Miltefosine Susceptibility and Resistance in Leishmania: From the Laboratory to the Field

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Abstract

Miltefosine is the first effective oral drug used in the chemotherapy of leishmaniasis. The drug is more effective than pentavalent antimonials that are still considered as the drug of choice for treatment of leishmaniasis in several endemic regions. Efficacy rates of miltefosine against visceral leishmaniasis are up to 95%, while against cutaneous leishmaniasis the rates vary between 53% and 91% depending on the species of the parasite and the endemic region. Recent reports have described an increased number of relapses in miltefosine-treated patients. This review describes the main findings associated with miltefosine susceptibility and resistance in Leishmania: two important factors involved in efficacy and failure in leishmaniasis treatment using this drug.

Keywords: Leishmania; Chemotherapy; Miltefosine; Drug resistance

Introduction

Species of genus Leishmania, the causative agents of leishmaniasis, are digenetic parasites transmitted by female sand flies that reside in cells of monocyte-phagocytic system of mammals as intracellular amastigotes. Parasites of genus Leishmania are classified into two subgenera: subgenus Leishmania (Leishmania) and subgenus Leishmania (Viannia). This subdivision is based on the localization of the promastigotes in the digestive tract of the sand fly. Around 20 species of the parasite are pathogenic to human and more than 30 species of sand flies are vectors of the disease. The disease has two patterns of transmission: anthroponotic and zoonotic. In the zoonotic leishmaniasis, several mammalian species (wild or domestic) have been implicated as reservoir hosts of parasite.

The leishmaniasis are responsible for two main clinical manifestations of the disease: tegumentary or visceral leishmaniasis (VL). In the visceral form, the parasite migrates to internal organs as the liver, spleen and bone narrow and may be lethal if not treated [1]. The tegumentary form may occur as ulcerative skin lesions developing at the site of the sand fly bite (localized cutaneous leishmaniasis [LCL]), multiple non-ulcerative nodules (diffuse cutaneous leishmaniasis [DCL]) or destructive mucosal inflammation (mucosal leishmaniasis [ML]) [2]. The clinical manifestation of each form of the disease depends on the species of infecting parasite and the host immunoinflammatory response. The disease occurs in tropical and subtropical regions of the world, particularly in Africa, Asia, America and in southern of Europe affecting about 12 million people worldwide with approximately 0.2 to 0.4 million VL cases and 0.7 to 1.2 million CL cases per year [3].

The chemotherapy for leishmaniasis is restricted in efficacy and in number of available drugs. Pentavalent antimonials are still the standard drug in some endemic areas despite its adverse reactions and progressive decreasing efficacy [4]. In the district of Bihar in India for example, dosages of the drug have been increased in the last decades, reaching the maximum acceptable toxicity [5]. Moreover, in this endemic region, reports show that up to 65% of patients do not respond to the treatment with pentavalent antimonials, indicating the emergence of drug resistant parasites [6-8]. An alternative to antimonials is amphotericin B. The liposomal formulation of amphotericin is widely used in Europe for example [9]. The cure rate can reach up to 95% of efficacy in patients with VL in just a single dose of the drug [10]. Despite its high toxicity, pentamidine is another alternative used in some endemic areas where antimonials are not more effective. Similar to the antimonials, pentamidine has significantly decreased its efficacy in more than 50% in the last decades. The drug is still used in some countries in South America against CL due to L. guyanensis [11,12]. The aminoglycoside paromomycin has recently approved for the treatment of VL in India [13]. Due to its low absorption in the gut, paromomycin requires parenteral administration, as it is also required for the other drugs mentioned before.

Miltefosine (MF) is the first and still the only oral drug used in the chemotherapy of leishmaniasis [14]. The drug is an analog of alkyl-lysophospholipid, initially developed against cancer. The anti-leishmanial activity was described in vitro and in vivo [15,16] and some years later registered for the use in India against VL. The half-life of MF is between 150 and 200 hours leading to a sub-optimal concentration in the plasma, which can lead to the emergence of resistant parasites [17]. The main side effects of MF are transient gastrointestinal discomfort, vomiting, diarrhea and increase of liver enzymes and serum creatinine [18]. MF is also teratogenic and therefore is strictly contraindicated in pregnant women.

Currently, the drug was already demonstrated as effective against VL and CL in South America [19,20]. MF has a rate cure of up to 95% against VL, with 100-150 mg/day (or 2.5 mg/kg body weight) for 28 days [14,21,22]. On the other hand, cure rates for CL in South America vary between 53% and 91% in infections caused by different species of the parasite [23-26].

Mechanism of action of Miltefosine

The mechanism of action of MF is not completely understood and it is proposed that the drug has more than one target in the...
parasite. The drug acts in cell membranes inhibiting phospholipid metabolism and the parasite's membrane composition by decreasing phosphatidylcholine and increasing phosphatidylethanolamine due to reduction of intracellular choline [27]. Additionally, changes in the length and level of unsaturation of fatty acids, as well as a reduction in ergosterol levels in promastigotes resistant to MF was found [28]. In the mitochondria, promastigotes treated with MF presented a significant reduction in the mitochondrial membrane potential and the enzyme cytochrome-c oxidase is inhibited in a dose dependent manner [29,30]. The drug binds to the outer leaflet of the plasma membrane and can be internalized by two possible mechanisms: (a) by endocytic pathway: the drug is internalized as member of an endocytic vesicle or (b) by flipase activity through the activity of an ATP-dependent mechanism mediated by the miltefosine transporter (MT) and its subunit Ros3 [31]. MT is an inward-directed lipid translocase that belongs to the P4 subfamily of P-type ATPases and Ros3 is a non-catalytic subunit of this transporter, which both play an important role in phosphocholine accumulation and in maintaining the phospholipid asymmetry of the parasite membrane [32-35]. Once accumulated, the drug can be eliminated by exocytosis or by flipase activity. Some members of ABC transporters subfamilies (ABC-B and ABC-G) can mediate this transport from the inner to the outer leaflet of the plasma membrane [36-38].

Miktefosine susceptibility in Leishmania

Molecular and biochemical differences among Leishmania spp. explain the differences in MF susceptibility. It is important to state that MF susceptibilities in promastigotes and intracellular amastigotes are correlated, what leads the choice of the promastigote form, that is easier to cultivate, for determination MF sensitivity in most of the studies. An intrinsic difference in MF susceptibility is observed for different species and clinical isolates of parasite in vitro [35,39-42]. For example, among Leishmania spp. pathogenic to man, L. (L.) donovani is considered one of the most sensitive in both stages of the parasite, while L. (V.) braziliensis is the less sensitive [40,42,43]. A large variation in MF susceptibility is also observed in clinical isolates for several Leishmania species [35,41,42]. In L. (L.) amazonensis for example, in vitro tests against promastigotes and intracellular amastigotes showed that a clinical isolate was less susceptible to MF than a type strain of this species. The intrinsic tolerance to MF in this clinical isolate was not due to polymorphisms in the MT and Ros3 genes [35]. Moreover, this differential susceptibility in vitro did not affect the clinical efficacy of infected mice with these two lines treated with MF [35]. Similarly, no correlation between the treatment outcome and MF susceptibility was observed in clinical isolates of L. (L.) donovani that presented large variation in drug sensibility [44]. Treatment with pentavalent antimonials has also demonstrated an ambiguous correlation between in vitro susceptibility of the parasite and treatment outcome in L. (L.) donovani and L. (V.) braziliensis infected patients [45,46].

For drug activity against Leishmania spp., it is essential the internalization of MF and a clear correlation between drug uptake and susceptibility is observed [32,47,48]. As mentioned before, the accumulation of MF is mediated by the MT and its subunit Ros3 [31]. Interestingly, the activity and substrate specificity of this machinery vary between Leishmania species and may be correlated with the differential susceptibility of these species to the MF [48]. According to these authors, the reduced expression of the complex MT and its subunit Ros3 is responsible for the low sensitivity of L. (V.) braziliensis to MF [48].

The variation in MF susceptibility could also be explained by differences in rate of division of the parasites, exposure to the drug, biochemical targets and drug metabolism and biochemical content of the plasma membrane [31]. MF susceptibility is a trait that can be summarized therefore as drug tolerance that is an innate feature due to intrinsic biochemical and molecular properties of the parasite. Differently from drug tolerance, drug resistance is a feature that emerges and spreads after parasite being exposed to the drug.

Miktefosine resistance in Leishmania

Parasites resistant to MF can be obtained as promastigotes in vitro by increasing drug concentration (stepwise selection) [32,35,41,49] or by chemical mutagenesis followed by selection of MF [32, 50]. In general, the mechanism of resistance is related to a defect in drug internalization due to mutations in the MT gene [32,35,49,51,52]. This defect in drug accumulation can be restored after functional expression of the MT gene in the resistant line [32,49]. Additionally, single mutations in Ros3 gene alleles were also observed in a MF resistant line leading to a high resistance level as observed in selected resistant lines containing mutations in MT alleles [33]. These data indicate that both proteins are selected during the drug pressure, although a higher recurrence of mutations in MT gene has been observed in resistant parasites [35,51-53]. Besides, once the MT gene is inactivated, the resistance phenotype persists in amastigotes in vitro and in vivo in animal models of VL and CL [35,54], indicating that this machinery is functional throughout the life cycle of the parasite. These findings showed that MT activity is essential for MF effectiveness and inactivation of this transporter becomes parasites completely refractory to MF.

Interestingly, when an alternative method using intracellular amastigotes in vitro to select MF resistant parasites, no change in MF susceptibility was found in amastigotes, although parasites were resistant when transformed back to promastigote [55,56]. This reason for this differential pattern of susceptibility in both stages of the parasite after MF selection in intracellular amastigotes is still unknown.

Miktefosine resistance can also be associated with an increase in efflux pumps through the overexpression of an ABC transporter. Some members of this family were already reported as able to mediate drug resistance: ABCB1 from the subfamily ABCB [38] and two members of the subfamily ABCG, ABCG4 and ABCG6 members [36,37] are implicated in phospholipid trafficking and reduction in MF accumulation.

Recently, whole genome sequencing of two resistant lines of L. (L.) major revealed that inactivating mutations at conserved residues were able to confer MF resistance [53]. Mutations were also observed in the gene encoding previously pyridoxal kinase. Pyridoxal kinase plays a vital role in the formation of pyridoxal-5′-phosphate but the mechanism involved in MF resistance is still unknown [53]. Whole genome sequencing of L. major mutants resistant to MF also revealed a homozygous mutation in the α-adaptin like protein gene in L. (L.) major and in two independent mutants of L. (L.) infantum [53]. This gene codes for the α subunit of Adaptor Protein 2 (AP2) complex involved in endocytosis of plasma membrane [57], suggesting a role in MF resistance in Leishmania. A role of this complex was already associated with suramin action and resistance in Trypanosoma brucei [58]. As mentioned before, MF may also enter in the parasite through the endocytic pathway, although this route is only important in a scenario in which the amount of drug bound to the membrane is extraordinarily high [31]. Overexpression of a functional copy of this gene in the resistant mutant and in wild-type parasites did not alter MF susceptibility [53]. Attempts to generate a double knockout of this gene were unsuccessful and no significant change in MF susceptibility was
observed in single knockout parasites of *L. (L.) donovani* (unpublished observations). These findings indicate that these mutations may be related to fitness compensatory mutations, differently from mutations in the *MT* gene that are directly involved in the primary resistance mechanism of the drug.

A global genomic expression by RNA microarray also revealed several differentially expressed genes in a MF resistant line of *L. (L.) donovani*, involved in DNA replication/repair mechanism, reduced protein synthesis and degradation, increased drug efflux, altered energy utilization and increased antioxidant defence mechanisms [51]. A point mutation was also found in the *MT* gene of this resistant line [51]. Two clinically derived *L. (L.) donovani* strains with different inherent antimonal sensitivities were also selected in vitro as promastigotes and changes in the number of copies of chromosomes, single-base mutations and deletions of *MT* gene were observed in these resistant lines [52]. Changes in the content of phosphatidylcholines and lysophosphatidylcholines were also associated with MF resistance in these two clinical lines [52].

As described above, most of knowledge about *Leishmania* MF resistance was performed in resistant parasites induced in vitro. Therefore, the molecular mechanisms and markers identified cannot be necessarily correlated with drug resistant parasites that emerge in endemic regions [59]. It might also be considered that anthroponotic disease in treated patients has a higher drug pressure on parasite population than the tegumentary disease in South America for example, that is zoonotic. All these aspects must be considered when clinical isolates from treated patients are studied. For example, a clinical decrease in the susceptibility of parasites to MF in vivo, a precursor of the emergence of drug resistance, has not yet been formally described, although several relapse cases after successful MF treatment have been reported for CL, DCL and VL [26, 60-65]. There is just one study that reports the selection of a *L. (L.) infantum* resistant line after treatment with MF in a HIV-coinfected patient [66]. The mechanism involved was associated with the occurrence of a mutation in the *MT* gene [66]. On the other hand, in patients infected with *L. (L.) donovani*, VL relapse cases were found up to 20% of patients after 6-12 months, but none of clinical isolates from these patients were resistant to MF in vitro [63]. Similarly, isolates of *L. (L.) donovani* from cured and failed patients showed a similar susceptibility to MF [43]. Otherwise, in clinical isolates of *L. (V.) panamensis* rescued before and after the treatment, an increase in MF susceptibility was found, indicating that resistant parasites were selected during the therapy with MF [41]. Finally, these studies indicated that the acquired resistance in leishmaniasis may or not be related with treatment failure using MF.

Miletefosine treatment failure in the field

As previously measured, the cure rate of MF in VL due to *L. (L.) donovani* is higher than 90%, but in the last years, an increase in the number of relapses after the end of treatment has been reported [63, 67]. In these studies, treatment failure was not due to intrinsic or acquired resistance and others factors recently reported in the literature have correlated relapse cases and treatment failure using MF; as for example: a lower exposure of the parasites to the drug (pharmacokinetics) [68], higher infectivity of the clinical isolates from these patients [69] and/or host-related factors (i.e. immunological factors) [70].

In South America, clinical studies for CL, show large efficacy variation between endemic regions and in infections caused by *L. (V.) braziliensis*, *L. (V.) guyanensis* and *L. (V.) panamensis* [23-26]. Despite this variation in efficacy, recent clinical trials have shown that the therapy using MF is more efficacious than the standard therapy with meglumine antimoniate [23-25]. In DCL patients infected with either *L. (L.) amazonensis* or *L. (L.) mexicana*, a high clinical efficacy at the end of the treatment is observed, followed by relapses [60,65]. Similar findings with *L. (L.) amazonensis* were observed in mice infected and treated with MF [35]. Mice relapsed some months after treatment and no change in drug susceptibility was observed in parasites recovered from lesions, indicating that parasites did not acquire resistance to MF (unpublished observations). In tegumentary leishmaniasis, other factors unrelated to drug susceptibility and resistance may also be correlated to MF treatment failure, as for example: localization of the parasites in tissues less accessible to drugs, quiescence and presence of *Leishmania* RNA virus-1 (LRV1 virus) in the parasites of the *Viannia* subgenus [70,71]. Recent studies have correlated the prevalence of LRV1 virus in *Leishmania* (Viannia) species and treatment failure due to the subversion of the host immune response [71,72].

Concluding Remarks

Studies based on drug susceptibility and resistance have been useful to decipher the molecular basis involved in the mechanisms of action of drugs in *Leishmania*. MF is the only oral effective drug available for leishmaniasis. The drug has a long half-life (150-200 h) and the treatment is long (28 days) what can induce the emergence of resistant parasites in case of inadequate use. Understanding MF resistance is also a prerequisite to monitor drug resistant parasites in the field and preserve the drug efficacy. Currently, the main goal is to correlate the recent knowledge obtained in vitro and MF unresponsiveness in the field. MF and Ros3 might be considered as the main molecular markers in case of clinical isolates that show low MF sensitivity, since this machinery is essential for MF activity and efficacy. In the last years, an increase in the number of treatment failure cases has been reported for all the clinical forms of the disease and it is urgent to understand this increase in the number of cases. Recent reports have not directly correlated clinical inefficacy with intrinsic or acquired differences of drug susceptibility what indicates that other aspects are involved in MF inefficacy in the field.

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References


