Leishmanicidal Activity of Edelfosine, Miltefosine and Ilmofosine

Samira Azzouz, Mimoun Maache, Ramon Gil Garcia and Antonio Osuna

Institute of Biotechnology, Department of Parasitology, Faculty of Sciences Campus, Fuentenueva
C.P. 18071, Granada, Spain

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Abstract: The anti-proliferative action of three alkyl-lysophospholipid derivatives, edelfosine (ET-OCH), miltefosine (Hexadecylphosphocholine), and ilmofosine (BM 14.440) has been studied on the promastigotes and amastigotes of Leishmania donovani. The effect of the three drugs has previously been studied, but the action mode was not clearly elucidated. In this study the effect on the intracellular amastigote forms was evaluated by two different methods: the traditional method, counting the amastigotes within the macrophages stained with Giemsa; and by a new method, staining the nuclear macrophages and amastigotes with ethidium bromide and counting the different population by flow cytometry. This new method, based on the flow cytometry, shows an advantage for evaluating the anti-proliferative effects in intracellular parasites. The ED50 were calculated for the drug activity after 72 hr, and for the three alkyl-lysophospholipid derivatives it were in the range of 26.73–33.31 µM against promastigotes and in the range of 16.46–23.16 against amastigotes. Also, studying the effect against macrophages J774A1, the ED50 were in the range of 24.28–26.38 µM. The effect of the alkyl-lysophospholipids in the macromolecular biosynthesis of the Leishmania donovani, was studied comparing the incorporation of labelled analogues ([3H] thymidine, [3H] uridine and [3H] leucine), respectively, in the DNA, RNA, and proteins of the flagellates treated. Miltefosine was the most active of the alkyl-lysophospholipids, especially in the inhibition of the RNA synthesis. The three compounds studied show high in vitro activity against L. donovani promastigotes and amastigotes.

The World Health Organization considers leishmaniasis one of the most serious diseases caused by protozoan parasites, with approximately 12 million cases in the world (http://www.who.int/emc/diseases/leish/index.html). The clinical manifestations of this parasitosis vary depending on the etiological agent (Herwaldt 1999). Visceral leishmaniasis, caused by L. donovani, L. infantum and L. chagasi, with the most characteristic symptoms, hepato- and splenomegalies, can cause 90% mortality when untreated.

At present, this disease is treated with pentavalent antimony and/or pentamidine salts (Manson-Bahr 1982), as well as with the antifungal drug amphotericin B, in patients who show resistance to antimonal treatments. However, although these treatments can be highly effective against the disease, the drawbacks include high toxicity and administration difficulties (Pearson & Sousa 1985). One major challenge of current research in the field of leishmaniasis is the development of new, effective molecules for chemotherapy. A new compound, derived from alkyl-lysophospholipids, has demonstrated its efficacy against leishmaniasis (Achterberg & Gerchen 1987; Croft et al. 1987, 1993 & 1996). Miltefosine was recently registered for the oral treatment of visceral leishmaniasis in India after successful clinical trials (Sundar et al. 2000) and has also been used in treating cutaneous leishmaniasis (Soto et al. 2001).

The present work provides an analysis of the action mode and the activity of three alkyl-lysophospholipid derivatives: edelfosine, and ilmofosine analogues to ether-lipids (Herrman & Bicher 1988; Kuhlencord et al. 1992), and the alkyl-phosphocholine derivative miltefosine (Daniel 1993), against Leishmania donovani.

Materials and Methods

Parasite. The strain of L. donovani used in this study was LCR-133 (Leishmania Reference Center, Jerusalem, Israel), isolated in 1967 from a human case of Kala-azar in Behenber (Ethiopia), and maintained in our laboratory since 1982 by successive passages in cultures of NNN medium, modified with a liquid phase in minimal essential medium (MEM) plus 10% of inactivated foetal bovine serum, kept in a moist air atmosphere at 28°.

Compounds tested. The compounds studied in the present work were three ether lipids, alkyl-lysophospholipid derivatives, with effective use in cancer therapy. The edelfosine and miltefosine were from Sigma Chemical Co., ilmofosine are from Dr. Simon Croft (Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London WC1E 7HT, UK). The chemical formulas and compositional structures of the three compounds are:

1- 1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphorylcholine.
Activities of edelfosine, miltefosine and ilmofosine against *Leishmania donovani* promastigotes and amastigotes, and against macrophages.

<table>
<thead>
<tr>
<th></th>
<th>Edelfosine</th>
<th>Miltefosine</th>
<th>Ilmofosine</th>
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<tr>
<td>Promastigotes</td>
<td>32.31 (29.11–35.51)</td>
<td>26.73 (23.28–30.19)</td>
<td>31.31 (27.42–35.21)</td>
</tr>
<tr>
<td></td>
<td>19.47b (18.33–20.61)c</td>
<td>16.46b (14.66–18.27)b</td>
<td>17.67b (16.76–18.59)b</td>
</tr>
</tbody>
</table>

ED50 values in micromolar with P95 confidence limits.

a Values obtained by flow cytometry.

b Values obtained by optic microscope.

Values of two independent experiments are given with 95% confidence intervals in brackets.
radioactive analogue at a 5 μCi/ml dose. At 0, 45, 75 and 145 min., the cultures were centrifuged, and the pellet, after three washes with cold medium, was precipitated with 10% trichloroacetic acid (TCA, Probus) for 2 hr at 4°C. Precipitates were filtered through Whatman GF/c filters, and washed first with TCA at 5%, and then with ethanol at 70%, and dried at 60°C under infrared lamps. Once dried, the filters were submerged in scintillation cocktail (2,5-diphenyloxazole [PPO], 4g; 1,4-bis(5-phenyloxazolyl)benzene [POPOP] 0.1 g; toluene 1,000 ml). The radioactivity incorporated was measured in a Liquid Scintillation System model LS 6000TA (Beckman). Radioactivity contained in the precipitable material was noted as a reflection of the radioactivity present in and incorporated by the precipitable material of the parasites. Inhibition of synthesis was determined by comparison to untreated controls.

Statistical analysis. ED50 values were calculated by a nonlinear regression to sigmoidal curves with the use of the Prism program (Graph Pad Software, Berkeley, CA, USA).

Values are expressed as the mean±S.D. The significance of the difference from the respective controls for each experimental test condition was assayed by using One-way Analysis of Variance (ANOVA), and Bonferroni post test was employed. Statistical differences having p<0.05 were considered to be significant.

Results

Antiproliferative effects.

In the 72 hr assays edelfosine, miltefosine and ilmofosine were more active against intracellular amastigotes than against extracellular promastigotes. The range of ED50 values for edelfosine, miltefosine and ilmofosine against promastigotes was respectively 32.66, 27.33 and 31.33 μM. Against intracellular amastigotes, the ED50 values for edelfosine, miltefosine and ilmofosine were respectively 23, 17 and 21 μM for the flow citometry method, and 19,16.66 and 17 μM for the microscopic method (table 1).

Effects of alkyl-lysophospholipids on macromolecule synthesis.

Fig. 1, 2 and 3 present the data for incorporation of thymidine, uridine and leucine in the precipitable material after incubation of the flagellates treated with the radioactive analogues for 145 min. The data referring to the control cultures show, in all cases, increased numbers of CPM, revealing an active synthesis of DNA, RNA and proteins.

The three compounds lowered the level of incorporation of the radiolabelled thymidine, uridine and leucine with respect to control. After 135 min. of incubation with the alkyl-lysophospholipids, the inhibition is more pronounced in RNA synthesis than in DNA and protein synthesis (table 2).
Alkyl-lysophospholipids action on the biosynthesis of DNA, RNA and protein.

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>Edelfosine</th>
<th>Miltefosine</th>
<th>Ilmofosine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DNA</td>
<td>RNA</td>
<td>Protein</td>
</tr>
<tr>
<td>0</td>
<td>0.114±0.001</td>
<td>0.084±0.0331</td>
<td>0.009±0.006</td>
</tr>
<tr>
<td>45</td>
<td>0.086±0.012</td>
<td>16.5±0.074</td>
<td>0.025±0.002</td>
</tr>
<tr>
<td>90</td>
<td>41.75±2.548</td>
<td>38.20±2.36</td>
<td>8.89±1.25</td>
</tr>
<tr>
<td>135</td>
<td>57.85±0.695</td>
<td>73.22±1.516</td>
<td>68.02±0.739</td>
</tr>
</tbody>
</table>

The results are expressed in percentage of synthesis inhibition (%IC) calculated by the formula % IC = (Tc/Tp/Tc)×100.

Tc being the CPM calculated in the control tubes, and Tp the CPM corresponding to the different products tested (n=3, ±S.D.).

Discussion

Antiproliferative effects.

According to the results of our study, the three alkyl-lysophospholipids assayed show an in vitro anti-proliferative activity which was very high against promastigotes and intracellular amastigotes of L. donovani. This confirms the results reported by other authors, who have demonstrated the leishmanicidal activity of different alkyl-lysophospholipids (Croft et al. 1987, 1993 & 1996).

The differences in the low activity of the alkyl-lysophospholipids against the promastigotes with respect to the amastigotes can be accounted for by the fact that the alkyl-lysophospholipids strengthen the cytotoxicity of the macrophages and consequently aid in the death of the parasite within the infected cell, either by the direct action of the compounds or by the secondary effect on the host cells (Berdel 1980; Andreesen et al. 1984; Daniel 1993).

It has been demonstrated that ether lysophospholipids stimulate the oxidative burst of peritoneal macrophages (Brachwitz & Vollgraf 1995) and boost glucose consumption in these cells (Hartung 1983). In activated tumour macrophages, increases result in the production of reactive-oxygen metabolites, such as H$_2$O$_2$ and superoxide O$_2^-$ (Hayashi et al. 1985).

The results of ED50 are different from those found by other authors who studied the effect of pentamidine, allopurinol, and amphoterin C on cell growth and in the parasite metabolism. Cell multiplication was studied by these authors by a dynamic analysis of DNA stained with propidium iodide. In another recent work (Di Giorgio et al. 2000), the authors applied cytometry to the detection of Leishmania amastigotes in macrophages, using an anti-leishmania monoclonal antibody to detect the parasite. The advantages of the method are diverse, because the technique has more reliability as microscopic counting while offering substantial time savings. The differences in the percentage of inhibition between the traditional microscopic method and flow cytometry may be due to human error in the former method.
Abdullah et al. (1999) used the flow cytometry technique for the estimation of the percentage of U-937 cells infected with different species of Leishmania. They used different dyes and they compared the results with those of the Giemsa staining method. Abdullah et al. (1999) were interested in the intracellular growth of leishmania within macrophages, while we are interested in detect and count the amastigotes of Leishmania donovani by flow cytometry after the infected treated cells were submitted to the hypotonic solution. Abdullah et al. founded that with the cells stained with PI, it was difficult to discriminate between infected and uninfected cells, PI like ethidium bromide are a DNA-binding fluorochromes. And the results they obtained by FCM analysis could not be reproduced by microscopy. In our case the treated and the infected treated cells, were lised and stabilized and stained before the FCM analysis. In the cytometer we count the nuclei of amastigotes and also those of cells, and we based on the size to differentiate between the cells and the amastigotes nuclei.

FACS-analysis itself is a rapid, accurate and reproducible method of analysis. Therfore, it has the potential to serve as a tool in Leishmania research.

Effects on the biosynthesis of macromolecules. The levels of macromolecular synthesis in L. donovani promastigotes treated with alkyl-lysophospholipids are reflected in fig. 1, 2 and 3. With respect to control, edelfosine depressed DNA synthesis by 57.85%, and RNA synthesis by 73.22%, and by 68.02% the protein synthesis. While miltefosine inhibited RNA synthesis by 96.32%, and DNA and protein synthesis by 73.46% and 83.97% respectively. Meanwhile, ilmofosine inhibited DNA synthesis by 40.32%, RNA synthesis by 73.72%, and protein synthesis by 65.60% (table 2). In the three cases RNA synthesis was first to be inhibited by the ALPs, followed by protein synthesis and lastly DNA synthesis.

Thus, DNA synthesis proved the least affected, implying either that these products do not act directly on DNA replication. Or, that they need more time to exert their activity, given that the incubation time with the drugs was only 135 min. The effectiveness of alkyl-lysophospholipids depends largely on contact time with the cells. Several studies have demonstrated that they are capable of inducing apoptosis in leukaemic cells, but this requires adequate incubation time, which can last days (Herrman & Bicher 1988; Engelmann et al. 1996).

On the other hand, RNA synthesis was the most strongly affected by the ether lipids, leaving protein synthesis in second place. The alkyl-lysophospholipids thus may exert indirect action on RNA, and therefore their activity can be recognized in the inhibition of many enzymes of phospholipid metabolism, and, in some proteins as well as enzymes responsible for the transduction signals, such as protein kinase C (Lux et al. 1996).

The role of alkyl-lysophospholipids in transduction signals previously demonstrated (Croft et al. 1996), and the mechanism of phophorylation/dephosphorylation that acts on these signals, could explain the synthesis of these new proteins (Daniel 1993).

The results in the present paper and by other authors indicate that alkyl-lysophospholipids represent an effective alternative to the treatment of leishmaniasis, showing a complex action mechanism which may be due primarily to direct action on the parasite membranes, the other effects observed being a direct consequence of the aforementioned action.

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References


