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Trypanocidal action of 2,4-dichloro-6-phenylphenoxyethyl diethylamine hydrobromide (Lilly 18947) on *Trypanosoma cruzi*[1]

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KEY WORDS Lilly 18947; *Trypanosoma cruzi*; trypanocidal agents; Vero cells

ABSTRACT

AIM: To study the effect of the inhibitor of cytochrome P450 known as Lilly 18947 (2, 4 dichloro-6 phenylphenoxy ethyl diethylamine) on *Trypanosoma cruzi*. **METHODS**: *Trypanosoma cruzi* epimastigotes were grown in culture, in absence or in presence of drug. The inhibition of its growth was followed by daily counting using a Neubauer chamber. The effect of Lilly 18947 on the parasite ultrastructure was examined by electron microscopy. To test the effect of different concentrations of drug on the parasite cycle, Vero cells were inoculated with trypomastigotes (RA strain) and after 72 h the percentage of infected cells and the number of intracellular parasites were estimated and expressed as the endocytic index. **RESULTS**: Growth of epimastigotes was inhibited by Lilly 18947. Concentrations as low as 50 μmol/L resulted in a complete disappearance of the parasites in culture by the fourth day. With lower concentrations, little growth was observed and total (25 μmol/L) or partial lysis (10 μmol/L) were registered by the eighth day of culture. Incubation of epimastigotes with 50 μmol/L of Lilly 18947 resulted in an early damage to cellular structures. Initial signs were dilatation of perinuclear membranes and mitochondria swelling. The infectivity of trypomastigotes to Vero cells in culture was nearly abolished at 15 and 30 μmol/L concentrations of the drug. **CONCLUSION**: Lilly 18947 was able to harm *Trypanosoma cruzi* membrane functions leading to the loss of its infective properties and its death.

INTRODUCTION

Chemotherapy and chemoprophylaxis of Chagas disease caused by a Trypanosomatid flagellate, *Trypanosoma cruzi*, are important public health issues for Latin American countries in view of the high social and economic impact of the disease which affects an estimated population of 16 million, with more at risk[1]. A number of drugs have been reported to be effective against *T cruzi in vitro* or in animal models, but no one has been found completely satisfactory for either the treatment of Chagasic patients or for treatment of blood to prevent infection via blood transfusion[2-10].

In fact, the two drugs in use for clinical treatment, nifurtimox (Nfx) and benznidazole (Bz), and Gentian violet which is being used for chemoprophylaxis against transfusional transmission of the disease, have serious toxic side effects or cast doubts about their safe use in clinical practice[2,3,7,11]. Clearly, new drugs and even new approaches for facing those needs are necessary.

We recently reported the trypanocidal effects of beta-diethylaminoethyl-diphenyl propyl acetate hydrochloride (SKF525A or proadifen)[12]. This drug is known to be a potent inhibitor of cytochrome P450 (P450)-mediated biotransformation processes[13-15], and also able to interact with the parasite P450[16,17]. Studies on the safety of its use for blood transfusion purposes are under way in one of our laboratories.

We report here experiments aimed at determining the potential trypanocidal properties of another very well established inhibitor of P450-mediated metabolic pathways, a compound known as Lilly 18947 (2,4 dichloro-6 phenylphenoxy ethyl diethylamine hydrobromide)[13-15].

This compound shares with SKF525A not only its ability to interact with P450 but also exhibits an

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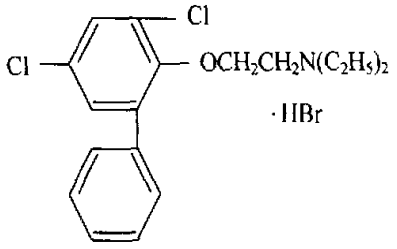
amphiphilic structure which is a characteristic feature of many drugs (including SKF525A) proven to be effective agents against the parasite[18].



Lilly 18947

MATERIALS AND METHODS

Chemicals Lilly 18947 (2,4 dichloro-6 phenyl-phenoxy ethyl diethylamine hydrobromide) (obtained from E Lilly Co), was dissolved in dimethylsulfoxide (Me_2SO). All other chemicals used were analytical reagents of the highest purity available.

Obtention of parasites Epimastigotes (CL Brener clone) were grown and harvested as previously described[19]. Cell-culture trypomastigotes (RA strain) were obtained from infected Vero cells[20].

Assay of inhibition of epimastigote growth Parasites were grown at 28 ℃, in the absence or in the presence of the drug concentrations stated in Fig 1, and growth was followed by daily counting using a Neubauer chamber.

Assay of the effect of Lilly 18947 on the parasite cycle in Vero cells Vero cells (4×10^5/L) were cultured at 37 ℃ in modified Eagle's medium (MEM) containing 5 % (v/v) fetal calf serum, in 24-well plate dishes containing glass coverslips[20]. After 48 h the cultures were inoculated with RA strain cell culture trypomastigotes (5×10^6/L) with or without Lilly 18947. After 24 h, the medium containing the non-internalized parasites, was removed; fresh medium, with or without drug, was added and the infected cells were incubated for 72 h and stained with May-Grünwald-Giemsa. The percentage of infected cells and the number of intracellular parasites were estimated by observing 500 cells in a Nikon Eclipse E400 microscope.

The results are expressed as the endocytic index (product of the percent of cells infected and the number of amastigotes per cell).

Electron microscopy Epimastigotes were fixed by suspension in 3 % glutaraldehyde in cacodylate buffer 0.1 mol/L, pH 7.2 for 120 min at room temperature. Afterwards samples were treated as described[21], and thin sections were observed in a Philips EM 300 electron microscope.

Statistics Experimental data were expressed as $\bar{x} \pm s$. The significance of the difference between mean values was assessed by unpaired t test. Calculations were performed using GraphPad Software (Instat

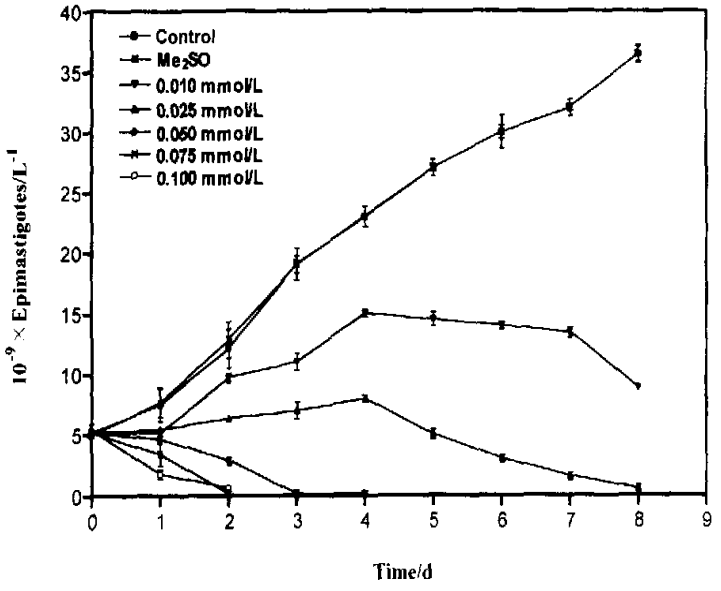


Fig 1. Effect of Lilly 18947 on growth of epimastigotes, CL Brener clone, in axenic culture. $n = 4$. $\bar{x} \pm s$.

Biostatistics, San Diego, USA).

RESULTS

Growth of CL Brener clone epimastigotes was inhibited by Lilly 18947; complete disappearance of the parasites was achieved by d 4 in culture by concentrations as low as 30 μmol/L (Fig 1). Little growth was observed in the presence of Lilly 18947 25 μmol/L with complete parasite lysis by d 8. Considerable inhibition of parasite growth and partial lysis on d 8 were observed in the presence of Lilly 18947 10 μmol/L.

The presence of the drug during culture of CL Brener clone epimastigotes led to a progressive parasite injury even at a concentration as low as 50 μmol/L. At 24 h of treatment, the cellular architecture of epimastigotes, compared with the untreated parasites (Fig 2A), exhibited severe dilatation of the nuclear envelope. Huge vacuoles not physically related to the perinuclear membrane were present in the cytoplasm. They might be highly swollen versions of the organelles previously described by other authors as autotrophic vacuoles[22,23], for they did not have externally attached ribosomes. Still another possibility, probably less likely, is that the poorly developed smooth endoplasmic reticulum of *T cruzi* was severely dilatated. The unlikelyhood of this possibility rests in the relatively extensive area covered by these vacuoles (Fig 2B) in relation to the small proportion of endoplasmic reticulum present in *T cruzi*. At 48 h of treatment, the dilatation of the nuclear envelope is even more intense. The mitochondria were also altered (swelling), but the subpellicular microtubules were relatively little affected (Fig 2C). After 72 h of treatment the mitochondria were severely altered with swelling, loss of its electron density and cristae. In the cytoplasm membrane bound vacuoles and an abundant number of ribosomes could be seen (Fig 2D). At this experimental condition as well as at higher concentrations, most of the parasites looked as empty bags, having extensive vacuoles covering almost the entire structure.

At Lilly 18947 100 μmol/L (60 min), the parasite showed also nuclear karyolysis and severe alterations of the subpellicular microtubules (Fig 2E). At 200 μmol/L of treatment (60 min) the alterations were even more intense, with nuclear karyorrhexis, swelling of the kinetoplast-mitochondrion complexes and also alterations of the subpellicular microtubules (Fig 2F).

When Lilly 18947 was tested on the parasite cycle in cultured Vero cells (Tab 1), the presence of the drug during infection and subsequent culture caused a marked decrease in the endocytic index even at concentrations as low as 15 μmol/L. Considerable decrease was also observed when the drug was present only during infection with Lilly 18947 15 μmol/L. When the drug was added 24 h after infection only a partial effect was observed. If the drug concentration added after infection is doubled (30 μmol/L) however the endocytic index remains very low. These results suggest that Lilly 18947 has a dual effect, on one hand on infecting trypomastigotes but also on the intracellular parasite growth.

Tab 1. Effect of Lilly 18947 on the parasite cycle in Vero cells. $n=4$. $\bar{x}\pm s$. $^bP<0.05$, $^cP<0.01$ *vs* control.

Treatment	Infected cells/%	Amastigotes per cell	Endocytic index
Control	19.1 ± 1.8	9.8 ± 1.6	187
Lilly 18947 15 μmol/L			
A	2.1 ± 0.3^c	1.00 ± 0.10^b	2
B	5.8 ± 1.0^c	3.5 ± 0.5^b	20
C	7.0 ± 2.5^c	6.4 ± 0.6^b	45
Lilly 18947 30 μmol/L			
A	0.50 ± 0.10^c	1.00 ± 0.10^b	0.5
B	4.3 ± 1.7^c	5.1 ± 0.6^b	22
C	1.50 ± 0.19^c	2.5 ± 0.4^b	4

A) Drug present during infection and subsequent culture; B) Drug present only during infection; C) Drug added 24 h after infection.

DISCUSSION

The behavior of Lilly 18947 acting on *T cruzi* has components which resemble those previously reported by us for trifluoperazine (TFP) and SKF525A. In fact, it shares with TFP the ability to harm mitochondria and with SKF525A the actions on *T cruzi* membranes. Effects of Lilly 18947 on liver mitochondrial function leading to their intensive swelling were previously reported[22,23] and they may also operate in the present case.

Concerning the effects of Lilly 18947, those resembling the effect of SKF525A on the parasite membranes[24] should not be unexpected since both are very well known general and potent inhibitors of cytochrome P450-mediated biotransformations. Both inhibitors, SKF525A and Lilly 18947, form metabolic intermediate complexes with cytochrome P450[13]. The secondary amine dealkylated metabolite of Lilly 18947 but not the fully dealkylated primary amine of this inhibitor (DPEA) also forms a reversible metabolic intermediate

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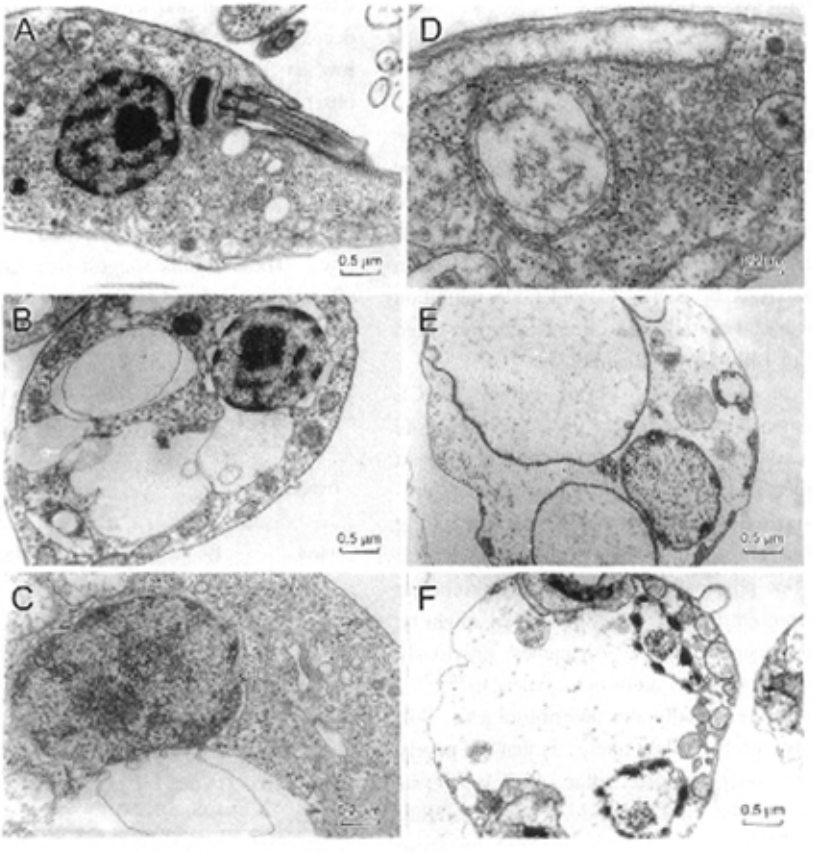

Fig 2. Effect of Lilly 18947 on the ultrastructure of *Trypanosoma cruzi* epimastigotes. A) Electron micrograph of a control *T cruzi* epimastigote. In the cytoplasm it can be observed: the nucleus surrounded by an intact perinuclear envelope, with a prominent karyosome and coarse heterochromatin patches interlaced into a network; the kinetoplast displaying tightly stacked K-DNA near the Golgi zone; the basal body with the emerging flagellum; numerous ribosomes; some membrane bound vesicles; the mitochondrion and the subpellicular microtubule network (×15400). B) Electron micrograph of *T cruzi* epimastigote incubated with Lilly 18947 50 μmol/L, 24 h. The ultrastructure of the parasite is altered. The cell becomes rounded although the subpellicular microtubule network has a normal appearance. The mitochondrion does not seem to be altered. Note the markedly enlarged perinuclear space (^) and the presence of huge vacuoles (TM) in the cytoplasm (×15400). C) Electron micrograph of *T cruzi* epimastigote incubated with Lilly 18947 50 μmol/L, 48 h. It is possible to observe a gross alteration of the perinuclear space and swelling of the mitochondrion (^), with loss of its electron density and cristae. The Golgi complex (TM) showes a sligt dilatation. The microtubules in parallel array with its characteristic structure are seen below the cell membrane (×38700). D) Electron micrograph of *T cruzi* epimastigote incubated with Lilly 18947 50 μmol/L, 72 h. It is possible to observe the mitochondrion (^) with an extremely loose matrix and only a few remnants of cristae projecting from the inner membrane into the matrix. There are also some membrane-bound vacuoles (TM) containing poorly defined material in the cytoplasm. Numerous free ribosomes occupy the cytoplasm. The subpellicular microtubules show its normal fine architecture (×38700). E) Electron micrograph of *T cruzi* epimastigote incubated with Lilly 18947 100 μmol/L, 60 min. It is possible to observe: some huge membrane-bound vacuoles (TM) in the cytoplasm of the parasite, nuclear karyolysis (^), and the absence of most of the subpellicular microtubules (×15400). F) Electron micrograph of *T cruzi* epimastigote incubated with Lilly 18947 200 μmol/L, 60 min. It is possible to observe: some small membrane-bound vacuoles near one side of the parasite, two nuclei with karyorrhexis (^), and also two altered kinetoplast-mitochondrion complexes (TM). The cytoplasm appeared to be extremely loose. The cell membrane is altered as well as the subpellicular layer of microtubules which disappeared in part of the cell or lost its regular disposition (×15400).

complex with cytochrome P450[13]. Whether these biotransformations of Lilly 18947 occur in *T cruzi* is not known at present. However, it is very well established that cytochrome P450 is involved in several steps of the synthesis of ergosterol which in turn, is a critical component of the parasite membranes[24].

The amphiphilic structure of Lilly 18947, however, might also play a role. In fact many drugs of amphiphilic structure proved in the hands of other workers to be effective against the parasite[19]. Detergent-like effects related to the amphiphilic nature of Lilly 18947 molecule or those of this drug on mitochondrial swelling or on lysosomal permeability previously described[23,25] could also play a role but only at the higher concentrations tested. Under those conditions, the above described inhibitory actions on cytochrome P450-mediated reactions would additionally have the contribution of the effects of the drug on different organelle membranes of the cell. Those concurrent synergistic contributions of Lilly 18947 might reasonably explain why at the higher concentrations tested, effects are far more intense and fundamentally why they are faster (they occur at 60 min after exposure rather than at 24 or 72 h after interaction of the drug with *T cruzi*).

Potential applications of this drug to the chemotherapy of Chagas' infection would require much deeper studies on its pharmacological-toxicological properties, as well as infection studies on animal models. Meanwhile the possibility of using amphiphilic drugs able to interact with cytochrome P450 should remain open as a possible new path to chemotherapeutic attack against Chagas disease.

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氢溴酸 2,4-二氯-6-苯基苯氧乙基二乙基胺(Lilly 18947)抗克氏锥虫的作用

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关键词 Lilly 18947; 锥虫, 克氏; 抗锥虫药; Vero 细胞

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