Comparative efficacy of two antimony regimens to treat *Leishmania braziliensis*-induced cutaneous leishmaniasis in rhesus macaques (*Macaca mulatta*)

Short title: CHEMOTHERAPY OF LEISHMANIASIS IN MACAQUES

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This study compares the efficacy of two N-methylglucomine antimoniate (MA) dose regimens for treating macaques with *L. braziliensis*-induced chronic skin disease. Whereas all animals treated with the full dose (20 mg MA/kg/day) were cured, 50% of monkeys receiving a low dose (5 mg MA/kg/day) regimen relapsed. The antimony concentrations in macaque plasma and tissue samples were greater in the full dose group compared to macaques receiving a sub-therapeutic MA regimen. Our data also suggest the presence of drug-induced hepatic pathology.

Leishmaniasis is a cause of significant morbidity and mortality throughout the world with two million new cases of human infection worldwide each year. However, an approved vaccine to prevent leishmaniasis does not exist (7). Treatment for leishmaniasis relies largely on pentavalent antimony (Sb\textsuperscript{V}) compounds (meglumine antimoniate and sodium stibogluconate). A number of other therapeutic agents may be employed, but high costs have limited the large scale use of the most potent drugs (4). Factors limiting the usefulness of Sb\textsuperscript{V} therapy include their adverse toxic effects (arthralgias, myalgias, cardiac arrhythmia, pancreatitis, and hepatic or renal function impairment) and the increasing occurrence of parasite resistance (2,4). Nevertheless, responses to Sb\textsuperscript{V} vary considerably depending on both the parasite's intrinsic drug sensitivity and the host's immune status (1,4,15). Poor clinical responses can also be attributed to inadequately dosed antimony regimens (1,2) and problems concerning drug pharmacokinetics (PK) or biodisposal (5,13).

The *Leishmania*-macaque model has proven to be a valuable in vivo system for drug and vaccine-development studies against infectious diseases (7,14). The aim of the
present study was to compare the pharmacological parameters (PK, toxicity, and
efficacy) of a low dose of MA with those of a standard dose of MA in macaques with *L.
braziliensis* infection. All animal studies were performed under the guidance and with
the approval of the Institutional Animal Care and Use Committee. Groups of six outbred
adult rhesus macaques (*Macaca mulatta*) were used in this study. A high dose (10^7
promastigotes) of virulent *L. braziliensis* (MHOM/BR/2000/CP13396 strain) was
injected intradermally above the left upper eyelid of each monkey. The infection was
allowed to proceed until the macaques reached skin disease progression. At nine weeks
post-infection (pi), macaques received a 21-day course of a low-dose (5 mg/kg/day) or
full dose (20 mg/kg/day) of MA, administered through an intramuscular route. A
vehicle-only treated control group was included to assess the development of a local
skin lesion caused by the infection. Animals were euthanized on days 55 (140, 142, O6,
L30, M2, O34) and 95 (S62, T32, U48, U12, U46, X53) after the completion of
treatment, and selected necropsy tissue specimens (liver, spleen, and kidneys) from
drug-cured and control macaques were removed to determine residual antimony tissue
concentrations and for histological examination to assess antimony-induced
histopathological changes. Antimony concentrations in macaque plasma and tissues
were determined by inductively Coupled Plasma Mass Spectrometry (ICP-MS) using
the optimized conditions previously described (11).

Although *L. braziliensis*-infected macaques showed a high degree of variability in
their lesion size (range 20-540 mm^2 in area) before antimonial therapy, the ulcerative
CL persisted in untreated animals until the end of the observation period (Fig. 1).
Treatment with both therapeutic schemes rapidly reduced the lesion size (in comparison
with untreated) after treatment. However, while complete healing was achieved in all
animals receiving a regular MA schedule, 3 out of 6 monkeys treated with a low-dose
regimen relapsed with the presentation of macroscopic wound inflammation after clinical cure and wound reopening 3-4 months after the cessation of therapy. The concentration-time profiles of Sb in macaque plasma (Fig. 2) confirmed that drug exposure was much lower in that group (range 11.3-149.3 ng Sb/g) compared to macaques treated with a full-dose regimen (range 36.4-150.5 ng Sb/g). Except for days 16, 23, 61, and 68, the differences in plasma Sb concentrations between the two groups were statistically significant ($p < 0.001 – 0.05$).

Sustained nadir blood levels of Sb gave rise to residual drug accumulation in different soft tissues. Approximately two and three months after the last dose of MA, Sb was detected in the liver, spleen, and kidneys of treated macaques, with concentrations in the renal tissue significantly lower than those found in hepatic tissue (Table). No overt clinical signs of toxicity were observed in any macaque, regardless of the administration scheme. Nevertheless, histological evidence of MA-induced hepatic injury (Fig. 3) was observed in all treated macaques. There was a clear correlation ($r = 0.94; p = 0.001$) between tissue Sb levels and the extent to which hepatocytes were affected. In contrast, no histopathological alterations were found in any other tissue examined.

Our main findings in macaques receiving a low Sb$^V$ dosage regimen are consistent with those obtained in our previous studies (14), but differ with the observations of Oliveira-Neto et al. (12) who recommended sub-therapeutic MA concentrations for treating $L. braziliensis$-infected patients. This is likely linked to variabilities in parasite drug susceptibility (1,4,15). In fact, taxonomic studies have shown a high degree of genetic variation in natural populations of $L. braziliensis$ from different geographic areas in Brazil (6). It should be noted that sublethal doses contribute to the selection of drug-resistant parasites (9) with the parasites that are inherently most drug resistant.
being favored (10). Moreover, drug-resistant clones of *Leishmania* spp. may exhibit cross-resistance (8).

The plasma PK profile of Sb in *L. braziliensis*-infected macaques treated with MA was similar to that reported for human cases receiving Sb\textsuperscript{V} drugs (3,11,13). Accordingly, most of the Sb absorbed from the injection site was eliminated rapidly, but long-lived nadir plasma concentrations of Sb caused a gradual accumulation of the drug in tissues after repeated daily dosing. With the exception of the spleen in groups treated with the standard dose, tissue concentrations of Sb in monkeys necropsied 95 days after the end of treatment were lower than levels found in monkeys examined 40 days earlier (Table). It remains to be determined if the observed variability in tissue Sb levels in macaques of the same dose group results from the out-bred genetics of these primates. The speciation of Sb in the organism and the mechanisms by which it is transported, distributed to tissues, and eliminated from the body remain unclear (4). We also provided evidence for an association between tissue Sb levels and the extent of hepatocyte damage. The drug-induced hepatic injury could be related to the conversion of Sb\textsuperscript{V} to Sb\textsuperscript{III}, which has been demonstrated to be considerably more toxic than Sb\textsuperscript{V} in different test systems (3).

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Legends to figures

Figure 1. Response of *Leishmania braziliensis* cutaneous infections in rhesus macaques to either a low dose (5 mg/kg/day) [group A] or a standard dose (20 mg/kg/day) [group B] treatment of MA. To assess therapy, lesion size development was scored weekly following infection and treatment. All values represent the mean ± SD. Before treatment, there was no statistical difference (*p* > 0.05) in the mean values of lesion sizes with time between macaque groups.

Figure 2. Time course of Sb plasma concentrations after intramuscular injection of either a low dose (group A) or a full-dose (group B) of MA in *L. braziliensis*-infected macaques. For detection of total Sb, heparin-anticoagulated blood samples were analyzed by ICP-MS as described (11). *Significant differences (*p* < 0.001 – 0.05) in plasma Sb concentrations between the two groups.

Figure 3. Histopathology of liver samples from representative drug-cured *Leishmania braziliensis*-infected macaques (A, L30; B, 140; C, U46; D, O34). Micrographs show focal hepatocellular acidophilic necrosis (indicated by arrows) with surrounding inflammatory infiltrates (shown in circles). These obliterate the sinusoids and protrude into the parenchyma and are associated with fatty changes in stellate cells (indicated by arrowheads). Hypotrophy of the hepatic parenchyma at the center of the lobules (shown in box) and numerous hemosiderin deposits within liver cells and Kupffer cells (inset in panel D) are also illustrated. H&E stain.
TABLE. Residual concentrations of antimony (ng Sb/g) found in selected necropsy tissue specimens from drug-cured *Leishmania braziliensis*-infected macaques

<table>
<thead>
<tr>
<th>Days after treatment</th>
<th>Group A</th>
<th>Group B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Monkey</td>
<td>Liver</td>
</tr>
<tr>
<td>140</td>
<td>2490</td>
<td>1800</td>
</tr>
<tr>
<td>55</td>
<td>142</td>
<td>2890</td>
</tr>
<tr>
<td>06</td>
<td>3360</td>
<td>1450</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>2913 ± 435.5</td>
<td>1433 ± 375.3</td>
</tr>
<tr>
<td>S62</td>
<td>831</td>
<td>370</td>
</tr>
<tr>
<td>95</td>
<td>T32</td>
<td>430</td>
</tr>
<tr>
<td>U48</td>
<td>747</td>
<td>700</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>669.3 ± 211.5</td>
<td>476.7 ± 193.5</td>
</tr>
</tbody>
</table>

*Primates were treated with either a low-dose (5 mg/kg/day) [group A] or a full-dose (20 mg/kg/day) [group B] of MA, administered intramuscularly. Necropsies were performed at different time points (as described in the Materials and Methods sections) after the completion of treatment. For this analysis, specimens from infected but untreated (n = 2) monkeys were also included as negative controls. N.D., not done. Residual levels of Sb were compared among organs (matched per monkey) for each dose group by the Friedman test followed by Dunn’s multiple comparisons test: Kidneys ≠ Liver in groups A and B (*P < 0.05).