Supplementary Material

Hyaluronic acid-Amphotericin B Nanocomplexes: a Promising Anti-Leishmanial Drug Delivery System

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Supplementary Methods

Parasites and *in vitro* axenic assays

L. amazonensis (MHOM/BR/LTB0016) were cultured at 25 ºC in Schneider's Insect medium supplemented with 10 % (v/v) heat inactivated fetal bovine serum (iFBS), 100 U/mL penicillin, 100 µg/mL streptomycin, 5 mM HEPES pH 7.4 and 5 µg/mL phenol red. *L. infantum* promastigotes (MHOM/MA/67/ITMAP-263) were cultured at 25 ºC in RPMI 1640 Glutamax medium supplemented with 10 % (v/v) iFBS, 50 U/mL penicillin, 50 µg/mL streptomycin and 20 mM HEPES sodium salt pH 7.4.

Promastigotes of *L. infantum* and *L. amazonensis* were seeded in 96-well plates at a density of 3 x 10^5 cells/well in complete RPMI medium or complete Schneider's Insect medium, respectively, containing increasing concentrations of free-AmB and HA-AmB nanocomplexes (0.0029 - 0.75 µM). After an incubation period of 24 h at 26 ºC, 20 µL of a filtered 2.5 mM resazurin solution prepared in PBS was added to each well, followed by a second incubation period of 24 h at 26 ºC. Using a SpectraMAX GeminiXS microplate reader (Molecular Devices LLC, California, USA) the fluorescence intensity was measured (λex 560/λem 590). The results were expressed as the mean percentage ± SD of viable parasites relatively to control and the assay was performed in triplicate at least three times.

Supplementary Data

In vitro cytotoxicity assay

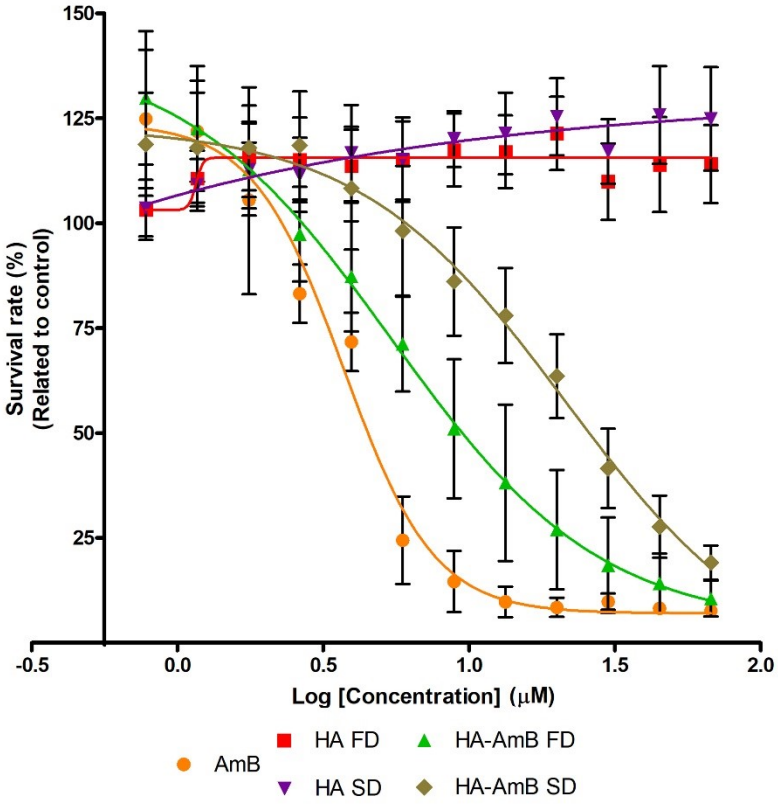

Figure S 1 - Dose-response curves of the *in vitro* cytotoxic activity of free-AmB, AmBisome® and HA-AmB nanocomplexes against BMMΦ after 24 h of treatment.

In vitro anti-leishmanial activity against promastigotes

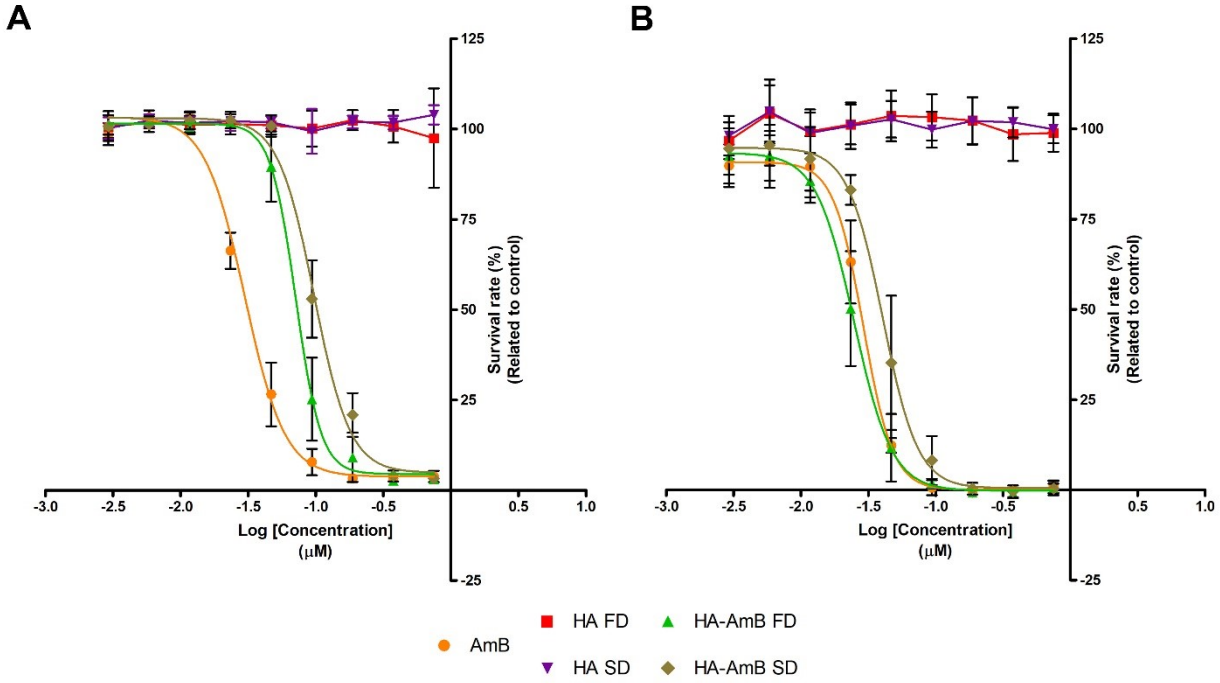

Figure S 2 – Dose-response curves of the in vitro anti-leishmanial activity of free-AmB and HA-AmB nanocomplexes against **(A)** *L. amazonensis* and **(B)** *L. infantum* promastigote cultures after 24 h of treatment with different concentrations (0.0029 - 0.75 µM) of the above referred formulations. Data is expressed as mean ± SD of at least three independent experiments.

Table S 1 – IC_{50} values of the anti-leishmanial reference compound AmB and of HA-AmB nanocomplexes against promastigotes of *L. amazonensis*.

Sample	*L. amazonensis* promastigotes IC_{50} (µM)
AmB	0.032 ± 0.007
HA-AmB FD	0.072 ± 0.037
HA-AmB SD	0.101 ± 0.052

Means ± SD (n = 3)

In vitro anti-leishmanial activity against *L. infantum*-infected macrophages

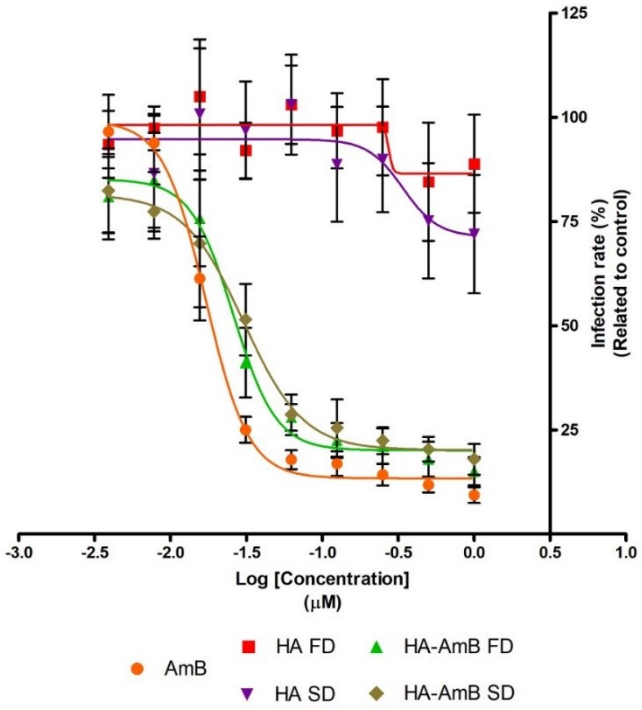

Figure S 3 - *In vitro* effect of AmB and HA-AmB nanocomplexes on intracellular amastigotes. *L. infantum*-infected BMMΦ were treated for 24 h with different concentrations (0.0039 to 1 μM) of the above referred formulations. Data (mean ± SD of at least three independent experiments) was obtained employing an automated image analysis protocol available for the IN Cell Analyzer system and used for the obtention of the dose-response curves.

In vivo systemic toxicity assessment

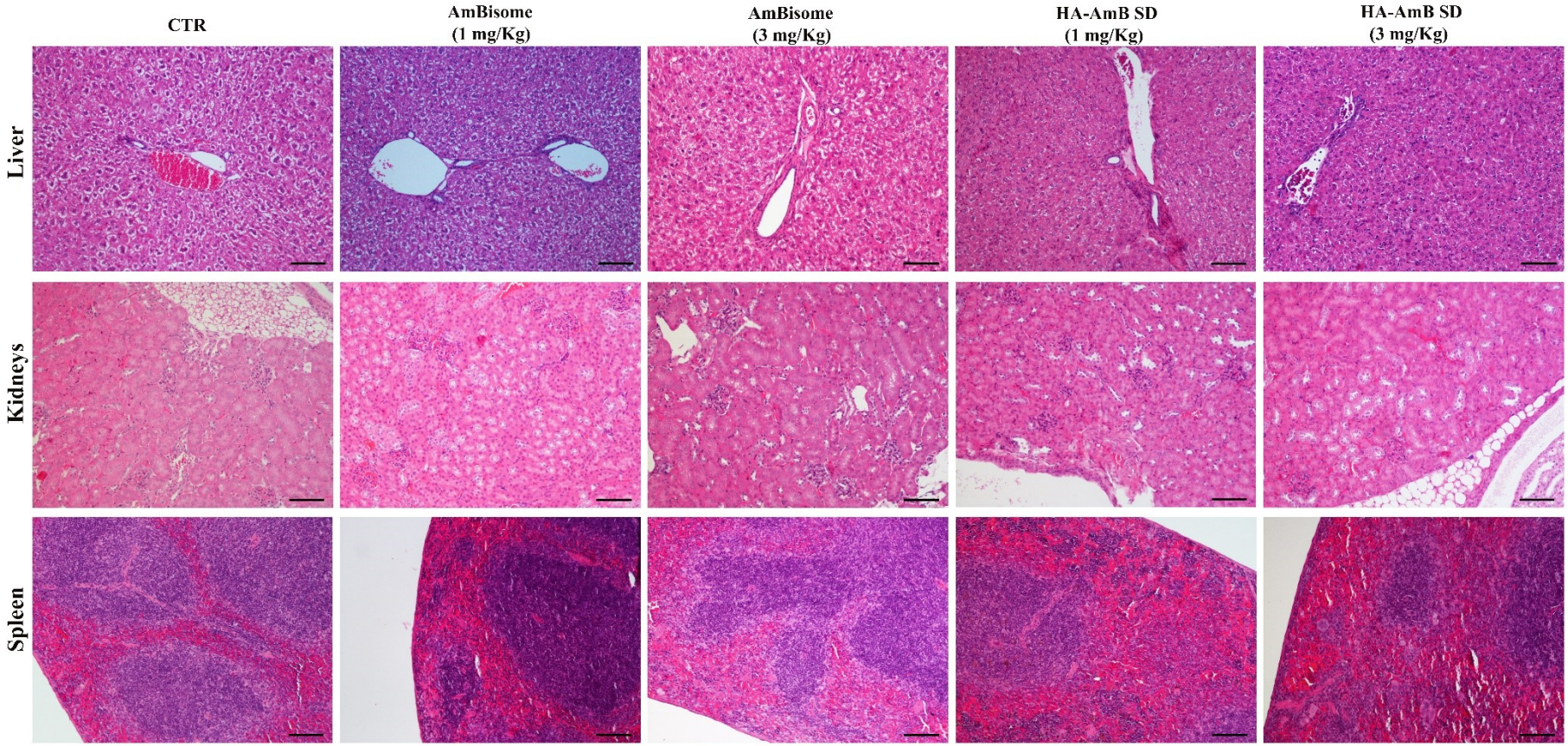

Figure S 4 – Representative H&E stainings of the mice's liver, kidney and spleen sections after treatment with AmBisome® (1 and 3 mg/Kg) and HA-AmB SD nanocomplex (1 and 3 mg/Kg) (100 x magnification, scale = 100 µm).

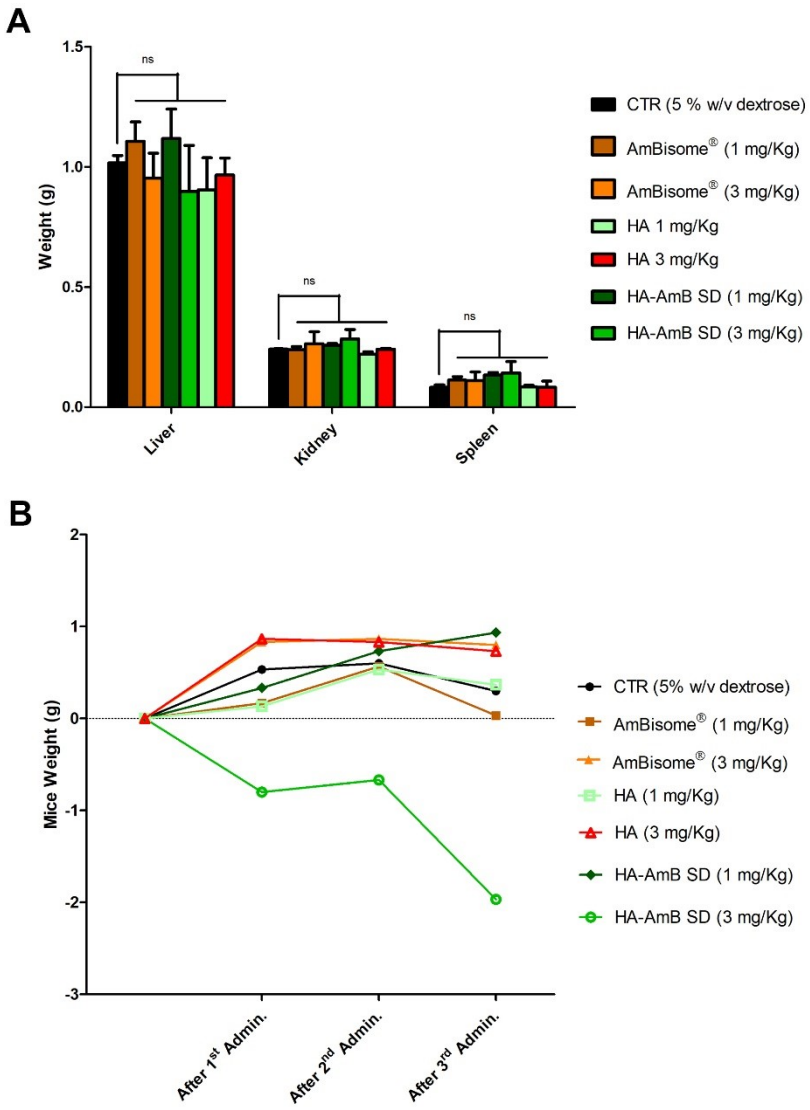

Figure S 5 - Weight of the **(A)** collected organs and **(B)** mice after intravenous treatment with different formulations at 1 and 3 mg/Kg. Data is expressed as mean ± SD. For the collected organs, bonferroni's multiple comparison post-test was used to evaluate differences between control and the treatment groups and no significant alterations were observed.

In vivo anti-leishmanial activity in infected mice

Table S 2 – Body weight changes (mean ± SD) according to each experimental group.

Treatment groups	Body weight (g)		
	Before infection	Before euthanasia	Change (%)
Non-infected	20.0 ± 1.2	21.0 ± 0.7	1.1 g (5.5 %)
CTR (5 % w/v dextrose)	20.6 ± 1.8	21.4 ± 1.1	0.8 g (3.9 %)
AmBisome® (1 mg/Kg)	20.8 ± 1.6	22.0 ± 1.9	1.2 g (5.8 %)
HA-AmB SD (1 mg/Kg)	20.9 ± 1.2	21.9 ± 1.7	1.0 g (5.0 %)

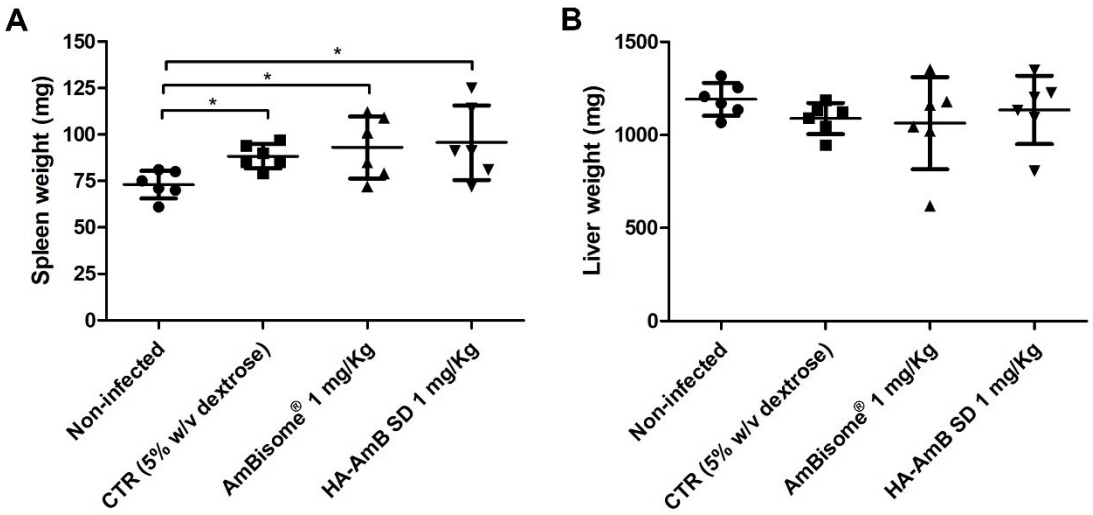

Figure S 6 - Weight of the **(A)** spleens and **(B)** livers collected from the treated mice. Each symbol represents one animal and the horizontal bars represent the mean weight of the organs. Bonferroni's multiple comparison post-test was used to evaluate differences between each group and only in the spleen was observed significant differences between the non-infected groups and the infected/treated groups (*$p < 0.05$).

Table S 3 - Histological alterations observed after intravenous treatment of *L. infantum*-infected mice with 1 mg/Kg of HA-AmB SD nanocomplex and AmBisome®.

Organ	Histological findings	Treatments			
		CTR (5 % w/v dextrose)	**Infected CTR (5 % w/v dextrose)**	**AmBisome**	**HA-AmB SD**
				1 mg/Kg	**1 mg/Kg**
Liver	Inflammatory infiltrate	Slight periportal (lymphoid) (1/3)	Slight multifocal (lymphoid) (1/4)	Slight multifocal (lymphoid) (1/3)	Slight multifocal and subacute (3/3)
	Lymphoid agglomerates	-	Multifocal to perivascular and periportal (2/4)	Multifocal to perivascular and periportal (2/3)	-
	Congestion	Generalized (1/3)	Generalized (1/4)	Generalized (1/3)	Generalized (1/3)
	Hydropic degeneration	Generalized (2/3)	Generalized (1/4)	-	-
	Vascular ectasia	Presence (1/3)	Presence (2/4)	Presence (2/3)	Presence (3/3)
Spleen	Follicular hyperplasia	-	Slight (2/4)	-	Slight (2/3)
	Hemosiderosis	Diffuse presence (1/3)	-	-	-