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

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Abstract

Miltefosine (hexadecylphosphocholine, MIL), registered as Impavido\textsuperscript{®}, has become the first oral drug for the treatment of visceral and cutaneous leishmaniasis. 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 minimize the risk for the appearance of drug failures and to ensure a long life span for this effective oral drug.

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1. Introduction

The Leishmaniases are a group of diseases with wide epidemiological and clinical diversity caused by the protozoon parasite *Leishmania*. The different clinical manifestations vary from cutaneous self-healing lesions (CL) to visceral disease (VL), fatal if left untreated. Leishmaniases 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 leishmaniases are available and control of the disease relies primarily on chemotherapy. The chemotherapy currently available for leishmaniases 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
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 in different areas. The search for an effective oral antileishmanial drug used in endemic areas where antimonials are inefficient, however from 1983 its activity was also demonstrated after oral administration to animals (Kühnencord 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 45% 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 (Schnarer et al., 2005).

MIL has a long-term 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, sub-therapeutic levels of MIL may remain for some weeks after a standard course of treatment. This characteristic might encourage the emergence of resistance (Bryceson, 2001).

**Fig. 1.** Chemical structure of MIL.

\[
\text{O} \quad \text{CH}_3 - \quad \text{CH}_3 \quad \text{O} \quad \text{P} \quad \text{O} \quad \text{CH}_3 \quad \text{N} \quad \text{CH}_3 \\
\text{CH}_2
\]

hexadecanol (Fig. 1), was synthesized at the Max-Planck-Institut für Biophysikalische Chemie in Gottingen (Germany) and successfully used for the therapy of cancer metastases. Specifically, a topical formulation of MIL (Millex) 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 (Kühnencord 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 45% 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 (Schnarer et al., 2005).

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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 (Capotillo 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 EC₅₀ 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 EC₅₀ 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 (EC₅₀ values 3.11–2.91 μM). L. major did respond to edelfosine as well as other species (EC₅₀ 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 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⁺ responders and Sb⁻ non-responders were intrinsically sensitive to MIL with EC₅₀ 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 EC₅₀ 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 (Crotch, 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 Bitman, 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 account-

(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-
Like effect (Croft et al., 2003). The metabolism of MIL inside \textit{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 flopp from the inner to the outer leaflet of the plasma membrane. Members of the ABC transporters family, such as \textit{PGP} (mdr1), are strong candidates to mediate this floppase activity (Pérez-Victoria et al., 2001; Rybczynska et al., 2001a). MIL being an oral drug and \textit{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 \textit{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 these 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 \textit{Leishmania} and \textit{Trypanosoma cruzi} (Lira et al., 2001; Santa-Rita et al., 2000), although a recent report has shown that inhibition of phosphatidyicholine 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 \textit{L. donovani} based on observed phenomena such as cell shrinkage, nuclear DNA condensation, DNA fragmentation into oligonucleosome-sized fragments and phosphatidyserine 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 Molinino, 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 \textit{Leishmania} infection.

4. Experimental MIL resistance in \textit{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 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 \textit{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 \textit{L. tropica} line previously generated was also shown to be cross-resistant to MIL and edelfosine (Pérez-Victoria et al., 2001). The common feature in all \textit{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 \textit{Leishmania} lines: the \textit{LbMT-LdHox3} dependent machinery

The characterization of \textit{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). \textit{Leishmania} promastigotes selected against high MIL concentrations (around 40 \textmu M) 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...
LdMT and LdRos3 proteins. 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 intra- cellular 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 also the specific function for these proteins. 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). 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. 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.

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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 flipase machinery at the plasma membrane. In an experimental resistant line to 40 μM MIL (M-40 R), which contains 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...
answer to this key question. However, LeDMT+/− and LeDMT mutated parasites are able to infect macrophages in vitro and obtained intracellular amastigotes are remarkably less sensitive to MIL. (Seiferli et al., unpublished results). Therefore, LeDMT (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 the phospholipid translocase subfamily, is much higher than that of LdRos3, and even this M-1M strain also contained an LeDMT 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) (Pérez-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 (Ryczynska 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 (Ryczynska 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, which is the case of LeDMT–LeRos3 parasites. 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 LeDMT under drug pressure is more likely to occur than that of LeRos3, 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 LeRos3. 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 LeRos3, and even this M-1M strain also contained an LeDMT allele inactivated (Pérez-Victoria et al., 2003b, manuscript in preparation).
fact that many new potential leishmanicidal agents, such as azoles, are known substrates of ABC transporters, strength-
ens the clinical relevance of ABC transporters. It also sup-
ports 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 pro-
teins seem to mediate the efflux of fluorescent phospholipid
analogues, although their overexpression did not yield sig-
ificant levels of MIL resistance (Parodi-Talice et al., 2003;
Arago-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 accu-
mulation 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; Perez-Victoria et al., 2003b, manuscip-
in preparation). Mechanistically, acquiring inactivating point
mutations in a single gene (but in two alleles) is much sim-
pler than down-regulating the expression of a gene product
or overexpressing a protein. Moreover, the resulting pheno-
type 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 inac-
tivated. 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 resis-
tance 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 probabili-
ties of developing resistance to either agent used on its own
(Anderson, 2005). Mechanisms of action are poorly under-
stood for anti-leishmanials, but it is important to note that
drugs against leishmaniasis, either on the market or on clini-
cal 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 (amino-
sidine) 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 combina-
tions for the therapy of VL. Using the standard amastigote-
macrophage model some degree of synergism was demon-
strated between MIL and sodium stibogluconate in vitro
against L. donovani. Interactions with amphotericin B, sita-
maquine and paromomycin were described as indifferent.
Similar patterns were observed when the promastigote stage
of the parasite was used (unpublished data). In vivo a dif-
fierent 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 intermedi-
ate dose of 21 mg/kg paromomycin. However, combination
with the maximal tolerable drug exposure seems more rel-
evant than combinations with suboptimal ones (Johnson et
al., 2004). Hence, ranking of potential partner drugs for MIL
favoured amphotericin B and paromomycin over sodium sti-
 bogluconate based on the in vivo data obtained in this study.
Findings also point out the importance of in vivo studies,

<table>
<thead>
<tr>
<th>Partner drug</th>
<th>Dose (mg/kg)</th>
<th>ED50 MIL (mg/kg)</th>
<th>AEI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium stibogluconate</td>
<td>0</td>
<td>5.06</td>
<td>0.98</td>
</tr>
<tr>
<td>30</td>
<td>2.38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>0</td>
<td>11.08</td>
<td>0.5</td>
</tr>
<tr>
<td>0.5</td>
<td>11.31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paromomycin</td>
<td>0</td>
<td>7.36</td>
<td>63</td>
</tr>
<tr>
<td>7.22</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Activity enhancement indices are calculated as follows: ED50 of MIL alone/ED50 of MIL in combination. Table taken and modified from Seifert
and Croft (2006).
Another approach was taken by Gupta et al. (2005) and MIL combined with picroliv, an immunostimulant isolated from the plant Picrorhiza kurrooa. 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 anti-leishmanial 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 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 anthropogenic 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):

1. **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, sub-therapeutic 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.
2. **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.

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