1 **Treatment of experimental visceral leishmaniasis with single-dose liposomal**
2 **amphotericin B – pharmacodynamics and biodistribution at different stages of disease**
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21
22 **Running title:** AmBisome® pharmacodynamics and biodistribution in EVL
23

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

75 liver and spleen were lower in infected mice after intravenous administration of AmBisome®
76 (9). A separate study showed that AmBisome®, when administered as single dose to *L.*
77 *donovani* infected BALB/c mice, was less effective in the chronic stage of infection compared
78 to the acute stage (10). In anti-infective therapy with drugs that directly kill pathogens, such as
79 amphotericin B, drug efficacy is driven by infection-site specific drug concentrations (11, 12).
80 We therefore hypothesised that the decrease in potency of AmBisome® observed in chronic
81 experimental VL (EVL) is linked to a decrease in organ-specific drug concentrations associated
82 with progression of infection. Here we show that i) decreased potency of AmBisome® during
83 progressive EVL is most pronounced in the spleen, ii) total drug amounts in liver and spleen
84 progressively decrease during EVL, iii) organ-specific drug distribution and elimination
85 patterns occur in EVL and iv) changes in drug levels in tissues are not reflected in the blood
86 compartment.
87

88 MATERIALS AND METHODS

89 **Drugs and reagents.** AmBisome® was purchased from Gilead (Cambridge, UK). The powder
90 was reconstituted in sterile water following the manufacturer's directions and further dilutions
91 prepared in 5% glucose. Amphotericin B (VETRANAL™ analytical standard), tolbutamide,
92 dimethyl sulfoxide (DMSO) and sodium dodecyl sulphate (SDS) were obtained from Sigma,
93 UK, and heparin from John Bell & Croyden, UK. Methanol (HPLC grade), 0.1% (v/v) formic
94 acid in water (LC-MS grade) and water (LC-MS grade) were purchased from Fisher Scientific
95 UK Ltd, UK.
96

97 ***In vivo* experiments and treatment.** Female BALB/c (Charles River, UK) and Rag-1 (B6)
98 K.O. mice (LSHTM breeding colony) were maintained under specific pathogen free conditions
99 in individually ventilated cages and exposed to 12 hour light – 12 hour dark cycles. Standard

100 rodent diet (RM No 1 Expanded) and filtered tap water were supplied *ad libitum*. Mice (6-10
101 weeks of age at the start of experiments) were infected by intravenous (i.v.) injection of 2 x 10^7
102 parasites (*L. donovani* MHOM/ET/67/HU3) as described (13). Parasites were maintained in
103 Rag-1 (B6) K.O. mice and amastigotes harvested from spleens >40 days after infection.
104 BALB/c mice were treated either 14 or 35 days after infection with a single i.v. dose of
105 AmBisome® in a 0.2 mL bolus injection. On the day of treatment, prior to the administration
106 of drugs, mice were weighed and randomised into the different treatment groups using a
107 random number generator. The average weight of mice in each experiment was used for dose
108 calculations. Untreated groups of mice were included as controls where appropriate. Age
109 matched uninfected mice were maintained under identical conditions for the same length of
110 time as their infected comparators. At experimental endpoints mice were weighed and
111 humanely killed by exsanguination under terminal anaesthesia. Blood was collected by cardiac
112 puncture in Eppendorf tubes containing heparin and plasma harvested by centrifugation. Livers
113 and spleens were removed and their weight recorded. Plasma and tissue samples were stored
114 at -80°C until further processing. For determination of parasite burdens tissue impression
115 smears were prepared, fixed in 100% methanol and stained in 10% Giemsa. Parasite burden
116 was determined microscopically and Leishman-Donovan Units (LDUs) calculated by the
117 formula number of parasites per host cell nucleus x organ weight in mg as described previously
118 (14). Microscopical evaluation of impression smears was carried out by a scientist unaware of
119 the treatment allocation.
120
121 **Processing of samples for drug quantification.** Samples were thawed at room temperature
122 immediately prior to processing. Livers and spleens were homogenised with an equal volume
123 of ZrO beads in 4 volumes of 0.1% aqueous formic acid in a Bullet Blender (Next Advance,
124 UK). For control matrix samples + internal standard (IS), calibration standards, quality control

125 (QC) samples and study samples 50 µl of tissue homogenate or plasma were diluted with 250µl
126 IS solution (200 ng/mL tolbutamide in a 84:16 (v/v) mixture of methanol : DMSO). For blank
127 samples (matrix sample without IS) 50 µl of tissue homogenate or plasma were diluted with a
128 84:16 (v/v) mixture of methanol: DMSO. After shaking for 10 minutes at 200 rpm at room
129 temperature dilutions were centrifuged at 4150 x g for 15 minutes at 4ºC. Supernatants were
130 transferred to 96 well plates and stored at -80°C.
131
132 **Preparation of calibration standards and QC samples.** An amphotericin B stock solution
133 (1.0 mg/mL) was prepared in DMSO and from that standard spiking solutions were prepared
134 by serial dilution in 1% SDS in water. Calibration standards at a minimum of 6 concentrations
135 were prepared by mixing 5 µL of the spiking solutions with 45 µL of blank tissue homogenate
136 or plasma, matrix matching that of the study samples to be analysed. QC samples at selected
137 concentrations were prepared in replicate in similar fashion and all samples processed as
138 described above.
139
140 **Preparation of study samples.** Concentrations of amphotericin B in study samples varied
141 widely, depending upon dosing and sampling regimens. Those samples expected to contain
142 high concentrations of amphotericin B were diluted with matching control matrix by a suitable
143 factor to reduce their concentration into a quantifiable range (i.e. within the range of calibration
144 standard concentrations).
145
146 **LC/MS analytical conditions.** All samples were analysed using an Agilent 1200 HPLC
147 combined with an Agilent 6410A triple quadrupole mass spectrometer (both Agilent, UK). A
148 mobile phase of water / 0.1% formic acid (channel A) and methanol / 0.1% formic acid (channel
149 B) was used to elute sample components from a Kinetex column packed with 5 µm XB-C_{18}

150 material (2.1mm x 50mm @50°C; Phenomenex, UK). The mobile phase composition was
151 initially 20% B, programmed to increase linearly to 90% B at 1.60 min. after injection; after
152 0.4 min. at 90% B the composition was returned to its initial 20% B at 2.10 min. post-injection.
153 Amphotericin B was detected monitoring the transition m/z 906.5 → m/z 743.2, the dehydrated
154 protonated molecule at m/z 906.5 being the most intense ion produced for this component in
155 the ion source.
156 Analyte concentrations were quantified against calibration standards prepared in matched
157 control matrix, with aliquots of sample and standard being injected typically in the range 1-5
158 μL, depending on expected study sample concentration.
159
160 **Pharmacokinetic analysis.** Noncompartmental analysis (NCA) was performed with Phoenix
161 Win Nonlin v6.3 (Certara, UK).
162
163 **Characterisation of serum protein profiles.** Blood was collected from *L. donovani* infected
164 or uninfected BALB/c mice by cardiac puncture under terminal anaesthesia and stored
165 overnight at 4ºC. Serum was harvested by centrifugation at 1500 x *g*, 4ºC for 15 minutes and
166 stored at -80ºC prior to analysis. Capillary electrophoresis and protein determinations were
167 carried out by LABOKLIN GmbH&Co.KG (Bad Kissingen, Germany).
168
169 **Statistical analysis.** Statistical significance between two groups was evaluated by a t-test and
170 between more than two groups by one-way ANOVA assuming Gaussian distribution, followed
171 by Sidak's multiple comparisons test for selected groups as applicable (GraphPad Prism 6). A
172 p value of ≤ 0.05 was considered statistically significant.
173

174 **Ethical statement.** Experiments involving animals were carried out under license in
175 accordance with the Animals (Scientific Procedures) Act 1986 (UK Home Office Project
176 Licences PPL70/6997 and PPL70/8207) following approval by the Animal Welfare and Ethics
177 Review Board at LSHTM.
178
179 **RESULTS**
180
181 **Efficacy of single dose liposomal amphotericin B in *L. donovani* infected BALB/c mice at**
182 **two different timepoints after infection.** Single dose AmBisome® was administered at doses
183 of 10 mg/kg, 2.5 mg/kg and 0.6 mg/kg 14 or 35 days after infection. Parasite burden was
184 evaluated 7 days after dosing. A reproducible decrease in drug efficacy between day 21 and
185 day 42 post infection was noted in the spleen, which was more pronounced at the lower doses.
186 In the liver decreased drug efficacy at the lower two doses was noted in one experiment,
187 whereas in another experiment an increase in drug efficacy was observed at the lowest dose
188 (Table 1).
189
190 **Time-to-kill studies.** Single dose AmBisome® was administered to *L. donovani* infected
191 BALB/c mice 14 days after infection at doses of 10 mg/kg, 2.5 mg/kg and 0.6 mg/kg. Parasite
192 burden was evaluated 1, 2, 3 or 7 days after dosing. In the liver effective parasite kill (%
193 inhibition >90%) was observed 2 days after administration of the highest dose. At the dose of
194 2.5 mg/kg the hepatic parasite burden was inhibited by >60% at 2 days, >70% at 3 days and
195 >80% (maximum kill) at 7 days after drug administration. The dose of 0.6 mg/kg was
196 ineffective (% inhibition <40%) and no change in parasite kill was observed over time (Fig.
197 1A). A similar pattern of effective kill was observed in the spleen after administration of 10
198 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

224 concentrations were observed in livers and spleens when equal drug doses were administered
225 on day 35 compared to day 14post infection. When expressed as ng/g tissue drug concentrations
226 in the liver were 2.6-, 2.2- and 5.6-fold lower on day 42 compared to day 21 post infection at
227 AmBisome® doses of 10 mg/kg, 2.5 mg/kg and 0.6 mg/kg. Respective tissue concentrations
228 in the spleen were 4.5-fold, 2.8-fold and 9.6-fold lower. Statistical significance was noted for
229 the 10 mg/kg dose group ($p \leq 0.0001$ for liver, $p \leq 0.01$ for spleen). Organ weights significantly
230 increased from day 21 to day 42 post infection ($p \leq 0.0001$ in all dose groups) with a 1.5-fold
231 increase in the liver and 2.3- to 2.9-fold increases in the spleen. When taking this increase in
232 organ weights into account and estimating total drug amount / organ the difference between
233 amphotericin B concentrations on day 21 and day 42 post infection decreased. Differences in
234 total amphotericin B amount / organ at AmBisome® doses of 10 mg/kg, 2.5 mg/kg and 0.6
235 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.
236 Statistical significance was noted for the 10 mg/kg dose group in the liver ($p \leq 0.0001$). Overall,
237 higher amphotericin B concentrations were measured in the liver compared to the spleen. Data
238 is summarised in Table 3A. Amphotericin B concentrations in plasma remained unchanged
239 when drug was administered on day 14 compared to day 35 post infection. A 0.3-fold difference
240 was noted in the 0.6 mg/kg dose group, but was not statistically significant (Table 3B).
241
242 **Comparative amphotericin B plasma and tissue concentrations in *L. donovani* infected**
243 **and uninfected BALB/c mice 7 days after administration of single dose AmBisome®.**
244 Amphotericin B concentrations were measured in *L. donovani* infected (14 or 35 days after
245 infection) and age and husbandry matched uninfected BALB/c mice after administration of 2.5
246 mg/kg AmBisome®. At both time points amphotericin B concentrations were significantly
247 lower (p value ≤ 0.0001) in livers and spleens from infected BALB/c mice compared to
248 uninfected ones. In the liver mean drug concentrations (ng amphotericin B / g tissue) on day

249 21 and day 42 post infection were 3.5- and 6.5-fold lower in infected compared to uninfected
250 BALB/c mice. This translated into 2.5- and 5.4-fold differences when taking organ weights
251 into account and estimating the total amphotericin B amount / organ (Fig. 2A and B). In the
252 spleen mean amphotericin B concentrations were 29.3- and 100.9-fold lower in infected
253 compared to uninfected mice on day 21 and day 42 post infection. Respective organ weight
254 adjusted differences were 10.3- and 16.5-fold (Fig. 2C and D). The opposite was observed in
255 plasma and significantly higher amphotericin B concentrations measured in infected BALB/c
256 mice compared to uninfected ones, with 2- and 1.9-fold differences on day 21 (p value $\leq$ 0.01)
257 and day 42 (p value $\leq$ 0.001) post infection (Fig. 2E). Tabulated results are provided in
258 Supplementary Table S1.
259 To investigate if differences in amphotericin B levels were also observed after administration
260 of higher drug doses *L. donovani* infected (day 33 post infection) or uninfected BALB/c mice
261 were treated with a single dose of 40 mg/kg AmBisome®. In line with above results
262 amphotericin B concentrations were significantly lower (p value < 0.0001) in livers and spleens
263 from infected BALB/c mice compared to uninfected ones. However, plasma concentrations
264 were similar between the two different groups (p value > 0.05). Data is shown in
265 Supplementary Table S1.
266
267 **Comparative amphotericin B plasma and tissue concentrations in *L. donovani* infected**
268 **and uninfected BALB/c mice up to 48 hours after administration of single dose**
269 **AmBisome®.** A single dose of 2.5 mg/kg AmBisome® was administered to *L. donovani*
270 infected (day 33 post infection) and age and husbandry matched uninfected BALB/c mice.
271 Plasma and tissue samples were collected 5 minutes, 4 hours, 24 hours and 48 hours after drug
272 administration and amphotericin B concentrations determined. Amphotericin B plasma levels
273 decreased within 24 hours in uninfected and *L. donovani* infected BALB/c mice and remained

274 at similar levels at 24 and 48 hours after drug administration (Fig. 3A and B). An increase in
275 amphotericin B concentration was observed in livers and spleens of uninfected BALB/c mice
276 from 5 minutes to 4 hours after drug administration. Amphotericin B concentrations at 24 and
277 48 hours after drug administration were comparable to those measured at 4 hours (Fig. 3C and
278 E). A different kinetic was observed in *L. donovani* infected BALB/c mice. In the liver a small
279 increase in amphotericin B concentration was noted from 5 minutes to 4 hours after drug
280 administration, followed by a decrease at 24 hours and, most notably, 48 hours after drug
281 administration (Fig. 3D). In the spleen amphotericin B concentrations decreased over the whole
282 observation period with no increase noted (Fig. 3F). Similar kinetics were observed when
283 estimating total drug amount per organ (Fig. 3 G – J). Tabulated results are provided in
284 Supplementary Table S2.
285
286 **Host responses to infection.** Serum samples from *L. donovani* infected (day 14 and 43 post
287 infection) and age and husbandry matched uninfected BALB/c mice were subjected to
288 electrophoresis and protein determination. Significant differences ($p \leq 0.05$) in serum protein
289 profiles were identified. In infected BALB/c mice serum concentrations of total protein (46.8
290 +/- 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
291 (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
292 43 post infection. At the same time % albumin levels decreased (61.4 +/- 0.7 *vs.* 49.3 +/- 1.1),
293 but no significant difference was found when comparing total albumin. The albumin:globulin
294 ratio decreased from day 14 to day 43 post infection (1.6 +/- 0.0 *vs.* 1.0 +/- 0.0). Compared to
295 uninfected BALB/c mice higher levels of total protein (56.6 +/- 1.0 *vs.* 47.6 +/- 0.8 g/L), alpha
296 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;
297 28.4 +/- 1.3 vs. 13.6 +/- 0.2 %) were measured in *L. donovani* infected mice on day 43 post
298 infection. At the same timepoint infected BALB/c mice had lower levels of albumin (27.8 +/-

299 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
300 infection increased levels of % gamma globulin were noted in infected compared to uninfected
301 BALB/c mice (16.3 +/- 0.5 *vs.* 12.3 +/- 0.6). On day 43 post infection infected BALB/c mice
302 had a lower albumin:globulin ratio than uninfected ones (1.0 +/- 0.0 *vs.* 1.7 +/- 0.1). Data is
303 summarised in Table 4.
304
305 **DISCUSSION**

306 The pharmacodynamics (PD) and pharmacokinetics (PK) of current anti-leishmanial drugs
307 have yet to be fully established in preclinical disease models. Here we investigated the potency
308 and biodistribution of AmBisome® in EVL after administration of three different single doses
309 and at two different timepoints. AmBisome® was well tolerated at all doses administered and
310 no signs of drug toxicity noted, based on observations of weight, fur condition, behaviour and
311 facial expression. We also determined the PK profile of AmBisome® in plasma of *L. donovani*
312 infected BALB/c mice in an exploratory study and compared trends to published data in
313 uninfected mice. The observed increase in C_{max} and AUC_{last} and the decrease in Vd and CL
314 with increasing doses is in line with this data (7).
315 After infection of BALB/c mice with *L. donovani* the parasite burden in the liver rapidly
316 increases for the first 2-4 weeks, followed by a decrease in parasite numbers and resolution of
317 infection. In the spleen the parasite burden increases from 2 weeks after infection and parasite
318 persistence is observed (15-17). Considering this organ-specific pattern of parasite growth is
319 important when analysing drug efficacy and potency data. In the spleen drug efficacy was
320 evaluated against an increasing parasite burden at both time points and a decrease in potency
321 observed when drug was administered on day 35 compared to day 14 post infection. However,
322 the magnitude of decrease and the dose at which this occurred differed between experiments.
323 It is notable that the decrease in drug concentrations / total drug amounts in the spleen following

324 administration of the same dose of AmBisome® on day 14 *vs.* day 35 post infection was less
325 pronounced in the third compared to the second experiment presented in Table 1
326 (Supplementary Table S3). This may explain the lack of decreased drug potency observed in
327 experiment 3. In the liver opposing trends were observed at the lowest dose. However, data
328 obtained in the liver on day 42 post infection is not indicative of direct drug kill only, it reflects
329 a mixture of parasite killing by drug and a mature granulomatous host response resolving the
330 infection (18-20). Hence, these opposing trends at a dose which lacked meaningful anti-
331 leishmanial drug efficacy, likely reflect differences in the efficiency of host response rather
332 than in drug potency.
333 In parallel to evaluating drug potencies we determined amphotericin B concentrations in
334 plasma, livers and spleens of infected BALB/c mice at the two different time points and
335 estimated the total drug amount per organ. In livers and spleens lower drug concentrations and
336 total drug amounts per organ were noted when drug was administered on day 35 compared to
337 day 14 post infection and the difference was highest at the lowest dose. The magnitude of
338 difference was lower for absolute (organ weight-adjusted) values than for relative values,
339 which suggests that differences in drug levels between the two time points can partly be
340 explained by the infection-associated increase in organ weights. A combined effect of organ
341 enlargement and other pathology-associated factors on drug distribution following
342 administration of AmBisome® to *L. donovani* infected BALB/c mice is also supported by
343 comparative studies in infected and uninfected mice. Again the magnitude of decrease in
344 amphotericin B levels in livers and spleens of infected mice was lower when organ weight-
345 adjustment was applied, but remained significant and increased from day 21 to day 42 post
346 infection in both organs. Interestingly, plasma levels of amphotericin B were higher in infected
347 compared to uninfected mice at this point.

348 To gain further insight into the kinetics of parasite kill and drug distribution we next examined
349 drug potency and distribution at multiple and earlier time points after drug administration. The
350 highest dose of 10 mg/kg AmBisome® exerted maximum kill within 48 hours of drug
351 administration. This window of time is consistent with drug distribution from blood to tissues
352 within 24 hours of drug administration, also observed in uninfected mice and other disease
353 models (21, 22), and a fast rate of parasite kill. Differences in amphotericin B levels between
354 *L. donovani* infected and uninfected tissues were also observed at earlier time points.
355 Importantly, these were indicative of organ-specific drug distribution and elimination in EVL.
356 Most notable was a lack of drug accumulation over time in *L. donovani* infected spleens. It is
357 currently not known to which extent the liposomal formulation contributes to the observed
358 differences. Comparative biodistribution studies between single dose AmBisome® and
359 Fungizone®, another clinically used non-liposomal amphotericin B formulation, are hampered
360 by the toxicity of Fungizone®. The maximum tolerated dose of 1mg/kg (i.v. bolus
361 administration) of this formulation precludes dosing at clinically meaningful dose levels (23).
362 Changes in drug distribution under pathological conditions have been reported for a number of
363 anti-microbials (9, 11, 12, 22, 24-27) and increased drug concentrations at inflammatory sites
364 believed to result from capillary endothelial damage and recruitment of drug-containing
365 phagocytic cells to sites of infection. However, tissue penetration of drugs is governed by a
366 number of factors, which include drug formulation, plasma protein binding and underlying
367 disease (12). In EVL different pathophysiological features are observed in the liver and spleen.
368 In the liver a Th1 dominated granulomatous response is characterised by an influx of T cells,
369 B cells, NK cells and monocytes, with a peak in the inflammatory response around 4 weeks
370 after infection (18-20). Kupffer cells (KCs), which in uninfected mice line the sinusoids and
371 form a uniformly distributed phagocytic network, are recruited into the core of the granuloma
372 in infected mice. Isolated KCs remaining in the sinusoidal network of infected mice have a

373 reduced cell volume (28), and loss of membrane activity of KCs has been reported within 2
374 hours of infection with *L. donovani* (29). Infection of the spleen is characterised by a
375 breakdown of marginal zone architecture, loss of marginal zone macrophages (MZMs) and
376 repositioning of marginal metallophilic macrophages (MMMs) (30), an increase in the number
377 of red pulp macrophages (31), destruction of the follicular dendritic cell and the $gp38^+$
378 fibroblastic reticular cell networks (15), and substantial changes to the vascular network
379 including sprouting of α-SMA+ vessels with active endothelial cell proliferation (32).
380 Following intravenous administration, liposomes interact with blood proteins and an inverse
381 relationship between the amount of protein bound and liposome clearance rate from blood has
382 been demonstrated (33). Here we show that chronically infected BALB/c mice display an
383 increase in serum gamma globulin and, to a lesser extent, alpha globulin, and a decrease in
384 albumin. Some of these proteins have been implicated in lipid-protein interactions (34, 35),
385 which may account for different rates in the distribution of AmBisome® from blood to tissues
386 between infected and uninfected mice. From the bloodstream liposomes distribute to highly
387 perfused tissues such as the liver and spleen, which regulate drug elimination (34), and cells of
388 the mononuclear phagocyte system (MPS) play a major role in this distribution (36). In the
389 liver also hepatocytes can participate in the uptake and metabolism of small and
390 phosphatidylcholine-based liposomes (37, 38), which is linked to their ability to exit
391 fenestrated vessels in this organ (34). In uninfected mice the diameter of liver sinusoidal
392 endothelial cell (LSEC)-fenestrae is on average 99 +/- 18 nm (39), which would allow
393 AmBisome® with a mean diameter of <100 nm (7) to pass through. However, fenestrae are
394 dynamic structures, which may undergo changes in response to local external stimuli (39) and
395 the impact of *L. donovani* infection on fenestration is as yet unknown. In the spleen of
396 uninfected mice most of the blood flows through the marginal zone (40) and MZMs display
397 preferential uptake of clodronate liposomes over other phagocytic cells in the spleen (41). It is

398 currently unknown if there are also differences in the extent of drug uptake between the
399 different cell types for AmBisome®. Future studies, utilising advanced techniques to
400 simultaneously image drug and cells, are needed to shed light on the spatial distribution of drug
401 in healthy and diseased organs over time (42).
402 Gershkovich et al. (9) hypothesised that reduced phagocytic activity of macrophages in EVL
403 or increased drug elimination, either through leaking capillaries in inflamed tissue or binding
404 of drug to fragments of killed parasites, may explain the differences in drug levels between
405 infected and uninfected tissues. Whilst the latter hypothesis cannot be ruled out it is unlikely
406 to fully account for the lack of drug accumulation in the spleen. Our data favours a model in
407 which i) increases in organ mass lead to decreased total drug amounts / organ, ii) increased
408 elimination and / or metabolism is the predominant feature in the liver and iii) drug distribution
409 to the spleen is progressively decreased during EVL.
410 A retrospective analysis of risk factors for VL relapse following treatment of patients with 20
411 mg/kg AmBisome®, administered as 4 doses of 5 mg/kg, in India showed that a slower
412 decrease in splenomegaly during treatment, but not spleen size at admission, was significantly
413 associated with relapse (43). In *L. donovani* infected BALB/ mice the granulomatous response
414 in the liver appears to follow many of the characteristics observed in subclinical human
415 infection, whereas the spleen shows hallmarks of progressive human disease (44). However, it
416 is currently unknown if the differences we observed in AmBisome® distribution and
417 elimination in this experimental model also exist at different stages in human VL. Nonetheless,
418 understanding the mechanisms of how the pathophysiology of EVL affects drug absorption,
419 distribution, metabolism and elimination (ADME) will improve the development and use of
420 anti-leishmanial drugs and drug delivery systems.
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Table 1.

		Percentage reduction parasite burden (mean +/- SEM)					
		Liver			Spleen		
Expt.	**Dose**	**Day 21 p.i.**	**Day 42 p.i.**	**Significance**	**Day 21 p.i.**	**Day 42 p.i.**	**Significance**
1	10 mg/kg	99 +/- 0	98 +/- 1	n.s.	99 +/- 0	83 +/- 4	$p \leq 0.05$
	2.5 mg/kg	78 +/- 2	78 +/- 5	n.s.	53 +/- 6	5 +/- 5	$p \leq 0.0001$
	0.6 mg/kg	21 +/- 8	41 +/- 4	$p \leq 0.01$	44 +/- 6	4 +/- 4	$p \leq 0.0001$
2	2.5 mg/kg	81 +/- 2	80 +/- 3	n.s.	71 +/- 2	36 +/- 11	$p \leq 0.05$
3	10 mg/kg	100 +/- 0	99 +/- 1	n.s.	99 +/- 0	93 +/- 2	n.s.
	2.5 mg/kg	94 +/- 1	80 +/- 5	$p \leq 0.05$	40 +/- 6	45 +/- 7	n.s.
	0.6 mg/kg	54 +/- 3	22 +/- 7	$p \leq 0.0001$	38 +/- 10	16 +/- 9	n.s.
		Parasite burden (LDU, mean +/- SEM) in untreated control groups					
1	N/A	966 +/- 66	518 +/- 48	$p \leq 0.01$	88 +/- 8	118 +/- 15	n.s.
2	N/A	415 +/- 32	176 +/- 11	$p \leq 0.001$	18 +/- 1	53 +/- 8	$p \leq 0.01$
3	N/A	682 +/- 54	194 +/- 44	$p \leq 0.001$	18 +/- 2	84 +/- 9	$p \leq 0.001$

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.

		Single i.v. dose of AmBisome® administered:		
PK parameter	**Unit**	**10 mg/kg**	**2.5 mg/kg**	**0.6 mg/kg**
$t_{1/2}$	Hr	3.4	5.0	NQ
Tmax	Hr	0.08	0.08	0.08
Cmax	ug/mL	240.3	29.8	3.3
AUClast	hr*ug/mL	368.5	50.5	10.8
CL	mL/hr/kg	27.0	48.1	41.9
Vd	mL/kg	104	252	681

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)

	Amphotericin B concentration [ng/g tissue], mean ± SD				**Ratio**		**Significance**	
	Liver		**Spleen**		**Liver**	**Spleen**	**Liver**	**Spleen**
Treatment group	**Day 21 p.i.**	**Day 42 p.i.**	**Day 21 p.i.**	**Day 42 p.i.**	**Day 21 *vs.* Day 42 p.i.**		**Day 21 *vs.* Day 42 p.i.**	
AmBisome 10 mg/kg	115 733 +/- 19 582	44 232 +/- 6 900	19 233 +/- 8 600	4 266 +/- 1 399	2.6	4.5	$p \leq 0.0001$	$p \leq 0.01$
AmBisome 2.5 mg/kg	12 220 +/- 1 308	5 492 +/- 862	1 770 +/- 412	624 +/- 205	2.2	2.8	n.s.	n.s.
AmBisome 0.6 mg/kg	1 430 +/- 923	257 +/- 58	490 +/- 146	51 +/- 12	5.6	9.6	n.s.	n.s.
	Organ weights [mg], mean ± SD				**Ratio**		**Significance**	
AmBisome 10 mg/kg	998 +/- 79	1 532 +/- 98	170 +/- 35	492 +/- 66	0.7	0.3	$p \leq 0.0001$	$p \leq 0.0001$
AmBisome 2.5 mg/kg	1 068 +/- 51	1 544 +/- 86	288 +/- 29	654 +/- 70	0.7	0.4	$p \leq 0.0001$	$p \leq 0.0001$
AmBisome 0.6 mg/kg	1 070 +/- 82	1 580 +/- 67	286 +/- 23	698 +/- 50	0.7	0.4	$p \leq 0.0001$	$p \leq 0.0001$
	Estimated amphotericin B amount [ng / organ], mean ± SD				**Ratio**		**Significance**	
AmBisome 10 mg/kg	114 548 +/- 12 631	67 456 +/- 9 000	3 071 +/- 699	2 046 +/- 455	1.7	1.5	$p \leq 0.0001$	n.s.
AmBisome 2.5 mg/kg	12 907 +/- 1 308	8 494 +/- 1 504	522 +/- 79	417 +/- 171	1.5	1.3	n.s.	n.s.
AmBisome 0.6 mg/kg	1 524 +/- 985	404 +/- 85	138 +/- 29	35 +/- 8	3.8	3.9	n.s.	n.s.

B)

	Amphotericin B concentration [ng/mL plasma], mean ± SD		**Ratio**	**Significance**
Treatment group	**Day 21**	**Day 42**	**Day 21 *vs.* Day 42**	**Day 21 *vs.* Day 42**
AmBisome 10 mg/kg	191 +/- 23	216 +/- 47	0.9	n.s.
AmBisome 2.5 mg/kg	116 +/- 18	129 +/- 28	0.9	n.s.
AmBisome 0.6 mg/kg	5 +/- 5	16 +/- 9	0.3	n.s.

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.

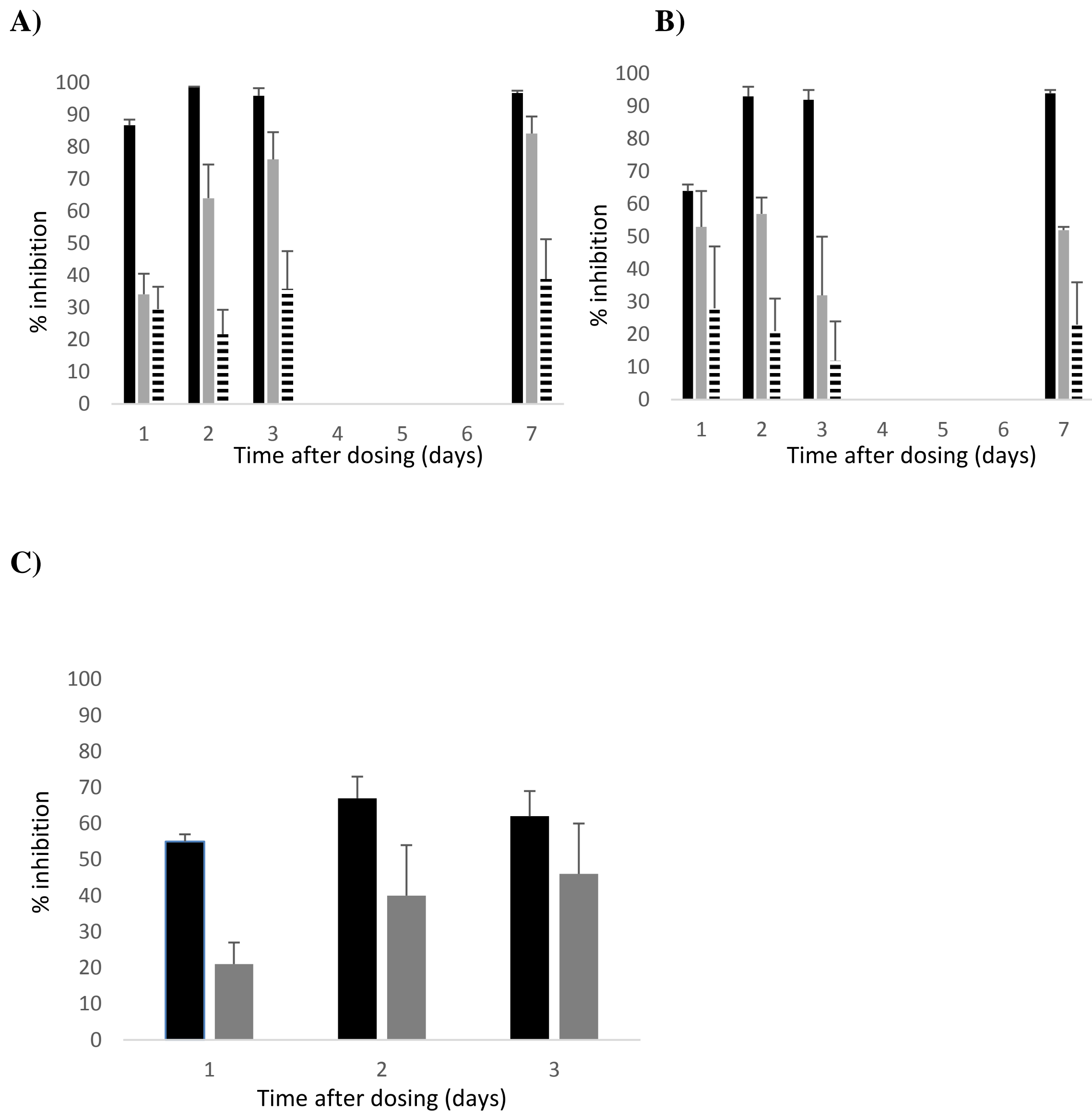
Table 4.

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

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 ($p \leq 0.05$).

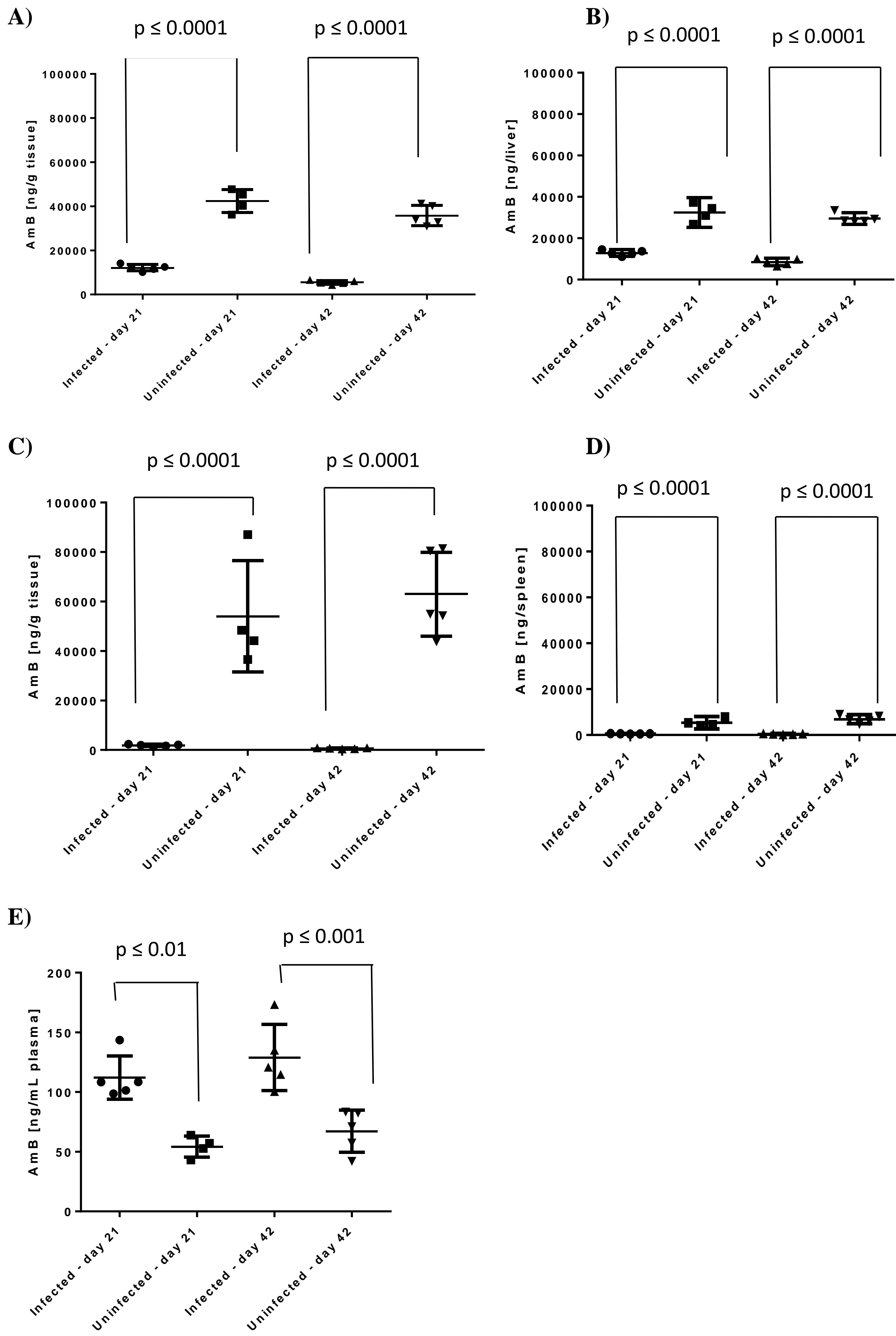
Fig. 1



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

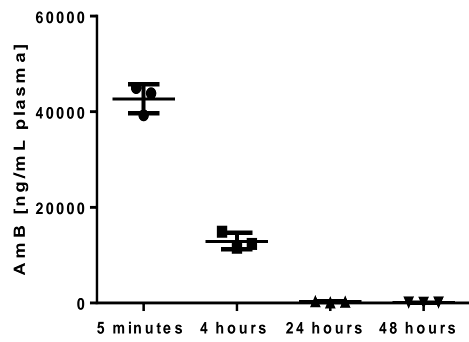


Comparative plasma and tissue concentrations in *L. donovani* infected and uninfected BALB/c mice 7 days after administration of single dose AmBisome®.

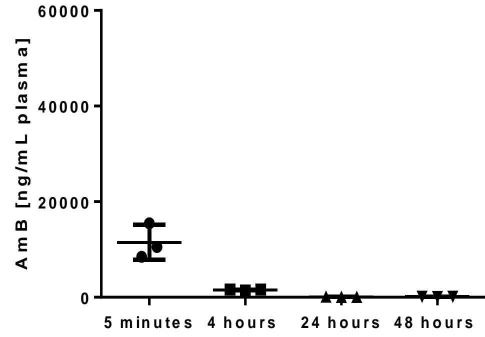
Amphotericin B concentrations were measured on day 21 or day 42 post infection (Infected - day 21, Infected – day 42) and in uninfected BALB/c mice, maintained under identical conditions (Uninfected - day 21, Uninfected – day 42). Amphotericin B concentrations are presented as ng/g tissue (A, C) or ng / 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

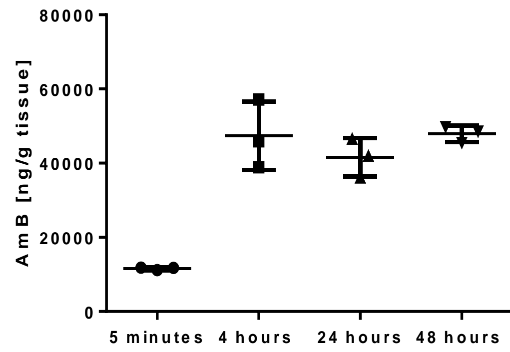
A)



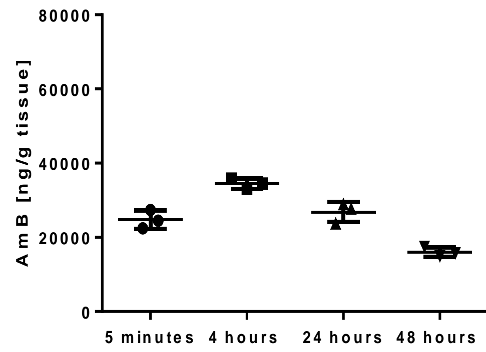
B)



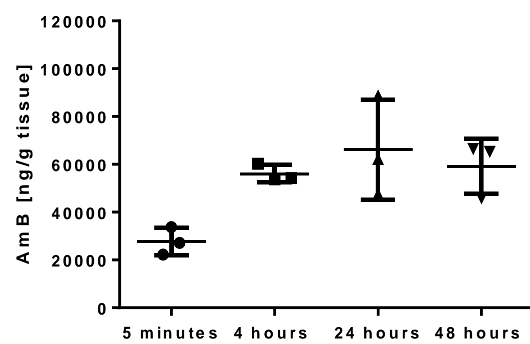
C)



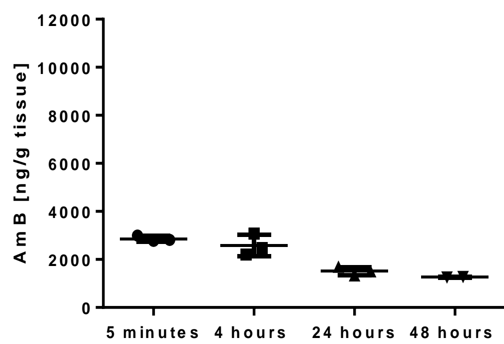
D)



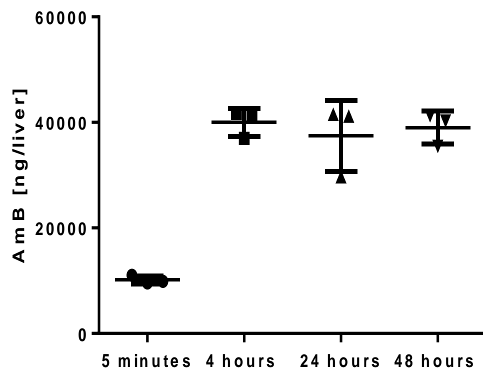
E)



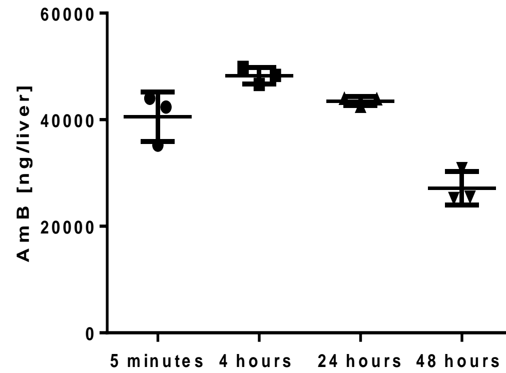
F)



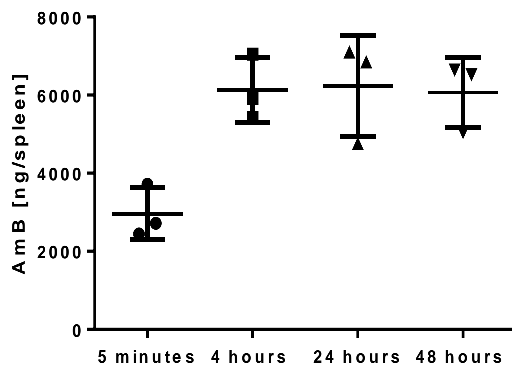
G)



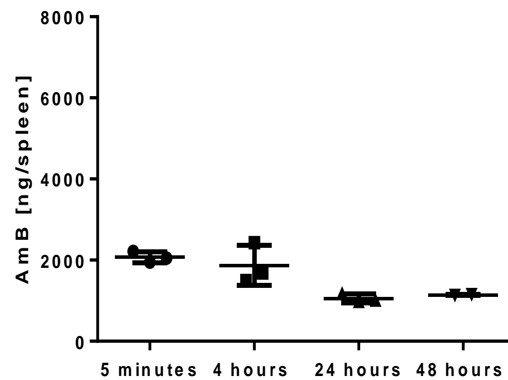
H)



I)



J)



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).