THE SENSITIVITY OF CLINICAL ISOLATES OF LEISHMANIA FROM PERU AND NEPAL TO MILTEFOSINE

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Abstract. Clinical isolates of Leishmania, from visceral leishmaniasis (VL) cases in Nepal and from cutaneous leishmaniasis (CL) cases in Peru, were cultured using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) to type species and strain. Promastigotes from 38 isolates, within eight passages from isolation, were used to infect mouse peritoneal macrophage cultures in vitro, and the amastigote sensitivity to miltefosine was determined. The concentration required to kill 50% of intracellular amastigotes from Nepalese VL isolates, all typed as Leishmania (L.) donovani (N = 24) from both Sh† responders and nonresponders, ranged from 8.7 to 0.04 μg/mL. In contrast, the concentration required to kill 50% intracellular amastigotes from isolates from Peru, typed as L. (V.) braziliensis (N = 8), was > 30 to 8.4 μg/mL, L. (V.) guyanensis (N = 2) > 30 to 1.9 μg/mL, L. (L.) mexicana (N = 1) >30 μg/mL, and L. (V.) lainsoni (N = 4) was 3.4 to 1.9 μg/mL. This demonstrates a notable difference in the intrinsic sensitivity of Leishmania species to miltefosine in vitro. If this model can be correlated to therapeutic outcome, it may have implications for the interpretation of clinical trials.

INTRODUCTION

The current chemotherapy for leishmaniasis is limited. Pentavalent antimonials have been the recommended drugs for the treatment of both visceral (VL) and cutaneous leishmaniasis (CL) for more than 50 years, but long courses, toxicity, and resistance in India limit their use. New drugs have become available in recent years for the treatment of both VL, including highly efficacious but expensive lipid amphotericin B formulations, of which AmBisome is the only one widely available. Oral miltefosine has recently been licensed for use in India for the treatment of VL. Topical formulations of paromomycin and oral miltefosine have been used to treat CL. Miltefosine is an alkylphosphocholine, initially developed as an anticancer agent, that also shows selective activity against Leishmania. Miltefosine has undergone successful clinical trials for anthroponotic VL in Bihar state, India, with 94% cure rate, including antimony-resistant cases, and is currently in Phase IV trials in India and Nepal. This oral drug has demonstrated some activity in the treatment of zoonotic CL in South America1,5 where it is currently in Phase III clinical trials. There are more than 17 species of Leishmania that cause leishmaniasis in human populations; these species have been shown to vary in sensitivity to a range of antileishmanial drugs including miltefosine. The current study is the first to evaluate the in vitro anti-leishmanial activity of miltefosine against recently isolated, typed Leishmania spp. from patients in the Old and New Worlds.

MATERIALS AND METHODS

Ethical clearance was obtained from the ethical committees of the Health Research Council, Kathmandu, Nepal, and Cayetano Heredia University, Lima, Peru. Patients were recruited from November 2002 until the beginning of 2004. Clinical cases of VL in Nepal were recruited at the B P Koirala Institute of Health Sciences, Dharan, Nepal, from a catchment area covering the eastern Terai. Individuals less than 2 years old were excluded from the study. Suspected VL cases, fever for 14 days or longer with splenomegaly, were confirmed by bone marrow aspiration and visual identification of parasites. Patients received 50 mg SbV kg⁻¹ day⁻¹ intramuscularly for 30 days (sodium antimony gluconate; Albert David Ltd, Calcutta, India). Unresponsive patients subsequently received amphotericin B (amphotericin B deoxycholate) treatment.

Cutaneous leishmaniasis patients were recruited at the Instituto Alexander von Humbolt, Lima, Peru, with geographical location noted in the patient data (Table 1). Pregnant women were excluded from the study. Patients presenting with an ulcerated or nodular lesion were considered. Infection by Leishmania was confirmed by direct examination of punch biopsy material with confirmation by parasite culture or PCR. Patients received 20 mg SbV kg⁻¹ day⁻¹ intravenously for 20 days, except for PER069 who received treatment of 30 days. Unresponsive patients received a repeat course of antimonials. If this was again unsuccessful, patients received intravenous amphotericin B (amphotericin B deoxycholate) treatment.

Patient material was isolated directly onto 3N° blood slopes with a saline/antibiotic overlay and sent to ITG, Antwerp. Parasites were cryopreserved in aliquots and typed within eight passages from isolation. Frozen stocks were sent to LSHTM, London, where the parasites were passed initially onto 3N slopes with M199 + 20% HIFCS overlay, then into M199 + 20% HIFCS alone. It was necessary to introduce the use of M199 to obtain a clean, bulk culture of promastigotes that would be sufficient for further evaluation. There is some evidence that the type of medium can affect the infectivity of the parasite, however, in this study all isolates were exposed to exactly the same growth conditions, and the work was carried out as close to the isolation as possible. Standard operating procedures were established between partners, which would minimize the effect of this variable between the strains. The parasites were tested for their in vitro sensitivity to miltefosine within eight passages from isolation.

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Late stage promastigotes were used to infect primary isolated mouse peritoneal macrophages at a ratio of 7 promastigotes to 1 macrophage in Labtek 16-well tissue culture well slides (VWR Ltd, Poole, UK), in quadruplicate. Infected cultures were maintained in RPMI 1640 medium (Sigma, Gillingham, UK) with 10% heat-inactivated fetal calf serum (Harlan SeraLab, Loughborough, UK) at 37°C (VL strains) or 34°C (CL strains) in a 5% CO₂/95% air mixture. Twenty-four hours after infection, one slide was methanol-fixed and Giemsa-stained to determine the initial level of infection. Cultures were then exposed to miltefosine (hexadecylphosphocholine, HPC; A G Scientific, San Diego, CA) over a dose range of 30, 10, 3, and 1 µg/mL, in quadruplicate at each concentration. Higher concentrations were not tested due to toxicity to macrophage host cells: at concentrations above 30 µg/mL, macrophages infected are destroyed and the slide cannot be evaluated. After 5 days, all slides were methanol-fixed and Giemsa-stained. The percent of infected macrophages in each well was determined by microscopy. From a comparison of counts from treated with untreated cultures, the percent inhibition was calculated by sigmoidal regression analysis (MS xsfit) and ED₅₀ (ED₉₀) values determined.

Leishmania (V.) braziliensis MHOM/BR/75/M2903 was the reference strain used for Peruvian isolates. L. (L.) donovani MHOM/ET/67/HU3 was used as the reference strains for Nepalese isolates. Both strains are known as WHO reference strains, as typed by isoenzyme analysis, and are sensitive to the standard antimonials, sodium stibogluconate and meglumine antimoniate.

**Leishmania species typing.** In Antwerp, Leishmania species typing was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of cysteine proteinase b (cbp) and gp63 genes for Nepalese strains and by multi-locus PCR-RFLP for Peruvian strains. Briefly, DNA was extracted from cultivated promastigotes with the QIAmp DNA mini Kit (Qiagen, Hilden, Germany), amplified and digested as reported elsewhere. Restriction patterns were resolved by capillary electrophoresis (2100 Bioanalyzer system, Agilent Technologies, Karlsruhe, Germany) in a micro-chip device (LabChip 1500, Caliper Technologies, Mountain View, CA) and compared with those of reference strains. Standard strains were also re-typed and confirmed to be L. (L.) donovani and L. (V.) braziliensis.

**RESULTS**

The range of activity of miltefosine against South American isolates of cutaneous leishmaniasis is shown in Table 1. L. (V.) la置于osis (PER105) was the most sensitive strain tested with an ED₅₀ value of 1.89 µg/mL. L. (V.) braziliensis and L. (V.) guyanensis isolates were insensitive to miltefosine over the range tested (30–0.3 µg/mL). The only isolates, for example PER127, which showed sensitivity to miltefosine were typed as L. (V.) la置于osis, with ED₅₀ values ranging from 3.37 to 1.89 µg/mL.

The activity of miltefosine against VL isolates from Nepal is shown in Table 2 with ED₅₀ values ranging from less than 0.04 µg/mL to 8 µg/mL. Results were similar in repeated experiments. Although the strains were identified as two L. (L.) donovani types (I and II, based on restriction pattern differences), there was no significant difference in sensitivity to miltefosine between them. The differences seen in the RFLP pattern were useful when tracing the genetic relationships among the strains. L. (L.) donovani HU3 is an Ethiopian strain of VL maintained in passage animals. An Indian reference strain would perhaps have been a more appropriate comparator but was not available to us at the time of this study.

**DISCUSSION**

This study supports previous work comparing species sensitivity to miltefosine, which found significant differences between New World and Old World CL and demonstrated sig-
TABLE 2
Sensitivity of Nepalese visceral leishmaniasis isolates to miltefosine

<table>
<thead>
<tr>
<th>Species</th>
<th>ID</th>
<th>ED$<em>{50}$ ± SEM µg miltefosine/mL (ED$</em>{50}$ ± SEM)</th>
<th>Patient details (Age; M/F)</th>
<th>Region (District)</th>
<th>Clinical response to antimonial treatment$^f$</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. (L.) donovani (I)</td>
<td>BPK025/0</td>
<td>$0.57 ± 0.1$ ($0.5 ± 0.1$)</td>
<td>32; M</td>
<td>Sunsari</td>
<td>Definite cure</td>
</tr>
<tr>
<td>L. (L.) donovani (I)</td>
<td>BPK026/0</td>
<td>$1.97 ± 0.1$ ($1.9 ± 0.1$)</td>
<td>48; M</td>
<td>Bhojpur</td>
<td>Definite cure</td>
</tr>
<tr>
<td>L. (L.) donovani (I)</td>
<td>BPK035/0</td>
<td>$1.27 ± 0.1$ ($1.3 ± 0.1$)</td>
<td>9; M</td>
<td>Saptari</td>
<td>Nonresponder</td>
</tr>
<tr>
<td>L. (L.) donovani (I)</td>
<td>BPK043/0</td>
<td>$1.97 ± 0.1$ ($2.0 ± 0.1$)</td>
<td>10; M</td>
<td>Sunsari</td>
<td>Nonresponder</td>
</tr>
<tr>
<td>L. (L.) donovani (I)</td>
<td>BPK077/0</td>
<td>$5.87 ± 0.1$ ($6.0 ± 0.1$)</td>
<td>47; M</td>
<td>Sunsari</td>
<td>Definite cure</td>
</tr>
<tr>
<td>L. (L.) donovani (I)</td>
<td>BPK090/0</td>
<td>$4.27 ± 0.1$ ($4.3 ± 0.1$)</td>
<td>46; M</td>
<td>Sunsari</td>
<td>Definite cure</td>
</tr>
<tr>
<td>L. (L.) donovani (I)</td>
<td>BPK177/0</td>
<td>$8.17 ± 0.1$ ($9.0 ± 0.2$)</td>
<td>40; M</td>
<td>Dhanusa</td>
<td>Nonresponder</td>
</tr>
<tr>
<td>L. (L.) donovani (I)</td>
<td>BPK187/0</td>
<td>$5.77 ± 0.1$ ($6.4 ± 0.6$)</td>
<td>45; M</td>
<td>Sunsari</td>
<td>Initial cure</td>
</tr>
<tr>
<td>L. (L.) donovani (I)</td>
<td>BPK277/0</td>
<td>$1.97 ± 0.1$ ($2.0 ± 0.1$)</td>
<td>10; M</td>
<td>Morang</td>
<td>Nonresponder</td>
</tr>
<tr>
<td>L. (L.) donovani (I)</td>
<td>BPK287/0</td>
<td>$2.27 ± 0.1$ ($2.3 ± 0.1$)</td>
<td>10; M</td>
<td>Morang</td>
<td>Nonresponder</td>
</tr>
<tr>
<td>L. (L.) donovani (I)</td>
<td>BPK297/0</td>
<td>$0.97 ± 0.1$ ($1.0 ± 0.1$)</td>
<td>10; M</td>
<td>Morang</td>
<td>Initial cure</td>
</tr>
<tr>
<td>L. (L.) donovani (I)</td>
<td>BPK307/0</td>
<td>$3.27 ± 0.1$ ($3.3 ± 0.1$)</td>
<td>12; M</td>
<td>Morang</td>
<td>Initial cure</td>
</tr>
<tr>
<td>L. (L.) donovani (I)</td>
<td>BPK407/0</td>
<td>$2.27 ± 0.1$ ($2.3 ± 0.1$)</td>
<td>12; M</td>
<td>Morang</td>
<td>Initial cure</td>
</tr>
<tr>
<td>L. (L.) donovani (I)</td>
<td>BPK417/0</td>
<td>$3.27 ± 0.1$ ($3.3 ± 0.1$)</td>
<td>12; M</td>
<td>Morang</td>
<td>Initial cure</td>
</tr>
<tr>
<td>L. (L.) donovani (I)</td>
<td>BPK427/0</td>
<td>$0.97 ± 0.1$ ($1.0 ± 0.1$)</td>
<td>10; M</td>
<td>Morang</td>
<td>Initial cure</td>
</tr>
<tr>
<td>L. (L.) donovani (I)</td>
<td>BPK437/0</td>
<td>$2.27 ± 0.1$ ($2.3 ± 0.1$)</td>
<td>12; M</td>
<td>Morang</td>
<td>Initial cure</td>
</tr>
</tbody>
</table>

*, $^f$ WHO reference strain, L. donovani MHOM/ET/67/HU3. $^f$ Isolates taken after completion of antimonial treatment. $^f$ Initial cure—resolution of clinical symptoms 6 months after treatment, negative bone marrow aspirate. Currently in follow-up period. Definite cure—complete resolution of clinical signs and symptoms with no evidence of relapse 12 months after treatment; negative bone marrow aspirate.

Table 2 shows the sensitivity of Nepalese visceral leishmaniasis isolates to miltefosine. The table includes species, identification (ID), and sensitivity data for each isolate. The sensitivity is measured as the effective dose 50 (ED$_{50}$) in µg miltefosine/mL, along with the 95% confidence interval. Patient details, including age and gender, and the region of origin (District) are also provided. The clinical response to antimonial treatment is indicated, with options for initial cure, definite cure, nonresponder, and relapse. The table highlights the significant differences in sensitivity between New World species, 8 with varying responses to miltefosine.

Significantly different sensitivities between New World species. 8 This report, one of the few that includes both molecular typing and sensitivity studies of a large number of recent isolates from patients, reflecting results seen in a recent clinical trial of miltefosine against cutaneous leishmaniasis. A study concluded that miltefosine, at 2.5 mg kg$^{-1}$ day$^{-1}$, had comparable efficacy to antimonials when treating presumed L. (V.) panamensis in Colombia, as previously reported. 7,16,17 That study concluded that miltefosine may be a useful drug for the treatment of South American CL. However, species identification, perhaps, should be a priority before treatment to give the best chance of efficacy and to reduce the possibility of either encouraging resistance or suppressing latent foci due to the potential necessity. Resistance mechanisms to miltefosine have been identified in laboratory-induced Leishmania. 16,17 It would be interesting to see if these were present in unresponsive “wild-type” parasites. 16

The utility of the in vitro test when assessing isolate drug sensitivity could be improved by testing parasites pre- and post-treatment, but to date, the number of samples where this is possible is not significant. Finally, the relevance of in vitro data to clinical outcome must also be considered as the two parameters may not necessarily correlate—confounding factors such as host immunity are absent from these tests. 16

The data serve to emphasize the extremely complex clinical picture of leishmaniasis.

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Sensitivity of *Leishmania* Isolates to Miltefosine


