Experimental Amphotericin B-Deoxycholate formulations for pulmonary aspergillosis: efficacy, biodistribution and nephrotoxicity.

Running Title: New Amphotericin B formula for pulmonary aspergillosis

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ABSTRACT

An experimental micellar formulation of amphotericin B (AMB) with sodium deoxycholate (DCH), AMB:DCH 1:1.5, was obtained and characterized to determine its aggregation state and particle size. Biodistribution, nephrotoxicity and efficacy against pulmonary aspergillosis in a murine model were studied and compared to the liposomal commercial amphotericin B after intravenous administration. The administration of 5 mg/kg AMB:DCH 1:1.5 presented 2.8-fold higher lung concentrations (18.125 ± 3.985 µg/g, after 6 daily doses) and lower kidney exposure (0.391 ± 0.167 µg/g) compared to liposomal commercial amphotericin B (6.567 ± 1.536 and 5.374 ± 1.157 µg/g, in lungs and kidneys, respectively). The different biodistribution of AMB:DCH micelle systems compared to liposomal commercial amphotericin B was attributed to their different morphology and particle size. The efficacy study has shown that both drugs administered at 5 mg/kg produced similar survival percentages and reduction of fungal burden. A slightly lower nephrotoxicity, associated to amphotericin B, was observed with AMB:DCH 1:1.5 than the one induced by the liposomal commercial formulation. However, AMB:DCH 1:1.5 reached higher AMB concentrations in lungs that could represents a therapeutic advantage over liposomal commercial amphotericin B-based treatment of pulmonary aspergillosis. These results are encouraging to explore the AMB:DCH 1:1.5 usefulness against this disease.

KEYWORDS: aspergillosis, Amphotericin B deoxycholate, pulmonary concentrations, efficacy, nephrotoxicity.

INTRODUCTION

The increase of immunosuppressive agents and the extensive usage of corticosteroids in chronic obstructive pulmonary disease have also led to a rising prevalence of invasive mycoses such as aspergillosis in recent years (1, 2). The initial site of most fungal infections as aspergillosis in humans is primarily the lungs, with hematogenous dissemination to the spleen, kidneys, liver, and brain as the disease progresses. So, high initial pulmonary concentrations of antifungal drugs are needed to avoid the dissemination of the pathogens that make the infection even more difficult to treat. Liposomal amphotericin B (LAMB) is the gold standard in aspergillosis therapy (1, 3). Several studies have demonstrated that large particle sizes of different AMB formulations are related to high nephrotoxicity (4-7). Commercial dimeric deoxycholate amphotericin B (D-AMB) shows a high percentage of small particles (56.2 ± 4.3 nm), and a small percentage of large particles (around 4.0 µm) (6) being the latter related to nephrotoxicity (4-7) LAMB, the reference commercial formulation, with a poly-aggregated AMB form, shows low particle size (around 100 nm) that enhances antifungal efficacy and diminishes drug toxicity (8) while other lipid complexes marketed as Abelcet® (ABLC) presents poly-aggregated AMB with a particle size of 1.6-11 µm (7). This larger particle size of ABLC formulations favors a greater pulmonary distribution but also increases its nephrotoxicity compared to LAMB (4).

However, the high costs of commercial lipid formulations limit their use. On the other hand, the less expensive commercial dimeric deoxycholate amphotericin B (D-AMB) has lower antifungal efficacy and causes serious side effects (9). Therefore, various new amphotericin B formulations such as emulsions, liposomes

and microspheres have been developed to increase efficacy and decrease side effects (10). In previous works, it has been observed that poly-aggregated systems of AMB with sodium deoxycholate are a safer and less toxic form than D-AMB, with the same proportion of DCH, when administered intravenously (6, 11). The AMB distribution to organs with high presence of macrophages and organs of the reticulo-endothelial system like liver and spleen is related to formulation parameters such as the aggregation state and particle size (11, 12). After the parenteral administration of LAMB, the highest concentrations appeared in organs of the reticulo-endothelial system, while lower drug concentrations appeared in other organs such as lungs (13). So, a lung concentration of 1.44 µg/g was obtained 24 hours after the administration of a 5 mg/kg dose of LAMB (14). These probably insufficient pulmonary concentrations make necessary to search new AMB formulations in order to guarantee higher efficacy against dangerous pulmonary pathogen agents like *Aspergillus* spp.

The aim of this project is to develop a lung-specific delivery system of AMB with a high pulmonary distribution and a low nephrotoxicity. A low renal dissemination pulmonary aspergillosis model was selected in order to correlate nephrotoxicity results with the new AMB formulation.

RESULTS

AMB formulations characteristics. The aggregation state of amphotericin B in the experimental formulations was evaluated by measuring UV-visible absorbance. A standard AMB formulation using methanol as solvent (M-AMB) and a formulation with no surfactant in water for injection (AMB:DCH 1:0) were used as

reference formulations and compared with deoxycholate containing formulation (AMB:DCH 1:1.5). The absorption spectrum of M-AMB showed four high pronounced peaks at 353, 372, 390 and 414 nm. The absorption spectrum of AMB:DCH 1:0 showed two faint peaks at 386 y 403 nm.

Absorption spectrum of AMB:DCH 1:1.5 formulation in water showed a shoulder around 330 nm and some faint peaks at higher wavelengths (393, 407 and 424 nm). The absorbance values at different wavelengths of this deoxycholate containing formulation was clearly different from the previously observed ones in the spectrum of M-AMB. Furthermore, AMB:DCH 1:1.5 showed 3-fold higher absorbance values than formulation with no DCH (AMB:DCH 1:0).

The particle size of experimental (AMB:DCH 1:1.5) and reference (AMB:DCH 1:0) formulations was performed and expressed as mean particle size (nm) ± standard deviation (SD). Both formulations in water presented polydispersity indexes less than 0.6.

The presence of sodium deoxycholate in AMB:DCH 1:1.5 formulation decreased significantly ($P<0.001$) the particle size (404.9 ± 1.7 nm) regarding the formulation without surfactant AMB:DCH 1:0 (514.8 ± 34.2 nm).

AMB biodistribution to kidneys and lungs. The study of AMB biodistribution consisted of the administration of a single and multiple doses of AMB in uninfected and immunosuppressed mice in order to evaluate the AMB concentration reached in lungs and kidneys.

AMB concentrations in renal and lung tissues 24 hours after a single dose of AMB:DCH 1:1.5 or LAMB formulations administered at 5 mg/kg dose are observed in Fig. 1. LAMB formulation showed AMB concentrations in kidneys 15 times

126 greater than AMB:DCH 1:1.5 (P<0.01). However, AMB concentrations reached in
127 lung tissues with AMB:DCH 1:1.5 formulation at 24 hours were significantly higher
128 (P<0.01) than the ones obtained with LAMB treatment (9.173 ± 0.498 µg/g versus
129 2.527 ± 0.386 µg/g, respectively). After 6 days of treatment, renal concentrations of
130 AMB showed an important cumulative effect (Fig. 1A). However, low kidney levels
131 of AMB were reached with AMB:DCH 1:1.5 formulation after 6 days of treatment
132 (0.391 ± 0.167 µg/g). Thus, AMB renal concentration for LAMB were 15-fold higher
133 than AMB:DCH 1:1.5 formulation (P<0.01). Also, lung concentrations of AMB
134 showed a cumulative effect with both AMB:DCH 1:1.5 and LAMB formulations (Fig.
135 1B). A higher concentration in lung tissue was observed with AMB:DCH 1:1.5
136 formulation at 5 mg/kg dose (18.125 ± 3.985 µg/g) compared with a dose of 5
137 mg/kg/day of LAMB formulation (6.567 ± 1.536 µg/g).

138 **Drug nephrotoxicity.** One of the most important problems of amphotericin B is
139 its nephrotoxicity. Therefore, renal toxicity of all AMB new experimental
140 formulations should be tested and compared with commercial formulations. In this
141 first preclinical study of toxicity, immunocompetent mice were used.

142 Serum nephrotoxicity biochemical parameters as creatinine and blood urea
143 nitrogen (BUN) were tested after six daily doses of each treatment at therapeutic or
144 elevated AMB doses (5 and 20 mg/kg/day). Table 1 shows the results of creatinine
145 and BUN serum levels. All creatinine concentrations obtained with experimental
146 and commercial formulations were slightly higher than the one obtained with
147 control group. These creatinine values were similar after the administration of
148 therapeutic or high doses of AMB. Commercial LAMB formulation presented the
149 highest values of creatinine concentrations.

Histopathological studies of renal tissue of all treated mice with 6 daily AMB 5 mg/kg/day doses showed tubular and glomerular morphologic abnormalities compared to the control group that maintained the normal morphology (Fig. 2). These changes in renal tissue of mice treated with experimental and commercial AMB formulations include extensive areas of inflammatory cells (surrounding tubules and glomeruli), extra-capillary proliferative glomerulonephritis and acute tubular epithelial damage with the presence of tubular necrosis, cell debris detachment, swelling and tubular dilation. The renal sample of LAMB formulation also showed protein casts in renal tubules. The photomicrographs of mice kidney sections regarding higher AMB doses (20 mg/kg/day) showed a small increase of renal tissue damages (data not shown) when this dose was administered as compared with the same formulations given at 5 mg/kg/day.

Drug efficacy. This study was performed in immunosuppressed and infected animals. Fig. 3A shows survival rates of untreated animals and of those treated with 5 mg/kg AMB:DCH 1:1.5 or LAMB. All animals from control group succumbed within 8 days post-challenge. Both treatments, AMB:DCH 1:1.5 and LAMB, significantly increased the survival of the animals with survival rates of 42.8% and 50.0% in comparison to the control group (P=0.077). However, no significant differences were found between treated groups (P>0.05).

Body weights showed a drop in all animals (Fig. 3B).This pattern was followed by a continuous drop in weight in control animals, in contrast to a progressive rise in weight of treated groups. Infected mice treated with AMB:DCH 1:1.5 and LAMB formulations at doses of 5 mg/kg showed similar percentages of weight loss as well as weight recovery starting on the day 8 and day 6 post infection, respectively.

Infection showed viable fungal elements in most of the lungs from control and treated animals with no significant differences between groups (P>0.05). Fungal burden in the group treated with experimental AMB:DCH 1:1.5 formulation showed the lowest values (log_{10} 2.58 ± 2.130 CFU/g lung tissue), with 40% of mice displaying non-detectable CFUs in this tissue. But, contrary to our expectations, all mice in the group receiving LAMB had high CFUs/g values (log_{10} 4.45 ± 0.40 CFU/g lung tissue). Infection showed low spread to kidneys in control animals but high clearance was observed in treated animals with undetectable CFUs in 80% of animals receiving AMB:DHC 1:1.5 and 100% of those receiving LAMB.

No CFUs were recovered from kidneys at the end of the experimental time i.e., 12 days after infection in treated animals in contrast to the results in lungs, where a few treated animals with AMB:DHC 1:1.5 and LAMB showed low fungal burden with no significant differences between them (P=0.371) (data not shown). Important changes on eradication percentages for each treatment group between days +8 and +12 were observed. Thus, the group that received 5 mg/kg of AMB:DCH 1:1.5 formulation presented similar eradication results in lung tissue 12 days after infection to the obtained on day 8. The group that received 5 mg/kg/day of LAMB formulation showed an important increase in eradication percentages between day +8 and day +12 (3 log_{10}).

DISCUSSION

The aggregation state study of the reference M-AMB formulation showed peaks corresponding to the monomeric form of AMB (15). However, absorption spectrum of AMB:DCH 1:1.5 formulation was clearly different from the previously

observed spectrum of the monomeric form and did not show the characteristic high peak of the dimeric form (15). Therefore, this spectrum of experimental AMB:DCH formulation was indicative of the presence of amphotericin B poly-aggregated form, which usually presents a lower nephrotoxicity than D-AMB (6, 9). AMB:DCH 1:1.5 showed higher absorbance values than the reference formulation with no deoxycholate (AMB:DCH 1:0). These higher absorbance values of AMB:DCH 1:1.5 formulation may be related with a poly-aggregated AMB form into a small size micellar system, induced by the surfactant action of the sodium deoxycholate (16, 17).

Particle size measurements were performed in triplicate and represented by the mean result (± standard deviation). Polydispersity indexes were similar to the ones presented by other authors in micellar formulations (6). In previous studies, the poly-aggregated formulation AMB:DCH 1:0 showed a higher particle size (1280 ± 216.0 nm) when using a shorter agitation time (2 minutes) (6). Therefore, stirring conditions has been proved to be a key issue in order to obtain small poly-aggregated AMB systems as other authors have also reported (17).

AMB:DCH 1:1.5 poly-aggregated formulation presented a small value of standard deviation when its particle size was studied and this low variability should be attributed to the stabilizing effect of DCH by means of ionic interactions with AMB (5). Thus, in this AMB formulation, sodium deoxycholate acts as a stabilizing agent inhibiting particle growth, so the surfactant effect is critical in AMB nanoparticle systems (16, 17). The use of surfactants as stabilizing agents has been previously reported by several authors (6, 18).

Finally, it is noteworthy that particle size of AMB in this poly-aggregated preparation was very different from the one observed with commercial AMB formulation (LAMB) that has been reported as about 100 nm by other authors and therefore may be related to different organ distribution (19).

AMB concentrations obtained in this work with the studied formulations showed different AMB distribution to kidneys and lungs as other authors have previously observed (13). Our results suggest that particle size and surface morphology of colloidal and liposomal systems may influence the distribution of AMB to different organs. Formulations with small particle size (LAMB) presented high concentrations in organs like kidney whilst the new poly-aggregated AMB:DCH formulation with higher particle size led to a greater lung distribution.

At renal level, the new poly-aggregated formulation developed in this work showed less AMB concentrations in kidneys than the ones observed with LAMB (20, 21) either after 1 or 6 days of treatment (5 mg/kg/day). The increase in AMB renal concentrations observed with the DCH formulation may be related to the influence of the hydrophilic sodium deoxycholate surfactant on micelles distribution. However, due to the important renal effects of AMB formulations (11), a toxicity study was performed with the experimental DCH formulation, employing LAMB and AMB:DCH 1:0 formulations as reference, to observe the possible influence of AMB and DCH in the nephrotoxicity.

The serum nephrotoxicity biochemical parameters study showed that BUN values for all the formulations were similar to the control group at both doses studied (5 and 20 mg/kg) and similar to those obtained in different treatments with

LAMB (22). Therefore, the high AMB renal concentrations after LAMB treatment did not translate into pathological BUN values.

All AMB formulations induced higher creatinine values than the control group. Similar increases in creatinine levels were observed with other poly-aggregated AMB formulations at elevated doses (15 mg/kg) (23). But, an increase in creatinine values was observed in LAMB group compared to AMB:DCH 1:1.5 at both doses studied (5 and 20 mg/kg). These high creatinine values were possibly related to high AMB redistribution into the kidneys observed with LAMB (22, 24). It is particularly noteworthy that the experimental AMB:DCH 1:1.5 formulation did not show pathological creatinine values and no significant difference ($P>0.05$) with the control group was observed. But there were significant differences between creatinine values after administration of LAMB and AMB:DCH 1:1.5 formulations at therapeutic and elevated AMB doses (5 and 20 mg/kg/day) ($P<0.001$ and $P<0.01$, respectively).

Nevertheless, all renal tissue samples of mice treated with all the formulations after 6 daily doses of 5 or 20 mg/kg/day presented inflammatory cells and tubular epithelial damage, whilst protein cast were only observed in renal tissue of mice treated with LAMB. So, all preclinical nephrotoxic studies with experimental AMB formulations should include histopathological studies, due to the fact that serum nephrotoxicity biochemical parameters (creatinine and BUN values) would be insufficient to confirm a prompt renal damage.

Furthermore, the renal histopathological study has shown the existence of renal damage (see Fig. 2) when AMB renal concentrations are equal to or greater than 0.372 µg/g, since this was the AMB renal concentration of mice treated i.v.

with 5 mg/kg/day of AMB:DCH 1:1.5 for six days (see Fig. 1). On the other hand, when renal damage was compared between DCH formulations (AMB:DCH 1:0 and AMB:DCH 1:1.5), it was observed that the presence of DCH in AMB formulations did not increase the nephrotoxicity after 6 daily doses of 5 and 20 mg/kg/day (see Table 1 and Fig. 2).

Nephrotoxicity values described in AMB:DCH 1:1.5 formulation for 6 days at high doses (20 mg/kg) were more related to the presence of large particles at this dose than with its high renal AMB concentration. These results are consistent with the nephrotoxicity values after prolonged treatments observed in AMB formulations with high particle size (25). However, nephrotoxicity values described with LAMB formulation are more related to a high AMB renal concentration. However, it must be emphasized that while LAMB formulation presented 15-fold higher AMB renal concentrations than DCH formulations, a 15-fold greater nephrotoxicity was not observed.

Lung AMB concentrations reached with experimental AMB:DCH 1:1.5 formulation at 5 mg/kg dose were higher than the ones obtained with LAMB. Differences in the particle size between AMB:DCH 1:1.5 and LAMB formulations may explain the differences observed in the distribution of AMB to the lungs because a higher particle size will allow a faster opsonization by macrophages (11). This may also explain the high AMB concentrations reached with both experimental AMB formulations in organs rich in macrophages like the lungs (14). These lung concentrations would be adequate to treat aspergillosis (MIC 0.5 - 8 µg/ml) (26). Furthermore, when experimental AMB:DCH 1:1.5 formulation was administered, the threshold pulmonary concentration (>4 µg/g) required for

292 therapeutic efficacy of LAMB treatment in a murine model of pulmonary
293 aspergillosis (27), was exceeded. Pulmonary AMB concentrations obtained with
294 LAMB after multiple doses were similar to the ones obtained with different AMB
295 formulations by other authors (27, 28). AMB:DCH 1:1.5 formulation had the
296 greatest cumulative effect on lung tissue compared to LAMB formulation (25).

297 Treatments consisting on AMB:DCH 1:1.5 and the gold standard formulation
298 (LAMB) have shown equivalent efficacy in terms of survival and reduction of tissue
299 burden despite AMB levels were higher in lungs of animals receiving AMB:DCH
300 than in those treated with LAMB. This may be due to the immunomodulatory effect
301 of the drug carrier since it has been demonstrated that empty liposomes show
302 efficacy against IPA modelled in corticosteroid-immunosuppressed mice. The role
303 of empty liposomes, as well as DCH, into the progression and outcome of the
304 infection deserves to be further explored (29). In addition, a comparable decrease
305 of the percentage of weight loss in the different groups was observed. A similar
306 relation between survival times and weight losses results has been previously
307 observed in different AMB aspergillosis treatments (7, 30).

308 Our pulmonary model has demonstrated high pulmonary affectation with viable
309 fungal elements at day 8 post infection with low renal affectation suggesting that
310 the A1160 strain has poorly disseminated to other organs+8 days post-challenge
311 (31). The absence of nephrotoxicity after treatment allow us to consider that
312 nephrotoxicity results in uninfected mice could be adequate to compare AMB:DCH
313 1:1.5 and LAMB at doses of 5 mg/kg. Previous studies showed similar BUN values
314 between infected and uninfected mice in LAMB and ABLC treatments (22).

315 The high mortality displayed by the infected control group was due to the
316 fungal burden (32, 33). In addition, tissue burden study performed 8 days post
317 infection showed great fungal clearance in lungs of those animals receiving 5
318 mg/kg of AMB:DCH 1:1.5 while all mice receiving with 5 mg/kg of LAMB showed
319 slightly higher levels of CFUs. These results were consistent with the lung
320 concentration data obtained in uninfected mice. Probably, the highest particle size
321 of AMB:DCH 1:1.5 formulation produced a faster biodistribution to lung tissue
322 (9.173 ± 0.498 µg/g at 24 h) and a greater fungal burden clearance (34). The drug
323 fast clearance of lung tissue for AMB:DCH 1:1.5 formulation might explain the
324 decrease in survival rate (+8 day) (see Fig 3). Nevertheless, LAMB at doses of 5
325 mg/kg had low lung concentration values after the initial dose in uninfected mice
326 (2.527 ± 0.386 µg/g at 24 h). This low initial biodistribution could be related to an
327 increase in fungal burden during the first stage of the infection in this group (27).
328 Doses of 10 mg/kg of LAMB formulation would be required in order to obtain a
329 significant reduction in *A. fumigatus* growth, during the first stage of the infection
330 (27, 35).

331 The fungal burden results obtained on day +12 confirm that this *A. fumigatus*
332 infection model by pulmonary inhalation poorly disseminated to kidneys (33).
333 Differences in lung tissue CFUs values for AMB:DCH 1:1.5 and LAMB treatment
334 may be related to the AMB concentrations due to the different particle size
335 between both formulations (11, 15). A decrease in CFUs for LAMB formulation at
336 the end of the study (+12 day) was related to a smaller particle size and
337 morphological characteristic of liposome form of AMB (30, 36). Thus, small spheres
338 of commercial AMB formulation (LAMB) remain in the lungs 12-48 hours after

administration (7, 28). These results from different formulations suggest that their morphology and particle size could affect the pharmacokinetic/pharmacodynamic characteristics of the different AMB formulations.

Nevertheless, a limitation of this study, performing the pharmacokinetic comparisons between uninfected mice and infected mice is the fact that the AMB concentrations that would be reached in infected mice would be higher than the ones obtained in this study in uninfected mice (22, 27). So, the similar efficacy observed with AMB:DCH 1:1.5 and LAMB would be related to concentrations higher than 4 µg/g in infected animals (AMB concentration associated with a significant reduction of lung fungal burden) with both formulations and not only the concentrations observed with uninfected animals (see Fig. 1) in the first 24 hours (27).

In conclusion, micellar poly-aggregated AMB:DCH 1:1.5 formulation showed 2.8-fold higher AMB concentration in lungs than commercial LAMB formulation after 6 doses of 5 mg/kg and a slightly lower nephrotoxicity due to the formulation characteristics. The improved pulmonary efficacy/toxicity ratio allows the micellar AMB:DCH 1:1.5 formulation to be considered a good alternative to LAMB liposomes for the treatment of pulmonary aspergillosis. Future studies with infected mice will be performed to evaluate the pharmacokinetic/pharmacodynamic efficacy of treatments with experimental AMB:DCH 1:1.5 and commercial LAMB formulations on its own compared to treatments combining different doses of these formulations.

MATERIALS AND METHODS

Drugs. Amphotericin B (AMB) (PubChem CID: 5280965) was supplied supply by Bristol Myers Squibb (Barcelona, Spain) and LAMB (Ambisome®) was supplied by UCB-Pharma (Brussels, Belgium).

Preparation of formulations. Experimental micellar formulation was prepared with AMB and DCH at a ratio of 1:1.5 w/w of drug to carrier proportions. The preparation method was as follows. 18.75 mg of DCH (PubChem CID: 23668196) purchased from Fluka-Biochemika (Bucks, Switzerland) were dissolved in 50 ml of water for injection and then 12.5 mg of AMB was added and vortexed during 4 minutes at 2400 rpm. These suspensions were diluted in 5% dextrose solution, as necessary, and vortexed during 4 minutes at 2400 rpm and stored in darkness at 25ºC. For the characterization of the aggregation state of the formulations, an AMB standard solution (M-AMB) was required. This formulation was prepared as follows. 10.0 mg of AMB were dissolved in 500 ml of methanol and vortexed during 4 minutes at 2400 rpm. M-AMB formulation was diluted in 5% dextrose solution, as necessary.

AMB formulations characterization. Different AMB:DCH formulations in water (AMB:DCH 1:0 and AMB:DCH 1:1.5) and M-AMB formulation in methanol were spectrophotometrically analyzed by UV absorption to determine the aggregation state of AMB. A scanning spectrum of each formulation was recorded by Shimadzu UV-1700 spectrophotometer (Kyoto, Japan) between 300-450 nm. Each sample was analyzed in triplicate.

The particle size of AMB:DCH formulations in water was analyzed by Microtrac® S-3500, (Microtrac Inc, Montgomeryville, USA). Mean size (nm) was

386 determined based on size distribution in number. Each sample was analyzed in
387 triplicate.

388 **Animals.** Four weeks-old OF-1 male mice weighting between 27-32 g were
389 used (Criffa S.A., Barcelona, Spain) in all experiments. All animals were housed
390 under standard conditions with water and food *ad libitum*. All animal care
391 procedures were supervised and approved by the Complutense University Animal
392 Welfare and Ethics Committee as well as the Universitat Rovira i Virgili Animal
393 Welfare and Ethics Committee.

394 **AMB biodistribution to kidneys and lungs.** This study was performed in
395 immunosuppressed uninfected mice in order to evaluate the AMB concentrations in
396 kidneys and lungs after single dose or 6 daily doses of AMB (5mg/kg/day).

397 Four groups of 6 animals each, were immunosuppressed by subcutaneous
398 injection of cortisone acetate (125 mg/kg) every 3 days (37). Five days after
399 immunosuppression began, animals received a single dose of AMB:DCH 1:1.5 or
400 LAMB both administered intravenously (i.v.) at doses of 5 mg/kg or multiple doses
401 administered once daily (QD) at doses of 5 mg/kg for six days.

402 To determine the concentrations of AMB in kidney and lung tissues, mice were
403 euthanized 24 hours after the last administration. Organs were aseptically removed
404 and frozen until used. Then, organs were homogenized in 0.5 ml of water for
405 injection and determination of total AMB concentration in tissue was performed by
406 the High Performance Liquid Chromatography (HPLC) method using a modification
407 of previously published assays (38). Briefly, all HPLC assays were performed using
408 the modular system Jasco® (Tokyo, Japan) that consisted of an automatic sample
409 injection (A5-2050), a high pressure pump (PU-1580) and an UV detector (1575),

operating at 406 nm. Analyses were performed on a Hypersil BDS® C18 column (5 µm, 250 mm x 4.6 mm). The column was isocratically eluted at a flow rate of 1 ml/min with 40:4.3:55.7 v/v/v acetonitrile/acetic acid/water mobile phase. The injection volume was 100 µl. Each sample was analyzed in triplicate.

Homogenized tissue samples were spiked with meloxicam (10 µl at 200 µg/ml). Two extractions of the homogenate aqueous tissue were carried out with methanol (400 µl × 2). After every extraction, the mixture was vortexed (2500 rpm, 2 min) and then centrifuged (4000 rpm, 4 min). The supernatant was filtered using a filter HVLP of 0.45 µm. Previous to the homogenization and analysis by HPLC, each organ was weighed weight and the results were expressed as µg/g of each organ. Under these conditions, the relative retention times of AMB and the internal standard were 8.5 and 6.1 min, respectively. Tissue AMB concentrations were calculated from linear regression calibration curves of the AMB/internal standard peak height ratio. The linear range in plasma was 0.01 to 10 µg/ml ($y=0.2191x-0.0026$; $R^2=0.9986$).

Drug nephrotoxicity. This study was performed in immunocompetent uninfected mice. Thirty five mice were divided in seven groups (n = 5) and treated i.v. with 5 or 20 mg/kg/day of LAMB, AMB:DCH 1:0, AMB:DCH 1:1.5 or placebo (5% dextrose) for six days. Blood samples were collected by cardiac puncture 24 h after the sixth treatment. Creatinine and blood urea nitrogen (BUN) were analysed in serum using a modular AutoAnalyzer Cobas 711 (Roche, Basel, Switzerland).

Histopathological evaluations were also performed on the renal tissues in these seven groups of mice. Kidneys were collected 24 h after the last drug treatment. Tissues were fixed in 4% paraformaldehyde (24 h) and paraffin-embedded. Cut

tissue sections of 5 µm were mounted on glass slides, rehydrated in water, and stained with haematoxylin and eosin (H&E, Sigma-Aldrich, St. Louis, MO). All tissues were examined and microphotographs of sections with x20 magnification were taken from the stained samples using an inverted IX70 microscope (Olympus, Hamburg, Germany).

Murine model of pulmonary aspergillosis. *A. fumigatus* strain A1160 was cultured on PDA and incubated at 37°C for 5 days. A conidial suspension was obtained by flooding the culture plate with 5 ml of saline with 0.05% Tween 20, scraping the fungal growth with a culture loop and drawing up the resultant suspension with a Pasteur pipette. The suspension was then filtered to remove clumps of hypha or agar and inoculum adjusted by hemocytometer count and serial dilution to the desired concentration. Animals were immunosuppressed by subcutaneous injection of cortisone acetate (125 mg/kg) starting 4 days prior infection and then every 3 days (37). Infection was performed by nasal instillation of $5x10^4$ CFUs/mouse in 20µl prior anesthesia with inhaled sevoflurane.

Efficacy study. Infection was performed by nasal instillation of $5x10^4$ CFUs/mouse in 20 µL prior anaesthesia with inhaled sevoflurane.

Groups of 16 animals each, received LAMB or AMB:DCH 1:1.5. Both formulations were administered i.v. at a dose of 5 mg/kg QD for 7 days. Control group received placebo (5% dextrose). Eight animals per group were used to CFU determination (+8 days) and the rest 8 animals to survival follow up for 12 days.

Twelve days post-infection, surviving animals from CFUs groups were euthanized by CO_2 inhalation followed by cervical dislocation. Kidneys and lungs

were aseptically removed, homogenized in 0.9% saline, 10-fold diluted and placed on PDA plates for CFU/g determination (+12 days).

Statistical analysis. Results related to particle size studies were expressed as mean values ± standard deviation of three measures and tested by the independent-sample *t* test. Results related to AMB concentrations were tested by Mann-Whitney test. Fungal burden data was analyzed by Mann-Whitney test and survival curves were compared using the log rank test. Statistical differences were performed via one-way ANOVA test using Minitab 15 (Minitab Ltd., Coventry, U.K.). Tukey's test was used for paired-group comparisons. A *P* value of less than 0.05 was considered to indicate statistical significance.

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REFERENCES

1. Fortún J, Meije Y, Fresco G, Moreno S. 2012. Aspergillosis. Clinical forms and treatment. Enferm Infecc Microbiol Clin 30(4):201-208.

2. Voltan AR, Quindós G, Medina KP, Fusco-Almeida AM, Soares MJ, Chorilli M. 2016. Fungal diseases: could nanostructured drug delivery systems be a novel paradigm for therapy? Int J Nanomedicine 11:3715-3730.
3. Walsh TJ, Anaissie EJ, Denning DW, Herbrecht R, Kontoyiannis DP, Marr KA. 2008. Treatment of aspergillosis: clinical practice guidelines of the Infectious Diseases Society of America. Clin Infect Dis 70(3):327-360.
4. Clemons KV, Schwartz JA, Stevens DA. 2012. Experimental central nervous system aspergillosis therapy: efficacy, drug levels and localization, immunohistopathology, and toxicity. Antimicrob Agents Chemother 56(8):4439-4449.
5. Wang Y, Ke X, Voo ZX, Yap SSL, Yang C, Gao S, Liu S, Venkataraman S, Obuobi SAO, Khara JS, Yang YY, Ee PLR. 2016. Biodegradable functional polycarbonate micelles for controlled release of amphotericin B. Acta Biomater 46:211-220.
6. Moreno-Rodríguez AC, Torrado-Durán S, Molero G, García-Rodríguez JJ, Torrado-Santiago S. 2015. Efficacy and toxicity evaluations of new amphotericin B micelle systems for brain fungal infections. Int J Pharm 494(1):17-22
7. Olson JA, Adler-Moore JP, Jensen GM, Schwartz J, Dignani MC, Proffitt RT. 2008. Comparison of the physicochemical, antifungal, and toxic properties of two liposomal amphotericin B products. Antimicrob Agents Chemother 52(1):259-268.

8. Nahar M, Mishra D, Dubey V, Kumar Jain N. 2008. Development, characterization and toxicity evaluation of amphotericin B-loaded gelatin nanoparticles. Nanomedicine 4(3):252-261.
9. Ishida K, Alves Castro R, Torrado JJ, Serrado DR, Pereira Borba-Santos L, Pereira Quintella L, de Souza W, Rozental S, Lopes-Bezerra LM. 2017. Efficacy of a poly-aggregated formulation of amphotericin B in treating systemic sporotrichosis caused by *Sporothrix brasiliensis*. Med Mycol 0:1-9.
10. Mariné M, Espada R, Torrado JJ, Pastor FJ, Guarro J. 2009. Efficacy of a new formulation of amphotericin B in murine disseminated infections by *Candida glabrata* or *Candida tropicalis*. Int J Antimicrob Agents 34(6):566-569.
11. Serrano DR, Hernández L, Fleire L, González-Alvarez I, Montoya A, Ballesteros MP, Dea-Ayuela MA, Miró G, Bolás-Fernández F, Torrado JJ. 2013. Hemolytic and pharmacokinetic studies of liposomal and particulate amphotericin B formulations. Int J Pharm 447(1-2):38-46.
12. Souza AC, Nascimiento AL, Vasconcelos NM, Jerônimo MS, Siqueira IM, R-Santos L, Cintra DO, Fuscaldi LL, Pires Júnior OR, Titze-de-Almeida R, Borin MF, Báo SN, Martins OP, Cardoso VN, Fernandes SO, Mortari MR, Tedesco AC, Amaral AC, Felipe MS, Bocca AL. 2015. Activity and *in vivo* tracking of Amphotericin B loaded PLGA nanoparticles. Eur J Med Chem 95:267-276.
13. Zhao M, Hu J, Zhang L, Zhang L, Sun Y, Ma N, Chen X, Gao Z. 2014. Study of amphotericin B magnetic liposomes for brain targeting. Int J Pharm 475(1-2):9-15.
14. Lewis RE, Albert ND, Liao G, Hou J, Prince RA, Kontoyiannis DP. 2010. Comparative pharmacodynamics of amphotericin B lipid complex and

liposomal amphotericin B in a murine model of pulmonary mucormycosis. Antimicrob Agents Chemother 54(3):1298-1304.

15. Espada R, Valdespina S, Alfonso C, Rivas G, Ballesteros MP, Torrado JJ. 2008. Effect of aggregation state on the toxicity of different amphotericin B preparations. Int J Pharm 36(1-2):64-69.
16. Faustino C, Serafim C, Ferreira I, Pinheiro L, Calado A. 2015. Solubilization power of amino acid-based gemini surfactant towards the hydrophobic drug amphotericin B. Colloids Surf A Physicochem Eng Asp 480:426-432.
17. Zu Y, Sun W, Zhao X, Wang W, Li Y, Ge Y, Liu Y, Wang K. 2014. Preparation and characterization of amorphous amphotericin B nanoparticles for oral administration through liquid antisolvent precipitation. Eur J Pharm Sci 53:109-117.
18. Tan SW, Billa N, Roberts CR, Burley JC. 2010. Surfactant effects on the physical characteristics of amphotericin B-containing nanostructured lipid carriers. Colloids Surf A Physicochem Eng Asp 372:73-79.
19. Jung SH, Lim DH, Jung SH, Lee JE, Jeong KS, Seong H, Shin BC. 2009. Amphotericin B-entrapping lipid nanoparticles and their *in vitro* and *in vivo* characteristics. Eur J Pharm Sci 37(3-4):313-320.
20. Clark JM, Whitney RR, Olsen SJ, George RJ, Swerdel MR, Kunselman L, Bonner DP. 1991. Amphotericin B lipid complex therapy of experimental fungal infections in mice. Antimicrob Agents Chemother 35(4):615-621.
21. Wasan KM, Grossie Jr VB, Lopez-Berestein G. 1994. Concentrations in serum and distribution in tissue of free and liposomal amphotericin B in rats during continuous intralipid infusion. Antimicrob Agents Chemother 38(9):2224-2226.

22. Olson JA, Adler-Moore JP, Schwartz J, Jensen GM, Proffitt RT. 2006. Comparative efficacies, toxicities, and tissue concentrations of amphotericin B lipid formulations in a murine pulmonary aspergillosis model. Antimicrob Agents Chemother 50:2122–2131.
23. Zia Q, Khan AA, Swaleha Z, Owais M. 2015. Self-assembled amphotericin B-loaded polyglutamic acid nanoparticles: preparation, characterization and in vitro potential against *Candida albicans*. Int J Nanomedicine 10:1769-1790.
24. Fernández-García R, de Pablo E, Ballesteros MP, Serrano DR. 2017. Unmet clinical needs in the treatment of systemic fungal infections: The role of amphotericin B and drug targeting. Int J Pharm 525:139-148.
25. Adler-Moore JP, Proffitt RT. 2008. Amphotericin B lipid preparations: what are the differences? Clin Microbiol Infect 14 (4): 25-36.
26. Lass-Flörl C, Alastruey-Izquierdo A, Cuenca-Estrella M, Perkhofer S, Rodriguez-Tudela JL. 2009. In Vitro Activities of Various Antifungal Drugs against *Aspergillus terreus*: Global Assessment Using the Methodology of the European Committee on Antimicrobial Susceptibility Testing. Antimicrob Agents Chemother 53(2):794-795.
27. Lewis RE, Liao G, Hou J, Chamilos G, Prince RA, Kontoyiannis DP. 2007. Comparative analysis of amphotericin B lipid complex and liposomal amphotericin B kinetics of lung accumulation and fungal clearance in a murine model of acute invasive pulmonary aspergillosis. Antimicrob Agents Chemother 51(4):1253-1258.

28. Takemoto K, Yamamoto Y, Ueda Y, Sumita Y, Yoshida K, Niki Y. 2006. Comparative study on the efficacy of AmBisome and Fungizone in a mouse model of pulmonary aspergillosis. J Antimicrob Chemother 57(4):724-731.
29. Lewis RE, Chamilos G, Prince RA, Kontoyiannis DP. 2007. Pretreatment with empty liposomes attenuates the immunopathology of invasive pulmonary aspergillosis in corticosteroid-immunosuppressed mice. Antimicrob Agents Chemother 51(3):1078-1081.
30. Olson JA, Schwartz JA, Hahka D, Nguyen N, Bunch T, Jensen GM, Adler-Moore JP. 2015. Toxicity and efficacy differences between liposomal amphotericin B formulations in uninfected and *Aspergillus fumigatus* infected mice. Med Mycol 53(2):107-118.
31. Barchiesi F, Santinelli A, Biscotti T, Greganti G, Giannini D, Manso E. 2016. Delay of antifungal therapy influences the outcome of invasive aspergillosis in experimental models of infection. J Antimicrob Chemother 71(8):2230-2233.
32. Lewis RE, Albert ND, Kontoyiannis DP. 2014. Comparative pharmacodynamics of posaconazole in neutropenic murine models of invasive pulmonary aspergillosis and mucormycosis. Antimicrob Agents Chemother 58(11):6767-6772.
33. Gavaldà J, Martín T, López P, Gomis X, Ramírez JL, Rodríguez D, Len O, Puigfel Y, Ruiz I, Pahissa A. 2005. Efficacy of high loading doses of liposomal amphotericin B in the treatment of experimental invasive pulmonary aspergillosis. Clin Microbiol Infect 11(12):999-1004.
34. Groll AH, Lyman CA, Petraitis V, Petraitiene R, Armstrong D, Mickiene D, Alfaro RM, Schaufele RL, Sein T, Bacher J, Walsh TJ. 2006.

597 Compartmentalized intrapulmonary pharmacokinetics of amphotericin B and its
598 lipid formulations. Antimicrob Agents Chemother 50(10):3418-3423.
599 35. Clemons KV, Espiritu M, Parmar R, Stevens DA. 2005. Comparative efficacies
600 of conventional amphotericin B, liposomal amphotericin B (AmBisome),
601 caspofungin, micafungin, and voriconazole alone and in combination against
602 experimental murine central nervous system aspergillosis. Antimicrob Agents
603 Chemother 49(12):4867-4875.
604 36. Adler-Moore JP, Gangneux JP, Pappas PG. 2016. Comparison between
605 liposomal formulations of amphotericin B. Med Mycol 54(3):223-231.
606 37. Dixon DM, Polak A, Walsh TJ. 1989. Fungus dose-dependent primary
607 pulmonary aspergillosis in immunosuppressed mice. Infect Immun 57(5):1452-
608 1456.
609 38. Corral MJ, Serrano DR, Inmaculada Moreno I, Torrado JJ, Domínguez M,
610 Alunda JM. 2014. Efficacy of low doses of amphotericin B plus allicin against
611 experimental visceral leishmaniasis. J Antimicrob Chemother 69(12):3268-
612 3274.

613

614 **FIGURE LEGENDS**

615 **Figure 1** Mean and standard deviation of AMB concentrations (µg/g) in (A) kidneys
616 and (B) lungs of immunosuppressed mice (n=6 mice/group) intravenously treated
617 with 1 or 6 daily doses of 5 mg/kg of AMB:DCH 1:1.5 or LAMB. * LAMB treatments
618 with 1 or 6 daily doses were significantly different from AMB:DCH 1:1.5 ($P<0.01$).

619 **Figure 2** Histopathology of kidneys from no-immunosuppressed, uninfected mice
620 following intravenous treatment with AMB formulations at a 6 daily therapeutic dose

of 5 mg/kg/day. (A) Appearance of normal renal tissue in a kidney from control group. (B) Tissue from mice treated with AMB:DCH 1:1.5. (C) Tissue from mice treated with AMB:DCH 1:0. (D) Tissue from mice treated with commercial formulation (LAMB). Arrow: inflammatory cells. Arrow head: tubular epithelial damage. Asterisk: intraluminal protein casts. Bar, 100 µm.

Figure 3 (A) Survival of mice (n= 8 mice/group) challenged intranasally with 20 µl of *A. fumigatus* (5 x 10^4 conidia/ml) and then intravenously treated with 5 mg/kg for 6 days with placebo (5% dextrose), 5 mg/kg LAMB, and 5 mg/kg AMB:DCH 1:1.5. (B) Mean percent weight change in uninfected immunosuppressed (n= 8/group): 5 mg/kg LAMB and 5 mg/kg AMB:DCH 1:1.5; and in infected mice (n= 8/group): 5 mg/kg LAMB, and 5 mg/kg AMB:DCH 1:1.5. * $P<0.05$ in comparison to control group.

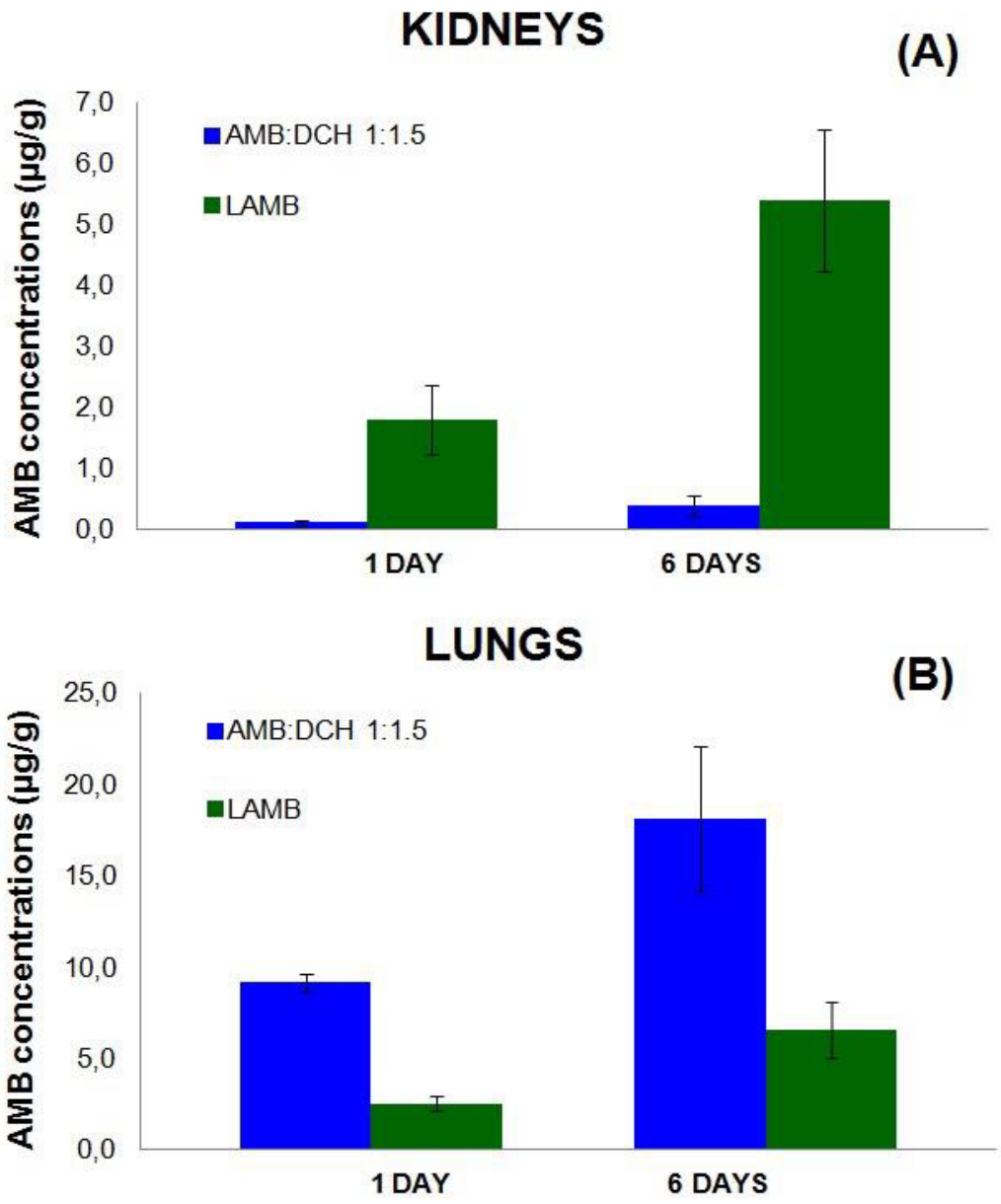

Figure 1 Mean and standard deviation of AMB concentrations (µg/g) in (A) kidneys and (B) lungs of immunosuppressed mice (n=6 mice/group) intravenously treated with 1 or 6 daily doses of 5 mg/kg of AMB:DCH 1:1.5 or LAMB. * LAMB treatments with 1 or 6 daily doses were significantly different from AMB:DCH 1:1.5 ($P<0.01$).

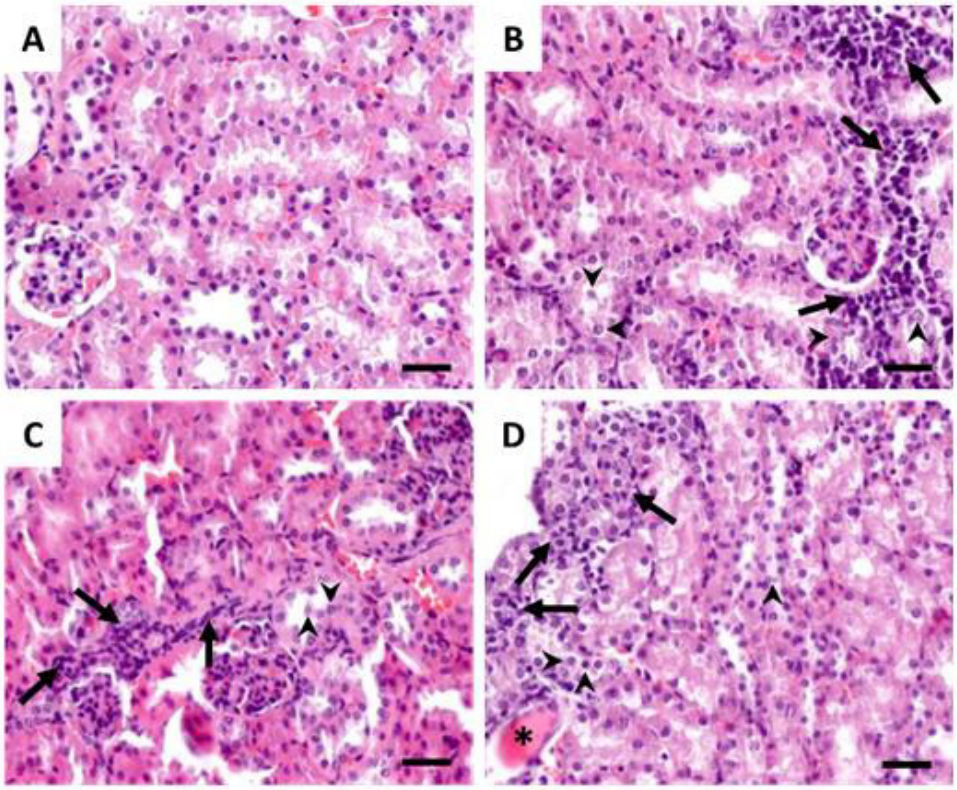

Figure 2 Histopathology of kidneys from no-immunosuppressed, uninfected mice following intravenous treatment with AMB formulations at a 6 daily therapeutic dose of 5 mg/kg/day. (A) Appearance of normal renal tissue in a kidney from control group. (B) Tissue from mice treated with AMB:DCH 1:1.5. (C) Tissue from mice treated with AMB:DCH 1:0. (D) Tissue from mice treated with commercial formulation (LAMB). Arrow: inflammatory cells. Arrow head: tubular epithelial damage. Asterisk: intraluminal protein casts. Bar, 100 µm.

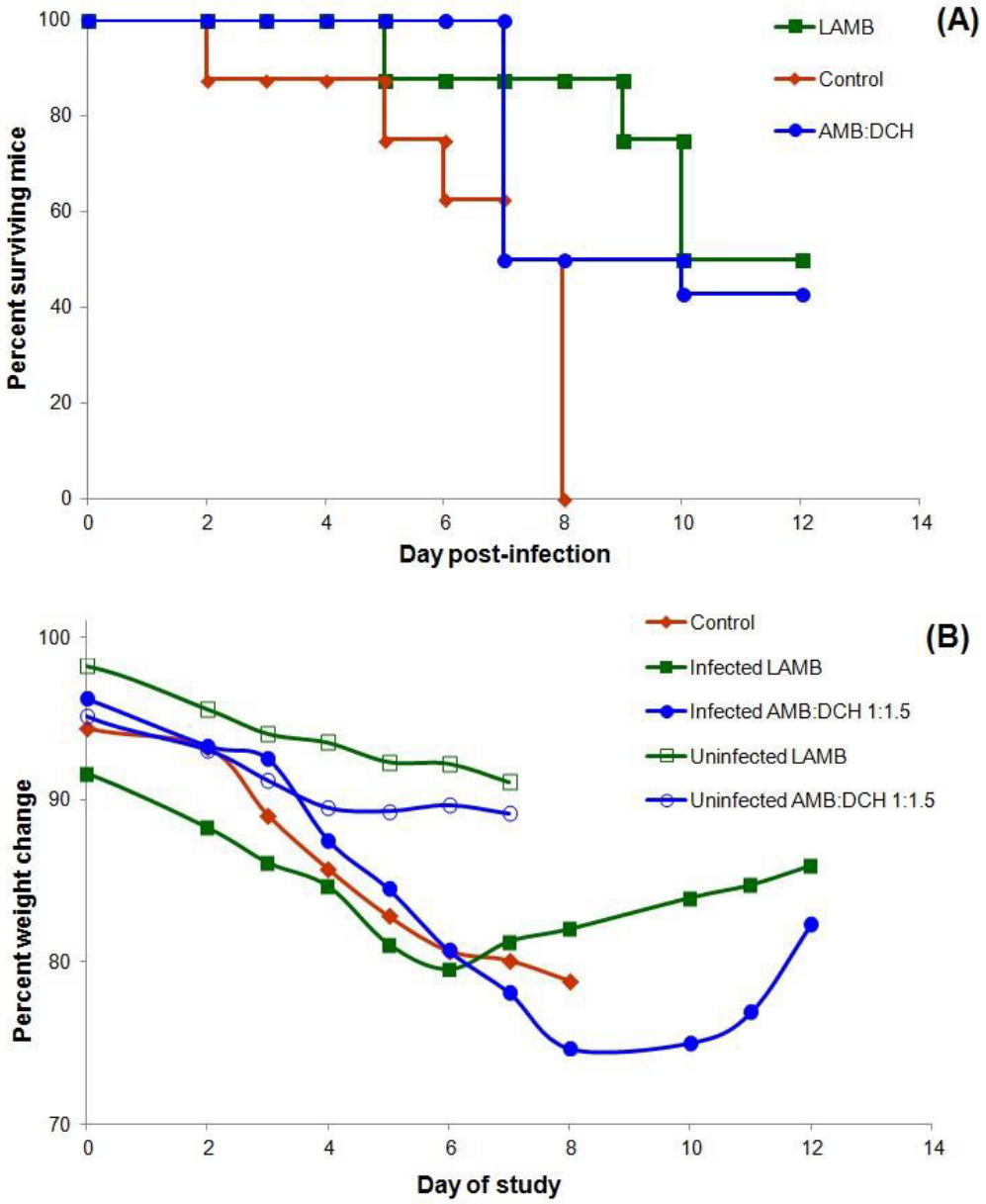

Figure 3 (A) Survival of mice (n= 8 mice/group) challenged intranasally with 20 µl of *A. fumigatus* (5 x 10^4 conidia/ml) and then intravenously treated with 5 mg/kg for 6 days with placebo (5% dextrose), 5 mg/kg LAMB, and 5 mg/kg AMB:DCH 1:1.5. (B) Mean percent weight change in uninfected immunosuppressed (n= 8/group): 5 mg/kg LAMB and 5 mg/kg AMB:DCH 1:1.5; and in infected mice (n= 8/group): 5 mg/kg LAMB, and 5 mg/kg AMB:DCH 1:1.5. * $P<0.05$ in comparison to control group.

Table 1 Creatinine and BUN concentrations (mg/dl) in uninfected mice 24 h after a 6 daily dose of the indicated amphotericin B formulation group: Control; LAMB (20 mg/kg), LAMB (5 mg/kg), AMB:DCH 1:0 (20 mg/kg), AMB:DCH 1:0 (5 mg/kg), AMB:DCH 1:1.5 (20 mg/kg), AMB:DCH 1:1.5 (5 mg/kg).

Formulation	Doses (mg/kg)	Creatinine $\bar{x}$ ± SD (mg/dl)	BUN $\bar{x}$ ± SD (mg/dl)
Control	---	0.18 ± 0.008	18.29 ± 0.609
LAMB	20	0.23 ± 0.034*	18.160 ± 0.963*
	5	0.24 ± 0.018*	18.29 ± 0.609*
AMB:DCH 1:0	20	0.22 ± 0.028*	17.27 ± 0.875*
	5	0.23 ± 0.005*	17.73 ± 1.276*
´AMB:DCH 1:1.5	20	0.20 ± 0.011*	18.20 ± 0.748*
	5	0.21 ± 0.005*	19.88 ± 0.762*

* LAMB treatments at 20 and 5 mg/kg doses were significantly different from control group ($P<0.01$ and $P<0.001$, respectively).

AMB:DCH 1:0 treatments at 20 and 5 mg/kg doses were significantly different from control group ($P=0.035$ and $P<0.01$, respectively).

AMB:DCH 1:1.5 treatments at 20 and 5 mg/kg doses were not significantly different from control group ($P=0.055$ and $P=0.057$, respectively).

LAMB treatments at 20 and 5 mg/kg doses were significantly different from AMB:DCH 1:1.5 ($P=0.005$ and $P=0.007$, respectively).

All treatments were not significantly different from control group ($P>0.05$).