Available online at www.sciencedirect.com
SCIENCE DIRECT
Drug Resistance Updates 9 (2006) 26–39
DRUG RESISTANCE Updates
www.elsevier.com/locate/drup

Mechanisms of experimental resistance of *Leishmania* to miltefosine: Implications for clinical use

F. Javier Pérez-Victoria [a], María P. Sánchez-Cañete [a], Karin Seifert [b], Simon L. Croft [c], Shyam Sundar [d], Santiago Castanys [a,1], Francisco Gamarro [a,*,1]

[a] *Instituto de Parasitología y Biomedicina "López-Neyra", Spanish Research Council (C.S.I.C.), Granada, Spain*
[b] *Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, UK*
[c] *Drugs for Neglected Diseases Initiative, Geneva, Switzerland*
[d] *Kala-azar Medical Research Center, Department of Medicine, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India*

Received 5 November 2005; received in revised form 27 March 2006; accepted 6 April 2006

Abstract

Miltefosine (hexadecylphosphocholine, MIL), registered as Impavido®, has become the first oral drug for the treatment of visceral and cutaneous leishmanasis. MIL is a simple molecule, very stable, relatively safe and highly efficient in clinical trials. However, MIL requires a long treatment course (28 days) and has a long half-life (around 150 h), which might accelerate the emergence of drug resistance in case of inadequate use. The mechanisms of MIL resistance have been studied in vitro with experimental resistant lines. Resistance was shown to develop quickly in *Leishmania* promastigotes. Interestingly, a decreased MIL accumulation has always accounted for the resistance phenotype. The lower MIL accumulation can be achieved by two independent mechanisms: (i) an increase in drug efflux, mediated by the overexpression of the ABC transporter P-glycoprotein, and (ii) a decrease in drug uptake, which is easily achieved by the inactivation of any one of the two proteins known to be responsible for the MIL uptake, the MIL transporter LdMT and its beta subunit LdRos3. Policies concerning a proper use of this drug should be followed and supervised by health authorities of endemic areas to minimalize the risk for the appearance of drug failures and to ensure a long life span for this effective oral drug.

Keywords: *Leishmania*; Miltefosine; Experimental drug resistance; Aminophospholipid translocase; CDC50 family; Control; Drug combinations; LdMT; LdRos3

1. Introduction

The Leishmaniases are a group of diseases with wide epidemiological and clinical diversity caused by the protozoan parasite *Leishmania*. The different clinical manifestations vary from cutaneous self-healing lesions (CL) to visceral disease (VL), fatal if left untreated. Leishmaniasis is considered to be the second most important protozoal disease and one of the neglected diseases that have become a special focus for WHO. From the estimated 500,000 new cases per year of VL or kala-azar, around 90% occur in endemic areas of the Indian, Bangladesh, Sudan and Brazil due to *Leishmania donovani*. These endemic areas constitute foci of anthroponotic transmission of the parasite, which increases the chances for the fast spreading of drug resistant parasites once these have been generated (Sundar, 2001). Unfortunately, as yet no effective vaccines against leishmaniasis are available and control of the disease relies primarily on chemotherapy. The chemotherapy currently available for leishmaniasis is far from satisfactory. New drugs are necessary and this requirement has been fed in recent years by the demonstration of acquired resistance to the pentavalent antimonial drugs, the first-line chemotherapy. One of the most significant recent

* Corresponding author at: Instituto de Parasitología y Biomedicina "López-Neyra". C.S.I.C. Parque Tecnológico de Ciencias de la Salud, Avda. del Conocimiento s/n, 18100 Armilla, Granada, Spain. Tel.: +34 958181667; fax: +34 958181632.
E-mail address: gamarro@ipb.csic.es (F. Gamarro).
[1] The authors are equal senior investigators in this study.

1368-7646/$ – see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.drup.2006.04.001

advances in this area has been the identification of miltefosine (MIL), an alkylphosphocholine originally developed as an anticancer drug, as an effective oral treatment for VL and CL. In the present review, we summarize recent information concerning MIL, focusing on experimental MIL resistance mechanisms. In the absence of new drugs, understanding the basis of treatment failure and developing tools for detecting this phenomenon will contribute to protect MIL and recommend the most adequate therapeutic procedures. Drug combinations and a rational use of MIL appear essential for an efficient and long life span of this promising antileishmanial drug.

2. Chemotherapy against leishmaniasis

Leishmaniasis control relies on chemotherapy. Available drugs are limited in number and efficacy. Pentavalent antimonials sodium stibogluconate (Pentostam) and meglumine antimoniate (Glucantime), the most standard drugs recommended 60 years ago, despite the low efficacy and adverse reactions, remain the first-line treatment in most parts of the world. Recently, antimonials have become almost obsolete in certain areas of India where unresponsiveness to these drugs has increased dramatically in the last years as demonstrated by a significant decrease in their efficacy (10-fold increase in dosage/duration of the usual treatment) and by the significant increase in the percentage of total therapeutic failure (around 60%) (Sundar, 2001). Amphotericin B is the second line treatment in India and has been used in different formulations, such as the liposomal formulation known as AmBisome, which has shown a >95% cure rate in patients of India suffering from the disease. However, the high cost of this treatment limits a wider use. Pentamidine is another antileishmanial drug used in endemic areas where antimonials are inefficient, however from 1983 its therapeutic efficacy has decreased significantly till 70%, decreasing its use in different areas. The search for an effective oral antileishmanial drug spans two decades. Allopurinol, the azoles, rifampicin and atovaquone showed activity in experimental systems, but proved disappointing in clinical trials. Apart from MIL, sitamaquine holds some promise and reports of several trials have been published recently. For other drugs treatments, see a recent review of Croft et al. (2005).

3. MIL for leishmaniasis

3.1. Miltefosine as the first oral treatment against leishmaniasis

MIL (hexadecylphosphocholine), originally developed as an anticancer drug, is the first oral drug that has proven to be highly effective against VL (Sundar et al., 2000a), including antimony-resistant cases (Jha et al., 1999), and against CL (Soto et al., 2001). MIL, a phosphorylcholine ester of hexadecanol (Fig. 1), was synthesized at the Max-Planck-Institut fur Biophysikalische Chemie in Gottingen (Germany) and successfully used for the therapy of cancer metastases. Specifically, a topical formulation of MIL (Miltex) is effective in cutaneous breast cancer metastases and was registered as the first anticancer drug for topical application (Burk et al., 1994). The in vitro and in vivo antileishmanial activity of MIL was first described by Croft et al. (1987); in 1992 its activity was also demonstrated after oral administration in animals (Kuhlencord et al., 1992). It moved from the laboratory through to registration in 6 years thanks to collaboration between the Government of India, the German biopharmaceutical company Zentaris and TDR (Tropical Diseases Research), a program co-sponsored by the World Bank, the U.N. Development Programme and the World Health Organization. Registered in India in 2002 as Impavido®, MIL is hopefully about to play an essential role in the control and treatment of this endemic disease (Ganguly, 2002). Furthermore, its use in the treatment for the increasing number of VL cases in Southern Europe is expected, as well as its use against CL in South America. So far, MIL has been registered in India, Germany and Colombia. MIL induced rapid clinical and parasitological cure (95% and 91% for VL and CL, respectively), doses of 100–150 mg/day (or 2.5 mg/kg body weight) for 28 days being the most effective. Strikingly, MIL is also effective and well tolerated in Indian children with VL and can be recommended as the first choice for treatment of childhood VL in India (Sundar et al., 2003; Bhattacharya et al., 2004). Paediatric VL constitutes one-half of the total VL cases (Bhattacharya et al., 2004). The pharmacokinetic of the drug and side effects were almost similar to adults. Cure rates in HIV co-infected patients seem to be similar to other drugs. A compassionate study using 39 HIV co-infected patients in Europe treated with MIL, resulted in initial parasitological cure of 43% patients, however, many relapses were observed (Sindermann et al., 2004). Its use as a single agent in co-infected patients could encourage the appearance of resistance. Recently, the successful treatment of disseminated CL in a severely immunocompromised patient infected with HIV-1 patient with MIL was reported (Schraner et al., 2005).

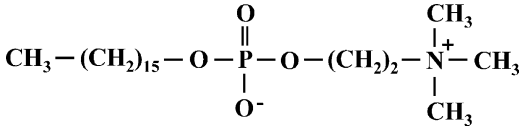

Fig. 1. Chemical structure of MIL.

MIL has a long-terminal half-life, which ranges between 150 and 200 h. Plasma levels of oral MIL are roughly dose proportional and urine excretion is negligible. About four half-lives (25–33 days) are required to reach more than 90% clearance of the plateau levels (at steady-state). Thus, subtherapeutic levels of MIL may remain for some weeks after a standard course of treatment. This characteristic might encourage the emergence of resistance (Bryceson, 2001).

The most commonly reported adverse reactions are transient gastrointestinal discomfort, vomiting, diarrhoea, and elevation of liver enzymes and serum creatinine. These effects are usually mild to moderate and transient or reversible at the end of treatment and therefore do not require discontinuation of treatment or dosage reduction. More importantly, MIL is an abortifacient and teratogenic in animals, which absolutely precludes its use in pregnant women. Indeed, reproduction contraception must be maintained for the period of MIL administration and about eight half-lives (2–3 months) in females with child bearing potential (Sundar et al., 2002).

3.2. Species variability on the sensitivity to MIL

There are over 20 species of *Leishmania* causing the different clinical forms of leishmaniasis in humans. Differences on the biochemical and molecular levels are used for phylogenetic analysis (Cupolillo et al., 2000; Quispe-Tintaya et al., 2005). Different sensitivities to various drugs including MIL have been demonstrated. In fact, sensitivities to MIL and edelfosine, another phospholipid analogue, have been assessed in vitro against different laboratory strains (Escobar et al., 2002). Six different *Leishmania* species, including *L. donovani*, *L. aethiopica*, *L. tropica*, *L. mexicana*, *L. panamensis* and *L. major*, were assayed and sensitivities compared between the promastigote and amastigote stage in standard assays. *L. donovani* was most sensitive in both life cycle stages with EC_{50} values of 4.6–3.3 μM for amastigotes and 0.5 and 0.4 μM for promastigotes (two different replicates each). The least sensitive species in both cases was *L. major* with significantly higher EC_{50} values ranging from 37.2 to 31.6 μM for amastigotes to 13.1 to 4.8 μM for promastigotes. The picture was similar for edelfosine with the exception of *L. mexicana* being the least sensitive species in the promastigote assay (EC_{50} values 3.11–2.91 μM). *L. major* did respond to edelfosine as well as other species (EC_{50} values 1.42–0.50 μM). It has also been reported that the lizard parasite *L. tarentolae* is 10 times less sensitive to MIL than the human parasite *L. donovani* (Pérez-Victoria et al., 2003b).

Differences were observed in clinical outcome between geographical regions in a placebo-controlled study of MIL against cutaneous leishmaniasis (CL) in Colombia and Guatemala (Soto et al., 2004). MIL was administered at a dose of 2.5 mg/kg/day to 133 patients, of whom 127 received the full 28-day course. The cure rate was 91% with MIL versus 38% with placebo in regions in Colombia where *L.* (*V.*) *panamensis* is common and described as similar to the historic antimony standard. A much lower cure rate, 53% with MIL versus 21% with placebo, was achieved in regions in Guatemala where *L.* (*V.*) *braziliensis* and *L. m. mexicana* are common compared to historical cure rates with antimony >90%. An initial uncontrolled open-label, dose-ranging study in Colombia where *L.* (*V.*) *panamensis* predominated had given a 94% cure rate with MIL at a dose of 2.5 mg/kg/day for 3–4 weeks (Soto et al., 2001). These results suggested that MIL cure rates might vary in different geographical areas depending on the specific sensitivity to MIL of the most prevalent species.

In a recent study, a significant variation in MIL sensitivity has been demonstrated on clinical isolates of different *Leishmania* spp. from Peru and Nepal (Yardley et al., 2005). Isolates taken from patients treated with antimonials were typed for species identification and sensitivity assessed in the standard amastigote-macrophage model. All *L. donovani* isolates, taken from Nepalese patients with VL both Sb^v responders and Sb^v non-responders were intrinsically sensitive to MIL with EC_{50} values ranging from 0.04 to 5.7 μg/ml (about 0.1–14 μM). Remarkably, most isolates from Peruvian CL patients, typed to the *L.* (*V.*) *braziliensis* complex were insensitive in the concentration range tested (up to 30 μg/ml, which corresponds to about 73 μM). The notable exception was *L.* (*V.*) *lainsoni*, which displayed comparable EC_{50} values to *L. donovani*, ranging from 1.89 to 3.37 μg/ml (4.6–8.3 μM) (Yardley et al., 2005). These data do establish a different sensitivity in natural *Leishmania* populations based on the species and the subtypes of the parasites, which agrees with the clinical outcome of MIL trials in different geographical areas (Soto et al., 2004).

The variability of different species in MIL sensitivity described in this paragraph is not due to acquired resistance but reflects differences in intrinsic susceptibility. This could as well have an important impact on clinical outcome. The greatest clinical significance is seen in Central and South America where distribution of *L. mexicana*, *L. amazonensis*, *L. panamensis*, *L. braziliensis* and other members of these groups overlap (Croft, 2004). Similar differences have been described with other drugs against leishmaniasis (Escobar et al., 2002; Neal et al., 1995; Grogl et al., 1992). This variation in sensitivity is difficult to interpret as it could be due to differences in the rate of division, or exposure of intracellular and extracellular stages to drugs, or biochemical targets or drug metabolism (Escobar et al., 2002). Because *Leishmania* species present significant differences in both membrane sterol (Goad et al., 1984, Beach et al., 1988.) and lipid content (Beach et al., 1979), another possibility is that the biochemical composition of these parasites might affect drug activity. However, considering that MIL internalization is a prerequisite for its action and the excellent correlation between MIL uptake and sensitivity levels, it could be plausible that the differences in susceptibility are related with the different ability to internalize the drug. Studies regarding the expression levels of LdMT and LdRos3, the proteins responsible for MIL uptake (see below), in different species deserve further attention. Indeed, overexpression of LdMT in *L. tarentolae*, a species refractory to MIL, increased MIL uptake values 20-fold and MIL sensitivity around 10-fold in promastigotes (Pérez-Victoria et al., 2003b), suggesting that parental *L. tarentolae* expresses low levels of LdMT.

In vitro drug sensitivity tests using the amastigote-macrophage model are suitable to compare sensitivities of species on the parasite level with the clinical outcome, but normal variation between assays has to be taken into account

when defining data obtained and comparing data from different studies. Importantly, MIL sensitivities in promastigotes and intracellular amastigotes correlate fairly well, indicating the use of the easy to grow promastigote form in vitro for the determination of drug sensitivity in clinical isolates.

3.3. MIL mechanisms of action

Although potential anti-tumor cell mechanisms of action of MIL and other phospholipid analogues have been elaborated (Brachwitz and Vollgraf, 1995; Arthur and Bittman, 1998), at present little is known about the leishmanicidal and trypanocidal mechanisms of MIL and other phospholipid analogues. Part of the knowledge for mechanisms of drug action comes from studies with experimental drug resistant cell lines. In the case of MIL, the elucidation of drug resistance mechanisms has clearly shown the intracellular drug accumulation as a prerequisite for MIL action.

We can differentiate three steps in the accumulation of short-chain phospholipids and derivatives such as MIL (Fig. 2):

(i) Binding of the drug to the outer leaflet of the plasma membrane. Under normal culture conditions, MIL is bound to albumin, which acts as a reservoir for the drug. Because MIL is water-soluble and able to bind to lipid monolayers (Rakotomanga et al., 2005), an equilibrium between the fraction bound to albumin and the fraction bound to cell membranes is rapidly achieved upon addition of the cell suspension (Fig. 2A). This equilibrium depends basically on the concentration of the drug, the amount of albumin (or the percentage of serum in the culture medium) and the number of cells (and plasma membranes) present (Pérez-Victoria et al., 2003a, unpublished observations).

(ii) Internalization of the drug inside the cell. Phospholipid molecules diffuse rapidly within a lipid monolayer. However, their flip–flop movement in a lipid bilayer (the movement from the outer to the inner leaflet or vice versa) is generally very slow, with $t_{1/2}$ typically of days (provided an intact plasma membrane is present) (Pomorski et al., 2004). Two possible mechanisms for the internalization of MIL exist. (a) Endocytic pathway: the MIL monomers integrated in the plasma membrane are internalized as members of the endocytic vesicle that is being budded. *Leishmania* parasites possess a high endocytic activity from the plasma membrane, which is restricted to the specialized area of the flagellar pocket (McConville et al., 2002). However, the endocytic pathway is only important in circumstances in which the amount of drug bound to the membrane is extraordinarily high. (b) Non-endocytic pathway or flippase activity: MIL monomers can also be translocated from the outer to the inner leaflet of the plasma membrane by the action of specific proteins (Fig. 2B). We have clearly shown that this mechanism is the most important one accounting for the uptake of MIL (Pérez-Victoria et al., 2003a). It depends on two proteins that likely form a complex at the plasma membrane: the MIL transporter LdMT and its beta subunit LdRos3 (see below).

(iii) Intracellular targeting and metabolism: once in the inner leaflet of the plasma membrane, MIL monomers can detach and equilibrate within the membranes (cytosolic leaflets) of internal organelles, due to their solubility in water. So far, nothing is known about the intracellular distribution of MIL, nor whether there is any organelle in which the drug tends to accumulate. The specific mechanisms of MIL action must take place inside the cell at this level. Electron microscopy studies of *Leishmania* cells following incubation with low MIL concentrations have shown the ability of the drug to disrupt the membranes of intracellular organelles, in a detergent-

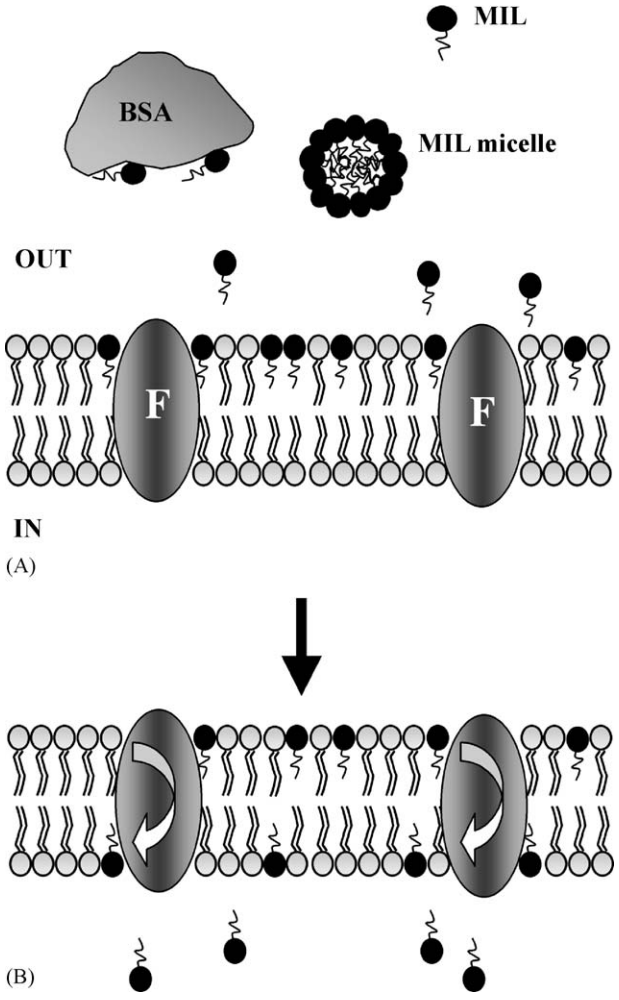

Fig. 2. Binding and uptake of MIL in *Leishmania* parasites. Sequential steps in the accumulation of MIL: (A) Binding of the drug to the outer leaflet of the plasma membrane. MIL is recruited by bovine serum albumin (BSA), which acts as a reservoir for the drug. (B) The fraction bound to cell membranes is internalized through a flippase protein (F) machinery present at the lipid bilayer. This translocation machinery includes, at least, two proteins at the plasma membrane: the MIL transporter LdMT and its beta subunit LdRos3 (see in the text).

like effect (Croft et al., 2003). The metabolism of MIL inside *Leishmania* cells is very slow or even negligible (Pérez-Victoria et al., 2003a), whereas the ability to internalize the drug is very high, which tends to generate high intracellular MIL concentrations able to kill the parasite. Finally, MIL could exit the cell by mechanisms opposite to those that determine internalization, namely exocytosis and protein-dependent flop from the inner to the outer leaflet of the plasma membrane. Members of the ABC transporters family, such as P-glycoprotein (mdr1), are strong candidates to mediate this floppase activity (Perez-Victoria et al., 2001; Rybczynska et al., 2001a).

MIL being an oral drug and *Leishmania* an obligate intracellular parasite of macrophages, the drug must pass a number of membrane barriers before reaching its target. It might be feasible that similar mechanisms account for the transport of MIL for any of them.

The specific mechanisms of MIL action inside *Leishmania* cells remain unknown. Perturbation of the alkyl-lipid metabolism and the biosynthesis of alkyl-anchored glycoproteins have been described (Lux et al., 2000). Nevertheless, MIL concentrations needed to inhibit the enzymes responsible for those activities were much higher than those needed to kill the parasite, suggesting that the primary target might be a different one. Damage to the flagellar membrane and defects in phospholipid biosynthesis have been reported in both *Leishmania* and *Trypanosoma cruzi* (Lira et al., 2001; Santa-Rita et al., 2000), although a recent report has shown that inhibition of phosphatidycholine synthesis is not the primary pathway leading to MIL-mediated apoptosis and cell death in mammalian cells (van der Sanden et al., 2004). Whatever the primary target is, MIL is known to induce apoptosis-like death in *L. donovani* based on observed phenomena such as cell shrinkage, nuclear DNA condensation, DNA fragmentation into oligonucleosome-sized fragments and phosphatidylserine exposure (Verma and Dey, 2004; Paris et al., 2004). Taking all data together, we favour a hypothesis in which MIL would produce numerous and different defects in the cell (likely from its detergent-like activity that disrupts intracellular membranes), which would finally result in an apoptosis-like parasite death. Thus, the basic requisite for the MIL antileishmanial action depends upon its internalization, as has been suggested for the specific antitumoral activity of MIL and other phospholipids analogues like edelfosine and ilmofosine in mammalian cells (Gajate and Mollinedo, 2002).

Regarding the toxicity of MIL in the context of the host-parasite relationship, further factors should be referred. Although the possible interference of MIL with immunological mechanisms cannot be completely ruled out, MIL does not induce the activation of natural killer cells, of cytotoxic spleen cells, the phagocytic activity of macrophages, or a humoral response per se (Hilgard et al., 1991). In isolated mononuclear cells and macrophages MIL was able to induce a variety of immunological and inflammatory effects (Beckers et al., 1994; Zeisig et al., 1995). Finally, it is remarkable that the leishmanicidal action of MIL did not require host T cell-dependent or activated macrophage-mediated mechanisms in in vivo animal models (Murray and Delph-Etienne, 2000; Escobar et al., 2002). Consequently MIL could be a drug potentially useful for treating T cell-deficient patients with kala-azar, including those with AIDS-associated *Leishmania* infection.

4. Experimental MIL resistance in *Leishmania*

Given that MIL has only recently started to be used for the treatment of visceral leishmaniasis cases in India and cutaneous leishmaniasis in Colombia, no drug resistance field isolate has so far been described. Before MIL starts to be widely used as leishmanicidal agent, the study of experimental MIL resistance in in vitro lines should provide a basic knowledge of the possible resistance mechanisms that might arise in the field. Such knowledge could be used for designing strategies leading to a more rational use of the drug, for determining molecular markers of MIL resistance able to monitor field resistant isolates and even to find interacting drugs able to overcome the MIL resistance phenotype.

Experimental *L. donovani* strains resistant to MIL are easily obtained by growing promastigotes in vitro with step-wise increasing drug pressure (Seifert et al., 2003) or by chemical mutagenesis followed by selection against a high concentration of MIL (Pérez-Victoria et al., 2003b). Furthermore, a multidrug-resistant *L. tropica* line previously generated was also shown to be cross-resistant to MIL and edelfosine (Perez-Victoria et al., 2001). The common feature in all *Leishmania* MIL resistant lines studied so far is a decrease in drug accumulation. Usually, lower intracellular concentrations of an active drug can be achieved through at least four different mechanisms: decreased uptake; increased efflux; faster metabolism (or its absence for inactive prodrugs); and altered plasma membrane permeability. The first two of these mechanisms have already been described in experimental MIL resistant lines.

4.1. Defective inward translocation of MIL in experimental resistant Leishmania lines: the LdMT-LdRos3 dependent machinery

The characterization of *L. donovani* strains selected for resistance to MIL has always shown a defective drug uptake as the mechanism responsible for the experimental drug resistance (Pérez-Victoria et al., 2003a, 2003b, manuscript in preparation). *Leishmania* promastigotes selected against high MIL concentrations (around 40 μM) showed a 15-fold reduction in sensitivity against MIL and edelfosine (Seifert et al., 2003; Pérez-Victoria et al., 2003b). Resistant parasites accumulated no more than 3% of the drug, compared to the plateau level of parental strains. A defect in the internalization step

on MIL must have occurred in the resistant line considering that binding of MIL to the parasite plasma membrane, efflux of preloaded drug and MIL metabolism were similar in sensitive and resistant parasites (Pérez-Victoria et al., 2003a). That same study established the basic mechanism for MIL uptake in *Leishmania* promastigotes. Resistant parasites were shown to be also defective in the internalization of fluorescent phospholipid analogues, but not in the endocytosis of different markers. Through biochemical studies in the parental line, the authors concluded that MIL is actively taken up by a protein-mediated and energy-dependent mechanism that consists of the specific translocation of MIL molecules from the outer to the inner leaflet of the parasite plasma membrane (Pérez-Victoria et al., 2003a). This activity has been given different names, such as "flippase activity", "translocation activity" or more accurately "inward-directed (phospholipid) translocation activity across the plasma membrane". A similar activity has been described in yeast (Grant et al., 2001; Hanson et al., 2003) and RAW 264.7 macrophages (Zoeller et al., 1995). Remarkably, those findings pointed out that drug uptake is a prerequisite for MIL activity against *Leishmania* and that the primary mechanism for MIL uptake is its specific translocation across the plasma membrane (the non-endocytic pathway) rather than its non-specific endocytosis, as it had been suggested in certain mammalian cell lines (Rybczynska et al., 2001b).

The biochemical characterization of the first in vitro MIL resistant parasites led the way for the identification of the proteins involved in MIL uptake and sensitivity (Pérez-Victoria et al., 2003b, manuscript in preparation). Functional complementation studies of different MIL resistant lines have identified two different proteins that are required for the uptake of MIL: the MIL transporter LdMT and its specific beta subunit LdRos3. Both proteins are essential but not individually sufficient for the translocation of MIL, likely because they form part of the same MIL translocation machinery present at the parasite plasma membrane.

The MIL transporter (LdMT) was first identified in an unbiased genetic screen as the protein mutated and thus inactive in MIL resistant parasites generated both by step-wise drug pressure selection and by mutagenesis (Pérez-Victoria et al., 2003b). LdMT is a large plasma membrane protein of 1097 amino acids that belongs to the P-type ATPase family of ion transporters. Specifically, LdMT is included in the phospholipid translocase subfamily of P-type ATPases, present in all eukaryota, which includes 5 members in yeast, among them the recently characterized Dnf1p, Dnf2p (the functional homologs of LdMT) (Pomorski et al., 2003) and 14 members in the human genome, including the ATPase II (Tang et al., 1996) and Fic1 (Bull et al., 1998) proteins. LdMT, as well as yeast Dnf1p and Dnf2p, is supposed to be the main protein responsible for MIL translocation at the plasma membrane, but definite evidence for its role as the MIL and phospholipid transporter is still lacking. However, it has been definitely shown that LdMT is essential for MIL uptake and sensitivity in *Leishmania* parasites. Targeted disruption of both *LdMT* alleles converted sensitive *L. donovani* parasites into highly resistant ones, through the inactivation of MIL uptake (Pérez-Victoria et al., manuscript in preparation). Similarly, the acquisition of inactivating point mutations within LdMT yields parasites unable to take up the drug and thus highly resistant to MIL (Pérez-Victoria et al., 2003b). A number of mutated and inactivated LdMT alleles has already been characterized (T420N; L856P; A653V; G276V; I914T; F414S + F430S + G824D; L366P + L780P + G824D; non-sense mutations at 145 and 210) (Pérez-Victoria et al., 2003b, unpublished results). It is noteworthy that many amino acids in different domains of the protein are essential for its activity. Overall, the highly conserved phosphorylation domain presents the maximal mutational pressure, and could be considered as a hot region for the generation of inactivating point mutations. However, we must emphasize that any mutation able to inactivate the transporter will have important and similar consequences regarding MIL sensitivity, and thus the whole gene should be analysed for monitoring drug resistance.

In order to be active, LdMT must be present at the parasite plasma membrane. Strikingly, the localization of LdMT and thus its activity depends on the presence of a specific beta subunit, LdRos3 (Pérez-Victoria et al., manuscript in preparation). LdRos3 belongs to the CDC50/Lem3 protein family. It contains two transmembrane segments separated by a large extracellular domain, with a total length of 366 amino acids. LdRos3 also localizes to the parasite plasma membrane, but only when an active LdMT is also present. Thus, both proteins are mutually dependent for their function and their localization at the plasma membrane (Fig. 3). LdRos3 was identified as the protein defective in M-1M *L. donovani* resistant parasites. This strain was generated by the treatment of a large number of parasites with a high MIL concentration. The clone had both *LdRos3* alleles inactivated, and also one of the *LdMT* alleles presented a non-sense mutation that disrupted the protein (but a second *LdMT* allele similar to the wild-type gene). These data indicate that both proteins are submitted to high selective pressure under MIL treatment, and that the ultimate phenotype when either LdRos3 or LdMT are disrupted is the same: the acquisition of high MIL resistance levels.

Remarkably, LdMT and LdRos3 only localize at the plasma membrane when both proteins are active and therefore able to mediate the internalization of MIL. Inactivating point mutations in LdMT recluded LdMT and LdRos3 at intracellular organelles (most likely the endoplasmic reticulum) (Pérez-Victoria et al., manuscript in preparation). Therefore, only the fraction of the LdMT-LdRos3 pool that reaches the plasma membrane accounts for the translocation activity of MIL. Antibodies directed against extracytoplasmic domains of either LdMT or LdRos3 should be effective to discriminate by simple experiments, such as flow cytometry, between the fraction of LdMT-LdRos3 present at the plasma membrane and the total protein pool.

The functional characterization of LdMT and LdRos3 has determined not only their requirement for MIL uptake, but

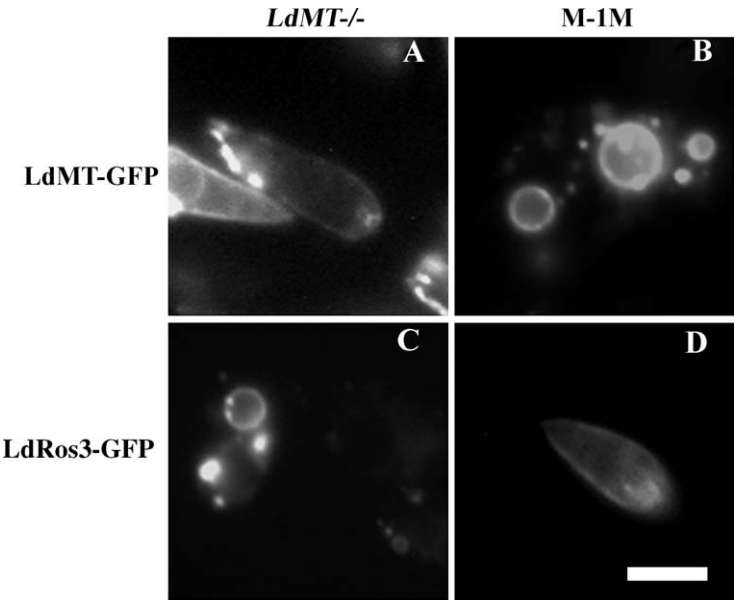

Fig. 3. LdMT and LdCDC50 are normally localized at the parasite plasma membrane. *LdMT−/−* parasites (lacking LdMT protein) (A and C) and M-1M parasites (lacking LdRos3) (B and D) were transfected with LdMT-GFP (A and B) or LdRos3-GFP (C and D) and the localization of the GFP chimeras were studied by fluorescence images of hypotonic buffer-treated parasites. LdMT and LdCDC50 are localized at the plasma membrane (A and D), but in the absence of the corresponding subunits (B, C) are localized in intracellular organelles (Bar, 5 μm). Adapted from Pérez-Victoria et al. (manuscript in preparation).

also the correlation between the expression levels of both proteins and the parasite response to the drug. MIL uptake correlates extraordinarily well with the sensitivity to the drug. The uptake levels – and therefore the sensitivity – depends on the expression levels of the functional flippase machinery at the plasma membrane. In an experimental resistant line to 40 μM MIL (M-40 R), which contain only inactivated LdMT polypeptides, the expression of a functional LdMT-GFP chimera restored MIL uptake and sensitivity. Furthermore, the level of MIL uptake correlated with the expression of LdMT-GFP (Fig. 4). When wild-type parasites overexpressed LdMT, they became hypersensitive to the drug, due to its ability to take up higher amounts of MIL (Pérez-Victoria et al., 2003b). But again, LdMT is only functional under the presence of LdRos3. Indeed, M1-M parasites (which lack LdRos3) were still deficient in MIL uptake after the overexpression of LdMT (Pérez-Victoria et al., manuscript in preparation). Introduction of a functional LdRos3-GFP chimera increased again MIL uptake and sensitivity in a manner that depended on the expression levels of LdRos3-GFP (Fig. 4). Nevertheless, when LdRos3 was overexpressed in wild-type parasites, no further increase in MIL uptake was observed. All these data indicate that either LdMT or LdRos3 can be the protein that limits MIL uptake, and that under normal circumstances (those of wild-type parasites) *L. donovani* promastigotes have an excess of LdRos3 in comparison of LdMT. It further suggests that both proteins form part of the same MIL translocation machinery present at the parasite plasma membrane, and therefore some kind of stoichiometric relationship may apply.

Does the inactivation of LdMT or LdRos3 produce resistant parasites in in vivo situations? We do not yet have an

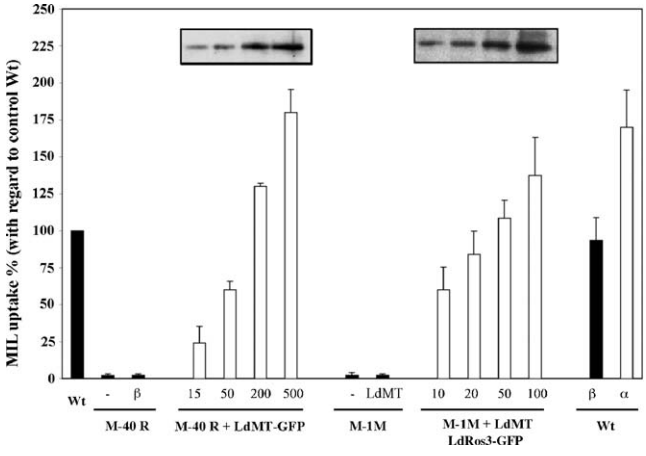

Fig. 4. Dependence of both LdMT and LdRos3 for the uptake of [^{14}C]MLF and correlation between their expression and uptake levels in *L. donovani*. The uptake of [^{14}C]MLF was measured after 60 min incubations for the following lines: wild-type parasites (Wt); M-40 R parasites (M-40 R), which contain inactivating mutations in LdMT; M-40 R parasites transfected with LdRos3 (β); M-40 R transfected with the pXG′-LdMT-GFP construction and selected against increasing concentrations of G-418 (15, 50, 100 and 500 μg/ml); M-1M parasites (M-1M), which contains inactive *LdRos3* alleles; M-1M parasites transfected with LdMT (M-1M LdMT); M-1M parasites double transfected, firstly with LdMT and selected with 100 μg/ml of hygromycin B and later with LdRos3-GFP (M-1M + LdMT + LdRos3-GFP) selected with increasing concentrations of G-418 (10, 20, 50 and 100 μg/ml); wild-type parasites transfected with either LdRos3 (Wt β) or LdMT (Wt α). Bars shown are the mean ± S.D. of three independent experiments. Correlation between uptake and expression levels is further highlighted by western blots with anti-GFP antibodies, shown above the bars. Adapted from Pérez-Victoria et al. (2003b, manuscript in preparation).

answer to this key question. However, *LdMT−/−* and LdMT mutated parasites are able to infect macrophages in vitro and obtained intracellular amastigotes are remarkably less sensitive to MIL (Seifert et al., unpublished results). Therefore, LdMT (and by extension LdRos3) is required for the activity of MIL against intracellular amastigotes of *L. donovani*. We are currently testing the situation in animal models of visceral leishmaniasis, infecting with either wild-type *L. donovani* parasites or with MIL-resistant ones. The outcome will depend on at least two factors: (i) the physiological role of LdMT–LdRos3 in intracellular amastigotes and in vivo circumstances (LdMT is supposed to aid in the maintenance of plasma membrane lipid asymmetry. The meaning of this feature remains unknown for the majority of eukaryotic cells. Promastigotes *Leishmania* parasites grown in vitro are not affected at all by the loss of LdMT function, but in vivo conditions are usually far more stringent). This physiological role might determine the parasites' infectivity and virulence; (ii) the role of MIL per se in infected macrophages.

To sum up, MIL uptake and sensitivity in vitro depends on the expression levels of the LdMT–LdRos3-dependent flippase machinery at the plasma membrane. Thus, any factor decreasing the steady-state levels of the LdMT–LdRos3-dependent machinery at the plasma membrane (i.e. protein expression levels, fate of these proteins, vesicular trafficking and endocytosis of the complex, efficiency in the folding, etc.) will generate parasites less sensitive to MIL. Selection of inactivating point mutations in LdMT or LdRos3 will have dramatic consequences. As *Leishmania* are diploid organisms, the inactivation of one allele for *LdMT* should produce a two-fold decrease in drug sensitivity, as demonstrated in *LdMT+/−* parasites, since LdMT is the limiting factor of the translocation machinery. The loss of the second allele will generate parasites that are defective in the translocation of the drug, and thus highly resistant to MIL. For the case of LdRos3, the inactivation of the first allele will likely not have noticeable effects in the MIL phenotype, but inactivation of the second allele will also produce parasites completely resistant to MIL. The inactivation of LdMT under drug pressure is more likely to occur than that of LdRos3, since the ATPase is three-fold larger and the number of essential amino acids, as predicted from their conservation in other members of the phospholipid translocase subfamily, is much higher than that of LdRos3. Indeed, out of eight different mutant in vitro strains highly resistant to MIL analyzed so far, only one (M-1M) contained inactivating mutations in both alleles of *LdRos3*, and even this M-1M strain also contained an *LdMT* allele inactivated (Pérez-Victoria et al., 2003b, manuscript in preparation).

4.2. Involvement of ABC transporters in experimental MIL resistance

Another mechanism to reduce the intracellular MIL accumulation, and consequently to acquire resistance to MIL by *Leishmania*, could be the overexpression of ABC transporters, acting as MIL floppases. The ABC (ATP-binding cassette) transporters constitute a family of large molecular weight membrane proteins that mediate the movement of molecules through the membranes in an ATP dependent process (reviewed in Borst and Elferink, 2002); specifically the P-glycoprotein MDR1 (ABCB subfamily) is able to export a wide range of hydrophobic drugs from the cell, decreasing their intracellular concentration and preventing their cytotoxic activity, thus conferring a multidrug resistance (MDR) phenotype. A *L. tropica* line overexpressing P-glycoprotein-MDR1 showed a significant cross-resistance to MIL and edelfosine (9.2- and 7.1-fold, respectively) (Perez-Victoria et al., 2001). It seems reasonable that P-glycoprotein was directly responsible of the resistance phenotype, because: (i) the resistance phenotype was dependent on the overexpression of P-glycoprotein; (ii) the MIL resistance phenotype could be overcome by specific P-glycoprotein inhibitors; (iii) MIL and edelfosine were able to modulate the P-glycoprotein-mediated resistance to daunomycin in the MDR line.

Some members of the ABC superfamily have recently been shown to mediate the translocation of specific lipid molecules from the cytosolic to the luminal leaflets of membranes. MDR3 is responsible of the specific translocation of phosphatidylcholine in the canalicular membrane of hepatocytes (van Helvoort et al., 1996). MDR1 and MRP1 have been shown to mediate the flop of certain phospholipids both in total cells and in reconstituted proteoliposomes (reviewed in Raggers et al., 2000). Furthermore, the overexpression of MDR1 has been linked to MIL and edelfosine resistance in certain cell lines, including KB and HeLa (Rybczynska et al., 2001a). Interestingly, cell lines in which MDR1 mediates MIL resistance were intrinsically more sensitive to MIL than those in which MDR1 overexpression did not significantly induce drug resistance (Rybczynska et al., 2001a). It might be possible that MDR1 is mediating the efflux of MIL, but only when a high intrinsic accumulation of MIL is achieved that efflux turns significant. Alternatively, overexpression of P-glycoprotein-like transporters could indirectly contribute to the MIL resistance as suggested by Hoffmann et al. (1997) by inducing changes in the physical properties of the cell membrane. Indeed, mdr1 gene transfections are described to alter the fluidity of the membrane in mammalian cells (Callaghan et al., 1992), and this change has been described to modify the effects of alkyl-lysophospholipids (Storme et al., 1985). Recent data support a role for *L. tropica* P-glycoprotein in the efflux of MIL from inside the cell (Pérez-Victoria et al., unpublished results). The *L. tropica* MDR line accumulated 5–10-fold less MIL than its parental line. This reduced accumulation was most likely due to an increase in the efflux, since P-glycoprotein inhibitors were able to restore the normal MIL uptake phenotype without affecting the parental line.

Until recently, the *Leishmania* multidrug transporters were thought to be involved in resistance to drugs not used to treat leishmaniasis in the field. The involvement of P-glycoprotein MDR1 in MIL resistance together with the

fact that many new potential leishmanicidal agents, such as azoles, are known substrates of ABC transporters, strengthens the clinical relevance of ABC transporters. It also supports the ever-increasing interest in the development of new specific inhibitors against the activity of these proteins (Perez-Victoria et al., 2001).

The role of other ABC transporters in MIL resistance has not been tested in *Leishmania* parasites, excepting the ABCA1 and ABCA2 proteins from *L. infantum*. Both proteins seem to mediate the efflux of fluorescent phospholipid analogues, although their overexpression did not yield significant levels of MIL resistance (Parodi-Talice et al., 2003; Araujo-Santos et al., 2005). It will be interesting to test the effects of overexpressing some other ABC transporters, specially MRP1, which has been localized to intracellular organelles (Legare et al., 2001) and could therefore promote MIL resistance by sequestration of the drug.

In summary, so far the only described mechanisms of MIL resistance in experimental lines involve a decreased accumulation of the drug, either by decreasing its uptake or by increasing its efflux. Other mechanisms that cope with high intracellular MIL concentrations have yet to be investigated. Remarkably, decreasing MIL uptake seems to be the easiest way to develop high levels of MIL resistance. Indeed, it is the mechanism that has always developed after MIL pressure (Seifert et al., 2003; Pérez-Victoria et al., 2003b, manuscript in preparation). Mechanistically, acquiring inactivating point mutations in a single gene (but in two alleles) is much simpler than down-regulating the expression of a gene product or overexpressing a protein. Moreover, the resulting phenotype is stable, being transferred to following generations (as opposed to gene expression regulation, which is usually lost over time). The only drawback for the parasite to follow this way (and the good news for the long-term use of MIL) would be an important physiological role for the protein being inactivated. Whether this is the case for LdMT–LdRos3 is not yet resolved. In any case it would be extremely important to prevent the generation of high MIL resistant parasites with a stable phenotype. Once generated, resistant parasites that show a defective uptake phenotype could expand rapidly in endemic areas, shortening the life span for an efficient use of MIL. Mechanisms for leishmaniasis control should then be put to work in that direction.

5. Experimental efficacy of MIL combinations with other drugs

Combining drugs to control the problem of resistance has been used in the treatment of pulmonary tuberculosis since the early 1950s (Ellard, 1984) and widely been adopted for the treatment of other infectious and parasitic diseases, such as fungal infections (Cuenca-Estrella, 2004), HIV/AIDs and malaria (White, 1999).

Combination therapy could impede the evolution of resistance by (i) reducing the population size (in case of synergistic interactions between drugs) and (ii) reducing the overall rate of resistance. The probability of simultaneously overcoming different types of inhibition is the product of the probabilities of developing resistance to either agent used on its own (Anderson, 2005). Mechanisms of action are poorly understood for anti-leishmanials, but it is important to note that drugs against leishmaniasis, either on the market or on clinical trial, belong to different chemical classes of compounds (Croft and Coombs, 2003). Furthermore, drug combinations could increase efficacy, shorten duration of treatment and increase compliance.

In VL combined drug treatments have been assessed in experimental models as well as clinical settings previously (Chunge et al., 1985, 1990; Seaman et al., 1993; Neal et al., 1995; Murray and Hariprashad, 1996; Thakur et al., 2000), mainly for sodium stibogluconate plus paromomycin (aminosidine) or sodium stibogluconate plus allopurinol.

Seifert and Croft (2006) recently investigated the in vitro and in vivo interactions between MIL and other standard anti-leishmanial drugs to identify suitable MIL combinations for the therapy of VL. Using the standard amastigote-macrophage model some degree of synergism was demonstrated between MIL and sodium stibogluconate in vitro against *L. donovani*. Interactions with amphotericin B, sitamaquine and paromomycin were described as indifferent. Similar patterns were observed when the promastigote stage of the parasite was used (unpublished data). In vivo a different picture emerged (Table 1). No significant interaction was observed when MIL was co-administered with sodium stibogluconate in the standard *L. donovani* BALB/c mouse model. An 11-fold increase of MIL activity was found when co-administered with the top dose of 0.5 mg/kg amphotericin B and vice versa. Paromomycin enhanced the activity of MIL by a factor of 7 when given at the top dose of 63 mg/kg. A decrease in MIL activity was observed at an intermediate dose of 21 mg/kg paromomycin. However, combination with the maximal tolerable drug exposure seems more relevant than combinations with suboptimal ones (Johnson et al., 2004). Hence, ranking of potential partner drugs for MIL favoured amphotericin B and paromomycin over sodium stibogluconate based on the in vivo data obtained in this study. Findings also point out the importance of in vivo studies,

Table 1
Summary of activity enhancement indices (AEIs) ± S.E.M. for in vivo interactions

Partner drug	Dose (mg/kg)	ED_{50} MIL (mg/kg)	AEI
Sodium stibogluconate	0	5.06	
	30	2.13	2.38
Amphotericin B	0	11.08	
	0.5	0.98	11.31
Paromomycin	0	7.36	
	63	1.02	7.22

Activity enhancement indices are calculated as follows: ED_{50} of MIL alone/ED_{50} of MIL in combination. Table taken and modified from Seifert and Croft (2006).

in which results can differ remarkably from in vitro assays, which lack the pharmacokinetic system and various other host factors.

Another approach was taken by Gupta et al. (2005) and MIL combined with picroliv, an immunostimulant isolated from the plant *Picrorhiza kurooa*. In hamsters infected with *L. donovani* the combination of 25 mg/kg × 5 days MIL plus 10 mg/kg × 33 days picroliv enhanced MIL efficacy significantly and close to a dose of 50 mg/kg × 5 days.

The need to consider drug combinations in antileishmanial therapy has been pointed out by several authors (Bryceson, 2001; Sundar, 2001; Croft, 2004). How experimental combination data translates into the clinical setting, the ultimate and most important one, remains open at the present. Leishmaniasis is a neglected disease and few drugs are in development. MIL is the latest drug to reach the market and it is essential not to jeopardize its life span.

6. A policy to prevent MIL resistance in leishmaniasis

6.1. Concepts in drug resistance: the example of antimony resistance in Bihar

The concept of drug resistance in clinical leishmaniasis is not straightforward, and sometimes it is confused with therapeutic failure, unresponsiveness or relapse. Therapeutic (or treatment) failure indicates that a patient did not fully recover from the disease during and after the treatment. Thus, it presents two different forms: (i) unresponsiveness, when the treatment fails from the beginning; (ii) relapse, when the patient initially recovers but sooner or later after completing the treatment, the disease starts to manifest again. Both cases may or may not be caused by resistance of the parasite to the drug. Cure rates depend not only on the efficacy of the drug, but on a number of host factors that aim to clear the parasites from the infected cells. Treatment failure due to drug resistance can similarly be divided into two groups (Bryceson, 2001): (i) one in which parasites causing the infection are resistant to the drug even before the treatment starts (which has been previously named as primary resistance); (ii) another in which parasites become resistant inside the patient during or after the treatment course (named as secondary resistance). Primary resistance is basically handled by controlling transmission. Preventing relapses and the generation of drug resistant mutants control secondary resistance (which will decrease primary resistances as well). Relative importance of primary or secondary resistances (or even both) depends on the drug considered and the features of the setting. In the case of antimonials, which have been widely and successfully used for decades, unresponsiveness is in principle likely to indicate resistance. Indeed, drug resistant field isolates leading to therapeutic failure have been widely described in endemic areas of the Indian subcontinent (Lira et al., 1999; Dube et al., 2005). It is important to take the lessons from the antimonial drugs case in India. Only in the state of North Bihar, highly endemic for VL, 60% of the patients do not respond to the standard antimonial treatment (Sundar et al., 2000). Antimonials were first used 60 years ago. Through the years, a 10-fold increase in dosage/duration of treatment has been implemented, starting in the 1980s. The first hints of the emergence of drug resistance were an increase in treatment failure due to the appearance of relapses. Then, relapses became more significant with time. In the early 1990s, unresponsive patients started to constitute significant numbers. Since then, a steady increase in unresponsive patients has been observed, until the 60% current prevalence (Sundar et al., 2000b; Sundar, 2001). Although impossible to establish with certainty, it looks likely that field isolates started to develop low levels of antimonials resistance (thus the increase in dosage/duration of treatment); then, higher resistance levels appeared in more and more parasite populations. Some of those resistant populations were fit enough as to infect the vector and continue the cycle in new infected human beings, causing the first unresponsive cases. From this point, the wide dissemination of drug resistant parasites was just a matter of time, considering the anthroponotic and endemic situation in north Bihar and the continuous use of antimonial drugs.

6.2. Determinants for the development of MIL resistance

A number of features can determine the likelihood and ease in the generation of resistant parasites that lead to treatment failure. We can differentiate between those factors that inherently come with a given drug (intrinsic determinants) from those that can be controlled by human behaviour (extrinsic determinants):

(i) *Intrinsic*. MIL shows a long half-life (150–200 h) and requires long treatment courses (28 days). Furthermore, the therapeutic ratio for MIL is very narrow. Thus, subtherapeutic levels may remain for some weeks after the standard treatment. Finally, the intrinsic mechanism of MIL action might implicate higher chances for developing drug resistance. Although the actual mechanism of MIL action is unknown, drug uptake is clearly a prerequisite for its action. In vitro studies with promastigotes have shown that the parasite responds to MIL pressure diminishing its uptake. Indeed, a fairly simple mechanism, selection of inactivating point mutations in any of the genes essential for MIL uptake, yields parasites highly resistant to MIL in a stable and transmissible phenotype. All these intrinsic features would indicate that chances for arising MIL resistant parasites are likely higher than for other drugs.

(ii) *Extrinsic*. A number of inappropriate human practices can make the difference in the initial gaining and further transmission of resistant parasites. The current situation in India highlights the importance of controlling these factors (Sundar and Murray, 2005). The most important one is the incomplete compliance, which is hampered by a number of additive problems: the high prices for MIL,

clearly not affordable for the majority of the population at certain endemic areas such as north Bihar (current treatment price is around US$ 150, whereas daily family income is approximately US$ 1); the current availability in the market, without any kind of regulation, which allows patients to purchase (and sell) small supplies of the drug and then discontinue treatment as symptoms disappear; the absence of medical control over compliance, which has been obvious even during the phase IV clinical trial. Many patients discontinued treatment or were lost to follow-up even though the drug was freely dispensed to enrolled patients once a week for 4 weeks (Sundar and Murray, 2005).

All of the above, together with the future MIL major use as a single agent in India might lead to the rapid emergence of widespread resistance, which would be a tragedy.

6.3. Policies to prevent the appearance and spread of MIL resistant mutants

(i) Access to the drug only through the public health system in endemic areas. This policy involves MIL prescription only by qualified physicians after a proper diagnosis has been established. MIL should then be given for free in a controlled manner under the auspicious of the government.

(ii) Control of compliance: Bound to the previous practice, MIL should constitute a directly observed therapy, similar to that already well established in India and other countries for tuberculosis with the DOT system.

(iii) Combination therapy: Together with complete compliance, this is the most important strategy to prevent drug resistances. It is not yet developed in the case of MIL (see above). Pilot studies should be followed with already marketed drugs. Positive drug combinations should be incorporated to the common practice as soon as the clinical data from the pilot studies are available.

(iv) Monitoring drug resistance: Ideally, parasite resistance should be monitored, rather than patient relapse rates. Endemic countries should be encouraged to set up reference laboratories capable of testing drug sensitivity of clinical isolates. For the case of MIL, tests in promastigote forms, easier to handle, could be sufficient, so even small clinical settings could have access to the tools required for the experiments. Thus, standardized in vitro sensitivity tests and control strains (specially a highly resistant in vitro line) should be made available for interested labs. Developing drug resistance markers and tools easy to use in the field should be encouraged. Given the already characterized genetic markers that determine high MIL resistance levels, namely *LdMT* and *LdRos3*, analysis of their mutations should be performed systematically for every parasite isolate that shows low MIL sensitivity. This strategy would facilitate tracking the level of their spread in affected populations, if ever generated. Collaborations between field physicians and researchers could help to implement this goal. It would then be possible to establish guidelines along the lines of those that exist for malaria or tuberculosis specific for each endemic region.

(v) Transmission control: Control of the vector; treatment of all patients early after the manifestations of the disease; in areas of zoonotic leishmaniasis, where the dog is the reservoir for the parasite, avoid the use of MIL for veterinary treatment.

7. Concluding remarks

MIL has been approved by Germany and Colombia, in addition to India. Three countries in South Asia (India, Nepal and Bangladesh) have decided to use MIL as the first line drug in their efforts to eliminate VL from the region by 2015. Unfortunately, the drug has been made available in the unregulated private sector of India at a high cost US$ ∼150. The most obvious solution to save this very important and only orally effective drug is to make it available free of cost through the public sector. At the same time, the compliance must be ensured through directly observed therapy. In India, one alternative is to link it through the revised national tuberculosis control programme (RNTCP). Further, the drug must be largely withdrawn from the private sector to prevent its misuse, and should be released in the private sector only in a regulated manner through trained/qualified physicians who should ensure compliance. Monitoring drug resistance and establishing combination therapies should come next.

Another important issue with MIL is its teratogenicity in animal models, as one mishap in humans could be sufficient to kill the drug. Level of literacy and awareness in endemic countries like those in Indian subcontinent demands additional measures to ensure that no pregnant women are prescribed MIL, and that contraception in women with child bearing age groups is made certain. In a recently concluded phase IV trial, several events of pregnancy were reported. Despite no malformation in pregnancy outcome, these events foretell the kind of chaotic situation one may land if proper precautions are not taken.

Acknowledgments

We are grateful to Zentaris (Frankfurt, Germany) for all the support and interest during the work of experimental characterization of mechanisms of MIL resistance in *Leishmania* and for providing us the MIL used in this study. This work was supported by EC grant QLRT-2000-01404 to S.L.C. and F.G., by the Spanish grant SAF2001-4562-E to F.G., by FIS Network RICET C03-04, by EU Marie Research Training Network grant MRTN-CT-2004-005330 to F.G. and by the Spanish grant SAF2005-01639 to S.C. María P. Sanchez-

Cañete was the recipient of a fellowship from the Ministerio de Educación y Cultura.

References

Anderson, J.B., 2005. Evolution of antifungal-drug resistance: mechanisms and pathogen fitness. Nat. Rev. Microbiol. 3, 547–556.

Araujo-Santos, J.M., Parodi-Talice, A., Castanys, S., Gamarro, F., 2005. The overexpression of an intracellular ABCA-like transporter alters phospholipid trafficking in *Leishmania*. Biochem. Biophys. Res. Commun. 330, 349–355.

Arthur, G., Bittman, R., 1998. The inhibition of cell signalling pathways by antitumor ether-lipids. Biochim. Biophys. Acta 1390, 85–102.

Bhattacharya, S.K., Jha, T.K., Sundar, S., Thakur, C.P., Engel, J., Sindermann, H., Junge, K., Karbwang, J., Bryceson, A.D., Berman, J.D., 2004. Efficacy and tolerability of miltefosine for childhood visceral leishmaniasis in India. Clin. Infect. Dis. 38, 217–221.

Beach, D.H., Holz G.G.Jr., Anekwe, G.E., 1979. Lipids of *Leishmania* promastigotes. J. Parasitol. 65, 201–216.

Beach, D.H., Goad, L.J., Holz, G.G., 1988. Effects of antimytotic azoles on growth and sterol biosynthesis of *Leishmania* promastigotes. Mol. Biochem. Parasitol. 31, 149–162.

Beckers, T., Voegeli, R., Hilgard, P., 1994. Molecular and cellular effects of hexadecylphosphocholine (miltefosine) in human myeloid leukaemia cell lines. Eur. J. Cancer 30A, 2143–2150.

Borst, P., Elferink, R.O., 2002. Mammalian ABC transporters in health and disease. Annu. Rev. Biochem. 71, 537–592.

Brachwitz, H., Vollgraf, C., 1995. Analogs of alkyllysophospholipids: chemistry, effects on the molecular level and their consequences for normal and malignant cells. Pharm. Ther. 66, 39–82.

Bryceson, A., 2001. A policy for leishmaniasis with respect to the prevention and control of drug resistance. Trop. Med. Int. Health 6, 928–934.

Bull, L.N., van Eijk, M.J., Pawlikowska, L., DeYoung, J.A., Juijn, J.A., Liao, M., Klomp, L.W., Lomri, N., Berger, R., Scharschmidt, B.F., Knisely, A.S., Houwen, R.H., Freimer, N.B., 1998. A gene encoding a P-type ATPase mutated in two forms of hereditary cholestasis. Nat. Genet. 18, 219–224.

Burk, K., David, M., Junge, K., Sindermann, H., 1994. Overview on the clinical development of miltefosine solution (Miltex) for the treatment of cutaneous breast cancer. Drugs Today 30, 59–72.

Callaghan, R., van Gorkom, L.C., Epand, R.M., 1992. A comparison of membrane properties and composition between cell lines selected and transfected for multi-drug resistance. Br. J. Cancer 66, 781–786.

Chunge, C.N., Gachihi, G., Muigai, R., Wasunna, K., Rashid, J.R., Chulay, J.D., Anabwani, G., Oster, C.N., Bryceson, A.D., 1985. Visceral leishmaniasis unresponsive to antimonial drugs. III. Successful treatment using a combination of sodium stibogluconate plus allopurinol. Trans. R. Soc. Trop. Med. Hyg. 79, 715–718.

Chunge, C.N., Owate, J., Pamba, H.O., Donno, L., 1990. Treatment of visceral leishmaniasis in Kenya by aminosidine alone or combined with sodium stibogluconate. Trans. R. Soc. Trop. Med. Hyg. 84, 221–225.

Croft, S.L., 2004. Drug Resistance in Leishmaniasis. World Health Organization, pp. 108–119.

Croft, S.L., Barrett, M., Urbina, J.A., 2005. Chemotherapy of trypanosomiasis and leishmaniasis. Trends Parasitol. 21, 508–512.

Croft, S.L., Coombs, G.H., 2003. Leishmaniasis—current chemotherapy and recent advances in the search for novel drugs. Trends Parasitol. 19, 502–508.

Croft, S.L., Neal, R.A., Pendergast, W., Chan, J.H., 1987. The activity of alkylphosphorylcholines and related derivatives against *Leishmania donovani*. Biochem. Pharmacol. 36, 263–2636.

Cuenca-Estrella, M., 2004. Combinations of antifungal agents in therapy—what value are they? J. Antimicrob. Chemother. 54, 854–869.

Cupolillo, E., Medina-Acosta, E., Noyes, H., Momen, H., Grimaldi Jr., G., 2000. A revised classification for *Leishmania* and *Endotrypanum*. Parasitol. Today 16, 142–144.

Dube, A., Singh, N., Sundar, S., 2005. Refractoriness to the treament of sodium stibogluconate in Indian kala-azar field isolates persist in in vitro and in vivo experimental models. Parasitol. Res. 96, 216–223.

Ellard, G.A., 1984. Rationale of the multidrug regimens recommended by a World Health Organization Study Group on Chemotherapy of Leprosy for Control Programs. Int. J. Lepr. Other Mycobact. Dis. 52, 395–401.

Escobar, P., Matu, S., Marques, C., Croft, S.L., 2002. Sensitivities of *Leishmania* species to hexadecylphosphocholine (miltefosine), ET-18-OCH(3) (edelfosine) and amphotericin B. Acta Trop. 81, 151–157.

Gajate, C., Mollinedo, F., 2002. Biological activities, mechanisms of action and biomedical prospect of the antitumor ether phospholipid ET-18-OCH(3) (edelfosine), a proapoptotic agent in tumor cells. Curr. Drug Metab. 3, 491–525.

Ganguly, N.K., 2002. Oral miltefosine may revolutionize treatment of visceral leishmaniasis. TDR News, World Health Organisation, vol. 68, p. 2.

Goad, L., Holz, G.G., Beach, D.H., 1984. Sterols of *Leishmania* species. Implication for biosynthesis. Mol. Biochem. Parasitol. 10, 161–170.

Grant, A.M., Hanson, P.K., Malone, L., Nichols, J.W., 2001. NBD-labeled phosphatidylcholine and phosphatidylethanolamine are internalized by transbilayer transport across the yeast plasma membrane. Traffic 2, 37–50.

Grogl, T., Thomason, N., Franke, E.D., 1992. Drug resistance in leishmaniasis: its implication in systemic chemotherapy of cutaneous and mucocutaneous disease. Am. J. Trop. Med. Hyg. 47, 117–126.

Gupta, S., Ramesh, S.C., Srivastava, V.M., 2005. Efficacy of picroliv in combination with miltefosine, an orally effective antileishmanial drug against experimental visceral leishmaniasis. Acta Trop. 94, 47.

Hanson, P.K., Malone, L., Birchmore, J.L., Nichols, J.W., 2003. Lem3p is essential for the uptake and potency of alkylphosphocholine drugs, edelfosine and miltefosine. J. Biol. Chem. 278, 36041–36050.

Hilgard, P., Kampherm, E., Nolan, L., Pohl, J., Reissmann, T., 1991. Investigation into the immunological effects of miltefosine, a new anticancer agent under development. J. Cancer Res. Clin. Oncol. 117, 403–408.

Hoffmann, J., Utz, I., Spitaler, M., Hofe, S., Rybczynska, M., Beck, W.T., Herrmann, D.B., Grunicke, H., 1997. Resistance to the new anti-cancer phospholipid ilmofosine (BM41 440). Br. J. Cancer 76, 862–869.

Jha, T.K., Sundar, S., Thakur, C.P., Bachmann, P., Karbwang, J., Fischer, C., Voss, A., Berman, J., 1999. Miltefosine, an oral agent, for the treatment of Indian visceral leishmaniasis. N. Engl. J. Med. 341, 1795–1800.

Johnson, M.D., MacDougall, C., Ostrosky-Zeichner, L., Perfect, J.R., Rex, J.H., 2004. Combination antifungal therapy. Antimicrob. Agents Chemother. 48, 693–715.

Kuhlencord, A., Maniera, T., Eibl, H., Unger, C., 1992. Hexadecylphosphocholine: oral treatment of visceral leishmaniasis in mice. Antimicrob. Agents Chemother. 36, 1630–1634.

Legare, D., Richard, D., Mukhopadhyay, R., Stierhof, Y.D., Rosen, B.P., Haimeur, A., Papadopoulou, B., Ouellette, M., 2001. The Leishmania ATP-binding cassette protein PGPA is an intracellular metal-thiol transporter ATPase. J. Biol. Chem. 276, 26301–26307.

Lira, R., Contreras, L.M., Rita, R.M., Urbina, J.A., 2001. 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. J. Antimicrob. Chemother. 47, 537–546.

Lira, R., Sundar, S., Makharia, A., Kenney, R., Gam, A., Saraiva, E., Sacks, D., 1999. Evidence that the high incidente of treatment failures in Indian kala-azar is due to the emergente of antimony resistant strains of *Leishmania donovani*. J. Infect. Dis. 180, 564–567.

Lux, H., Heise, N., Klenner, T., Hart, D., Opperdoes, F.R., 2000. Ether-lipid (alkyl-phospholipid) metabolism and the mechanism of action of ether-lipid analogues in *Leishmania*. Mol. Biochem. Parasitol. 111, 1–14.

McConville, M.J., Mullin, K.A., Ilgoutz, S.C., Teasdale, R.D., 2002. Secretory pathway of trypanosomatid parasites. Microbiol. Mol. Biol Rev. 66, 122–154.

Murray, H.W., Delph-Etienne, S., 2000. Visceral leishmanicidal activity of hexadecylphosphocholine (miltefosine) in mice deficient in T cells and activated macrophage microbicidal mechanisms. J. Infect. Dis. 181, 795–799.

Murray, H.W., Hariprashad, J., 1996. Activity of oral atovaquone alone and in combination with antimony in experimental visceral leishmaniasis. Antimicrob. Agents Chemother. 40, 586–587.

Neal, R.A., Allen, S., McCoy, N., Olliaro, P., Croft, S.L., 1995. The sensitivity of *Leishmania* species to aminosidine. J. Antimicrob. Chemother. 35, 577–584.

Paris, C., Loiseau, P.M., Bories, C., Breard, J., 2004. Miltefosine induces apoptosis-like death in *Leishmania donovani* promastigotes. Antimicrob. Agents Chemother. 48, 852–859.

Parodi-Talice, A., Araujo, J.M., Torres, C., Pérez-Victoria, J.M., Gamarro, F., Castanys, S., 2003. The overexpression of a new ABC transporter in Leishmania is related to phospholipid trafficking and reduced infectivity. Biochim. Biophys. Acta 1612 (2), 195–207.

Pérez-Victoria, F.J., Castanys, S., Gamarro, F., 2003a. Resistance to miltefosine in *Leishmania donovani* involves a defective inward translocation of the drug. Antimicrob. Agents Chemother. 47, 2397–2403.

Pérez-Victoria, F.J., Gamarro, F., Ouellette, M., Castanys, S., 2003b. Functional cloning of the miltefosine transporter: a novel P-type phospholipid translocase from *Leishmania* involved in drug resistance. J. Biol. Chem. 278, 49965–49971.

Perez-Victoria, J.M., Pérez-Victoria, F.J., Parodi-Talice, A., Jimenez, I.A., Ravelo, A.G., Castanys, S., Gamarro, F., 2001. Alkyl-lysophospholipid resistance in multidrug-resistant *Leishmania tropica* and chemosensitization by a novel P-glycoprotein-like transporter modulator. Antimicrob. Agents Chemother. 45, 2468–2474.

Pomorski, T., Lombardi, R., Riezman, H., Devaux, P.F., van Meer, G., Holthuis, J.C., 2003. Drs2p-related P-type ATPases Dnf1p and Dnf2p are required for phospholipid translocation across the yeast plasma membrane and serve a role in endocytosis. Mol. Biol. Cell. 14, 1240–1254.

Pomorski, T., Holthuis, J.C.M., Herrmann, A., van Meer, G., 2004. Tracking down lipid flippases and their biological functions. J. Cell Sci. 117, 805–813.

Quispe-Tintaya, K.W., Laurent, T., Decuypere, S., Hide, M., Banuls, A.L., De Doncker, S., Rijal, S., Canavate, C., Campino, L., Dujardin, J.C., 2005. Fluorogenic assay for molecular typing of the *Leishmania donovani* complex: taxonomic and clinical applications. J. Infect. Dis. 192, 685–692.

Raggers, R.J., Pomorski, T., Holthuis, J.C., Kalin, N., van Meer, G., 2000. Lipid traffic: the ABC of transbilayer movement. Traffic 1, 226–234.

Rakotomanga, M., Saint-Pierre-Chazalet, M., Loiseau, P.M., 2005. Alteration of fatty acid and sterol metabolism in miltefosine-resistant *Leishmania donovani* promastigotes and consequences for drug–membrane interactions. Antimicrob. Agents Chemother. 49, 2677–2686.

Rybczynska, M., Liu, R., Lu, P., Sharom, F.J., Steinfels, E., Pietro, A.D., Spitaler, M., Grunicke, H., Hofmann, J., 2001a. MDR1 causes resistance to the antitumour drug miltefosine. Br. J. Cancer 84, 1405–1411.

Rybczynska, M., Spitaler, M., Knebel, N.G., Boeck, G., Grunicke, H., Hofmann, J., 2001b. Effects of miltefosine on various biochemical parameters in a panel of tumor cell lines with different sensitivities. Biochem. Pharmacol. 62, 765–772.

Santa-Rita, R.M., Santos Barbosa, H., Meirelles, M.N., de Castro, S.L., 2000. Effect of the alkyl-lysophospholipids on the proliferation and differentiation of *Trypanosoma cruzi*. Acta Trop. 75, 219–228.

Schraner, C., Hasse, B., Hasse, U., Baumann, D., Faeh, A., Burg, G., Grimm, F., Mathis, A., Weber, R., Gunthard, H.F., 2005. Successful treatment with miltefosine of disseminated cutaneous leishmaniasis in a severely immunocompromised patient infected with HIV-1. Clin. Infect. Dis. 40, 120–124.

Seaman, J., Pryce, D., Sondorp, H.E., Moody, A., Bryceson, A.D., Davidson, R.N., 1993. Epidemic visceral leishmaniasis in Sudan: a randomized trial of aminosidine plus sodium stibogluconate versus sodium stibogluconate alone. J. Infect. Dis. 168, 715–720.

Seifert, K., Matu, S., Pérez-Victoria, F.J., Castanys, S., Gamarro, F., Croft, S.L., 2003. Characterisation of *Leishmania donovani* promastigotes resistant to hexadecylphosphocholine (miltefosine). Int. J. Antimicrob. Agents 22, 380–387.

Seifert, K., Croft, S.L., 2006. In vitro and in vivo interactions between miltefosine and other anti-leishmanial drugs. Antimicrob. Agents Chemother. 50, 73–79.

Sindermann, H., Engel, K.R., Fischer, C., Bommer, W., 2004. Oral miltefosine for leishmaniasis in immunocompromised patients: compassionate use in 39 patients with HIV infection. Clin. Infect. Dis. 39, 1520–1523.

Soto, J., Arana, B.A., Toledo, J., Rizzo, N., Vega, J.C., Diaz, A., Luz, M., Gutierrez, P., Arboleda, M., Berman, J.D., Junge, K., Engel, J., Sindermann, H., 2004. Miltefosine for new world cutaneous leishmaniasis. Clin. Infect. Dis. 38, 1266–1272.

Soto, J., Toledo, J., Soto, J., Toledo, J., Gutierrez, P., Nicholls, R.S., Padilla, J., Engel, J., Fischer, C., Voss, A., Berman, J., 2001. Treatment of American cutaneous leishmaniasis with miltefosine, an oral agent. Clin. Infect. Dis. 33, 57–61.

Storme, G.A., Berdel, W.E., van Blittterswijk, W.J., Bruyneel, E.A., De Bruyne, G.K., Marcel, M.M., 1985. Antiinvasive effect of racemic 1-*O*-octadecyl-2-*O*-methlglycero-3-phosphocholine on MO4 mouse fibrosarcoma cells in vitro. Cancer Res. 45, 351–357.

Sundar, S., 2001. Drug resistance in Indian visceral leishmaniasis. Trop. Med. Int. Health 6, 654–849.

Sundar, S., Jha, T.K., Sindermann, H., Junge, K., Bachmann, P., Berman, J., 2003. Oral miltefosine treatment in children with mild to moderate Indian visceral leishmaniasis. Pediatr. Infect. Dis. J. 22, 434–438.

Sundar, S., Jha, T.K., Thakur, C.P., Engel, J., Sindermann, H., Fischer, C., Klaus, K., Anthony Bryceson, A., Berman, J., 2002. Oral miltefosine for Indian visceral leishmaniasis. N. Engl. J. Med. 347, 1739–1746.

Sundar, S., Makharia, A., More, D.K., Agrawal, G., Voss, A., Fischer, C., Bachmann, P., Murray, H.W., 2000a. Short-course of oral miltefosine for treatment of visceral leishmaniasis. Clin. Infect. Dis. 31, 1110–1113.

Sundar, S., More, D.K., Singh, M.K., Singh, V.P., Sharma, S., Makharia, A., Kumar, P.C., Murray, H.W., 2000b. Failure of pentavalent antimony in visceral leishmaniasis in India: report from the center of the Indian epidemic. Clin. Infect. Dis. 31, 1104–1107.

Sundar, S., Murray, H.W., 2005. Availability of miltefosine for the treatment of kala-azar in India. Bull. World Health Organ. 83, 394–395.

Tang, X., Halleck, M.S., Schlegel, R.A., Williamson, P., 1996. A subfamily of P-type ATPases with aminophospholipid transporting activity. Science 272, 1495–1497.

Thakur, C.P., Kanyok, T.P., Pandey, A.K., Sinha, G.P., Zaniewski, A.E., Houlihan, H.H., Olliaro, P., 2000. A prospective randomized, comparative, open-label trial of the safety and efficacy of paromomycin (aminosidine) plus sodium stibogluconate versus sodium stibogluconate alone for the treatment of visceral leishmaniasis. Trans. R. Soc. Trop. Med. Hyg. 94, 429–431.

van der Sanden, M.H., Houweling, M., Duijsings, D., Vaandrager, A.B., van Golde, L.M., 2004. Inhibition of phosphatidycholine sinthesis is not the primary pathway in hexadecylphosphocholine-induced apoptosis. Biochim. Biophys. Acta 1636, 99–107.

van Helvoort, A., Smith, A.J., Sprong, H., Fritzsche, I., Schinkel, A.H., Borst, P., van Meer, G., 1996. MDR1 P-glycoprotein is a lipid translocase of broad specificity, while MDR3 P-glycoprotein specifically translocates phosphatidylcholine. Cell 87, 507–517.

Verma, N.K., Dey, C.S., 2004. Possible mechanism of miltefosine-mediated death of *Leishmania donovani*. Antimicrob. Agents Chemother. 48, 3010–3015.

White, N.J., 1999. Delaying antimalarial drug resistance with combination chemotherapy. Parassitologia 41, 301–308.

Yardley, V., Croft, S.L., De Doncker, S., Dujardin, J.C., Koirala, S., Rijal, S., Miranda, C., Llanos-Cuentas, A., Chappuis, F., 2005. The sensitivity of clinical isolates of *Leishmania* from Peru and Nepal to miltefosine. Am. J. Trop. Med. Hyg. 73, 272–275.

Zeisig, R., Rudolf, M., Eue, I., Arndt, D., 1995. Influence of hexadecylphosphocholine on the release of tumor necrosis factor and nitroxide from peritoneal macrophages in vitro. J. Cancer Res. Clin. Oncol. 121, 69–75.

Zoeller, R.A., Layne, M.D., Modest, E.J., 1995. Animal cell mutants unable to take up biologically active glycerophospholipids. J. Lipid Res. 36, 1866–1875.