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 (PBVΦ) 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 PBVΦ and differentiated THP-1 cells (EC50 0.08–0.40 µM) and miltefosine was more active in PBVΦ (EC50 0.16–0.74 µM) compared with PEMs and BMMΦ (EC50 2.60–7.67 µM). Sodium stibogluconate displayed highest activity in PBVΦ (EC50 1.38–1.89 µg Sbv/mL), followed by PEMs (EC50 21.75–27.79 µg Sbv/mL) and BMMΦ and differentiated THP-1 cells (EC50 28.96–112.77 µg Sbv/mL). Paromomycin showed highest activity in PBVΦ (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 promonoctytic 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 female BALB/c mice (Charles River Ltd, Margate, UK) by lavage 24 h after intraperitoneal injection of 2% soluble starch (Sigma).

Bone marrow-derived macrophages (BMMΦ) 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 at 300 g for 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 incubated in Petri dishes at 37°C and 5% CO2 for 8 days with addition of fresh medium after 4–5 days to obtain macrophage monolayers.

Peripheral blood monocyte-derived macrophages (PBMMΦ) were obtained from heparinized venous blood from healthy volunteers. Blood was diluted 1:1 in RPMI 1640 medium, layered onto Histopaque®-1077 (Sigma) and centrifuged for 30 min at 400 g. Mononuclear cells were aspirated from the Histopaque®-plasma interface, washed three times with warm RPMI 1640 medium and resuspended in RPMI 1640 medium plus penicillin (100 U/mL), streptomycin (100 µg/mL) and 10% autologous serum. Cells were incubated at 37°C and 5% CO2 for 6 days and the medium was replaced twice during this period, which produced macrophage monolayers.

THP-1 cells were incubated in RPMI 1640 medium plus 10% hi-FCS and 20 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma) at 37°C and 5% CO2 for 72 h to induce maturation. Differentiated THP-1 cells were washed three times with fresh medium containing no PMA prior to infection.

Macrophages were plated in Lab-tek 16-well chamber slides (VWR International Ltd, UK) at a density of 5 x 104 cells/well in RPMI 1640 medium plus 10% hi-FCS and left to adhere overnight at 37°C and 5% CO2 before infection.

All animal experiments were conducted under licence in accordance with UK Home Office approval.

Parasites and infection

L. donovani strain MHOM/ET/67/L82 was used and amastigotes harvested from the spleen of infected Syrian hamsters. Host cells were infected at a ratio of seven amastigotes to one macrophage. After incubating cultures overnight at 37°C and 5% CO2 non-phagocytosed amastigotes were washed off and drug dilutions added as described below.

Preparation of drug stocks and drug assays

A 5.4 mM stock of amphotericin B (Fungizone®; E.R. Squibb and Sons, Hounslow, UK) was obtained following the manufacturer's instructions. Miltefosine (Zentaris GmbH) and paromomycin sulphate (Sigma) stock solutions were prepared at 20 mM in purified water (Milli-Q gradient A10 system) followed by sterile filtration (Minisart® syringe end filters, pore size 0.2 µm). Sodium stibogluconate (GlaxoSmithKline, UK) was prepared at 20 mg Sbv/mL in purified water and dissolution of the powder aided by incubation at 37°C, followed by sterile filtration. All aqueous drug solutions appeared clear and fully dissolved.

Drugs were 3-fold serially diluted over six concentrations in RPMI 1640 medium plus 10% hi-FCS, in quadruplicate at each concentration. The PBMMΦ assay in Experiment 1 used only four concentrations due to limited macrophage numbers. Stock solutions and dilutions were prepared fresh for each use. Concentration ranges were adjusted to cover EC50 and EC90 values where possible. Infected macrophages were incubated with drug dilutions for a total of 5 days at 37°C and 5% CO2; medium was changed once after 3 days. Untreated macrophages received medium plus 10% hi-FCS and infection was determined on days 1, 4 and 6 post-infection. The 6 day post-infection points also served as untreated controls.

Evaluation of drug activity and data analysis

At endpoints slides were fixed with 100% methanol, stained with 10% Giemsa and examined microscopically. One hundred macrophages per well were scored as infected or uninfected. The average of the quadruplicates of untreated cultures was taken to serve as 100% control against which the percentage inhibition of infected macrophages in treated cultures was calculated. EC50 and EC90 values were estimated with the non-linear sigmoidal curve-fitting Levenburg Marquardt algorithm (Microsoft xfit; ID Business Solution, Guildford, UK). Counting and data analysis were performed by two different investigators, one blinded to all conditions. Three separate experiments were performed, each with macrophages infected at the same time with the same batch of parasite. A one-way analysis of variance (ANOVA) was performed to test for statistical significance (GraphPad Prism).

Results

Analysis of anti-leishmanial drug activity

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 EC50 level amphotericin B displayed higher activity in PEMs and PBMMΦ (EC50 0.02–0.06 µM) than in differentiated THP-1 cells and BMMΦ (EC50 0.08–0.40 µM). Miltefosine displayed highest activity against amastigotes in PBMMΦ (EC50 0.16–0.74 µM), followed by differentiated THP-1 cells (EC50 0.81–5.46 µM), BMMΦ (EC50 2.60–5.83 µM) and PEMs (EC50 5.73–7.67 µM). Paromomycin sulphate showed poor activity in all macrophage models studied. The highest activity was noted in PBMMΦ (EC50 80.03–104.38 µM) and PEMs (EC50 75.42–201.63 µM). Sodium stibogluconate was most active against amastigotes in PBMMΦ (EC50 0.138–1.89 µg Sbv/mL), followed by PEMs (EC50 21.75–27.79 µg Sbv/mL), differentiated THP-1 cells and BMMΦ (EC50 28.96–112.77 µg Sbv/mL). A similar picture emerged at the EC90 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 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 PBMMΦ. In PBMMΦ 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>
<thead>
<tr>
<th>Host cell/% infection</th>
<th>Amphotericin B</th>
<th>Miltefosine</th>
<th>Paromomycin&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sodium stibogluconate (μg Sb&lt;sup&gt;III&lt;/sup&gt;/mL)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>EC&lt;sub&gt;90&lt;/sub&gt;</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>EC&lt;sub&gt;90&lt;/sub&gt;</td>
</tr>
<tr>
<td>PE &lt;sup&gt;+&lt;/sup&gt; 90</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>
<td>22.25 (15.79–28.71)</td>
</tr>
<tr>
<td>PE &lt;sup&gt;+&lt;/sup&gt; 79</td>
<td>0.03 (0.02–0.03)</td>
<td>0.05 (0.04–0.06)</td>
<td>5.73 (4.88–6.57)</td>
<td>15.65 (7.67–23.63)</td>
</tr>
<tr>
<td>BM &lt;sup&gt;+&lt;/sup&gt; 80</td>
<td>0.06 (0.04–0.08)</td>
<td>0.14 (0.03–0.25)</td>
<td>5.60 (4.60–6.59)</td>
<td>32.74 (15.45–50.02)</td>
</tr>
<tr>
<td>BM &lt;sup&gt;+&lt;/sup&gt; 37</td>
<td>0.04 (0.03–0.06)</td>
<td>0.06 (0.03–0.09)</td>
<td>5.83 (2.45–9.21)</td>
<td>9.47 (9.32–9.62)</td>
</tr>
<tr>
<td>BM &lt;sup&gt;+&lt;/sup&gt; 68</td>
<td>0.02 (0.02–0.03)</td>
<td>0.04 (0.03–0.04)</td>
<td>2.60 (1.66–3.45)</td>
<td>14.32 (10.48–18.15)</td>
</tr>
<tr>
<td>THP-1 75</td>
<td>0.26 (0.22–0.29)</td>
<td>0.35 (0.28–0.43)</td>
<td>2.47 (1.66–3.27)</td>
<td>5.41 (1.89–8.94)</td>
</tr>
<tr>
<td>THP-1 65</td>
<td>0.11 (0.07–0.15)</td>
<td>0.37 (0.17–0.58)</td>
<td>0.81 (0.55–1.07)</td>
<td>6.99 (0–16.65)</td>
</tr>
<tr>
<td>THP-1 96</td>
<td>0.13 (0.12–0.15)</td>
<td>0.25 (0.21–0.30)</td>
<td>5.46 (3.34–7.58)</td>
<td>&gt;900&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>PB &lt;sup&gt;+&lt;/sup&gt; 50</td>
<td>0.40 (0.38–0.42)</td>
<td>0.45 (0.44–0.46)</td>
<td>&lt;1.11</td>
<td>1.52 (0.97–2.06)</td>
</tr>
<tr>
<td>PB &lt;sup&gt;+&lt;/sup&gt; 74</td>
<td>0.16 (0.14–0.17)</td>
<td>0.21 (0.17–0.24)</td>
<td>0.74 (0.67–0.81)</td>
<td>2.02 (1.63–2.41)</td>
</tr>
<tr>
<td>PB &lt;sup&gt;+&lt;/sup&gt; 59</td>
<td>0.08 (0.05–0.11)</td>
<td>0.13 (0.10–0.15)</td>
<td>0.16 (0.10–0.21)</td>
<td>0.72 (0.51–0.93)</td>
</tr>
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</table>

NO, not obtained.

Data represent the geometric mean of quadruplicate EC<sub>50</sub>/EC<sub>90</sub> values for each experiment and three separate experiments. Values are in μM unless indicated otherwise and 95% confidence intervals are given in brackets.

% infection gives the percentage of infected macrophages at endpoint ± SEM.

<sup>a</sup>Based on the salt (paromomycin sulphate).

<sup>b</sup>Inhibition close to 50%, 90%.
Similar observations have been reported previously,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 against L. donovani in PEMs.3,7 However, interpretation of data for paromomycin is limited by its low activity. Studies with longer incubation times emphasized that interpretation of results and comparisons in drug activity are based on heterogeneity of macrophage populations. The graph is based on the percentage of infected macrophages in a panel of different macrophages. We report a clear host cell dependence for sodium stibogluconate, which is demonstrated by the up to 56-fold difference in the activity of sodium stibogluconate against L. donovani with differentiated THP-1 cells and BMMϕ (68%) and PBMϕ (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 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.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.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 L. donovani in PBMϕ compared with differentiated THP-1 cells. In parallel we assessed the development of L. donovani infection in the different macrophages, based on the same criteria as used to determine drug activity. Similar observations have been reported previously,3,9 including lower infection levels in PBMϕ compared with PEs and BMMϕ for Leishmania infantum.10 Such differences may reflect differences in previous exposure to tissue-specific environmental stimuli, which may influence cell metabolism and function,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.3

In conclusion we have demonstrated that anti-leishmanial drug activity in vitro can be host cell dependent. Within the framework of harmonization our data favour the use of PBMϕ 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**


