Article available at http://www.parasite-journal.org or http://dx.doi.org/10.1051/parasite/2009163215

STUDY OF THE STRESS PROTEINS SECRETED BY *LEISHMANIA DONOVANI* AFTER TREATMENT WITH EDELFOSINE, MITELFOSINE AND ILMOFOSINE, AND MORPHOLOGICAL ALTERATIONS ANALYZED BY ELECTRONIC MICROSCOPY

AZZOUZ S.*, MAACHE M.**, SARCIRON M.E.*, PETAVY A.F.* & OSUNA A.**

Summary:

We studied the stress proteins induced in protozoa *Leishmania donovani* after treatment with edelfosine, miltefosine and ilmofosine. We studied the morphological and structural modifications caused in the promastigote forms of the parasite after treatment with the three alkyl-lysophospholipids (ALPs). A resistant strain of *L. donovani* to miltefosine was obtained and the morphological modifications were observed. The stress proteins induction was studied in promastigote forms and also in amastigote-like forms obtained *in vitro*. The proteins synthesized with the three alkyl-lysophospholipids were compared to those obtained by heat shock. The axenic amastigote forms synthesized a pattern of different proteins for those observed in the promastigote forms. The morphological alterations were observed under electronic microscopy. The membrane and mitochondria were the organs most affected by the three ALPs. We noted an apparition of vacuoles and vesicles in the treated promastigotes. In the resistant strain, we noted myelin bodies in the treated and untreated parasites.

KEY WORDS : *Leishmania donovani*, alkyl-lysophospholipids, amastigote-like forms, stress proteins, morphological alteration, electronic microscopy.

Résumé : ÉTUDE DES PROTÉINES DE STRESS SECRÉTÉES PAR *LEISHMANIA DONOVANI* APRÈS TRAITEMENT PAR EDELFOSINE, MILTEFOSINE ET ILMOFOSINE, ET ANALYSE DES ALTÉRATIONS MORPHOLOGIQUES PAR MICROSCOPIE ÉLECTRONIQUE

Le protozoaire Leishmania donovani, *quand il est traité avec l'edelfosine, la miltefosine et l'ilmofosine, synthétise de nouvelles protéines appelées "protéines de stress". Ces protéines ont fait l'objet de ce travail, en plus de l'étude des modifications morphologiques et structurelles provoquées chez le parasite traité avec ces trois alkyl-lysophospholipides (ALPs). Une souche de* L. donovani *résistante à la miltefosine a été créée et les modifications causées après traitement avec cette molécule ont été comparées à celles de la souche résistante non traitée. Les protéines de stress ont été étudiées chez les formes promastigotes mais aussi chez les formes amastigotes axéniques obtenues* in vitro. *Ces dernières synthétisent des protéines différentes à celles synthétisées par les formes promastigotes. Les protéines synthétisées après traitement avec les ALPs sont comparées à celles synthétisées par "heat shok". Les altérations morphologiques ont été observées par microscopie électronique. La membrane et la mitochondrie sont les organes les plus affectés par les trois produits. On note l'apparition de vésicules et de vacuoles chez les promastigotes traités. Chez la souche résistante, on note la présence de corps myéliniques chez les parasites traités comme chez les parasites non traités.*

MOTS CLÉS : Leishmania donovani, *alkyl-lysophospholipides, forme amastigote axénique, protéine de stress, altération morphologique, microscopie électronique.*

INTRODUCTION

The World Health Organization considers leishmaniasis as one of the most serious diseases caused by protozoan parasites. This parasite can cause cutaneous or visceral lesions, with 0.5 million cases reported annually (http://www.who.int/leishmaniasis/en/). Visceral leishmaniasis (VL) caused by *L. donovani*, *L. infantum* and *L. chagasi*, which when untreated can cause 70,000 deaths per year (Murray, 2005).

* Département de Parasitologie et Mycologie médicale, Université Claude Bernard - Lyon I, 8, av. Rockefeller, 69373 Lyon Cedex 08, France.
** Institute of Biotechnology, Department of Parasitology, Faculty of Sciences, Campus Fuentenueva, CP 18071, Granada, Spain.
Correspondence: Samira Azzouz.
Tel.: + 33 (0) 4 78 77 72 77 – Fax: + 33 (0) 4 78 77 71 58.
E-mail: sazzouz@ugr.es

Treatment of visceral leishmaniasis (VL) has been based for the last 50 years upon pentavalent antimonial drugs. Development of resistance to antimonials and lack of efficacy against VL/HIV co-infections have emphasised the need for new drugs.

New compounds, derived from alkyl-lysophospholipids (ALPs) – edelfosine, miltefosine and ilmofosine – that were originally developed as an anti-cancer drug, have a high activity against all stages of *L. donovani* (Croft *et al.*, 1996). Miltefosine was recently registered for the oral treatment of VL in India following successful clinical trials (Sundar, 2000) and has also been used in treatment of cutaneous leishmaniasis (Soto, 2001).

Although, various mechanisms of action of ALPs against tumour cells have been identified, including inhibition of enzymes of cell signalling pathways and induction of apoptosis (Wieder, 1999). Mechanism(s) of action of miltefosine against *Leishmania* so far identified

include effects on perturbation of the alkyl-lipid metabolism, phospholipid biosynthesis and membranes (Lira, 2001).

Promastigotes and amastigotes morphologically clearly differ (Bates, 1994). The two stages also differ on the basis of bioenergetics, including the utilization of fatty acids (Hart, 1982), enzymes of fatty acid oxidation, glycolytic enzymes and pathways (Castilla, 1995). Unsurprisingly, the susceptibilities of amastigotes and promastigotes to antileishmanial compounds are also different (Neal, 1978).

Aiming at a better understanding of the mechanism of action of ALPs, we studied the proteins synthesized by *L. donovani* when it is treated with edelfosine, miltefosine and ilmofosine. The proteins synthesised by promastigotes treated with the three drugs were compared to those synthesised by axenic amastigote forms of *L. donovani*. Since the principal action of the ALPs begins in the membrane, we studied the morphological and structural alterations caused on the parasite by the compounds. Structural modifications were also studied on a resistant strain to miltefosine of *L. donovani*. Theses modifications were evaluated under electronic microscopy.

MATERIALS AND METHODS

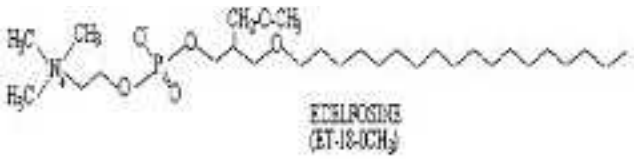
Parasite

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 calf serum (IFCS) (Sigma, France), kept in a moist air atmosphere at 28 °C.

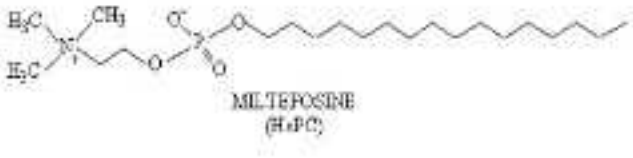
Drugs

The compounds studied in the present work were three ether lipids – ALPs derivatives – with effective use in cancer therapy. The edelfosine and miltefosine were purchased from Sigma Chemical Co; ilmofosine was offered kindly by Dr Simon Croft (Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine). The chemical formulas and compositional structures of the three compounds are:

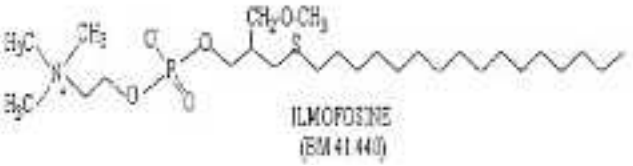
1-octadecyl-2-methyl-sn-glycero-3-phosphocholine



hexadecylphosphocholine



1-hexadecitio-2-methoxymehyl-rac-glycero-3-phosphocholine



Transformation from promastigote to amastigote-like forms

The amastigote-like forms were obtained following the method of Castilla (1995). Briefly, metacyclic promastigote forms in the exponential-growth-phase cultures at 28 °C in TC-199 medium supplemented with 20 % IFCS, with a density of 3×10^6 cell/mL were used, and then were transferred at 37 °C in a moist atmosphere enriched with 5 % CO_2. To obtain the amastigote forms, the culture was incubated in TC-199 medium supplemented with 30 % IFCS and adjusted to pH 6.7. After 72 hours of incubation, 90 % of the forms displayed oval shape without extracellular flagellum.

Obtention and maintaining of the resistant strain of *L. donovani* to miltefosine

Promastigotes of *L. donovani* were cultivated with growing concentrations of hexadecylphosphocholine, in the TC-199 medium supplemented with 30 % of IFCS and stabilized with Hepes buffer. The initial concentration was of 0.019 µM, and once the exponential growth was reached, the hexadecylphosphocholine concentration was incremented until the maximum one of 47.5 µM. This product was previously dissolved in 70 % of ethanol to a final concentration of 10 mg/ml, and stored at - 20 °C for its next use.

Induction of the different types of stress

Both, flagellate forms and amastigotes obtained *in vitro* and cultivated in an amino-acid-free medium were incubated for 120 min with 47.5 µg/ml of the three different ALPs. Logarithmic promastigote cultures (1×10^6 flagellates/mL) obtained at 28 °C in 75 ml flasks (Costar, Cambridge, Mass, USA), (10 ml per culture flask) were incubated for 120 min in a 37 °C water bath, to induce heat shock. Amastigote-like form cultures (10×10^6 parasites/mL), cultivated at 37 °C in 75-ml flat flasks

(10 ml per culture flask), were incubated for 120 min in a 42 °C water bath.

PROTEIN LABELLING AND ELECTROPHORESIS

In order to eliminate the excess of added compounds, after treatment with the three drugs and heat, the promastigotes and axenic amastigotes were washed with Hank's balanced solution by three centrifugations. The protein labelling was performed with 10 µCi/ml of Tran35S-Label [63-68-3] 1132 Ci/mmol (41,88 TBq/mmol) (ICN, Irvine, CA 92717 USA) for two hours of pulse. Then, the parasites were harvested, washed twice in Hank's balanced solution by centrifugation at 16,000 g for 30 sec. at 4 °C. The supernatant was discared and the pellet was suspended in sample buffer (20 % glycerol; 10 % β-mercaptoethanol; 4.6 % SDS; 0.125M Tris HCl pH 6.8 and 0.1 % bromophenol blue). The samples were subjected to two freeze-thaw cycles in liquid nitrogen and heated at 90 °C for 5 min. The protein concentration was determined by the Bio-Rad method and stored at a final concentration of 1 mg/mL. Untreated promastigote and axenic amastigote cultures were used as a control.

The SDS PAGE was performed in denaturing conditions, according to Laemmli by using a 12.5 % acrylamide-bisacrylamide slab gels. Electrophoresis was carried out at 250 V, 10 mA, 3 W, 70 v/h. For all cases, 1 µg of protein was used. After each run, the gels were fixed in TCA for 30 min, washed in distilled water and immersed in Dupon Enhancer for 30 min, and then they were washed twice in distilled water and dried. Protein molecular weights were estimated according to a protein mixture (Pharmacia, France) used as a standard. The gels were autoradiographed by using X-Otomat Kodak film for 72 h at - 80 °C.

ELECTRONIC MICROSCOPY

Promastigotes of *L. donovani* (1×10^7 parasites/mL) were cultivated in TC-199 medium supplemented with 20 % of IFCS, at which previously 38 µM of the edelfosine or miltefosine or ilmofosine had been added. After five days, the parasites were spun to 2,000 g for 10 minutes, and the pellet was washed in PBS and fixed for two hours in cacodylate buffer (0.05 M, pH 7.4) containing p-formaldehyde and glutaraldehyde (in equal shares) 2 % (v/v). Then, it was transferred for eight hours in 0.1 M cacodylate buffer with 2 % of glutaraldehyde. Postfixation was carried out for two hours with 2 % of osmium tetroxide (p/v) and potassium ferrocyanide 2 % (p/v). Once the fixation was completed, the staining of the blocks was performed with 1 % of uranyl acetate solution (p/v) for 30 minutes. Then, the blocks were dehydrated in a growing series of alcohols. The infiltration was carried out in resin Spurr for one hour and the polymerization was done in 12 hours. The courts were mounted on covers copper grids with Formvar and they were stained with uranyl acetate solution and lead citrate.

The grids were observed by using an EM10C ZEISS microscope located in Electron microscopy service (Scientific Center of University of Granada.)

RESULTS

PROTEIN ELECTROPHORESIS AND FLUOROGRAPHS IN PROMASTIGOTE FORMS

Auto-radiography results indicated that, the promastigotes incubation with a sub-toxic concentration of each of three ALPs derivatives at 28 °C induced the synthesis of new proteins. As shown in Figure 1, and in the presence of the ALPs derivatives, the promasigotes synthesized two proteins of low molecular weight of 17 and 12 kDa. This not observed in parasites submitted to a heat shock. An important protein band of approximately 50 kDa and others with relative molecular weights of 125, 112, 94, 90, 27, 20 kDa appeared in all cases, as well as in promastigotes treated with edelfosine, miltefosine, and ilmofo-

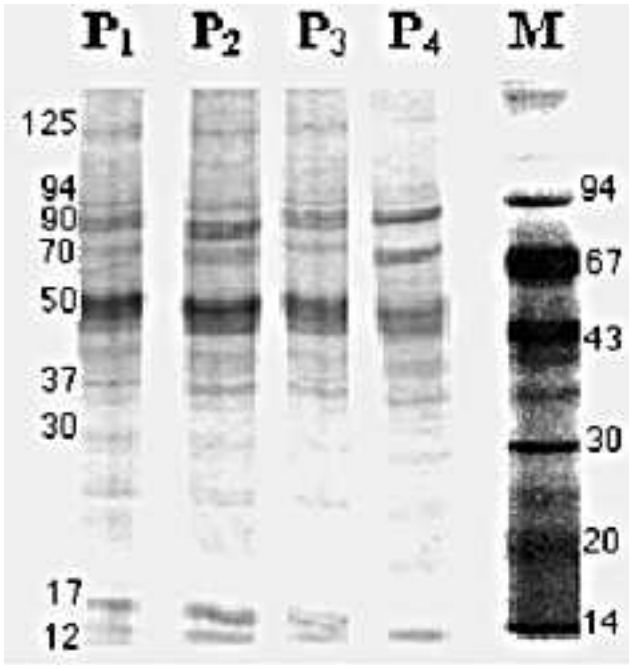


Fig. 1. – Autoradiograph of the proteins expressed by the promastigotes labelled with methionine (trans35S-label). P1: promastigotes treated with edelfosine for 2 h; P2: promastigotes treated with ilmofosine for 2 h; P3: promastigotes treated with miltefosine for 2 h; P4: promastigotes cultured at 37 °C for 2 h; M: standard proteins. Numbers on the right indicate relative molecular mass markers (in kDa).

Bands of 17 and 12 kDa appear in the promastigotes treated with ALPs.

Bands of 125, 70, 94, 90, 40, 37, 30 appear in all cases.

sine, and in promastigotes submitted to a heat shock. The 70 kDa band appeared in all cases. Untreated promastigotes were used as a control. The autography results showed that any band was labeled.

Protein electrophoresis and fluorographs in axenic amastigote forms

The auto-radiography analysis of the *in vitro* cultivated amastigotes (Fig. 2) showed the presence of an important protein band of 43 kDa in all cases. However, the cultivated axenic amastigotes at 42 °C for two hours showed a pattern of new proteins with 100 and 60 kDa. The treatment of axenic amastigote forms with edelfosine involves the synthesis of new protein bands with molecular weights of 100, 98, 90 kDa, and those treated with miltefosine synthesized a protein of 60 kDa. While, in the axenic amastigotes treated with ilmofosine, new proteins with a molecular weight of 100, 94, 90 kDa were appeared. In addition, the protein band of 70 kDa was observed in all cases of the treatment.
Untreated amastigotes were used as a control. The autography results showed that any band was labeled.

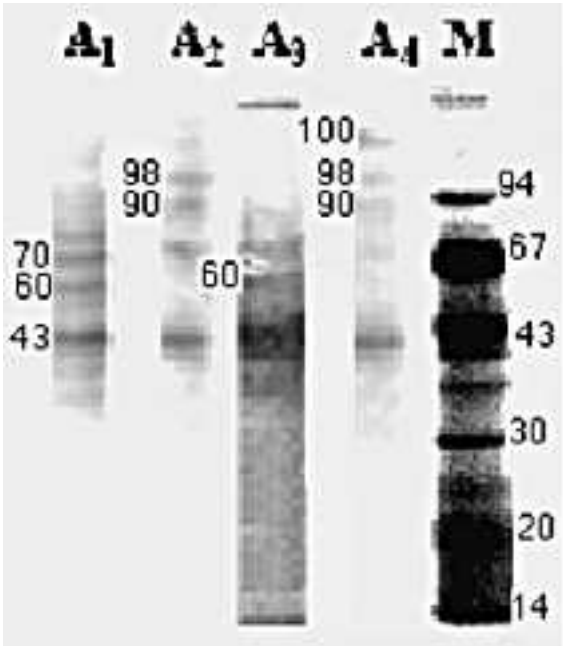


Fig. 2. – Autoradiograph of the proteins expressed by the amastigotes labelled with methionine (tran35S-label). A1: amastigotes cultured at 42 °C for 2 h; A2: amastigotes treated with edelfosine for 2 h; A3: amastigotes treated with miltefosine; A4: amastigotes treated with ilmofosine for 2 h; M: standard proteins. Numbers on the left indicate relative molecular mass markers (in kDa). The molecular mass of new synthesized protein was marked aside the bands.
A band of 43 and 70 kDa appear in all the cases.
Band of 70 and 60 kDa appear when the parasites were cultivated at 42 °C.
Band of 98 and 90 kDa appear when the parasites were trated with edelfosine.
Band of 60 kDa appear when the parasites were trated with miltefosine.
Bands of 100, 98 and 90 kDa appear in the parasite treated with ilmofosine.

Electronic microscopy results

In the sensitive strain of *L. donovani* treated with edelfosine, miltefosine and ilmofosine, the electronic microscopy results showed an important alteration in the promastigote structures (Fig. 3) according to the untreated control (Fig. 3A). The treated parasites showed severe structural damages (Figs 3B, C, D). Nevertheless, no clear differences among drug types were found. For parasites treated with edelfosine, extensive cytoplasm vacuolization was observed (Fig. 3B), while, with miltefosine, a presence of cytoplasmic vesicles was observed (Fig. 3C). In contrast, a sever condensation of the nuclear chromatin, and the membrane appeared ruffled were observed in the parasite treated with ilmofosine (Fig. 3D).
In the resistant strain, the treatment with miltefosine, did not cause any important modification in the parasite structures. The myelin bodies were present in the treated and untreated parasites (Fig. 4).

DISCUSSION

Our results showed that the incubation of the parasite with ALPs at subtoxic doses and according to the time periods, prompt the synthesis of a number of new proteins with similar molecular weight to those that the parasite undergoes under thermal-stress conditions. Similar situations arise in other parasites, such as *Giardia* sp., where the treatment with some drugs such as metronidazole or quinacrine can induce the synthesis of these stress proteins (Lindley, 1988).
This kind of proteins has been studied primarily as the organism's response to heat, and three families of genes have been identified in *Drosophila* as being responsible for this response. These genes are not only involved in other processes of cell stress: hypoxia; hypoglycaemia; treatment with heavy metals, oxidant or anti-tumour agents; changes in hydrostatic pressure; changes of free intracellular Ca^{2+} in the cytosol (Kiang, 1994), but also in normal processes of cell differentiation, such as haematopoiesis, embryogenesis, sexual differentiation, and phagocytosis (Biswas, 1994). The expression of these proteins can be considered as normal physiological process that enables the survival of the cell under normal conditions (Maresca, 1994). To stress caused by heat or by the treatment with ALPs, promastigote and axenic amastigote forms responded differently by synthesising different kind of proteins (Figs 1, 2). After the treatment with ALPs, promastigotes

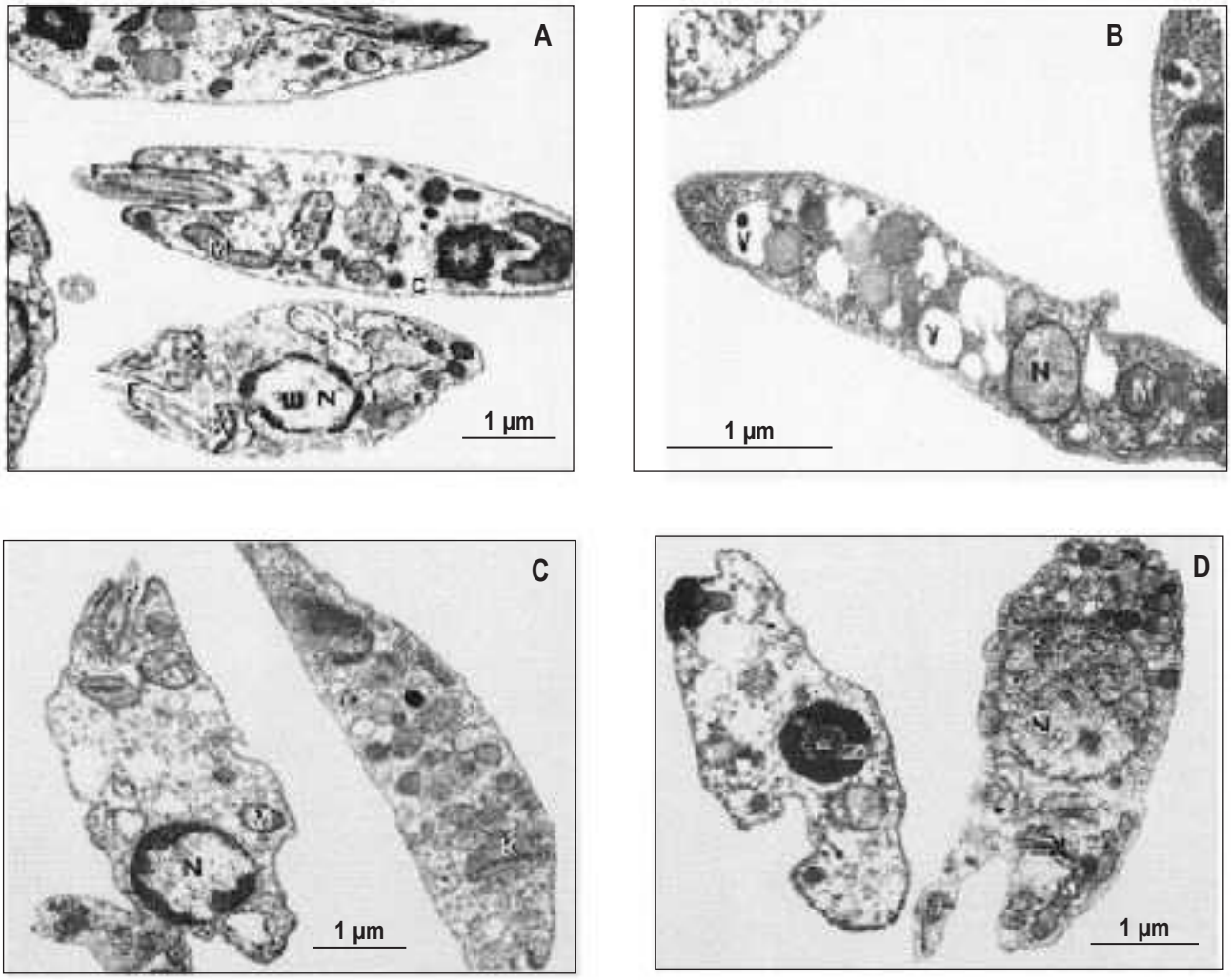


Fig. 3. – Illustartions of promastigotes of *L. donovani* to electronical microscopy. A: promastigotes of *L. donovani* no treated (control); B: promastigotes of L. donovani treated with edelfosine; C: promastigotes of *L. donovani* treated with miltefosine; D: promastigotes of *L. donovani* treated with ilmofosine.
N: nucleus; K: kynetoplast; C: cytosquelet; M: mitochondria; V: lipidic vacuole. F: whip.

synthesized specifically certain proteins, in particular protein bands of 17 and 12 kDa.

Rametti *et al.* (2004) show that during staurosporine-induced neuronal apoptosis, tau protein first underwent transient hyperphosphorylation which was followed by dephosphorylation and cleavage. This cleavage generates 10 kDa protein fragment in addition to those of 17 kDa and 50 kDa. The role of ALPs in signals transduction has been previously demonstrated (Croft, 1993), and the mechanism of phophorylation/dephosphorylation that acts on these signals could explain the synthesis of these new proteins (Daniel, 1993). The protein band of 70 kDa appears in almost all cases. Otherwise, the heat shock protein 70 kDa sequences (HSP70) are of great importance as molecular chaperones in protein folding and transport (Karlin, 1998). These proteins seem to be abundant when the cells are under stress conditions. Amastigotes synthesis different proteins from those synthesized by promastigotes, especially the protein band of 100 kDa, and that of 43 kDa which appears practically in all cases. The synthesis of the new proteins may be explained as mechanism response to the produced changes in intracellular environment which affect the metabolic way of the parasite. In addition, this may be explained again, as the expression of different iso-enzymes able to act in different intracellular environments (Morimoto, 1994). This result is similar to findings reported by Krobitch *et al.* (1998).

Ruiz-Ruano *et al.* (1991) studied the changes in the expression dynamic of polypeptides following the differentiation from infective trypomastigote to multiplicative amastigote forms of *Trypanosoma cruzi* responsible of Chagas's disease. They found that, after complete differentiation from trypomastigotes to amas-

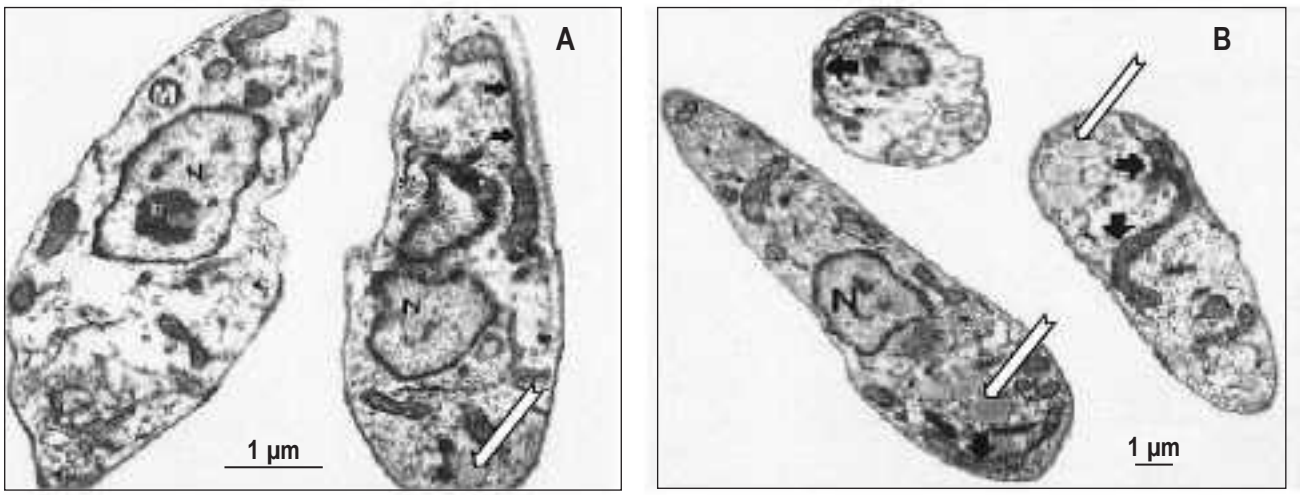


Fig. 4. – Illustartions of miltefosine's resistantes promastigotes of *L. donovani* to electronical microscopy. A: resistant's promastigotes to miltefosine no treated (control); B: resistant's promastigotes to miltefosine treated with the ether lipid.
N: nucleus; K: kynetoplast; C: cytosquelet; M: mitochondria; V: lipidic vacuole; F: whip.
➡ mitochondria with deformed crests (× 14,868).
⇨ myelinics bodies.

tigotes, the expression of the polypeptide 43 kDa among of others is up-regulated in amastigotes.
In the axenic amastigotes treated with edelfosine and ilmofosine, the presence of a protein band of 90 kDa was observed. Otherwise, Gausdal *et al.* (2004) show that Hsp90 "client proteins" involved in oncogenic survival signalling could therefore be important for leukemic cell survival, and it is important in antileukemic treatment. The presence of this Hsp 90 protein in the amastigotes of *L. donovani* treated with edelfosine and ilmofosine could suggest that the ALPs induce the same reaction in *L. donovani* like occurred in leukemic cells.
The studies of the ultra-structural changes suffered by the parasite under the effects of the ALPs have been carried out by electronic microscopy, and have shown that the ALPs affect mainly the plasmatic and mitochondrial membranes, the structure of the mitochondria, and the chromatin organization. In addition of the structural alterations studied in promastigote sensitive strain, we also studied them in the strain resistant to miltefosine. In the resistant strain, we observed that the flagellated forms seem not be affected by the compounds, but curiously we observed an apparition of deformed mitochondria with a crest, both in treated and untreated one. In contrast, in the promastigote sensitive strain, we observed a series of alterations, like: form loss, stability lack in cytosquelet, diffused and broken mitochondrial membranes, structure and organelle lyses, and the apparition of disorganized lipidic vacuoles. The membrane alterations have been previously described in the cells cultivated or treated in the presence of the ALPs (Herrmann, 1986). In addition of the damages suffered in the membrane structures, it seems that the mitochondria is one of the organelles most affected and altered by the ALPs actions, appears hypertrophied with a widened crests. In addition of the mentioned structures, the nucleus appears affected by these molecule actions, especially after the processing with the ilmofosine, where we observed the separation between nuclear membrane and nuclear headquarters and the very condensed chromatin. This may be due to the programmed death of the parasite, which is a very studied phenomenon and considered as one of the action mechanisms of ALPs and other ether lipids as shown previously in the tumour cells (Engelmann, 1996). Besides all these alterations, we observed a lysis in the cytosol and the reticulum.
The results found by our research group and by other authors indicate that alkyllysophospholipids represent an effective alternative to treat leishmaniasis disease, due to there complex action mechanisms which target primarily the parasite membranes, the other effects observed being a direct consequence of the aforementioned action. The axenic amastigote and promastigote forms of *L. donovani* showed a different behaviour when they are submitted to stress conditions. With these results, we confirm all the previous works about the axenic amastigotes and their differences with the promastigote forms.
Miltefosine, an alkylphosphocholine, was recently registered for the oral treatment of visceral leishmaniasis in India following successful clinical trials (Sundar, 2002). Edelfosine, an alkylglycerophosphocholine, and ilmofosine, the thioether substituted phosphatidylcholine analogue, show a high activity against promastigotes of *L. donovani*. The different structures of the three ALPs

can explain the variations in their effects. And we think that, by doing some modifications in the structures of edelfosine and ilmofosine can be the key for their future possible use for the treatment of leishmaniasis.

ACKNOWLEDGEMENTS

This study was supported by CEE Antileishmanial and antitrypanosomal activities of alkyllysophosopholipids project from the European Union (INCO-CT96-0084) and by the Spanish project number SAF 1998-0158

The authors declare that the experiments comply with the current laws of the country in which the experiments were performed.

REFERENCES

BATES P. Complete developmental cycle of *Leishmania mexicana* in axenic culture. Parasitology, 1994, *108*, 1-9.

BISWAS S. & SHARMA Y.D. Enhanced expression of *Plasmodium falciparum* heat shock protein PFHSP70-I at higher temperatures and parasite survival. *FEMS Microbiology Letters*, 1994, *124*, 425-429.

CASTILLA J.J., SANCHEZ-MORENO M., MESA C. & OSUNA A. *Leishmania donovani*: *in vitro* culture and [1H] NMR characterization of amastigote-like forms. *Molecular and Cellular Biochemistry*, 1995, *142*, 89-97.

CROFT S.L., NEAL R.A., THORNTONN E.A. & HERRMANN D.B.J. Antileishmanial activity of ether phospholipid ilmofosine. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 1993, *87*, 217-219.

CROFT S.L., SNOWDON D. & YARDLEY V. The activities of four anticancer alkyllysophospholipids against *Leishmania donovani, Trypanosoma cruzi* and *Trypanosoma brucei*. *Journal of Antimicrobial Chemotherapy*, 1996, *38*, 1041-1047.

DANIEL J. Ether lipids in experimental cancer chemotherapy, *in*: Cancer Chemotherapy. Hichman J.A. & Tritton T.R. (eds), Blackwell, Oxford, 1993.

ENGELMANN J., HENKE J., WILLKER W., KUTSCHER B., NOSSNER G., ENGEL J. & LEIBFRITZ D. Early stage monitoring of miltefosien induced apoptosis in KB cells by mulktinuclear NMR spectroscopy. *Anticancer Research*, 1996, *16*, 1429-1439.

GAUSDAL G., GJERTSEN B.T., FLADMARK K.E., DEMOL H., VANDEKERCKHOVE J. & DOSKELAND S.O. Caspase-dependent, geldanamycin-enhanced cleavage of co-chaperone p23 in leukemic apoptosis. *Leukemia*, 2004, *18*, 1989-1996.

HART D. & COOMBS G. *Leishmania mexicana*: energy métabolisme amastigotes and promastigotes. *Experimental Parasitology*, 1982, *54*, 397-409.

HERRMANN D.B. & NEUMANN H.A. Cytotoxic ether phospholipids. *Journal of Biological Chemistry*, 1986, *261*, 7742-7747.

KARLIN S. & BROCCHIERI L. Heat shock protein 70 family: multiple sequence comparisons, function, and evolution. *Journal of Molecular Evolution*, 1998, *47*, 565-577.

KIANG J.G., CARR F.E., BURNS M.R. & MCCLAIN D.E. HSP-72 synthesis is promoted by increase in $[Ca^{2+}]i$ or activation of G proteins but not pHi or cAMP. *American Journal of Physiology*, 1994, *267*, 104-114.

KROBITSCH S., BRANDAU S., HOYER C., SCHMETZ C., HUBEL A. & CLOS J. *Leishmania donovani* heat shock protein 100. Characterization and function in amastigote stage differentiation. *Journal of Biological Chemistry*, 1998, *273*, 6488-6494.

LINDLEY T.A., CHAKRABORTY P.R. & EDLIND T.D. Heat shock and stress response in *Giardia lamblia*. *Molecular and Biochemical Parasitology*, 1988, *28*, 135-143.

LIRA R., CONTRERAS L.M., SANTA-RITA R.M. & URBINA J.A. Mechanism of action of anti-proliferative lysophospholipid analogues against the protozoan parasite *Trypanosoma cruzi*: potentiation of *in vitro* activity by the sterol biosynthesis inhibitor ketoconazole. *Journal of Antimicrobial Chemotherapy*, 2001, *47*, 537-546

MARESCA B. & KOBAYASHI G.S. Hsp70 in parasites: as an inducible protective protein and as an antigen. *Experientia*, 1994, *50*, 1067-1074.

MORIMOTO R.I., JURIVICH D.A., KROEGER P.E., MATHUR S.K., MURPHY S.P., NAKAI A., SARGE K., ABRAVAYA K. & SISTONEN L.T. *In*: The biology of heat shock proteins and molecular chaperones, Morimoto R.I., Tissières A. & Georgopoulos C. (eds), Cold Spring Harbor Laboratory Press, Plainview, NY, 1994, pp. 417-455.

MURRAY H.W., BERMAN J.D, DAVIES C.R. & SARAVIA N.G. Advances in leishmaniasis. *Lancet*, 2005, *366*, 1561-1577.

NEAL R.A. Experimental chemotherapy, *in*: The leishmaniases in biology and medicine. Peters W. & Killick-Kendrick R. (eds), Academic Press, London, 1978, pp. 793-845.

RAMETTI A., ESCLAIRE F., YARDIN C. & TERRO F. Linking alterations in tau phosphorylation and cleavage during neuronal apoptosis. *Journal of Biological Chemistry*, 2004, *279*, 54518-54528.

RUIZ-RUANO A., VILLALTA F. & LIMA M.F. Changes in polypeptide expression following *Trypanosoma cruzi* differentiation from trypomastigotes to amastigotes. *Biochemistry International*, 1991, *25*, 101-108.

SOTO J., TOLEDO J., GUTIERREZ P *et al*. Treatment of American cutaneous leishmaniasis with miltefosine, an oral agent. *Clinical Infectious Diseases*, 2001, *33*, 57-61.

SUNDAR S., JHA T.K., THAKUR C.P., ENGEL J., SINDERMANN H., FISCHER C. *et al.* Oral miltefosine for Indian visceral leishmaniasis. *New England Journal of Medicine*, 2002, *347*, 1739-1746.

SUNDAR S., MAKHARIA A., MORE D.K. *et al*. Short-course of oral miltefosine for treatment of visceral leishmaniasis. *Clinical Infectious Diseases*, 2000, *31*, 1110-1113.

WIEDER T., REUTTER W., ORFANOS C.E. & GEILEN C.C. Mechanism of action of phospholipid analogs as anticancer compounds. *Progress in Lipid Research*, 1999, *38*, 249-259.

Reçu le 14 janvier 2009
Accepté le 18 mai 2009