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## AmBisome® treatment of murine cutaneous leishmaniasis: relation between skin pharmacokinetics and efficacy

Gert-Jan Wijnant<sup>1,2</sup>, Katrien Van Bocxlaer<sup>1</sup>, Vanessa Yardley<sup>1</sup>, Andy Harris<sup>3</sup>, Sudaxshina Murdan<sup>2</sup> and Simon L. Croft<sup>1\*</sup>

1. Department of Immunology and Infection, Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, United Kingdom
2. Department of Pharmaceutics, UCL School of Pharmacy, London, United Kingdom.
3. Pharmidex Pharmaceutical Services Ltd, 3<sup>rd</sup> floor, Hanover Street, London W1S 1YH, UK

Corresponding author [simon.croft@lshtm.ac.uk](mailto:simon.croft@lshtm.ac.uk).

### ABSTRACT

AmBisome® (LAmB), a liposomal formulation of amphotericin B (AmB), is a second-line treatment for the parasitic skin disease cutaneous leishmaniasis (CL). Little is known about its tissue distribution and pharmacodynamics to inform clinical use in CL. Here, we compared the skin pharmacokinetics of LAmB with Fungizone® (DAmB), the deoxycholate form of AmB, in murine models of *Leishmania major* CL. Drug levels at the target site (the localized lesion) 48 hours after single intravenous (IV) dosing of the individual AmB formulations (1 mg/kg of body weight) were similar, but were 3-fold higher for LAmB than for DAmB on day 10 after multiple administrations (1 mg/kg on days 0, 2, 4, 6 and 8). After single and multiple dosing, intralesional concentrations were respectively 5- and 20-fold higher compared to those in the healthy control skin of the same infected mice. We then evaluated how drug levels in the lesion after LAmB treatment relate to therapeutic outcomes. After five administrations of the drug at 0, 6.25 or 12.5 mg/kg (IV), there was a clear correlation between dose level, intralesional AmB concentration and relative reduction in parasite load and lesion size ( $R^2$  values > 0.9). This study confirms the improved efficacy of the liposomal over the deoxycholate AmB formulation in experimental CL, which is related to higher intralesional drug accumulation.

### KEYWORDS

Pharmacokinetics, pharmacodynamics, amphotericin B, cutaneous leishmaniasis

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## 41 INTRODUCTION

42 Cutaneous leishmaniasis (CL) is a vector-borne neglected tropical disease caused by  
43 intracellular protozoan *Leishmania* parasites. Current estimates suggest 350 million people at  
44 risk, 12 million cases per year and 1-1.5 million new cases annually in more than 98  
45 countries, of which the majority occurs in Latin America and the Middle East (1). While  
46 mortality is limited for the most common form, localized CL, morbidity is serious due to  
47 ulceration, disfigurement and often permanent scarring after healing of the lesion, which are  
48 all associated with social stigmatization. More complex and potentially dangerous forms of  
49 CL are diffuse (diffuse cutaneous leishmaniasis, DCL), chronic (*leishmaniasis recidivans*,  
50 CCL) or destructive to the nasopharyngeal mucosa (mucocutaneous leishmaniasis, MCL).  
51 Current treatments are hampered in their clinical value by toxicity, side effects, variable  
52 efficacy, high cost or invasive administration route. First-line treatment consists of  
53 pentavalent antimonials, second-line chemotherapeutic options include paromomycin,  
54 miltefosine and amphotericin B (AmB). AmB, a macrocyclic polyene antibiotic and important  
55 antifungal agent derived from *Streptomyces nodosus*, is active due to complexation with  
56 ergosterol in leishmanial cell membranes, leading to the formation of pores and ultimately  
57 pathogen death (2). Due to infusion-related and acute (nephro)toxicity issues of the classic  
58 colloidal dispersion with deoxycholate (Fungizone®, DAmB), lipid formulations with an  
59 improved tolerability profile and different physicochemical properties were developed,  
60 including a phospholipid complex (Abelcet®), a dispersion with cholesteryl esters  
61 (Amphocil™), a multilamellar liposome (Fungisome®) and a unilamellar liposome  
62 (AmBisome®, LAmB) (3).

63 No standard dose regimens have been established for LAmB in the treatment of CL, as  
64 published data are limited to small case series or individual case reports (4), but clinical  
65 success has been achieved with a course of daily 3 mg/kg for a total dose of 18-21 mg/kg.  
66 Due to the need for intravenous administration of LAmB and the related risk of systemic

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67 adverse effects, it is typically reserved as a 2<sup>nd</sup> line treatment for complex CL. This includes  
68 patients with (or at risk of) MCL, DCL or CCL, but also cases where lesions are large,  
69 numerous, potentially disfiguring, unresponsive to earlier therapeutic attempts and  
70 aesthetically or practically unfeasible to cure locally. General limitations of LAmB include  
71 the high price as well as the requirements for cold chain, slow infusion and hospitalization  
72 (5). Despite the relative safety and efficacy of LAmB in CL, fundamental questions about its  
73 pharmacology for this disease remain unanswered. Evaluation of pharmacokinetics (PK) and  
74 pharmacodynamics (PD) in preclinical models is important to inform optimal clinical use and  
75 learn lessons for drug development. A number of studies have looked at the difference in PK  
76 and PD properties of AmB formulations in the treatment of invasive fungal pathologies (6-  
77 11), but none have done so for CL. Here, we report (i) the single dose pharmacokinetics of  
78 LAmB and DAmB in both healthy and *Leishmania major*-infected BALB/c mice, (ii) skin  
79 distribution after multiple dosing of LAmB and DamB in murine CL and (iii) the relationship  
80 between dose, intralesional AmB concentrations and response after LAmB treatment at three  
81 dose levels.

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## 85 MATERIALS AND METHODS

86 **Drugs.** AmBisome® (LAmB, Gilead, UK) and Fungizone (DAmB, Bristol-Myers Squibb,  
87 UK) were reconstituted with sterile water as per the manufacturer's instructions to yield stock  
88 solutions of respectively 4 mg/ml and 5 mg/ml. These were diluted in 5% aqueous dextrose to  
89 a dose of 1 mg/kg (0.02 mg per dose of 200 µl for mice of a mean weight of 20 g). For  
90 LAmB, additional doses of 6.25, 12.5 and 25 mg/kg were similarly prepared. The dilutions  
91 were prepared one day before starting the experiment and stored at 4 °C.

92 **Parasites.** *L. major* MHOM/SA85/JISH118 parasites were cultured in Schneider's insect  
93 medium (Sigma, UK) supplemented with 10% heat-inactivated fetal calf serum (HiFCS,  
94 Sigma, UK). These parasites were passaged each week at a 1:10 ratio of the existing culture  
95 to fresh medium in 25-ml culture flasks without a filter and incubated at 26 °C. For infection  
96 of mice, stationary-phase parasites (as confirmed by light microscopy) were centrifuged for  
97 10 min at 2100 rpm at 4 °C. The supernatant was removed, and the pellet was resuspended in

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98 pure Schneider's insect medium. The number of cells was estimated by microscopic counting  
99 with a Neubauer hemocytometer.

100 ***In vivo L. major* models of CL.** Female BALB/c mice around 6 to 8 weeks old were  
101 purchased from Charles River Ltd. (Margate, UK). These mice were kept in humidity- and  
102 temperature controlled rooms (55 to 65% and 25 to 26 °C, respectively) and fed water and  
103 rodent food *ad libitum*. After acclimatization for 1 week, mice were randomized and  
104 subcutaneously (s.c.) injected in the shaven rump above the tail with 200 µl of a parasite  
105 suspension containing  $4 \times 10^7$  low-passage-number ( $p < 5$ ), stationary-phase *L. major*  
106 promastigotes in RPMI medium. Uninfected mice received a similar, but parasite-free  
107 injection of 200 µl RPMI medium instead. Twelve days later, when a 4- to 5-mm non-  
108 ulcerating nodule had formed on the rump of infected animals, mice were allocated to the  
109 different experimental groups to ensure comparable lesion sizes.

110 **Ethics statement.** All animal experiments were conducted under license X20014A54  
111 according to UK Home Office regulations under the Animals (Scientific Procedures) Act  
112 1986 and EC Directive 2010/63/E.

113 **Single-dose PK study.** Uninfected and *L. major*-infected BALB/c mice ( $n = 4-5$  per group)  
114 each received LAmB or DAmB at 1 mg/kg of body weight over a 1-2 minute period by an  
115 intravenous bolus (200 µl). Plasma, rump (lesion site) and back (control site) skin samples  
116 were collected at 0.5, 2, 6, 24 and 48 hours post-infusion.

117 **Multiple-dose PK and PD study.** *L. major*-infected BALB/c mice ( $n = 4-5$  per group) each  
118 received LAmB or DAmB at 1 mg/kg or 5% dextrose over a 1-2 minute period by an  
119 intravenous bolus (200 µl) on days 0, 2, 4, 6 and 8. Skin samples from rump (lesion site) and  
120 back (control site) were collected on day 10 (48 hours after the 5<sup>th</sup> and last drug  
121 administration). This day 10 time point of sacrifice allowed direct comparison with the  
122 outcomes of the single-dose PK study (last time point: 48 hours). The alternate day dosing  
123 regimen was based on earlier data on the efficacy of LAmB in the *L. major*-BALB/c model  
124 of CL (12). The PD methodology can be found in the following section.

125 **Dose-concentration-response study.** *L. major*-infected BALB/c mice ( $n = 4-5$  per group)  
126 each received LAmB (IV) at 0, 6.25, 12.5 or 25 mg/kg on day 0, 2, 4, 6 and 8. Lesion size  
127 was measured daily in two dimensions (length and width) using digital callipers and the mean  
128 size (average of length and width) was calculated. On day 10, rump (lesion site) and back  
129 (control site) skin samples were collected and parasite load was evaluated. The methodology

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130 to extract parasite DNA from lesions and quantify parasite load by qPCR has already been  
131 described in full detail earlier (13).

132 **Skin sample collection and preparation.** After sacrificing mice (CO<sub>2</sub>), skin was harvested  
133 by surgical removal from the areas containing the localized CL lesion (at the parasite  
134 inoculation site on the rump above the tail, ‘lesion-site’) and CL-uninfected skin on the back  
135 (‘control site’). The skin tissue was cut into fine, long pieces and placed into SureLock  
136 microcentrifuge tubes (StarLab, UK) together with 1 spatula (about 100 mg) of 2 mm  
137 zirconium oxide beads (Next Advance, UK) and 1 ml phosphate buffered saline (PBS, 0.9%  
138 NaOH and pH 7.4, Sigma, UK). Samples were ground using a Bullet Blender Storm 24  
139 (NextAdvance, UK) set at speed 12 for 20 minutes to obtain a smoothly flowing homogenate  
140 and stored at -80 °C until further use. The homogenate (50 µl) was added to 250 µl of a  
141 mixture of 84:16 methanol:DMSO (HPLC grade, Fisher Chemical, UK) containing 200  
142 ng/ml tolbutamide (analytical standard, Sigma, UK) internal standard for drug extraction and  
143 protein precipitation in 96-well plates. Plates were shaken for 10 minutes at 200 rpm and  
144 centrifuged for 15 minutes at 6600 rpm at 4 °C. 150 µl supernatant was collected and stored  
145 at -80 °C until analysis. Blanks with and without internal standard as well as calibration  
146 samples with known concentrations of AmB (similarly extracted and prepared after spiking  
147 45 µl blank skin homogenate (derived from untreated BALB/c mice) with 5 µl working  
148 solutions of known AmB concentrations in 1% SDS (Sigma)) were included.

149 **Plasma sample collection and preparation.** Blood samples were taken from live animals by  
150 needle pricks in the lateral tail veins and collected in Eppendorf tubes preloaded with heparin  
151 (2 µl of a 1000 units/ml stock (aq)). After centrifugation at 6500 rpm at 4 °C for 10 minutes,  
152 the supernatant plasma was collected in new tubes. Plasma samples for which concentrations  
153 of AmB above the upper limit of quantification were expected, were first diluted with drug-  
154 free blank plasma derived from untreated BALB/c mice. 20 µl plasma was added to 100 µl of  
155 a 200 ng/ml tolbutamide internal standard in 84:16 methanol:DMSO. Supernatant (60 µl) was  
156 collected and further treated as described for skin samples. Again, blanks with and without  
157 internal standard and calibration standards (similarly extracted and prepared after spiking 18  
158 µl blank plasma (derived from untreated BALB/c mice) with 2 µl working solutions of  
159 known AmB concentrations in 1% SDS (Sigma)) were included.

160 **LC-MS/MS quantification of AmB.** The LC-MS/MS methodology to quantify AmB levels  
161 in experimental leishmaniasis samples has been described earlier by Voak *et al.* (14).

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162 Analysis was conducted at Pharmidex Pharmaceutical Services Ltd. (Stevenage, UK). The  
163 lower limit of quantification was 1 ng/ml.

164 **Pharmacokinetic parameters.** Single dose PK parameters were estimated assuming  
165 noncompartmental analysis in WinNonlin. AUC<sub>0-48h</sub> values for skin were calculated using  
166 GraphPad Prism version 7.02.

167 **Statistical analysis.** Differences among lesion sizes and parasite loads in the groups were  
168 assessed by using one-way analysis of variance (ANOVA) assuming Gaussian distribution  
169 followed by Tukey's multiple-comparison test. Data is presented as means and standard error  
170 of the mean (SEM). A p-value < 0.05 was considered statistically significant. All analyses  
171 were performed using GraphPad Prism version 7.02.

## 172 **RESULTS**

### 173 **Single dose plasma and skin PK in healthy and *L. major*-infected mice**

174 Plasma concentration-versus-time plots after intravenous (IV) administration of a single dose  
175 of 1 mg/kg LAmB or DAmB to uninfected and *L. major*-infected mice are shown in figure 1a  
176 and 1b, respectively. 1 mg/kg was used as it is the highest tolerated single dose of DAmB  
177 which does not cause signs of acute toxicity (data from pilot studies not shown). Plasma PK  
178 were similar between uninfected and infected mice for the two AmB formulations, with  
179 comparable C<sub>max</sub>, AUC, Cl, T<sub>1/2</sub> and Vd (table 1). However, the plasma profiles for LAmB  
180 and DamB individually were significantly different. Compared to DAmB, LAmB achieved a  
181 higher plasma peak and systemic exposure (C<sub>max</sub> and AUC around 10- and 3-fold greater,  
182 respectively), but showed a shorter half life and lower clearance and volume of distribution. It  
183 should be noted that the terminal phase for LAmB was not clearly defined.

184 AmB exposure in the rump (lesion site) and back (control site) skin, expressed as AUC<sub>0.5-48h</sub>,  
185 are shown in table 2. In uninfected animals, similar drug distribution profiles in the healthy  
186 rump (fig 1c) and back (figure 1e) tissues were obtained. Compared to DAmB, LAmB gave  
187 similar drug peak levels around 60 ng/g, but at earlier time points (after 30 minutes *versus* 2-  
188 6 hours) and only half the total exposure. The rump-to-back AUC<sub>0.5-48h</sub> ratios (1.3 for DAmB,  
189 1.5 for LAmB) indicate that there are limited differences in skin drug exposure based on  
190 anatomical location in uninfected mice. In contrast, in *L. major*-infected animals, the  
191 presence of the localized cutaneous lesion on the rump (figure 1d) strongly enhanced drug  
192 accumulation for both formulations in comparison to the CL-uninfected back skin of the



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193 same mice (figure 1f). Based on the rump-to-back  $AUC_{0.5-48h}$  ratios, AmB levels are 6-fold  
194 higher for LAmB and 8-fold higher for DAmB. Compared to DAmB, LAmB had a similar  
195 peak concentration in skin ( $132 \pm 28$  versus  $159 \pm 8$   $\mu\text{g/g}$ ) at later time points (24 h versus 6  
196 h), showing a trend of slower drug accumulation into and elimination from the lesion. AmB  
197 levels in the rump and back tissue for both formulations in infected mice was around 5-fold  
198 higher than in uninfected mice. Changes in AmB plasma concentrations after 1 mg/kg LAmB  
199 or DAmB infusion are not reflective for those in skin tissues. No adverse effects at this dose  
200 level were observed for either formulation.

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#### 211 **Multiple dose skin PK and PD in *L. major*-infected mice**

212 Skin distribution after multiple dosing of either LAmB or DAmB (1 mg/kg on day 0, 2, 4, 6  
213 and 8) in CL-infected mice is shown in figure 2. On day 10, intralesional levels for LAmB  
214 ( $542 \pm 46$  ng/g) were 3-fold higher than for DAmB ( $170 \pm 18$  ng/g,  $p < 0.0001$ ). Comparing  
215 these concentrations 48 hours after the last dosing to those found during earlier single dose  
216 PK studies at the same time point (see figure 1c and 1d – LAmB:  $110 \pm 17$  ng/g; DAmB:  $92 \pm$   
217  $4$  ng/g), a gradual and linear drug accumulation in the target tissue during treatment can be  
218 assumed for LAmB but not for DAmB. Again, AmB levels in the lesion were significantly  
219 higher compared to those in the healthy back skin for LAmB (x 20,  $p < 0.0001$ ) and DAmB (x  
220 12,  $p < 0.0001$ ).

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221 We then compared the resulting efficacy outcomes for LAmB and DAmB after complete 5 x  
222 1 mg/kg treatment. A small reduction in day 10 lesion size compared to the untreated (5%  
223 dextrose) group ( $9.9 \pm 0.8$  mm) was found for LAmB ( $9.4 \pm 0.2$  mm) and DAmB ( $8.7 \pm 0.6$ ),  
224 but in both cases the difference was not significant ( $p=0.83$  and  $0.34$ , respectively). A lower  
225 relative parasite load was also found for LAmB ( $2.0 \pm 0.6 \times 10^7$  parasites/g) and DAmB ( $6.1$   
226  $\pm 3.4 \times 10^7$  parasites/g), but again without a statistically significant difference compared to  
227 the control ( $1.6 \pm 0.5 \times 10^8$  parasites/g,  $p=0.12$  and  $0.23$  respectively). As expected, both  
228 formulations show some antileishmanial efficacy at 5 x 1 mg/kg, but the toxicity limit of  
229 DAmB (1 mg/kg) does not allow a meaningful comparison at clinically relevant dose levels.  
230 Because of this, we only further investigated the dose-concentration-response relationship at  
231 higher doses for LAmB.

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### 233 **Dose-concentration-response of LAmB in *L. major*-infected mice**

234 After *L. major*-infected mice received 5 doses of LAmB at either 0, 6.25, 12.5 or 25 mg/kg  
235 LAmB (on days 0, 2, 4, 6 and 8), the dose level was related to the resulting day 10  
236 intralesional AmB concentrations (figure 3a) as well as response indicated by lesion size and  
237 parasite load (figure 3b and 3c respectively). Figure 3d shows the non-linear fit sigmoidal  
238 dose-response curve plotting the logarithm of these intralesional AmB levels *versus* relative  
239 reductions in parasite load and lesion size compared to the untreated controls (0 mg/kg). The  
240 calculated dose required to achieve 50% ( $ED_{50}$ ) and 90% of maximum effect ( $ED_{90}$ ) was 9.16  
241 and 16.73 mg/kg for lesion size. For parasite load,  $ED_{50}$  was 7.55 and  $ED_{90}$  was 9.16 mg/kg.

242 We observed a linear dose-concentration-response relationship up to 12.5 mg/kg. Between  
243 the 0 and 12.5 mg/kg range, correlation was strong between dose-concentration (linear  
244 regression goodness of fit  $R^2=0.99$ ) and concentration-response ( $R^2=0.99$  and  $0.91$  for  
245 relative reduction in parasite load and lesion size, respectively). Little additional efficacy was  
246 found by doubling the dose from 12.5 to 25 mg/kg, while intralesional AmB levels increased  
247 nonlinearly by 5-fold; this resulted in only a small additional reduction in lesion size and  
248 parasite load. This indicates that at 25 mg/kg, the near-maximum efficacy of LAmB for this  
249 specific treatment regimen had been reached. Significant reductions in parasite load and  
250 lesion size ( $P<0.05$ ) were found between the control and treated groups at all three dose  
251 levels. Doubling of the LAmB dose from 6.25 to 12.5 to 25 mg/kg range resulted in a further



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252 decrease in parasite load and lesion size, but the differences among the groups were not  
253 significant ( $p>0.05$ ).

## 254 **DISCUSSION**

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256 The pharmacokinetics and pharmacodynamics of many drugs currently used in the treatment  
257 of CL, including different formulations of AmB, are poorly understood (15). We have  
258 investigated the single- and multiple-dose skin distribution of AmB following dosing with  
259 either the unilamellar liposome AmBisome® (LAmB) or the micellar deoxycholate salt form  
260 Fungizone® (DAmB). Significant differences in pharmacokinetics were observed between *L.*  
261 *major*-infected and uninfected animals, as well as between the two drug formulations.

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264 We observed an important impact of the CL infection on skin accumulation for both LAmB  
265 and DAmB. Drug levels in the localized lesion were over 5-to-20 fold elevated compared to  
266 those in the healthy skin tissue of the same infected mice, as well as in uninfected animals.  
267 The pathological condition of CL-infected skin, mainly caused by the severe localized  
268 inflammatory immune response against the *Leishmania* parasites multiplying within dermal  
269 macrophages, may explain this phenomenon. After intravenous administration, DAmB  
270 dissociates from the colloidal micelles and over 95% of AmB binds to plasma proteins (16) to  
271 from a high molecular weight association. LAmB also interacts with proteins and while 90%  
272 of AmB remains stably intercalated in the 60-80 nm sized liposomes (4, 16), coating by  
273 opsonins makes the liposomes prone to ingestion by phagocytes in systemic circulation and  
274 the reticuloendothelial system in liver and spleen (17). While these complexes have impaired  
275 extravasation in healthy skin (continuous endothelium with small vessel pores of a 6-12 nm  
276 diameter (18)), the leaky vasculature at the infection site (increased permeability, disease-  
277 inflicted capillary damage) could enhance local drug accumulation (19). Another factor,  
278 especially for LAmB, is the migration of phagocytic monocytes, which can serve as potential  
279 drug reservoirs, from the bloodstream to the infection site. This is a characteristic of the  
280 early-stage and acute immune response against *Leishmania* (20, 21), causing small, non-  
281 ulcerated CL nodules (as observed in our *L. major*-infected mice 12-days post-inoculation).  
282 Little is known about the elimination of AmB from the target site by local metabolism or

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283 lymphatic drainage. However, the latter has been hypothesized as a reason behind the much  
284 lower activity of liposomal formulations of AmB (12) and sodium stibogluconate (22) when  
285 injected intralesionally compared to intravenously. The impact of these individual  
286 physiological processes on local drug distribution in skin is difficult to estimate using the  
287 current methodology, which is based on total drug levels and unable to distinguish between  
288 intra- or extracellular, as well as free, protein-bound or liposome-encapsulated AmB.  
289 Furthermore, the general limitations of tissue homogenates apply, such as loss of spatial drug  
290 disposition within the compartments of the organ of interest. Novel techniques, such as  
291 microdialysis and MALDI MS imaging, have untapped potential in pharmacological CL  
292 research to respectively measure unbound concentrations in the dermal interstitial fluid (23)  
293 or study drug disposition within the cellular architecture of infected skin (24). These findings  
294 about AmB accumulation in diseased tissue could also be relevant in the treatment of deep  
295 cutaneous mycoses (such as invasive candidiasis), where the pathogen, like *Leishmania*, is  
296 located in the dermis (25), instead of the superficial portions of the epidermis where most  
297 fungi typically reside.

298 Comparing the pharmacokinetics of the individual two AmB formulations, we saw significant  
299 differences between LAmB and DAmB, consistent with previous studies (14, 26-28). Plasma  
300 concentrations and exposure were much higher for LAmB over DAmB and not reflective of  
301 changes in skin tissue levels for either formulation. Drug concentrations at the target site  
302 were similar after single intravenous dosing of the individual AmB formulations, but 3-fold  
303 higher for LAmB than for DAmB following 5-time administration of the same dose.  
304 Recently, Imam and colleagues (28) also investigated the distribution of LAmB and DAmB  
305 in *L. major*-infected BALB/c mice, but skin was not evaluated in this study. Increased  
306 accumulation of liposomes in inflammatory over healthy sites has also been described for  
307 subcutaneous tumours (29), bacterial skin abscesses (30, 31) and fungal infections (32). The  
308 so-called ‘enhanced permeation and retention effect’, increased drug accumulation at sites of  
309 leaky vasculature and defective lymphatic drainage, has been coined as the rationale behind  
310 nanoparticle-based drug delivery in cancer and inflammation (19). The data and our  
311 understanding of CL histopathology suggest that this effect can also be exploited as a passive  
312 targeting strategy in this context, by encapsulation of antileishmanial drugs in small (< 100  
313 nm), stable (tightly packed phospholipids with cholesterol), unilamellar liposomes (17)  
314 similar to AmBisome®. Indeed, several promising results have already been achieved with  
315 nanoparticles of AmB and other drugs for the treatment of CL (28, 33-38).

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316 Finally, we evaluated how drug concentrations at the infection site after LAmB treatment  
317 relate to outcomes. After administration of five consecutive doses, the 1 mg/kg dose of  
318 LAmB (as well as DAmB, for which this is the tolerated maximum) proved to be too low to  
319 be therapeutic, but a linear dose-concentration-response effect was found for 6.25 and 12.5  
320 mg/kg. The clear correlation between intralesional drug levels and treatment outcomes can be  
321 explained by the known concentration-dependent manner in which AmB exerts its  
322 antimicrobial activity (39). Interestingly, for doubling the LAmB dose from 12.5 to 25  
323 mg/kg, intralesional AmB levels increased by over 5-fold. This could be due to the known  
324 phenomenon of saturation of AmB uptake and clearance mechanisms in the organs of the  
325 reticuloendothelial system, possibly resulting in higher plasma exposure and increased  
326 distribution to other tissues (40). However, little additional efficacy for 25 compared to 12.5  
327 mg/kg was observed. Both these doses were able to achieve a near-100 % reduction in  
328 parasite load but not lesion size, indicating the need for longer treatment as the host's  
329 response to parasite elimination in the skin appears to be delayed. Results are in line with  
330 published data (12, 41) and suggest the clinical superiority of LAmB over DAmB in CL  
331 based on enhanced intralesional accumulation of the liposome, as well as already known  
332 factors such as better tolerability and potentially shorter treatment courses. Further PK PD  
333 analysis of LAmB is required to inform optimized clinical dose regimens, especially for the  
334 different complex forms of CL, as there are known differences in species-specific drug  
335 sensitivity (42), histopathology (20) and immunology (21). It is currently unknown to what  
336 degree our observations about skin accumulation of LAmB in the *L. major*-BALB/c model  
337 are translatable to human CL, but understanding of preclinical PK and PK PD relationships  
338 should improve the use and development of antileishmanial drugs.

339 In summary, intravenous LAmB has potent and dose-dependent *in vivo* activity against CL  
340 due to relatively high drug accumulation within the lesion, which is enhanced by the inflamed  
341 state of the infected target tissue and the pharmacokinetic properties of the liposomal  
342 formulation.

### 343 **FIGURE LEGENDS**

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345 Figure 1: Single dose pharmacokinetics of Fungizone® (DAmB, ●) and AmBisome®  
346 (LAmB, ○). Uninfected and *L. major*-infected BALB/c mice received one intravenous dose  
347 (1 mg/kg of body weight) of a formulation, after which amphotericin B (AmB) levels in  
348 plasma (a, b) and skin at multiple time points were determined. Two skin sites per animal  
349 were included: the rump (parasite inoculation site where the localized CL lesion is present in

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350 infected (d), but not in uninfected (c) mice) and the back (lesion-free control site in both  
351 infected (f) and uninfected (e) animals). Each point represents the mean  $\pm$  SEM (n=4-5 per  
352 group).

353 Figure 2: Multiple dose skin pharmacokinetics of Fungizone® (DAmB) and AmBisome®  
354 (LAmB). *L. major*-infected BALB/c mice received intravenous doses of 1 mg/kg of body  
355 weight on days 0, 2, 4, 6 and 8. On day 10 (48 hours after the last dosing), skin samples were  
356 collected for amphotericin B (AmB) analysis. The CL lesion was localized on the rump,  
357 while the back skin served as a lesion-free, healthy control site. Each point represents the  
358 mean  $\pm$  SEM (n=4-5 per group). Differences were analysed using 1-way ANOVA followed  
359 by Turkey's multiple comparison tests and considered significant if  $p < 0.05$  (\*) or not  
360 significant (ns) if not ( $p < 0.0001$ : \*\*\*\*).

361 Figure 3: Dose-concentration-response relationship of AmBisome® (LAmB) in experimental  
362 CL. *L. major*-infected mice received five doses of either 5% dextrose (0 mg/kg, untreated  
363 control), 6.25, 12.5 and 25 mg/kg LAmB (IV). On day 10, resulting intralesional amphotericin  
364 B levels (3a), lesion size (3b) and parasite load (3c) were evaluated. Outcomes are linked in a  
365 logarithmic-scale dose-response curve plotting drug concentrations against relative reduction  
366 in lesion size and parasite load (3d, non-linear fit with variable slope). Each point represents  
367 the mean  $\pm$  SEM (n=4-5 per group). Differences among day 10 outcomes were analysed  
368 using 1-way ANOVA followed by Turkey's multiple comparison tests and considered  
369 significant if  $p < 0.05$  (\*),  $p < 0.01$  (\*\*),  $p < 0.001$  (\*\*\*) and  $p < 0.0001$  (\*\*\*\*) or not significant  
370 (ns) if not.

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### 383 TABLES

384

385 **TABLE 1** Pharmacokinetic profile of Fungizone® and AmBisome® in uninfected and *L.*  
386 *major*-infected mice after a single intravenous 1 mg/kg dose. Values for pharmacokinetic  
387 parameters are calculated from the plasma PK profiles seen in figure 1 (a, b).

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PK parameter	Unit	Fungizone® (DAmB)		AmBisome® (LAmB)	
		Uninfected	Infected	Uninfected	Infected
C <sub>max</sub>	ug/ml	1.1	1.0	11.1	8.2
AUC	h · ug/ml	21.5	30.2	62.7	71.0
Cl	ml/h/kg	29.6	18.9	14.2	13.5
T <sub>1/2</sub>	h	36.1	39.7	10.7	8.5
Vd	ml/kg	1458	1075	225	143

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391 **TABLE 2** Skin distribution of Fungizone® and AmBisome® in uninfected and *L. major*-  
 392 infected mice after a single intravenous 1 mg/kg dose. AUC<sub>0.5-48h</sub> values are calculated from  
 393 skin profiles seen in figure 1 (c, d, e, f).

Skin site	Fungizone® (DAmB)		AmBisome® (LAmB)	
	Uninfected	Infected	Uninfected	Infected
Rump (lesion site)	1586 ± 495	6035 ± 273	863 ± 365	5270 ± 1003
Back (control site)	1269 ± 190	710 ± 194	573 ± 142	915 ± 312
Rump-to-back ratio	1.3	8.5	1.5	5.8

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