Isradipine and Lacidipine: Effects *In Vivo* and *In Vitro* on *Trypanosoma Cruzi* Epimastigotes

Luis J. Núñez-Vergara,[1]* *J. A. Squella,*[1] *Soledad Bollo-Dragnic,*[1] *R. Marín-Catalán,*[2] *L. Pino,*[2] *G. Díaz-Araya*[2] *and M. E. Letelier*[2]

[1]Laboratory of Bioelectrochemistry, [2]Laboratory of Pharmacology, Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile, PO Box 233, Santiago-Chile [Tel: (56-2)6782887; Fax: (56-2)7378920]

ABSTRACT. 1. Isradipine and lacidipine, two new drugs that are members of the nitro-aryl-1,4-dihydropyridine family, produced inhibition of both growth cultures and oxygen consumption on epimastigotes of *Trypanosoma cruzi* Tulahuen strain, at micromolar concentrations.

2. Isradipine was found to be the most potent derivative in both, in growth cultures (I_{50}=20.8 μM) and *in vivo* oxygen uptake (I_{50}=31.1 μM).

3. Diltiazem and verapamil, two well-known calcium channel antagonists, lacked inhibitory activity, even at a 100 μM concentration.

4. The present findings indicate that the trypanocide effects exerted by isradipine and lacidipine are not related with a disruption of the calcium homeostasis of the parasite.

KEY WORDS. *Trypanosoma cruzi*, isradipine, lacidipine, culture growth inhibition, oxygen consumption, *in situ* mitochondria

INTRODUCTION

Since the discovery of nifedipine in 1971, major efforts have been directed towards the development of new 1,4-dihydropyridine calcium channel antagonists. In the course of a systematic manipulation of the dihydropyridine structure, targeted to the identification of novel, potent, and long-acting antihypertensive agents, lacidipine and isradipine have emerged as the most promising drug candidates (Carpi *et al.*, 1986).

Trypanosoma cruzi has 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 environment. Considering the changes in Ca^{2+} during the life cycle of this parasite it is evident that calcium homeostasis should be efficient to support these changes (Docampo, 1993; Moreno *et al.*, 1991). Two intracellular calcium transport systems have been detected in *T. cruzi*. Ca^{2+} uptake by the first pool occurs by an electrophoretic mechanism, which is inhibited by antimycin A and ruthenium red, and stimulated by respiratory substrates, phosphate and acetate. This pool has a high capacity and low affinity for calcium and is able to buffer external Ca^{2+} at concentrations in the range of 0.6-0.7 μM (Moreno *et al.*, 1991; Vercesi *et al.*, 1991), being typical characteristics of mitochondria (Carafoli, 1989). Ca^{2+} uptake by other intracellular pools is inhibited by high concentrations of vanadate and anticalmodulin agents, and stimulated by ATP. This pool has a low capacity and high affinity for Ca^{2+} and is able to buffer external Ca^{2+} in the range of 0.05–1.0 μM (Carafoli, 1989; Moreno *et al.*, 1991).

In a recent paper (Oz *et al.*, 1992) it has been demonstrated that the epimastigote forms of *T. cruzi* maintain $[Ca^{2+}]$, by uptake, sequestration, and extrusion mechanisms, with properties common to eukaryotic organisms. On the other hand, in this same paper, the effects of several calcium channel antagonists on the intracellular calcium homeostasis of this parasite were assessed. It was demonstrated that verapamil and isradipine inhibited the uptake of Ca^{2+} by greater than 50%, whereas diltiazem, nifedipine, and nicardipine were ineffective.

We took into account that *T. cruzi*, the agent that causes Chagas disease, represents a serious problem in Latin American countries, not only at a socioeconomic level, but also in health and that up to now there is no suitable and effective treatment for this disease, and we have explored the possibility that drugs which interfere with the calcium homeostasis of the parasite, i.e., calcium channel antagonists, could have significant effects on *T. cruzi* culture growth. In conclusion, in this paper we explored the effects of isradipine and lacidipine, two novel 1,4-dihydropyridine calcium channel antagonists, on *T. cruzi* growth cultures and oxygen uptake. Also, for comparative purposes the effects of verapamil and diltiazem, two non-structural related drugs with the above-mentioned derivatives are included.

MATERIALS AND METHODS

Materials

Tryptose, tryptone, yeast extract, and fetal calf serum were obtained from Difco (Detroit, MI, USA). Sucrose digitonin, hemin, EDTA, phenazine metasulphate, 2,6-dichlorophenol-indophenol (DCIP), succinic acid, ADP, serum albumin, and all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

DRUGS. Isradipine: 4-(4-benzofurazanyl)-1,4-dihydro-2,6-demethyl-3,5-pyridinedicarboxylic acid methyl 1-methyl ethyl ester was obtained from Sandoz Laboratories (Santiago, Chile). Lacidipine, Diethyl(E)-4-[2-(*tert*-butoxycarbonyl)vynil]-phenyl]-2,6dimethyl-1,4-dihydropyridine 3,5-dicarboxylate was obtained from Glaxo (Santiago, Chile). Verapamil, α-[3[2-(3,4--dimethoxyphenyl)ethyl]methylamino]propyl]-3,4-dimethoxy-α-(1-methylethyl)-benzeneacetonitrile was obtained

*To whom correspondence should be addressed.

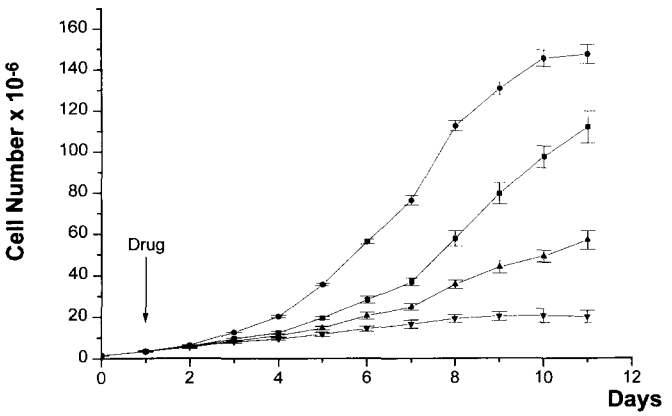

FIGURE 1. Effect of isradipine (■) 10 μM, (▲) 20 μM, and (▼) 30 μM on *T. cruzi* epimastigotes growth. Control (●). Each curve represents the mean of three independent experiments ±SD.

from Chile Laboratories (Santiago, Chile). Diltiazem, (2S-cis)-3-(Acetyloxy)-5-[2-(dimethylamino)ethyl]-2,3-dihydro-2-(4-metoxyphenyl)-1,5-benzothiazepin-4(5H)-one acetate was obtained from Parke Davis Laboratories (Santiago, Chile).

Trypanosomes

Tulahuén and LQ strains and clone Dm 28c of *T. cruzi* epimastigotes were grown at 28°C in Diamond monophasic medium with blood replaced by 4 μM hemin; the pH was adjusted to 7.2 before sterilization. Fetal calf serum was added at 4% final concentration. *T. cruzi* epimastigotes growth was followed by nephelometry using culture flasks with a side-arm tube (Aldunate *et al.*, 1986). The parasites were harvested on the fifth day of growth by centrifugation at 500 *g* for 10 min. They were washed twice with 0.17 M NaCl–0.052 M potassium phosphate (pH 7.2). Protein values were determined according to Lowry *et al.* (1951).

Isolation of T. cruzi *epimastigotes in situ mitochondria*

T. cruzi epimastigotes were resuspended in 200 mM sucrose, 2 mM $MgCl_2$, 1 mM EDTA, 10 mM potassium phosphate buffer, pH 7.4 (buffer solution A) and preincubated with digitonin 70 μg/mg of protein for 5 min (Aldunate *et al.*, 1992).

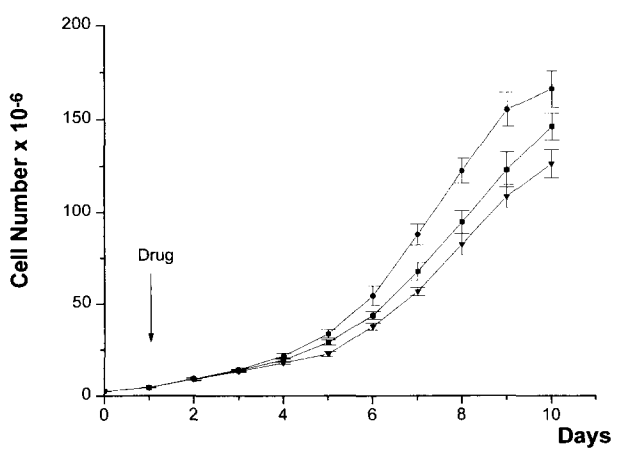

FIGURE 2. Effect of lacidipine (■) 10 μM and (▼) 30 μM on *T. cruzi* epimastigotes growth. Control (●). Each curve represents the mean of three independent experiments±SD.

TABLE 1. Comparative effects of isradipine and lacidipine on the culture growth of *T. cruzi* epimastigotes (Tulahuén strain)

Drug	I_{50},* μM
Isradipine	20.8 ± 0.9
Lacidipine	33.5 ± 1.2
Nifurtimox‡	20
Benznidazol‡	40
Diltiazem	No inhibition
Verapamil	No inhibition

*I_{50} values represent the concentration of drug required to inhibit 50% of growth cultures (7 days after drug addition). Data were calculated from curves of growth *vs* drug concentration.
‡Data taken from Goijman *et al.* (1985).

Oxygen uptake

Oxygen uptake measurements were carried out polarographically with a Clark electrode (N°. 5331, Yellow Springs Instruments, Yellow Springs, OH, USA) in a Gilson 5/6 oxygraph. Intact and digitonin permeabilized epimastigotes, 2.0 and 3.0 mg of protein/ml, respectively, were suspended in buffer solution A in a final volume of 2.0 ml. All drugs were added in dimethyl sulfoxide. No effect of dimethyl sulfoxide was observed at the concentration used.

RESULTS AND DISCUSSION

The two tested 1,4-dihydropyridine derivatives inhibited the culture growth and oxygen consumption, both *in vivo* and *in vitro*.

Figure 1 shows the effect of isradipine on the growth of *T. cruzi* epimastigotes. This drug inhibited by 66.3% the growth of the epimastigotes when it was added at a 30 μM final concentration to the culture of parasites. However, lacidipine used in the same concentration only inhibited 29.9% the growth (Fig. 2).

As can be seen in Tables 1 and 2, isradipine and lacidipine inhibited concentration-dependently both the rate of growth and the rate of oxygen consumption of a culture of intact *T. cruzi* epimastigotes, Tulahuén strain. The above results denote a significant inhibitory activity for isradipine and lacidipine. These effects are comparable with those exhibited by nifurtimox and benznidazol (Table 1). It is noteworthy that these two drugs are until now the only therapeutic tools for the acute phase treatment of the Chagas disease. As can be seen from Table 1, the I_{50} for isradipine (20.8 μM) and lacidipine

TABLE 2. Effects of some calcium channel antagonists on the oxygen consumption of *T. cruzi* epimastigotes (Tulahuén strain)

	I_{50},* μM	
1,4-DHP	*In vivo*‡	*In vitro*§
Isradipine	32.0 ± 1.3	136.1 ± 2.2
Lacidipine	112.5 ± 1.8	46.8 ± 1.9
Verapamil	No inhibition	No inhibition
Diltiazem	No inhibition	No inhibition

*I_{50} values represent the concentration of drug required to inhibit 50% of respiration. Data were calculated from curves of oxygen consumption *vs* drug concentration. Mean of four independent experiments ± standard deviation.
‡Intact parasites.
§*In situ* mitochondria, see experiment for details.

(33.5 μM) are within the range of inhibitory concentration, previously found by Goijman *et al.* (1985) for nifurtimox (20 μM) and benznidazol (40 μM). In order to test an eventual relationship between the calcium channel blocking activity and the effects here found, two well-known calcium channel antagonists nonstructurally related with the tested 1,4-dihydropyridine derivatives, verapamil and diltiazem, were also assessed in the same experimental conditions previously used with the other drugs. As is shown in Table 1, both calcium channel antagonists (verapamil and diltiazem) completely lack of any inhibitory activity on culture growth, even at higher concentrations than 100 μM.

The I_{50} of isradipine and lacidipine on the rate of oxygen uptake both *in vivo* and *in vitro* are shown in Table 2. The results indicate that isradipine was the most potent derivative (I_{50} ratio=3.59) *in vivo* experiments. The difference between the I_{50} for the *in vivo* and *in vitro* effects (4.25-fold) on O_2 consumption of isradipine could be explained as an action of this drug at levels other than the respiratory chain of the parasite. Apparently, the toxic effect of isradipine is not limited by its permeability.

In contrast, lacidipine exhibits the most potent effects in *in vitro* experiments (I_{50} ratio=2.90). Also, in Table 2 it can be seen that diltiazem and verapamil lack effects on oxygen uptake, both *in vitro* and *in vivo*.

On the other hand, taking into account the susceptibility differences shown by strains and clones of *T. cruzi* to the action of drugs, studies on the effects of isradipine and lacidipine in strains other than Tulahuén (LQ and clone Dm 28c) were also conducted. Results of such studies did not show differential sensitivities of the strains to the drugs, both in culture growth and oxygen uptake, when compared with the results obtained with the Tulahuén strain.

In conclusion, results from this paper provide experimental support to substantiate trypanocide effects of two novel 1,4-dihydropyridine derivatives. Such effects appear not directly related with a disruption of calcium homeostasis of the parasite by the tested drugs. Thus, as was previously demonstrated by Oz *et al.* (1992) verapamil and isradipine inhibited the uptake of Ca^{2+} by *T. cruzi* epimastigotes at levels greater than 50%, whereas diltiazem and other 1,4-dihydropyridine derivatives such as nifedipine and nicardipine were ineffective. In our study, isradipine had a significant trypanocide effect, but verapamil completely lacked inhibitory effects. In spite of the above results, future work must be conducted to establish the actual role of this target for trypanocide action. Concerning the future of the 1,4-dihydropyridine derivatives in Chagas disease treatment, we think at present its potential trypanocide usefulness is precluded by the significant human cardiovascular effects of these derivatives. However, a strategy that leads to a variation in the dihydropyridine structure and synthesis of the distomers (enantiomer with lower cardiovascular activity) could result in sterically pure and racemic dihydropyridines with low hypotensive properties. At present, we are assessing the potential trypanocide activities on *T. cruzi* culture growth with oxidized dihydropyridine derivatives (i.e., pyridine derivatives), which are well-known for their lack of cardiovascular effects.

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