Effect of the lysophospholipid analogues edelfosine, ilmofosine and miltefosine against Leishmania amazonensis

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Objectives: Analysis of the effect of edelfosine, ilmofosine and miltefosine on Leishmania amazonensis and of potential targets of these lysophospholipid analogues.

Methods: Quantification and ultrastructural analysis of the effect of lysophospholipid analogues on promastigote forms and on infected peritoneal macrophages, and flow cytometry analysis of treated promastigotes labelled with propidium iodide and rhodamine 123 (Rh123).

Results: The lysophospholipid analogues presented potent antiproliferative activity with IC50/3 days of 1.9–3.4 μM for promastigotes and 4.2–9.0 μM for intracellular amastigotes. Treatment with these analogues in Schneider medium for 1 day led to a dose-dependent decrease in Rh123 fluorescence, an effect more accentuated in edelfosine-treated parasites, suggesting interference with the potential of the mitochondrial membrane. In both forms of L. amazonensis, edelfosine induced extensive mitochondrial damage, multinucleation and, in promastigotes, also led to plasma membrane alterations, formation of autophagic structures and membranous arrangements inside the flagellar pocket.

Conclusions: The alkylglycerophosphocholines edelfosine and ilmofosine were more active than the alkylphosphocholine miltefosine against promastigotes and intracellular amastigotes of L. amazonensis, and ultrastructural and flow cytometry data indicate the mitochondrion as a target of edelfosine.

Keywords: L. amazonensis, alkylglycerophosphocholines, flow cytometry, chemotherapy, electron microscopy

Introduction

Leishmania amazonensis is a species of the New World, which causes cutaneous leishmaniasis and, as a second pathology, a diffuse cutaneous form of the disease.1 Leishmaniasis in its different forms is endemic in 88 countries of tropical and subtropical regions, affecting more than 12 million people.2 Moreover, its clinical treatment is inadequate and expensive.3,4 Lysophospholipid analogues, such as edelfosine, ilmofosine and miltefosine, have been extensively studied as agents for the chemotherapy of cancer and of diseases caused by pathogenic trypanosomatids.5,6 As far as we know, there is only one report dealing with the activity of edelfosine and miltefosine against two of the New World species, Leishmania mexicana and Leishmania panamensis.7 Most experimental studies with lysophospholipid analogues have been performed with Old World Leishmania species,8–11 leading clinical studies12 to approve miltefosine for use in India for the treatment of visceral leishmaniasis caused by Leishmania donovani. In this context, we have investigated the activity of these analogues against L. amazonensis, comparing

the effect of three analogues against promastigotes and intracellular amastigotes, and investigating potential targets in the parasite using the techniques of electron microscopy and flow cytometry.

Materials and methods

Parasites and drugs

L. amazonensis (strain MHOM/BR/77/LTB0016) amastigote forms were obtained from lesions of infected Swiss mice.13 The parasites were added to Schneider medium supplemented with 10% fetal calf serum (FCS), 100 IU penicillin, 100 μg/mL streptomycin and 1 mM L-glutamine, pH 7.2, at 25°C, leading to differentiation to promastigotes, which were maintained for up to four passages in this same medium.14

Edelfosine (1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphocholine) was obtained from Bachem Ltd (St Helens, UK); ilmofosine (1-hexadecylthio-2-methoxymethyl-rac-glycero-3-phosphocholine) and miltefosine (hexadecylphosphocholine) were from...
Sigma Chemical Co. (St Louis, MO, USA). Stock solutions of the lysophospholipid analogues were prepared in phosphate buffered saline (PBS), pH 7.2.

**Effect of the lysophospholipid analogues on the proliferation of *L. amazonensis***

Culture promastigotes were resuspended in Schneider medium (5×10^6 cells/mL) and plated in 24-well plates at 25°C, in the absence or presence of different concentrations of the analogues. Cell counts were performed up to 3 days of treatment and the corresponding IC_{50} value was calculated. This parameter expresses the concentration of each analogue that leads to 50% inhibition of parasite proliferation, and was obtained from at least three experiments and expressed as mean±S.D. After 1 day of treatment with edelfosine, the parasites were processed for transmission and scanning electron microscopy (TEM and SEM, respectively).

**Effect of the lysophospholipid analogues on *L. amazonensis*-infected macrophages***

Mouse peritoneal macrophages were infected with promastigotes at a ratio of 10:1 parasites/host cell. After interaction for 1 h, non-internalized parasites were removed by washing with Dulbecco’s modified Eagle’s medium plus 10% FCS. Fresh medium, with or without the analogues, was added to the cultures and changed every 2 days. After 1–3 days of treatment, the cultures were fixed, stained with Giemsa and the percentage of infection and the number of intracellular parasites quantified. Alternatively, the cultures were processed for TEM.

**Ultrastructural analysis***

Promastigotes or infected cell cultures treated with edelfosine and the corresponding controls were washed with PBS and processed for electron microscopy. For TEM, the parasites were adhered to poly-L-lysine-coated coverslips, fixed with 2.5% glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.2) at room temperature for 40 min and post-fixed with 1% OsO₄ in the same buffer for 30 min. The cells were dehydrated in an ascending acetone series, dried by the critical point method with CO₂, mounted on silver Cellotape on aluminium stubs, coated with a 20 nm thick layer of gold and examined in a 940 DSM Zeiss microscope (Oberkochen, Germany). For TEM, after washing in PBS, the parasites or macrophage cultures were fixed with 2.5% glutaraldehyde and 2.5 mM CaCl₂ in a cacodylate buffer for 1 h at 4°C, post-fixed in 1% OsO₄, 0.8% potassium ferricyanide and 2.5 mM CaCl₂ in a 0.1 M cacodylate buffer for 1 h, dehydrated as described above and embedded in Polybed 812 resin. Ultrathin sections (Leica Ultracuts, UCT, Vienna, Austria) were stained with uranyl acetate and lead citrate and then examined in an EM10C Zeiss microscope.

**Flow cytometry analysis***

Promastigotes (5×10^6 cells/mL) were treated with the lysophospholipid analogues at 25°C for 10 min in PBS or else for 1–3 days in Schneider medium. Thereafter, 500 μL aliquots of the cultures were added to the same volume of a solution containing 20 μg/mL propidium iodide (PI) plus 12 μg/mL rhodamine 123 (Rh123) and then incubated at 25°C for 20 min. The material was kept on ice until analysis. Data acquisition and analysis were performed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with Cell Quest software (Joseph Trotter, Scripps Research Institute, San Diego, CA, USA). A total of 10,000 events were acquired in the region previously established as that corresponding to the parasites. Alterations in the fluorescence for Rh123 were quantified using an index of variation (IV) obtained by the equation [(MT–MC)/MC], where MT is the median of fluorescence for treated parasites and MC that for control parasites. Negative IV values correspond to a depolarization of the mitochondrial membrane.

**Results***

Treatment of the parasites with all three lysophospholipid analogues resulted in a dose-dependent inhibition effect on the proliferation of *L. amazonensis* (Figures 1 and 2, and Table 1). The IC_{50}/3 days for intracellular amastigotes was 4.2±0.7, 4.5±0.3 and 9.0±0.2 μM for edelfosine, ilmofosine and miltefosine, respectively. Concentrations up to 60 μM of the three analogues caused no damage to murine peritoneal macrophages.

To investigate which organelles were potentially the initial targets of edelfosine, TEM and SEM techniques were employed.

**Figure 1.** Effect of lysophospholipid analogues on the proliferation of *L. amazonensis* promastigotes: (a) edelfosine; (b) ilmofosine; (c) miltefosine.
Based on the IC₅₀ values for 1 day of treatment, promastigote forms were treated with this analogue. Whereas control parasites presented their characteristic organization (Figure 3a), treatment with 1–6 mM edelfosine for 1 day led to multinucleation (Figure 3b), formation of autophagic structures (Figure 3c), blebbing and ruffling of the flagellar and body membranes (Figure 3d), and to the appearance of a large number of vesicles and myelin-like figures inside the flagellar pocket (Figure 3d and e). The mitochondria suffered several alterations, with swelling of the organelle, disorganization of the inner membrane, reduction of the electron density of the matrix and the presence of concentric membranous arrangements (Figure 3b, g and h). Treated promastigotes also presented damaged mitochondrion partially surrounded by an endoplasmic reticulum profile (Figure 3f). Shortening of both the flagellum and the cell body were observed by SEM, when compared with untreated parasites (Figure 3i and j).

Ultrastructural analysis of L. amazonensis-infected macrophages treated with 10 or 20 mM edelfosine up to 3 days showed several alterations in intracellular amastigotes, when compared with untreated cultures (Figure 4a–c). The parasites presented a large number of vesicles in the cytoplasm and inside the flagellar pocket (Figure 4d and e), as well as swelling of the mitochondrion with loss of the inner membrane organization (Figure 4f). The parasitophorous vacuole presented intense membrane vesiculation (Figure 4d), membranous structures and parasite debris (Figure 4d, e and g). Multinucleated parasites were found inside the host cell (Figure 4g). These alterations were progressive with time of treatment and drug concentration, leading to disintegration of organelles and to the appearance of parasite ghosts (data not shown). Treatment with edelfosine did not affect the kinetoplast, the Golgi complex or the subpellicular microtubules of both promastigotes and intracellular amastigotes.

Labelling of treated promastigotes with PI and Rh123 was performed to further evaluate damages at, respectively, the membrane and mitochondrion. Incubation of promastigotes with 0.5–10 mM edelfosine in PBS for 10 min caused a dose-dependent permeabilization of the plasma membrane, as monitored by PI (Figure 5a). The percentage of PI-labelled parasites after treatment with 10 mM edelfosine was 51.2%, and the addition of 10% FCS to PBS prevented this effect, even at a four-fold higher concentration of edelfosine (data not shown). Treatment of the parasites with the lysophospholipid analogues in Schneider medium for 1 day led to no important permeabilization of the plasma membrane, whereas a dose-dependent decrease in Rh123 fluorescence was detected (Table 2); this effect was more accentuated for edelfosine (Figure 5b).

**Discussion**

The three lysophospholipid analogues inhibited the proliferation of promastigotes and intracellular amastigotes of L. amazonensis; edelfosine and ilmofosine (alkylglycerophosphocholines) were more active than the alkylphosphocholine miltefosine. The IC₅₀ values for intracellular parasites were in the range 4.2–9.0 μM after 3 days, whereas macrophages were affected.
Figure 3. Ultrastructural alterations in L. amazonensis promastigotes treated with edelfosine for 1 day: (a) control parasite, note the elongated body and the normal aspect of the intracellular organelles (k, kinetoplast; F, flagellum; FP, flagellar pocket; M, mitochondrion; N, nucleus); (b–d) 1 μM induced multinucleation (N), mitochondrion swelling with membranous structures inside the organelle (small arrow), formation of autophagic structures (asterisk) and of vesicles inside the flagellar pocket (arrows), and alterations at the plasma membrane (arrowheads); (e and f) 3 μM induced formation of myelin-like figures inside the flagellar pocket (arrow) and partial surrounding of a damaged mitochondrion by an endoplasmic reticulum profile (star); (g and h) 6 μM led to alterations in the mitochondria (small arrows); (i) control parasites observed by SEM with elongated body and emerging flagellum; (j) 6 μM caused shortening of the parasite body (white arrows) and of the flagellum. Bars: 0.25 μm (a–g) and 1 μm (h–j).
only at concentrations above 60 μM. Comparison with other New World *Leishmania* species showed that promastigotes of *L. amazonensis* presented susceptibility to miltefosine and edelfosine similar to that of *L. panamensis* and about two-fold higher than that of *L. mexicana.*

In promastigotes of *L. amazonensis*, edelfosine induced alterations at the plasma membrane and formation of autophagic structures, which could have been the result of recycling of abnormal membranes during the processing of damaged organelles, as described in suramin-treated *Trypanosoma rhodesiense.*

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**Figure 4.** Ultrastructural alterations in *L. amazonensis* amastigotes interiorized in peritoneal macrophages, after treatment of the infected cultures with edelfosine for different times: (a,b,c) control cultures after, respectively, 1, 2 and 3 days of infection with parasites inside parasitophorous vacuoles (PV) (NM, nucleus of the macrophage); (d) 10 μM/1 day induced vacuolization of the parasite’s cytoplasm (small arrows), intense vesiculation of the PV’s membrane (arrowheads) and formation of membranous structures (arrows) inside this organelle; (e) 20 μM/2 days induced in the parasite vacuolization of the cytoplasm (small arrow) and inside the flagellar pocket (curved arrow), and also formation of membranous structures (arrows) inside PV; (f) 10 μM/3 days led to alterations in the mitochondrion of the parasite with formation of membranous structures (white arrow); (g) 20 μM/3 days induced the formation of a large number of membranous bodies into PV (arrows), which encloses trinucleated parasites (N). Bars: 1 μm (a–c) and 0.25 μm (d–g).
Blebbing and ruffling of the plasma membrane have been described in *Trypanosoma cruzi* and HL-60 leukaemic cells after treatment with edelfosine, in similar experimental conditions. Since ultrastructural analysis demonstrated that treatment with lysophospholipid analogues induced alterations at the membrane and mitochondrion of promastigotes, we incubated treated parasites with PI and Rh123. Treatment with edelfosine at concentrations above 1 µM in PBS for 10 min led to a gradual increase in membrane permeabilization, whereas the addition of 10% FCS prevented such an effect, even at 40-fold higher concentrations. This ‘protective effect’ of serum has been reported in experiments with tumour cells and *T. cruzi* and is associated with a decrease in free analogue, due to its interaction with serum components. The decrease in Rh123 fluorescence after treatment of promastigotes in Schneider medium for 1 day with the analogues, especially edelfosine suggests interference with the hydrogenionic potential of the mitochondrial membrane, as reported in paromomycin-treated *L. donovani*. The reduced retention of Rh123 is not due to secondary plasma membrane permeabilization; since in our experiments the percentage of PI-labelled parasites after treatment was similar to that of untreated ones. These results are in agreement with the extensive damage of the parasite mitochondrion, as detected by TEM.

The multinucleation observed in promastigotes and amastigotes of *L. amazonensis* has been reported in mammalian tumour lineages treated with edelfosine, in which supplementation with phosphatidylcholine precursors partially reverted the observed polyploidy induced by blockage of cytokinesis. The mitochondrial damage, with membranous arrangements inside this organelle and in the flagellar pocket, and the formation of autophagic structures observed in edelfosine-treated *L. amazonensis* have also been described when this parasite or *T. cruzi* were treated with inhibitors of sterol synthesis. Such alterations are associated with depletion of ergosterol and alteration of the physical properties of the membranes. We have previously shown that lysophospholipid analogues inhibit the proliferation and the synthesis of phospholipids and sterols in *T. cruzi*. Further experiments on lipid characterization and quantification of treated *L. amazonensis* are needed in order to ascertain if a similar mode of action is also operative in this trypanosomatid, with the morphological alterations in membranous systems linked with interference in the biosynthesis of lipids.

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**References**


