Identification of a *Leishmania infantum* gene mediating resistance to miltefosine and Sb^{III}

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Abstract

Resistance to treatment is a growing problem in efforts to control Old World leishmaniasis. Parasites resistant to new therapeutics such as miltefosine have not been reported from the field yet but based on experimental evidence, may appear soon. Therefore, we attempted to identify genetic markers that may correlate with miltefosine resistance. Using a functional cloning approach, we have isolated a gene from *Leishmania infantum* that, upon over-expression, confers protection not only against miltefosine, but also against Sb^{III}, the active principle of anti-leishmanial antimonials. The gene encodes a very large putative polypeptide of 299 kDa that shows no similarities to known proteins or functional motifs. Database mining and karyotyping experiments suggest that in *L. infantum* this gene is part of a 44-kbp duplicated region that is found on two separate chromosomes, CHR08 and CHR29.
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1. Introduction

Visceral leishmaniasis (VL) is a disease caused by protozoan parasites of the *Leishmania donovani* complex, with an estimated 500,000 new cases each year. The parasites are transmitted by sandflies of the genera *Phlebotomus* and *Lutzomyia*. Most of VL cases (90%) occur in the Indian sub-continent, Sudan and Brazil. The increasing incidence of VL in Mediterranean countries is mainly due to co-infection with HIV (Alvar et al., 1997; Desjeux and Alvar, 2003). If left untreated, VL is almost invariably fatal.

No vaccines for VL are available yet and vector control in endemic regions is inadequate. Thus, control of this disease relies exclusively on chemotherapy. A serious problem in the control of VL is the development of resistance to standard anti-leishmanials, such as pentavalent antimonials. In northern Bihar (India), a highly endemic region, up to 60% of VL-patients do not respond to pentavalent antimony treatment (Sundar, 2001a, b; Sundar and Rai, 2005). The increasing ineffectiveness of standard anti-leishmanials urgently requires a search for new anti-parasitic compounds and the introduction of combination therapies against VL (Sundar et al., 2000).

Miltefosine (hexadecylphosphocholine) was originally developed as an anti-cancer agent. It is now approved for treatment of VL in India (Sundar et al., 2006). In clinical trials, cure rates of 95% (Jha et al., 1999) were achieved, including antimony-resistant cases and cutaneous leishmaniasis (CL) (Sundar et al., 1998, 1999; Soto et al., 2001). It is the first oral drug against VL and is now marketed under the brand name "Impavido^{TM}".

Several studies have been carried out on the mode of action of miltefosine in tumour cell lines and trypanosomatids, however there is no consensus concerning the molecular target. Perturbation of the alkyl-lipid metabo-

lism, the glycosylphosphatidylinositol anchor biosynthesis, signal transduction and the inhibition of an enzyme (alkyl-specific-acyl-CoA acyltransferase) involved in lipid-remodelling, have been described in *Leishmania mexicana* promastigotes (Lux et al., 1996, 2000). It was also reported that miltefosine induces cell death in *Leishmania* promastigotes and amastigotes, with many features of metazoan programmed cell death (Paris et al., 2004; Verma and Dey, 2004; Verma et al., 2007).

Since the recent introduction of this drug for clinical use, no cases of therapy-resistance have been reported. However, the ease with which a miltefosine-resistant *Leishmania* cell line was selected *in vitro* (Seifert et al., 2003) and the long plasma half-life of this drug, suggest that there is a high risk that parasites may develop resistance, especially when the use of miltefosine as a single anti-leishmanial becomes widespread (Sundar et al., 2006). To counter the possible emergence of resistance, a combination of drugs in anti-leishmanial therapy needs to be considered (Sundar and Rai, 2005). For the design of simultaneous and sequential combination therapy, the resistance mechanisms of the individual drugs must be known to avoid cross-resistance. Therefore, it is important to identify the determinants of miltefosine resistance in *Leishmania*. Furthermore, the identification of resistance markers will help in monitoring the spread of resistance once it emerges in the field.

A miltefosine-resistant strain was generated in the laboratory (Seifert et al., 2003) and characterised. In this strain, resistance to miltefosine was linked to impaired translocation of the drug from the outer to the inner leaflet of the plasma membrane in a resistant *L. donovani* strain (Perez-Victoria et al., 2003a). Using functional cloning, the miltefosine translocase, LdMT, a novel P-type ATPase, was identified (Perez-Victoria et al., 2003b). Two distinct point mutations in this membrane-bound protein were found to be responsible for reduced translocation of the drug.

In another study, the same group identified the ß-sub-unit of LdMT, dubbed LdRos3. Inactivation of either protein leads to decreased translocase activity, resulting in miltefosine resistance (Perez-Victoria et al., 2006a, b).

Another putative transporter protein, ABCG4, was recently found to induce moderate (twofold) resistance to miltefosine when over-expressed in *Leishmania infantum*. The protein localised to the plasma membrane, further supporting a role as transporter (Castanys-Munoz et al., 2007).

Cross-resistance to miltefosine was observed in a pentavalent antimony-resistant *L. donovani* field isolate. Comparative proteome analysis revealed a number of differentially expressed proteins in the resistant cell line, including heat shock protein 83 (HSP83) and small kinetoplast calpain-related protein 1 (SKCRP1) (Vergnes et al., 2007). Transfection of either gene into the corresponding sensitive parasite strain led to an increase in miltefosine-resistance, confirming their role in resistance.

In another study, a *Leishmania tropica* strain, selected for daunomycin resistance, over-expressed a P-glycoprotein gene, *MDR1*. This cell line was cross-resistant to miltefosine, suggesting that *MDR1* may contribute to miltefosine resistance (Perez-Victoria et al., 2001, 2006c).

The aim of this study was to identify additional molecular markers of miltefosine resistance in *L. infantum*, the causative agent of VL in the Mediterranean region and South America. This was done by using functional cloning, i.e. genetic complementation. *Leishmania* spp. are uniquely suited for this kind of approach as they allow the stable maintenance of episomes from a cosmid-based genomic DNA library. This method allows the rapid selection of genes that mediate selectable traits and the functions of several genes have been elucidated using this approach (Clos and Choudhury, 2006).

Here we describe the result of a selection screening process for miltefosine resistance marker genes by challenging random cosmid-bearing *L. infantum* cells with inhibiting concentrations of miltefosine. We have identified a gene encoding a 299-kDa protein of unknown function. Transfection and concomitant over-expression of this gene confers resistance to miltefosine and to the trivalent antimony compound, antimonyl tartrate, but not to pentamidine.

2. Materials and methods

2.1. Parasite culture

Leishmania infantum clone 3511, derived from strain MHOM/FR/91/LEM2259 (Garin et al., 2001), was used as donor and recipient for the cosmid library DNA. Promastigotes were routinely cultivated at 25 °C in supplemented M199 medium (Hubel et al., 1997). Cells were counted using a Schaerfe System CASY Cell Counter.

Strains MHOM/SU/73/5ASKH (*Leishmania major*), and *L. donovani 1SR* were gifts from David Evans (London School of Hygiene and Tropical Medicine, UK) and Daniel Zilberstein (Technion Haifa, Israel), respectively, and were previously described (Brandau et al., 1995; Krobitsch et al., 1998). Strain MCAN/ES/98/LLM-877 (*L. infantum*) was provided by Martin Wiese (Bernhard Nocht Institute, Hamburg, Germany).

Recombinant promastigotes were cultured in the presence of G418 geneticin throughout this study. Prior to certain experiments (flow cytometry, determination of resistance) G418 was removed from cultures by washing cells several times in G418-free medium and cultivation in the absence of G418 for 24 h.

2.2. Drugs

Miltefosine was a gift from Aeterna Zentaris GmbH (Frankfurt, Germany); potassium antimonyl tartrate (Sb^{III}) and pentamidine isethionate were purchased from Sigma–Aldrich (Munich).