

Supporting information

Oral particle Uptake and Organ Targeting Drives the Activity of Amphotericin B Nanoparticles

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1. Methods

1.1 Materials

AmB was supplied by Azelis (Barcelona, Spain). AmBisome[®] was purchased from Gilead Sciences S.L. (Madrid, Spain). All reagents and chemicals used without further purification were obtained from Sigma Aldrich Chemical Co (Poole, UK or Madrid, Spain) and solvents (HPLC grade) and acids from Fisher Scientific (Loughborough, UK) or VWR (Lutterworth, UK) unless otherwise stated. Visking dialysis membranes were obtained from Medicell International Ltd. (London, UK). Palmitic acid D31.98% and methanol-d₆ were purchased from Cambridge Isotope Laboratories, Inc (Cheshire, UK). Sodium deoxycholate was supplied by Fluka Chemie AG (Buchs, Switzerland) and dibasic sodium phosphate and monobasic sodium phosphate were purchased from Panreac S.A. (Barcelona, Spain).

1.2 Polymer synthesis and characterization

Briefly, acid degradation of glycol chitosan (GC) was carried out dissolving GC (5 g) in hydrochloric acid (4 M, 380 mL) at 50 °C for 24 h. The purification of the product was performed by exhaustive dialysis (MWCO = 3500 Da) against water over 24 h. After dialysis, the product was lyophilised obtaining 2.57 g of a cream-colored cotton-like solid (GC). GC (2.57 g) and sodium bicarbonate (1.93 g) were dissolved in 515 mL of ethanol: water (1:3.167). Palmitic acid N-hydroxysuccinimide (4.07 g) was dissolved in ethanol (772 mL) and then was added drop wise into the GC solution mixture with continuous stirring for 72 h protected from light. Afterwards, ethanol was removed by evaporation and the remaining aqueous phase was extracted with diethyl ether (x 3) followed by exhaustive dialysis (MWCO = 12000 Da) against water over 24 hours. After dialysis, the product was freeze-dried resulting in 4.67 g of white cotton-like solid (PGC). PGC (4.67 g) was dispersed in N-methyl-2-pyrrolidone (392 mL) for 1 h at room temperature. Sodium hydroxide (625.4 mg), sodium iodide (696.9 mg) and methyl iodide (7.819 mL or 17.82 g) were added and the reaction mixture was left stirring at 36 °C under nitrogen flow for 3 h. Then, diethyl ether was added to the reaction mixture to precipitate GCPQ. The resulting solid was dispersed in water and dialysed (MWCO 7000 Da) exhaustively against water over 24 hours. After that, the quaternary ammonium iodide salt was passed through an Amberlite column (IRA-96 Cl-1) previously packed and washed firstly with hydrochloric acid (1 M) and then with water until a neutral pH had been achieved. After passing through the column, the clear eluate (GCPQ) was lyophilised resulting in a light brown fibrous solid (2.43 g).

Gel Permeation Chromatography- Multiangle Laser Light Scattering. The molecular weights of GC24 and GCPQ were determined as previously described^{6, 10} using a Wyatt gel permeation chromatography-multi-angle light scattering instrument

equipped with Dawn Heleos II MALLS detector ($\lambda = 658$ nm), Optilab rEX interferometric refractometer ($\lambda = 658$ nm) and quasielastic light scattering (QELS) detectors (Wyatt technology Corporation, Santa Barbara, CA, USA).

Fourier-Transformed Infrared spectroscopy (FTIR). The infrared absorption spectra was recorded as previously described⁷ by using a Perkin-Elmer Spectrum 100 FTIR spectrometer equipped with a Universal Attenuated Total Reflectance accessory and a zinc selenide crystal (4000-650 cm^{-1}) and Spectrum FTIR software.

Transmission Electron Microscopy (TEM). TEM was performed using a FEI CM120 BioTwin transmission electron microscope (Philips, Eindhoven, The Netherlands) operating at 120 KV. AMT digital camera was used to capture the images. A drop of sample was placed onto a Formvar/carbon coated grid, blotting off the excess sample with Whatman No. 1 filter paper. Samples were negatively stained with 1% w/v uranyl acetate aqueous solution.

Photon Correlation Spectroscopy (PCS). Particle size was measured by PCS on a Malvern Zetasizer 3000 HSA (Malvern Instruments, Malvern, UK) at 25 °C, a wavelength of 633 nm and a detection angle of 90°. Data was analysed by the Contin method of size distribution analysis. Mean size (nm) was determined based on size distribution in volume. Prior to measurements, polystyrene standards (diameter = 100 nm) were measured; size results were in accordance with the nominal size of the standard particles.

Spontaneous Raman Scattering Spectroscopy. Spontaneous Raman spectra were acquired with a Renishaw RM1000 Raman microscope (Renishaw, Wotton-Under-Edge, UK) using a 1200-line/mm grating. This yielded a spectral resolution of 1cm^{-1} . A

diode laser provided up to 300 mW power excitation light at 785 nm. Calibration of the system was undertaken using the Raman band of a silicon wafer at 520 cm^{-1} . Spectral data were acquired with Renishaw v.1.2 WiRE software.

Multimodal Multiphoton Microscopy. An optical parametric oscillator (OPO) (Levante Emerald, APE, Berlin) pumped with a frequency-doubled Nd:Vanadium picosecond oscillator (High-Q Laser Production GmbH) was used to give the dual wavelength picosecond excitation required for Coherent Anti-Stokes Raman Spectroscopy (CARS) microscopy. The frequency-doubled 6 ps, 76 MHz pulse train pump laser generated light at 532 nm, while the pump laser fundamental at 1064 nm was also available as a separate output. For CARS, the signal beam from the OPO was used as the pump, with output wavelengths ranging from 670 to 980 nm and the idler was used as the Stokes beam, ranging between 1130 nm and 1450 nm. For SRS, the 1064 nm laser fundamental was used as the pump beam and was passed through an acousto-optic modulator which provided amplitude modulation at 1.7 MHz, while the signal output from the OPO was used as the Stokes beam. The total combined power of both the signal and idler beams was approximately 2 W, while the average power at the sample was kept between 15 mW and 30 mW through the use of attenuating filters.

Two-photon auto-fluorescence (TPF) and second harmonic generation (SHG) signals were generated with a mode-locked femtosecond Ti:sapphire oscillator (Mira 900D; Coherent, USA) which produced 100-fs pulses at 76 MHz. The central wavelength of the fs beam was 800 nm with an average power at the sample that was attenuated to between 5 and 30 mW. The femtosecond and picosecond beams were directed into a confocal laser scanner (FV300, Olympus UK). The beams were passed into an inverted microscope (IX71, Olympus UK) which was modified to optimize transmission of near

infra-red light, equipped with a 60X, 1.2 NA water immersion objective (UPlanS Apo, Olympus UK)

The CARS, TPF and SHG signals were collected using the objective lens in the epi-direction. The epi-CARS signal was separated from the pump and Stokes beams by a long-wave pass dichroic mirror (z850rdc-xr, Chroma Technologies, USA) and directed onto a R3896 photomultiplier tube at the rear microscope port. The anti-Stokes signal was isolated by a single band-pass filter centered at 750 nm (HQ750/210, Chroma Technologies USA). To detect TPF or SHG, the signal was first spectrally separated from the 800 nm excitation beam by a dichroic mirror (670dcxr; Chroma Technologies). After this, different bandpass filters were used to enable either TPF signal (CG-BG-39-1.00-1 and F70-500-3-PFU; CVI Melles Griot UK) or SHG signal (F10-400-5-QBL; CVI Melles Griot UK) to reach the photomultiplier tube.

SRS was detected in the forward direction by a 1.0NA condenser lens (LUMFI, Olympus) and detected by a large area photodiode (FDS1010, Thorlabs). A bandpass filter (850/90 nm, Chroma) was mounted in front of the detector to block the modulated 1064 nm beam. A lock-in amplifier (SR844, Stanford Research Systems) was used to detect the SRS signal with a time constant of 100 μ s. SRS images were generated by recording either the X or R outputs from the lock-in.

SRS Images of 512×512 pixels were acquired with pixel dwell time of 200 μ s, resulting in a total of acquisition time of 53 s per frame. All CARS, TPF and SHG images were acquired with a frame size of 512×512 pixels at a rate of ~ 11 seconds per frame. Depending on the field of view, pixel widths were between 0.33 μ m and 0.5 μ m while the step size in image z-stacks yielded voxels of 0.5 μ m in depth (see supporting information Figure S5).

1.3 Preparation, characterization, dissolution and stability studies of AmB formulations

Preparation of AmB deoxycholate (AMBd). AMBd was prepared with the same composition as the marketed reference formulation Fungizone[®]. Briefly, AmB (50 mg supplied by Azelis, Spain) was dispersed in 5 mL of an aqueous solution formed by adding sodium deoxycholate (41 mg, Fluka Chemie A.G., Buchs, Switzerland), dibasic sodium phosphate (10 mg) and monobasic sodium phosphate (0.9 mg, Panreac S.A., Barcelona, Spain) previously adjusted to pH 12.0 with sodium hydroxide (2 M, Panreac S.A., Barcelona, Spain). Once the drug was homogeneously dispersed, it was acidified to pH 7.4 adding orthophosphoric acid (2 M, Panreac S.A., Barcelona, Spain). Water was added to a final AmB concentration of 5 mg mL⁻¹.

Isocratic HPLC quantification of AmB. A validated analytical method utilising an Agilent 1200 series HPLC was used. Briefly, AmB was isocratically eluted using a Thermo Hypersil BDS C18 reverse-phase column (200 × 4.6 mm, 5 µm) and a mobile phase consisted of acetonitrile: acetic acid: water (52:4.3:43.7, v/v/v) with a flow rate of 1 mL min⁻¹. Absorbance was monitored at 406 nm, and the injection volume was set at 40 µL. The retention time of AmB was 6.9 min and linear regression calibration curve was obtained between 0.05 to 50 µg mL⁻¹ ($y=12.424x + 1.0095$; $R^2 = 1$).

Flow-through cell dissolution study (USP 4)

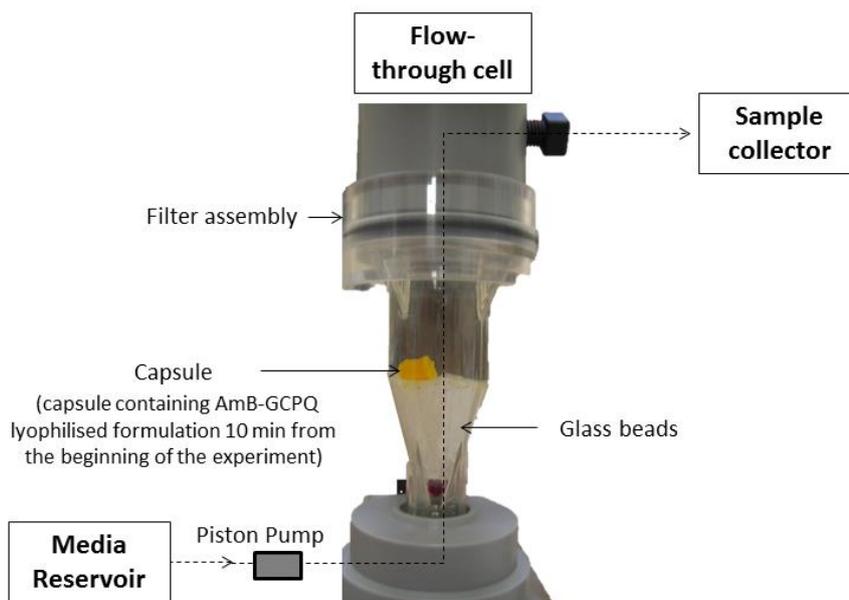


Figure S1. Schematic diagram of the open-loop configuration for the flow-through cell apparatus used for dissolution studies

Long-term stability studies

At various time intervals, the contents of each capsule were redispersed in deionised water (at 1 mg mL^{-1}) and drug content and particle size analysis were performed (Figure S3).

Drug content analysis. The redispersed AmB lyophilized formulation ($15 \mu\text{L}$) was further diluted up to 5 mL with a mixture (1:1) of methanol and mobile phase (acetonitrile: acetic acid: water, 53:4.3:43.7 v/v/v). AmB content was analysed by the isocratic HPLC method above described.

Particle size analysis. The redispersed AmB lyophilized formulation was further diluted (1:12) with deionized water and analysed by PCS, as described above.

1.4 Efficacy study in a systemic murine model of visceral leishmaniasis

Animals. BALB/c male inbred mice of 6-8 weeks of age were purchased from Harlan Iberica S.A. (Barcelona, Spain) and were allocated according to the standards of the committee of animal Welfare in plastic cages in the House Unit of the Complutense University of Madrid in controlled laboratory animal conditions (22 °C ambient temperature, 60% humidity and 12 h light and dark cycle). Food and water were available *ad libitum*. All experiments were approved by the Complutense University Institutional Animal Care and Ethics Committee.

Preparation of *Leishmania infantum* parasites for experimental infection. The preparation of the parasites and the experimental infection were performed as previously described. Briefly, *L. infantum* amastigotes M/CAN/ES/96/BCN150 (Zymodeme MON-1) were harvested from spleens of infected hamsters and cultured in NNN medium supplemented with penicillin (100 µg mL⁻¹) and streptomycin (100 mg mL⁻¹) for 5 days up to the time of transformation into promastigotes. Afterwards, they were transferred to Schneider medium supplemented with 0.4 g L⁻¹ sodium bicarbonate, 25 mM HEPES, 10% heat-inactivated foetal bovine serum (FBS), 100 µg mL⁻¹ penicillin and 100 µg mL⁻¹ streptomycin, at pH 6.8. Under these conditions, the resistance to complement lysis was determined to quantify the maximum number of metacyclic forms which was achieved at day 7. After 7 days (stationary phase), from the primary culture, promastigotes were harvested by centrifugation (3500 rpm, 15 min, 4°C). Finally promastigotes were washed with sterile phosphate buffer saline (PBS) at pH 7.2 and then resuspended in PBS. Promastigotes were counted using a Neubauer haemocytometer to provide the appropriate number of promastigotes per inoculum.

Infection. Each BALB/c mouse was infected with 10^7 promastigotes by intracardiac route using syringe with 26 G needle.

Treatment. BALB/c mice (20 - 25 g) were randomly split into five groups of eight animals. Treatment was started on day 24 post-infection. Group A received AmBisome[®] intraperitoneal (i.p.) at a single dose of 5 mg kg^{-1} body weight. Prior to administration, AmBisome[®] was reconstituted with water for injection up to 4 mg mL^{-1} and then further diluted with glucose 5% w/v administering a final volume of 0.2 mL. Group B received orally using oral gavage (p.o.) AmB-GCPQ AmBisome[®] formulation (AmB-GCPQ, 1:5) at 5 mg kg^{-1} body weight daily for five consecutive days. Group C was kept as an untreated control for groups A and B. Group D was treated orally with the same formulation as group B (AmB-GCPQ) at 5 mg kg^{-1} body weight daily for ten consecutive days. Group E was kept as untreated control for group D. Animals were killed on day 31 (groups A, B and C) or day 36 (groups D and E) post- infection. Spleen and liver from each animal were aseptically removed and weighted to quantify the parasite burdens. Plasma and kidneys were collected to quantify the concentration of AmB. Plasma was separated by centrifugation and then both plasma and kidneys were stored at $-20 \text{ }^\circ\text{C}$ until assayed.

Tissue burden. Estimation of parasite burden was quantified by the limit dilution assay as described previously. Briefly the spleen (0.15 g) and liver (0.5 g) were homogenised in 5 mL of PBS – 50 mM glucose – 2 mM ethylene diamine tetraacetic acid (EDTA) solution at $4 \text{ }^\circ\text{C}$ using a sterilized steel stainless tissue grinder. Cell debris was removed by passage through a glass wool column. The suspension obtained was centrifuged at $2000 \times g$ for 15 min at $4 \text{ }^\circ\text{C}$. Afterwards, the supernatants were discarded and the pellets were collected and resuspended in Schneider medium supplemented as described above

and then 200 μL of this suspension were transferred to the first well of a 96-well microtiter plate containing NNN medium supplemented with antibiotics as described above. Serial dilutions were repeated transferring 100 μL from the previous well to the next one and adding 100 μL of Schneider medium. After incubation at 26 °C for 7 days, microplates were examined using an inverted microscope at a magnification of X40 and the presence or absence of mobile promastigotes in each well recorded. The last dilution at which mobile promastigotes could be detected was considered as the off-spring from one single parasite cell. The percentage of suppression of parasite replication (PS) was calculated using the following equation modified from Manandhar et al.:

$$\text{PS} = (\text{PC} - \text{PT}) / \text{PC} \times 100 \quad (\text{Equation 1});$$

where PC is the number of parasites in the control group per tissue weight (g) and PT is the number of parasites after treatment per tissue weight (g).

2. Results

Table 1. Characterization of AmB formulations. Particle size is reported as volume distribution of the different populations and its corresponding percentage. Zeta potential values are reported as mean \pm SD.

Formulation	Composition	Appearance	Size (nm - %)	Zeta potential (mV)
GCPQ	GCPQ (5 mg mL ⁻¹)	Transparent	16.7 nm – 98.4% >500 nm – 1.6%	+16.0 \pm 0.98
AmB in dextrose 5%	AmB (1 mg mL ⁻¹)	Opaque yellow	1656 nm – 72.3% 438.9 nm – 27.7%	-39.5 \pm 3.1
AmB-GCPQ before centrifugation	AmB (1 mg mL ⁻¹) GCPQ (5 mg mL ⁻¹)	Transparent yellow	5610 nm – 4.7% 170.5 nm - 28.3% 25.2 nm - 67%	+3.0 \pm 0.1
AmB-GCPQ after centrifugation	AmB (1 mg mL ⁻¹) GCPQ (5 mg mL ⁻¹)	Transparent yellow	216 nm - 40.9% 34.7 nm – 59.6%	+3.0 \pm 0.1
AMBd (AmB-desoxycholate)	AmB (1 mg mL ⁻¹) Sodium deoxycholate (0.82 mg mL ⁻¹) Dibasic sodium phosphate (0.02 mg mL ⁻¹) Monobasic sodium phosphate (0.016 mg mL ⁻¹)	Translucent orange	759.9 nm – 96.2% 182.5 nm – 3.8%	-27.7 \pm 5.8

Table 2. Pharmacokinetic parameters following the oral administration of AmB formulations in CD-1 mice. C_{max} values are expressed as mean \pm SD ($\mu\text{g mL}^{-1}$ or $\mu\text{g g}^{-1}$). AUC₀₋₂₄ values are expressed as $\mu\text{g h mL}^{-1}$ for plasma and $\mu\text{g h g}^{-1}$ for tissues. Mean Ratio values were calculated based on the ratio between the C_{max} in the corresponding organs and the C_{max} in kidneys. Key: NA- Not applicable; BID – twice a day.

Formulation	AmB dose (no. of days)	Collection of the samples	Plasma		Liver			Spleen			Lungs			Kidney	
			C _{max} (mean \pm SD, $\mu\text{g mL}^{-1}$)	AUC ₀₋₂₄ ($\mu\text{g h mL}^{-1}$)	C _{max} (mean \pm SD, $\mu\text{g g}^{-1}$)	AUC ₀₋₂₄ ($\mu\text{g h g}^{-1}$)	Ratio Liver/Kidney	C _{max} (mean \pm SD, $\mu\text{g g}^{-1}$)	AUC ₀₋₂₄ ($\mu\text{g h g}^{-1}$)	Ratio Spleen/kidney	C _{max} (mean \pm SD, $\mu\text{g g}^{-1}$)	AUC ₀₋₂₄ ($\mu\text{g h g}^{-1}$)	Ratio Lung/kidney	C _{max} (mean \pm SD, $\mu\text{g g}^{-1}$)	AUC ₀₋₂₄ ($\mu\text{g h g}^{-1}$)
AmB-GCPQ	5 mg kg ⁻¹ (single dose)	NA	0.308 \pm 0.022	5.808	0.985 \pm 0.095	11.718	0.880	1.364 \pm 0.173	25.979	1.219	1.608 \pm 0.423	26.535	1.437	1.119 \pm 0.053	20.390
AMBd	5 mg kg ⁻¹ (single dose)	NA	0.255 \pm 0.023	5.169	0.908 \pm 0.058	9.873	0.928	0.763 \pm 0.221	13.826	0.780	0.827 \pm 0.142	15.22	0.845	0.978 \pm 0.168	18.041
AmB in dextrose	5 mg kg ⁻¹ (single dose)	NA	0.251 \pm 0.021	4.408	0.344 \pm 0.201	4.459	0.404	0.686 \pm 0.150	13.147	0.806	0.733 \pm 0.185	10.5799	0.861	0.851 \pm 0.149	17.014
AmB-GCPQ	5 mg kg ⁻¹ once a day (for 5 days)	24 h after last dose	0.229 \pm 0.010	NA	0.892 \pm 0.272	NA	0.635	1.716 \pm 0.563	NA	1.221	1.548 \pm 0.844	NA	1.102	1.405 \pm 0.054	NA
AmB-GCPQ	5 mg kg ⁻¹ BID (for 5 days)	12 h after last dose	0.462 \pm 0.051	NA	6.281 \pm 1.175	NA	1.877	7.884 \pm 2.621	NA	2.356	7.456 \pm 2.584	NA	2.228	3.346 \pm 0.725	NA

Figure S2. Transmission electron micrographs with negative staining: (a) GCPQ micelles (40 mg mL⁻¹) in deionized water. Micelles are 5-20 nm in diameter. (b) AmB (8 mg mL⁻¹)-GCPQ (40 mg mL⁻¹) nanoparticles in deionized water. (c) AMBd diluted with deionized water to 1 mg mL⁻¹. (d) AmB crystals (8 mg mL⁻¹) in dextrose (5%, w/v). Crystals are 0.5–2 microns in diameter. (e) Schematic representation of the molecular organization of AmB and GPCQ.

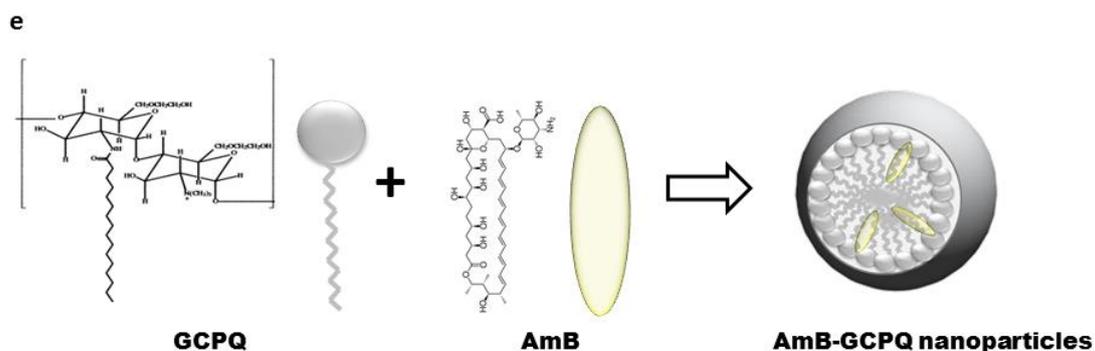
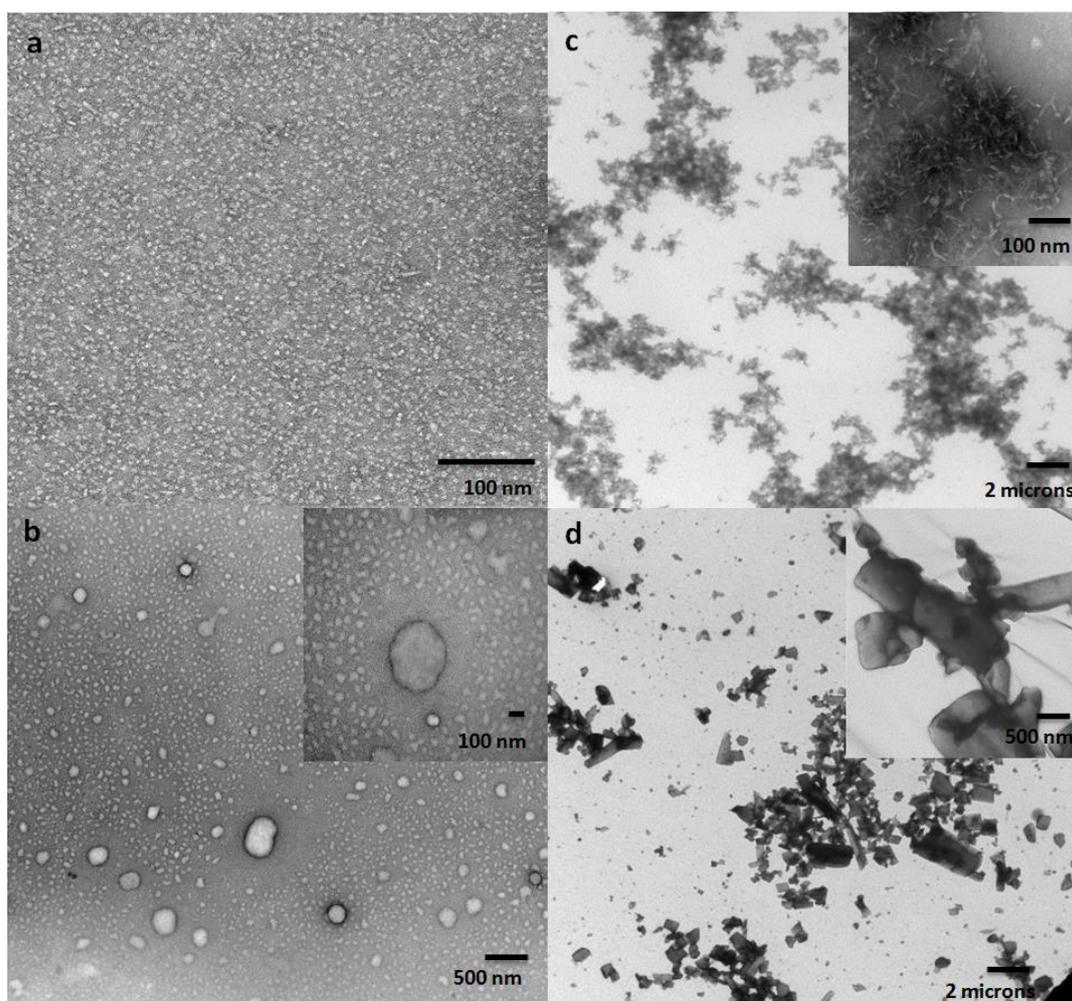
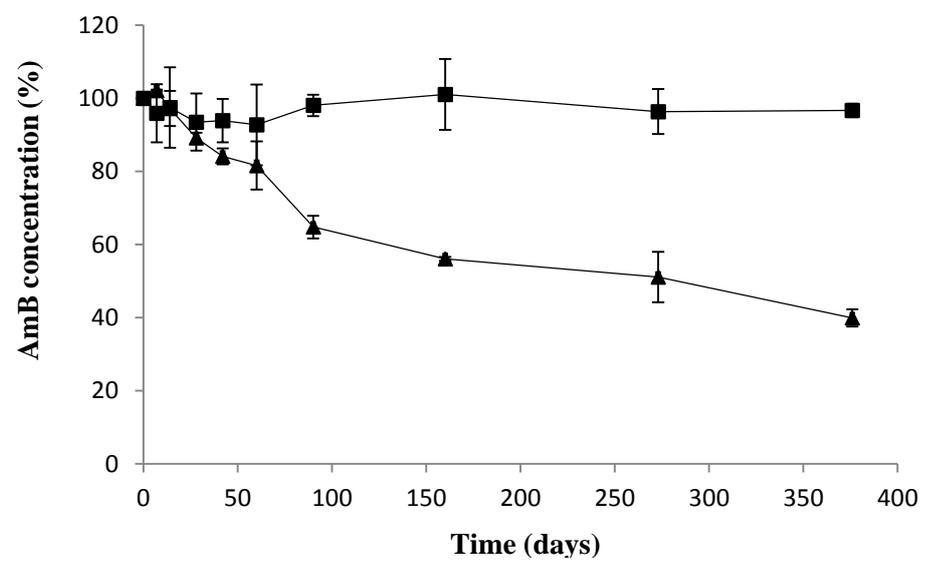
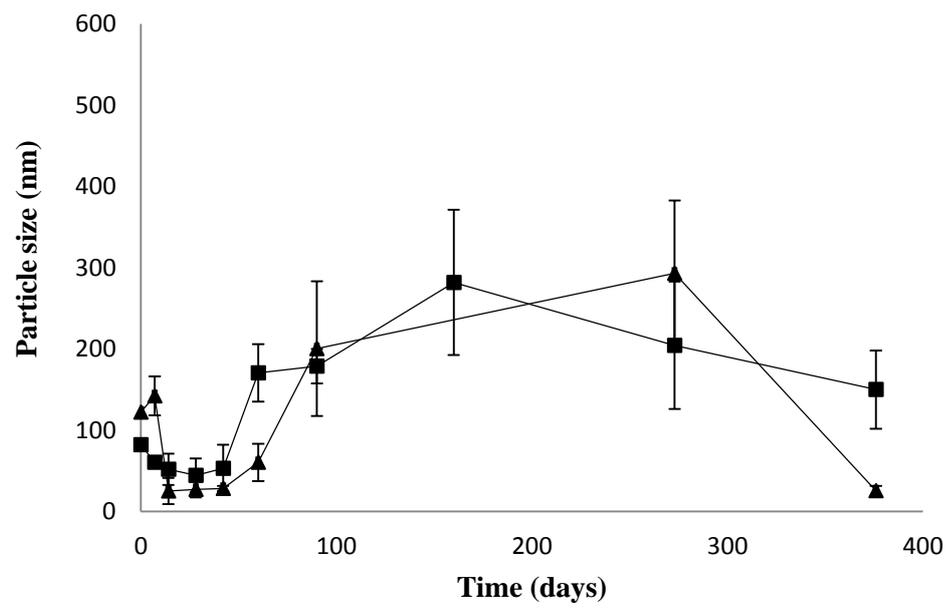


Figure S3. Long-term stability studies of AmB-GCPQ formulation at 5 ± 3 °C. (a) AmB concentration (%). (b) Particle size (nm). (c) Physical appearance: i) Lyophilised formulation at day 0; ii) liquid formulation at day 0; iii) formulations at day 7; iv) formulations at day 60; v) formulations at day 270 and vi) formulations after one year. Key: Liquid formulation (-▲-) and capsules filled with lyophilised formulation (-■-).

a)



b)



c)

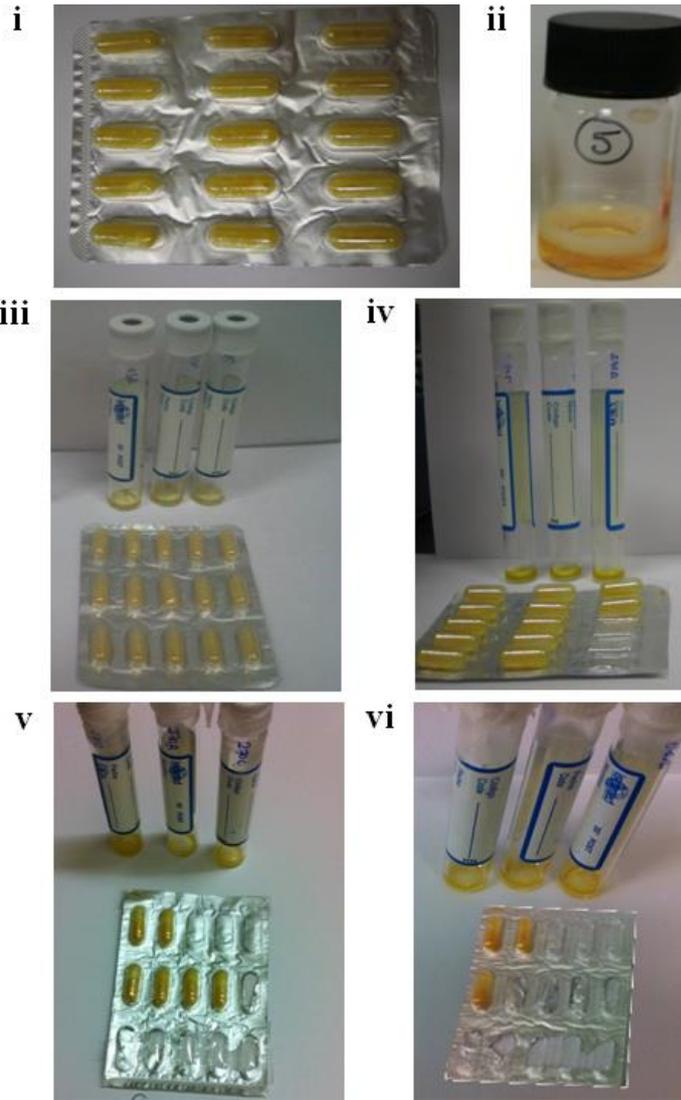
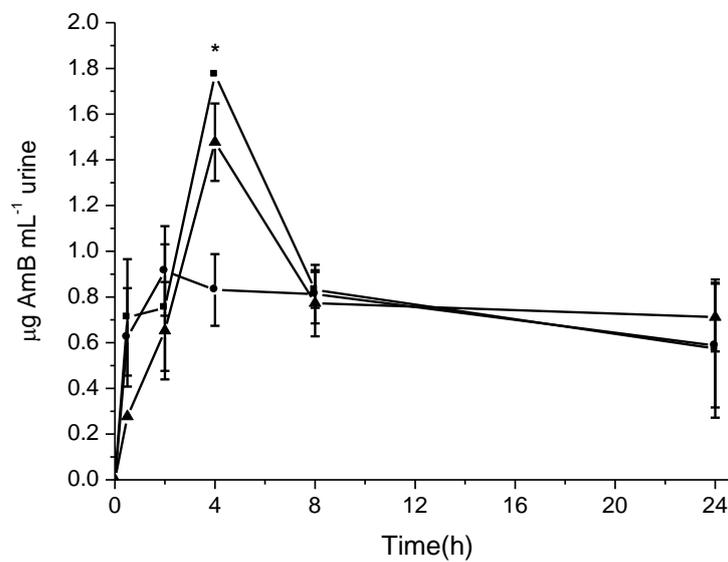


Figure S4. AmB pharmacokinetics (mean \pm SD) after a single oral administration of AmB at 5 mg kg⁻¹ in CD-1 mice. Key: AmB in dextrose (-■-); AmBd (Amphotericin B – deoxycholate formulation (-▲-); and AmB (5 mg kg⁻¹)- GCPQ (25 mg kg⁻¹) formulation (-●-). (a) AmB concentration in urine (μ g mL⁻¹). (b) AmB concentration in bile (μ g g⁻¹). Statistical significant differences: * = p < 0.05 AmB-GCPQ versus AmB in dextrose; # = p < 0.05 AmB-GCPQ versus AMBd; + = p < 0.05 AmB in dextrose versus AmBd.

a)



b)

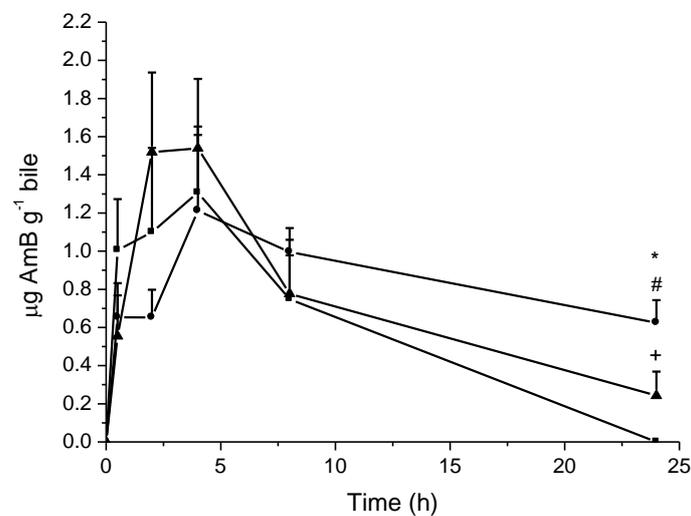
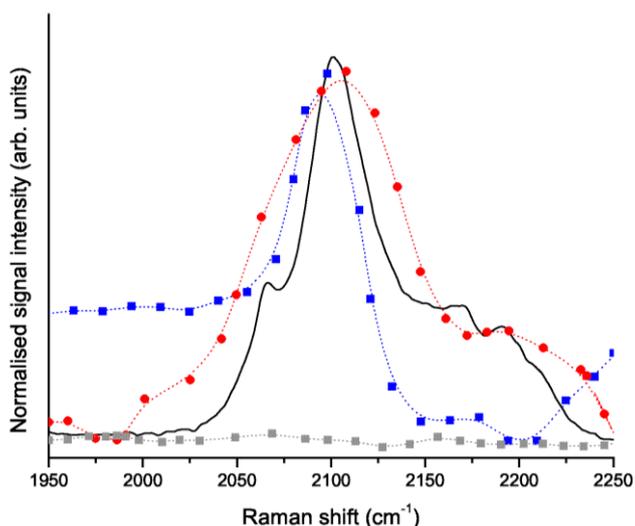


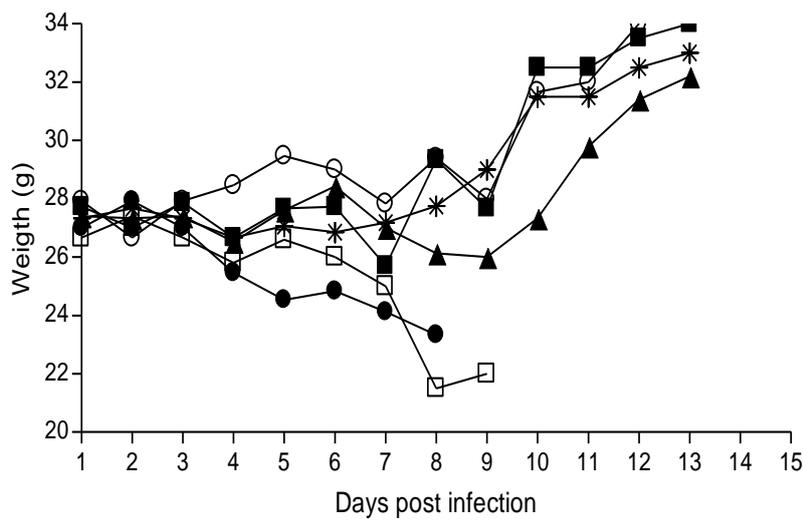
Figure S5. Stimulated Raman Scattering (SRS) and epi-detected CARS hyperspectral profiles of dGCPQ particles in-situ in liver tissue (red and blue markers, respectively) in comparison with a spontaneous Raman spectrum of dGCPQ (solid black line). The grey markers relate to the SRS spectral profile of liver tissue devoid of dGCPQ particles, illustrating the chemical specificity of this technique.



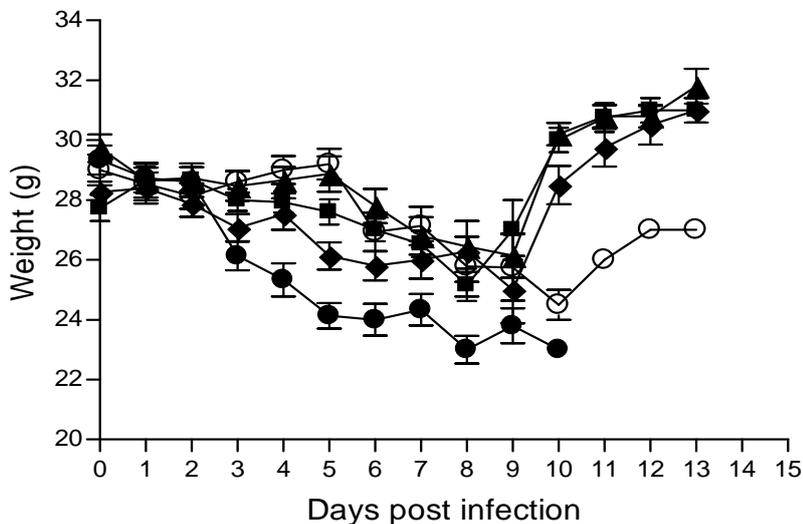
Successful deuteration of GCPQ was ascertained by using spontaneous Raman spectroscopy. When the hydrogen atoms in the palmitoyl chain are replaced with deuterium atoms as in dGCPQ, a characteristic shift in the CH₂ stretching mode at ~ 2845 cm⁻¹ to ~ 2100 cm⁻¹ is observed. As also illustrated by the grey data points in Figure 3, SRS microspectroscopy of liver samples yielded no endogenous signal when the pump and Stokes wavelengths were tuned to 2100 cm⁻¹, since this peak lies within the so-called biologically ‘silent region’

Figure S6. Clinical assessment of gastrointestinal toxicity recorded as body weight of OF-1 mice infected i.v. with 1×10^4 CFU of *A. fumigatus*. Drugs were administered intravenously (i.v.) or orally by gavage (p.o) 24h after infection for 10 days. **(a)** Groups of animals (n = 15) received: no treatment (-●-), AMBd i.v. at $0.5 \text{ mg kg}^{-1} \text{ day}^{-1}$ (-○-), AMBd p.o. at $2.5 \text{ mg kg}^{-1} \text{ day}^{-1}$ (-□-), AmBisome® i.v. at a dose of $2.5 \text{ mg kg}^{-1} \text{ day}^{-1}$ (-▲-), AmB-GCPQ p.o. at $2.5 \text{ mg kg}^{-1} \text{ day}^{-1}$ (-*-) or AMBd i.v. at a dose of 0.5 mg kg^{-1} at day 1 followed by AmB-GCPQ p.o. at $2.5 \text{ mg kg}^{-1} \text{ day}^{-1}$ (-■-). **(b)** Groups of animals (n = 15) received: no treatment (-●-), AMBd i.v. at $0.8 \text{ mg kg}^{-1} \text{ day}^{-1}$ (-○-), AmBisome® i.v. at a dose of $5 \text{ mg kg}^{-1} \text{ day}^{-1}$ (-▲-), AmB-GCPQ p.o. at $7.5 \text{ mg kg}^{-1} \text{ day}^{-1}$ (-◆-) or AmB-GCPQ p.o. at $15 \text{ mg kg}^{-1} \text{ day}^{-1}$ (-■-).

a)



b)



Video S1. Three-dimensional reconstructions of multiphoton images obtained from a lung data stack. Some dGCPQ particles can be observed at the very edge of the blood vessels and within type 1 and 2 cells. Interestingly, some of the red blood cells are echinocytic (top right and top middle) which probably happened during the fixation process rather than in the animal.