Treatment of experimental visceral leishmaniasis with single-dose liposomal amphotericin B – pharmacodynamics and biodistribution at different stages of disease

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Running title: AmBisome\textregistered pharmacodynamics and biodistribution in EVL
Abstract
Visceral leishmaniasis is a neglected tropical disease, which causes significant morbidity and mortality worldwide. Characterising the pharmacokinetics and pharmacodynamics of anti-leishmanial drugs in pre-clinical models is important for drug development and use. Here we investigated the pharmacodynamics and drug distribution of AmBisome® in *L. donovani* infected BALB/c mice at three different dose levels and two different time points after infection. We additionally compared drug levels in plasma, liver and spleen in infected and uninfected BALB/c mice over time. At the highest administered dose of 10 mg/kg AmBisome®, >90% parasite inhibition was observed within 2 days after drug administration, consistent with drug distribution from blood to tissue within 24 hours and a fast rate of kill. Decreased drug potency was observed in the spleen when AmBisome® was administered on day 35 after infection, compared to day 14 after infection. Amphotericin B concentrations and total drug amounts per organ were lower in liver and spleen when AmBisome® was administered at the advanced stage of infection and when compared to uninfected BALB/c mice. However, the magnitude of difference was lower when total drug amounts per organ were estimated. Differences were also noted in drug distribution to *L. donovani* infected livers and spleens. Taken together our data suggests that organ enlargement and other pathophysiological factors cause infection- and organ-specific drug distribution and elimination after administration of single dose AmBisome® to *L. donovani* infected mice. Plasma levels were not reflective of changes in drug levels in tissues.
INTRODUCTION

Visceral leishmaniasis (VL) is a vector-borne neglected tropical disease (NTD) caused by protozoan parasites of the genus *Leishmania*. VL has a high mortality rate if untreated. Current estimates suggest 200,000 – 400,000 cases and 20,000 – 40,000 deaths per year worldwide with over 90% of cases occurring in India, Bangladesh, Sudan, South Sudan, Brazil and Ethiopia (1).

Major clinical manifestations of disease are fever, enlargement of the liver and spleen, weight loss and pancytopenia. Available treatment options include pentavalent antimonials, paromomycin in combination with pentavalent antimony (in East Africa), miltefosine and amphotericin B in different formulations (2). Liposomal amphotericin B (AmBisome®) has emerged as the preferred treatment for VL in Europe and the US (3) and South Asia (2). In India a cure rate of 95.7% was achieved with a single infusion of 10 mg/kg of AmBisome® in a trial carried out at an urban referral centre (4). Feasibility of this treatment regime was confirmed in a rural setting in Bangladesh at primary health care level (5). Single-dose AmBisome® was also one component in recently trialled short-course multidrug treatment regimens for VL in India (6).

AmBisome® was initially developed to improve efficacy and reduce toxicity of amphotericin B as an anti-fungal agent. Its favourable therapeutic index is attributed to prolonged circulation times due to a small liposome size, allowing distribution to tissues, and a tight association of amphotericin B with the liposome bilayer, providing stability under physiological conditions (7). AmBisome® also remains the anti-leishmanial agent with the widest therapeutic window for treatment of VL (3).

Earlier studies have established the pre-clinical pharmacokinetics of AmBisome® in mice, rats, rabbits and dogs (7, 8). In addition, a comparative, single time-point biodistribution study in *L. donovani* infected and uninfected BALB/c mice showed that amphotericin B concentrations in
liver and spleen were lower in infected mice after intravenous administration of AmBisome® (9). A separate study showed that AmBisome®, when administered as single dose to L. donovani infected BALB/c mice, was less effective in the chronic stage of infection compared to the acute stage (10). In anti-infective therapy with drugs that directly kill pathogens, such as amphotericin B, drug efficacy is driven by infection-site specific drug concentrations (11, 12). We therefore hypothesised that the decrease in potency of AmBisome® observed in chronic experimental VL (EVL) is linked to a decrease in organ-specific drug concentrations associated with progression of infection. Here we show that i) decreased potency of AmBisome® during progressive EVL is most pronounced in the spleen, ii) total drug amounts in liver and spleen progressively decrease during EVL, iii) organ-specific drug distribution and elimination patterns occur in EVL and iv) changes in drug levels in tissues are not reflected in the blood compartment.

MATERIALS AND METHODS

Drugs and reagents. AmBisome® was purchased from Gilead (Cambridge, UK). The powder was reconstituted in sterile water following the manufacturer's directions and further dilutions prepared in 5% glucose. Amphotericin B (VETRANAL™ analytical standard), tolbutamide, dimethyl sulfoxide (DMSO) and sodium dodecyl sulphate (SDS) were obtained from Sigma, UK, and heparin from John Bell & Croyden, UK. Methanol (HPLC grade), 0.1% (v/v) formic acid in water (LC-MS grade) and water (LC-MS grade) were purchased from Fisher Scientific UK Ltd, UK.

In vivo experiments and treatment. Female BALB/c (Charles River, UK) and Rag-1 (B6) K.O. mice (LSHTM breeding colony) were maintained under specific pathogen free conditions in individually ventilated cages and exposed to 12 hour light – 12 hour dark cycles. Standard
rodent diet (RM No 1 Expanded) and filtered tap water were supplied ad libitum. Mice (6-10 weeks of age at the start of experiments) were infected by intravenous (i.v.) injection of $2 \times 10^7$ parasites ($L. \text{donovani} \text{ MHOM/ET/67/HU3}$) as described (13). Parasites were maintained in Rag-1 (B6) K.O. mice and amastigotes harvested from spleens >40 days after infection. BALB/c mice were treated either 14 or 35 days after infection with a single i.v. dose of AmBisome® in a 0.2 mL bolus injection. On the day of treatment, prior to the administration of drugs, mice were weighed and randomised into the different treatment groups using a random number generator. The average weight of mice in each experiment was used for dose calculations. Untreated groups of mice were included as controls where appropriate. Age matched uninfected mice were maintained under identical conditions for the same length of time as their infected comparators. At experimental endpoints mice were weighed and humanely killed by exsanguination under terminal anaesthesia. Blood was collected by cardiac puncture in Eppendorf tubes containing heparin and plasma harvested by centrifugation. Livers and spleens were removed and their weight recorded. Plasma and tissue samples were stored at $-80^\circ$C until further processing. For determination of parasite burdens tissue impression smears were prepared, fixed in 100% methanol and stained in 10% Giemsa. Parasite burden was determined microscopically and Leishman-Donovan Units (LDUs) calculated by the formula number of parasites per host cell nucleus x organ weight in mg as described previously (14). Microscopical evaluation of impression smears was carried out by a scientist unaware of the treatment allocation.

**Processing of samples for drug quantification.** Samples were thawed at room temperature immediately prior to processing. Livers and spleens were homogenised with an equal volume of ZrO beads in 4 volumes of 0.1% aqueous formic acid in a Bullet Blender (Next Advance, UK). For control matrix samples + internal standard (IS), calibration standards, quality control
(QC) samples and study samples 50 µl of tissue homogenate or plasma were diluted with 250µl IS solution (200 ng/mL tolbutamide in a 84:16 (v/v) mixture of methanol : DMSO). For blank samples (matrix sample without IS) 50 µl of tissue homogenate or plasma were diluted with a 84:16 (v/v) mixture of methanol: DMSO. After shaking for 10 minutes at 200 rpm at room temperature dilutions were centrifuged at 4150 x g for 15 minutes at 4°C. Supernatants were transferred to 96 well plates and stored at -80°C.

**Preparation of calibration standards and QC samples.** An amphotericin B stock solution (1.0 mg/mL) was prepared in DMSO and from that standard spiking solutions were prepared by serial dilution in 1% SDS in water. Calibration standards at a minimum of 6 concentrations were prepared by mixing 5 µL of the spiking solutions with 45 µL of blank tissue homogenate or plasma, matrix matching that of the study samples to be analysed. QC samples at selected concentrations were prepared in replicate in similar fashion and all samples processed as described above.

**Preparation of study samples.** Concentrations of amphotericin B in study samples varied widely, depending upon dosing and sampling regimens. Those samples expected to contain high concentrations of amphotericin B were diluted with matching control matrix by a suitable factor to reduce their concentration into a quantifiable range (i.e. within the range of calibration standard concentrations).

**LC/MS analytical conditions.** All samples were analysed using an Agilent 1200 HPLC combined with an Agilent 6410A triple quadrupole mass spectrometer (both Agilent, UK). A mobile phase of water / 0.1% formic acid (channel A) and methanol / 0.1% formic acid (channel B) was used to elute sample components from a Kinetex column packed with 5 μm XB-C18
material (2.1mm x 50mm @50°C; Phenomenex, UK). The mobile phase composition was initially 20% B, programmed to increase linearly to 90% B at 1.60 min. after injection; after 0.4 min. at 90% B the composition was returned to its initial 20% B at 2.10 min. post-injection. Amphotericin B was detected monitoring the transition m/z 906.5 → m/z 743.2, the dehydrated protonated molecule at m/z 906.5 being the most intense ion produced for this component in the ion source.

Analyte concentrations were quantified against calibration standards prepared in matched control matrix, with aliquots of sample and standard being injected typically in the range 1-5 µL, depending on expected study sample concentration.

**Pharmacokinetic analysis.** Noncompartmental analysis (NCA) was performed with Phoenix Win Nonlin v6.3 (Certara, UK).

**Characterisation of serum protein profiles.** Blood was collected from *L. donovani* infected or uninfected BALB/c mice by cardiac puncture under terminal anaesthesia and stored overnight at 4°C. Serum was harvested by centrifugation at 1500 x g, 4°C for 15 minutes and stored at -80°C prior to analysis. Capillary electrophoresis and protein determinations were carried out by LABOKLIN GmbH&Co.KG (Bad Kissingen, Germany).

**Statistical analysis.** Statistical significance between two groups was evaluated by a t-test and between more than two groups by one-way ANOVA assuming Gaussian distribution, followed by Sidak’s multiple comparisons test for selected groups as applicable (GraphPad Prism 6). A p value of ≤ 0.05 was considered statistically significant.
Ethical statement. Experiments involving animals were carried out under license in accordance with the Animals (Scientific Procedures) Act 1986 (UK Home Office Project Licences PPL70/6997 and PPL70/8207) following approval by the Animal Welfare and Ethics Review Board at LSHTM.

RESULTS

Efficacy of single dose liposomal amphotericin B in *L. donovani* infected BALB/c mice at two different timepoints after infection. Single dose AmBisome® was administered at doses of 10 mg/kg, 2.5 mg/kg and 0.6 mg/kg 14 or 35 days after infection. Parasite burden was evaluated 7 days after dosing. A reproducible decrease in drug efficacy between day 21 and day 42 post infection was noted in the spleen, which was more pronounced at the lower doses. In the liver decreased drug efficacy at the lower two doses was noted in one experiment, whereas in another experiment an increase in drug efficacy was observed at the lowest dose (Table 1).

Time-to-kill studies. Single dose AmBisome® was administered to *L. donovani* infected BALB/c mice 14 days after infection at doses of 10 mg/kg, 2.5 mg/kg and 0.6 mg/kg. Parasite burden was evaluated 1, 2, 3 or 7 days after dosing. In the liver effective parasite kill (% inhibition >90%) was observed 2 days after administration of the highest dose. At the dose of 2.5 mg/kg the hepatic parasite burden was inhibited by >60% at 2 days, >70% at 3 days and >80% (maximum kill) at 7 days after drug administration. The dose of 0.6 mg/kg was ineffective (% inhibition <40%) and no change in parasite kill was observed over time (Fig. 1A). A similar pattern of effective kill was observed in the spleen after administration of 10 mg/kg AmBisome® (>80% and >90% inhibition in repeat experiments at 2 days and >90% at
3 days after drug administration) and a static pattern (% inhibition <40%) after administration of 0.6 mg/kg. Maximum kill at the dose of 2.5 mg/kg varied from 57% to 71% inhibition in repeat experiments with peaks at 2 and 3 days after drug administration (Fig. 1B). In an additional experiment *L. donovani* infected BALB/c mice were treated with a single dose of 2.5 mg/kg AmBisome® 33 days after infection. The hepatic parasite burden was inhibited by 55%, 67% and 62% on days 1, 2 and 3 after drug administration. In the spleen parasite kill was low on day 1 after drug administration (<40% inhibition) and increased on days 2 and 3 after drug administration to a maximum of 46% inhibition (Fig. 1C).

**Plasma pharmacokinetics of single dose AmBisome® in *L. donovani* infected BALB/c mice.** Single dose AmBisome® was administered at dose levels of 10 mg/kg, 2.5 mg/kg and 0.6 mg/kg 14 days after infection. Plasma samples were obtained at 5 minutes, 30 minutes, 1 hour, 3 hours, 6 hours and 24 hours after drug administration, followed by determination of drug concentrations and estimation of PK parameters. The volume of distribution (Vd) and clearance (CL) increased with decreasing doses of AmBisome®. The Vd increased from 104 mL/kg at 10 mg/kg to 252 and 681 mL/kg at 2.5 mg/kg and 0.6 mg/kg respectively. The CL was 27 mL/hr/kg at a dose of 10 mg/kg and 48.1 and 41.9 mL/hr/kg at doses of 2.5 mg/kg and 0.6 mg/kg. The AUC (0-24 hours) was highest at 10 mg/kg (368.5 hr*µg/mL) and lower at doses of 2.5 mg/kg and 0.6 mg/kg (50.5 and 10.8 hr*µg/mL respectively). PK parameters are summarised in Table 2.

**Tissue distribution of amphotericin B in *L. donovani* infected BALB/c mice following administration of AmBisome® at two different timepoints after infection.** Single dose AmBisome® was administered 14 or 35 days after infection. Amphotericin B concentrations in plasma, liver and spleen were measured 7 days after dosing. Lower amphotericin B
concentrations were observed in livers and spleens when equal drug doses were administered on day 35 compared to day 14 post infection. When expressed as ng/g tissue drug concentrations in the liver were 2.6-, 2.2- and 5.6-fold lower on day 42 compared to day 21 post infection at AmBisome® doses of 10 mg/kg, 2.5 mg/kg and 0.6 mg/kg. Respective tissue concentrations in the spleen were 4.5-fold, 2.8-fold and 9.6-fold lower. Statistical significance was noted for the 10 mg/kg dose group (p \leq 0.0001 for liver, p \leq 0.01 for spleen). Organ weights significantly increased from day 21 to day 42 post infection (p \leq 0.0001 in all dose groups) with a 1.5-fold increase in the liver and 2.3- to 2.9-fold increases in the spleen. When taking this increase in organ weights into account and estimating total drug amount / organ the difference between amphotericin B concentrations on day 21 and day 42 post infection decreased. Differences in total amphotericin B amount / organ at AmBisome® doses of 10 mg/kg, 2.5 mg/kg and 0.6 mg/kg were 1.7-, 1.5- and 3.8-fold in the liver and 1.5-, 1.3- and 3.9-fold in the spleen. Statistical significance was noted for the 10 mg/kg dose group in the liver (p \leq 0.0001). Overall, higher amphotericin B concentrations were measured in the liver compared to the spleen. Data is summarised in Table 3A. Amphotericin B concentrations in plasma remained unchanged when drug was administered on day 14 compared to day 35 post infection. A 0.3-fold difference was noted in the 0.6 mg/kg dose group, but was not statistically significant (Table 3B).

**Comparative amphotericin B plasma and tissue concentrations in L. donovani infected and uninfected BALB/c mice 7 days after administration of single dose AmBisome®.**

Amphotericin B concentrations were measured in L. donovani infected (14 or 35 days after infection) and age and husbandry matched uninfected BALB/c mice after administration of 2.5 mg/kg AmBisome®. At both time points amphotericin B concentrations were significantly lower (p value \leq 0.0001) in livers and spleens from infected BALB/c mice compared to uninfected ones. In the liver mean drug concentrations (ng amphotericin B / g tissue) on day
21 and day 42 post infection were 3.5- and 6.5-fold lower in infected compared to uninfected BALB/c mice. This translated into 2.5- and 5.4-fold differences when taking organ weights into account and estimating the total amphotericin B amount / organ (Fig. 2A and B). In the spleen mean amphotericin B concentrations were 29.3- and 100.9-fold lower in infected compared to uninfected mice on day 21 and day 42 post infection. Respective organ weight adjusted differences were 10.3- and 16.5-fold (Fig. 2C and D). The opposite was observed in plasma and significantly higher amphotericin B concentrations measured in infected BALB/c mice compared to uninfected ones, with 2- and 1.9-fold differences on day 21 (p value ≤ 0.01) and day 42 (p value ≤ 0.001) post infection (Fig. 2E). Tabulated results are provided in Supplementary Table S1.

To investigate if differences in amphotericin B levels were also observed after administration of higher drug doses *L. donovani* infected (day 33 post infection) or uninfected BALB/c mice were treated with a single dose of 40 mg/kg AmBisome®. In line with above results amphotericin B concentrations were significantly lower (p value < 0.0001) in livers and spleens from infected BALB/c mice compared to uninfected ones. However, plasma concentrations were similar between the two different groups (p value > 0.05). Data is shown in Supplementary Table S1.

**Comparative amphotericin B plasma and tissue concentrations in *L. donovani* infected and uninfected BALB/c mice up to 48 hours after administration of single dose AmBisome®.** A single dose of 2.5 mg/kg AmBisome® was administered to *L. donovani* infected (day 33 post infection) and age and husbandry matched uninfected BALB/c mice. Plasma and tissue samples were collected 5 minutes, 4 hours, 24 hours and 48 hours after drug administration and amphotericin B concentrations determined. Amphotericin B plasma levels decreased within 24 hours in uninfected and *L. donovani* infected BALB/c mice and remained
at similar levels at 24 and 48 hours after drug administration (Fig. 3A and B). An increase in amphotericin B concentration was observed in livers and spleens of uninfected BALB/c mice from 5 minutes to 4 hours after drug administration. Amphotericin B concentrations at 24 and 48 hours after drug administration were comparable to those measured at 4 hours (Fig. 3C and E). A different kinetic was observed in *L. donovani* infected BALB/c mice. In the liver a small increase in amphotericin B concentration was noted from 5 minutes to 4 hours after drug administration, followed by a decrease at 24 hours and, most notably, 48 hours after drug administration (Fig. 3D). In the spleen amphotericin B concentrations decreased over the whole observation period with no increase noted (Fig. 3F). Similar kinetics were observed when estimating total drug amount per organ (Fig. 3 G – J). Tabulated results are provided in Supplementary Table S2.

**Host responses to infection.** Serum samples from *L. donovani* infected (day 14 and 43 post infection) and age and husbandry matched uninfected BALB/c mice were subjected to electrophoresis and protein determination. Significant differences (p ≤ 0.05) in serum protein profiles were identified. In infected BALB/c mice serum concentrations of total protein (46.8 +/- 0.6 vs. 56.6 +/- 1.0 g/L), alpha globulin (6.7 +/- 0.2 vs. 8.5 +/- 0.2 g/L) and gamma globulin (7.6 +/- 0.2 vs. 16.1 +/- 1.0 g/L; 16.3 +/- 0.5 vs. 28.4 +/- 1.3 %) increased from day 14 to day 43 post infection. At the same time % albumin levels decreased (61.4 +/- 0.7 vs. 49.3 +/- 1.1), but no significant difference was found when comparing total albumin. The albumin:globulin ratio decreased from day 14 to day 43 post infection ( 1.6 +/- 0.0 vs. 1.0 +/- 0.0). Compared to uninfected BALB/c mice higher levels of total protein (56.6 +/- 1.0 vs. 47.6 +/- 0.8 g/L), alpha globulin (8.5 +/- 0.2 vs. 7.3 +/- 0.2 g/L) and gamma globulin (16.1 +/- 1.0 vs. 6.5 +/- 0.2 g/L; 28.4 +/- 1.3 vs. 13.6 +/- 0.2 %) were measured in *L. donovani* infected mice on day 43 post infection. At the same timepoint infected BALB/c mice had lower levels of albumin (27.8 +/-
0.4 vs. 29.9 +/- 0.5 g/L; 49.3 +/- 1.1 vs. 62.8 +/- 1.6 %) than uninfected ones. On day 14 post infection increased levels of % gamma globulin were noted in infected compared to uninfected BALB/c mice (16.3 +/- 0.5 vs. 12.3 +/- 0.6). On day 43 post infection infected BALB/c mice had a lower albumin:globulin ratio than uninfected ones (1.0 +/- 0.0 vs. 1.7 +/- 0.1). Data is summarised in Table 4.

**DISCUSSION**

The pharmacodynamics (PD) and pharmacokinetics (PK) of current anti-leishmanial drugs have yet to be fully established in preclinical disease models. Here we investigated the potency and biodistribution of AmBisome® in EVL after administration of three different single doses and at two different timepoints. AmBisome® was well tolerated at all doses administered and no signs of drug toxicity noted, based on observations of weight, fur condition, behaviour and facial expression. We also determined the PK profile of AmBisome® in plasma of *L. donovani* infected BALB/c mice in an exploratory study and compared trends to published data in uninfected mice. The observed increase in C\textsubscript{max} and AUC\textsubscript{last} and the decrease in Vd and CL with increasing doses is in line with this data (7).

After infection of BALB/c mice with *L. donovani* the parasite burden in the liver rapidly increases for the first 2-4 weeks, followed by a decrease in parasite numbers and resolution of infection. In the spleen the parasite burden increases from 2 weeks after infection and parasite persistence is observed (15-17). Considering this organ-specific pattern of parasite growth is important when analysing drug efficacy and potency data. In the spleen drug efficacy was evaluated against an increasing parasite burden at both time points and a decrease in potency observed when drug was administered on day 35 compared to day 14 post infection. However, the magnitude of decrease and the dose at which this occurred differed between experiments. It is notable that the decrease in drug concentrations / total drug amounts in the spleen following
administration of the same dose of AmBisome® on day 14 vs. day 35 post infection was less pronounced in the third compared to the second experiment presented in Table 1 (Supplementary Table S3). This may explain the lack of decreased drug potency observed in experiment 3. In the liver opposing trends were observed at the lowest dose. However, data obtained in the liver on day 42 post infection is not indicative of direct drug kill only, it reflects a mixture of parasite killing by drug and a mature granulomatous host response resolving the infection (18-20). Hence, these opposing trends at a dose which lacked meaningful anti-leishmanial drug efficacy, likely reflect differences in the efficiency of host response rather than in drug potency.

In parallel to evaluating drug potencies we determined amphotericin B concentrations in plasma, livers and spleens of infected BALB/c mice at the two different time points and estimated the total drug amount per organ. In livers and spleens lower drug concentrations and total drug amounts per organ were noted when drug was administered on day 35 compared to day 14 post infection and the difference was highest at the lowest dose. The magnitude of difference was lower for absolute (organ weight-adjusted) values than for relative values, which suggests that differences in drug levels between the two time points can partly be explained by the infection-associated increase in organ weights. A combined effect of organ enlargement and other pathology-associated factors on drug distribution following administration of AmBisome® to L. donovani infected BALB/c mice is also supported by comparative studies in infected and uninfected mice. Again the magnitude of decrease in amphotericin B levels in livers and spleens of infected mice was lower when organ weight-adjustment was applied, but remained significant and increased from day 21 to day 42 post infection in both organs. Interestingly, plasma levels of amphotericin B were higher in infected compared to uninfected mice at this point.
To gain further insight into the kinetics of parasite kill and drug distribution we next examined drug potency and distribution at multiple and earlier time points after drug administration. The highest dose of 10 mg/kg AmBisome® exerted maximum kill within 48 hours of drug administration. This window of time is consistent with drug distribution from blood to tissues within 24 hours of drug administration, also observed in uninfected mice and other disease models (21, 22), and a fast rate of parasite kill. Differences in amphotericin B levels between L. donovani infected and uninfected tissues were also observed at earlier time points. Importantly, these were indicative of organ-specific drug distribution and elimination in EVL. Most notable was a lack of drug accumulation over time in L. donovani infected spleens. It is currently not known to which extent the liposomal formulation contributes to the observed differences. Comparative biodistribution studies between single dose AmBisome® and Fungizone®, another clinically used non-liposomal amphotericin B formulation, are hampered by the toxicity of Fungizone®. The maximum tolerated dose of 1mg/kg (i.v. bolus administration) of this formulation precludes dosing at clinically meaningful dose levels (23). Changes in drug distribution under pathological conditions have been reported for a number of anti-microbials (9, 11, 12, 22, 24-27) and increased drug concentrations at inflammatory sites believed to result from capillary endothelial damage and recruitment of drug-containing phagocytic cells to sites of infection. However, tissue penetration of drugs is governed by a number of factors, which include drug formulation, plasma protein binding and underlying disease (12). In EVL different pathophysiological features are observed in the liver and spleen. In the liver a Th1 dominated granulomatous response is characterised by an influx of T cells, B cells, NK cells and monocytes, with a peak in the inflammatory response around 4 weeks after infection (18-20). Kupffer cells (KCs), which in uninfected mice line the sinusoids and form a uniformly distributed phagocytic network, are recruited into the core of the granuloma in infected mice. Isolated KCs remaining in the sinusoidal network of infected mice have a
reduced cell volume (28), and loss of membrane activity of KCs has been reported within 2 hours of infection with *L. donovani* (29). Infection of the spleen is characterised by a breakdown of marginal zone architecture, loss of marginal zone macrophages (MZMs) and repositioning of marginal metallophilic macrophages (MMMs) (30), an increase in the number of red pulp macrophages (31), destruction of the follicular dendritic cell and the gp38+ fibroblastic reticular cell networks (15), and substantial changes to the vascular network including sprouting of α-SMA+ vessels with active endothelial cell proliferation (32).

Following intravenous administration, liposomes interact with blood proteins and an inverse relationship between the amount of protein bound and liposome clearance rate from blood has been demonstrated (33). Here we show that chronically infected BALB/c mice display an increase in serum gamma globulin and, to a lesser extent, alpha globulin, and a decrease in albumin. Some of these proteins have been implicated in lipid-protein interactions (34, 35), which may account for different rates in the distribution of AmBisome® from blood to tissues between infected and uninfected mice. From the bloodstream liposomes distribute to highly perfused tissues such as the liver and spleen, which regulate drug elimination (34), and cells of the mononuclear phagocyte system (MPS) play a major role in this distribution (36). In the liver also hepatocytes can participate in the uptake and metabolism of small and phosphatidylcholine-based liposomes (37, 38), which is linked to their ability to exit fenestrated vessels in this organ (34). In uninfected mice the diameter of liver sinusoidal endothelial cell (LSEC)-fenestrae is on average 99 +/- 18 nm (39), which would allow AmBisome® with a mean diameter of <100 nm (7) to pass through. However, fenestrae are dynamic structures, which may undergo changes in response to local external stimuli (39) and the impact of *L. donovani* infection on fenestration is as yet unknown. In the spleen of uninfected mice most of the blood flows through the marginal zone (40) and MZMs display preferential uptake of clodronate liposomes over other phagocytic cells in the spleen (41). It is
currently unknown if there are also differences in the extent of drug uptake between the
different cell types for AmBisome®. Future studies, utilising advanced techniques to
simultaneously image drug and cells, are needed to shed light on the spatial distribution of drug
in healthy and diseased organs over time (42).

Gershkovich et al. (9) hypothesised that reduced phagocytic activity of macrophages in EVL
or increased drug elimination, either through leaking capillaries in inflamed tissue or binding
of drug to fragments of killed parasites, may explain the differences in drug levels between
infected and uninfected tissues. Whilst the latter hypothesis cannot be ruled out it is unlikely
to fully account for the lack of drug accumulation in the spleen. Our data favours a model in
which i) increases in organ mass lead to decreased total drug amounts / organ, ii) increased
elimination and / or metabolism is the predominant feature in the liver and iii) drug distribution
to the spleen is progressively decreased during EVL.

A retrospective analysis of risk factors for VL relapse following treatment of patients with 20
mg/kg AmBisome®, administered as 4 doses of 5 mg/kg, in India showed that a slower
decrease in splenomegaly during treatment, but not spleen size at admission, was significantly
associated with relapse (43). In *L. donovani* infected BALB/ mice the granulomatous response
in the liver appears to follow many of the characteristics observed in subclinical human
infection, whereas the spleen shows hallmarks of progressive human disease (44). However, it
is currently unknown if the differences we observed in AmBisome® distribution and
elimination in this experimental model also exist at different stages in human VL. Nonetheless,
understanding the mechanisms of how the pathophysiology of EVL affects drug absorption,
distribution, metabolism and elimination (ADME) will improve the development and use of
anti-leishmanial drugs and drug delivery systems.
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Table 1.

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<td></td>
<td>0.6 mg/kg</td>
<td>21 +/- 8</td>
<td>41 +/- 4</td>
<td>p ≤ 0.01</td>
<td>44 +/- 6</td>
<td>4 +/- 4</td>
<td>p ≤ 0.0001</td>
</tr>
<tr>
<td>2</td>
<td>2.5 mg/kg</td>
<td>81 +/- 2</td>
<td>80 +/- 3</td>
<td>n.s.</td>
<td>71 +/- 2</td>
<td>36 +/- 11</td>
<td>p ≤ 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>10 mg/kg</td>
<td>100 +/- 0</td>
<td>99 +/- 1</td>
<td>n.s.</td>
<td>99 +/- 0</td>
<td>93 +/- 2</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>2.5 mg/kg</td>
<td>94 +/- 1</td>
<td>80 +/- 5</td>
<td>p ≤ 0.05</td>
<td>40 +/- 6</td>
<td>45 +/- 7</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>0.6 mg/kg</td>
<td>54 +/- 3</td>
<td>22 +/- 7</td>
<td>p ≤ 0.0001</td>
<td>38 +/- 10</td>
<td>16 +/- 9</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

Parasite burden (LDU, mean +/- SEM) in untreated control groups

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>N/A</td>
<td>966 +/- 66</td>
<td>518 +/- 48</td>
<td>p ≤ 0.01</td>
<td>88 +/- 8</td>
<td>118 +/- 15</td>
<td>n.s.</td>
</tr>
<tr>
<td>2</td>
<td>N/A</td>
<td>415 +/- 32</td>
<td>176 +/- 11</td>
<td>p ≤ 0.001</td>
<td>18 +/- 1</td>
<td>53 +/- 8</td>
<td>p ≤ 0.01</td>
</tr>
<tr>
<td>3</td>
<td>N/A</td>
<td>682 +/- 54</td>
<td>194 +/- 44</td>
<td>p ≤ 0.001</td>
<td>18 +/- 2</td>
<td>84 +/- 9</td>
<td>p ≤ 0.001</td>
</tr>
</tbody>
</table>

Efficacy of single dose AmBisome® in *L. donovani* infected BALB/c mice.

Parasite burden was evaluated 7 days after drug administration on day 21 or 42 post infection (p.i.) and is given as mean +/- standard error of the mean (SEM). Expt. refers to the number of separate experiments, n.s. not significant, LDU Leishman-Donovan Units.
Table 2.

<table>
<thead>
<tr>
<th>PK parameter</th>
<th>Unit</th>
<th>10 mg/kg</th>
<th>2.5 mg/kg</th>
<th>0.6 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>t₁/₂</td>
<td>Hr</td>
<td>3.4</td>
<td>5.0</td>
<td>NQ</td>
</tr>
<tr>
<td>Tmax</td>
<td>Hr</td>
<td>0.08</td>
<td>0.08</td>
<td>0.08</td>
</tr>
<tr>
<td>Cmax</td>
<td>ug/mL</td>
<td>240.3</td>
<td>29.8</td>
<td>3.3</td>
</tr>
<tr>
<td>AUClast</td>
<td>hr*ug/mL</td>
<td>368.5</td>
<td>50.5</td>
<td>10.8</td>
</tr>
<tr>
<td>CL</td>
<td>mL/hr/kg</td>
<td>27.0</td>
<td>48.1</td>
<td>41.9</td>
</tr>
<tr>
<td>Vd</td>
<td>mL/kg</td>
<td>104</td>
<td>252</td>
<td>681</td>
</tr>
</tbody>
</table>

Pharmacokinetic profile of single dose AmBisome® in plasma of *L. donovani* infected BALB/c mice.

Mice had been infected for 14 days at the time of dosing. Parasite burden in untreated controls (mean +/- standard deviation) was 644 +/- 25 LDU in the liver and 17 +/- 1 LDU in the spleen (n=3). Data is representative of two separate experiments (n = 1 mouse / timepoint). NQ not quoted.
### Table 3.

#### A)

<table>
<thead>
<tr>
<th>Amphotericin B concentration [ng/g tissue], mean ± SD</th>
<th>Ratio</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>AmBisome 10 mg/kg</td>
<td>115 733 +/- 19 582</td>
<td>44 232 +/- 6 900</td>
</tr>
<tr>
<td>AmBisome 2.5 mg/kg</td>
<td>12 220 +/- 1 308</td>
<td>5 492 +/- 862</td>
</tr>
<tr>
<td>AmBisome 0.6 mg/kg</td>
<td>1 430 +/- 923</td>
<td>257 +/- 58</td>
</tr>
</tbody>
</table>

#### B)

<table>
<thead>
<tr>
<th>Amphotericin B concentration [ng/mL plasma], mean ± SD</th>
<th>Ratio</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment group</td>
<td>Day 21</td>
<td>Day 42</td>
</tr>
<tr>
<td>AmBisome 10 mg/kg</td>
<td>191 +/- 23</td>
<td>216 +/- 47</td>
</tr>
<tr>
<td>AmBisome 2.5 mg/kg</td>
<td>116 +/- 18</td>
<td>129 +/- 28</td>
</tr>
<tr>
<td>AmBisome 0.6 mg/kg</td>
<td>5 +/- 5</td>
<td>16 +/- 9</td>
</tr>
</tbody>
</table>
Tissue and plasma concentrations of amphotericin B in *L. donovani* infected BALB/c mice 7 days after administration of single dose AmBisome®.

Amphotericin B concentrations were determined 7 days after drug administration on day 21 or day 42 post infection (p.i.) in livers and spleens (A) and plasma (B). Data is presented as group mean (n = 5 mice / group) +/- standard deviation (SD). Ratios were calculated as follows: mean drug concentration Day 21 p.i. / mean drug concentration Day 42 p.i.. Total amphotericin B concentrations / organ were calculated for individual mice as follows: organ weight in g (as determined at sacrifice) * amphotericin B concentration in ng/g tissue (as measured after processing of whole organs). N.s. not significant. Drug concentrations presented here were determined in the same samples as drug potency was evaluated in experiment 3 in Table 1.
Table 4.

<table>
<thead>
<tr>
<th></th>
<th>Infected Day 14</th>
<th>Uninfected Day 14</th>
<th>Infected Day 43</th>
<th>Uninfected Day 43</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total protein [g/L]</td>
<td>46.8 +/- 0.6a</td>
<td>46.2 +/- 0.6</td>
<td>56.6 +/- 1.0a,b</td>
<td>47.6 +/- 0.8b</td>
</tr>
<tr>
<td>Albumin [g/L]</td>
<td>28.7 +/- 0.4</td>
<td>29.5 +/- 0.6</td>
<td>27.8 +/- 0.4b</td>
<td>29.9 +/- 0.5b</td>
</tr>
<tr>
<td>Albumin %</td>
<td>61.4 +/- 0.7a</td>
<td>64.0 +/- 1.1</td>
<td>49.3 +/- 1.1a,b</td>
<td>62.8 +/- 1.6b</td>
</tr>
<tr>
<td>Alpha globulin [g/L]</td>
<td>6.7 +/- 0.2a</td>
<td>6.8 +/- 0.2</td>
<td>8.5 +/- 0.2a,b</td>
<td>7.3 +/- 0.2b</td>
</tr>
<tr>
<td>Alpha globulin %</td>
<td>14.4 +/- 0.3</td>
<td>14.7 +/- 0.4</td>
<td>15.1 +/- 0.3</td>
<td>15.3 +/- 0.3</td>
</tr>
<tr>
<td>Beta globulin [g/L]</td>
<td>3.7 +/- 0.3</td>
<td>4.2 +/- 0.4</td>
<td>4.1 +/- 0.1</td>
<td>4.0 +/- 0.8</td>
</tr>
<tr>
<td>Beta globulin %</td>
<td>7.9 +/- 0.5</td>
<td>9.0 +/- 0.7</td>
<td>7.2 +/- 0.2</td>
<td>8.2 +/- 1.6</td>
</tr>
<tr>
<td>Gamma globulin [g/L]</td>
<td>7.6 +/- 0.2a</td>
<td>5.7 +/- 0.3</td>
<td>16.1 +/- 1.0a,b</td>
<td>6.5 +/- 0.2b</td>
</tr>
<tr>
<td>Gamma globulin %</td>
<td>16.3 +/- 0.5a,c</td>
<td>12.3 +/- 0.6c</td>
<td>28.4 +/- 1.3a,b</td>
<td>13.6 +/- 0.2b</td>
</tr>
<tr>
<td>Albumin:globulin ratio</td>
<td>1.6 +/- 0.0a</td>
<td>1.8 +/- 0.1</td>
<td>1.0 +/- 0.0a,b</td>
<td>1.7 +/- 0.1b</td>
</tr>
</tbody>
</table>

Serum protein profile in *L. donovani* infected and uninfected BALB/c mice.

BALB/c mice (n = 6-7/group) were infected with *L. donovani* for 14 days (Infected Day 14) or 43 days (Infected Day 43) or left uninfected, but maintained under identical conditions as infected ones (Uninfected Day 14 and Uninfected Day 43). Data is presented as mean +/- standard error of the mean (SEM). Parasite burden (mean +/- standard deviation) in infected livers was 396 +/- 61 and 273 +/- 52 LDU on day 14 and day 43 post infection, and in infected spleens 8 +/- 2 and 93 +/- 18 respectively. Values indicated with the same letter (a, b or c) are significantly different (p ≤0.05).
Time-to-kill of single dose AmBisome® in *L. donovani* infected BALB/c mice.

Percentage inhibition of parasite burden in liver (A) and spleen (B) 1, 2, 3 and 7 days after a single dose of AmBisome® at dose levels of 10 mg/kg (black bars), 2.5 mg/kg (grey bars) and 0.6 mg/kg (striped bars). C: Percentage inhibition of parasite burden in liver (black bars) and spleen (grey bars) 1, 2 and 3 days after a single dose of 2.5 mg/kg AmBisome®. Treatment was given 14 days (A and B) or 33 days (C) after infection. Data are presented as group mean (n=5), error bars represent standard error of the mean (SEM). Data in A and B is representative of two separate experiments.
Fig. 2

A)  

B)  

C)  

D)  

E)
Comparative plasma and tissue concentrations in *L. donovani* infected and uninfected BALB/c mice 7 days after administration of single dose AmBisome®.

Amphotericin B concentrations were measured on day 21 or day 42 post infection (Infected – day 21, Infected – day 42) and in uninfected BALB/c mice, maintained under identical conditions (Uninfected - day 21, Uninfected – day 42). Amphotericin B concentrations are presented as ng/g tissue (A, C) or ng / organ (B, D) in livers (A, B) and spleens (C, D) and as ng/mL in plasma (E). Each symbol represents data from an individual mouse. Horizontal lines indicate the mean (n = 4-5 mice / group) and error bars standard deviations (SD). Total amphotericin B concentrations / organ were calculated for individual mice as follows: organ weight in g (as determined at sacrifice) * amphotericin B concentration in ng/g tissue (as measured after processing of whole organs). Tissue concentrations in infected mice were measured in samples from experiment 3 in Table 1. Data is representative of two separate experiments.
Fig. 3

A)

B)

C)

D)

E)

F)
Comparative plasma and tissue concentrations in *L. donovani* infected and uninfected BALB/c mice up to 48 hours after administration of single dose AmBisome®.

AmBisome® was administered to BALB/c mice, naive (A, C, E, G, I) or infected with *L. donovani* for 35 days (B, D, F, H, J) and amphotericin B concentrations determined in plasma (A, B), liver (C, D, G, H) and spleen (E, F, I, J) at 5 minutes, 4 hours, 24 hours or 48 hours after drug administration. Amphotericin B concentrations are presented as ng/g tissue (C – F) or total amphotericin B concentration / organ (G – J). Total amphotericin B concentrations / organ were calculated as follows: organ weight in g (as determined at sacrifice) * amphotericin B concentration in ng/g tissue (as measured after processing of whole organs). Each symbol represents data from an individual mouse. Horizontal lines indicate the mean (n = 3 / group) and error bars standard deviation (SD).