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Research Paper

Hemisynthetic trifluralin analogues incorporated in liposomes for the treatment of leishmanial infections

Manuela Carvalheiro [a,*], M. Alexandra Esteves [b], David Santos-Mateus [d], Rui M. Lopes [a], M. Armanda Rodrigues [d], Carla V. Eleutério [a], Effie Scoulica [c], Gabriela Santos-Gomes [d], M. Eugénia M. Cruz [a,*]

[a] Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, Universidade de Lisboa, Av. Prof. Gama Pinto, 1649-003 Lisbon, Portugal
[b] Solar Energy Unit, National Laboratory for Energy and Geology (LNEG), Estrada do Paço do Lumiar, 1649-038 Lisbon, Portugal
[c] Laboratory of Clinical Bacteriology, School of Medicine, University of Crete, 71003 Heraklion, Greece
[d] Unidade de Ensino e Investigação de Parasitologia Médica and Centro de Malária e Outras Doenças Tropicais, Instituto de Higiene e Medicina Tropical (IHMT), Universidade Nova de Lisboa, Rua da Junqueira 100, 1349-008 Lisbon, Portugal

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ABSTRACT

Leishmaniasis, a vector-borne parasitic disease caused by *Leishmania* protozoa, is one of the most neglected tropical diseases in terms of drug discovery and development. Current treatment is based on a limited number of chemotherapeutic agents all of which present either/or resistance issues, severe toxicities and adverse reactions associated with extended treatment regimens, and high cost of therapy. Dinitroanilines are a new class of drugs with proven *in vitro* antileishmanial activity. In previous work a liposomal formulation of one dinitroaniline (TFL) was found to be active against *Leishmania* parasites in a murine model of visceral leishmaniasis (VL) and in the treatment of experimental canine leishmaniasis. In this study we have investigated the use of dinitroaniline analogues (TFL-A) associated to liposomes, as means to further improve TFL antileishmanial activity. The potential of the liposomal formulations was assessed *in vitro* against *Leishmania infantum* promastigotes and intracellular amastigotes and *in vivo* in a murine model of zoonotic VL. Free and liposomal TFL-A were active *in vitro* against *Leishmania* parasites, and they also exhibited reduced cytotoxicity and haemolytic activity. Treatment of infected mice with liposomal TFL-A reduced the amastigote loads in the spleen up to 97%, compared with the loads for untreated controls. These findings illustrate that chemical synthesis of new molecules associated with the use of Nano Drug Delivery Systems that naturally target the diseased organs could be a promising strategy for effective management of VL.

1. Introduction

Leishmaniasis is the collective name of a group of infectious diseases caused by protozoan parasites of the genus *Leishmania*. The visceral, cutaneous or mucocutaneous forms can cause a broad spectrum of clinical outcomes, ranging from self-healing skin ulcers, to severe, life-threatening manifestations depending on the virulence of the infecting species and the host's immune response [1]. Leishmaniasis is endemic throughout many tropical and subtropical countries where an estimated 12 million people are currently infected and 50,000 deaths occur each year [2,3]. In the absence of an approved vaccine for the human population treatment relies almost exclusively on chemotherapy. First-line chemotherapy based on pentavalent antimonial compounds and second-line recommended treatment that may include Amphotericin B and pentamidine present serious limitations such as toxicity, lack of efficacy and the emergence of resistant strains in some areas [4,5]. Miltefosine, a phosphocholine analogue, has the advantage of being the first efficient oral antileishmanial drug, however the existence of severe signs of toxicity (e.g. teratogenicity), and the easily induced parasite resistance has restricted its use [6,7]. AmBisome®, a liposomal formulation of amphotericin B is currently the most effective treatment available. It minimizes the

Abbreviations: VL, visceral leishmaniasis; TFL, trifluralin; TFL-A, trifluralin analogues; NanoDDS, nano drug delivery systems; DMPC, dimyristoylphosphatidylcholine; DMPG, dimyristoylphosphoglycerol; FBS, foetal bovine serum; THP-1, human monocytic cell line; I.E., incorporation efficacy; L.C., loading capacity.

* Corresponding authors.
E-mail addresses: mcarvalheiro@ff.ulisboa.pt (M. Carvalheiro), eugeniacruz@ff.ulisboa.pt (M. Eugénia M. Cruz).

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toxic effects of the free drug and requires a shorter course of therapy (5–10 days). However, its prohibitive cost precludes the widespread use in endemic countries [8]. Thus, there is an urgent need to develop novel, effective, safe non-conventional and cost-effective chemotherapies to fight this neglected disease.

The association with Nano Drug Delivery Systems (NanoDDS) to direct active drugs to the sites of infection promoting intracellular delivery is part of a strategy to improve the pharmaceutical efficacy and lessen the toxic effects of several antileishmanial drugs. Liposomes are the ideal NanoDDS as they are rapidly ingested by macrophages together with the incorporated drugs [9,10]. Over the last decades, many attempts have been made to treat leishmaniasis with liposomal drugs [11–15]. In general, these liposomal formulations have proved to be superior, allowing the administration of considerably larger doses without revealing toxicity and reducing the dosing schedules [16,17]. However most of the drugs have some resistance associated that may not be overcome by the use of these new formulations. The shortcomings of the available antileishmanial drugs, prompt the search for novel therapies.

Dinitroanilines are widely used in herbicidal formulations that have also revealed antiparasitic properties [18]. The mechanism of action of dinitroanilines is determined by their specific binding to parasite tubulins (the main structural component of microtubules), which causes an antimitotic activity [19]. An additional attractive feature of these agents is their lack of binding affinity to animal tubulins, meaning that they are not toxic to mammals [18]. However unfavourable physicochemical properties (low water solubility and instability) have compromised their development as antiparasitic agents. We have demonstrated that the incorporation of a dinitroaniline, Trifluralin (TFL), in liposomes led to an increased solubility and stability of the drug. Moreover, TFL liposomes were able to deliver the drug *in vivo*, to the sites of infection (liver and spleen) where it proved to be active against *Leishmania donovani* parasites [20]. The TFL liposomes also improved the clinical condition of dogs and reduced the density of parasites. Nevertheless a complete elimination of the parasites was never achieved [21]. In recent work we have incorporated another dinitroaniline (Oryzalin – ORZ) in two different NanoDDS: liposomes and Solid Lipid Nanoparticles (SLN). Both systems caused an *in vitro* reduction of ORZ cytotoxicity, abolished the haemolytic activity and kept its antileishmanial intracellular activity [22,23]. The incorporation in liposomes caused the preferential accumulation of ORZ in the liver and spleen, and *in vivo* studies demonstrated that both NanoDDS significantly improved the pharmacological performance of this drug [22,24].

In this work we discuss the use of hemi-synthetic TFL analogues (TFL-A) in which the amine group was modified with different substituents [25] either in free or liposomal form. The potential clinical development of these new molecules may be seriously limited due to their low aqueous solubility. In this context, we have proved that the association to tailor made NanoDDS exhibiting high TFL-A loading capacities, has overcome these limitations and improved *in vivo* efficacy.

2. Materials and methods

2.1. Materials

The phospholipids dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol (DMPG) were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and were used without further purification. Polycarbonate membranes were from Nuclepore (Pleasanton, CA, USA). PD-10 columns were purchased from Bio-Rad (Hercules, CA, USA). RPMI 1640 media; Schneider's Drosophila medium, penicillin–streptomycin, and foetal bovine serum (FBS) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Histopaque 1077 was purchased from Invitrogen (Paisley, UK). LIVE/DEAD viability kit was obtained from Molecular Probes (Eugene, OR, USA). Glucantime® (*N*-meglumine antimoniate) in 5 mL ampoules was from Merial (Lyon, France). Acetonitrile (HPLC grade) was from Merck (Darmstadt, Germany) and all other reagents were analytical grade.

2.1.1. TFL analogues

The TFL analogues 2-((2,6-dinitro-4-trifluoromethyl-phenyl)-butylamino)-ethanol (TFL-A3) and 4-(2,6-dinitro-4-trifluoromethyl-phenylamino)-phenol (TFL-A6) were synthesized by a general procedure previously reported [25].

2.2. Cell lines, parasite strains and animals

The human monocytic cell line THP-1 was used as the host cell for *Leishmania* parasites. THP-1 cells were grown in RPMI 1640 medium, supplemented with 10% heat inactivated FBS, L-glutamine, Penicillin 100 U/mL and Streptomycin 100 μg/mL, pH 7.4 at 37 °C, 5% CO_2. *Leishmania infantum* promastigotes were cultured in RPMI 1640 medium, supplemented with 10% heat inactivated FBS, L-glutamine, and Penicillin 100 U/mL plus Streptomycin 100 μg/mL, pH 7.4 at 26 °C.

BALB/c mice (6–8 weeks old, 25–30 g) were obtained from Gulbenkian Institute of Science, Portugal. Animals were fed with standard laboratory food and water *ad libitum*. All animal experiments were carried out with the permission of the Portuguese veterinary authorities (DGAV) and the local animal ethical committee, and in accordance with the Declaration of Helsinki, the EEC Directive (86/609/EEC) and the Portuguese laws D.R. no. 31/92, D.R. 153 IA 67/92, and all following legislations.

2.3. Methods

2.3.1. Preparation and characterization of TFL-A liposomes

Liposomes composed of DMPC and DMPG were prepared by the thin lipid film-hydration method followed by extrusion, as previously described [20]. For the two TFL-A tested, different DMPC:DMPG molar ratios (7:3 or 9:1) were chosen. Briefly, lipids and the TFL-A (3 μmol/mL) were dissolved in chloroform and dried under a nitrogen stream to a thin lipidic layer. The Liposomal suspension was formed from this film in two steps; first a trehalose–citrate buffer (10 mM sodium citrate, 135 mM NaCl, 29 mM trehalose, pH 5.5) was added with continuous stirring until a homogenous suspension was obtained. The hydration was completed with the addition of the remaining citrate buffer (10 mM sodium citrate, 145 mM NaCl, pH 5.5) so that the final lipid concentration was 20 mM for the incorporation of TFL-A3 and 16 mM for TFL-A6. The multilamellar vesicle suspension was successively extruded at 30 °C, through polycarbonate membranes (Nucleopore, Whatman plc, Kent, UK) of 0.8, 0.4 and 0.2 μm pore sizes in a thermobarrel extruder (Lipex Biomembranes, Vancouver, Canada). The non-incorporated TFL-A was removed by gel filtration on an Econo-Pac® 10DG column (Bio-Rad Laboratories, Hercules, CA, USA). Final liposomal suspensions were obtained after ultra-centrifugation at 180,000*g* for 2 h at 20 °C in a Beckman L8-60M ultracentrifuge (Beckman Instruments, Inc., Fullerton, CA, USA).

The TFL-A liposomal formulations used for both *in vitro* and *in vivo* studies were concentrated by suspending the pellet after ultra-centrifugation in around 5 fold lower volume. Concentrated liposomal formulations were then freeze-dried in an Edwards Modulyo Freeze Dryer (Crawley, UK). The freeze-drying process took place overnight (12–18 h) at a pressure of about 10 Pa and

at an initial temperature of −40 °C. The lyophilized cakes were reconstituted with distilled water to the appropriated volume and the potential released TFL-A was removed by gel filtration.

Phospholipid content of the vesicles was determined using the method described by Rouser et al. [26] based on quantification of inorganic phosphorous. An HPLC method was set-up for the quantification of free and liposomal TFL-A using a Beckman System Gold Nouveau (Beckman Instruments, Inc.,) with a 126 Pump Direct Control and a Midas type 830 autosampler with a 20 μL sample loop. This system is connected to specific computer software (32 Karat, version 7.0) for the integration of chromatograms. All the samples were dissolved in the mobile phase consisting of 0.02 M sodium acetate (pH 6.55): acetonitrile (40:60). The chromatographic run was performed on a Supelco Nucleosil C18, 5 μm (150 × 4.6 mm) analytical column (Bellefonte, PA, USA) starting with a gradient from 60% to 80% acetonitrile in 8 min and held for 5 min thereafter returning to the initial conditions in 2 min. The instrumental settings were: flow rate 1 mL/min; column temperature 25 °C and detection at 280 nm for TFL-A6 and at 360 nm for TFL-A3 in a diode-array Detector Module model 168 (Beckman Instruments, Inc.).

TFL-A liposomes size and size distribution were determined by dynamic light scattering in a Zetasizer 1000HSA (Malvern Instruments, Malvern, UK). The zeta potential was calculated by dynamic light scattering in a Zetasizer 2000 (Malvern Instruments).

TFL-A liposomes were characterized in terms of incorporation efficacy (I.E.), loading capacity (L.C.) and TFL-A and lipid yield determined as described by the equations below:

$$\text{L.C. (g/mol)} = [\text{TFL} - \text{A}]_f/[\text{Lip}]_f$$

$$\text{TFL-A yield}(\%) = ([\text{TFL-A}]_f/[\text{TFL-A}]_i) \times 100$$

$$\text{Lipid yield } (\%) = ([\text{Lip}]_f/[\text{Lip}]_i) \times 100$$

$$\text{I.E. } (\%) = ([\text{TFL-A}]_f/[\text{Lip}]_f)/([\text{TFL-A}]_i/[\text{Lip}]_i) \times 100$$

where $[\text{Lip}]_f$ and $[\text{TFL-A}]_f$ represent lipid and TFL-A concentration in the final liposomal formulations and $[\text{Lip}]_i$ and $[\text{TFL-A}]_i$ represent lipid and TFL-A concentration in the initial liposomal suspension.

2.3.2. Cytotoxicity on THP1 cell line

To quantitatively estimate the degree of cell damage, THP-1 cell cultures were incubated at 1×10^6 cells/mL during 72 h at 37 °C with free and liposomal forms of TFL-A, in concentrations ranging from 50 to 6.25 μM. After incubation, cells were suspended in labelling buffer (10 mM HEPES, 150 mM NaCl, 10% BSA, pH 7.4) containing 12 μM of Propidium iodide (PI) and 100 nM of SYBR-14 (LIVE/DEAD Sperm Viability kit, Molecular Probes). The cytotoxicity was analysed by flow cytometry on an Epics Elite model flow cytometer (Coulter, USA) equipped with a 488 nm argon laser. Differential monitoring of the dyes was achieved by reading the green fluorescence of SYBR-14 at 545 nm and the red fluorescence PI at 645 nm. At least 10,000 cells were analysed per sample and data analysis was performed on fluorescence intensities that excluded cell autofluorescence and cell debris. The CC_{50} values were calculated using sigmoidal regression analysis from the data of three independent experiments [27].

2.3.3. Haemolytic activity on human whole blood

Liposomal and free TFL-A were diluted with PBS buffer and distributed in 96 well plates in concentrations ranging from 250 to 6.25 μM TFL. An equal volume of red blood cells (RBC) obtained from EDTA-preserved peripheral blood of healthy volunteers washed twice and suspended in PBS (pH 7.4) was added. After 1 h of incubation at 37 °C, RBC was centrifuged at 800*g* for 10 min. The absorbance of the supernatant was measured at 540 nm in an ELISA counter with the reference filter set at 620 nm. The percentage haemolytic activity of each formulation at different concentrations was estimated as $(A - A_0/A_{max} - A_0) \times 100$ where A_0 is the background haemolysis obtained by the incubation of RBC with PBS and A_{max} is the 100% haemolysis achieved upon incubation of RBCs in distilled water. The HC_{50}, concentration that exhibited 50% haemolysis was also determined.

2.3.4. Antileishmanial activity against intracellular L. infantum

Differentiated THP-1 cells (RPMI with 1 μM retinoic acid, 72 h, 37 °C, 5% CO_2) were infected with *L. infantum* (LEM 235) promastigotes at a 1:4 parasite/cell ratio and incubated overnight at 37 °C, 5% CO_2. The cellular suspension was centrifuged at 400*g* for 10 min and the pellet collected in RPMI medium was carefully layered on 4 mL of Histopaque 1077 (Sigma, Greece). Free promastigotes were removed by centrifugation at 1000*g* for 20 min. The opaque cell layer containing the infected mononuclear cells was collected and washed twice with PBS (pH 7.4) and resuspended in RPMI at 4×10^5 cells/mL. Liposomal and free TFL-A, in concentrations ranging from 50 to 0.4 μM, were incubated with the infected THP-1 cells in 24 well tissue culture plates (Cellstar, Greiner) for 48 h at 37 °C, 5% CO_2. After incubation cells were smeared onto glass slides and spin in a cytocentrifuge (Cytospin, Japan) at 45*g* for 1 min, air-dried, fixed with methanol and stained with Giemsa stain. The percentage of infected THP-1 cells was observed microscopically at 1000× magnification. The IC_{50} values were calculated using sigmoidal regression analysis from the data of three independent experiments [27].

2.3.5. Animal experiments

BALB/c mice weighing between 25 and 30 g were separated randomly into groups and infected intraperitoneally (i.p.) with 10^7 *L. infantum* (MHOM/PT/89/IMT151) virulent promastigotes freshly transformed. At 45 days postinfection, mice were randomly sorted into groups prior to dosing. The non-treated (control) group was injected i.p. with trehalose/citrate buffer once a day for 10 days. The two treated groups were injected i.p. with a dose of 25 mg TFL-A/kg/day of either free TFL-A (dissolved in trehalose/citrate buffer containing 5% Tween 80) or liposomal TFL-A (freshly reconstituted from freeze-dried cakes) once a day for 10 days. Two additional control groups were included: Glucantime® (meglumine antimoniate), used as a positive control was administered subcutaneously (s.c.) in a dose of 15 mg SbV/kg for 5 consecutive days and empty liposomes (20 μmol Lip/mouse/day) used as control of the lipid vesicle, were administered i.p. once a day for 10 days. Mice were sacrificed 3 days post-treatment and spleens were aseptically collected and weighted. Viable parasite loads in control and treated animals were estimated by limiting dilution assay [28]. The spleen and liver, from each mouse, were homogenized individually in Schneider's Drosophila medium supplemented with 10% heat-inactivated FBS. The tissue suspensions were diluted to a total volume of 3 mL. An additional 1:2 dilution was made for the spleen suspensions. A volume of 200 μL of the tissue suspensions was placed into the first well and four-fold serial dilutions were distributed in 96 well plates and incubated at 24 °C. Two weeks after incubation a sample of each well was examined under the microscope and labelled as positive or negative depending on the presence or absence of promastigotes. The final titre was set as the highest dilution for which the well contained at least one parasite and the number of parasites per gram of tissue was calculated as follows:

$$\frac{\left[\frac{(\text{Reciprocal titre of last positive well} \times \text{Total volume of homogenized tissue})}{\text{Weight(g) of homogenized tissue}}\right]}{(\text{Volume of first well} \times \text{Dilution Factor})}$$

The viable parasitic load was expressed as the number of *Leishmania* per gram of homogenized organ.

2.3.6. Statistical analysis

Data from *in vitro* studies are expressed as mean values (±) standard deviation (SD) or as mean values (±) standard error (SEM) according to samples in each study. Statistical analysis was performed using ANOVA Single Factor. The acceptable probability for a significant difference between mean values was $p < 0.05$ or as stated.

The non-parametric Mann–Whitney *U* test was used to compare parasite load from treated and non-treated infected mice. Differences were considered significant with a 5% significance level ($p < 0.05$). Statistical analysis was performed with the SPSS 13.0 for Windows software (SPSS Inc., USA) using values from at least three independent experiments.

3. Results

3.1. TFL-A liposomal formulations

Two TFL-A (designated TFL-A3 and TFL-A6) synthetized by our group [25] (Fig. 1) were incorporated in liposomes composed of DMPC:DMPG with 7:3 or 9:1 molar ratios (determinant of surface charge properties).

The L.C. of these formulations, a crucial factor for the preparation of highly loaded TFL-A liposomes to be used in animal experiments, was optimized by increasing the amount of the TFL-A in the initial bulk suspension ($[TFL\text{-}A/Lip]_i$). This was reached by the preparation of formulations with different ($[TFL\text{-}A/Lip]_i$ molar ratios (1:4; 1:5 and 1:10). The dependence of $[TFL\text{-}A/Lip]_f$, I.E. and TFL-A yield on the initial experimental conditions was evaluated. Physicochemical parameters for all the TFL-A liposomal formulations are shown in Table 1.

The incorporation parameters for TFL-A3 were dependent from the experimental conditions. In fact, all 9:1 molar ratio formulations showed higher parameters than the corresponding ones with the 7:3 ratio. Considering only the former formulations, the saturation of the lipid membrane with TFL-A3 was reached at a $[TFL\text{-}A3/Lip]_i$ of 83 g/mol corresponding to a L.C. of about 75 g of this analogue per mol of lipid. For higher initial ratios the L.C. remains constant. The I.E. is dependent on the $[TFL\text{-}A3/Lip]_i$, increasing up to 91% for a $[TFL\text{-}A3/Lip]_i$ of 83 g/mol and then decreasing to 50%. The TFL-A3 yield decreases from 64% to 32% over the range of $[TFL\text{-}A3/Lip]_i$ assayed.

The TFL-A6 containing liposomes present also acceptable incorporation parameters for all the experimental conditions used. For this analogue the L.C. is also strongly dependent on the $[TFL\text{-}A6/Lip]_i$, increasing from 30 to about 80 g/mol with the increase in $[TFL\text{-}A6/Lip]_i$ between 42 and 105 g/mol. The TFL-A6 yield values remain constant through the range of $[TFL\text{-}A6/Lip]_i$ used. The high I.E. values indicate that DMPC:DMPG are appropriate lipid mixtures for incorporation of this analogue regardless of the lipidic mixture molar ratio.

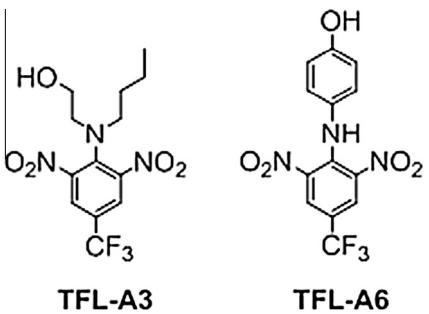

Fig. 1. Chemical structures of TFL-A3 and TFL-A6.

The size of the vesicles was found to be independent from the amount of TFL-A incorporated in the range studied, showing mean average values of about 185 nm.

The zeta potential of the DMPC:DMPG liposomes, which is closely related to the percentage of PG in the formulation, was not significantly affected by the type (TFL-A3 or TFL-A6) or amount of the incorporated analogue. Nevertheless as referred above, the TFL-A3 shows higher incorporation parameters for formulations containing 10% DMPG (DMPC:DMPG 9:1). Conversely, the TFL-A6 shows a preference for more negative bilayer structures containing 30% DMPG (DMPC:DMPG 7:3).

The TFL-A liposomal formulations selected from the above results (9:1 molar ratio for TFL-A3 and 7:3 for TFL-A6) were freeze-dried and stored in small aliquots (data not shown). These liposomes prepared in laboratory scale batches were used for the *in vitro* and *in vivo* biological evaluation assays. Their stability was assessed, after reconstitution of the freeze-dried cakes with water by the determination of TFL-A yield and vesicle sizes.

The results obtained for both analogues were similar with an overall TFL-A yield over 80% for all formulations. The size of liposomes after the freeze drying process and reconstitution was consistently similar, with negligible variations (less than 5%). The yield values of incorporate drug and sizes are representative of stable liposomes.

The characteristics observed for all formulations show that it was possible to optimize the incorporation of both TFL-A in liposomes using the same experimental conditions and lipid composition changing only the phospholipid and TFL-A:Lip molar ratios. These results point out that the TFL-A3 analogue (containing a linear alkyl hydroxyl group) required a less negative conditions allowing higher incorporation of TFL-A3 correspond to DMPC:DMPG liposomes in a 9:1 molar ratio and with a 1:5 TFL-A:Lip molar ratio. For TFL-A6 the best DMPC:DMPG molar ratio was 7:3 with a TFL-A:Lip 1:4 molar ratio. These incorporations resulted in 75 g of TFL-A3 and 77 g of TFL-A6 per mol of lipid (corresponding to approx. 0.11 g TFL-A/g lipid).

3.2. In vitro biological evaluation of TFL-A liposomes

The *in vitro* biological behaviour of the TFL-A either free or incorporated in liposomes was estimated by several studies: cytotoxicity; haemolytic activity and antileishmanial activity assays. Table 2 summarizes the results of all the assays performed providing a general analysis.

The results of the *in vitro* cytotoxicity assessment show that both TFL-A3 and TFL-A6 liposomal formulations as well as free TFL-A3 are non-cytotoxic in the range of concentrations tested, exhibiting IC_{50} values higher than 50 μM. On the opposite free TFL-A6 presents a potential cytotoxicity, with an IC_{50} value of 40 μM.

The haemolytic activity for both liposomal and free TFL-A at 100 μM was lower than 4% (data not shown). The HC_{50} values were higher than 500 μM, indicating that both TFL-A forms did not exhibit any detectable toxicity for mammalian cells as opposed to miltefosine (HC_{50} = 38.3 μM; CC_{50} = 28.6 μM).

The antileishmanial activity of free and liposomal TFL-A was assessed *in vitro* against the intracellular amastigote form of *Leishmania* parasites by infecting human monocytic leukaemia THP-1 cells. As displayed in Table 2, after treatment with concentrations up to and including 50 μM of both liposomal and free formulations of TFL-A3 and TFL-A6 all remained just as potent against intracellular amastigotes with IC_{50} values range between 1.2 and 3.2 μM. In addition, the intracellular activity of both TFL-A3 and

Table 1
Incorporation parameters of TFL-A containing liposomes as a function of lipid composition and $[TFL\text{-}A/Lip]_i$.

TFL-A	DMPC:DMPG (molar ratio)	TFL-A:Lip (molar ratio)	$[TFL\text{-}A/Lip]_i$ (g/mol)[a]	L.C. (g/mol)	TFL-A yield (%)	Lip yield (%)	I.E. (%)	Zeta potential (mV)
TFL-A3	7:3	1:10	62 ± 8	43 ± 4	57 ± 6	81 ± 12	71 ± 9	−47 ± 2
		1:5	99 ± 3	49 ± 7	32 ± 5	65 ± 2	49 ± 6	−45 ± 7
		1:4	121 ± 5	55 ± 5	35 ± 9	77 ± 4	45 ± 8	−46 ± 4
	9:1	1:10	68 ± 3	58 ± 2	64 ± 8	75 ± 6	86 ± 4	−36 ± 2
		1:5	83 ± 7	75 ± 9	63 ± 4	69 ± 4	91 ± 4	−31 ± 2
		1:4	151 ± 4	75 ± 2	32 ± 9	64 ± 2	50 ± 4	−34 ± 5
TFL-A6	7:3	1:10	42 ± 1	30 ± 2	62 ± 4	90 ± 8	70 ± 4	−42 ± 7
		1:5	77 ± 13	56 ± 5	63 ± 9	85 ± 9	74 ± 11	−42 ± 6
		1:4	105 ± 3	77 ± 3	69 ± 4	95 ± 1	73 ± 3	−45 ± 5
	9:1	1:10	42 ± 2	35 ± 5	67 ± 2	80 ± 4	84 ± 6	−34 ± 7
		1:5	90 ± 2	61 ± 3	63 ± 4	93 ± 1	68 ± 3	−33 ± 6
		1:4	104 ± 2	75 ± 8	66 ± 2	92 ± 7	72 ± 8	−30 ± 5

Values are mean ± SD of at least three independent experiments. Vesicles were sized to a mean diameter between 170 and 200 nm in all formulations, with a polydispersity index (P.I.) < 0.2. The zeta potential measurements were determined in citrate buffer, pH 5.5, 300 mOsm.
TFL-A6: $[Lip]_i$ = 16 μmol/mL; TFL-A3: $[Lip]_i$ = 20 μmol/mL.
[a] $[TFL\text{-}A/Lip]_i$ = Initial TFL-A to lipid ratio.

Table 2
In vitro biological evaluation of free and liposomal TFL-A.

Formulation	Cytotoxicity IC_{50} (μM)	Haemolysis HC_{50} (μM)	Intracellular amastigotes *L. infantum* IC_{50} (μM)
TFL-A3	>50	>500	1.2 ± 0.4
L-TFL-A3	>50	>500	1.4 ± 0.2
TFL-A6	40	>500	1.8 ± 1.3
L-TFL-A6	>50	>500	3.2 ± 0.9

The cytotoxicity of free (TFL-A3 and TFL-A6) or liposomal (L-TFL-A3 and L-TFL-A6) analogues was evaluated in the THP-1 cell line and the haemolytic activity in human RBC. The antiparasitic activity was evaluated against intracellular amastigotes of *L. infantum*. The data are means ± SEM of three independent experiments.

TFL-A6 liposomal formulations presented an analogous profile (data not shown).

3.3. In vivo antileishmanial activity of free and liposomal TFL-A

The therapeutic efficacy of free and liposomal TFL-A was evaluated in a rodent model for zoonotic visceral leishmaniasis (*L. infantum*) in different and representative sets of experiments. Treatments started at day 45 post-infection. All formulations were administered i.p. as a multiple-dose regimen. The amastigote reduction was used as a measure for drug efficacy as compared with vehicle-treated (unloaded liposomes) control animals.

The first set of experiments (Table 3), in which mice were treated with free and liposomal TFL-A3 and TFL-A6 formulations, showed that all formulations were more active in the spleen and the liposomal form of both TFL-A were significantly more effective than the corresponding free analogue in both organs. Treatment with L-TFL-A3 resulted in an almost complete removal of the parasites from the spleen with a 97% amastigote reduction while causing only a 48% reduction in the liver. Free TFL-A3 was significantly less active achieving only 42% ($p < 0.01$) and 18% ($p < 0.05$) parasite reductions in the spleen and liver respectively.

Table 3
Free and liposomal TFL-A *in vivo* efficacy in a murine zoonotic VL model.

Formulation	% Amastigote reduction in:			
	Spleen		Liver	
	Mean	SD	Mean	SD
TFL-A3	42	8	18	6
L-TFL-A3	97	6	48	11
TFL-A6	27	12	ND[a]	ND
L-TFL-A6	72	10	ND	ND
Vehicle[b]	15	5	ND	ND

BALB/c mice infected with 10^7 *L. infantum* (MHOM/PT/89/IMT151) promastigotes on day 0 were treated with 25 mg/kg/day of free (TFL-A3 and TFL-A6) or liposomal (L-TFL-A3 and L-TFL-A6) formulations during days 45–56 post-infection. Amastigote burdens in spleen and liver were determined 3 days after the last treatment by the limiting dilution assay. Results are expressed as the per cent reduction of amastigotes relative to infected control animals. Numbers show the mean and SD values of three independent experiments.
[a] ND – not determined.
[b] Vehicle – unloaded liposomes.

Treatment with liposomal TFL-A6 caused a 72% amastigote reduction in the spleen, not significantly different ($p < 0.05$) from that of liposomal TFL-A3. However, treatment with free TFL-A6 displayed very irregular results, inducing either no reduction in the number of viable parasite or similar inhibition values as the liposomal form (data not shown). Results for this analogue in the liver were also very inconsistent showing an absolute lack of reproducibility (data not shown).

In a second set of *in vivo* studies (Fig. 2), the effect of lipids on the therapeutic activity of the TFL-A3 liposomal formulation was evaluated with unloaded liposomes of the same lipid composition (vehicle). In these studies Glucantime® (SbV) was also included as an antileishmanial standard drug. The therapeutic activity of these formulations was estimated by comparing the amastigote loads in infected spleens of treated animals with those of untreated mice.

Treatment with liposomal TFL-A3 caused about 2.5 fold higher reduction of amastigote burden compared to the inhibition

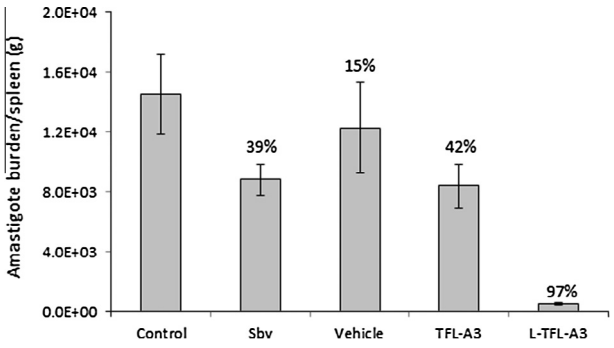

Fig. 2. *In vivo* therapeutic effect of Glucantime® (SbV), unloaded DMPC:DMPG (vehicle), free TFL-A3 and TFL-A3 loaded liposomes (L-TFL-A3) against *L. infantum* amastigotes in the spleen of BALB/c mice. Mice infected with *L. infantum* (MHOM/PT/89/IMT 151) received 10 doses (i.p.) of 25 mg TFL-A3/kg of L-TFL-A3 or 20 μmol Lipid/mouse of unloaded liposomes. Glucantime® (SbV) was administered s.c., 15 mg SbV/kg, 5 doses. Untreated infected BALB/c mice (control) received 10 doses of citrate/trehalose buffer. The parasite load in the spleen was determined by the limiting dilution assay. Results are expressed as the mean values of viable parasites per gram (g) of homogenized spleen ± SEM of at least five mice per group. Numbers correspond to the% of amastigote reduction when compared to control group.

induced by Glucantime® (39%). In contrast, treatment with the vehicle had little effect on *Leishmania* growth, generating only 15% of parasite reduction. These results confirm the therapeutic activity of the TFL-A3 in liposomal formulations and the reduced effect of the vehicle in zoonotic visceral leishmaniasis.

4. Discussion

Since the pioneer work of Chan and Fong [18], dinitroanilines, in particular TFL, have been considered as promising candidates for the treatment of *Leishmania* infections. Their preclinical development has been hindered by low water solubility and poor pharmacokinetics. Recent research efforts were focused on chemical modification studies [25,29–31] and development of NanoDDS aiming to increase dinitroanilines solubility and improve their activity against parasite [22–24]. Our previous work demonstrated the successful development of conventional and long-circulating liposomes containing TFL as the formulations were active against different species of *Leishmania* infecting both mice and dogs [20,21]. However, no complete elimination of parasites was reached in these studies. The search for new effective dinitroaniline compounds, driven by the need to improve TFL properties and antileishmanial activity, has led to the synthesis of new TFL analogues (TFL-A) [25].

In this work we describe and characterize the pharmaceutical optimization of DMPC:DMPG liposomal formulations to incorporate and stabilize two chemically synthesized TFL-A molecules with proven *in vitro* antileishmanial activity [25]. The same lipidic composition was previously used for the incorporation and stabilization of ORZ [24]. The authors proved that liposomes composed of lipids with Tc above 20 °C and containing the negatively charged PG moiety, targeted the incorporated drugs to macrophages [22]. Liposomes with similar composition were also able to stabilize rifabutin (a hydrophobic drug) and target it to the sites of infection [32]. With similar objectives, rigid lipids were chosen to immobilize the antileishmanial hydrophobic drug Amphotericin B [33,34].

The incorporation profiles obtained for the TFL-A formulations (Table 1) support the idea that the experimental conditions required to reach maximal incorporation were dependent of each TFL-A, suggesting a different interaction of the molecules with the phospholipid matrix. This was also confirmed by the preference of each analogue to a different TFL A:Lip molar ratio and liposome surface charge properties. The observed independency of the zeta potential from the amount of TFL-A incorporated, indicates that these molecules are inserted in the lipidic matrix and do not expose any charged portion to the external medium. Moreover, our results further demonstrate that the incorporation into liposomes resulted in a significant increase of the aqueous solubility of both analogues (35 fold for TFL-A3 and 70 fold for TFL-A6) improving the physicochemical properties for future development as antileishmanial drugs.

The maintenance of TFL-A loadings and homogeneous small sizes after freeze-drying of the formulations adds yet another advantage as this technique is well accepted for long-term storage of liposomes [35]. In this study it was also verified that 1% (w/v) trehalose was adequate to lyoprotect the DMPC:DMPG formulation incorporating TFL-A. In our studies with TFL the presence of 10% trehalose was needed to stabilize the drug incorporated into DOPC:DOPG liposomes during freeze-drying [20]. The reduction of the trehalose amount represents a technological advantage as it allows for an easier manipulation of the formulations. The TFL-A freeze-dried aliquots can be stored during a desired period of time (up to 2 years). When needed, the ready to use preparations are reconstituted by the simple addition of water.

The *in vitro* biological evaluation of TFL-A liposomes was focused on the potential toxic effects and on the antileishmanial activity. The incorporation of TFL-A6 in liposomes represents an advantage over the free analogue documented by the elimination of its cytotoxicity against THP-1 cells within the range of concentrations used (IC50 > 50 μM). The negligible haemolytic activity in RBC (<10% at 500 μM) demonstrated by both TFL-A either free or incorporated in liposomes, can be considered as an additional important advantage of these molecules with regard to the standard and commercial drug miltefosine which lyses 96% RBC at 100 μM [25].

The *in vitro* antileishmanial activity results demonstrate that both the free and liposomal forms of the TFL-A are able to target the intracellular amastigote form of *L. infantum* in infected macrophages, considered the more relevant parasitic stage for the biological evaluation of new drugs [36]. It was also confirmed that these molecules retain their activity when incorporated in liposomes. Moreover, liposomal TFL-A3 was more potent than miltefosine (IC_{50} = 2.7 μM) whereas liposomal TFL-A6 appeared to have similar activity as the commercial drug [25].

Considering that the aim of this study was to deliver TFL-A to *Leishmania* infected mononuclear phagocyte system cells, the activity of free and liposomal TFL-A3 and TFL-A6 was investigated in a murine model of zoonotic visceral leishmaniasis. The liposomal formulations used were specifically designed to possess the characteristics that guarantee their accumulation in the target organs of the disease (liver and spleen) as demonstrated before [22].

This strategy allowed to prove that it is possible to translate the *in vitro* biological behaviour of TFL-A into an efficient *in vivo* activity against *L. infantum* infection by the incorporation of these analogues into conventional liposomes. Specifically, the TFL-A3 analogue after being incorporated in liposomes was responsible for more than 95% reduction of parasite growth in the spleen of treated mice, and 48% in the liver. These results are in accordance with the biodistribution profile of conventional liposomes after intraperitoneal administration, as it was reported a higher accumulation in spleen as compared to liver [37]. This tendency leads to an increase amount of TFL-A in this organ which was translated into a stronger reduction of parasite growth. This result confirms not only the role of liposomes as delivery systems, but also the potential of TFL-A3 analogue as an antiparasitic drug. As for TFL-A6 the incorporation in liposomes represents the best option to solubilize this drug and to assess its antileishmanial activity since the activity of the free compound revealed a very irregular behaviour, probably due to solubility problems. The different behaviour observed by the two TFL-A tested *in vivo*, may be due to the contribution of the substituent introduced in the TFL molecule (phenol in TFL-A6 or linear alkyl hydroxyl in TFL-A3) on the therapeutic activity. However to establish a structure/activity relationship, a systematic study of a series of related compounds would be necessary. Moreover it was shown that the phospholipids constituents of the liposomes (vehicle) had no direct influence on therapeutic effect of the TFL-A formulations, their only role was as specific NanoDDS to target the analogues to the sites of infection. In addition both TFL-A liposomal formulations were more active than the standard drug Glucantime® which presents toxicity issues [38].

These studies proved that treatment of *L. infantum* infection with the new synthetic TFL-As reduces the parasite load in the spleen and the liver of treated mice. The higher efficacy, demonstrated by the TFL-A molecules when incorporated in conventional liposomes as compared to the free analogues, represents a therapeutic advantage in delivering these compounds.

5. Conclusion

Our data demonstrated the increased effectiveness of TFL-A liposomal formulations in a murine model of zoonotic leishmaniasis, compared to the free analogues. The construction of tailor made liposomes containing the TFL-A proved to be a determinant strategy for the efficient intracellular delivery of TFL-A with a reduction of their potential negative side effects relative to the free molecules (i.e. TFL-A6). These liposomal formulations display other important advantages: strong increase of the TFL-A aqueous solubility and elimination of the surfactant for the solubilization of the analogues. Furthermore, the findings reported here have established that the design and synthesis of chemical compounds, derived from classes of bioactive agents with proven antileishmanial activity, is an alternative approach for the search of new active formulations for the treatment of *Leishmania* infections. The important reduction of viable parasites induced by the treatment with TLF-A3 incorporated into liposomes in this mouse model indicates that this compound could be valuable for the treatment of zoonotic visceral leishmaniasis.

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Transparency declarations

None to declare.

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