *T. cruzi* and its efficient hydrolysis *in situ* into the active HADH inhibitor. Similar results were obtained with some ornithine decarboxylase (ODC) inhibitors used for the treatment of African trypanosomiasis that explain why the α-monofluoromethyldehydroornithine methyl ester was more potent than α-difluoromethylornithine against mouse trypanosomiasis.[25]

These studies strongly suggest that inhibition of HADH-isozyme II in *T. cruzi* has an important effect on the motility and survival of this parasite. However, these results are very preliminary and there is certainly no guarantee that ethyl N-isopropyl oxamate itself will be therapeutically useful in field studies. Nevertheless inhibitors of HADH-isozyme II may have a strong potential for use in this parasitic disease. In keeping with this, we designed and synthesized two gossypol derivatives, 4-isopropyl salicylaldehyde and 4-isopropylsalicylic acid, and found that these compounds also inhibited α-HADH-isozyme II from *T. cruzi* and showed trypanocidal activity in the two studied *T. cruzi* mexican strains, MIGUS and NINOA strains. These substances were active *in vitro* on the motility and survival of intact parasites and, *in vivo*, reduced parasitemia in laboratory mice.[26]

Nonetheless, additional studies on the action of the ethyl N-isopropyl oxamate on experimental infections of mammalian cell cultures and of animals are necessary to test its possible use as a therapeutic agent in trypanosomiasis. The results presented here are encouraging and relevant, since there is presently no satisfactory drug for use in Chagas' disease, a very serious endemic affecting millions of people in South America so that the search for new pharmacological agents is amply justified.

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