In the Indian subcontinent, the spread of antimony resistance has enforced a shift in visceral leishmaniasis (VL) therapy. Miltefosine (MIL) was licensed for VL in 2002 and is now being used as a first-line therapy within the Kala-azar elimination program in India, Nepal, and Bangladesh (1). Quite recently, increased MIL treatment failure rates have been reported (2) that have been endorsed by the first reports of laboratory-confirmed primary field resistance (3,4). Paromomycin (PMM), an aminoglycoside antibiotic with a confirmed efficacy against VL, was licensed in 2006 mainly for use in combination therapy (5). For now, its use is still limited and widespread field resistance has not yet been reported, although some naturally PMM-resistant strains have already been documented (4). Given the paucity of other affordable VL therapeutic options and the increasing pressure on MIL therapy, more widespread use of PMM may logically ensue. Conversely, laboratory studies already demonstrated that MIL and PMM resistance can be selected in vitro using axenic promastigotes (6–8). Considering the debatable relevance of promastigote-based studies, our group developed an in vitro resistance selection protocol on intracellular amastigotes, revealing a process-dependent outcome (4,9). Rapid generation of PMM-resistant amastigotes for several Leishmania donovani and Leishmania infantum strains was obtained, while the derived promastigotes remained fully PMM susceptible. In contrast, selection of MIL resistance consistently failed as reflected by the unchanged MIL susceptibilities at the promastigote and amastigote levels (4). To validate these unexpected in vitro findings and in an alternative attempt to obtain MIL-resistant strains, the present study in hamsters established a resistance selection process on in vivo amastigotes. This model was chosen based on the previous observations that Syrian golden hamsters do not fully clear Leishmania infection after MIL treatment at 40 mg/kg of body weight orally for 5 days (10) and that intraperitoneal PMM treatment at 150 mg/kg for 5 days only resulted in 80% reduction of amastigote burdens (S. Hendrickx, unpublished data); this is in contrast to the study performed by Sane et al. (11). Since these two treatment regimens resulted in incomplete parasite clearance, this treatment approach was exploited in the present study to monitor relapse and recurrence of disease in treated animals and assess the effect of successive treatment cycles on drug susceptibility, thereby fully mimicking clinical drug use in the patient. Using this strategy, strains generated in vivo and in vitro can be compared phenotypically, validating our resistance selection procedures.

**MATERIALS AND METHODS**

**Ethics statement.** The use of laboratory rodents was carried out in strict accordance to all mandatory guidelines (European Union directives, including the Revised Directive 2010/63/EU on the protection of animals used for scientific purposes that went into effect on 1 January 2013 and the Declaration of Helsinki in its latest version) and was approved by the ethical committee of the University of Antwerp, Belgium (UA-ECD 2010–17 [18 August 2010]).

**Animals.** Female golden hamsters (body weight, 80 to 100 g) were purchased from Janvier (France) and kept in quarantine for at least 5 days before infection. Food for laboratory rodents (Carfil, Arendonk, Belgium) and drinking water were available ad libitum. The animals were randomly allocated to experimental units of 3 animals each.

**Leishmania parasites and infection.** Leishmania infantum (MHOM/MA[BE]/67/ITMAP263) and L. donovani (MHOM/ET/67/L82) ex vivo amastigotes were obtained from the spleen of heavily infected donor hamsters and purified using two centrifugation steps. After determination of
FIG 1 Schematic representation of the in vivo drug resistance selection protocol in the Syrian golden hamster. (1) Hamsters were infected with ex vivo amastigotes. (2) After 21 days, infected hamsters were treated with PMM or MIL. (3) Upon treatment, hamsters were closely monitored until they relapsed showing clinical signs. (4) A liver biopsy specimen was taken to assess the presence of substantial amastigote burdens. (5 and 6) Hamsters were treated again with MIL or PMM (5) or they were euthanized for harvesting ex vivo spleen-derived amastigotes to be used to infect a new hamster, thereby initiating the next selection cycle (6). Sid, once daily; IP, intraperitoneal; PO, per oral; d, day.

the Stauber index (12), the amastigote suspension was diluted to prepare an infection inoculum containing $2 \times 10^7$ amastigotes/100 μl phosphate-buffered saline (PBS). Hamsters were anesthetized by isoflurane inhalation and infected by intracardial injection. Promastigotes were cultured in promastigote hemoflagellate minimal essential medium (HOMEM) supplemented with 20% inactivated fetal calf serum (Invitrogen, Ghent, Belgium) and 20% spent promastigote medium (13).

Treatment relapse schedule and evaluation parameters. Two treatment groups were set up for each Leishmania species: one orally treated with MIL (Sigma, Diegem, Belgium) at 20 mg/kg body weight for 5 consecutive days and one intraperitoneally treated with PMM (Sigma, Diegem, Belgium) at 350 mg/kg body weight for 5 consecutive days. MIL (molecular weight [MW], 407.57) was formulated in distilled water at 20 mg/ml, and PMM (MW, 713.71) was formulated in distilled water at 150 mg/ml. Infected animals were treated starting from 21 days postinfection (dpi). Animals were then closely monitored, since the time-to-relapse would most likely vary between individual hamsters and might shorten upon subsequent selection cycles owing to decreasing parasite susceptibility. Their general condition and body weight were checked twice weekly. When an animal presented clinical signs of recurrent disease (body weight loss and deteriorating overall appearance), a liver biopsy specimen was taken to confirm the presence of adequate parasite burden, and a next round of treatment was initiated. Whenever possible, a promastigote back-transformation assay was performed on the biopsy specimen to evaluate the PMM-MIL susceptibility in vitro. To minimize animal suffering, hamsters were euthanized with a CO2 overdose after the second relapse. At that time, they were used to collect spleen-derived ex vivo amastigotes to allow infection of a new hamster (Fig. 1). Subsequent selection rounds were terminated either when drug susceptibility values indicated that a resistant endpoint was reached or after 3 successive treatment/relapse cycles.

Susceptibility determination. Amastigote and promastigote susceptibility was determined as described previously (14). For promastigote susceptibility testing, procyclic promastigotes were exposed to 2-fold serial drug dilutions. After 4 days incubation at 25°C, promastigote viability was measured using resazurin and 50% inhibitory concentration (IC50) values were determined. For amastigote susceptibility testing, primary peritoneal macrophages were infected with metacyclic promastigotes. After 24 h, the medium was discarded and replaced by 2-fold drug dilutions in medium. Cells were incubated at 37°C in the presence of 5% CO2 for 4 days, upon which the plates were Giemsa-stained for microscopic determination of the IC50 values. Cutoff values for resistance were set at 150 μM for PMM and 15 μM for MIL, as described earlier (4). The activity index (AI) as introduced by Yardley et al. (15) was used to normalize IC50 values between different strains by direct comparison with the susceptible reference strain. Based on previous studies on antimonials, strains with an AI of >4 can be considered fully resistant (16).

Statistical analysis. Statistical differences between the different passages were analyzed using two-way analysis of variance (ANOVA). Results were considered statistically significant if $P$ was <0.05.

RESULTS

Promastigote back-transformation assay. On the liver biopsy specimens and at autopsy, promastigote back-transformation was performed in an attempt to recover residual parasites that could be used for in vitro susceptibility determination. However, due to difficulties of adaptation of the amastigotes derived in vivo to adjust to the in vitro conditions, this assay unfortunately was not always successful. When the promastigote back-transformation did become positive, promastigotes were collected and transferred to routine promastigote culture with modifications to the promastigote medium to contain a higher concentration of fetal calf serum (20%) and spent medium to boost promastigote growth.

Amastigote and promastigote susceptibility data. Already af-
TABLE 2 Amastigote and promastigote susceptibility of L. infantum upon PPM selection rounds

<table>
<thead>
<tr>
<th>L. infantum type</th>
<th>PMM (µM)</th>
<th>PMM (µM)</th>
<th>AI</th>
<th>AI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>amastigote susceptibility (mean ± SEM)</td>
<td>promastigote susceptibility (mean ± SEM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>65.7 ± 5.9</td>
<td>17.3 ± 2.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1</td>
<td>397.7 ± 30.4</td>
<td>49.5 ± 7.2</td>
<td>6.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.9 (ns&lt;sup&gt;b&lt;/sup&gt;)</td>
</tr>
<tr>
<td>P2</td>
<td>430.9 ± 24.5</td>
<td>31.2 ± 8.1</td>
<td>6.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.8 (ns)</td>
</tr>
</tbody>
</table>

<sup>a</sup> WT, wild type; P1, after 1 drug treatment round; P2, after 2 drug treatment rounds.
<sup>b</sup> IC<sub>50</sub> values and corresponding AI were the result of at least three independent in vitro assays run in duplicate.
<sup>c</sup> P < 0.001.

TABLE 3 Amastigote and promastigote susceptibility of L. infantum upon MIL selection rounds

<table>
<thead>
<tr>
<th>L. infantum type</th>
<th>MIL (µM)</th>
<th>MIL (µM)</th>
<th>AI</th>
<th>AI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>amastigote susceptibility (mean ± SEM)</td>
<td>promastigote susceptibility (mean ± SEM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>1.8 ± 0.4</td>
<td>0.8 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1</td>
<td>2.3 ± 0.3</td>
<td>5.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>P2</td>
<td>1.9 ± 0.6</td>
<td>4.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>P3</td>
<td>3.0 ± 0.3</td>
<td>7.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.2 ± 0.2</td>
<td>4.0&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> WT, wild type; P1, after 1 drug treatment round; P2, after 2 drug treatment rounds; P3, after 3 drug treatment rounds.
<sup>b</sup> IC<sub>50</sub> values and corresponding AI were the result of at least three independent in vitro assays run in duplicate.
<sup>c</sup> P < 0.001.
<sup>d</sup> ND, not done.

TABLE 4 Amastigote and promastigote susceptibility of L. donovani upon MIL selection rounds

<table>
<thead>
<tr>
<th>L. donovani type</th>
<th>MIL (µM)</th>
<th>MIL (µM)</th>
<th>AI</th>
<th>AI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>amastigote susceptibility (mean ± SEM)</td>
<td>promastigote susceptibility (mean ± SEM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>1.7 ± 0.2</td>
<td>1.7 ± 0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1</td>
<td>1.7 ± 0.2</td>
<td>0.9 (ns&lt;sup&gt;e&lt;/sup&gt;)</td>
<td>ND&lt;sup&gt;f&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>P2</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P3</td>
<td>3.6 ± 0.5</td>
<td>2.0&lt;sup&gt;f&lt;/sup&gt;</td>
<td>5.0 ± 0.5</td>
<td>2.9&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> WT, wild type; P1, after 1 drug treatment round; P2, after 2 drug treatment rounds; P3, after 3 drug treatment rounds.
<sup>b</sup> IC<sub>50</sub> values and corresponding AI were the result of at least three independent assays run in duplicate.
<sup>c</sup> ns, nonsignificant.
<sup>d</sup> ND, not done.
<sup>e</sup> P < 0.001.

Amastigote and promastigote susceptibility after amastigote-to-promastigote back-transformation. was relatively challenging due to the lack of promastigote growth. increased MIL susceptibility after successive MIL treatment rounds, although the cutoff value for resistance was merely reached. Therefore, two additional treatment/relapse cycles were carried out, finally resulting in a generation of fully MIL-resistant amastigotes and MIL-susceptible promastigotes (Table 2). Although the selection of MIL resistance proved to be straightforward, successive MIL treatment of L. infantum and L. donovani did not result in the anticipated shift in MIL IC<sub>50</sub> values toward an MIL-resistant phenotype (Tables 3 and 4). Moreover, determination of MIL susceptibility after successive MIL treatment rounds was relatively challenging due to the lack of promastigote growth capability after amastigote-to-promastigote back-transformation.

DISCUSSION

Due to the spread of primary antimony resistance in the Indian subcontinent, alternative drugs like MIL and PPM are now recommended for the treatment of VL (17). Considering some intrinsic traits of the two drugs, repetitive use in areas where VL is endemic will ultimately lead to the development of primary resistance (5, 17). For example, MIL has a long elimination half-life and requires a 4-week treatment course affecting proper compliance, hence causing lasting suboptimal drug levels in the patient (17). A drop in clinical efficacy has been reported worldwide, resulting in increasing numbers of MIL treatment failure with levels reaching up to 20% in the Indian subcontinent (2). Surprisingly, very few of these relapse isolates actually were MIL resistant in the standard intracellular amastigote laboratory assay. Although a decreased MIL susceptibility was observed in a number of Brazilian L. infantum relapse isolates, susceptibility results of Indian relapse isolates still yield controversy (2, 18, 19). Since the first MIL-resistant isolate was reported in 2012, a second naturally resistant strain was found by our research group (3, 4). As for PPM resistance, this was already reported to develop fairly readily (4, 7, 9, 20). Moreover, numerous in vitro studies demonstrated quick selection of MIL and PPM resistance on promastigotes (6–8). To target the more relevant amastigote stage in mammalian infection, our research group previously developed an in vitro resistance selection protocol on intracellular amastigotes (9). For PPM, several strains with PPM-resistant amastigotes but still fully PPM-susceptible promastigotes were obtained. In contrast, selection on promastigotes caused the two stages to become resistant, showing that in vitro selection data should be interpreted with some caution. For MIL, in vitro selection on intracellular amastigotes failed.
to induce a susceptibility shift in most *L. donovani* and *L. infantum* isolates (4).

Previous experiments already indicated that treatment relapses can be evoked in the Syrian golden hamster, which provides an ideal tool to validate our *in vitro* selection results *in vivo*, hereby more closely mimicking the actual field situation (10). Since the Syrian hamster model also has fairly good predictive value for human VL, it was logically selected for our drug resistance selection design *in vivo* (21, 22). For PMM, implementation of this protocol resulted in a prompt reduction of the FMM susceptibility for *L. infantum* and *L. donovani* (Tables 1 and 2). Although amastigote susceptibility declined based on the predetermined susceptibility cutoff value of 150 μM, a drug-susceptible phenotype was conserved at promastigote level, which fully corresponded to the phenotypic outcome after *in vitro* selection (4). Hence, the present study endorses the applicability and predictivity of our *in vitro* selection protocol on intracellular amastigotes to generate PMM-resistant strains and once more emphasizes the need to use intracellular amastigotes for the selection of drug resistance. After successive cycles of MIL exposure *in vivo* and adopting the predetermined susceptibility cutoff IC₅₀ value of 15 μM, no resistant phenotype was revealed on either parasite species or stage (Tables 3 and 4), despite the observation that posttreatment relapse did occur systematically. Using statistical analysis, calculation of the activity index for each passage suggested the appearance of a “resistant” phenotype. However, considering that substantial susceptibility variations may occur between different “susceptible” strains, hence influencing the actual calculated AI value, it may be more rational to use the predetermined susceptibility cutoff values to define “clinical resistance” (4). Conversely, using AI values may still provide a useful tool to indicate more subtle nuances for fundamental drug susceptibility research purposes. After the initial selection cycles, the anticipated MIL relapses mainly resulted in a rapid increase of amastigote burdens in the liver, while the spleen-derived *ex vivo* amastigotes remained rather scarce. However, adequate purification of liver-derived amastigotes proved to be difficult likely due to the presence of metabolizing enzymes, and only spleen-derived amastigotes were used for subsequent infection. For PMM, this was never an issue, as subtoxic doses resulted in an 80% clearance rate at best, leading to equally rapid increases in parasite burdens in the two target organs.

A remarkable finding was the reduced promastigote growth rate observed after MIL treatment, which conflicts with the higher metacyclogenesis and *in vitro* infection potential observed for *L. donovani* MIL relapse isolates from the Indian subcontinent (23), and the increase in parasite fitness observed in promastigote-selected MIL-resistant *L. donovani* (24). It is evident that additional research is still needed on the impact of MIL resistance on parasite fitness. Although the present *in vivo* protocol may indeed avoid adaptive phenomena to the *in vitro* culture system with subsequent virulence loss, *in vitro* susceptibility testing was practically hampered by the low conversion level of amastigotes into promastigotes. Remarkably, promastigote back-transformation and subsequent growth appeared more problematic after MIL exposure, leaving susceptibility determination mostly entirely dependent on the use of *ex vivo* amastigotes upon necropsy of MIL-treated animals, which explains the lack of susceptibility data for several passages (Tables 1 to 4). Although the present study offers convincing arguments to investigate phenotypic and genotypic strain-specific alterations by repeated drug exposure, such research will unfortunately be restrained by this problem of *in vitro* adaptation and will, therefore, be limited to studies on *ex vivo* amastigotes, if available.

In conclusion and until fully characterized resistant field isolates become available, “laboratory-selected” resistant strains may be a good proxy to study features associated with drug resistance and become an asset in uncovering the underlying dynamics and mechanisms of PMM or MIL resistance. It also needs to be reemphasized that *in vitro* resistance selection should run on intracellular amastigotes rather than on extracellular promastigotes, while *in vivo* selection models may have even better predictive value. The present study also highlights why PMM must be avoided in monotherapy and strongly recommends more in-depth research on the underlying mechanisms of MIL relapse to clarify the missing link between treatment failure and the unintentional evolution toward intrinsic MIL resistance.

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