Evidence of a drug-specific impact of experimentally selected paromomycin and miltefosine resistance on parasite fitness in *Leishmania infantum*

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Objectives: Although miltefosine and paromomycin were only recently introduced to treat visceral leishmaniasis, increasing numbers of miltefosine treatment failures and occasional primary resistance to both drugs have been reported. Understanding alterations in parasite behaviour linked to drug resistance is essential to assess the propensity for emergence and spread of resistant strains, particularly since a positive effect on fitness has been reported for antimony-resistant parasites. This laboratory study compared the fitness of a drug-susceptible parent WT clinical *Leishmania infantum* isolate (MHOM/FR/96/LEM3323) and derived paromomycin and paromomycin drug-resistant lines that were experimentally selected at the intracellular amastigote level.

Methods: Parasite fitness of WT, paromomycin-resistant and miltefosine-resistant strains, in vitro and in vivo parasite growth, metacyclogenesis, infectivity and macrophage stress responses were comparatively evaluated.

Results: No significant differences in promastigote fitness were noted between the WT and paromomycin-resistant strain, while clear benefits could be demonstrated for paromomycin-resistant amastigotes in terms of enhanced in vitro and in vivo growth potential and intracellular stress response. The miltefosine-resistant phenotype showed incomplete promastigote metacyclogenesis, decreased intracellular growth and weakened stress response, revealing a reduced fitness compared with WT parent parasites.

Conclusions: The rapid selection and fitness advantages of paromomycin-resistant amastigotes endorse the current use of paromomycin in combination therapy. Although a reduced fitness of miltefosine-resistant strains may explain the difficulty of miltefosine resistance selection in vitro, the growing number of miltefosine treatment failures in the field still requires further exploratory research.

Introduction

The spread of primary antimony resistance in the Indian subcontinent has enforced the introduction of miltefosine and paromomycin for the treatment of visceral leishmaniasis (VL). Despite the increasing number of miltefosine treatment failures,¹ clinical reports on primary miltefosine or paromomycin resistance in the field are still very scarce.²,³ Contrary to *Leishmania donovani*, miltefosine relapse isolates from *Leishmania infantum*-infected patients display a decreased susceptibility, which may possibly also be related to its veterinary use to treat canine leishmaniasis and *L. infantum*-associated HIV coinfections. Since incomplete parasite eradication is the rule in both groups, the large residual parasite reservoir will promote selection of miltefosine resistance upon repeated drug exposure.²,⁴ To support strategies concerning treatment and emergence of drug resistance, experimental selection of drug resistance can facilitate applied and fundamental ‘drug resistance’ research with the particular advantage that the drug-susceptible WT can be directly compared with matched derived resistant lines. While in the past resistance has mostly been selected in promastigotes, an earlier study by our group demonstrated that selection of drug resistance strongly depends on the selection protocol leading to the recommendation to use intracellular amastigotes whenever possible.⁵ Although former research mainly focused on unravelling resistance mechanisms, parasite fitness must be considered a relevant factor as well, potentially influencing the spreading potential of resistant strains. Comparison of unmatched antimony-susceptible and -resistant *L. donovani* strains from the Indian subcontinent indicated an enhanced fitness of antimony-resistant isolates.⁶–¹¹ Likewise, a large-scale field study on *L. donovani* miltefosine cure and relapse isolates from Nepal suggested a higher in vitro infectivity of
mitofoxine relapse isoltes. Recently, evidence for increased fitness was obtained after selection of paromomycin resistance in L. donovani promastigotes. The present laboratory study aimed to evaluate the impact of experimental mitofoxine and paromomycin resistance on parasite fitness in an L. infantum strain isolated from an HIV-infected patient. Promastigotes were grown in L. infantum strains. While a decreased fitness could be demonstrated for the miltefosine-resistant and paromomycin-resistant derived strains. While a decreased fitness could be demonstrated for the miltefosine-resistant strain, the paromomycin-resistant isolate displayed enhanced intracellular amastigote growth and survival.

**Materials and methods**

**Parasite strains**

The L. infantum field isolate (MHOM/FR/96/LEM3323) used for the experimental selection of both mitofoxine and paromomycin resistance was obtained from the Centre National de Référence des Leishmania and was isolated from a French HIV patient. Promastigotes were grown in HOMEM medium (Gibco™, Life Technologies, Ghent, Belgium) at 25°C and subcultured twice weekly. Resistance was selected in intracellular amastigotes as previously described. Promastigote and amastigote susceptibilities were determined as previously described and are summarized in Table 1.

**Promastigote growth**

The growth profile of WT, mitofoxine-resistant and paromomycin-resistant promastigotes was assessed by flow cytometry (FCM). Promastigotes were diluted in PBS (Gibco™, Life Technologies) for FCM counting, using a FACSCalibur™ flow cytometer (BD Biosciences, NJ, USA) with addition of CountBright absolute counting beads (Molecular Probes™, OR, USA) as internal standard for quantification of the exact volume analysed. To generate growth curves, promastigotes of each strain were inoculated into 5 mL of HOMEM at exactly 5 × 10⁴ promastigotes/mL. Every 24 h, three biological replicates were quantified in duplo for up to 240 h and analysed using BD CellquestPro™ software. The average promastigote density at each timepoint was calculated and used to draw the final growth curves.

**In vitro intracellular amastigote growth**

To evaluate the intracellular amastigote growth of WT, paromomycin-resistant and mitofoxine-resistant promastigotes, their macrophage infection potential was determined. Promastigotes were counted by FCM and used to infect primary mouse peritoneal macrophages at an infection ratio of 5:1. Life/dead staining with TO-PRO®-3 iodide (Molecular Probes™) was used to correct for the variable number of dead promastigotes in the different cultures. At 24 h post-infection, the macrophages were fixed with methanol, Giemsa-stained and light microscopically evaluated for level of infection. To determine the number of intracellular amastigotes per macrophage and the percentage of infected macrophages, the intracellular amastigote burden of 100 macrophages was counted in 10 randomly selected microscopic fields and used to calculate the infection index:

\[
\text{Infection index} = \frac{\text{total no. of amastigotes counted}}{\text{total no. of cells counted}} \times \text{total no. of macrophages}
\]

### Table 1. Promastigote and intracellular amastigote susceptibilities of the WT, mitofoxine-resistant and paromomycin-resistant strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Intracellular amastigote susceptibility</th>
<th>Promastigote susceptibility</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mitofoxine (μM) IC₅₀±SEM</td>
<td>paromomycin (μM) IC₅₀±SEM</td>
</tr>
<tr>
<td>WT</td>
<td>2.3±0.5</td>
<td>89.3±4.5</td>
</tr>
<tr>
<td>Mitofoxine resistant</td>
<td>&gt;20</td>
<td>78.5±9.2</td>
</tr>
<tr>
<td>Paromomycin resistant</td>
<td>0.5±0.1</td>
<td>212.6±31.0</td>
</tr>
</tbody>
</table>

For promastigote susceptibility, 10⁵ log-phase promastigotes were exposed to serial dilutions of mitofoxine or paromomycin for 72 h at 25°C. Parasite multiplication was assessed after addition of resazurin and fluorimetric reading. For amastigote susceptibility, primary peritoneal mouse macrophages were infected with metacyclic promastigotes at an infection ratio of 15 stages per cell. Total parasite burdens were microscopically assessed on Giemsa-stained wells after 5 days of incubation. The results are expressed as percentage reductions in parasite burden compared with untreated infected controls and IC₅₀±SEM were calculated. Susceptibility values are the result of at least three independent replicates and are expressed as the mean IC₅₀ value ± SEM.
In vivo amastigote multiplication

To evaluate the in vivo infectivity and growth of each strain, 12 female BALB/c mice were infected intracardially with $2 \times 10^7$ metacyclic promastigotes. Up to 28 days post-infection, three animals per group were sacrificed at weekly intervals to determine the parasite burdens in liver, spleen and bone marrow. Amastigote burdens are expressed as Leishman–Donovan units (LDUs) after microscopic quantification of the Stauber index\(^{18}\) and by SYBR Green-based real-time PCR targeting the cysteine protease b (cpb) gene. Both the forward primer (5′-ATG TCT TAC CAG AGC GGC G-3′) and the reverse primer (5′-TCA CCC CAC GAG TTC TTG AT-3′) were purchased from Integrated DNA Technologies (Leuven, Belgium). To assess amastigote viability, a small piece of organ was placed in HOMEM medium and incubated at 25°C for 2 weeks to assess promastigote back-transformation.

Intracellular amastigote stress resistance

To evaluate the capacity of intracellular amastigotes to cope with intracellular stress, infected macrophages were exposed to either Escherichia coli-derived LPS and IFN-γ at concentrations ranging from 0.05 to 100 ng/mL (Sigma–Aldrich, Diegem, Belgium) or S-nitroso-N-acetyl-DL-penicillamine (SNAP) (Sigma–Aldrich, Diegem, Belgium) at concentrations ranging from 0 to 800 μM for 48 h, as described previously.\(^7,13,20,21\) Macrophage stress responses were determined by microscopic assessment of the percentage amastigote burden reduction compared with unstimulated infected control cells. Since previous research on WT and paromomycin-resistant strains suggested differences in host cell IL-10 production upon infection,\(^13\) the production of endogenous IL-10 in the supernatant of promastigote preconditioning cut-off value for both strains was reached after 192 h. Although some significant differences could be observed for WT parasites (Figure 2a), the paromomycin-resistant strain by adaptations to the culture medium and enforced metacyclogenesis by promastigote preconditioning,\(^18\) no increase in percentage metacyclic promastigotes could be obtained (data not shown).

The following formula was used to calculate the amastigote multiplication ratio:

$$\text{Amastigote multiplication ratio} = \frac{\text{no. of amastigotes at } T_x}{\text{no. of amastigotes at } T_0}$$

Figure 1. Promastigote growth curves of WT, paromomycin-resistant (PMM-R) and miltefosine-resistant (MIL-R) matched isolates. (a) WT and PMM-R parasites show comparable growth. (b) MIL-R parasites show significantly decreased growth compared with WT starting at 96 h in culture ($P < 0.001$). Results are expressed as mean ± SEM and are based on three independent replicates run in duplicate.

Statistical analysis

All statistical analyses were performed using GraphPad Prism version 4.00 software. Statistical differences between WT and resistant parasites and between the different timepoints within one group were determined using two-way ANOVA with Bonferroni post hoc comparisons for parasite growth, parasite morphology and infection indices. Morphological and infection indices intergroup comparisons were done using the non-parametric Friedman test followed by Dunn’s post hoc comparisons. Tests were considered statistically significant if $P < 0.05$.

Ethics statement

The use of laboratory rodents was carried out in strict accordance to all mandatory guidelines (EU directives, including the Revised Directive 2010/63/EU on the Protection of Animals used for Scientific Purposes that came into force on 1 January 2013, and the Declaration of Helsinki in its latest version) and was approved by the Ethics Committee of the University of Antwerp, Belgium (UA-ECD 2010-17, 18 August 2010).

Results

Promastigote growth

No statistical differences could be demonstrated between paromomycin-resistant and WT parasites (Figure 1a), whereas the miltefosine-resistant strain showed a significantly decreased growth pattern starting after 96 h of cultivation (Figure 1b). All strains reached the stationary phase leading to metacyclogenesis at $\sim 144$ h.

Promastigote metacyclogenesis

Although some significant differences could be observed for WT and paromomycin-resistant parasites at 144 and 168 h in favour of paromomycin-resistant parasites, the predetermined metacyclogenesis cut-off value for both strains was reached after 192 h. At that timepoint, no differences in percentage metacyclics (~64%) were observed between WT and paromomycin-resistant parasites (Figure 2a). The metacyclogenesis process in miltefosine-resistant parasites was much less evident (Figure 2b) and did not exceed 16% (data not shown). Despite several efforts to enhance metacyclogenesis and thus increase infectivity of the miltefosine-resistant strain by adaptations to the culture medium and enforced metacyclogenesis by promastigote preconditioning,\(^18\) no increase in percentage metacyclic promastigotes could be obtained (data not shown).
Promastigote infectivity

All strains reached their highest infectivity after 144 h of cultivation. At that timepoint, the infection indices of WT and paromomycin-resistant parasites were not statistically different (Figure 3a), whereas the infection index of the miltefosine-resistant strain was markedly lower (Figure 3b), corresponding to its lower level of metacyclogenesis.

In vitro intracellular amastigote growth

Although an initial increase in intracellular amastigote burden was observed for all strains, paromomycin-resistant amastigotes did show a notable advantage over WT and miltefosine-resistant parasites (Figure 4).

In vivo amastigote multiplication

Female BALB/c mice were infected with WT, miltefosine-resistant and paromomycin-resistant promastigotes for comparative monitoring of the amastigote multiplication ratio in the target organs. Every 7 days, three mice per strain were sacrificed and intracellular amastigote burdens in liver and spleen were determined by microscopic counting and real-time PCR. As bone marrow yields were insufficient to allow PCR, only microscopic counting was performed. Both in liver (Figure 5a) and spleen (Figure 5b), paromomycin-resistant parasites reached the highest burdens while no significant differences were noted between WT and paromomycin-resistant parasites in the bone marrow (Figure 5c). As could be expected based on their in vitro metacyclogenesis profile, miltefosine-resistant parasites displayed significantly lower burdens in all target organs.

Intracellular amastigote stress resistance

While paromomycin-resistant parasites showed an enhanced tolerance towards nitrosative stress, no significant differences could be detected between miltefosine-resistant and WT parasites (Figure 6a). Measurement of the endogenous IL-10 production by infected macrophages upon stimulation with LPS and IFN-γ revealed significantly lower IL-10 production of miltefosine-resistant infected macrophages, whereas no significant differences
on three independent replicates run in duplicate.

In summary, we demonstrated that in vitro selection for miltefosine-resistant phenotype on both promastigote and amastigote levels.14 To assess the possible impact on fitness, LEM3323 and its miltefosine- and paromomycin-resistant derived counterparts were subjected to the same battery of 'virulence' assays. Although increased parasite fitness for a promastigote-selected paromomycin-resistant strain has been described,16 no apparent benefit of paromomycin resistance could be demonstrated for the promastigote stage. When focusing on the intracellular amastigote stage, the paromomycin-resistant strain did show marked benefits over WT parasites, e.g. enhanced in vitro and in vivo amastigote replication and resistance towards macrophage-induced stress responses.

After comparison of the WT and miltefosine-resistant strain, all results suggested a decrease in parasite fitness upon acquisition of miltefosine resistance under the stated experimental conditions. This decline was evident at both the promastigote and intracellular amastigote stages and was reflected by a reduced replication potential, metacyclogenesis and stress tolerance. Although it was hypothesized that miltefosine treatment outcome could be related to the enhanced infectivity and metacyclogenesis of miltefosine relapse isolates,1,2 the present study does not support this. Indeed, if relapse isolates did in fact display an enhanced infectivity, experimental selection of drug resistance would be more straightforward. The association between high infectivity and facilitated resistance development may explain why our selection protocol failed for most L. infantum and L. donovani isolates, and was successful only for the LEM3323 whose promastigotes were shown to cause massive macrophage

![Figure 4.](https://academic.oup.com/jac/article-abstract/71/7/1914/1751521)
infection and intracellular replication, even at an infection ratio of 1 promastigote/macrophage (S. Hendrickx, unpublished results). Another natural miltefosine-resistant clinical isolate (LEM5159) \(^2\) shared almost identical in vitro infectivity, though its amastigote replication profile was less pronounced (S. Hendrickx, unpublished results).

Although the fitness decrease in miltefosine-resistant \(L.\ infantum\) should be confirmed in other miltefosine-resistant isolates, additional research by our group has already suggested a decline in fitness upon repeated miltefosine exposure of amastigotes in vivo as a reduced amastigote-to-promastigote transformation ability.\(^3\)\(^6\) Despite the fact miltefosine resistance will certainly not be the sole factor contributing to the increasing levels of miltefosine treatment failures worldwide,\(^1\)\(^4\) it is encouraging to know that actual full-blown miltefosine resistance comes with a fitness cost for the parasite and may to some extent restrict extensive spread of primary resistance. With regard to the latter, parasite fitness in the vector should be explored as well.

**Figure 5.** In vivo amastigote growth of WT, paromomycin-resistant (PMM-R) and miltefosine-resistant (MIL-R) matched isolates in liver (a), spleen (b) and bone marrow (c) of infected BALB/c mice. Results are expressed as mean ± SD and are based on three independent replicates. The bar graphs represent the infection indices based on microscopic counting of Giemsa-stained smears and are expressed either as LDU for the liver or as the average number of amastigotes per nucleus when infection was limited in spleen and bone marrow. The broken line graphs represent the amastigote burden as determined by RT–PCR.
cells stimulated with LPS and IFN-γ macrophages (*tolerance towards nitrosative stress inside primary peritoneal mouse parasites, while PMM-R parasites showed a significantly enhanced No significant differences could be observed between WT and MIL-R parasites. None to declare. Transparency declarations

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