In Vitro Susceptibilities of *Leishmania donovani* Promastigote and Amastigote Stages to Antileishmanial Reference Drugs: Practical Relevance of Stage-Specific Differences

Marieke Vermeersch, Raquel Inocêncio da Luz, Kim Toté, Jean-Pierre Timmermans, Paul Cos, and Louis Maes

Laboratory of Microbiology, Parasitology, and Hygiene (LMPH) and Laboratory of Cell Biology and Histology, Faculty of Pharmaceutical, Biomedical, and Veterinary Sciences, University of Antwerp, Groenenborgerlaan 171, 2020 Antwerp, Belgium

Received 23 April 2009/Returned for modification 10 June 2009/Accepted 15 June 2009

The in vitro susceptibilities of the reference strain *Leishmania donovani* MHOM/ET/67/L82 to sodium stibogluconate, amphotericin B, miltefosine, and the experimental compound PX-6518 were determined for extracellular log-phase promastigotes, established axenic amastigotes, fresh spleen-derived amastigotes, and intracellular amastigotes in primary mouse peritoneal macrophages. Susceptibility to amphotericin B did not differ across the various axenic models (50% inhibitory concentrations [IC₅₀], 0.6 to 0.7 μM), and amphotericin B showed slightly higher potency against intracellular amastigotes (IC₅₀ 0.1 to 0.4 μM). A similar trend was observed for miltefosine, with comparable efficacies against the extracellular (IC₅₀ 0.4 to 3.8 μM) and intracellular (IC₅₀ 0.9 to 4.3 μM) stages. Sodium stibogluconate, used either as Pentostam or as a crystalline substance, was inactive against all axenic stages (IC₅₀ >64 μg Sb/V/ml) but showed good efficacy against intracellular amastigotes (IC₅₀ 22 to 28 μg Sb/V/ml); the crystalline substance was about two to three times more potent (IC₅₀ 9 to 11 μg Sb/V/ml). The activity profile of PX-6518 was comparable to that of sodium stibogluconate, but at a much higher potency (IC₅₀ 0.1 μg/ml). In conclusion, the differential susceptibility determines which in vitro models are appropriate for either drug screening or resistance monitoring of clinical field isolates. Despite the more complex and labor-intensive protocol, the current results support the intracellular amastigote model as the gold standard for in vitro *Leishmania* drug discovery research and for evaluation of the resistance of field strains, since it also includes host cell-mediated effects. Axenic systems can be recommended only for compounds for which no cellular mechanisms are involved, for example, amphotericin B and miltefosine.

Current first-line chemotherapy of leishmaniasis relies on a rather limited arsenal of drugs including sodium stibogluconate, meglumine antimoniate, amphotericin B, and miltefosine, but these entail either problems of emerging resistance, severe side effects, or high costs (5). Since vaccines are not yet on the horizon (23), maintenance and improvement of existing treatment regimens, combined with new drug discovery initiatives, appear to be the only ways to guarantee continued control of this important tropical disease (3, 11, 27).

Both for drug screening and for determination of the susceptibility of field strains, different laboratory methods are being used that focus on the promastigote, the axenic amastigote, or the intracellular amastigote stage. However, it remains unclear how these models cross-validate each other. The differences in environmental conditions between promastigotes and amastigotes in vivo are reflected in their needs for in vitro cultivation. While promastigotes are easily cultured in suspension (8), amastigotes are more difficult to maintain in vitro, since they require macrophages as host cells to meet the highly acidic intracellular environment (15). Cultivation of axenic amastigotes in suspension has been successful, enabling promising new opportunities for drug screening and mode-of-action studies (31, 38). For field strains, infection of macrophages with metacyclic promastigotes is generally used but is subject to a high degree of variability in infectivity depending on the growth characteristics of the isolate and the level of in vitro metacyclogenesis (6). Despite the large body of literature on the subject, the central question remains which laboratory system is optimally suited for either drug-screening purposes or field strain susceptibility testing against the current first-line antileishmanial drugs.

The aim of the present study was to compare the different in vitro test systems and the intrinsic susceptibilities of the various stages of a single reference strain of *Leishmania donovani* (MHOM/ET/67/L82) to pentavalent antimony (Sb⁵⁺), amphotericin B, miltefosine, and the experimental “antileishmanial lead” compound PX-6518, a saponin mixture isolated from the Vietnamese plant *Maesa balansae* (19) and included in the test to underline the importance of current findings for drug screening and mode-of-action studies. To our knowledge, this is a first report on the in vitro sensitivities of all *Leishmania* stages (including freshly collected ex vivo amastigotes) to a broad range of standard reference drugs in a single integrated experimental setup.

MATERIALS AND METHODS

Culture media, products, reagents, and animals. Adenosine, folic acid, d-biotin, hemin, NaHCO₃, potato starch, dimethyl sulfoxide, Giemsa stain, and...
resazurin were purchased from Sigma, whereas minimal essential medium (MEM), RPMI-1640 medium, l-glutamine, fetal calf serum (FCS), and trypsin-EDTA (0.25%) were supplied by Invitrogen. The powder form of sodium stibogluconate and the marketed injectable formulation Pentostam (GSK) were included along with miltefosine (pure crystalline compound obtained from WHO-TDR) and amphotericin B-deoxycholate (Fungizone; Squibb). The experimental drug PX-6518 was available from previous investigations (19).

Golden hamsters and Swiss mice were supplied by Janvier (France). Animal experiments were approved by the ethical committee of the University of Antwerp.

Parasite and cell cultures. Leishmania donovani MHOM/ET/67/L82 (also known as LV9 or HU3) was obtained as a cryostable from the Institute of Tropical Medicine in Antwerp, Belgium, and was used to infect golden hamsters serving as donor animals. The adapted axenic amastigote culture of the same strain was obtained from the Swiss Tropical Institute and further maintained in Schneider’s medium. For the specific purpose of this experiment, the axenic amastigotes were converted back to promastigotes and were maintained in MEM supplemented with 200 mM l-glutamine, 16.5 mM NaHCO3, 10% heat-inactivated FCS, 40 mg/liter adenine, 3 mg/liter folic acid, 2 mg/liter D-biotin, and 2.5 mg/liter hemin. Fresh ex vivo amastigotes were purified from the spleen of a 6-week-infected donor hamster and were immediately kept at 37°C on acidic amastigote medium. Primary mouse peritoneal macrophages (PMM) were collected from Swiss mice 2 days after peritoneal stimulation with a 2% potato starch suspension. Cells were collected and grown in RPMI-1640 medium supplemented with 200 mM l-glutamine, 16.5 mM NaHCO3, and 5% inactivated FCS. Human simian virus 40-immortalized lung fibroblasts (MRC-5 SV2) were maintained in MEM supplemented with 200 mM l-glutamine, 16.5 mM NaHCO3, 10% heat-inactivated FCS, 40 mg/liter adenine, 3 mg/liter folic acid, 2 mg/liter D-biotin, and 2.5 mg/liter hemin. Fresh ex vivo amastigotes were purified from the spleen of a 6-week-infected donor hamster and were immediately kept at 37°C on acidic amastigote medium. Primary mouse peritoneal macrophages (PMM) were collected from Swiss mice 2 days after peritoneal stimulation with a 2% potato starch suspension. Cells were collected and grown in RPMI-1640 medium supplemented with 200 mM l-glutamine, 16.5 mM NaHCO3, and 5% inactivated FCS. Human simian virus 40-immortalized lung fibroblasts (MRC-5 SV2) were obtained from the European Collection of Cell Cultures (United Kingdom) and maintained in MEM supplemented with 5% FCS and 1% l-glutamine. All amastigote cultures and assays and the cytotoxicity assay were conducted at 37°C under 5% CO2. The promastigote cultures and assay mixtures were kept at 25°C under normal atmospheric conditions.

Compound solutions/dilutions. Stock solutions of crystalline sodium stibogluconate (NaSbV [31.3% SbV]) were prepared in preheated phosphate-buffered saline at 37°C and were immediately used in the various tests. Pentostam (100 mg SbV/ml), miltefosine, and semipurified PX-6518 were dissolved/diluted in MilliQ-water. All results with crystalline NaSbV and Pentostam are presented as SbV μg/ml equivalents. The stock solutions were kept at −20°C prior to use, except for miltefosine and Pentostam, which were stored at 4°C. For axenic tests, twofold serial dilutions of the reference drugs were spotted in 96-well flat-bottom microplates (Greiner) prior to addition of the biological test before infection with either promastigotes, axenic amastigotes, or ex vivo amastigotes. To assure optimal macrogolycenosis prior to infection, promastigotes were preconditioned for 24 h at 37°C in acidified promastigote medium (pH 5.4).

For promastigotes, an infection ratio of 15 stages per macrophage was used. Since axenic and ex vivo amastigotes tend to multiply slightly more extensively in PPM, the infection ratio was kept at 5. Compound dilutions were added 2 h after infection, except for promastigotes, which were left undisturbed for another 24 h to allow proper internalization and transformation into amastigotes. At this stage, the medium was replaced to remove the noninternalized stages. After 5 days of incubation, total parasite burdens were microscopically assessed on Giemsa-stained wells and were compared to those for untreated infected controls. The results are expressed as the percentages of reduction in the parasite burden from that in wells with untreated infected controls, and the IC50s were calculated. At least six independent tests were performed for each observation.

Electron microscopic studies. A small-scale ultrastructural study was performed with the knowledge that long-term cultivation of axenic amastigotes may promote the reappearance of promastigote-like features (16, 24). Samples for transmission electron microscopy were obtained after centrifugation of extracellular stages or trypsinized PMM with intracellular stages, fixation in a 0.1 M sodium cacodylate-buffered (pH 7.4) 2.5% glutaraldehyde solution for 2 h, rinsing (three times, for 10 min each time) in 0.1 M sodium cacodylate-buffered (pH 7.4) 7.5% saccharose, and postfixation in 1% OsO4 solution for 1 h. After dehydration in an ethanol gradient (70% ethanol [20 min], 96% ethanol [20 min], and 100% ethanol [twice, for 20 min each time]), samples were embedded in Durcupan ACM. Ultrathin sections were stained with uranyl acetate and lead citrate and were examined in a Philips CM 10 microscope at 80 kV.

Intracellular drug sensitivity assays. PMM were seeded in 96-well microtiter plates at 3 × 104 cells/well and were left for adhesion and differentiation for 48 h before infection with either promastigotes, axenic amastigotes, or ex vivo amastigotes. The 50% inhibitory concentration (IC50) was calculated by Probit analysis. At least six independent replicates were performed for each observation.

Results

In vitro susceptibility testing. The activity of amphotericin B did not differ much across the different models and stages (Table 1). Mean IC50 against axenic log-phase promastigotes, established axenic, and ex vivo amastigotes ranged from 0.6 to 0.4 μM. Slightly higher potency was observed against the intracellular amastigote stage (IC50, 0.1 to 0.4 μM), irrespective of the stages that were used for infection.

A similar trend was observed for miltefosine, with almost comparable efficacies against the extracellular and intracellular stages; established axenic and ex vivo amastigotes were slightly more sensitive (IC50, 0.4 to 0.9 μM).

Pentostam and NaSbV were not active against promastigotes (IC50 >64 μg SbV/ml), but surprisingly, no activity was observed against established axenic amastigotes or freshly collected spleen-derived ex vivo amastigotes (IC50 >64 μg SbV/ml). Against intracellular amastigotes, Pentostam yielded IC50 of 22 to 28 μg SbV/ml, although with a higher degree of variability among the different replicates. It is noteworthy that

### Table 1. IC50 of antileishmanial reference compounds against extracellular stages and intracellular amastigotes of *L. donovani* MHOM/ET/67/L82 in PPM after infection with either promastigotes, axenic amastigotes, or ex vivo amastigotes

<table>
<thead>
<tr>
<th>Reference compound (unit)</th>
<th>Axenic evaluation model</th>
<th>Cellular (PPM) evaluation model</th>
<th>Cytotoxicity (MRC-5 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Log-phase promastigotes</td>
<td>Axenic amastigotes</td>
<td>Ex vivo amastigotes</td>
</tr>
<tr>
<td>Amphotericin B (µM)</td>
<td>0.7 ± 0.2</td>
<td>0.7 ± 0.3</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>Miltefosine (µM)</td>
<td>3.8 ± 2.1</td>
<td>0.4 ± 0.2</td>
<td>0.9 ± 0.5</td>
</tr>
<tr>
<td>PX-6518 (µg/ml)</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
</tr>
<tr>
<td>Pentostam (µg/ml)</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td>&gt;64</td>
</tr>
<tr>
<td>NaSbV (µg/ml)</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td>&gt;64</td>
</tr>
</tbody>
</table>

*Promastigotes, axenic amastigotes, and ex vivo amastigotes.*
crystalline NaSb\textsuperscript{V} showed about a two- to threefold increase in potency, with IC\textsubscript{50}s ranging from 8.8 to 10.5 \(\mu\)g Sb\textsuperscript{V}/ml.

Like Pentostam and NaSb\textsuperscript{V}, PX-6518 showed no efficacy against promastigotes, established axenic amastigotes, or ex vivo amastigotes. A consistent, high activity was obtained against intracellular stages after infection with either axenic or ex vivo amastigotes (IC\textsubscript{50} 0.1 \(\mu\)g/ml). Infection with metacyclic promastigotes resulted in marginally lower efficacy (IC\textsubscript{50} 0.7 \(\mu\)g/ml), which may reflect incomplete intracellular transformation of promastigotes into amastigotes.

The applicability of the different models for either drug screening or determination of the resistance of field strains is summarized in Table 2.

**Morphological evaluation.** Specific attention was given to the established axenic amastigotes in an attempt to ascertain whether the parasite stages were either amastigote-like or promastigote-like. Light microscopy showed amastigote-like organisms, i.e., clear, immobile, rounded stages with dense cell masses, about 2 \(\mu\)m in diameter. However, ultrastructural characteristics indicated the presence of organisms that were more promastigote-like, i.e., more-oval cell bodies, the presence of a strong protruding flagellum, and the absence of megasomes (Fig. 1a and b). In contrast, fresh ex vivo and intracellular amastigote stages (Fig. 1c) did not possess flagella beyond the margins of the cell body, were clearly rounded, and exhibited structures resembling megasomes.

### DISCUSSION

The *Leishmania* life cycle comprises different developmental stages, all of which have been used for either drug-screening purposes or determination of the resistance of field isolates. However, specific recommendations are needed depending on the experimental objective. To address this, the comparative in vitro sensitivities of all possible stages of a single *L. donovani* reference strain (MHOM/ET/67/L82) to all current first-line drugs and one experimental drug, PX-6518, were determined (Table 1).

The main observation for all reference drugs was that log-phase promastigotes tended to be less sensitive than the other extracellular or intracellular stages and were fully refractory to Sb\textsuperscript{V} and PX-6518. These findings and the fact that promastigotes are the vector stage, differing considerably from the intracellular mammalian amastigote target, provide a convincing argument for excluding them for drug-screening purposes (Table 2). On the other hand, susceptibility to amphotericin B and miltefosine, which are known to affect membrane integrity directly or indirectly and which are able to exert antileishmanial action independently of cell-mediated parasiticidal mechanisms, has been clearly demonstrated (20, 26, 40). It is therefore not too speculative to propose that promastigotes could indeed be used for determination of the resistance of field isolates to amphotericin B and miltefosine (18, 25). This procedure will become particularly relevant for the large-scale monitoring of resistance to miltefosine in the Indian subcontinent, in view of its recent introduction as a first-line medication in the current “Kala-Azar Elimination Program” (36; WHO Regional Office for South-East Asia, meeting on guidelines and standard operating procedures for kala-azar elimination, Kolkata, India, 16 April 2007).

### TABLE 2. Suitability of different *L. donovani* in vitro laboratory models for drug screening and drug susceptibility determination

<table>
<thead>
<tr>
<th>Test system (<em>L. donovani</em>)</th>
<th>Drug screening</th>
<th>Determination of susceptibility to the following drug(^b):</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log-phase promastigotes</td>
<td>–</td>
<td>AMB + MILT – Sb – PX-6518 –</td>
</tr>
<tr>
<td>Established axenic amastigotes</td>
<td>–</td>
<td>AMB – MILT – Sb – PX-6518 –</td>
</tr>
<tr>
<td>Ex vivo axenic amastigotes</td>
<td>–</td>
<td>AMB + MILT – Sb – PX-6518 –</td>
</tr>
<tr>
<td>Intracellular amastigotes</td>
<td>+</td>
<td>AMB + MILT + Sb + PX-6518 +</td>
</tr>
</tbody>
</table>

\(^a\) +, suitable; –, not suitable; NA, not applicable.

\(^b\) AMB, amphotericin B; MILT, miltefosine; Sb, antimony (Pentostam or Sb\textsuperscript{V}).

FIG. 1. Ultrastructural details of in vitro-established axenic and intracellular amastigotes. Shown are *Leishmania donovani* L82 axenic amastigotes in cell-free medium (a and b) and 72 h after infection of PMM (c). Established axenic amastigotes show oval cell bodies with a clear nucleus (n) and kinetoplast (k) and with flagella (f) protruding from the flagellar pocket. In contrast, intracellular axenic amastigotes are more rounded and possess short flagella and megasome-like (m) structures.
Established axenic amastigotes have been positioned as an attractive alternative to the more complicated and labor-intensive cellular amastigote test in drug screening, but they obviously cannot be proposed for monitoring of the resistance of clinical field strains (Table 2). Publications on similarities between axenic and intracellular amastigotes in terms of morphology, gene regulation, enzyme profile, and sensitivity to some reference drugs may strengthen the use of established axenic amastigotes as a promising alternative drug-screening model (4, 9, 37). However, other investigators have drawn attention to marked differences between axenic amastigotes and lesion-derived ex vivo or intracellular amastigotes (17). In addition, long-term axenic cultivation may promote the reappearance of promastigote-like features, indicating the need for caution use and continued follow-up of the amastigote-like characteristics (16, 24). Based on the drug sensitivity profile (Table 1) and the small-scale transmission electron microscopic study, our axenic amastigote model exhibited distinct promastigote-like characteristics, despite all precautions to prevent the culture from drifting back to the promastigote stage. Under light microscopy, the axenic amastigotes appeared as immobile round stages with dense cell masses (diameter, about 2 μm). Ultrastructurally, flagella protruding from the flagellar pocket were frequently observed (Fig. 1a and b). On the other hand, internalized axenic amastigotes were clearly rounded, without protruding flagella, and contained megasome-like structures (Fig. 1c), which are membrane-bound compartments with lysosomal properties described for amastigotes of Leishmania chagasi (1) and Leishmania mexicana complex (39). Apart from these ultrastructural indications, our in vitro results with SbV and PX-6518 also confirm that the axenic amastigotes behaved as promastigotes. A general consensus is that SbV acts as a prodrug, exerting its parasiticial effects through reduced SbIII, which is highly toxic to all stages (2, 7). Even though several reports describe selective activity of SbV on established axenic amastigotes (13, 32, 33), other researchers argue that cellular mechanisms are indeed required for the antileishmanial action of SbV (12, 22, 28). Although the mechanism of action of PX-6518 is not yet known, our results indicate that its action is mainly cell-mediated as well, as shown by its lack of efficacy in the axenic models (IC50 >32 μg/ml) and its high potency against intracellular amastigotes (IC50, 0.1 μg/ml).

Besides PMM, other cells, such as U937, J774, THP-1, and bone marrow-derived and human monocyte-derived macrophages, have been used in the intracellular amastigote model. Recently, it was shown that the macrophage cell type may influence the levels of activity that are obtained with current antileishmanial drugs, necessitating harmonization if results from different laboratories are to be compared (29). No differences in the IC50S of the reference drugs were found upon infection of PPM with metacyclic promastigotes, established axenic amastigotes, or fresh spleen-derived ex vivo amastigotes (Table 1). Amphotericin B showed slightly higher potency in the cellular model than in the axenic model, a difference that is likely related to accumulation in the phagolysosome. Miltefosine showed no clear trend to support any theory of differential sensitivity. Stibogluconate was active only in the cellular model, with a higher potency for the crystalline substance than for Pentostam (IC50S, 22.0 to 28.4 μg SbV/ml). A similar differential susceptibility was observed for PX-6518, but at a much higher potency (IC50, 0.1 μg/ml). The slightly lower activity after infection with metacyclic promastigotes (IC50, 0.7 μg/ml) may be related to incomplete intracellular transformation into amastigotes. The latter phenomenon has been shown to occur frequently with clinical field isolates during their adaptation to in vitro culture and will affect the outcome of in vitro Sb resistance determinations (L. Maes, unpublished data).

Overall, the results obtained in the present study are within the range of published IC50S for L. donovani strain L82 (10, 14, 30, 35): IC50S of 4.8 to 34.5 μg SbV/ml were reported for sodium stibogluconate against intracellular amastigotes; miltefosine exhibited IC50S of 0.18 to 13.6 μM on promastigotes and 2.8 to 5.8 μM on intracellular amastigotes, while amphotericin B showed IC50S of 0.003 to 0.15 μM and 0.026 to 0.076 μg/ml against promastigotes and intracellular amastigotes, respectively.

In summary, this study clearly demonstrates that the intracellular amastigote model is the only suitable approach for drug screening. The same cellular model is also required for the determination of resistance to Sb in view of its cell-mediated mode of action. For the latter, particular care must be taken to ascertain adequate intracellular transformation into amastigotes, a problem frequently encountered with poorly infective field strains. If field strains are infective to hamsters, it may sometimes be helpful to use ex vivo amastigotes as an infection inoculum for macrophages. For amphotericin B and miltefosine, the simpler axenic models could theoretically be used for resistance detection, but validation with a large set of field strains is still needed.

ACKNOWLEDGMENTS

This work was supported by a grant from FWO Flanders (project G.0103.06) and the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR). M. Vermeersch is a BOF-NOI 20282 Ph.D. student at the University of Antwerp. P. Cos is a postdoctoral researcher awarded a grant by the Fund for Scientific Research (FWO Flanders, Belgium).

REFERENCES


