In vitro ‘time-to-kill’ assay to assess the cidal activity dynamics of current reference drugs against Leishmania donovani and Leishmania infantum

L. Maes*, J. Beyers, A. Mondelaers, M. Van den Kerkhof, E. Eberhardt, G. Caljon and S. Hendrickx

Laboratory for Microbiology, Parasitology and Hygiene, University of Antwerp, Antwerp, Belgium

*Corresponding author. Tel: +32.3.265.25.27; Fax: +32.3.265.26.81; E-mail: louis.maes@uantwerpen.be

Received 28 April 2016; returned 26 July 2016; revised 3 August 2016; accepted 26 August 2016

Objectives: Despite a continued search for novel antileishmanial drugs, treatment options remain restricted to a few standard drugs, e.g. antimonials, miltefosine, amphotericin B and paromomycin. Although these drugs have now been used for several decades, their mode of action still remains partly hypothetical and their dynamics of cidal action and time-to-kill are still poorly documented.

Methods: An in vitro time-to-kill assay on intracellular amastigotes of the laboratory reference strains Leishmania donovani (MHOM/ET/67/L82) and Leishmania infantum (MHOM/MA(BE)/67/ITMAP263) was set up to evaluate the cidal activity dynamics of the listed reference drugs at three different concentrations: at IC_{50}, 2×IC_{50} and the near cytotoxic dose level (CC_{90} determined on MRC-5 cells). This assay focused on identifying the minimal exposure time needed to completely eliminate viable intracellular amastigotes, using the standard microscopic Giemsa assay and the promastigote back-transformation assay.

Results: While 100% reduction was microscopically apparent for most drugs, the promastigote back-transformation assay clearly demonstrated a concentration- and time-dependent cidal mechanism. The time-to-kill at 2×IC_{50} was >240 h for pentavalent antimony (77 μg eq./mL), 96 h for trivalent antimony (44 μg eq./mL), 168 to >240 h for miltefosine (10 μM), 168 h for paromomycin (100 μM) and >240 h for amphotericin B (2 μM). No differences were noted between both Leishmania species.

Conclusions: Evaluation of the concentration- and time-dependent cidal activity using the promastigote back-transformation assay revealed striking differences in efficacy of the different antileishmanial reference drugs. This assay should allow in-depth pharmacodynamic evaluation of novel drug leads in comparison with the existing antileishmanial drug repertoire.

Introduction

Although used in different combinations and diverse treatment regimens, antimonials, miltefosine, amphotericin B and paromomycin comprise the mainstay of current drug options against visceral leishmaniasis.1,2 Despite being in use for several decades, their mode of action still remains partly hypothetical and surprisingly little is known about the pharmacodynamics and the minimal exposure time required to exert full leishmanicidal potential. Knowledge about the ‘time-to-kill’ may help refine the design of combination therapies that are currently being implemented to combat drug resistance.1,3 Next to the usage of different cell types resulting in variations in drug activity,4 the wide range of drug exposure time from 48 to 120 h for intracellular amastigotes in in vitro susceptibility assays5-8 makes it virtually impossible to directly compare literature data, hence emphasizing once more the need for enhanced harmonization of standard operating procedures in Leishmania drug research.9 Since there is no adequate information on how long intracellular amastigotes must be exposed to a drug to result in full cidal efficacy, the present in vitro study on Leishmania donovani and Leishmania infantum measured the minimal time that is needed to eliminate all viable parasites when exposed to 1×IC_{50} and 2×IC_{50} in addition to the cytotoxic (CC_{90}) drug concentration as a measure for maximal effect. Two techniques were used, namely standard Giemsa-based microscopy and the promastigote back-transformation assay.10

Unravelling the cidal dynamics (‘time-to-kill’) of novel drug lead candidates is considered an essential part of preclinical pharmacokinetic–pharmacodynamic evaluation to support decisions on projected dosing regimens in humans based on in vitro and in vivo laboratory data.11 Surprisingly, the cidal dynamics of the current antileishmanial reference drugs are poorly documented. Based on the pivotal need for a fully standardized in vitro cell-based assay, ex vivo (spleen-derived) amastigotes of reference laboratory species/strains are preferably used to ensure optimal infection dynamics, which are much more variable among clinical
isolates. Moreover, this assay may provide a rating system to better position novel drug leads against the existing spectrum of current antileishmanial drugs.

Materials and methods

Test substances and formulations

For each reference drug, the IC₅₀ (Table 2) and the CC₉₀ as determined by the standard intracellular amastigote susceptibility assay (Table 1) were included for evaluation of ‘time-to-kill’. Potassium antimony tartrate trihydrate (trivalent antimony), miltefosine and paromomycin were purchased from Sigma-Aldrich (Diegem, Belgium); sodium stibogluconate (pentavalent antimony) was obtained from Calbiochem (EMD Millipore Corporation, Billerica, MA, USA). Stock solutions (20 mM) were prepared in PBS for the antimonials and miltefosine and in demineralized water for paromomycin. Amphotericin B (Fungizone®; Gilead, CA, USA) was dissolved in 5% dextrose in water.

Experimental design

To identify ‘time-to-kill’, primary peritoneal mouse macrophages were collected and infected with L. donovani (MHOM/ET/67/L82) and L. infantum (MHOM/MA(BE)/67/ITMAP263) spleen-derived amastigotes at an infection ratio of 15:1.⁴ After 2 h of incubation at 37°C and 5% CO₂, all the parasites have become internalized and the infected cells were exposed to serially diluted concentrations of the antileishmanial reference drugs. The culture medium with drug was renewed every 3 days to ensure optimal macrophage viability and adequate drug exposure during the 10 day (240 h) follow-up period. The infection index (number of intracellular amastigotes/number of cells counted) was determined microscopically every 24 h after Giemsa staining. The percentage reduction of intracellular amastigote burdens in the drug-exposed wells was compared with infected non-treated control wells and used as a measure of drug activity. For each treatment duration, a duplicate assay was run in parallel to assess the viability of the residual amastigote burden using the promastigote back-transformation assay as described previously.³ Briefly, infected macrophages are mechanically disrupted to release any remaining viable amastigotes after drug exposure, thereby allowing back-transformation to the promastigote form. After 2 weeks of incubation at room temperature, the presence of extracellular promastigotes is evaluated microscopically. Based on the number of wells remaining negative upon drug treatment, a percentage reduction can be calculated. The cytotoxic concentration (CC₉₀) was determined on MRC-5 cells, as previously described.⁵ The ‘time-to-kill’ was defined as the shortest time needed for a drug at a particular concentration (IC₅₀, 2 × IC₅₀, CC₉₀) to microscopically clear >95% of the intracellular infection combined with a complete absence of promastigotes (e.g. 100% reduction) in the promastigote back-transformation assay. As control for parasite viability, the increase in parasite burden in untreated infected control cells was monitored microscopically. Macrophage viability was evaluated every 24 h by trypan blue staining of both uninfected and infected macrophages.

Results and discussion

A maximum reduction in macrophage viability of 8.2% ± 0.9% was observed in the non-infected control cultures (data not shown), while infection with L. donovani and L. infantum showed a steady increase in infection index, reflecting adequate macrophage and parasite viability throughout the 10 day course of the experiment (Figure S1 available as Supplementary data at JAC Online). To monitor parasite burdens, and in particular amastigote viability, the standard Giemsa-based microscopy assay was used in tandem with the promastigote back-transformation assay, which shows a much higher sensitivity.⁴ The time-to-kill for each drug is presented in Table 1. The dynamics of amastigote elimination (microscopy) and cidal efficacy (promastigote back-transformation) are presented in detail for each drug (available as Supplementary data at JAC Online).

The experimental protocol specifically used spleen-derived amastigotes of reference laboratory strains known to become fully internalized by the macrophage host cell. However, running the same protocol on clinical isolates using metacyclic promastigotes is likely to produce false results since non-internalized promastigotes may carry over in the back-transformation assay. The use of horse serum has been suggested to minimize this particular problem.¹³

Pentavalent antimonials (Figure S2) failed to eliminate all viable amastigotes since full cidal efficacy could not be obtained within 240 h of drug exposure. On the other hand, trivalent antimony (Figure S3) showed much better efficacy since time-to-kill was reached within 96 h at 2 × IC₅₀ and within 72 h at CC₉₀ (Table 1).

Table 1. In vitro time-to-kill for current antileishmanial reference compounds

<table>
<thead>
<tr>
<th>Drug</th>
<th>Leishmania²</th>
<th>MRC-5</th>
<th>L. infantum</th>
<th>L. donovani</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC₅₀</td>
<td>CC₉₀</td>
<td>IC₅₀</td>
<td>2 × IC₅₀</td>
<td>CC₉₀</td>
</tr>
<tr>
<td>Sb⁴</td>
<td>38.5 eq.</td>
<td>77 eq.</td>
<td>&gt;240</td>
<td>&gt;240</td>
</tr>
<tr>
<td>Sb⁷</td>
<td>22 eq.</td>
<td>88 eq.</td>
<td>120</td>
<td>96</td>
</tr>
<tr>
<td>MLL</td>
<td>5 µM</td>
<td>20 µM</td>
<td>&gt;240</td>
<td>168</td>
</tr>
<tr>
<td>PPM</td>
<td>50 µM</td>
<td>500 µM</td>
<td>&gt;240</td>
<td>168</td>
</tr>
<tr>
<td>AMB</td>
<td>1 µM</td>
<td>16 µM</td>
<td>&gt;240</td>
<td>168</td>
</tr>
</tbody>
</table>

Sb⁴, pentavalent antimonials; Sb⁷, trivalent antimonials; MLL, miltefosine; PPM, paromomycin; AMB, amphotericin B. Results are expressed as the time (h) needed to exert a cidal effect that is based on (i) >95% reduction in amastigote burden observed microscopically following Giemsa staining and (ii) 100% reduction in viability as calculated by the percentage of wells remaining negative for promastigote back-transformation. Time-to-kill values are the result of two independent experiments performed in quadruplicate for the microscopic assay and in octuplet for the promastigote back-transformation assay. The susceptibility in the standard in vitro assays was determined as previously described.⁴

²No difference in IC₅₀ value for L. infantum and L. donovani.
The latter may be explained by the fact that the pentavalent form is actually a pro-drug that requires transformation into the trivalent form to become active. For miltefosine (Figure S4) it took at least 168 h at 2×IC50 to completely eliminate amastigotes, while clearance at CC90 of L. donovani took marginally longer (144 h) compared with L. infantum (96 h). For paromomycin (Figure S5), no significant differences were observed between the two species (time-to-kill 168 h at 2×IC50 and 120 h at CC90), and compared with miltefosine, slightly longer exposure times were required for full elimination of viable amastigotes. The latter may possibly explain the surprising outcome of our in vitro resistance selection protocol in which a larger number of paromomycin-resistant isolates could be obtained compared with only one miltefosine-resistant L. infantum isolate. While amphotericin B is commonly known to act rapidly based on the standard Giemsa method, current results (Figure S6) demonstrate that under the stated experimental conditions, it was difficult to obtain cidal efficacy in vitro, except at near-cytotoxic (CC90) concentrations (168 h) (Table 1). Even though relapses are reported in immunocompromised patients, amphotericin B is still considered the most effective treatment option.

Immunocompromised patients can only be cured after prolonged exposure to higher drug concentrations and their clinical isolates generally do not show a decreased drug susceptibility in the standard assays, suggesting that factors other than drug resistance are definitely involved in amphotericin B treatment failure. Although this in vitro time-to-kill assay was primarily developed to assess and compare the cidal action dynamics of the different reference drugs on laboratory-adapted reference strains, the relation of the time-to-kill assay to drug resistance and treatment outcome involving clinical isolates deserves further exploration.

Acknowledgements
We acknowledge An Matheeussen and Pim-Bart Feijens for their technical assistance.

Funding
This work was funded by the Research Fund Flanders (FWO: project G051812N) and the Research Council of the University of Antwerp (TT-ZAPBOF 33049). The Laboratory for Microbiology, Parasitology and Hygiene (LMPH) is a partner of the Antwerp Drug Discovery Network (ADDN, www.addn.be).

Transparency declarations
None to declare.

Supplementary data
Figures S1–S6 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References