Activities of Hexadecylphosphocholine (Miltefosine), AmBisome, and Sodium Stibogluconate (Pentostam) against *Leishmania donovani* in Immunodeficient *scid* Mice

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In both *scid* and BALB/c mouse-*Leishmania donovani* models, hexadecylphosphocholine (miltefosine) and AmBisome had similar levels of activity. In contrast, sodium stibogluconate (Pentostam) was significantly less active against *L. donovani* in *scid* mice than in BALB/c mice. The in vitro anti-leishmanial activity of miltefosine was similar in peritoneal macrophages derived from both *scid* and BALB/c mice, whereas Pentostam and AmBisome were significantly more active in the latter.

Visceral leishmaniasis (VL) is caused by the obligate intracellular protozoan parasites *Leishmania donovani*, *Leishmania infantum*, and *Leishmania chagasi*. Currently, there are an estimated 500,000 new cases of VL/annum, with particularly important foci in northeast India and Sudan (World Health Organization). The recommended drugs for the treatment of VL are the pentavalent antimonials (SbVs) sodium stibogluconate (Pentostam) and meglumine antimonate (Glucantime), with amphotericin B (Fungizone), lipid formulations of amphotericin B, and paromomycin (aminosidine) used as alternative therapies (3, 9). All these drugs have limitations of the need for parenteral administration and long courses of treatment, toxicity, and/or cost. There is increased concern over the rising number of cases of VL in India that fail to respond to standard antimonials (28) and the emergence of VL as an opportunistic infection in human immunodeficiency virus (HIV)-infected and AIDS patients (1), especially in the *L. infantum* focus in southwestern Europe (10). Treatment of Leishmania-HIV coinfections is problematic, as the conventional antimonial treatment is less effective in immunocompromised patients (1). Courses of high-dose antimonial regimens and liposomal amphotericin B have limited efficacy, most patients relapse, and maintenance therapy is necessary (1, 9).

Recently, hexadecylphosphocholine (HPC; miltefosine), an alkylphosphocholine originally developed as an anticancer drug (29), has proved to be an effective treatment for VL in clinical trials in India (15, 27). Oral treatment with HPC at 100 mg/day for 4 weeks was effective in treating 114 of 120 VL cases (15) and cases that had not responded to prior antimonial therapy (27). These clinical studies followed experimental studies that demonstrated that alkylphosphocholines, including HPC, are active against *L. donovani* in vitro macrophage and mouse models of infection (7) and by oral administration against murine VL (8, 16, 17, 30). The molecular mechanism of action of HPC against cancer cells has been linked to apoptosis as well as different lipid-dependent cell signaling pathways (2), but its mode of action against *Leishmania* parasites remains unclear. It has also been suggested that HPC has immunomodulatory properties (12, 13, 31); however, some studies have shown that HPC retains its antitumor properties in immunodeficient mice, suggesting that activity is not dependent on a T-cell-mediated immune response, although increases in macrophage, T-cell, and B-cell numbers were observed (25).

Recently, the antileishmanial activity of HPC was shown to be retained in mouse models deficient in T-cell, endogenous gamma interferon (IFN-γ), and macrophage killing (reactive nitrogen and oxygen radicals) mechanisms (18). As part of a project on the antileishmanial activities of alkyllysophospholipids, we have examined the activity of HPC in *scid* mice which are functionally deficient in T and B cells (4) and compared it with those of AmBisome and sodium stibogluconate, two drugs frequently used in the treatment of VL in immunosuppressed individuals.

HPC (Sigma, Poole, United Kingdom), Pentostam and sodium stibogluconate (GlaxoWellcome, Dartford, United Kingdom), Fungizone (E. R. Squibb & Sons, Hounslow, United Kingdom), and AmBisome (generously donated by R. Proffitt, Gilead Biosciences, San Dimas, Calif.) were used in the study. Drugs were tested in either C.B-17 *scid* mice (from a colony maintained at the London School of Hygiene and Tropical Medicine) or BALB/c mice (Charles River Ltd., Margate, United Kingdom). As described previously (7) 6- to 8-week-old mice were infected intravenously with 2 × 10⁷ *L. donovani* MHOM/ET/67/L82 amastigotes derived from a hamster and randomly sorted into groups of five. In the first experiment mice were dosed 7 days after infection with 30 mg of HPC per kg of body weight per dose (orally [p.o.]) or 45 mg of SbV as sodium stibogluconate per kg per dose (subcutaneously [s.c.]) for 5 consecutive days. In a second experiment, groups of mice were dosed 14 days after infection with HPC at 30, 10, 3, or 1 mg/kg/dose (p.o.) or with sodium stibogluconate at 45, 15, and 5 mg of SbV/kg/dose (s.c.) for 5 consecutive days. The activity of AmBisome was compared with that of Fungizone in the two mouse models in two experiments by using 5, 1, and 0.2 mg of...
AmBisome per kg per dose in a single dose given intravenously (i.v.) and Fungizone at 1 mg/kg/dose as a standard in the first experiment and at 5, 1, and 0.2 mg/kg/dose three times on alternate days in the second experiment. In all experiments, mice were weighed and necropsied 3 days after the completion of treatment. Impression smears, prepared from weighed livers, were methanol fixed and Giemsa stained. Drug activity was determined by comparing the number of amastigotes per 500 liver cells \( \times \) organ weight (in milligrams) (Leishman Donovan unit [LDU]) in mice from the treated and the untreated groups. The 50% effective doses (ED\(_{50}\)) and ED\(_{90}\) were calculated by sigmoidal regression analysis (M$\text{S}$lf$t$, ID Business Solution, Guilford, United Kingdom). The activities of these drugs were further tested in vitro with infected peritoneal macrophages (PM\(_f\)) from scid mice and BALB/c mice. PM\(_f\) were obtained by abdominal lavage with Dulbecco’s minimal essential medium (DMEM; Life Technologies, Paisley, United Kingdom). A total of 10\(^6\) cells/ml were plated in 16-well Labtek tissue culture slides (Life Technologies) and allowed to adhere for 16 h at 37°C in a 5% CO\(_2\)-95% air mixture in DMEM with 10% heat-inactivated fetal calf serum (Harlan Sera-Lab, Loughborough, United Kingdom). Adherent PM\(_f\) were infected with \textit{L. donovani} amastigotes at a ratio of 10 parasites:1 macrophage. After 12 h, nonphagocytosed parasites were removed by washing with serum-free DMEM. Infected cultures were incubated for 72 h with the drugs in a threefold dilution series in quadruplicate at each concentration. Drug activity was determined microscopically by counting the percentage of infected cells in methanol-fixed and Giemsa-stained preparations. The ED\(_{50}\) and ED\(_{90}\) were calculated and analyzed as described above.

Treatment with HPC at 30 mg/kg/dose p.o. for 5 days was previously shown to be effective against \textit{L. donovani} in BALB/c mice (8, 16, 17). In an initial study with this dose, HPC proved to be equally active in BALB/c and scid mice, with >95% parasite inhibition during the second week of infection (data not shown). In comparison, sodium stibogluconate at a similarly chosen effective dose (45 mg of Sh\(^V\)/kg/dose for five days) was significantly (\(P < 0.05\)) more active in BALB/c mice (87.35\% \(\pm\) 9.15\% parasite inhibition) than in scid mice (41.00\% \(\pm\) 14.35\% parasite inhibition) (data not shown). HPC, AmBisome, and sodium stibogluconate were then tested over a dose range during the 3rd week of infection, a period in which the parasite burden in the liver has been shown to be similar in both strains of mice (11). The LDUs before treatment (2 weeks after infection) were 1,804 \(\pm\) 85 and 2,088 \(\pm\) 78 in scid and BALB/c mice, respectively. At the end of the treatment (3 weeks after infection), untreated control mice from both groups presented with similar liver parasite burdens, with LDUs of 2,335 \(\pm\) 185 (scid mice) and 2,375 \(\pm\) 270 (BALB/c mice). HPC showed similar dose-response effects in both BALB/c and scid mice (Fig. 1A), with ED\(_{50}\) and ED\(_{90}\) of 3.98 and 14.35 mg of Sh\(^V\)/kg/dose, respectively. There was no significant difference between the values (\(P > 0.45\)). In contrast, sodium stibogluconate at a significantly (\(P < 0.05\)) higher level of activity in BALB/c mice than in scid mice, with ED\(_{50}\) and ED\(_{90}\) of 2.91 and 42.66 mg/kg/dose, respectively, with no significant difference between the values (\(P > 0.45\)). The percentages of parasite killing by HPC at 30 mg/kg were similar in both BALB/c and scid mice (98.68 and 94.7%, respectively); this is in contrast to the results of treatment with sodium stibogluconate at 45 mg of Sh\(^V\)/kg, with which there was a higher percentage of parasite killing in BALB/c mice than in scid mice (96.26 and 28.8%, respectively). The drugs were well tolerated by the mice at the top doses, and no weight reductions were recorded in the mice in the treated groups. AmBisome at a single dose was active in both models (Fig. 1C), with ED\(_{50}\) and ED\(_{90}\) of 2.91 and >5 mg/kg/dose and 1.51 and 3.1 mg/kg/dose in BALB/c and scid mice, respectively. There was no significant difference between the two models in the present study (\(P > 0.5\)). The standard amphotericin B formulation (Fungizone) was inactive at 1 mg/kg/dose i.v. in both models. In a second experiment, multiple dosing (on 5 alternate days) with AmBisome gave lower ED\(_{50}\) and ED\(_{90}\) in both models: <0.2 and <0.2 mg/kg/dose, respectively,
BALB/c mice (98.5% inhibition at the lowest dose of 0.2 mg/kg) and 0.3 and 0.19 mg/kg/dose, respectively, in scid mice. Multiple doses of Fungizone at 1 mg/kg gave 65.5% inhibition in scid mice and 79.5% inhibition in BALB/c mice.

To extend these in vivo observations that suggested the T- and B-cell independence of the antileishmanial activities of HPC and AmBisome and the immunodependence of sodium stibogluconate, all compounds were tested in vitro against L. donovani-infected PMφ. In two experiments, no difference was observed between the activities of HPC in scid and BALB/c mouse PMφ. In contrast, significant differences (P < 0.05) were observed in the antileishmanial activity of Pentostam, with ED50 values for scid mouse-derived infected PMφ being approximately threefold higher than those for BALB/c mouse-derived infected PMφ. AmBisome was significantly more active in BALB/c mouse PMφ than in scid mouse PMφ (P < 0.05). At 0.25 μM, AmBisome caused 97.18% ± 0.97% parasite inhibition in infected BALB/c mouse PMφ, whereas it caused 76.22% ± 1.51% parasite inhibition in infected PMφ from scid mice (Table 1). In the two studies the levels of infection in untreated control PMφ were 92 and 95%, respectively, at 72 h. The ED50 and ED90 for BALB/c mouse-derived macrophages are higher in the present study than the values reported previously (8) due to the 3-day drug exposure in the present study compared to the 5 days used in the screening study.

The immunomodulatory effects of HPC have been described previously, including its activity as a costimulatory signal for T-cell and macrophage activation in vitro (12, 25, 31), an enhancer of IFN-γ production and granulocyte-macrophage colony-stimulating factor expression from peripheral mononuclear human cells in combination with interleukin-2 (13, 31), and an inducer of nitric oxide when used in a liposomal HPC preparation on the human histiocyte cell line U937 (12) or an inducer of nitric oxide when used in a liposomal HPC formulation on the human histiocyte cell line U937 (12) or an inducer of nitric oxide when used in a liposomal HPC formulation on the human histiocyte cell line U937 (12). More recently, it has been demonstrated that IL-12 regulates the in vivo effect of sodium stibogluconate by regulation of IFN-γ production by T cells (21). To extend these studies and confirm that the immune dependency of drug activity was at the T- or B-cell level, the activities were also determined in vitro against amastigotes in PMφ from both strains. Whereas the activity of HPC was similar in both macrophage models, sodium stibogluconate was significantly more active against PMφ from BALB/c mice than those from scid mice. This result suggests that macrophage type or status has a role in the activity of this drug, as there are no intrinsic differences between macrophages from both species of mice (14, 19). Interestingly, AmBisome was also significantly more active against L. donovani in PMφ from BALB/c mice than against L. donovani in PMφ from scid mice (P > 0.05). Variations in the activities of amphotericin B formulations against Leishmania

<table>
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<th>Compound</th>
<th>BALB/c mice</th>
<th>scid mice</th>
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<tr>
<td></td>
<td>ED50a</td>
<td>ED90a</td>
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<tr>
<td>HPC</td>
<td>7.48 (7.29)b</td>
<td>10.85 (10.31)</td>
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<tr>
<td>AmBisome</td>
<td>0.05</td>
<td>0.2</td>
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<tr>
<td>Fungizone</td>
<td>0.03</td>
<td>0.06d</td>
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a The ED50a and ED90a are in micromolar for HPC, AmBisome, and Fungizone. b The values in parentheses are results of a second experiment. c ED50 and ED90 are in micromolar for HPC, AmBisome, and Fungizone and are in micrograms of Sb V per milliliter for Pentostam. d P > 0.05.
in macrophage models have been reported previously, including differences between the activities in peritoneal and THP-1-derived macrophages (32).

There have been increasing numbers of patients with *L. infantum* and HIV coinfections, especially in Mediterranean countries, during the past decade (1, 10). These immunocompromised patients generally have a poor response to antimonials, and failure or relapse rates of 52% within 1 to 36 months are commonly reported (10). Experimental studies with mice previously suggested that Fungizone could be an effective antileishmanial agent in immunocompromised patients. However, this drug has not been so successful, even in lipid formulations, for the treatment of patients with *L. infantum* and HIV coinfections (10). The dissemination of parasites in these patients away from the usual sites of infection does not favor the pharmacokinetic profile of amphotericin B or its lipid formulations. However, HPC is well absorbed following oral administration, is distributed throughout the body (5), and offers opportunities for further study of the treatment of VL in immunocompromised patients.

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