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ORIGINAL ARTICLE

Intracellular amastigote replication may not be required for successful *in vitro* selection of miltefosine resistance in *Leishmania infantum*

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Abstract

Although miltefosine (MIL) has only recently been positioned as a first-line therapeutic option for visceral leishmaniasis, field reports note an increasing trend in treatment failures. Study of laboratory selected MIL-resistant strains is needed in the absence of confirmed resistant clinical isolates. In contrast to promastigotes, experimental *in vitro* selection of MIL-resistance on intracellular amastigotes has not yet been documented. This study reports for the first time the selection of MIL-resistance in *Leishmania infantum* LEM3323, a strain which clearly shows active intracellular replication. Starting from the hypothesis that active multiplication may be essential in the resistance selection process, several other *L. infantum* strains were evaluated. Although strain LEM5269 showed only marginally lower intracellular multiplication, selection for resistance failed, as was also the case for several other strains showing poor or no intracellular replication. These results suggest that intracellular multiplication may not be an absolute prerequisite for the outcome of experimental *in vitro* MIL-resistance selection in clinical field isolates.

Introduction

Miltefosine (MIL) has been positioned in 2005 as first-line treatment for visceral leishmaniasis (VL) as part of the Kala-azar elimination program, launched to tackle the widespread antimony-resistance in the Indian subcontinent and to reduce the number of VL cases to less than 1/10,000 by 2015 (Dhillon et al. 2008). Since MIL has a long elimination half-live and requires a long treatment regimen, development of drug resistance appears unavoidable (Dorlo et al. 2012). MIL is now roughly one decade in widespread use and treatment failures have indeed started to appear in the Indian subcontinent (Rijal et al. 2013). However, despite the trend towards a decreasing MIL-susceptibility, Indian *L. donovani* isolates from MIL-relapse patients do not display a clear MIL-resistant phenotype in the laboratory, raising questions on the true nature of these relapses (Bhandari et al. 2012; Rijal et al. 2013). In other parts of the world where VL infection is generally caused by *L. infantum,* emergence of MIL-resistance is even more probable due to the substantial number of HIV co-infected patients receiving MIL-treatment and because of its veterinary use to treat canine leishmaniasis, known to be zoonotic (Noli and Saridomichelakis 2014; van Griesven et al. 2014). Since both HIV co-infected patients and dogs have difficulties clearing all parasites upon drug treatment, they actually may harbor a huge parasite reservoir under potential continuous drug selection pressure linked to multiple treatment courses, hence facilitating emergence of drug resistance. Most relapse isolates from Brazil where treatment of dogs is not uncommon actually display a decreased MIL-susceptibility (Carnielli et al. 2014), in contrast to the Indian *L. donovani* isolates mentioned earlier. Of particular interest are two laboratory confirmed MIL-resistant *L. infantum* isolates isolated from HIV co-infected patients in Europe (Cojean et al. 2012; Hendrickx et al. 2014).

Experimental selection of MIL-resistance in the laboratory may offer insights into putative resistance mechanisms and contribute in designing strategies that cope with emergence and spread of resistance. Previously, we reported an *in vitro* drug resistance selection protocol using intracellular amastigotes whereby several paromomycin (PMM) resistant *L. donovani* strains could relatively easy be generated (Hendrickx et al. 2012). Quite surprisingly, implementation of this protocol for the experimental selection of MIL-resistance did not result in a susceptibility drop (Hendrickx et al. 2014). Considering a possibly enhanced likelihood of MIL-resistance in *L. infantum,* this laboratory study specifically aimed to explore the experimental selection of MIL-resistance in several *L. infantum* field isolates (Table 1). Here, we report for the first time the successful selection of MIL-resistance in a *L. infantum* field isolate (LEM3323) adopting our *in vitro* selection protocol on intracellular amastigotes. In an attempt to explain the discrepancy in resistance-selection efficiency between MIL (poor) and PMM (high), it became clear

that LEM3323 was the only strain that displayed high intracellular replication while other strains lacking obvious intracellular multiplication failed to develop MIL-resistance. Evaluation of the role of intracellular multiplication for the successful selection of MIL-resistance was subsequently checked in another *L. infantum* isolate (LEM5269) displaying moderate intracellular growth, however, no resistance could be demonstrated.

Materials and methods

Strain selection

The selection of *L. infantum* strains showing various backgrounds of resistance for antimony (Sb), miltefosine (MIL) and paromomycin (PMM) is summarized in Table 1. Being aware of the loss of virulence in promastigotes after long-term *in vitro* cultivation, *ex vivo* amastigotes were used for infection with our reference laboratory strain (MHOM/MA/67/ITMAP263) that had already been established in Balb/c mice and hamsters. All other isolates were only available as axenic promastigotes. The field isolates for experimental selection of MIL-resistance were obtained from 'Centre National de Référence des Leishmania (CNRL)' (Dr. L. Lachaud). MHOM/FR/96/LEM3323 originated from a French HIV-positive patient whereas MCAN/DZ/2006/ENV1 (LEM5269) was isolated from an Algerian dog. MCAN/BR/2002/BH402/60 was part of a large efficacy trial with a liposomal meglumine-antimonate formulation (Costa Val 2004) and was isolated from an infected Brazilian mongrel dog. MCAN/ES/--/MILT-200 and MCAN/ES/--/MILT-153 were isolated from infected dogs in Spain (kindly provided by Carmen Cañavate, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Spain). Isolates MHOM/BR/2007/WC (L3015) and MHOM/BR/2007/AS-8 (L3034) were obtained from Brazilian HIV-positive patients from the FIOCRUZ *Leishmania* collection (Inocencio da Luz et al. 2011). Species identification was done previously using isoenzyme electrophoresis and pteridine-reductase 1 (PTR1) sequencing (Inocencio da Luz et al. 2011). Promastigotes of all strains were grown in HOMEM medium (Gibco®, Life technologies, Ghent, Belgium) at 25°C and further cryopreserved until use.

Microscopic assessment of intracellular amastigote multiplication

Primary peritoneal mouse macrophages were collected from female Swiss mice, seeded in 96-well plates at 30,000 macrophages/well in 100 µl of RPMI-1640 (Invitrogen, UK) (da Luz et al. 2009) and infected 24 hours later with metacyclic promastigotes adopting an infection level of 15 promastigotes per macrophage. Metacyclogenesis was carried out by spontaneous acidification of the culture medium

as described earlier (Vermeersch et al. 2009). Metacyclic promastigotes were enumerated based on their morphological changes (elongation of the flagellum and shortening of the cell body). Non-internalized promastigotes were removed by successive washing with PBS. Amastigote growth was evaluated by staining infected macrophages every 24h with Giemsa. A minimum of a hundred at random macrophages was evaluated in ten independent microscopic fields to assess the total number of macrophages, the number of infected macrophages (infection rate) and the number of intracellular amastigotes. The infection index was determined according to the formula:

$$Infection\ index\ = \frac{\#\ amastigotes\ counted}{total\ \#\ macrophages\ counted}$$

A correction for differences in baseline infectivity was made to allow comparison between different strains. The infectivity 24h post-infection was used as an internal baseline control (T0). Amastigote multiplication ratios were calculated using the formula:

$$Amastigote\ multiplication\ ratio\ = \frac{infection\ ratio\ at\ Tx}{infection\ ratio\ at\ T0}$$

Resistance selection

Resistance was experimentally induced on intracellular amastigotes, as previously described (Hendrickx et al. 2014) and terminated after five successive selection cycles. Only the two field strains showing increasing intracellular amastigote burdens (LEM3323 and LEM5269) were included for resistance selection.

Amastigote and promastigote susceptibility testing

After subsequent MIL-selection cycles, the susceptibility of both promastigotes and amastigotes was determined as described earlier (Vermeersch et al. 2009). Briefly, amastigote susceptibility was determined by exposing infected primary peritoneal mouse macrophages to two-fold drug dilutions for 96h and microscopic evaluation of intracellular amastigote burdens upon Giemsa staining for calculation of IC_{50}-values. Promastigote susceptibility was determined by exposing procyclic promastigotes to two-fold drug dilutions for 72h and adding resazurin for fluorimetric reading and calculation of IC_{50}-values.

Results

Microscopic assessment of intracellular amastigote multiplication

Results of the microscopic assessment of the intracellular parasite burden upon infection of primary macrophages with metacyclic promastigotes or *ex vivo* amastigotes are presented in Figure 1 and corresponding macrophage infection rates are shown in Table 3. While infection of macrophages with *ex vivo* amastigotes from our laboratory reference strain (ITMAP263) resulted in an increasing intracellular parasite burden over time, infection with metacyclic promastigotes consistently resulted in decreasing burdens compared to the initial (24h) intracellular burden, with the exception of two strains, i.e. LEM3323 and LEM5269.

Resistance selection

A previous study from our research group already reported particular difficulties for successfully establishing a MIL-resistant phenotype of *L. donovani* when using intracellular amastigotes during successive selection cycles (Hendrickx et al. 2014). The two *L. infantum* field isolates showing increasing intracellular amastigote burdens in primary peritoneal macrophages (Fig. 1) were selected to check the role of replication in the selection outcome of MIL-resistance. Both strains were exposed to five successive *in vitro* selection cycles (Table 2) and remarkably only resulted in the generation of MIL-resistant LEM3323 (amastigote IC_{50} >20 µM; promastigote IC_{50} >40 µM). Strain LEM5269, showing intracellular multiplication only to a slightly lower extend compared to LEM3323, remained fully MIL-susceptible both at promastigote and amastigote level.

Discussion

Within about one decade of its use as first-line therapy, miltefosine (MIL) is confronted with an increase in treatment failure rates in the Indian subcontinent (Rijal et al. 2013). However, while isolation of *L. donovani* strains with laboratory confirmed MIL-resistance has failed up till now, two MIL-resistant field isolates of *L. infantum* were obtained from HIV co-infected patients in Europe (Cojean et al. 2012; Hendrickx et al. 2014). The fact that its pharmacokinetic profile favors the selection of MIL-resistance (Dorlo et al. 2012) in addition to its regular use in HIV co-infected patients and in veterinary practice to treat canine leishmaniasis, is an argument to monitor the emergence and spread of MIL-resistance even more. After all, both dogs and HIV-patients may function as parasite reservoirs as complete parasite clearance is difficult to achieve upon drug treatment (Noli and Saridomichelakis 2014; van Griesven et al. 2014). So far, research on MIL-resistance has mainly focused on the identification of resistance mechanisms in laboratory-induced strains using *in vitro* selection protocols on axenic promastigotes

(Pérez-Victoria et al. 2006; Seifert et al. 2007). However, promastigotes cannot be regarded as the relevant parasite stage for infection in mammals, hereby underlining the need to focus on intracellular amastigotes for *in vitro* resistance selection (Hendrickx et al. 2012). Moreover, previous research already demonstrated that in contrast to the straightforward selection of MIL-resistance on promastigotes, selection of MIL-resistance on intracellular amastigotes could not be achieved for a variety of *L. donovani* and *L. infantum* isolates (Pérez-Victoria et al., 2003; Hendrickx et al. 2014). This study now reports for the first time experimental selection of actual MIL-resistance in an *L. infantum* field isolate (LEM3323), resulting in a MIL-resistant phenotype both on promastigote and amastigote level, with susceptibility levels reaching macrophage cytotoxic concentrations (Table 1).

In-depth characterization of strain-specific traits revealed an unexpected decline in initial (24h) intracellular amastigote burdens for most *L. infantum* field isolates, except for LEM3323 and LEM5269 which both displayed increasing intracellular parasite burdens (Fig. 1). Since strain LEM3323 had been the only isolate to acquire a MIL-resistant phenotype so far, intracellular amastigote replication was assumed to be a decisive feature for selection of MIL-resistance in our *in vitro* selection protocol. To challenge this hypothesis, LEM5269 was also subjected to successive MIL-selection cycles. However, no shift in MIL-susceptibility could be observed, which strongly contrasts with the complete MIL-resistant phenotype of LEM3323 within 5 selection cycles. Failure to select for resistance occurred with *L. infantum* L3015 and various *L. donovani* isolates that also displayed decreasing amastigote burdens (Fig. 1) (Hendrickx et al. 2014; unpublished data). As such, no formal causal relationship between *in vitro* intracellular amastigote replication and successful selection of MIL-resistance could be identified. The findings with LEM3323 still remain unique and deserve further investigation both at genomic and phenotypic level. Since infection with *ex vivo* amastigotes also results in *in vitro* amastigote replication (Fig. 1), follow-up research will include this as well, together with the dynamics of resistance selection in MIL-treated laboratory animals. Above all, it is encouraging to learn that development of full-blown MIL-resistance may be much less probable than originally assumed, and actually may explain the current inability to isolate resistant parasites from MIL-treated relapse patients. However, seeing the limited number of alternatives in the drug discovery pipeline, additional research to understand the increasing treatment failure rates remains pivotal to safeguard MIL-treatment for future use.

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Tables

Table 1: Overall susceptibility profile of the clinical isolates to evaluate intracellular amastigote replication potential. The amastigote susceptibility profile based on the routine intracellular susceptibility assay of the selected strains is shown for pentavalent antimonials (Sb^V), trivalent antimonials (Sb^{III}), miltefosine (MIL) and paromomycin (PMM). Actual MIL IC_{50}-values (shown right) are the result of at least three independent replicates (SEM: standard error of mean; isolates were typed R: resistant; S: sensitive or I: intermediate resistant, as described previously (Inocêncio da Luz et al. 2011)).

Strain	Country	Origin	Sb^V	Sb^{III}	MIL	PMM	Amastigote IC_{50} ± SEM
MHOM/MA/67/ITMAP263	Marocco	reference lab strain	S	S	S	S	1.0 ± 0.1
BH 402/60	Brazil	dog isolate	S	S	S	S	1.4 ± 0.6
L3034	Paraguay	HIV-pos patient	R	I	S	S	1.6 ± 0.3
MILT200	Spain	dog isolate	S	S	S	S	0.6 ± 0.3
LEM4023	France	HIV-pos patient	R	S	S	S	1.2 ± 0.7
LEM5269	Algeria	dog isolate	R	R	S	S	1.4 ± 0.1
L3015	Brazil	HIV-pos patient	R	I	S	S	0.8 ± 0.1
MILT153	Spain	dog isolate	R	S	S	S	0.4 ± 0.2
LEM3323	France	HIV-pos patient, failure AmB	R	R	S	S	2.3 ± 0.5

Table 2: Amastigote and promastigote susceptibility data of two strains before and after five successive MIL-resistance selection cycles (MIL). IC_{50}-values for both amastigotes and promastigotes are presented. All assays were run in triplicate. (mean ± standard error of the mean (SEM), 3 replicates).

Strain	Amastigote IC_{50}	±	SEM	Promastigote IC_{50}	±	SEM
LEM3323	2.3	±	0.5	5.3	±	0.3
LEM5269	1.4	±	0.1	11.8	±	1.0
LEM3323 MIL	>20			>40		
LEM5269 MIL	1.1	±	0.1	11.5	±	1.1

Table 3: Mean macrophage infection rates of different *L. infantum* clinical isolates. Infection rates are the percentage of infected macrophages in two independent repeats and are presented as the mean ± standard error of the mean (SEM).

Strain	Infection rate (%)		
	Mean	±	SEM
ITMAP263	97.8	±	2.2
BHU402/60	92.2	±	7.8
L3034	82.2	±	0.9
MILT200	60.1	±	13.0
LEM4023	87.5	±	2.5
LEM5269	77.5	±	9.5
L3015	59.0	±	2.8
MILT153	81.5	±	8.5
LEM3323	87.5	±	3.3

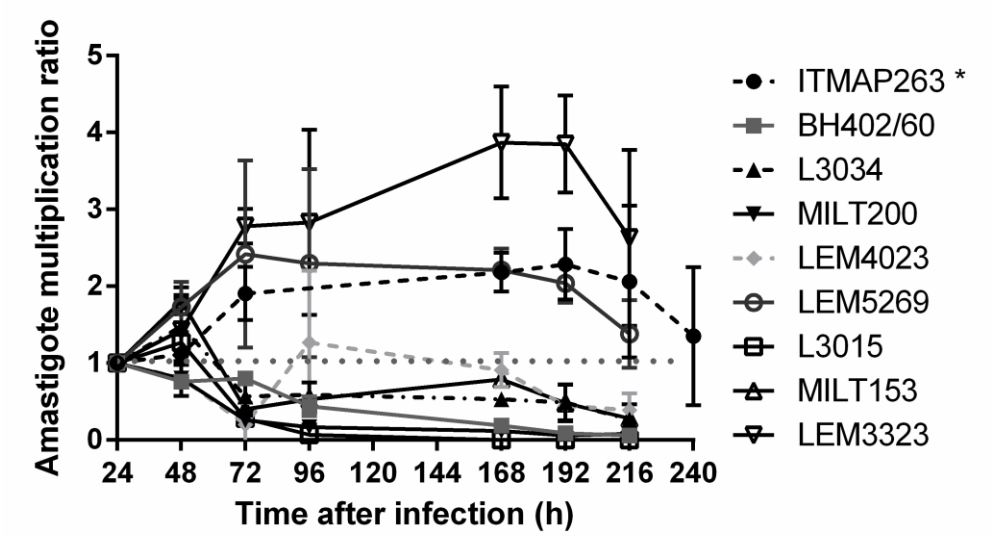

Figure 1. Intracellular growth curves of *L. infantum* field isolates and a reference lab strain. A decline in intracellular amastigote burden was observed for most *L. infantum* strains, except for LEM3323 and initially also for LEM5269. When *ex vivo* amastigotes* of the lab strain (ITMAP263) were used to infect host cells, increasing parasite burdens were observed. Amastigote multiplication ratios were the result of at least two independent replicates and are presented as mean ± standard deviation.