Antileishmanial Activity, Uptake, and Biodistribution of an Amphotericin B and Poly(α-Glutamic Acid) Complex

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A noncovalent, water-soluble complex of amphotericin B (AMB) and poly(α-glutamic acid) (PGA), with AMB loadings ranging from 25 to 55% (wt/wt) using PGA with a molecular weight range of 50,000 to 70,000, was prepared as a potential new treatment for visceral leishmaniasis (VL). The AMB-PGA complex was shown to be as active as Fungizone (AMB deoxycholate) against intracellular Leishmania donovani amastigotes in differentiated THP-1 cells. The in vitro uptake of the AMB-PGA complex by differentiated THP-1 cells was similar to that of Fungizone and higher than that of AmBisome (liposomal AMB). The AMB-PGA complex also displayed a dose-response profile similar to that of AmBisome in vivo in BALB/c mice against L. donovani, with 50% effective doses (ED_{50}) of 0.24 ± 0.03 mg/kg of body weight for the AMB-PGA complex and 0.24 ± 0.06 mg/kg for AmBisome. A biodistribution study with mice indicated that the AMB-PGA complex cleared more rapidly from plasma than AmBisome, with a comparable low level of distribution to the kidneys.

Leishmaniasis is a disease complex caused by obligate intracellular protozoa of the genus Leishmania. The most serious form of this disease is the systemic infection visceral leishmaniasis (VL), which is fatal if untreated. There are an estimated 0.5 million cases of VL every year and more than 50,000 deaths (1). The intracellular amastigote stage, which survives and multiplies in tissue macrophages, is the target for chemotherapy. The current treatments for VL include pentavalent antimonials (Pentostam and Glucantime), miltefosine, and paromomycin, and amphotericin B (AMB) liposomal formulation (AmBisome) is also used treatment in India, where antimony resistance is prevalent (2).

AMB is a polyene antibiotic used for the treatment of systemic fungal infections that is also highly effective for leishmaniasis. Fungizone, the conventional form of AMB that is used clinically, is a colloidal dispersion of AMB with sodium deoxycholate (3). However, Fungizone exhibits severe toxic side effects, especially nephrotoxicity, and requires hospitalization and a dosing regimen that lasts up to 30 days (4). Several lipid-based formulations, for example, Amphocil, AmBisome, and Abelcet, have been developed and used clinically to overcome the limitations of Fungizone (5). The liposomal formulation of AMB (AmBisome) is most commonly used to treat VL (6), as it has a high therapeutic index with significantly fewer side effects than Fungizone. Although the use of AmBisome is frequently limited by high cost (7), a recent donation program could improve access (8).

Colloidal (liposomes and niosomes) and particulate (microparticles, nanoparticles, nanospheres, and microspheres) carriers have been investigated for passive drug targeting to the mononuclear phagocyte system (MPS) of the liver and spleen (9). Polymers have also been used in the lysosomotropic delivery of anticancer drugs (10), being taken up by endocytosis and trafficked through the endosome to the lysosome. Examples of polymeric amphotericin B drug delivery systems that have been developed to the preclinical stage include (i) conjugates of N-(2-hydroxypropyl)methacrylamide (HPMA) (11), polyethylene glycol (PEG) (12), and arabinogalactan (13) and (ii) complexes, e.g., poly-(methacrylic acid) (5, 14).

We have developed a noncovalent complex of AMB with poly(α-glutamic acid) (PGA) (Fig. 1) (15). PGA was selected because it (i) is a biodegradable, nontoxic, and biocompatible polymer (16), (ii) contains carboxylic groups that can form ionic complexes with a charged compound such as AMB, and (iii) has been used in clinical trials in a covalently bound conjugate to paclitaxel (Opaxio; Cell Therapeutics) (17). Our AMB-PGA complexes did not display hemolytic toxicity over a 24 h of incubation at concentrations up to 100 μg/ml of AMB equivalents or toxicity to KB and THP-1 cells after 72 h of incubation (15). These complexes also proved to be active against intracellular amastigotes of Leishmania major in differentiated THP-1 cells (15). The AMB-PGA complex remained stable, nontoxic, and active in vitro after storage either as a solid for 30 days or in solution for 7 days at 37°C (15). In this paper, we describe the efficacy of AMB-PGA complex against intracellular amastigotes of Leishmania donovani in vitro and in vivo together with data on cell uptake and biodistribution.

MATERIALS AND METHODS

Materials. Poly-γ-glutamic acid sodium salt (molecular weight of 50,000 to 70,000) was purchased from Sigma-Aldrich (catalogue no. G0421). Amphotericin B with 98% purity (injectable grade; batch no. HAN0604301) was purchased by the Drugs for Neglected Diseases initiative (DNDi; Geneva, Switzerland) from EgChemicals (China). Dimethyl sulfoxide (DMSO), 99.9% (anhydrous grade), was purchased from Sigma-Aldrich. Sodium hydroxide (1 M) was purchased from Fisher Scientific. AmBisome was a gift from Gilead Sciences. Fungizone was purchased from AAH Pharmaceuticals.
Antileishmanial Activity of Amphotericin B Complex

Methods. (i) Preparation of AMB-PGA complex. The AMB-PGA complex was prepared following dissolution of a defined amount of AMB (30 mg) in dry DMSO (0.6 ml) overnight in a glass vial (7 ml) (15). Depending on the desired AMB loading, a specified amount of AMB was dissolved in a separate solution of dry DMSO (0.6 ml) for 1 h. For example, to prepare AMB-PGA complex having 30 or 50% AMB loading, 20 or 45 mg of AMB was dissolved in DMSO (0.6 ml) in a glass vial (14 ml) (15). The PGA solution was added dropwise to the AMB solution. The mixture was stirred for 1 h, and then sodium hydroxide (2 equivalents to polymer, 1 M, 464 μl) was divided into two portions. The first portion of 232 μl of sodium hydroxide was added dropwise (1 drop every 5 s), and then the second portion (232 μl) was diluted in water (1 ml). The diluted sodium hydroxide solution was then added dropwise, followed by the addition of water (12 ml) (15). The reaction mixture was left to stir at room temperature for 1 h and then purified by dialysis (molecular mass cutoff, 12 to 14 kDa). The solution was filtered using a microsyringe filter (polyethersulfone [PES], 0.22 μm). The solution was freeze-dried to yield a yellow fluffy product. The fabrication process produced AMB-PGA complexes with AMB in an aggregated form that is similar to AmBisome and at a size of ~120 to 170 nm (measured by dynamic light scattering) (15). The AMB-PGA complex was prepared with weight percentages of AMB ranging from ~25 to 55%, with AMB water solubility ranging from 1.5 to 3 mg/ml.

(ii) In vitro antileishmanial activity against intracellular amastigotes. L. donovani (MHOM/ET/67/HU3) was maintained in RAG-1 mice (London School of Hygiene & Tropical Medicine colony maintained at Harlan, United Kingdom). Amastigotes were harvested from the spleens of infected mice. THP-1 cells were differentiated (18) and infected by L. donovani amastigotes as described previously (11). Stock solutions of the complexes (1 mg/ml of AMB equivalents) were prepared in sterile double-distilled water (Sigma-Aldrich, United Kingdom). Fungizone and AmBisome were reconstituted according to the manufacturers’ protocols. Complexes were tested from a starting concentration of 5 μg/ml of AMB equivalents in a 3-fold dilution series over six concentrations. Polymer (PGA) alone was included as a control at the highest concentration used in the drug complexes. Serial dilutions of the complexes and controls were tested in duplicate. Infected cultures were incubated for 72 h at 37°C, after which medium was removed and slides were fixed with 100% methanol and stained with Giemsa (10% in water) for 10 min. The level of infection per well was evaluated by counting the infected macrophages per 100 macrophages by microscope, and results were expressed as percent reduction in infected macrophages compared to untreated control wells. Data were analyzed with Microsoft Excel using a nonlinear sigmoidal curve-fitting Levenberg-Marquardt logarithm, and 50 and 90% effective concentrations (EC50 and EC90) were estimated.

(iii) Macrophage uptake. For the uptake of AMB-PGA complexes, differentiated THP-1 cells in a monolayer (5 × 10^4 cells/ml) were used (19). Fungizone and AmBisome were included as controls. Stock solutions of AMB-PGA complex (51% AMB loading) were prepared. The formulations were tested in triplicate cultures from the highest AMB concentration, 25 μg/ml, over four concentrations in a 5-fold dilution series with incubation periods of 1, 4, and 24 h. At the end of the incubation period, cells were washed rapidly with cold medium three times to remove free AMB, while minimizing release of the internalized AMB, and then lysed by adding Triton X (300 μl, 0.1% in phosphate-buffered saline [PBS]) and shaking for 20 min at room temperature. An aliquot of cell lysate (100 μl) was taken and mixed with methanol (100 μl) to solubilize AMB as a monomer. The methanolic mixtures of cell lysates were centrifuged at 4,500 rpm for 10 min, and the supernatant was withdrawn for quantification of AMB. Absorbance of AMB was determined at 407 nm by high-performance liquid chromatography (HPLC). AMB concentrations were extrapolated from a calibration curve of AMB in aqueous methanol (50%, vol/vol) obtained in the absence of cells under the same experimental conditions.

An aliquot of cell lysate was withdrawn for determination of protein concentration by micro-bicinchoninic acid (microBCA) assay (Fisher Scientific) by following the manufacturer’s protocol. The absorbance was read at 570 nm, and the protein concentration was determined from a bovine serum albumin (BSA) calibration curve. The results were presented graphically as AMB uptake (nmol of AMB/mg of protein) versus AMB concentration (μg/ml) or time (h).

(iv) In vivo antileishmanial activity. Female BALB/c mice were infected by injection of 2 × 10^5 L. donovani amastigotes (0.2 ml of RPMI medium) via the tail vein. The AMB-PGA solutions were prepared in a double-distilled sterile water (Sigma-Aldrich) stock of 1.25 mg/ml (AMB equivalents) and filtered using a 0.22 μm PES membrane (Millipore). Complex solutions were diluted using water for injection at the required dose. AmBisome was used as control and reconstituted in dextrose (5%) in water according the manufacturer’s protocol. Groups of 5 mice were dosed intravenously (0.2 ml via the tail vein) on days 7, 9, and 11 postinfection. Mice were sacrificed on day 14 postinfection. Livers were removed, weighed, and impression smears were taken, fixed in 100% methanol, and stained with 10% aqueous Giemsa stain for 45 min. The number of amastigotes per 500 host cell nuclei was determined. The parasite burden was calculated in Leishman-Donovan units (LDU) (number of amastigotes per liver cell nuclei multiplied by weight of the liver [n = 5]) (20). The reduction in parasite burden in each mouse was calculated relative to the mean LDU (n = 5) of the untreated control group and expressed as percent inhibition. Dose-response analysis and 50 and 90% effective doses (ED50 and ED90) were calculated using XLfit (ID Business Solution, Guildford, United Kingdom).

(v) In vivo distribution of the complex. Female BALB/c mice were used to determine the distribution of AMB-PGA complex (29% AMB loading). AmBisome was included as a control and was reconstituted in 5% dextrose (aqueous) according to the manufacturer’s protocol; the complex was reconstituted as described above. The complex and AmBisome were administered in a 0.2 ml bolus by intravenous injection at an AMB equivalent dose of 1.25 mg/kg (body weight). AMB concentrations in plasma and organs were determined by HPLC by following an established method (21). Blood samples were obtained by cardiac puncture at 30 min and 2, 4, 6, and 24 h after drug injection. The plasma was separated by centrifugation at 10,000 rpm for 10 min. AMB was extracted from the plasma by addition of 2 parts methanol to 1 part plasma and vortexed for 10 min. The plasma-methanol mixtures were centrifuged at 4,500 rpm for 10 min. Mice were sacrificed and the liver, spleen, and kidneys were removed and homogenized in double-distilled water. AMB was extracted from the homogenate by addition of 2 parts methanol to 1 part homogenate. The methanol-homogenate mixtures were vortexed for 1 min and centrifuged at 6,000 rpm for 20 min. The supernatants were filtered using a microsyringe filter (0.22-μm PES membrane; Millipore). Three mice were used for each time point. The residual blood in the organs was calculated according to organ weight (22). The amount of AMB in the residual blood was calculated and subtracted from amount of AMB in the tissues. Data from the repeated experiment are shown in Fig. S2 and S3 in the supplemental material.

(vi) HPLC analysis for quantification of AMB. High-performance liquid chromatography (Jasco) analysis was conducted using a C18 column (particle size, 5 μm; length, 250 mm) (Fisher Scientific) by following an established analytical method (21). The mobile phase used was aceto-
nitrile (52%), water (43.7%), and acetic acid (4.3%). The injection volume was 100 μl, and the flow rate was 1 ml/min. AMB was detected at 407 nm with a run time of 20 min. Under these conditions, the retention time of AMB was 8 min. The internal standard was 1-amino-4-nitronaphthalene. The sensitivity of the system was 0.016 μg/ml, and the detection limit was 0.054 μg/ml of AMB.

Ethics statement. Studies using animals were performed under license according to United Kingdom government (home office license 70/6997) and the requirements of the ethical review committee of London School of Hygiene & Tropical Medicine.

Statistical analysis. Data analysis was performed using Graphpad Prism 4 software, and results are expressed as means ± standard deviations. Statistical analysis was conducted using Graphpad Instat. P values were determined using two-tailed t test assuming equal variance, and significance was set at a P value of <0.05.

RESULTS

Evaluation of in vitro antileishmanial activity of AMB-PGA complex. The in vitro activities of AMB-PGA complexes against L. donovani intracellular amastigotes in differentiated THP-1 cells, with AMB loadings ranging from ~25 to 55%, are shown in Table 1. All AMB-PGA complexes were equivalent in activity to Fungizone irrespective of the AMB loading. The in vitro relative potency of the AMB-PGA complexes was, on average, ~1.3 compared to Fungizone (Table 1). The PGA polymer was inactive (4.64% ± 3.65% inhibition) against amastigotes at a concentration of 14.6 μg/ml, the polymer concentration at the highest concentration of the AMB-PGA complex evaluated.

Macrophase uptake. The amount of AMB accumulated in the differentiated THP-1 cells was concentration dependent for both Fungizone and AMB-PGA complex (51% AMB loading) at 1, 4, and 24 h (Fig. 2). At an extracellular AMB concentration of 25 μg/ml, the amount of AMB accumulated in the cells increased with incubation time for both Fungizone and AMB-PGA complex (Fig. 2D). The uptake of Fungizone was higher (1.3-fold; P = 0.1023) than that of the complex after 24 h of incubation (Fig. 2C and D). In contrast, AmBisome displayed a significantly lower uptake than both the complex and Fungizone AMB after 24 h of incubation (P < 0.0001) (Fig. 2C and D). Similar results were obtained with differentiated THP-1 cells infected with L. donovani (Fig. 3). There was no significant difference between AMB uptake in uninfected and infected differentiated THP-1 cells (P > 0.05) (Fig. 2 and 3). Data from the repeat experiment are shown in Fig. S1 in the supplemental material.

In vivo antileishmanial activity of AMB-PGA complex in a mouse model. In the initial experiments, two AMB-PGA complexes with AMB loadings of 31 and 51% were tested by intravenous administration of doses of 1.25 and 2.5 mg/kg of AMB equivalents administered on three alternate days over a 5-day period. All the complexes were active against L. donovani in vivo, with no significant difference in activity between complexes with 31 and 51% AMB loading (P = 0.51) (Table 2). The effect of AMB loading using a higher AMB single dose (2.5 mg/kg) on the activity of

<table>
<thead>
<tr>
<th>Formulation</th>
<th>AMB loading (%)</th>
<th>EC_{50} (μg/ml)</th>
<th>EC_{90} (μg/ml)</th>
<th>Relative potency</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25.5</td>
<td>0.33 ± 0.08</td>
<td>0.95 ± 0.28</td>
<td>1.5</td>
</tr>
<tr>
<td>2</td>
<td>35</td>
<td>0.23 ± 0.04</td>
<td>0.74 ± 0.23</td>
<td>1.1</td>
</tr>
<tr>
<td>3</td>
<td>51</td>
<td>0.23 ± 0.05</td>
<td>0.82 ± 0.32</td>
<td>1.1</td>
</tr>
<tr>
<td>Fungizone</td>
<td>45</td>
<td>0.22 ± 0.04</td>
<td>0.42 ± 0.04</td>
<td></td>
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</table>

*Infection levels in the untreated cells were 80.25% ± 1.89% and 80.75% ± 4.35% after 24 and 72 h postinfection. The EC_{50} is an average of quadruplicates ± standard deviations. Relative potency is EC_{50} for drug/EC_{50} for standard drug.
AMB-PGA complex was examined (Table 2). There was no significant difference in the \textit{in vivo} antileishmanial activity between AMB-PGA complexes with different AMB loading (31 and 51\%) even at the higher dose of 2.5 mg/kg ($P = 0.15$). However, there was a significant increase in the \textit{in vivo} antileishmanial activity of both AMB-PGA complexes (31 and 51\% AMB loading) when the dose was increased from 1.25 to 2.5 mg/kg ($P = 0.06$ and 0.03) (Table 2).

The dose-response effect of AMB-PGA complex was demonstrated following administration of three dose levels, 0.1, 0.5, and 2.5 mg/kg of AMB equivalents, as single doses on three alternate days over a 5-day period. The AMB-PGA complex (29\% AMB loading) displayed an \textit{in vivo} antileishmanial activity similar to that of AmBisome (Fig. 4), with an ED$_{50}$ of 0.24 ± 0.03 mg/kg (ED$_{90}$, 0.76 ± 0.06 mg/kg) for the AMB-PGA complex, in comparison to 0.24 ± 0.06 mg/kg (ED$_{90}$, 0.76 ± 0.06 mg/kg) for AmBisome, indicating a relative potency of the AMB-PGA complex to AmBisome of 1. The polymer alone displayed 11.78\% ± 2.2\% inhibition of hepatic parasite burden at a total dose of 18.36 mg/kg, equivalent to the total amount of the polymer administered in a dose of 7.5 mg/kg of the AMB-PGA complex. There was no inhibition of hepatic parasite burden at a total polymer-alone dose of 3.66 mg/kg (1.5 mg/kg of AMB equivalents). There was no sign of overt toxicity in mice, as indicated by absence of weight loss in all mouse groups receiving either the polymer alone, the AMB-PGA complex, or AmBisome in both experiments.

**Distribution of the complex in the plasma and organs.** The distribution of AMB-PGA complex in plasma, liver, spleen, and kidneys was determined at different time points (Fig. 5). The complex was cleared from the blood circulation (Fig. 5D), with an extensive uptake of the complex by the liver and spleen (Fig. 5A and B). The highest plasma concentrations for AmB-PGA complex were 22.34 ± 1.40 and 0.90 ± 0.04 g/ml, respectively, at 30 min postinjection. Most of the AmB-PGA complex was cleared

### TABLE 2

<table>
<thead>
<tr>
<th>Group</th>
<th>AMB loading (%)</th>
<th>Dosing regimen</th>
<th>% parasite burden inhibition</th>
<th>Parasite burden (LDU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>31</td>
<td>i.v. × 3 (mg/kg)</td>
<td>80.94 ± 8.78</td>
<td>213.97 ± 98.64</td>
</tr>
<tr>
<td>2</td>
<td>31</td>
<td>2.50</td>
<td>90.65 ± 4.64</td>
<td>105.06 ± 52.10</td>
</tr>
<tr>
<td>3</td>
<td>51</td>
<td>1.25</td>
<td>84.44 ± 7.22</td>
<td>174.74 ± 81.07</td>
</tr>
<tr>
<td>4</td>
<td>51</td>
<td>2.50</td>
<td>94.4 ± 2.94</td>
<td>57.45 ± 33.03</td>
</tr>
<tr>
<td>5</td>
<td>AmBisome</td>
<td>1.00</td>
<td>95.32 ± 1.31</td>
<td>50.36 ± 14.68</td>
</tr>
</tbody>
</table>

*The hepatic parasite burden was 1,123 ± 161 LDU in the untreated control group. Parasite burden inhibition and LDU are averages of quintuplicates ± standard deviations. i.v., intravenous.*
from the plasma within 30 min, while AmBisome displayed a prolonged circulation (Fig. 5D).

The complex showed similar AMB concentration profiles in the liver and spleen (Fig. 5A and B). The maximum concentrations of AMB in the liver and spleen were 11.35 ± 1.23 μg/g of tissue (at 24 h) and 12.32 ± 4.11 μg/g (at 24 h) for the complex and 13.97 ± 1.86 μg/g (at 6 h) and 22.88 ± 5.42 μg/g (at 4 h) for AmBisome, respectively (Fig. 5A and B). The complex displayed an AMB concentration profile in the liver similar to that of AmBisome (Fig. 5A). The uptake of the complex by the spleen was lower than that of AmBisome (Fig. 5B). There was negligible disposition of both the complex and AmBisome in the kidneys even at 24 h (Fig. 5C).

**DISCUSSION**

There is an urgent need for an improved delivery system for AMB that is cheap, effective, and stable under high-temperature and high-humidity conditions. Although liposomal AMB (AmBisome) has proved to be highly effective in the treatment of VL in India, this formulation is expensive and not stable at temperatures above 25°C (23). Poly(α-glutamic acid) (PGA) was evaluated for the preparation of a nontoxic, stable, and soluble form of AMB (15).

The in vitro antileishmanial activity of all the AMB-PGA complexes was similar to that of AMB-deoxycholate (Fungizone), showing that complexation of AMB with PGA did not affect its activity against intracellular amastigotes in differentiated THP-1 cells. These complexes had activity superior to those reported in other studies with AmBisome (EC_{50} of 1.0 ± 0.05 μg/ml) (24) and AMB polymeric conjugates with N-(2-hydroxypropyl)-methacrylamide (AMB-HPMA) (EC_{50} of 0.63 to 1.09 μg/ml) in a similar model (11). Additional studies showed similar uptake of the AMB-PGA complex and Fungizone by differentiated THP-1 cells. The uptake of AMB-PGA complexes by the differentiated THP-1 cells might be via endocytosis, and it can be related to (i) particle size (>100 nm) and highly aggregated state (15, 25), (ii) negative surface charge (15, 26), and (iii) the colloidal nature of the complex (25). Heat-induced AMB superaggregates were extensively taken up by macrophages (J774) in vitro via endocytosis due to their large size (600 nm) (27). The insignificant uptake of AmBisome by the differentiated THP-1 cells reported here is consistent with studies using J774 cells, another macrophage cell line (19). The reduced internalization of AmBisome can be partially explained by its small size (28) and the presence of cholesterol in lipid bilayer (29).

Importantly, AMB-PGA complexes displayed (i) a potent and reproducible in vivo antileishmanial activity equal to that of AmBisome, (ii) reduced toxicity in vitro and in vivo compared to that of Fungizone (the reported 50% lethal dose [LD_{50}] is 2.3 mg/kg) (30), (iii) increased selectivity for intracellular L. donovani amastigotes, and (iv) high stability due to tight association of AMB with the polymer (15). Furthermore, the polymer alone showed insignificant antileishmanial activity in vitro and in vivo, indicating that the activity of the complex is caused by AMB.
The *in vivo* antileishmanial activity of the complex was not related to AMB loading of the complex and was shown to depend on the dose. This differs from other studies on AMB-HPMA conjugates which showed that higher AMB loading led to an increase in *in vivo* antileishmanial activity (31). Our AMB-HPMA complexes were more active than Fungizone (ED$_{50}$ of 0.95 to 4.9 mg/kg) (32) and AMB-HPMA conjugates (27.5% AMB) (ED$_{50}$ of 0.97 and ED$_{50}$ of $>1$ mg/kg) (11) and equally as active as heat-induced AMB superaggregates (ED$_{50}$ of 0.144 ± 0.018 to 0.37 ± 0.14 mg/kg) (33) against *L. donovani* in BALB/c mice. Differences in the antileishmanial activities of AMB-HPMA complex and AMB-HPMA conjugates could result from the nature of the bond between AMB and the polymer (31). In the case of AMB-HPMA conjugates, AMB is attached to the polymer covalently via a biodegradable linker (GlyPhelLeuGly) (11), while in our AMB-HPMA complex there is noncovalent association between AMB and the PGA.

The potent *in vivo* activity of AMB-HPMA complex can be related to both AMB aggregation and the size of the AMB-HPMA complex. AMB is present in the form of nontoxic and water-soluble aggregates in the AMB-HPMA complex because of its association with the polymer (15). Polyaggregates of AMB were observed to be active *in vivo* against *L. infantum* in a Syrian hamster model (35). This *in vivo* activity was attributed to the large size of the polyaggregates, which favors their uptake by phagocytic cells and therefore their activity against *Leishmania* amastigotes in the macrophages (35).

The *in vivo* activity of the complex can also be explained by its distribution, especially in the infected organs (liver and spleen). Previous studies also reported increased accumulation of AMB particulate systems, including nanospheres (36), heat-induced superaggregates (37), and polyaggregates (34), in the liver and spleen. Our complex had low plasma AMB concentrations, possibly due to rapid clearance by the MPS. This contrasts to studies on AMB-polyethylene glycol conjugates (AMB-PEG), where an increase in the plasma concentration of AMB (~6 mg/ml after a 2-mg/kg single intravenous injection in mice) was reported (12), possibly due to a reduction of phagocytosis of the AMB-PEG conjugates by the MPS due to the stealth effect of PEG. In the case of an AMB-arabinogalactan conjugate, the plasma AMB level was reported to be mainly dependent on the molecular weight of the polymer (13).

Liposomal AMB (AmBisome) displayed a high plasma AMB concentration, consistent with previous studies (30), which was attributed to the small particle size (<100 nm), tightly packed stable phospholipids, and the presence of cholesterol resulting in reduced opsonization and uptake of AmBisome by the MPS via phagocytosis (28, 30). The *in vivo* distribution of AmBisome (Fig. 5) was mainly to the liver and spleen, and the level in the kidneys was negligible. Similar results have been reported with uninfected animal models (28, 30).

The extensive uptake of our AMB-HPMA complex by the MPS was confirmed in uninfected mice, in which high levels of AMB were measured in the liver and spleen (Fig. 5). A similar profile was reported for AMB polyaggregates and AMB encapsulated in albumin microspheres (34). Lipid-based formulations such as Abelcet and Amphocil have large particles (1 to 10 μm and 115 to 120 nm) and highly aggregated AMB that enables their rapid accumulation in the liver, spleen, and bone marrow (38, 39). The high AMB accumulation in the liver explains the potent antileishmanial activity of the AMB-HPMA complex in this BALB/c mouse model. Further studies are required to determine the activity of the complex in the spleen and bone marrow, the other key sites of *L. donovani* infection, in either mouse or hamster models of infection.

In conclusion, AMB-HPMA complexes described here have demonstrated potent *in vivo* antileishmanial activity against *L. donovani* and reduced AMB toxicity similar to the values for AmBisome. This complex had an AMB distribution profile in the blood circulation and spleen different from that of AmBisome. The activity and distribution of the complex remain to be determined in the hamster chronic infection model. Our results indicate the potential of AMB-HPMA complexes for the treatment of VL and the need for more extensive studies using this approach.

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