

# Anti-proliferative synergy of lysophospholipid analogues and ketoconazole against *Trypanosoma cruzi* (Kinetoplastida: Trypanosomatidae): cellular and ultrastructural analysis

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**Objectives:** Investigation of the antiproliferative synergy of the lysophospholipid analogues (LPAs) edelfosine, ilmofosine and miltefosine with the ergosterol biosynthesis inhibitor ketoconazole against *Trypanosoma cruzi*.

**Methods:** The effect of LPAs, ketoconazole and their combination was evaluated against epimastigotes and intracellular amastigotes by the parameter IC<sub>50</sub> leading to construction of isobolograms, for determination of a synergic effect. For epimastigotes, ultrastructural damage induced by these treatments was evaluated by transmission and scanning electron microscopy.

**Results:** Synergy was confirmed against both epimastigotes and amastigotes of the parasite. Edelfosine or ketoconazole alone induced morphological alterations in the plasma membrane and reservosomes of the parasites, while in combination, they also led to severe mitochondrial damage, formation of autophagic structures and multinucleation. Scanning electron microscopy confirmed the effect at the plasma membrane and also revealed alterations in the shape of the parasites.

**Conclusions:** Our results describe the synergic anti-proliferative effect of LPAs and ketoconazole against epimastigotes and intracellular amastigotes and suggest that in epimastigotes, plasma membrane, reservosomes and mitochondria are targets of these drugs, possibly by interference with lipid metabolism.

Keywords: *T. cruzi*, chemotherapy, edelfosine, ilmofosine, miltefosine, synergy

## Introduction

Lysophospholipid analogues (LPAs), designed as potential immunomodulators, have been shown to display anti-tumour activity, and several mechanisms have been proposed for their cytotoxic activity.<sup>1</sup> The most studied analogues are the alkylglycerophosphocholine, edelfosine, the thioether substituted phosphatidylcholine analogue, ilmofosine and the alkylphosphocholine, miltefosine.

There is an urgent need for more efficient and safe drugs for the treatment of Chagas' disease caused by *Trypanosoma cruzi*.<sup>2</sup> In this context, several studies on the effect of LPAs against *T. cruzi* have been conducted.<sup>3–5</sup> In the present work, we

extended this investigation analysing the effect of the combination of LPAs with ketoconazole on intracellular amastigotes and, at an ultrastructural level on epimastigotes, using the Y strain of the parasite.

## Materials and methods

### Drugs

Edelfosine, an alkylglycerophosphocholine, was purchased from Bachem (UK). Ilmofosine, miltefosine and ketoconazole were purchased from Sigma Chemical Co. (USA).

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*Combined effects of LPAs and ketoconazole on epimastigote and amastigote proliferation*

The Y strain of *Trypanosoma cruzi* was used in all experiments. Epimastigotes, maintained in LIT medium, were harvested during the exponential phase of growth and resuspended in the same medium at  $1 \times 10^7$  cells/mL. An aliquot of 500  $\mu$ L of the suspension was added to the same volume of the drugs, alone or in combination, prepared at twice the desired final concentration in 24-well plates, followed by incubation at 28°C, and daily countings were carried out up to 120 h. Bloodstream trypomastigotes were resuspended to a concentration of  $10 \times 10^6$  cells/mL in Dulbecco's modified Eagle's medium plus 10% fetal calf serum. This suspension (100  $\mu$ L) was added to the same volume of the drugs, alone or in equimolar combinations (8–70  $\mu$ M), previously prepared at twice the desired final concentrations in 96-well plates, followed by incubation at 4°C and counting after 24 h. The effect on intracellular amastigotes was analysed after infection of Vero cells with trypomastigotes for 2 h, followed by treatment with the LPAs for 96 h, as described previously.<sup>6</sup>

*Synergy calculations*

Classical isobolograms were constructed by plotting drug concentrations that either alone or in combination inhibited by 50% the proliferation of epimastigotes and intracellular amastigotes (IC<sub>50</sub>). The fractional inhibitory concentration index (FIC) was calculated as described by Hallander *et al.*<sup>7</sup>

*Ultrastructural analysis*

Culture epimastigotes ( $5 \times 10^6$  cells/mL in LIT medium) were treated for up to 120 h at 28°C with combinations of edelfosine and ketoconazole or with each drug alone.

The parasites were fixed for 60 min at 4°C with 2.5% glutaraldehyde and 2.5 mM CaCl<sub>2</sub> in 0.1 M cacodylate buffer, pH 7.2 and post-fixed for 1 h at 4°C with 1% OsO<sub>4</sub>, 0.8% potassium ferricyanide and 2.5 mM CaCl<sub>2</sub> in this same buffer. Thereafter, they were routinely processed for transmission electron microscopy (TEM) and examined in a Zeiss EM10C electron microscope (Oberkochen, Germany).

For scanning electron microscopy (SEM), epimastigotes were washed with PBS, adhered to poly-L-lysine coated coverslips, and fixed for 30 min at room temperature with 2.5% glutaraldehyde in 0.1 M cacodylate buffer. After post-fixation for 30 min at room temperature with 1% OsO<sub>4</sub>, the coverslips were dehydrated in an ascending acetone series, critical-point dried with CO<sub>2</sub>, mounted with silver cellulotape on aluminium stubs and coated with a 20 nm thickness gold layer. The samples were then examined in a Zeiss DSM940 scanning electron microscope (Oberkochen, Germany).

**Results**

Combination of sub-lethal equimolar concentrations (0.1  $\mu$ M) of edelfosine with ketoconazole gave  $44.3 \pm 2.2\%$  inhibition of epimastigote proliferation after 72 h, and the corresponding FIC value was  $0.23 \pm 0.03$ . For trypomastigotes, each drug at 35  $\mu$ M led to about 15% lysis, while using the drugs at the same equimolar concentration, this percentage increased to 29%. Against intracellular amastigotes, combinations of LPAs with ketoconazole were also synergic, presenting concave isobolograms, with FIC values of  $0.05 \pm 0.04$ ,  $0.45 \pm 0.04$  and  $0.45 \pm 0.05$  after

96 h of treatment with edelfosine, ilmofosine and miltefosine, respectively.

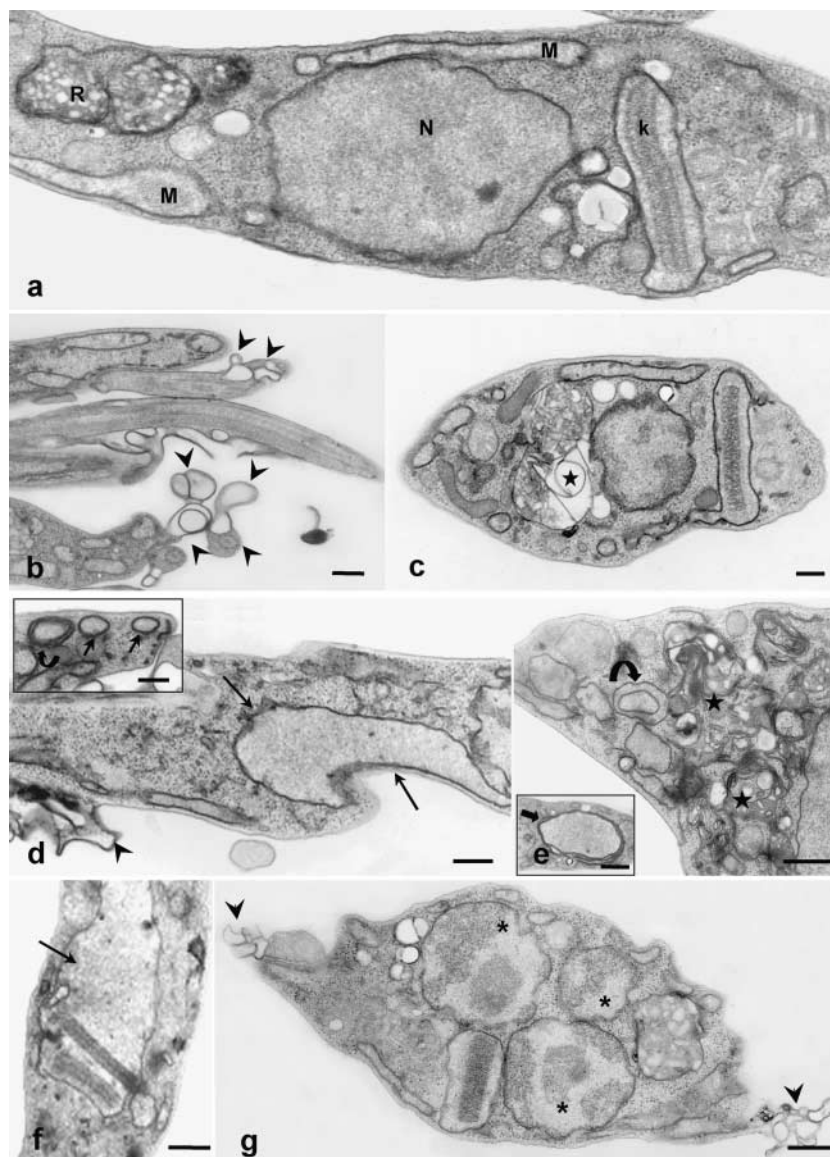
Treatment of epimastigotes for 48 h with 0.3  $\mu$ M edelfosine (Figure 1b) or 0.1  $\mu$ M ketoconazole (Figure 1c) caused alterations in reservosomes and plasma membrane. The combination of 0.1  $\mu$ M edelfosine with 0.1  $\mu$ M ketoconazole, besides similar alterations, induced mitochondrial swelling, with a decrease in electron density of the mitochondrial matrix (Figure 1d). Treatment with 0.3  $\mu$ M edelfosine plus 0.03  $\mu$ M ketoconazole led to the appearance of membranous structures inside the mitochondrion (inset, Figure 1d). Increasing the time of treatment to 120 h, parasites treated with each drug alone presented more intense damage of reservosomes (data not shown), and 0.1  $\mu$ M edelfosine + 0.1  $\mu$ M ketoconazole induced swelling of the mitochondrion, which contained concentric membranous structures and the appearance of membrane arrangements in the cytoplasm resembling autophagic structures (Figure 1e and inset). Treatment with 0.3  $\mu$ M edelfosine + 0.03  $\mu$ M ketoconazole for 120 h led to alterations similar to those observed with their equimolar combination (0.1  $\mu$ M), and also the formation of multinucleated parasites (Figure 1f and g).

Analysis by SEM of epimastigotes treated for 48 h with 0.3  $\mu$ M edelfosine showed the formation of large blebs at the plasma membrane (Figure 2b), and with 0.1  $\mu$ M ketoconazole showed alterations in the parasite morphology (Figure 2c). Combination of 0.3  $\mu$ M edelfosine with 0.03  $\mu$ M ketoconazole led to both formation of blebs and alterations in the body shape (Figure 2d and e). After 120 h of treatment, each drug alone (Figure 2g and h) or in combination (Figure 2i) induced more pronounced alterations in the morphology of the parasite, including flattened parasites and detachment of the flagellum from the cell body.

**Discussion**

In this work, we showed that when LPAs are combined with ketoconazole, synergic effects are produced against epimastigotes and intracellular amastigotes (Y strain), a fact probably related to the effects of both types of compound on the sterol and phospholipid composition of the parasites. In epimastigotes, LPAs are potent inhibitors of phosphatidylcholine synthesis and edelfosine leads to replacement of ergosterol and its 24-ethyl derivative by their  $\Delta^{22}$ -saturated analogues.<sup>5</sup> Equimolar combinations of edelfosine and ketoconazole increased the lytic activity against trypomastigotes in comparison with each drug alone.

For the ultrastructural analysis of the synergic effect, two combinations of edelfosine with ketoconazole were used—0.1  $\mu$ M+0.1  $\mu$ M and 0.3  $\mu$ M+0.03  $\mu$ M—based on previous results with the EP strain.<sup>5</sup> Treatment of epimastigotes with edelfosine, ketoconazole, or combinations, led to alterations in the plasma membrane and a progressive alteration of the reservosomes. Reservosomes are acidic storage compartments in epimastigotes which are consumed during the metacyclogenesis process.<sup>8</sup> Sub-fractionation of this organelle revealed, besides the presence of cruzipain, large amounts of ergosterol and esterified cholesterol, and as the major membrane components, phosphatidylcholine and phosphatidylethanolamine.<sup>9</sup> Since this organelle is a storage site, alteration of its lipid content could induce alterations in its composition and structure, as already reported after treatment with edelfosine<sup>5</sup> and ketoconazole.<sup>10</sup>



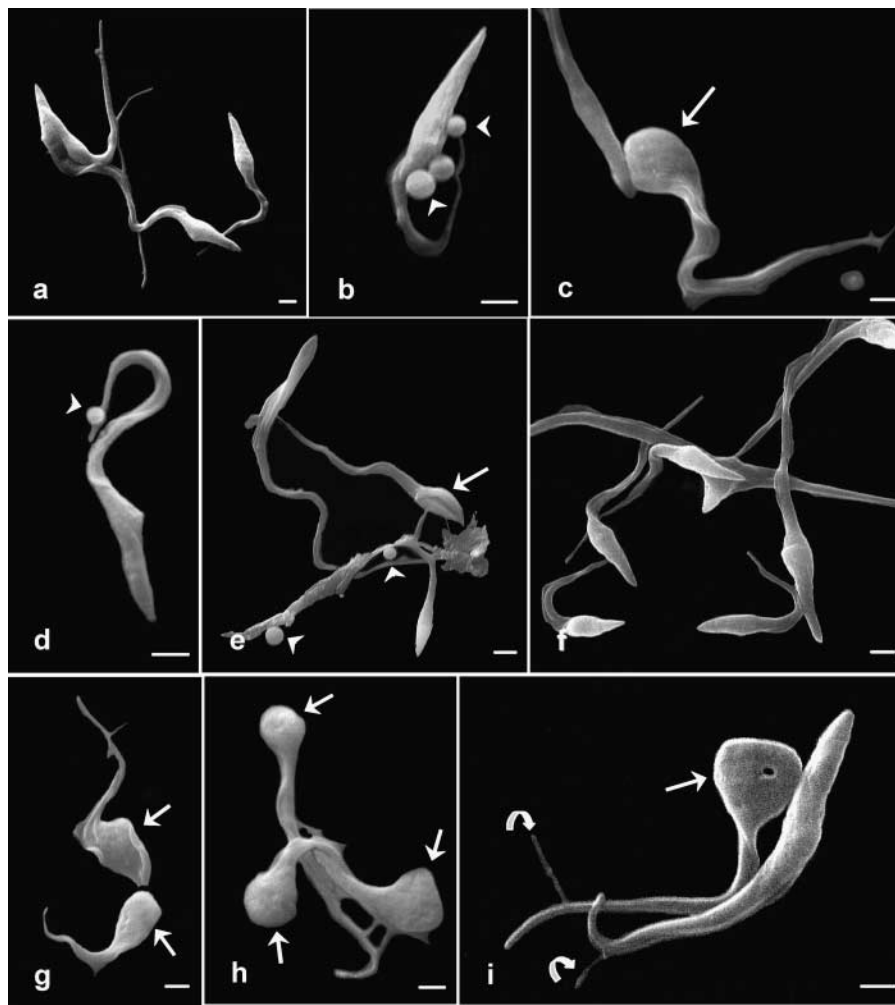
**Figure 1.** Transmission electron microscopy of *T. cruzi* epimastigotes treated with edelfosine (ET-18), ketoconazole (KET) or combinations of both: (a) control parasite showing the kinetoplast (k), mitochondrion (M), nucleus (N) and reservosome (R); (b) treatment with  $0.3 \mu\text{M}$  ET-18/48 h or (c)  $0.1 \mu\text{M}$  KET/48 h caused alterations both in reservosomes (star) and in the plasma membrane (arrowheads); (d)  $0.1 \mu\text{M}$  ET-18+ $0.1 \mu\text{M}$  KET/48 h induced alterations in the membrane (arrowheads), mitochondrial swelling (arrows), inset:  $0.3 \mu\text{M}$  ET-18+ $0.03 \mu\text{M}$  KET/48 h showing also alterations in the mitochondrion (arrows) with formation of membranous structures inside the organelle (curved arrow); (e)  $0.1 \mu\text{M}$  ET-18+ $0.1 \mu\text{M}$  KET/120 h induced extensive alterations in the reservosomes (star) and presence of membranous structures inside the mitochondrion (curved arrow); inset: same combination of drugs led to formation of autophagic vacuoles (large arrows); (f and g)  $0.3 \mu\text{M}$  ET-18+ $0.03 \mu\text{M}$  KET induced alterations in the mitochondrion (arrow), plasma membrane (arrowheads) and the appearance of multinucleated parasites (asterisks). Bars =  $0.25 \mu\text{m}$ .

Equimolar amounts of ketoconazole plus terbinafine ( $1 \mu\text{M}$ ) induce mitochondrial alterations in epimastigotes much earlier than the azole alone.<sup>10</sup> In our experiments, edelfosine or ketoconazole induced marked alterations in the parasite, mainly at the plasma membrane and reservosomes, but their combination also caused mitochondrial damage, including the presence of membranous structures inside the organelle. SEM confirmed that these drugs, alone or combined, induced alterations in the plasma membrane detected by TEM, and revealed progressive changes in the shape of the parasites.

Swelling of the mitochondrion, with decreased electron density in the matrix, in parasites treated with edelfosine and

ketoconazole has been already described when *T. cruzi* were incubated with ICI-195,739 or 22,26-azasterol, which are known sterol biosynthesis inhibitors (SBIs).<sup>11,12</sup> The formation of concentric membranous structures inside the mitochondrion, observed with edelfosine plus ketoconazole, has been previously described after treatment with SBIs.<sup>13</sup> It has been reported that exogenous ergosterol, but not cholesterol, reversed the effect of ketoconazole against epimastigotes.<sup>11</sup> The present data reinforce the concept that sterols are essential in trypanosomatids for the maintenance of the structural organization of the mitochondrion.

Combined treatment of epimastigotes for 120 h resulted in the formation of autophagic vacuoles and multinucleation.



**Figure 2.** Scanning electron microscopy of *T. cruzi* epimastigotes treated with edelfosine (ET-18), ketoconazole (KET) or combinations of both: (a) control parasites (48 h), showing the elongated body and the anterior flagellum; (b) treatment with 0.3  $\mu\text{M}$  ET-18/48 h induced the formation of blebs at the plasma membrane (arrowheads); (c) 0.1  $\mu\text{M}$  KET/48 h led to alteration in the shape (arrow); (d and e) 0.3  $\mu\text{M}$  ET-18+0.03  $\mu\text{M}$  KET/48 h led both to formation of blebs (arrowheads), as well as rounding of the parasite body (arrow); (f) control parasites (120 h), showing the elongated body and the anterior flagellum; (g) treatment for 120 h with 0.3  $\mu\text{M}$  ET-18, (h) 0.1  $\mu\text{M}$  KET and (i) 0.3  $\mu\text{M}$  ET-18+0.03  $\mu\text{M}$  KET led to parasites with abnormal shape (arrows) and detachment of the flagella from the cell body (curved arrows). Bars = 1  $\mu\text{m}$ .

The appearance of autophagic structures is probably due to recycling of abnormal membranes during the processing of damaged organelles. In mammalian tumour lineages treated with edelfosine, multinucleation has been reported,<sup>14</sup> and ICI 195,739 induced blockage of cytokinesis in *T. cruzi*.<sup>11</sup> In this context, the anti-proliferative effect of edelfosine and/or SBIs could also be associated with interruption of cell division at the cytokinesis stage.

In conclusion, our present results demonstrate that edelfosine and ketoconazole induce alterations in the plasma membrane, reservosomes and mitochondrion of *T. cruzi*, indicating that these organelles are potential targets of both drugs, probably through interference with lipid metabolism. It has been previously reported that oral treatment of *T. cruzi*-infected mice in the acute phase with edelfosine was ineffective,<sup>5</sup> and that combination of ketoconazole with terbinafine<sup>15</sup> led to reduction in the doses of each drug. In view of our results, together with data from the literature, further experiments will be carried out *in vivo* with edelfosine and ketoconazole, aiming at an effective scheme of treatment of *T. cruzi* infection.

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