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Research paper

Trifluralin liposomal formulations active against *Leishmania donovani* infections

Manuela Carvalheiro [a,c], João Jorge [a], Carla Eleutério [a,c], Ana F. Pinhal [a], Ana C. Sousa [a,c], José G. Morais [b,c], M. Eugénia M. Cruz [a,c,*]

[a] Unit of New Forms of Bioactive Agents, National Institute of Engineering Technology and Innovation, Lisbon, Portugal
[b] Faculty of Pharmacy, University of Lisbon, Lisbon, Portugal
[c] Research Institute for Medicines and Pharmaceutical Sciences, University of Lisbon, Lisbon, Portugal

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ABSTRACT

The purpose of this study was to increase the therapeutic index of the antiparasitic drug, trifluralin (TFL), to allow its parenteral administration without the need of toxic solvents. This was achieved by incorporating TFL in liposomes with high loading capacity. These formulations were stable in freeze-dried form during at least one year and in frozen form during at least three months. Therapeutic activity, assessed on a visceral model of infection, showed that TFL liposomes reduced the number of parasites by up to one third or one half as compared to negative control and to free TFL, respectively.

1. Introduction

Leishmaniasis is a disease caused by an intracellular parasite of the genus *Leishmania*, with a prevalence of at least 12 million infections and about 2 million new cases every year [1,2]. The visceral form of leishmaniasis, infecting macrophages of the liver, spleen and bone marrow, is usually fatal if not treated. Cutaneous and mucocutaneous forms have significant morbidity and occurrence of deforming lesions [1].

Several different classes of drugs have been used for the treatment of leishmaniasis. However, first line treatments, including pentavalent antimonial drugs, are limited by efficacy, long course and severe adverse reactions. These effects and the emergence of several resistant strains are responsible for their progressive failure and discontinuation. A number of other drugs has been reported, but none of them can conjugate high efficacy, acceptable toxicity and low cost [1,3].

Trifluralin (TFL) is a dinitroaniline active *in vitro* against several protozoan parasites, including *Leishmania*, acting by binding to parasite tubulins [2,4]. The *in vivo* topical administration of TFL was effective against murine cutaneous leishmaniasis [2]. The use by parenteral route has not been reported probably due to its low water solubility (0.22 mg/L) and consequently to the need of either large volumes of aqueous solvents or solvents not compatible with parenteral administration, to reach the therapeutic doses [5].

Our strategy to overcome the difficulties in handling TFL and to increase its therapeutic activity was the incorporation into liposomes. Liposomes seem to be the appropriate carriers to deliver antileishmanial drugs as, after intravenous administration, they are preferentially taken up by macrophages from liver and spleen [6,7], where *Leishmania* replicates.

Due to TFL specific mechanism of action and the consequently low toxicity in mammals [2,4] it seems a good candidate for the treatment of leishmaniasis providing that correct formulation and route of administration is achieved.

2. Methods

2.1. Liposome preparation and characterization

Liposomes were prepared by lipid film hydration [6,8] with some modifications. Briefly, lipidic components (Lip) (10 μmol/mL) and TFL (335–450 μg/mL) were dissolved in chloroform and dried under N_2. Liposomes were formed in three steps: first a 154 mM NaCl or a 300 mM trehalose solution (1/10 of the final volume) was added until the lipid film was dispersed. This step was repeated and 10 min later the hydration was completed with 154 mM NaCl or 300 mM trehalose. Liposomes were down-sized by extrusion. Non-incorporated TFL was removed by centrifugation (5000*g*, 10 min). For stability studies, formulations were ultra-cen-

* Corresponding author. Dep. de Biotecnologia, Instituto Nacional de Engenharia Tecnologia e Inovação, Unidade de Novas Formas de Agentes Bioactivos, I.P., Estrada do Paço do Lumiar 22, 1649-038 Lisboa, Portugal. Tel.: +351 210924722; fax: +351 217163636.
E-mail address: eugenia.cruz@ineti.pt (M. Eugénia M. Cruz).

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trifuged (180,000*g*, 1 h) and the pellets suspended either in 154 mM NaCl or 300 mM trehalose. Aliquotes of 1 mL were either freeze-dried, frozen (slow freezing at −70 °C) or kept at 4 °C. TFL liposomes were characterized for TFL content (HPLC using a Bio-Sil C18 HL-90-5 (150 × 4.6 mm) column), mean particle size (Ø) (quasi-elastic laser light scattering) and zeta potential.

Abbreviations and equations

$[TFL/Lip]_i$ Initial TFL to Lip ratio (μg/μmol)
$[TFL/Lip]_f$ Loading capacity: final TFL to Lip ratio (μg/μmol)
$([TFL]_f/[TFL]_i) \times 100$ TFL recovery (%)
$([Lip]_f/[Lip]_i) \times 100$ Lipid recovery (%)
$([TFL/Lip]_f)/([TFL/Lip]_i) \times 100$ I.E. = Incorporation (%)

2.2. Stability studies in suspension

Freshly made liposomes or freeze-dried liposomes reconstituted with water were kept at 4 °C. At selected time points samples were taken and centrifuged at 5000*g* for 10 min to remove lipid aggregates and non-incorporated TFL. The supernatant containing TFL liposomes was analysed as in Section 2.1.

2.3. Stability studies in freeze-dried form

At selected time points lyophilized liposomes were hydrated using either 154 mM NaCl (0% trehalose) or 300 mM trehalose (10% trehalose) or trehalose in the first two hydration steps and NaCl completed the hydration (5% final trehalose). Samples were centrifuged at 5000*g* for 10 min. The supernatant containing TFL liposomes was analysed as in Section 2.1.

2.4. Stability studies in frozen form

At selected time points frozen liposomes were thawed to room temperature and centrifuged at 5000*g* for 10 min. The supernatant containing TFL liposomes was analysed as in Section 2.1.

2.5. Visceral animal model studies

Eight to 10-week-old female BALB/c mice (Charles River Laboratories) were infected with 10^7 *Leishmania donovani* MHOM/ET/67/HU3 amastigotes. One week postinfection groups of 5 mice received 15 mg/kg/day of TFL either free or liposomal for 5 consecutive days. Another group received 1 dose of glucantime (15 mg Sb^v/kg). Two weeks postinfection animals were sacrificed, livers weighed and impression smears prepared. Parasite numbers were determined and parasitemia suppression (inhibition percentage relative to parasite load of negative control) calculated [3].

3. Results and discussion

3.1. Effect of lipid composition on the incorporation of TFL in liposomes

The incorporation of TFL in liposomes made from different lipid composition, containing phosphatidylcholines and either phosphatidylglycerol (PG) or lipids bound to polyethyleneglycol (PEG) is shown in Table 1. The rationale for PG selection was based on the fact that this lipid has been described to target macrophages of liver and spleen, organs where *Leishmania* parasites reside [9]. As for PEG-modified lipids, including distearoylphosphatidylethanolamine (DSPE-PEG), they were selected as PEG promotes prolonged circulation times of liposomes in the blood stream [7].

Low TFL incorporation parameters were obtained with egg phosphatidylcholine (PC) (F_1). The addition of PG promotes good incorporation to PC (F_2). Several other lipid mixtures were evaluated, including dioleoylphosphatidylglycerol (DOPG), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG).

Liposomes with a fluid membrane at room temperature (phase transition temperature (p.t.t.) below 0 °C) containing 30% (mol/mol) of PG (F_2 and F_3) showed good TFL incorporation parameters. These values were strongly reduced in liposomes with rigid membrane at room temperature (p.t.t. + 42 °C) (F_4). This is probably due to the hydrophobicity of TFL and the difficult insertion and stabilisation in rigid lipid bilayers, as described for other hydrophobic drugs [6]. When DSPE-PEG was added to lipid mixtures based on PC and PG (F_5–F_7) incorporation parameters comparable with those of liposomes without PEG (F_2) were obtained. A loading capacity of 23 μg/μmol was obtained for the three formulations corresponding, in a typical preparation, to 280 μg TFL/mL. Smaller TFL and lipid recoveries were observed for formulation F_6 containing both PG and DSPE-PEG. The increase on $[TFL/Lip]_i$ from around 27 (F_5–F_7) to around 52 μg/μmol (F_8–F_{10}), increased the loading capacity in the PG based formulations (F_8 *versus* F_5; and F_9 *versus* F_6), indicating that saturation was not yet reached. The inclusion of cholesterol (Chol) in one of these mixtures reduced by 50% the incorporation parameters including the loading capacity (F_{11} *versus* F_{10}). This can be explained by the competition of TFL and Chol for the same domain in the lipid bilayer. The zeta potential of TFL formulations containing PG (−35 to −38 mV) changed to around neutrality (−5 mV) for formulations containing PEG, due to its shielding effect.

3.2. Saturation studies

In order to determine under which conditions the liposomal membrane is saturated with TFL, the systematic influence of $[TFL/Lip]_i$ on incorporation parameters was studied (Fig. 1). The loading capacity increases with increasing $[TFL/Lip]_i$ reaching a plateau at a $[TFL/Lip]_i$ above 36 μg/μmol. Under these conditions about 30 μg of TFL were incorporated per μmol of lipid, corresponding to a recovery of 72% and an I.E. of 82%. The zeta potential was not significantly affected by the amount of TFL incorporated, indicating that TFL does not contribute to the superficial charge of the vesicles, as expected due to its neutrality (pKa 5.34). The size of the vesicles (118 ± 2 nm) was also independent of the amount of drug incorporated. Further tests were performed with a $[TFL/Lip]_i$ of 33.5 μg/μmol (corresponding to 1 μmol of initial TFL and 10 μmol initial lipid), as it maximizes the I.E. and the loading capacity. Typically, formulations with about 260 μg of TFL/mL were obtained. All the formulations prepared for the animal studies were concentrated up to 10-fold.

3.3. Stability in suspension

TFL liposomal formulations that presented good incorporation parameters in Table 1 were selected and screened for their stability in suspension. The formulations were stored in saline at 4 °C during 11 days. The formulations with a loss in TFL less than 5%, are conventional liposomes composed of fluid lipids (F_2 and F_3) and long circulating liposomes (F_{10}) (data not shown). The stability of these formulations in lyophilized and frozen form was studied.

3.4. Stability in lyophilized form

We have investigated the protective effect of trehalose during freeze-drying of TFL liposomes, namely if its presence either in the inner or the outer space of liposomes affects the retention of

Table 1
Incorporation parameters of various TFL formulations

	Phospholipid composition (molar ratio)	$[TFL]_i/[Lip]_i$ (μg/μmol)	$[TFL]_f/[Lip]_f$ (μg/μmol)	$[TFL]_f/[TFL]_i$ (%)	$[Lip]_f/[Lip]_i$ (%)	I.E. (%)	Zeta potential (mV)
F_1	PC	30 ± 4	2 ± 1	5 ± 1	68 ± 5	8 ± 1	−2 ± 2
F_2	PC:PG (7:3)	34 ± 2	27 ± 1	66 ± 8	81 ± 4	80 ± 6	−38 ± 2
F_3	DOPC:DOPG (7:3)	31 ± 3	24 ± 4	71 ± 12	52 ± 18	81 ± 12	−35 ± 2
F_4	DPPC:DPPG (7:3)	35 ± 2	9 ± 1	11 ± 4	44 ± 20	25 ± 3	−35 ± 2
F_5	PC:PG:DSPE-PEG (12:3:1)	28 ± 5	23 ± 3	71 ± 14	85 ± 8	84 ± 17	−5 ± 3
F_6	PG:DSPE-PEG (15:1)	27 ± 1	23 ± 1	56 ± 1	65 ± 1	86 ± 2	−5 ± 1
F_7	PC:DSPE-PEG (15:1)	26 ± 6	23 ± 6	74 ± 12	84 ± 8	88 ± 9	−5 ± 2
F_8	PC:PG:DSPE-PEG (12:3:1)	53 ± 2*	28 ± 1	41 ± 1	77 ± 2	53 ± 1	−5 ± 3
F_9	PG:DSPE-PEG (15:1)	51 ± 4*	39 ± 8	43 ± 6	56 ± 1	76 ± 7	−6 ± 2
F_{10}	PC:DSPE-PEG (15:1)	53 ± 1*	27 ± 1	40 ± 1	78 ± 2	50 ± 2	−5 ± 1
F_{11}	PC:Chol:DSPE-PEG (12:3:1)	54 ± 2*	15 ± 1	19 ± 1	68 ± 1	27 ± 1	−4 ± 1

Liposomes were extruded until is achieved a mean diameter of 100–120 nm. The data shown is an average ± SD from at least three independent experiments.
$[TFL]_i$ = TFL concentration before extrusion: (335 μg/mL) or *(450 μg/mL); $[TFL]_f$ = TFL concentration after centrifugation.
$[Lip]_i$ = 9,03–13,5 μmol/mL. Corresponding to 6.8–10.1 mg/mL.

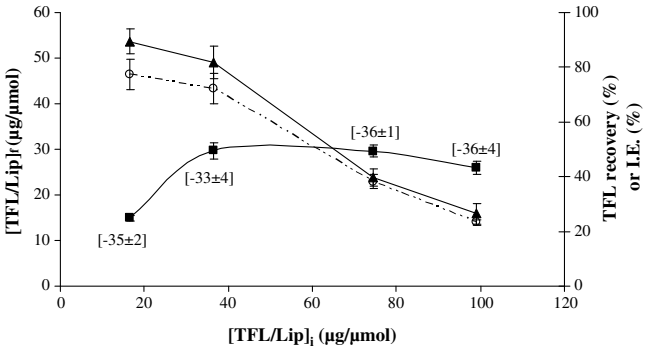

Fig. 1. Saturation profile for formulation F_3. Influence of $[TFL/Lip]_i$ (TFL to lipid ratio after the hydration of the lipidic film) on the incorporation parameters: $[TFL/Lip]_f$ = TFL to lipid ratio after ultra-centrifugation [■]; TFL recovery [○]; I.E. [▲]; and zeta potential values (mV) into brackets. Formulations of about 10 μmol/mL total lipid and about 0.5; 1; 2 and 3 μmol/mL TFL, corresponding to $[TFL/Lip]_i$ of 17, 36, 74 and 99 μg/μmol, were prepared as described in Section 2.1. The data points represent the average ± SD of at least three independent experiments.

TFL and liposomes properties. Table 2a shows that TFL recovery in freeze-dried liposomes was not substantially affected by the presence of trehalose in the inner space (hydration medium). However its presence on the outer space (suspension medium) is crucial for TFL retention and for the preservation of liposome size and surface properties. When TFL liposomes were freeze-dried in the absence of trehalose there was no TFL recovered after rehydration, which could be explained by drug sublimation and/or spun-down drug aggregates or precipitates. When trehalose was present only in the inner space, TFL recovery was null or very small (up to 8%). However, if trehalose was present in the outer space before freeze-drying, TFL recovery in lyophilized/rehydrated liposomes varied from 80% to 96%. This finding was independent from either the presence or the concentration of trehalose in the inner space and from the lipid compositions studied (F_2 or F_3). The size of liposomes was also affected by the presence of trehalose before freeze-drying. In the absence of trehalose in the outer medium, liposomes increased their sizes up to 155% of the initial value. Smaller reductions (up to 14%) were observed when liposomes were freeze-dried in the presence of trehalose. Small changes in zeta potentials (15–25%) were also observed.

To better understand this behaviour we analysed TFL recovery after the ultra-centrifugation step used to change the suspension medium before lyophilisation (Table 2b). At this step TFL recovery was not affected by the presence or the concentration of trehalose.

When lyophilisation took place in the presence of saline, changes occurring in the vesicle structure and in lipids distribution in the liposome outer layer, must be responsible for the TFL leakage. The discolouration of the freeze-dried cake from the typical yellow colour of TFL to a white colour confirms that the loss of drug occurs during lyophilisation. In the absence of a lyoprotectant, the association of TFL with the acyl chains of the phospholipids is not strong enough to avoid leakage and sublimation of the drug. This is probably caused by an increase in competing interactions between acyl chains resulting from the drying of the bilayers [10]. The presence of trehalose protects TFL in liposomes by allowing the establishment or maintenance of the original TFL-acyl chains interaction, thus acting as an anti-sublimating agent [8]. The lipid compositions that allowed higher TFL recoveries and higher I.E. (F_2

Table 2a
Evaluation of the protective effect of trehalose on TFL recovery and particle size variation in freeze-dried liposomes

Formulation	Hydration medium	TFL recovery (%) Suspension medium		Size variation (%) Suspension medium	
		NaCl	Trehalose	NaCl	Trehalose
F_2	0%	0	96 ± 4	86 ± 8	−3 ± 10
	5%	0	95 ± 3	155 ± 63	−10 ± 1
	10%	8 ± 1	89 ± 10	123 ± 65	−13 ± 1
F_3	0%	0	90 ± 7	50 ± 13	−12 ± 4
	5%	0	94 ± 1	38 ± 45	−14 ± 3
	10%	0	80 ± 15	76 ± 35	−9 ± 1

(0%) = 154 mM NaCl; (5%) = 154 mM NaCl and 300 mM trehalose. (10%) = 300 mM trehalose; TFL recovery (in percentage) = $[TFL]_f/[TFL]_i \times 100$; $[TFL]_i$ and $[TFL]_f$ represent the TFL concentration before and after lyophilisation; size variation is the increase or decrease size of the vesicles before and after lyophilisation as percentage of the respective value before lyophilisation; the diameters before lyophilisation were 192 ± 7 nm for F_2 and 187 ± 17 nm for F_3; Zeta potential values were 40 ± 5 and 38 ± 4 mV, for F_2 and F_3, respectively. The data shown are the average ± SD from three to nine independent experiments.

Table 2b
Evaluation of the presence of trehalose on TFL recovery before lyophilisation (after ultra-centrifugation)

Formulation	Hydration medium	TFL recovery (%) Suspension medium	
		NaCl	Trehalose
F_2	0%	74 ± 13	75 ± 13
	5%	87 ± 22	95 ± 27
	10%	79 ± 6	45 ± 12
F_3	0%	78 ± 14	71 ± 17
	5%	70 ± 21	75 ± 14
	10%	73 ± 15	62 ± 12

(0%) = 154 mM NaCl; (5%) = 154 mM NaCl and 300 mM trehalose. (10%) = 300 mM trehalose; TFL recovery (in percentage) = $[TFL]_f/[TFL]_i \times 100$; $[TFL]_i$ and $[TFL]_f$ represent the TFL concentration before and after ultra-centrifugation.

and F_3) also retained TFL in higher percentage in lyophilized form with trehalose, during at least two weeks, while keeping constant the size of liposomes.

Fig. 2 shows long term stability of F_2 and F_3 lyophilized formulations. These were reconstituted at various times, up to 12 months. Formulation F_3 retained more than 95% of TFL whereas formulation F_2 retained a lower amount, nonetheless more than 95% was still incorporated during the first 6 months, decreasing by 20% in the following months. Variations in the particle size smaller than 20% for both formulations were observed.

The presence of trehalose in the preparation process of TFL liposomes also allowed a better manipulation of the liposomal suspension. This made the extrusion easier, prevented the shrinkage of the freeze-dried cake assuring a good and instantaneous rehydration and homogenization by the simple addition of water.

3.5. Stability after reconstitution

The stability of the above reconstituted lyophilized formulations kept at 4 °C during 72 h was studied (Table 3). Both formulations kept more than 99% of the incorporated TFL 24 h after reconstitution and still maintaining 87–90% of the drug after 72 h. Size is maintained during this period of time. The maintenance of the liposome properties 3 days after reconstitution will allow their safe usage in a number of other experiments, e.g. *in vivo* studies.

3.6. Stability frozen at low temperature

The stability of frozen TFL liposomes, at low temperature, over more than 80 days, was also studied (Fig. 3). The vesicle size variations were up to 11% of the original values, with no changes of the zeta potentials. The finding that it is possible to keep certain TFL formulations frozen with no further treatment after preparation is important due to the convenience of this procedure.

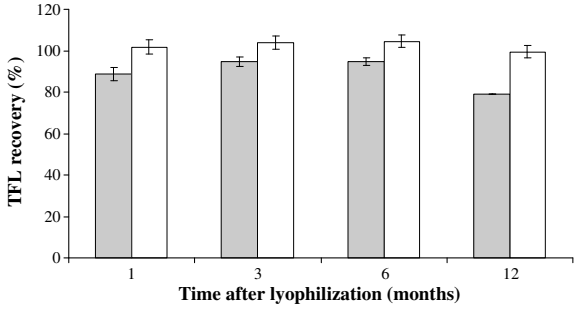

Fig. 2. Long-term storage stability of freeze-dried TFL liposomes: F_2 [■] and F_3 [□] (1 μmol TFL: 10 μmol Lip). TFL recovery was evaluated as function of time after lyophilisation. TFL/Lipid ratio before lyophilisation was about 31 μg/μmol. Sizes before lyophilisation were 123 ± 4 nm for F_2 and 140 ± 6 nm for F_3. After one year storage the mean diameters were 108 ± 1 nm and 115 ± 2 nm, respectively, for F_2 and F_3. Lipid recoveries were between 92% and 95% for both formulations. The data points represent the average ± SD of at least three independent experiments.

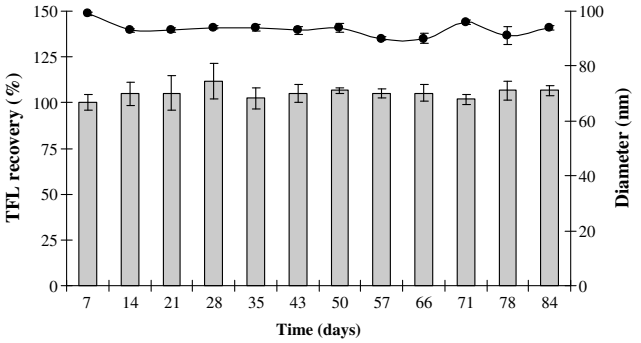

Fig. 3. Stability of the liposomal formulation F_3, stored at low temperature (−70 °C). Left axis represents TFL recovery (bars), where [TFL]i and [TFL]f are TFL before freezing and after thawing and centrifugation respectively. $[TFL]_i = 190 \pm 4$ μg/mL $[Lip]_i = 10 \pm 1$ μmol/mL. The right axis (round symbols) represents the mean diameter of the liposomes. The initial zeta potential was −42 ± 6 mV. The data points represent the average ± SD of, at least, three independent experiments.

3.7. Therapeutic activity

The therapeutic activity of TFL formulations was evaluated in a murine visceral model of infection (*Leishmania donovani*). TFL liposomal formulations were concentrated in such a way that the needed dose (15 mg/kg/day) could be administered in an appropriated volume (up to 200 μL/mouse). All formulations (free and liposomal) significantly reduced the parasite load as compared to the negative control. Liposomal formulations studied (F_2 and F_3) were significantly more active than free TFL ($p < 0.05$), presenting a parasitemia inhibition ranging from 55 to 68% while the free drug shows a value of only 33% (data not shown). The therapeutic activity of TFL liposomes is not statistically dependent on the lipid composition, with all liposomal formulations displaying equal activity to the standard drug (glucantime).

Fig. 4 shows the parasitemia inhibition in mice after treatment either with free TFL or a TFL liposomal formulation selected as the most stable (F_3). Formulation F_3 is 2-fold more active than the non-incorporated drug and presents a similar pattern as glucantime.

Liposomal formulations have the advantage of not needing organic solvents to be administered as is the case of the free TFL. It is expected that these results can be improved by changing the treatment schedule. In fact, the dose and/or treatment duration can be increased without major problem, taking into consideration the low toxicity of TFL in mammals [2,4]. Treatment with TFL liposomes of the same lipid composition (F_3) improved the clinical condition of dogs and reduced the density of parasites [4].

Table 3
Reconstitution of a freeze-dried cake of TFL liposomes: characteristics and stability

Formulation	24 h		48 h		72 h	
	TFL recovery (%)	Size variation (%)	TFL recovery (%)	Size variation (%)	TFL recovery (%)	Size variation (%)
F_2	98 ± 10	−4	95 ± 7	−2	90 ± 8	−3
F_3	92 ± 5	+6	87 ± 14	+8	84 ± 5	+8

At the time of reconstitution, TFL varied between 237 and 323 μg/mL for F_2 and 204 and 300 μg/mL for F_3. Liposomal suspensions were kept at 4 °C. $[TFL]_f$ was determined after rejection of the non-incorporated material by low speed centrifugation.
Size variation is the increase or decrease of vesicle size after respectively 24, 48 and 72 h, as a percentage of the value at the time of reconstitution. These values were 106 ± 2 nm for F_2 and 117 ± 8 nm for F_3 with a PIn smaller than 0.2.

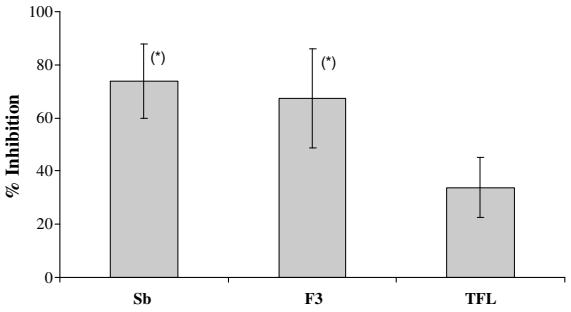

Fig. 4. *In vivo* activity of TFL liposomal formulation (F_3) in a visceral animal model of *Leishmania donovani*, in comparison with free TFL (dissolved in ethanol) and the standard drug glucantime (Sb). Results are expressed as inhibition percentage relative to parasite load of non-treated control animals (injected with liposomes suspension medium (300 mM trehalose). Results are from one experiment representative of three independent experiments. (*) Statistically different from free TFL as determined by ANOVA single factor $p < 0.05$ (Fcrit < F).

4. Conclusion

The work presented in this paper represents a successful attempt to incorporate TFL in liposomes, providing simultaneously an appropriate solvent for the drug, a stabilising system for its maintenance/storage during a substantial time after production and a drug targeting to *Leishmania* infected organs. Liposomal formulations containing TFL showed advantage on the reduction of parasite loads in mice, without the need of toxic solvents.

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References

[1] S.L. Croft, G.H. Coombs, Leishmaniasis – current chemotherapy and recent advances in the search for novel drugs, Trends Parasitol. 19 (11) (2003) 502–508.
[2] M. Chan, M. Grogl, C.C. Chen, E.J. Bienen, D. Fong, Herbicides to curb human parasitic infections: *in vitro* and *in vivo* effects of trifluralin on the trypanosomatid protozoan, Proc. Natl. Acad. Sci. USA 90 (12) (1993) 5657–5661.
[3] M. Larabi, V. Yardley, P.M. Loiseau, M. Appel, P. Legrand, A. Gulik, C. Bories, S.L. Croft, G. Barratt, Toxicity and antileishmanial activity of a new stable lipid suspension of amphotericin B, Antimicrob. Agents Chemother. 47 (12) (2003) 3774–3779.
[4] C. Marques, M. Carvalheiro, M.A. Pereira, J. Jorge, M.E.M. Cruz, G.M. Santos-Gomes, Efficacy of liposome trifluralin in the treatment of experimental canine leishmaniosis, The Veterinary Journal 178 (1) (2008) 133–137.
[5] L. Mamy, E. Barriuso, B. Gabrielle, Environmental fate of herbicides trifluralin, metazachlor, metamitron and sulcotrione compared with that of glyphosate, a substitute broad spectrum herbicide for different glyphosate-resistant crops, Pest Manag. Sci. 61 (2005) 905–916.
[6] M.M. Gaspar, S. Neves, F. Portaels, J. Pedrosa, M.T. Silva, M.E.M. Cruz, Therapeutic efficacy of liposomal rifabutin in a *Mycobacterium avium* model of infection, Antimicrob. Agents Chemother. 44 (9) (2000) 2424–2430.
[7] H. Pinto-Alphandary, A. Andremont, P. Couvreur, Targeted delivery of antibiotics using liposomes and nanoparticles. Research and applications, Int. J. Antimicrob. Ag. 13 (3) (2000) 155–168.
[8] M.E.M. Cruz, M.C. Carvalheiro, J.C.S. Jorge, Dinitroaniline liposomal formulations and processes for their preparation, European Patent EP 1.030.653 B1 (2005).
[9] T. Daemen, M. Velinova, J. Regts, M. de Jager, R. Kalicharan, J. Donga, J.J.L. van der Want, G.L. Scherphof, Different intrahepatic distribution of phosphatidylglycerol and phosphatidylserine liposomes in the rat, Hepatology 26 (2) (1997) 416–423.
[10] E.C.A. van Winden, N.J. Zuidam, D.J.A. Crommelin, Strategies for large scale production and optimised stability of pharmaceutical liposomes developed for parenteral use, in: D.D. Lasic, D. Papahadjopoulos (Eds.), Medical Applications of Liposomes, Elsevier Science B.V., Amsterdam, 1998, pp. 567–604.