In vitro activity of anti-leishmanial drugs against Leishmania donovani is host cell dependent

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Objectives: To evaluate the in vitro activity of anti-leishmanial drugs against intracellular Leishmania donovani amastigotes in different types of macrophages.

Methods: Mouse peritoneal macrophages (PEMs), mouse bone marrow-derived macrophages (BMMΦ), human peripheral blood monocyte-derived macrophages (PBМΦ) and differentiated THP-1 cells were infected with L. donovani. Cultures were incubated with sodium stibogluconate, amphotericin B deoxycholate (Fungizone®), miltefosine or paromomycin sulphate over six concentrations in 3-fold serial dilutions for 5 days. Analysis was based on percentage inhibition of infected macrophages and EC50/EC90 values estimated using sigmoidal curve-fitting.

Results: The rank order of drug activity was the same in the different macrophage populations: amphotericin B > miltefosine > sodium stibogluconate > paromomycin. However, significant (P < 0.05) differences were observed between populations. Amphotericin B was more active in PEMs and BMMΦ (EC50 0.02–0.06 μM) compared with PBМΦ and differentiated THP-1 cells (EC50 0.08–0.40 μM) and miltefosine was more active in PBМΦ (EC50 0.16–0.74 μM) compared with PEMs and BMMΦ (EC50 2.60–7.67 μM). Sodium stibogluconate displayed highest activity in PBМΦ (EC50 1.38–1.89 μg Sb/μL), followed by PEMs (EC50 21.75–27.79 μg Sb/μL) and BMMΦ and differentiated THP-1 cells (EC50 28.96–112.77 μg Sb/μL). Paromomycin showed highest activity in PBМΦ (EC50 80.03–104.38 μM) and PEMs (EC50 75.42–201.63 μM).

Conclusions: In vitro activity of anti-leishmanial drugs is host cell dependent. This has implications for: (i) the evaluation of in vitro drug activity; (ii) the evaluation of drug susceptibility of clinical isolates; and (iii) the standardization of anti-leishmanial drug assays.

Keywords: leishmaniasis, macrophages, biological activity

Introduction

Leishmaniases are a spectrum of diseases caused by trypanosomatid parasites of the genus Leishmania. Parasites survive and multiply within phagolysosomes of macrophages as intracellular amastigotes. Pathologies range from self-limiting cutaneous leishmaniasis (CL) to fatal visceral leishmaniasis (VL) with 1.5–2 million new cases per year, 0.5 million of which are VL. Treatment options include pentavalent antimony (sodium stibogluconate or meglumine antimoniate), the polyene amphotericin B (as deoxycholate salt or a liposomal formulation, AmBisome®), the alkylphosphocholine miltefosine and the aminoglycoside paromomycin. Despite recent advances safety, resistance and cost issues necessitate the continued effort to identify improved anti-leishmanial drugs. Studies have also been undertaken to test the drug susceptibility of clinical isolates.

Biological evaluations rely on in vitro models that simulate the environment of the intracellular amastigote stage. Models have utilized primary macrophages and cell lines as host cells, including mouse peritoneal and human blood monocyte-derived macrophages, human monocytic THP-1 cells, human promonocytic U937 cells and murine J774.1 cells.

We aimed to assess the host cell dependence of anti-leishmanial drug activity through a direct comparative analysis by evaluating the in vitro activity of four anti-leishmanial drugs against Leishmania donovani amastigotes in four different host cells.
Materials and methods
Preparation of macrophages

Mouse peritoneal macrophages (PEMs) were harvested from CD1 mice (Charles River Ltd, Margate, UK) by lavage 24 h after intraperitoneal injection of 2% soluble starch (Sigma).

Bone marrow-derived macrophages (BMMFs) were obtained from femurs of female BALB/c mice (Charles River Ltd). Briefly, cavities were flushed with Dulbecco's modified Eagle's medium (DMEM) plus 10% heat-inactivated fetal calf serum (hi-FCS), 100 U/mL penicillin, 100 μg/mL streptomycin and 8 mM glutamine (all Sigma). Cells were pelleted by centrifugation (300 \( \times \) g, 10 min, 4°C) and resuspended in the above medium plus 15% L-929 fibroblast culture supernatant (source of macrophage colony-stimulating factor; M-CSF). The suspension was flushed with Dulbecco's modified Eagle's medium (DMEM) plus 10% hi-FCS, in quadruplicate at each concentration. The activity-based rank order of anti-leishmanial drugs was the same for all macrophages: amphotericin B > miltefosine > sodium stibogluconate > paromomycin. However, significant \( (p<0.05) \) quantitative differences in drug activity between the different macrophages were observed.

At the EC\(_{50}\) level amphotericin B displayed higher activity in PEMs and BMMFs (EC\(_{50}\) 0.02–0.06 μM) than in differentiated THP-1 cells and PBMMFs (EC\(_{50}\) 0.08–0.40 μM). Miltefosine displayed highest activity against amastigotes in PBMMFs (EC\(_{50}\) 0.16–0.74 μM), followed by differentiated THP-1 cells (EC\(_{50}\) 0.81–5.46 μM), BMMFs (EC\(_{50}\) 2.60–5.83 μM) and PEMs (EC\(_{50}\) 5.73–7.67 μM). Paromomycin sulphate showed poor activity in all macrophage models studied. The highest activity was noted in PBMMFs (EC\(_{50}\) 80.03–104.38 μM) and PEMs (EC\(_{50}\) 75.42–201.63 μM). Sodium stibogluconate was most active against amastigotes in PBMMFs (EC\(_{50}\) 1.38–1.89 μg Sbv/mL), followed by PEMs (EC\(_{50}\) 21.75–27.79 μg Sbv/mL), differentiated THP-1 cells and BMMFs (EC\(_{50}\) 28.96–112.77 μg Sbv/mL). A similar picture emerged at the EC\(_{50}\) level, but fewer data were obtained for sodium stibogluconate and paromomycin sulphate due to their low activity and higher variability in this region of the dose–response curve. Results were consistent and reproducible in three separate experiments (Table 1).

Analysis of anti-leishmanial drug activity

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Analysis of course of infection between different macrophage populations

The course of infection in the different macrophages was analysed based on the percentage of infected macrophages 1 day, 4 days and 6 days post-infection. Statistically significant differences \( (p<0.05) \) were observed.

Pooled analysis from all experiments (Figure 1) showed a decrease in the infection level between day 1 and day 6 post-infection for PEMs and BMMFs. In PBMMFs increases were observed from day 1 to day 4 and day 4 to day 6 post-infection. In differentiated THP-1 cells infection increased from day 1 to day 4 and then decreased until day 6 post-infection. Initial infection levels...
### Table 1. Drug activity against *L. donovani* in four different macrophage populations

<table>
<thead>
<tr>
<th>Host cell/% infection</th>
<th>Amphotericin B</th>
<th>Miltefosine</th>
<th>Paromomycin(^a)</th>
<th>Sodium stibogluconate ((\mu g) Sb(^b)/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC(_{50})</td>
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<tr>
<td>PEmS</td>
<td>90 ± 3</td>
<td>0.06 (0.05–0.07)</td>
<td>0.19 (0.13–0.24)</td>
<td>7.67 (6.53–8.81)</td>
</tr>
<tr>
<td></td>
<td>79 ± 4</td>
<td>0.03 (0.02–0.03)</td>
<td>0.05 (0.04–0.06)</td>
<td>5.73 (4.88–6.67)</td>
</tr>
<tr>
<td></td>
<td>90 ± 3</td>
<td>22.25 (15.79–28.71)</td>
<td>75.42 (65.48–85.35)</td>
<td>&gt;300</td>
</tr>
<tr>
<td></td>
<td>79 ± 4</td>
<td>201.63 (177.26–226.00)</td>
<td>&gt;900</td>
<td></td>
</tr>
<tr>
<td>BM(\Phi)</td>
<td>80 ± 4</td>
<td>5.60 (4.60–6.59)</td>
<td>32.74 (15.45–50.02)</td>
<td>&gt;300</td>
</tr>
<tr>
<td></td>
<td>37 ± 7</td>
<td>5.83 (2.45–9.21)</td>
<td>9.47 (9.32–9.62)</td>
<td>&gt;300</td>
</tr>
<tr>
<td></td>
<td>68 ± 7</td>
<td>2.60 (1.66–3.45)</td>
<td>14.32 (10.48–18.15)</td>
<td>&gt;900(^b)</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>321.61 (167.25–475.98)</td>
<td>&gt;900(^b)</td>
<td></td>
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<tr>
<td>THP-1</td>
<td>75 ± 4</td>
<td>2.47 (1.66–3.27)</td>
<td>5.41 (1.89–8.94)</td>
<td>&gt;300</td>
</tr>
<tr>
<td></td>
<td>65 ± 4</td>
<td>0.81 (0.55–1.07)</td>
<td>6.99 (0–16.65)</td>
<td>&gt;300</td>
</tr>
<tr>
<td></td>
<td>96 ± 1</td>
<td>5.46 (3.34–7.58)</td>
<td>&gt;300(^b)</td>
<td></td>
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<tr>
<td></td>
<td>300</td>
<td>165.74 (140.99–190.49)</td>
<td>&gt;900</td>
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<tr>
<td>PB(\Phi)</td>
<td>50 ± 8</td>
<td>0.40 (0.38–0.42)</td>
<td>0.45 (0.44–0.46)</td>
<td>&lt;1.11</td>
</tr>
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<td></td>
<td>74 ± 5</td>
<td>0.74 (0.67–0.81)</td>
<td>7.02 (1.63–2.41)</td>
<td>&gt;300</td>
</tr>
<tr>
<td></td>
<td>59 ± 5</td>
<td>0.16 (0.10–0.21)</td>
<td>0.72 (0.51–0.93)</td>
<td>&gt;900</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>80.03 (59.66–100.40)</td>
<td>&gt;900</td>
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</tr>
</tbody>
</table>

\(^a\)Based on the salt (paromomycin sulphate).
\(^b\)Inhibition close to 50%, 90%.

No, not obtained.

Data represent the geometric mean of quadruplicate EC\(_{50}\)/EC\(_{90}\) values for each experiment and three separate experiments. Values are in \(\mu M\) unless indicated otherwise and 95% confidence intervals are given in brackets.

% infection gives the percentage of infected macrophages at endpoint ± SEM.
Similar observations have been reported previously,\textsuperscript{3,9} including based on the same criteria as used to determine drug activity. Studies with longer incubation times reported higher activities for paromomycin and sodium stibogluconate,\textsuperscript{3,7} which gives data for two experiments with four points each (\textit{n}=8). Values next to the bars indicate the level of infection. Error bars represent SEM.

were highest in PEMs (92%), followed by differentiated THP-1 cells and BMM\textdelta (68%) and PBMM\textdelta (35%).

Discussion

This is the first report focused on a direct comparative analysis of drug activity for all four standard anti-leishmanial drugs against intracellular \textit{L. donovani} in a panel of different macrophages. We report a clear host cell dependence for sodium stibogluconate, intermediate for miltefosine and less so for amphotericin B and paromomycin. However, interpretation of data for paromomycin is limited by its low activity. Studies with longer incubation times reported higher activities for paromomycin and sodium stibogluconate in PEMs.\textsuperscript{3,7}

The differences in amphotericin B activity between different macrophages is in the same order of magnitude as previously described by one of the authors.\textsuperscript{8} Observations on differences in drug activity are based on heterogeneity of macrophage populations well known in immunological studies but poorly defined in drug studies.

In vitro anti-leishmanial activity against the intracellular stage is a critical decision point in drug discovery and important when analysing drug susceptibility of clinical isolates. Our data emphasize that interpretation of results and comparisons between studies need to take the host cell into account, best demonstrated by the up to 56-fold difference in the activity of sodium stibogluconate against \textit{L. donovani} in PBMM\textdelta compared with differentiated THP-1 cells. In parallel we assessed the development of \textit{L. donovani} infection in the different macrophages, based on the same criteria as used to determine drug activity. Similar observations have been reported previously,\textsuperscript{3,9} including lower infection levels in PBMM\textdelta compared with PEMs and BMM\textdelta for \textit{Leishmania infantum}.\textsuperscript{10} Such differences may reflect differences in previous exposure to tissue-specific environmental stimuli, which may influence cell metabolism and function,\textsuperscript{9} and macrophages studied were derived from different origins and by different methods. Infection level and intracellular parasite burden can also affect drug activity, well known for sodium stibogluconate in PEMs.\textsuperscript{3}

In conclusion we have demonstrated that anti-leishmanial drug activity \textit{in vitro} can be host cell dependent. Within the framework of harmonization our data favour the use of PBMM\textdelta and PEMs for the evaluation of anti-leishmanial drug activity and drug susceptibility of clinical isolates. Further studies are required to understand the differences in phenotypes.

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Transparency declarations

None to declare.

References